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Murine B7-H3 Is a Negative Regulator of T Cells

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T cell activation is regulated by the innate immune system through positive and negative costimulatory molecules. B7-H3 is a novel B7-like molecule with a putative receptor on activated T cells. Human B7-H3 was first described as a positive costimulator, most potently inducing IFN-γ production and cellular immunity. In this study, we examined the expression and function of mouse B7-H3. B7-H3 is mostly expressed on professional APCs; its expression on dendritic cells appears to be up-regulated by LPS. In contrast to human B7-H3, we found that mouse B7-H3 protein inhibited T cell activation and effector cytokine production. An antagonistic mAb to B7-H3 enhanced T cell proliferation in vitro and led to exacerbated experimental autoimmune encephalomyelitis in vivo. Therefore, mouse B7-H3 serves as a negative regulator of T cell activation and function. The Journal of Immunology, 2004, 173: 2500–2506.
Materials and Methods

Generation of anti-B7-H3 mAbs

A female Lewis rat (3–4 mo old) was immunized with 100 μg of B7-H3-Ig, and hybridoma was generated as previously described (29). ELISA was performed to identify the clones that reacted with B7-H3-Ig fusion protein, but not with control human IgG1.

Flow cytometric analysis

Purified anti-B7-H3 and anti-B7-S1 Abs and a rat IgG (Sigma-Aldrich, St. Louis, MO) were biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, IL). Anti-CD4, -CD8, -CD11b, -CD11c, -CD25 -CD44, and -B20 Abs were obtained from BD Pharmingen (San Diego, CA). Fc block (BD Pharmingen) and rat IgG were used to reduce nonspecific staining. Peritoneal macrophages were collected by extracting peritoneal lavage. Bone marrow cells from C57BL/6 mice femurs were extracted and cultured in complete medium and GM-CSF (20 ng/ml; PeproTech, Rocky Hill, NJ) for 7 days.

In vitro T cell assays

CD4+ T cells from C57BL/6 or OT-II mice were purified as previously described (13, 30). The cells were treated with plate-bound anti-CD3 in the absence or the presence of human IgG or B7-H3-Ig. IL-2 production was measured by ELISA after 24 h after incubation with [3H]thymidine in the last 8 h. For in vitro Ab blocking assays, splenocytes from OT-II mice were stimulated 24 h after T cell activation, and cell proliferation was measured 72 h later.

Luciferase assay

DO11.10 T cell hybridoma cells were maintained in complete RPMI 1640 supplemented with 10% FBS (HyClone, Logan, UT). Cells transfected with 0.25 μg of PRL null and 1 μg of luciferase promoter reporter plasmids of NFAT, AP-1, or NF-κB were stimulated with 1 μg/ml plate-bound anti-CD3 Ab with or without B7-H3-Ig for 4 h. Supernatants were used to measure IL-2 by ELISA. Cell extracts were prepared, and luciferase activity was measured by using the Dual-Luciferase system (Promega, Madison, WI).

EAE induction and analysis

EAE was induced in C57BL/6 mice with myelin oligodendrocyte gp35–55 (MOG35–55) peptide by immunizing once with the peptide in CFA on day 0 and boosting once with peptide in IFA on day 7. Two treatments with pertussis toxin, 1 day after each immunization (days 1 and 8), were performed. Control or blocking Ab for B7-H3 (100 μg) was injected on days 1, 4, and 7. Mice were observed daily and were scored on a scale of 0–5 with gradations of 0.5 for intermediate scores: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, hind- and forelimb paralysis; and 5, death. Preparation and stimulation of mononuclear cells from brain tissues were performed as previously described (13). Statistic analysis was performed by t test using PRISM version 2.01 (GraphPad, San Diego, CA).

Results

Expression of B7-H3 molecule by immune cells

B7-H3-Ig fusion protein was used to immunize a female Lewis rat to generate anti-B7-H3 Abs. Anti-B7-H3 mAb stained 293 cells transiently transfected with mouse B7-H3, but not B7S1 (Fig. 1A). B7S1 expression was confirmed with an anti-B7S1 mAb (Fig. 1B). Anti-B7-H3 stained splenic B cells from wild-type as well as B7.1/ B7.2- or B7h-deficient mice (Fig. 1C). This staining could not be inhibited by PD-L2-Ig (data not shown). These results indicate that we have generated a B7-H3-specific Ab.

