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研究代表者
橋口 昌章 (HASHIGUCHI MASAOKI)
東京医科歯科大学・大学院医歯学総合研究科・助教
研究者番号：20372443

研究成果の概要：補助シグナル分子は細胞間相互作用において正あるいは負に応答を制御することが知られている。本研究により、これまで詳細な機能が不明であった B7 ファミリー分子 B7-H3 のカウンターレセプターが Trem ファミリーに属する Trem-like transcript 2 (Tlt2, