IMMUNOLOGY. For the article “Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses,” by Masaaki Hashiguchi, Hiroko Kobori, Patcharee Ritprajak, Yosuke Kamimura, Haruo Kozono, and Miyuki Azuma, which appeared in issue 30, July 29, 2008, of Proc Natl Acad Sci USA (105:10495–10500; first published July 23, 2008; 10.1073/pnas.0802423105), the authors note that, due to a printer’s error, in Fig. 3C, the two lower graphs were distorted. The corrected figure and its legend appear below.

Fig. 3. B7-H3 costimulates CD8+ T cell responses. (A) WT P815 and B7-H3/P815 transfectants were stained with isotype control Ab or FITC-anti-B7-H3 (MIH32) mAb. Cell surface B7-H3 on WT P815 and B7-H3/P815 cells is presented as histograms with the control staining (shaded). (B) CD8+ or CD4+ T cells were cocultured with the indicated ratio of WT P815 or B7-H3/P815 in the presence of anti-CD3 mAb (0.4 μg/ml for CD8+ T cells and 0.1 μg/ml for CD4+ T cells) for 3 days. Proliferative responses in the final 18 h were assessed by [3H]thymidine incorporation. The IFN-γ levels in the culture supernatants at 48 h were measured by ELISA. The cpm counts and IFN-γ production for CD8+ or CD4+ T cells stimulated with anti-CD3 mAb alone and CD8+ or CD4+ T cells cocultured with P815 cells in the absence of anti-CD3 mAb were ~2,500 cpm and ~0.015 ng/ml, respectively. Values shown are the mean ± SD. The data are representative of three independent experiments. (C Left) TLT-2 expression on OT-I CD8+ cells. OT-I CD8+ cells were stained for TLT-2, as described in Fig. 2C. (Right) B7-H3 and B7–1 expression on E.G7 and E.G7 transfectants. E.G7, B7-H3/E.G7, and B7–1/E.G7 were stained with FITC-anti-B7-H3 mAb or PE-anti-B7–1 mAb or the appropriate fluorochrome-conjugated control Ig. The data are presented as histograms, with the respective control staining (shaded). (D) Enhanced IFN-γ production (Upper) and cytotoxicity (Lower) of OT-I CD8+ T cells costimulated with B7-H3-transfectants. OT-I CD8+ cells were stimulated with the indicated ratio of control E.G7, B7-H3/E.G7, or B7–1/E.G7 cells for 48 h. The IFN-γ levels in the supernatants were measured by ELISA. OT-I CD8+ T cells were cocultured with E.G7 or B7-H3/E.G7 for 3 days, and cytotoxicity against E.G7 was measured by the JAM test. Values shown are the mean specific lysis ± SD. The data are representative of three independent experiments. *, statistically different from the WT control (P < 0.05).
Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses

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The B7 family member B7-H3 (CD276) plays important roles in immune responses. However, the function of B7-H3 remains controversial. We found that murine B7-H3 specifically bound to Triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2, TREML2). TLT-2 was expressed on CD8+ T cells constitutively and on activated CD4+ T cells. Stimulation with B7-H3 transfectants preferentially up-regulated the proliferation and IFN-γ production of CD8+ T cells. Transduction of TLT-2 into T cells resulted in enhanced IL-2 and IFN-γ production via interactions with B7-H3. Blockade of the B7-H3:TLT-2 pathway with a mAb against B7-H3 or TLT-2 efficiently inhibited contact hypersensitivity responses. Our results demonstrate a direct interaction between B7-H3 and TLT-2 that preferentially enhances CD8+ T cell activation.

The B7 family of costimulatory proteins controls both positively and negatively T cell-mediated immune responses by binding to counterreceptors expressed on the T cells (1–3). B7-H3 (CD276) is a new member of the B7 family; it was originally identified as a costimulatory molecule that induces T cell proliferation and IFN-γ production in humans (4). B7-H3 is not expressed in significant amounts on freshly isolated lymphocytes and is weakly induced on dendritic cells (DCs) and monocytes/macrophages upon activation (4–7). In addition to its expression on activated lymphocytes (7), B7-H3 is detected on various human and murine tumor cells (8–12), nasal and airway epithelial cells (13–15), and osteoblasts (16).

The immunologic function of B7-H3 is controversial, with conflicting costimulatory and coinhibitory functions being reported. Several groups have demonstrated that soluble human B7-H3 costimulates anti-CD3-mAb-induced T cell proliferation and the production of effector cytokines such as IFN-γ and IL-10 (4), and that cell surface B7-H3 introduced on tumor cells enhances CTL generation in vitro (4) and antitumor immunity in vivo (17–20). The acute and chronic cardiac allograft rejection seen in B7-H3-deficient mice can be reduced by a subtherapeutic regimen of immunosuppressants (21). These results support the notion that B7-H3 promotes T cell-mediated immune responses. In contrast, other groups have proposed the opposite functions for B7-H3. Soluble mouse and human B7-H3 inhibit anti-CD3 mAb-induced T cell proliferation, cytokine production, and activation of transcriptional factors such as NFAT, NF-κB, and AP-1 (6, 7, 22). In B7-H3-deficient mice Th1-mediated hyperactivity and onset of experimental autoimmune encephalomyelitis (EAE) are promoted, and treatment with a blocking anti-B7-H3 mAb exacerbates EAE (6, 7). Administration of anti-B7-H3 mAb during the induction phase augments the severity of Th2-mediated experimental allergic conjunctivitis (23), DC-associated B7-H3 induced by CD4+CD25+ regulatory T cells (Tregs) impairs T cell stimulatory function (24). These results suggest that B7-H3 is a negative regulator that preferentially affects CD4 T cell responses.

Although the counter receptor for B7-H3 has not been identified, soluble B7-H3 protein binds a putative counter receptor on activated T cells that is distinct from CD28, CTLA-4, ICOS, and PD-1 (4, 25). In the present study, we demonstrate that Triggering receptor expressed on myeloid cells (TREM)-like Transcript 2 (TLT-2, TREML2) is a receptor for B7-H3. We describe the generation of functional blocking mAbs against B7-H3 and TLT-2, as well as the expression and function of the B7-H3:TLT-2 pathway in vitro and in vivo.

Results

B7-H3 Binds TLT-2. To identify the counterreceptor for B7-H3, we performed a BLASTp search on the National Center for Biotechnology Information (NCBI) database for proteins homologous to CD28 family members. Several candidate proteins were extracted based on the EST expression patterns. The cDNAs for candidate proteins, including TLT-2, TLT-4, TLT-6, CD300A, and CD300D as well as CD82 family members, were transduced into J558L cells that lacked B7-H3 by using the IRES-eGFP bicistronic retroviral vector. B7-H3 Ig bound specifically to TLT-2-transfected J558L cells that expressed higher levels of eGFP (Fig. 1A). Next, we assessed by flow cytometry the relative affinity of serially diluted B7-H3 Ig chimeric protein to the cell surface by using a TLT-2-transduced DO11.10 hybridoma. The mean fluorescence intensity (MFI) was assessed, and the dissociation constant (Kd) was determined by Scatchard plot analysis. The Kd value for B7-H3 Ig binding to cell surface TLT-2 was 90 ± 44 nM (Fig. 1B), which was comparable to Kd values for human B7-DC binding to cell surface human PD-1 (89 nM) (26) and mouse B7-DC binding to cell surface mouse PD-1 (143 nM) (unpublished data), as assessed in flow cytometric binding studies. To investigate the functional roles of B7-H3 and TLT-2, we established mAbs against B7-H3 (MIH32 and MIH35, both 

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2/eGFP-transduced J558L cells (Fig. S1B), TLT-2 on T cell surfaces was stained more sensitively by MIH47 (Fig. S1C). B7-H3Ig binding to TLT-2 was inhibited by preincubation with MIH49 (Fig. 1C), but not with MIH47 (data not shown). Therefore, we used MIH47 for T cell staining and MIH49 for the functional blocking analyses. The inhibition of binding between B7-H3 and TLT-2 by anti-B7-H3 mAb or anti-TLT-2 mAb suggests direct and specific binding of B7-H3 to TLT-2.

