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Blockade of B7-H1 on Macrophages Suppresses CD4$^+$ T Cell Proliferation by Augmenting IFN-γ-Induced Nitric Oxide Production[1]

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PD-1 is an immunoinhibitory receptor that belongs to the CD28/CTLA-4 family. B7-H1 (PD-L1) and B7-DC (PD-L2), which belong to the B7 family, have been identified as ligands for PD-1. Paradoxically, it has been reported that both B7-H1 and B7-DC costimulate or inhibit T cell proliferation and cytokine production. To determine the role of B7-H1 and B7-DC in T cell-APC interactions, we examined the contribution of B7-H1 and B7-DC to CD4$^+$ T cell activation by B cells, dendritic cells, and macrophages using anti-B7-H1, anti-B7-DC, and anti-PD-1 blocking mAbs. Anti-B7-H1 mAb and its Fab markedly inhibited the proliferation of anti-CD3-stimulated naive CD4$^+$ T cells, but enhanced IL-2 and IFN-γ production in the presence of macrophages. The inhibition of T cell proliferation by anti-B7-H1 mAb was abolished by neutralizing anti-IFN-γ mAb. Coculture of CD4$^+$ T cells and macrophages from IFN-γ-deficient or wild-type mice showed that CD4$^+$ T cell-derived IFN-γ was mainly responsible for the inhibition of CD4$^+$ T cell proliferation. Anti-B7-H1 mAb induced IFN-γ-mediated production of NO by macrophages, and inducible NO synthase inhibitors abrogated the inhibition of CD4$^+$ T cell proliferation by anti-B7-H1 mAb. These results indicated that the inhibition of T cell proliferation by anti-B7-H1 mAb was due to enhanced IFN-γ production, which augmented NO production by macrophages, suggesting a critical role for B7-H1 on macrophages in regulating IFN-γ production by naive CD4$^+$ T cells and, hence, NO production by macrophages. *The Journal of Immunology,* **2005, 175: 1586–1592.**

T cell activation is regulated by a balance between positive and negative signals. This balance is mainly kept by the interaction of costimulatory or coinhibitory receptors on T cells with their ligands on APCs. Programmed death-1 (PD-1)[3] is a type I transmembrane protein that was originally identified in a T cell line undergoing programmed cell death (1), but additional studies have shown that its expression is associated with lymphocyte activation rather than cell death (2, 3). Structurally, PD-1 belongs to the CD28/CTLA-4 subfamily of the Ig superfamily and contains a single Ig V-like domain in its extracellular region (4, 5). The PD-1 cytoplasmic region contains two tyrosine residues, with the N-terminal tyrosine being located in an ITIM and the C-terminal tyrosine being located in an immunoreceptor tyrosine-based switch motif (6). In vivo studies using PD-1-deficient mice have shown that PD-1 plays an important role in the regulation of peripheral tolerance and prevention of autoimmunity. PD-1-deficient C57BL/6 mice developed a lupus-like disease and arthritis (7). In addition, PD-1-deficient BALB/c mice developed autoantibody-mediated dilated cardiomyopathy (8). Moreover, PD-1-deficient mice crossed with H-2L^d-specific TCR transgenic mice on an H-2$^{b/d}$ background exhibited graft-vs-host disease-like pathologies (7). All these results have suggested that PD-1 acts as an important negative regulator of autoimmune responses.

Two new members of the B7 family, B7-H1 (PD-L1) and B7-DC (PD-L2), have been identified to be the ligands for PD-1 (9–12). In vitro studies have shown that the engagement of PD-1 by B7-H1 or B7-DC inhibited TCR-mediated T cell proliferation and cytokine (IFN-γ, IL-10, IL-4, and IL-2) production (10, 11). These results indicated that the engagement of PD-1 by B7-H1 or B7-DC led to down-regulation of T cell responses. However, not all studies have supported the inhibitory role for B7-H1 and B7-DC. A previous report has shown that naive T cells stimulated with immobilized anti-CD3 and B7-DC-Ig exhibited enhanced proliferation and IFN-γ production (12). Moreover, when T cells were stimulated with low doses of anti-CD3 and immobilized B7-H1-Ig, proliferation and production of IFN-γ, GM-CSF, and IL-10 were enhanced (9, 13). These results indicated that B7-H1 and B7-DC could costimulate T cell proliferation and cytokine production. The reason for these contradictory results remains unknown. One possible explanation may be the presence of a second receptor for B7-H1 and B7-DC, which delivers a costimulatory signal.