All known members of the B7 family are expressed by professional APC. In addition, the new members of this family, i.e., B7h, PD1L1, PD1L2, and B7S1, are broadly distributed in nonlymphoid tissues and cells. We previously reported the expression of B7-H3 mRNA by Northern blot analysis in lymphoid tissues thymus and spleen, and also in a number of nonlymphoid organs (26). To understand the immune regulation by B7-H3, we used anti-B7-H3 Ab for FACS analysis (Fig. 2). In spleen, B7-H3 is expressed on a minor fraction of CD4+ and CD8+ cells (8.7 and 11%, respectively; Fig. 2B). These B7-H3+ T cells appear to coexpress CD44, but not CD25, marker (Fig. 2B). In contrast, nearly all B220+ splenic B cells (Fig. 2A) and splenic CD11c+ dendritic cells (Fig. 2C) constitutively express B7-H3. Bone marrow–derived dendritic cells and peritoneal macrophages all express B7-H3 (Fig. 2, C and D). Therefore, mouse B7-H3 is mostly expressed by all professional APCs and a subpopulation of T cell population that we have examined.

Members of the B7 family are differentially regulated in professional APC by various stimuli. For instance, CD80 and CD86 expression can be induced by innate activation (3). B7h is downregulated on B cells by IgM engagement and up-regulated on fibroblasts by TNF-α (11, 31). Human B7-H3 was shown to be an inducible molecule on the surface of DCs, monocytes, and T cells (25). Therefore, we examined the regulation of B7-H3 expression on dendritic cells by an innate activator. After LPS stimulation for 24 h, bone marrow–derived DCs exhibited up-regulation of B7-H3 expression (Fig. 2C). However, B7-H3 expression on purified B cells and macrophages was not altered after the same treatment (Fig. 2, A and D).
Inhibition of T cell activation and function by B7-H3-Ig

The expression of B7-H3 on professional APC suggests a role for B7-H3 in the regulation of T cell immune responses. We previously showed using B7-H3-Ig fusion protein that a putative receptor for B7-H3 was induced on activated T cells (26). Naive CD4$^+$ and CD8$^+$ T cells from C57BL/6 lymph nodes were not strongly bound by the biotinylated B7-H3-Ig; after Con A activation for 48 h, B7-H3 receptor was up-regulated on activated T cells. B7-H3-Ig can bind to CD28$^+$ and ICOS$^+$ T cells activated in the same fashion (data not shown).

As a first step to assess the function of B7-H3 on T cell activation and function, we stimulated purified CD4$^+$ cells from C57BL/6 mice with different doses of anti-CD3 in the absence or the presence of B7.1-Ig or B7-H3-Ig and measured cell proliferation (Fig. 3A). B7.1-Ig, as expected, strongly enhanced T cell stimulation, whereas B7-H3-Ig inhibited T cell proliferation. An irrelevant protein containing the human IgG1 tag in the C terminus, expressed and purified in the same fashion, did not alter the proliferation of anti-CD3-stimulated T cells (21), indicating that this inhibitory effect by B7-H3-Ig was not due to our method of protein preparation.

The hallmark of T cell activation is the production of IL-2, which drives T cell clonal expansion. We thus examined whether IL-2 production is affected by B7-H3-Ig costimulation. Although B7.1-Ig strongly enhanced IL-2 production, B7-H3-Ig inhibited, after 24 h of treatment (Fig. 3B). To assess whether inhibition of T cell proliferation by B7-H3-Ig was the result of IL-2 reduction, we added exogenous IL-2 to the OT-II T cells treated with anti-CD3 with or without B7-H3-Ig. Addition of IL-2 fully restored the proliferation of T cells costimulated with...
B7-H3-Ig (Fig. 3C). Therefore, murine B7-H3 appears to inhibit T cell activation via reducing IL-2 production, whereas human B7-H3 costimulation was reported to increase T cell proliferation and IFN-γ production (25).

Similar to other newly identified B7 molecules, B7h, PDL1, PDL2, and B7S1, B7-H3 is expressed in lymphoid and nonlymphoid tissues (23, 24). This expression pattern suggests its role in regulating both naive T cell activation in lymphoid tissues and effector T cell function in the periphery. To assess the role of B7-H3 in effector T cell regulation, we tested the effect of B7-H3-Ig treatment on in vitro activated OT-II cells by both ELISA and intracellular staining (data not shown). We found during the secondary stimulation with plate-bound anti-CD3 that B7-H3-Ig treatment reduced the expression of both Th1 and Th2 effector cytokines by OT-II effector cells (Fig. 3D). Therefore, in accordance with its expression pattern, this result further suggests B7-H3 as a negative regulator of effector CD4 T cells.