**TLT-2 Is Expressed Constitutively on CD8⁺ T Cells and Induced on CD4⁺ T Cells After Activation.** Flow cytometry analysis revealed that TLT-2 was expressed on freshly isolated CD8⁺ T cells but not on CD4⁺ T cells (Fig. 2A). However, stimulation with anti-CD3 mAb (Fig. 2A) or Concanavalin A (data not shown) clearly induced TLT-2 on CD4⁺ T cells. The TLT-2 expressed on CD8⁺ T cells was not changed to any great extent after activation. These expression patterns were confirmed by RT-PCR (Fig. 2A). The CD4⁺/CD25⁺ Treg cells expressed a low level of TLT-2 mRNA. In splenocytes, the B220⁺ B cells and CD11b⁺ macrophages expressed TLT-2 (Fig. 2B). Low-level TLT-2 expression was detected in CD49b⁺ NK cells and CD11c⁺ DCs. Peritoneal B-1 (CD11b⁺Ly6C⁻Ly6G⁺), B-2 (CD11b⁺Ly6C⁺Ly6G⁻), and CD11b⁺ macrophages expressed substantial levels of TLT-2. Immature and mature BM-DCs expressed low and similar levels of TLT-2. Although the above results were obtained by staining with MIH47, staining by using MIH49 showed higher levels of TLT-2 expression on B cells (Fig. S1C) and myeloid cells (data not shown), which was consistent with results presented in a previous report (27). Differential reactivities of these mAbs may be because of differences in epitopes on TLT-2 and glycosylation in respective cell types. TLT-2 expression on various cell lines was examined by RT-PCR (data not shown) and flow cytometry (Fig. S2). High-level cell surface expression of TLT-2 was observed in MBL-2 (a T cell line), A20.2J and BAL17 (B cell lines), WEHI231.7, J774A.1, and WEHI13B cells (monocytic/macrophage cell lines), and weak expression was detected in DO11.10 (a T cell hybridoma), RAW264.7 (a macrophage cell line), and DC2.4 cells (a DC-like cell line). Consistent with the results obtained for freshly isolated cells, cell surface expression of TLT-2 was seen for most of the B and myeloid cell lines.

**B7-H3 Augments CD8⁺ T Cell Responses.** To investigate the functional roles of B7-H3 in T cell activation, we generated several B7-H3 transfectants that expressed high levels of B7-H3 and performed T cell costimulation assays. Wild type (WT) P815 cells spontaneously expressed a low level of B7-H3, whereas B7-H3-transduced P815 (B7-H3/P815) cells expressed an ~50-fold higher level of B7-H3 (Fig. 3A). CD4⁺ and CD8⁺ T cells were cocultured with either FcγR-bearing WT P815 or B7-H3/P815 in the presence of anti-CD3 mAb. Although CD8⁺ T cells, the anti-CD3-induced proliferation and IFN-γ production induced by B7-H3/P815 were efficiently enhanced, as compared with the responses induced by WT P815 (Fig. 3B). However, CD4⁺ T cells did not show clear differences in these responses induced by B7-H3/P815 or WT P815 stimulation. Similar results were obtained in a cell division assay involving CFSE-labeled T cells. B7-H3-transduced P815 (B7-H3/P815) cells expressed an ~50-fold higher level of B7-H3 (Fig. 3A). CD4⁺ and CD8⁺ T cells were cocultured with either FcγR-bearing WT P815 or B7-H3/P815 in the presence of anti-CD3 mAb. Although CD8⁺ T cells, the anti-CD3-induced proliferation and IFN-γ production induced by B7-H3/P815 were efficiently enhanced, as compared with the responses induced by WT P815 (Fig. 3B). However, CD4⁺ T cells did not show clear differences in these responses induced by B7-H3/P815 or WT P815 stimulation. Similar results were obtained in a cell division assay involving CFSE-labeled T cells.
CD4+ and CD8+ T cells (data not shown). To confirm the effects of B7-H3-mediated costimulation on CD8+ T cells, we examined the antigen-specific responses by using OT-I TCR transgenic CD8+ T cells. OT-I CD8+ T cells expressed a substantial level of TLT-2, comparable to freshly isolated CD8+ T cells from BALB/c mice (Fig. 3C). OVA-expressing E.G7 cells were transduced with B7-H3 or B7−1 (Fig. 3C). OT-I CD8+ T cells cocultured with B7-H3-transduced E.G7 (B7-H3/E.G7) showed dramatically increased IFN-γ levels in the culture supernatants, as compared with coculturing with the control E.G7. The enhanced level of IFN-γ production was slightly lower than that achieved by stimulation with B7−1/E.G7 (Fig. 3D). Furthermore, cytotoxicity of OT-I CD8+ T cells was clearly enhanced by B7-H3/E.G7 cells preactivated for 3 days, as compared with the control E.G7 (Fig. 3D). These results suggest that B7-H3 is a potent costimulator for CD8+ T cells.

**Interaction of B7-H3 with TLT-2 Enhances T Cell Activation.** We next investigated whether the interaction of B7-H3 with TLT-2 induces T cell activation. The transduction of TLT-2 resulted in prominent expression of TLT-2 on the DO11.10 cells (Fig. 4A). When TLT-2-transduced DO11.10 (TLT-2/DO11.10) cells were stimulated with B7-H3/P815 plus anti-CD3 mAb, IL-2 production was markedly enhanced, compared with the control vector-transduced DO11.10 (GFP/DO11.10) (Fig. 4A). These results suggest that the interactions with TLT-2 and B7-H3 between DO11.10 and P815 cells augment IL-2 production. Next, to examine the involvement of TLT-2-mediated costimulation in splenic T cells, we transduced TLT-2 into preactivated CD8+ and CD4+ T cells by using a retroviral transduction system. Transduction of TLT-2 resulted in higher levels of TLT-2 on both CD4+ and CD8+ T cells, as compared with GFP-transduced control cells (Fig. 4B). TLT-2-transduced CD8+ T cells produced remarkably high levels of IFN-γ as compared with the control CD8+ T cells, regardless of stimulation with either WT P815 or B7-H3/P815 (Fig. 4B). Similar results were obtained for TLT-2-transduced CD4+ T cells, although the levels of IFN-γ production were clearly lower than those seen for CD8+ T cells. These results indicate that TLT-2 expressed on both CD8+ and CD4+ T cells positively costimulates T cells via the binding of B7-H3.
Fig. 4. Interaction of B7-H3 with TLT-2 enhances T cell activation. (A) Enhanced IL-2 production as a result of TLT-2:B7-H3 binding. (Left) TLT-2 expression on DO11.10 cells. GFP/DO11.10 and TLT-2/DO11.10 cells were stained as described in Fig. 2C. (Right) IL-2 production by TLT-2/DO11.10. The DO11.10 cells were cocultured with the indicated ratio of WT P815 or B7-H3/P815 cells in the presence of 1 μg/ml anti-CD3 mAb for 24 h. The IL-2 levels in the supernatants were measured by ELISA. The IL-2 levels in the culture without stimulator cells were <0.015 ng/ml. (B) Enhanced IFN-γ production by CD4+ and CD8+ T cells as a result of TLT-2:B7-H3 binding. (Left) Activated CD4+ and CD8+ T cells were retrovirotransduced with pMux-eGFP (GFP) or TLT-2:eGFP (LTL-2) and stained for TLT-2 expression. GFP+ cells were sorted by flow cytometry and used as responder cells (indicated by squared section). (Right) Control GFP+ or TLT-2+ GFP+ cells were stimulated with WT P815 or B7-H3/P815 at an R/S ratio of either 2 or 72 h and the IFN-γ production was determined by ELISA. The IFN-γ levels from CD8+ or CD4+ T cells stimulated with anti-CD3 mAb alone and CD8+ or CD4+ T cells cocultured with P815 cells in the absence of anti-CD3 mAb were <0.015 ng/ml. Values shown are mean ± SD. All data are representative of three independent experiments.