B7-H1 was expressed on immunocompetent cells such as T cells, B cells, dendritic cells (DCs), and macrophages (14). In contrast, B7-DC expression was highly restricted to DCs and activated macrophages (14). PD-1 was expressed on activated T and B cells and a subset of thymocytes (2, 14). These results have suggested that B7-H1 and B7-DC probably contribute to T cell-APC interactions. To address this possibility, we examined the contributions of B7-H1 and B7-DC to CD4$^+$ T cell activation by B cells, DCs, and macrophages using blocking mAbs against B7-H1, B7-DC, and PD-1. We found that the blockade of B7-H1 and PD-1 markedly inhibited the proliferation, but enhanced the IL-2 and IFN-γ

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[3] Abbreviations used in this paper: PD-1, programmed death 1; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible NO synthase; 1-MT, 1-methyl-D,L-tryptophan; L-NIO, N^5-(1-iminoethyl)-L-ornithine dihydrochloride; L-NMMA, N^G-monomethyl-L-arginine acetate.

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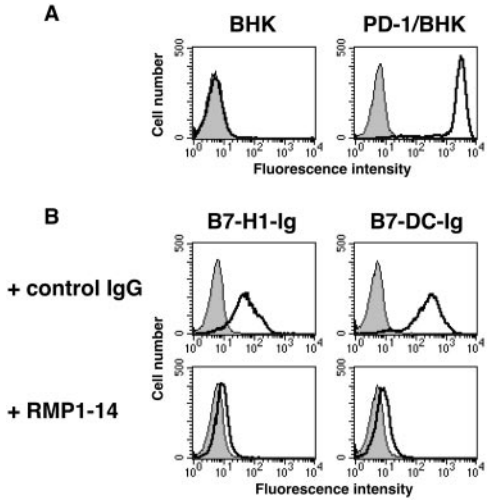

FIGURE 1. Characterization of RMP1-14 mAb. *A*, Reactivity of RMP1-14 to mouse PD-1 transfectant. BHK-derived transfectant (PD-1/BHK) and parental cells were stained with biotinylated RMP1-14 or control rat IgG2a, followed by PE-labeled streptavidin. The blank histograms indicate staining with RMP1-14, and the shaded histograms indicate background staining with control rat IgG2a. B, RMP1-14 inhibits B7-H1-Ig and B7-DC-Ig binding to PD-1 transfectant. PD-1/BHK cells were pretreated with control rat IgG2a or RMP1-14 and then stained with B7-H1-Ig, B7-DC-Ig, or control human IgG1, followed by PE-labeled goat anti-human IgG Ab. The blank histograms indicate staining with B7-H1-Ig or B7-DC-Ig. The shaded histograms indicate background staining with control human IgG1.

production, of naive $CD4^+$ T cells when costimulated by macrophages. This inhibition of $CD4^+$ T cell proliferation was due to accumulation of NO produced by macrophages in response to $CD4^+$ T cell-derived IFN-γ. These results suggested that B7-H1 on macrophages might play a critical role in the control of NO production by macrophages through the regulation of IFN-γ production by $CD4^+$ T cells. The pathophysiological relevance of this finding is discussed.

Materials and Methods

Mice and cell lines

Male BALB/c and C57BL/6 mice were purchased from Charles River Breeding Laboratories Japan. IFN-γ-deficient C57BL/6 mice were provided by Dr. Y. Iwakura (University of Tokyo, Tokyo, Japan). IFN-γR-deficient C57BL/6 mice were provided by Dr. T. Taniguchi (University of Tokyo). All mice were maintained under specific pathogen-free conditions and used at 6–7 wk of age according to institutional guidelines. A baby hamster kidney cell line, BHK, was purchased from American Type Culture Collection. The cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME.

Abs and reagents

Purified anti-mouse CD3 mAb (145-2C11), anti-mouse IFN-γ mAb (R4-6A2), rat IgG2a isotype control, and PE-labeled streptavidin were purchased from BD Pharmingen. Goat anti-mouse IgM $F(ab')_2$ Ab was purchased from Jackson ImmunoResearch Laboratories. Anti-mouse B7-H1 mAb (MIH6), anti-mouse B7-DC mAb (TY25), and anti-mouse CD40 mAb (HM40-3) were generated in our laboratory as previously described (14, 15). Fabs of anti-B7-H1 mAb were prepared using immobilized papain and a protein A column (Pierce). The purity of Fab was verified by SDS-PAGE analysis. A new anti-mouse PD-1 mAb (RMP1-14) was also generated in our laboratory. A Sprague Dawley rat was immunized with mouse PD-1-transfected BHK cells (2). Three days after the final immunization, spleen cells were fused with P3U1 myeloma cells as previously described (14). After hypoxanthine-aminopterin-thymidine selection, one hybridoma producing RMP1-14 mAb (rat IgG2a, κ) was identified by its strong reactivity with PD-1-transfected BHK cells, but not with parental BHK cells, by flow cytometry (Fig. 1*A*). RMP1-14 blocked the binding of both B7-H1-Ig and B7-DC-Ig fusion proteins (14) to PD-1-transfected BHK cells (Fig. 1B). The inducible NO synthase (iNOS) inhibitors, N^G-monomethyl-L-arginine acetate (L-NMMA) and N^5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO), were purchased from Wako Pure Chemicals. LPS and the indoleamine 2,3-dioxygenase (IDO) inhibitor, 1-methyl-D,L-tryptophan (1-MT), were purchased from Sigma-Aldrich. Recombinant mouse IFN-γ was purchased from BD Biosciences.

Preparation of $CD4^+$ T cells, B cells, DCs, and macrophages

$CD62L^+$ $CD4^+$ naive T cells were purified from splenocytes by passage through nylon wool columns (Wako Biochemicals) and by using autoMACS columns with a $CD4^+$ T cell isolation kit and anti-CD62L-coupled microbeads (Miltenyi Biotec) according to the manufacturer's instructions (>95% $CD4^+CD62L^+$). Small resting B cells were also purified from splenocytes as previously described (16). Briefly, spleen cells were treated with a mixture of hybridoma supernatants (anti-Thy-1.2, anti-CD4, and anti-CD8) and Low-Tox rabbit complement (Cedarlane Laboratories). After Percoll (Amersham Biosciences) gradient centrifugation, small B cells were collected from the 60/70% interface. Purified B cells (>95% $B220^+$; 3×10^6/ml) were cultured with anti-IgM Ab (5 μg/ml) and anti-CD40 mAb (5 μg/ml) for 24 h, then used as APCs. For isolating splenic DCs, spleens were digested with 400 U/ml collagenase (Wako Biochemicals) in the presence of 5 mM EDTA and separated into low and high density fractions on Optiprep gradient (Axis-Shield). Low-density cells were incubated with anti-CD11c-coupled microbeads (Miltenyi Biotec), and the bound cells were isolated using autoMACS columns (>95% $CD11c^+$). Peritoneal macrophages were obtained from mice that had received 2 ml of 4% thioglycolate (Sigma-Aldrich) i.p. 4 days previously. Peritoneal exudate cells were harvested by peritoneal lavage with ice-cold PBS and were depleted of nonadherent cells after 1-h culture on a plastic dish (>95% $CD11b^+$).

T cell proliferation and cytokine assay

Purified naive $CD4^+$ T cells (1×10^5/well) were cocultured with irradiated B cells (1×10^5/well), DCs (1×10^4/well), or macrophages (1×10^5/well) in the presence or the absence of anti-CD3 mAb (2 μg/ml) in 96-well, flat-bottom plates for 24–72 h. Each blocking mAb (5 μg/ml) or control rat IgG (5 μg/ml) and iNOS inhibitors (100 μM) or IDO inhibitor (1 mM)

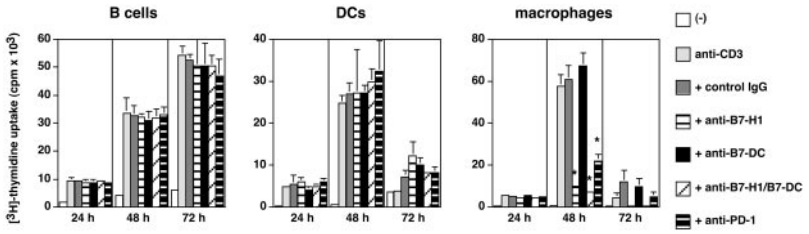

FIGURE 2. Effects of anti-B7-H1, anti-B7-DC, and anti-PD-1 mAbs on T cell proliferation costimulated by B cells, DCs, or macrophages. Naive $CD4^+$ T cells were cocultured with anti-IgM and anti-CD40-stimulated B cells, splenic DCs, or peritoneal macrophages in the presence of anti-CD3 mAb (2 μg/ml) and the indicated mAbs (5 μg/ml). The proliferative response was assessed at the indicated time points by pulsing the cultures with [^{3}H]thymidine for the last 6 h. The data are expressed as the mean $\pm$ SD of triplicate wells. Similar results were obtained from three independent experiments. *, $p <$ 0.01 compared with control IgG.

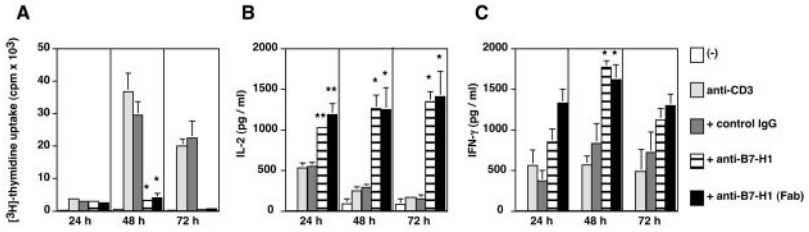

FIGURE 3. Effect of anti-B7-H1 mAb on $CD4^+$ T cell proliferation and cytokine production costimulated by macrophages. Naive $CD4^+$ T cells were cocultured with peritoneal macrophages in the presence of anti-CD3 mAb (2 μg/ml) and intact or Fab of anti-B7-H1 mAb (5 μg/ml). *A*, The proliferative response was assessed at the indicated time points by pulsing the cultures with [^{3}H]thymidine for the last 6 h. Production of IL-2 (*B*) and IFN-γ (*C*) in culture supernatants was measured by ELISA. The data are expressed as the mean $\pm$ SD of triplicate wells. Similar results were obtained from three independent experiments. *, $p < 0.01$; **, $p < 0.05$ (compared with control IgG).

were added at the start of the cultures. For estimating proliferative responses, the cultures were pulsed with 0.5 μCi/well [^{3}H]thymidine (DuPont-NEN) for the last 6 h and then harvested using a Micro 96 Harvester (Skatron). Incorporated radioactivity was measured in a Micro β counter (Micro β Plus). To determine the production of cytokines, cell-free supernatants were collected at 24–72 h and assayed for IL-2, IL-4, IL-5, and IL-10 by ELISA using OptEIA kits (BD Pharmingen) and for IFN-γ using the mouse IFN-γ ELISA Ready-SET-Go kit (eBioscience) according to the manufacturer's instructions.

Measurement of NO production

The nitrite concentration in the culture supernatants was measured by a colorimetric assay using the Griess reagent. Cell-free supernatants (100 μl) were added to a microtiter plate containing 100 μl of 1% sulfanilamide and 0.1% naphthylethylenediamine in 2.5% H_3PO_4. After 10 min, the OD was measured at 550 nm and compared with a standard curve of $NaNO_3$.

Statistical analysis

Significant differences between two experimental groups were analyzed by unpaired Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Involvement of B7-H1 and B7-DC in T cell activation by B cells, DCs, and macrophages

To investigate the roles of B7-H1 and B7-DC in T-APC interactions, we first examined the contributions of B7-H1 and B7-DC to T cell activation by B cells, DCs, and macrophages. Purified naive $CD4^+$ T cells from the spleens of BALB/c mice were cocultured with preactivated B cells, splenic DCs, or thioglycolate-elicited peritoneal macrophages in the presence of anti-CD3 mAb and anti-B7-H1 mAb, anti-B7-DC mAb, anti-PD-1 mAb, or control rat IgG for 24–72 h, and then the proliferative response was assessed by [^{3}H]thymidine uptake (Fig. 2). In the absence of APCs, no significant proliferation (<1000 cpm) of naive $CD4^+$ T cells was observed upon anti-CD3 stimulation (data not shown). The addition of activated B cells, DCs, or macrophages markedly costimulated the proliferation at 48 h. DCs were the most potent, in that the costimulatory effect of 1×10^4 DCs was almost comparable to that of 1×10^5 B cells or macrophages. The anti-B7-H1, anti-B7-DC, and anti-PD-1 blocking mAbs did not apparently affect the $CD4^+$ T cell proliferation when costimulated by B cells or DCs. In contrast, when costimulated by macrophages, the proliferative response was markedly inhibited by either anti-B7-H1 or anti-PD-1 mAb alone, but not by anti-B7-DC mAb. Similar inhibitory effects of anti-B7-H1 and anti-PD-1 mAbs were also observed when $CD4^+$ T cells were cocultured with resident peritoneal macrophages or splenic macrophages from naive mice (data not shown). These results suggested a critical contribution of PD-1/B7-H1 interaction to the costimulation of $CD4^+$ T cell proliferation by macrophages.

To exclude a possible contribution of FcRs on macrophages to the inhibitory effect of anti-B7-H1 mAb, naive $CD4^+$ T cells were cocultured with macrophages in the presence of anti-CD3 mAb and intact or Fab of anti-B7-H1 mAb. As shown in Fig. 3*A*, $CD4^+$ T cell proliferation was comparably inhibited by both intact and Fab of anti-B7-H1 mAb. We also examined cytokine production in these cultures. Unexpectedly, the production of IL-2 and IFN-γ was enhanced by either intact or Fab of anti-B7-H1 mAb (Fig. 3, *B* and *C*). IL-4, IL-5, and IL-10 were not detectable in this experiment (data not shown). These results indicated that the inhibitory effect of anti-B7-H1 mAb on $CD4^+$ T cell proliferation was not due to a lack of IL-2 production.