NFAT, NF-xB, and AP-1 factors are the major transcriptional regulators of T cell activation and function (25, 26). We therefore assessed whether B7-H3 could inhibit any of these signaling pathways. DO11.10 T cell hybridoma cells, which express B7-H3 receptor after activation, are capable of producing IL-2 upon anti-CD3 stimulation, and this IL-2 expression can be inhibited when a negative costimulator receptor was simultaneously engaged (24). We transfected DO11.10 with NFAT, AP-1, or NF-xB luciferase reporter constructs. Treatment with B7-H3-Ig modestly reduced the activity of NF-xB and NFAT (Fig. 4). AP-1 activation in DO11.10 cells required CD28 signal, and B7-H3-Ig strongly inhibited AP-1 activation by anti-CD3 and CD28 (Fig. 4). Reduction of NFAT, NF-xB, and AP-1 transcriptional activities correlated with a reduction in IL-2 production and activation-induced cell death (data not shown). B7-H3-Ig on its own did not induce cell death (data not shown). The global blockade of T cell activation is consistent with the finding that all negative costimulatory receptors in the CD28 superfamily, CTLA-4, PD-1, and B and T lymphocyte attenuators, can recruit SHP-1 and SHP-2 to the membrane and inhibit tyrosine phosphorylation of early TCR signaling components (24, 25, 26). This work substantiates the above data using B7-H3-Ig and indicates that B7-H3 is a physiological negative regulator of T cell activation and IL-2 expression.

To assess the physiological function of B7-H3 in T cell regulation, we examined whether the anti-B7-H3 Ab we generated can block binding of B7-H3 to its receptor. Biotinylated B7-H3-Ig was incubated with a rat control IgG (no blocking) or anti-B7-H3 (blocking) before staining of Con A-activated mouse lymph node cells. The anti-B7-H3 Ab greatly inhibited the binding of B7-H3-Ig to its receptor on T cells, indicating that it is a blocking Ab for B7-H3 (Fig. 5A).

We used the anti-B7-H3 blocking Ab in our in vitro and in vivo analyses. We first examined the function of this blocking Ab in vitro by activating splenocytes from C57BL/6 mice with different doses of anti-CD3. In this experiment, positive and negative costimulation is provided by different splenic APC. Although a control rat IgG did not alter T cell proliferation, treatment with the anti-B7-H3 blocking Ab greatly enhanced it (Fig. 5B). We also measured IL-2 production within the first 24 h of treatment and found that B7-H3 blocking Ab also greatly increased the levels of IL-2 production by T cells (Fig. 5C). This work substantiates the above data using B7-H3-Ig and indicates that B7-H3 is a physiological negative regulator of T cell activation and IL-2 expression.

To examine whether negative regulation of T cells by B7-H3 has any important immune function in vivo, we immunized C57BL/6 mice with MOG35-55 peptide to induce EAE. Control rat Ig or anti-B7-H3 blocking Ab was injected into mice during the T cell priming phase, i.e., between the first and second immunizations. Mice treated with anti-B7-H3 Ab consistently developed accelerated disease, with an earlier onset and more robust EAE than those treated with a control rat Ab (Fig. 6A). Four of five mice in the B7-H3 Ab-treated group developed EAE, with maximal disease score of 3, whereas in the control group, only one mouse had a disease score of 1.5. We examined infiltrating mononuclear cells in the brains of mice that developed disease from each group on day 16 and found that B7-H3 Ab treatment in mice resulted in a greatly increased number of CD4+ cells (Fig. 6B). This experiment supports the idea that B7-H3 is a negative regulator of T cell activation and function.
Discussion

The development of a productive T cell response depends upon appropriate costimulatory signals provided by APC in addition to MHC presentation of antigenic peptides to T cells. Costimulatory molecules, after binding to their specific receptors on T cells, regulate intracellular signaling pathways that may lower or increase the threshold of T cell activation. Recent studies have revealed an increased number of B7 family members with exquisite costimulatory functions (34). In this study we characterized the expression and function of the murine B7-H3 molecule. We show that mouse B7-H3 functions as a negative regulator for T cell activation and function.

Human B7-H3 was first reported as an inducible molecule on dendritic cells and monocytes by inflammatory cytokines and a combination of PMA and ionomycin (25). Contrary to human B7-H3, whose expression is not found on resting APC, murine B7-H3 is widely expressed on almost all resting and activated B cells, macrophages, and dendritic cells (Fig. 2). B7-H3 is also expressed by a minor subset of CD4+ and CD8+ T cells (Fig. 2B); the functional significance of this T cell expression remains to be determined. LPS activation of purified B cells and the RAW macrophage cell line did not affect B7-H3 expression. However, the expression of B7-H3 on dendritic cells seems to be further up-regulated upon LPS activation (Fig. 2C). LPS regulation of B7-H3 expression has also recently been described by Suh et al. (35), although the physiological significance of this regulation is unclear at this stage. It is noteworthy that other B7 family members are up-regulated differently in B cells and dendritic cells. CD80 and CD86 are well known to be up-regulated in APC by a variety of innate stimuli. Activation of dendritic cells by TLR-4 (through LPS) regulates the expression of B7.2, a costimulatory molecule that is important for T cell activation (36). In contrast, B7h, the ligand for ICOS, is down-regulated in B cells after IgM cross-linking (31). All these findings suggest a combinatorial model for costimulation of T cells: each B7 ligand is regulated differentially, which reflects the natural history of APC, and the combination of these ligands regulates the threshold of T cell activation. It is significant to note that the new B7 family members, B7h, PDL1/2, B7S1, and B7-H3, are also widely distributed in nonlymphoid tissues, whereas their receptors are expressed on activated T cells. It is possible that they may possess important functions in modulating effector T cell function once activated T cells migrate into the nonlymphoid tissues. At this effector stage, the combinatorial signals presented by B7 ligands, which are tissue specific and regulated by inflammatory cytokines, may influence the nature and extent of T cell function.

Human B7-H3 was previously reported to increase T cell proliferation and IFN-γ production (25). Recently, other studies also demonstrated an inhibitory function for mouse and human B7-H3 (35, 37), whereas different groups observed an enhancing effect (25, 38). In this study we tested the function of mouse B7-H3 on T cells, first using the B7-H3-Ig fusion protein and then a B7-H3 blocking Ab. B7-H3-Ig reduced CD4+ T cell proliferation (Fig. 3A). We further showed that B7-H3 regulation of T cell proliferation occurs through an IL-2-dependent mechanism. B7-H3-Ig reduced IL-2 production in vitro (Fig. 5A). B7-H3-Ig inhibited NFAT, AP-1, and NF-κB activities. DO11.10 T cell hybridoma was transfected with NFAT, AP-1, or NF-κB luciferase reporter constructs. Cells were activated for 4 h with the indicated treatments and were analyzed for luciferase activity. The data shown are representative of at least three independent experiments with similar results.

FIGURE 4. B7-H3-Ig inhibited NFAT, AP-1, and NF-κB activities. DO11.10 T cell hybridoma was transfected with NFAT, AP-1, or NF-κB luciferase reporter constructs. Cells were activated for 4 h with the indicated treatments and were analyzed for luciferase activity. The data shown are representative of at least three independent experiments with similar results.

FIGURE 5. Anti-B7-H3 blocking Ab enhanced T cell proliferation and IL-2 production in vitro. A, Biotinylated B7-H3-Ig was incubated with a rat control IgG or anti-B7-H3 before staining with Con A-activated mouse lymph node cells. The filled histogram represents control staining with biotinylated human IgG. B and C, Spleen cells from C57BL/6 mice were incubated with the indicated doses of anti-CD3 at the presence of 5 μg/ml control rat IgG or anti-B7-H3 Ab. Cell proliferation (B) was measured by [3H]thymidine uptake after 72 h, and IL-2 (C) was assayed by ELISA 24 h after treatment. The data shown are representative of at least two independent experiments with similar results.
production by activated T cells, and addition of exogenous IL-2 restored the proliferation of T cells by B7-H3-Ig-treated cells (Fig. 3A). B7-H3-Ig inhibited effector cytokine expression by in vitro differentiated OT-II cells (Fig. 3D), suggesting a role in the regulation of T cell effector function. In support of negative regulation of T cells by B7-H3, we observed diminished activity of NFAT, NF-κB, and AP-1 transcriptional factors (Fig. 4). We then examined the physiological significance of B7-H3 costimulation using a blocking Ab. Treatment of anti-CD3 activated splenocytes with this Ab greatly enhanced IL-2 production by activated T cells, and addition of exogenous IL-2 expression by activated T cells and hence the inhibition of T cell proliferation and interleukin 2 mRNA accumulation. J. Exp. Med. 173:721.