Materials and Methods

Treatment of Either Anti-B7-H3 or Anti-TLT-2 mAb Attenuates Contact Hypersensitivity (CH) Responses. To investigate the functions of B7-H3 and TLT-2 in vivo, we examined the effects on CH responses to dinitrofluorobenzene (DNFB) by treating with the anti-B7-H3 or anti-TLT-2 mAb. Administration of the anti-B7-H3 mAb at sensitization significantly decreased ear swelling, and this inhibitory effect was found to be long-lasting when rechallenge was performed at 28 days after the primary sensitization. The differences in ear thickness before and after challenge are displayed as ear swelling values (mean ± SD for each group of five mice). The data shown are representative of three independent experiments. (B) Anti-B7-H3 mAb treatment decreases CD8+ T cell expansion and hapten-specific IFN-γ production. Regional LN s from mice treated with control Ig or anti-B7-H3 mAb at sensitization were analyzed 3 days after the final sensitization. Cells were analyzed by flow cytometry. The percentages of CD3+, CD4+ CD3+ 5, CD8+ CD3+ cells are presented as CD3+ T, CD4+ T, and CD8+ T cells, respectively. CD3+ T cells from control Ig- or anti-B7-H3 mAb-treated mice were stimulated with DNBS-pulsed splenocytes for 48 h, and IFN-γ production was measured. Values shown are the mean ± SD for each group of three mice. The data are representative of two independent experiments. (C) Anti-TLT-2 mAb treatment at sensitization or challenge inhibits ear swelling. CH was induced and treated with anti-TLT-2 (MIH49) mAb and rechallenge were performed as described in A. Values shown are the mean ± SD for each group of five mice. Data are representative of two independent experiments. Groups showing statistically significant differences from the control Ig-treated group are marked (*, *P < 0.05).

Discussion

In the present study, we demonstrate a direct and specific interaction between the B7 family member B7-H3 and the TREM family member TLT-2. TLT-2 is expressed constitutively on CD8+ T cells and induced on CD4+ T cells after activation. The binding of B7-H3 to TLT-2 on T cells enhances T cell effector functions, such as proliferation, cytokine production, and cytotoxicity. The augmentation of T cell activation via the TLT-2:B7-H3 pathway was particularly prominent in CD8+ T cells. Blockade of the TLT-2:B7-H3 pathway by using mAbs against B7-H3 or TLT-2 efficiently inhibited both the induction and effector phases of the CH responses. Our results indicate that TLT-2 is a counterreceptor for B7-H3, and that the TLT-2:B7-H3 pathway costimulates T cell activation.