Involvement of IFN-γ in the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 and anti-PD-1 mAbs

The inhibitory effect of anti-B7-H1 mAb on $CD4^+$ T cell proliferation might be explained by a down-regulation of IL-2R expression on $CD4^+$ T cells. However, the expression levels of IL-2R α-, β-, and γ-chains on anti-CD3-stimulated $CD4^+$ T cells in the presence of macrophages were not significantly affected by anti-B7-H1 mAb compared with control IgG (data not shown). Alternatively, the enhanced IFN-γ production might be responsible for the inhibition of $CD4^+$ T cell proliferation. To address this possibility, we examined the effect of neutralizing anti-IFN-γ mAb on the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 and anti-PD-1 mAbs. Purified naive $CD4^+$ T cells were cocultured with macrophages in the presence of anti-CD3 mAb and anti-B7-H1 or anti-PD-1 mAb with or without anti-IFN-γ mAb for 48 h, and then the proliferative response was assessed. As shown in Fig. 4*A*, anti-IFN-γ mAb alone significantly enhanced the proliferation of

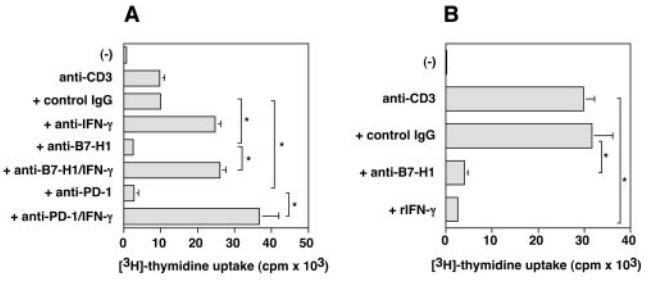

FIGURE 4. Involvement of IFN-γ in the inhibitory effects of anti-B7-H1 and anti-PD-1 mAbs on T cell proliferation costimulated by macrophages. *A*, Naive $CD4^+$ T cells were cocultured with peritoneal macrophages in the presence of anti-CD3 mAb (2 μg/ml) and anti-B7-H1 or anti-PD-1 mAb (5 μg/ml) with or without anti-IFN-γ mAb (50 μg/ml) for 48 h. *B*, Naive $CD4^+$ T cells were cocultured with macrophages in the presence of anti-CD3 mAb (2 μg/ml) and anti-B7-H1 mAb (5 μg/ml) or rIFN-γ (2 ng/ml) for 48 h. The proliferative response was assessed by pulsing the cultures with [^{3}H]thymidine for the last 6 h. The data are expressed as the mean $\pm$ SD of triplicate wells. Similar results were obtained from three independent experiments. *, $p < 0.01$.

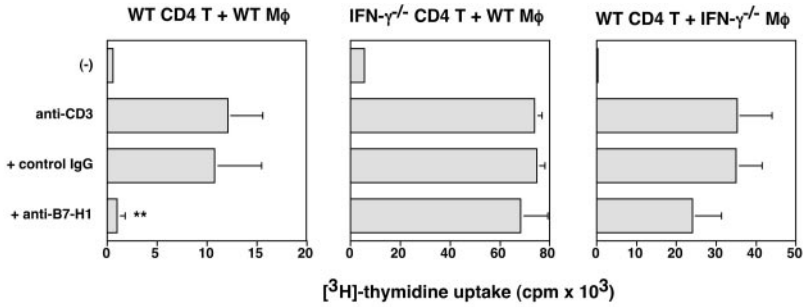

FIGURE 5. The inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb is mostly dependent on $CD4^+$ T cell-derived IFN-γ. Naive $CD4^+$ T cells and peritoneal macrophages were purified from IFN-γ-deficient or wild-type mice and cultured with anti-CD3 mAb (2 μg/ml) and anti-B7-H1 mAb (5 μg/ml) for 48 h. The proliferative response was assessed by pulsing the cultures with [^{3}H]thymidine for the last 6 h. The data are expressed as the mean ± SD of triplicate wells. Similar results were obtained from three independent experiments. **, $p < 0.05$.

$CD4^+$ T cells, indicating a suppressive effect of endogenously produced IFN-γ. More importantly, the inhibitory effects of both anti-B7-H1 mAb and anti-PD-1 mAbs were totally abrogated by anti-IFN-γ mAb. These results indicated that the inhibitory effects of anti-B7-H1 and anti-PD-1 mAbs on $CD4^+$ T cell proliferation were dependent on the increased IFN-γ production. To further substantiate the inhibitory effect of the increased IFN-γ, we added a physiological concentration of rIFN-γ (2 ng/ml). As shown in Fig. 4*B*, the addition of rIFN-γ inhibited the proliferation of $CD4^+$ T cells to a comparable level with anti-B7-H1 mAb.

$CD4^+$ T cell-derived IFN-γ is primarily responsible for the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb

To determine the source of IFN-γ, $CD4^+$ T cells and macrophages were prepared from IFN-γ-deficient or wild-type mice and cocultured with anti-CD3 mAb in the presence of anti-B7-H1 mAb or control IgG. The proliferative response was assessed on 48 h. As shown in Fig. 5, anti-B7-H1 mAb markedly inhibited the proliferation when both $CD4^+$ T cells and macrophages were prepared from wild-type mice. In contrast, this inhibitory effect was mostly abrogated when IFN-γ-deficient $CD4^+$ T cells were cocultured with wild-type macrophages. In contrast, anti-B7-H1 mAb still partially inhibited $CD4^+$ T cell proliferation when wild-type $CD4^+$ T cells were cocultured with IFN-γ-deficient macrophages. These results indicated that $CD4^+$ T cells, rather than macrophages, were the major source of IFN-γ responsible for the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb.

IFN-γR on macrophages is responsible for the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb

We also examined the involvement of IFN-γR in the anti-B7-H1 mAb-mediated suppression. As shown in Fig. 6, when macrophages were prepared from IFN-γ$R^{-/-}$ mice, the proliferation of $CD4^+$ T cells was not significantly inhibited by anti-B7-H1 mAb, indicating a critical contribution of IFN-γR to macrophages.

Anti-B7-H1 mAb induces IFN-γ-mediated NO production by macrophages

It is known that macrophages produce NO after induction of iNOS by IFN-γ, and that NO inhibits T cell proliferation (17–19). Therefore, we next examined NO production in the coculture of $CD4^+$ T cells and macrophages in the presence of anti-CD3 mAb. As shown in Fig. 7*B*, the addition of anti-B7-H1 mAb markedly induced NO production, which was totally abrogated by anti-IFN-γ mAb. NO production was apparently associated with the inhibition of $CD4^+$ T cell proliferation (Fig. 7*A*). Moreover, NO production was not observed when macrophages were derived from IFN-γ$R^{-/-}$ mice (data not shown). These results indicated that the B7-H1 blockade induced IFN-γ-dependent NO production by macrophages.

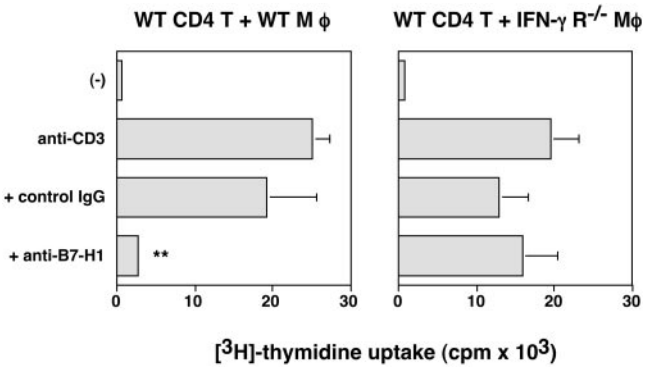

FIGURE 6. The inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb is dependent on IFN-γR on macrophages. Naive $CD4^+$ T cells and peritoneal macrophages were purified from IFN-γR-deficient or wild-type mice and cultured with anti-CD3 mAb (2 μg/ml) and anti-B7-H1 mAb (5 μg/ml) for 48 h. The proliferative response was assessed by pulsing the cultures with [^{3}H]thymidine for the last 6 h. The data are expressed as the mean ± SD of triplicate wells. Similar results were obtained from three independent experiments. **, $p < 0.05$.

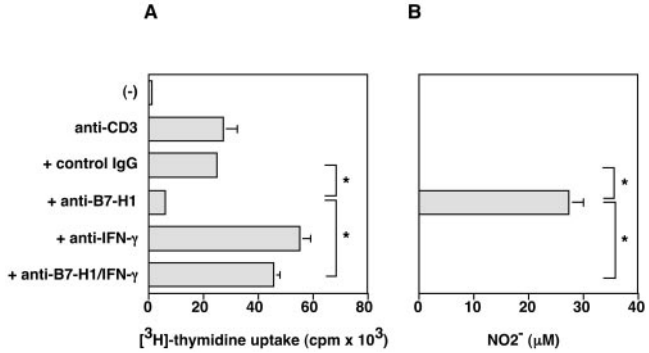

FIGURE 7. Anti-B7-H1 mAb induces IFN-γ-mediated NO production. Naive $CD4^+$ T cells were cocultured with peritoneal macrophages in the presence or the absence of anti-CD3 mAb with or without anti-B7-H1 and/or anti-IFN-γ mAbs for 48 h. *A*, The proliferative response was assessed by pulsing the cultures with [^{3}H]thymidine for the last 6 h. *B*, NO production in culture supernatants was analyzed by the Griess method. The data are expressed as the mean ± SD of triplicate wells. Similar results were obtained from three independent experiments. *, $p < 0.01$.