Continuing the observation that the CD28 family member BTLA interacts with the TNF receptor family molecule TNFRSF14 (herpesvirus entry mediator, HVEM) (28), we now show that a CD28-B7 family member binds to another family
molecule. The TREM cluster includes the genes that encode TREM-1, TREM-2, and murine TREM-3, as well as the ‘TREM-like’ genes that encode TLT-1, TLT-2, TLT-4, the human TLT-3 and murine TLT-6 (29). All TREM family proteins are type I transmembrane glycoproteins that consist of a single extracellular Ig-like domain of the V-type, a transmembrane domain, and a short cytoplasmic domain (30, 31). TREM-1 is expressed by neutrophils and macrophages and amplifies inflammatory responses to pathogens. In contrast, TREM-2 mainly controls the differentiation and development of other myeloid cells, including DCs, osteoclasts, and microglia (30). Although research on TREM proteins has focused on myeloid lineage cells, in the present study, we highlight the expression and function of TREM family members in T cells. Consistent with a previous report (27), TLT-2 was expressed predominantly on B cells and macrophages, although we observed significant expression on freshly isolated unstimulated CD8+ T cells and activated CD4+ and CD8+ T cells after short-term stimulation. The augmentation of proliferation and cytokine production costimulated with B7-H3-transfectants were consistently observed in both types of T cells that expressed endogenous TLT-2 and exogenously introduced high levels of TLT-2 (Figs. 3 and 4). However, the augmentation of proliferation and cytokine production costimulated with B7-H3-transfectants was more evident for CD8+ T cells than for CD4+ T cells. The efficiency of the B7-H3 pathway to the CD8+ T cell responses has been confirmed by previous reports describing the successful induction of CTL and antitumor immunity by B7-H3-introduced tumors (17–20). The study using B7-H3-deficient mice also demonstrated a costimulatory function of B7-H3 in both CD4+ and CD8+ T cells in acute and chronic allograft rejection (21). However, as described in the introduction, the coinductive function of B7-H3 has also been reported (6, 7, 23, 24). How can we explain the previous reports of an opposite function of B7-H3 in immune responses? There may be several possibilities. The first possibility is the existence of second receptor other than TLT-2. The previous report (6) demonstrated the involvement of negative function of B7-H3 in Th1 responses and preferential induction of B7-H3 by a Th1 cytokine, IFN-γ. An undefined negative receptor might be dominantly induced on T1 type of CD4+ T cells and regulated Th1 responses. Unknown receptor:B7-H3-mediated coinductive pathway may contribute to the negative feedback for TLT-2:B7-H3-mediated activation of Th1 and CTL responses. A second possibility is an involvement of regulatory role of IFN-γ in Th2 responses. B7-H3-mediated costimulation in TLT-2-expressing CD8+ T cells initially enhances IFN-γ production and this may result in the regulation of Th2-mediated immune responses. This may account for the development of severe airway inflammation observed in B7-H3-deficient mice (6). A third possibility is that TLT-2 expressed on myeloid cells plays roles in innate immunity and inflammatory responses. In the present study, we focused on the functions of TLT-2 expressed on T cells, whereas in reality TLT-2 is broadly expressed on myeloid cells and its ligand, B7-H3, is also abundantly expressed on various cell types including immune cells and tissue cells. TLT-2 expressed on myeloid cells may play different roles in immune responses. Although we observed similar effects on CH responses by treatment with either anti-B7-H3 or TLT-2 mAb, we cannot exclude the possibility that B7-H3 expressed on myeloid cells may play different roles in immune responses. Although we observed similar effects on CH responses by treatment with either anti-B7-H3 or TLT-2 mAb, we cannot exclude the possibility that B7-H3 expressed on myeloid cells may play different roles in immune responses.

In summary, we show that TLT-2 is a counterreceptor for B7-H3, and that the interaction of B7-H3 with TLT-2 on T cells enhances T cell activation. Among the B7 family of T cell costimulatory pathways, the B7-H3:TLT-2 pathway appears to have a unique role in CD8+ T cell activation. Although TLT-2 has immune functions other than as a T cell costimulatory molecule, intervention with B7-H3:TLT-2 may represent a target for the regulation of immune responses.

Materials and Methods

Mice. Female 6-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). OVA-specific TCR transgenic OT-I mice (32) were kindly provided by W. R. Heath (The Walter and Eliza Hall Institute, Melbourne) through H. Udono (RIKEN, Yokohama City, Japan). Mice were maintained under specific pathogen-free conditions and used at 6–10 weeks of age. All mouse procedures were reviewed and approved by the Animal Care and Use Committee of Tokyo Medical and Dental University.