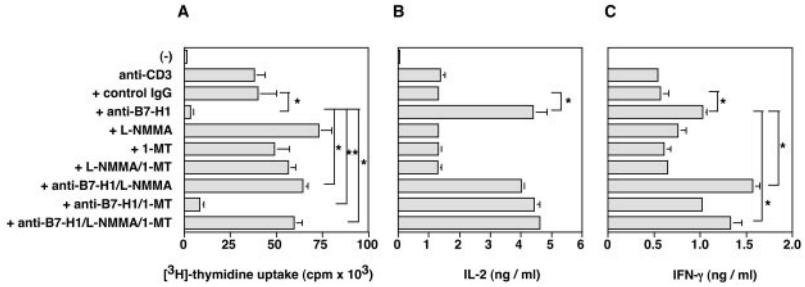

FIGURE 8. Effects of iNOS inhibitor and/or IDO inhibitor on the $CD4^+$ T cell proliferation and cytokine production. Naive $CD4^+$ T cells were cocultured with peritoneal macrophages in the presence or the absence of anti-CD3 mAb with or without control IgG or anti-B7-H1 mAb and iNOS inhibitor (L-NMMA) and/or IDO inhibitor (1-MT) for 48 h. *A*, The proliferative response was assessed by pulsing the cultures with [^{3}H]thymidine for the last 6 h. The production of IL-2 (*B*) and IFN-γ (*C*) in culture supernatants was measured by ELISA. The data are expressed as the mean $\pm$ SD of triplicate wells. Similar results were obtained from three independent experiments. *, $p < 0.01$; **, $p < 0.05$.

Effects of iNOS inhibitor and IDO inhibitor on the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb

We next examined the effects of iNOS inhibitors, L-NMMA and L-NIO, on the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb. As shown in Fig. 8*A*, L-NMMA alone significantly enhanced the proliferation, indicating a suppression by endogenously produced NO. More importantly, the inhibition by anti-B7-H1 mAb was totally abrogated by L-NMMA. L-NIO exhibited a similar effect (data not shown). The expression level of B7-H1 on macrophages was not affected by L-NMMA, as estimated by flow cytometry (data not shown). These results indicated that the inhibitory effect of anti-B7-H1 mAb on $CD4^+$ T cell proliferation was mediated by iNOS-induced NO production by macrophages.

It has recently been reported that IDO is a tryptophan-catabolizing enzyme expressed by IFN-γ-stimulated macrophages that inhibits T cell proliferation by tryptophan depletion (20–23). Thus, we also examined the effect of a specific inhibitor of IDO, 1-MT, on the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb. As shown in Fig. 8*A*, 1-MT partially reversed the inhibition of $CD4^+$ T cell proliferation by anti-B7-H1 mAb, suggesting a minor contribution of IDO. However, the combination of 1-MT with L-NMMA did not exert an additive effect, indicating the predominant contribution of iNOS, rather than IDO.

We also examined the effects of L-NMMA and/or 1-MT on cytokine production. As shown in Fig. 8*B*, the increased IL-2 level caused by anti-B7-H1 mAb was not significantly affected by L-NMMA and/or 1-MT. In contrast, the increased IFN-γ level caused by anti-B7-H1 mAb was further increased by L-NMMA, suggesting the expansion of IFN-γ-producing T cells (Fig. 8*C*).

Discussion

In this study we found that the blockade of the PD-1/B7-H1 interaction markedly inhibited the proliferation of anti-CD3-stimulated naive $CD4^+$ T cells when costimulated by macrophages in vitro. This inhibition was not due to blockade of a positive costimulation by PD-1/B7-H1 for priming T cell activation, because the production of IL-2 and IFN-γ was, instead, enhanced by the blockade of B7-H1. Additional experiments showed that the enhanced IFN-γ production by $CD4^+$ T cells induced iNOS-mediated NO production by macrophages, which, in turn, inhibited the proliferation of $CD4^+$ T cells. These results suggest that the PD-1/B7-H1 interaction plays a critical role in regulating IFN-γ production, NO production, and T cell expansion through the cognate interaction between $CD4^+$ T cells and macrophages.

The blockade of B7-H1 enhanced IFN-γ production by $CD4^+$ T cells, suggesting a critical role for B7-H1 on macrophages to suppress IFN-γ production by $CD4^+$ T cells. We have previously demonstrated that B7-H1 is constitutively expressed on macrophages at a high level and is further up-regulated by T cell-derived cytokines, including IFN-γ (14). Therefore, the B7-H1-mediated suppression may be a negative feedback mechanism to prevent overproduction of IFN-γ. The inhibition of IFN-γ production by B7-H1 might be directly mediated by negative signaling via PD-1, because it has been shown that the immunoreceptor tyrosine-based switch motif of PD-1 recruits tyrosine phosphatases Src homology protein tyrosine phosphatase-1 and -2, which counteract the TCR-mediated T cell activation, in primary human T cells (24). Alternatively, the suppression of IFN-γ production by B7-H1 might be indirectly mediated by IL-10, because it has been reported that B7-H1 enhances IL-10 production by $CD4^+$ T cells (9). However, we could not detect IL-10 in the coculture of $CD4^+$ T cells and macrophages in the present study. Therefore, the negative signaling via PD-1 seems to be more likely to be responsible for the suppression of IFN-γ production by B7-H1. The critical role of B7-H1 in regulating IFN-γ production by naive $CD4^+$ T cells is consistent with a recent study using B7-H1-deficient mice (25).

The inhibition of T cell proliferation by anti-B7-H1 mAb was abolished by anti-IFN-γ mAb and iNOS inhibitors, indicating that it was mediated by IFN-γ-induced and iNOS-mediated NO production by macrophages. It has previously been reported that NO is a potent inhibitor of T cell proliferation, but does not affect the secretion of IL-2 or the expression of IL-2R (26). The NO-mediated inhibition of T cell proliferation was associated with markedly reduced tyrosine phosphorylation of Jak3 and STAT5, suggesting a blockade of IL-2R signaling for proliferation (27). This is consistent with our present observation that the B7-H1 blockade resulted in the accumulation of IL-2 in culture supernatants, suggesting a blockade of the IL-2/IL-2R-mediated proliferation pathway. Actually, the tyrosine phosphorylation of STAT5 in anti-CD3-stimulated naive $CD4^+$ T cells in the presence of macrophages was markedly decreased by the addition of anti-B7-H1 mAb, as estimated by Western blotting (data not shown). Moreover, it has been reported that activated T cells were arrested in the G_0/G_1 phase of the cell cycle in the presence of NO-producing macrophages (28). We confirmed that anti-CD3-stimulated $CD4^+$ T cells in the presence of macrophages were mostly arrested in the G_0/G_1 phase when anti-B7-H1 mAb was added, as estimated by nuclear staining with propidium iodide and flow cytometry (data not shown). We also noticed that sub-G_0/G_1 population, representing apoptotic nuclei, was not increased by the addition of anti-B7-H1 mAb (data not shown). These results indicated that the blockade of cell cycle progression, but not the induction of apoptosis, was primarily responsible for the inhibition of T cell proliferation by anti-B7-H1 mAb.

The $CD4^+$ T cell-derived, IFN-γ-mediated NO production by macrophages plays a pivotal role in the protective immunity against microbial pathogens (29). We have previously demonstrated that the B7-H1 expression on macrophages is also up-regulated by microbial products such as LPS (14). If the PD-1/B7-H1 interaction down-regulates NO production by macrophages through cognate interaction with Ag-specific $CD4^+$ T cells, the blockade of PD-1/B7-H1 interaction may be beneficial for augmenting the protective immunity against microbial pathogens. In contrast, IFN-γ, macrophages, and NO have been also implicated in the pathogenesis of various inflammatory diseases, such as rheumatoid arthritis (30–32), multiple sclerosis (33), psoriasis (34), and type 1 diabetes (35–41). It has been reported that PD-1-dificient mice develop lupus-like arthritis (7) and that B7-H1-dificient mice develop exacerbated experimental autoimmune encephalomyelitis (25). We have also demonstrated that the blockade of PD-1 and B7-H1 exacerbated contact hypersensitivity (42) and autoimmune diabetes (43) in mice. If the PD-1/B7-H1-mediated down-regulation of IFN-γ and NO is involved in the suppression of inflammatory responses, blockade of the PD-1/B7-H1 interaction may exacerbate the infection-associated pathologies, such as pneumonitis and hepatitis. Therefore, the potentially beneficial or detrimental effect of the PD-1/B7-H1 blockade remains to be determined by additional studies using mouse models of microbial infection.

The original descriptions of cytokine-inducible, NO-mediated inhibition of lymphocyte proliferation were published over a decade ago (44, 45). These observations have been verified in various systems (27, 46–49). It has been reported that in murine *Trypanosoma brucei* infection, the activation of macrophages to produce NO leads to impaired lymphocyte responses and immunosuppression (50). Moreover, NO modulates the severity of autoimmune diseases by exerting a protective effect through inhibition of autoreactive T cells. In experimental autoimmune encephalomyelitis, mice with a targeted disruption of the iNOS gene show increased severity due to increased proliferation of pathogenic T cells (51, 52). In a rat model of autoimmune nephritis, pharmacologic inhibition of iNOS with 1-N^6-(1-iminoethyl)-lysine intensified the renal injury, suggesting that inhibition of iNOS may alter the expansion of nephritogenic T cells (53). The production of large amounts of NO by iNOS is a double-edged sword, eliciting pro- or anti-inflammatory effects in different situations. We have demonstrated in this study that the PD-1/B7-H1 interaction plays an important role in regulating NO production by macrophages. Therefore, more beneficial effects of the PD-1/B7-H1 blockade to augment immune responses against pathogens and tumors may be achieved by combination with the iNOS blockade.

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Disclosures

The authors have no financial conflict of interest.

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