Homology Search and TLT-2 Plasmids. BLASTp search of the NCBI database for amino acid homologies to CD28 family molecules identified several candidates based on the EST expression profiles. The cDNAs for these candidate molecules were amplified from BALB/c splenocytes by using RT-PCR, ligated into pcCR2.1-TOPO vector (Invitrogen), and sequenced. cDNAs were inserted into pMXs-IG and pMXs-neo to generate TLT-2:pmXs-IG and TLT-2:pmXs-neo, respectively. TLT-2 was retrovirally transduced as described in SI Materials and Methods.

Anti-CD3 mAb-Induced Costimulation Assay. DO11.10 cells (1 × 10^5 per well), freshly isolated T cells (2 × 10^5 per well) or retrovirally transduced GFP+ T cells (1 × 10^5 per well) were cocultured with mitomycin C-treated WT P815 or B7-H3/P815 cells at the indicated R/S ratios in the presence of anti-CD3 mAb (145-2C11) in 96-well flat-bottom plates for 1 day (for DO11.10 cells) or 2–3 days (for T cells). For the measurement of proliferative responses, the cultures were pulsed with [^3]H]thymidine for the final 18 h of culture and the incorporation of radioactivity was measured by using a microplate beta counter, as described previously (33). Culture supernatants were collected at the indicated time-points, and the IL-2 and IFN-γ levels were measured by ELISA, as described previously (33).

IFN-γ Production and CTL Assay for OT-I Cells. OT-I CD8+ T cells (1 × 10^5 per well) were stimulated with a graded ratio of mitomycin C-treated E.G7 (control), B7-H3/E.G7 or B7-1/E.G7 cells, the culture supernatants were collected after 48 h, and IFN-γ production was measured by ELISA. The cytotoxicity of OT-I CD8+ T cells for E.G7 cells was measured by the 6-h JAM test described previously (34). Briefly, OT-I CD8+ T cells were cocultured with mitomycin C-treated E.G7 or B7-H3/E.G7 cells for 3 days and the harvested CD8+ T cells were used as effector cells. The E.G7 cells were labeled with [^3]H]thymidine and then used as target cells (5 × 10^3 per well). The incorporated radioactivity was measured.

Contact Hypersensitivity (CH) Reaction. CH to DNFB was induced as described previously (35). Briefly, 20 μl of 0.5% DNFB (Sigma) dissolved in acetone:olive oil (4:1) was painted onto shaved abdominal skin on days 0 and 1, followed on day 5 by the application of 0.2% DNFB to both sides of the ear. Ear thickness was measured before challenge and 24, 48, and 72 h after challenge. For the antibody treatments, mice received i.p. injections of 200 μg per mouse of control rat IgG (MP Biomedical), rat IgM (MP Biomedical), anti-B7-H3 (MHI35) mAb or anti-TLT-2 (MHI29) mAb, 2 h before each sensitization or challenge. The secondary challenge (rechallenge) was performed 28 days after the primary challenge.

LN cells were collected from the draining LN (inguinal, cervical, and axillary lymph nodes) of control Ig- or anti-B7-H3 mAb-treated sensitized mice 3 days after the final sensitization. LN cells were analyzed by flow cytometry. To measure the T cell responses to DNFB, LN T cells were purified by using the MACS separation system with biotinylated anti-B220, anti-MHC class II and anti-CD49b mAbs followed by streptavidin-microbeads (Miltenyi Biotec). Purified LN T cells (2 × 10^5 per well; >97% CD3+ T cells) were cocultured with DNFB-pulsed splenocytes as described previously (36). IFN-γ production after 3 days of culture was measured by ELISA.

Statistics. Statistical analysis was performed by using the Student’s t test and Mann–Whitney U test for in vitro and in vivo analyses, respectively. Values of P < 0.05 were considered significant.

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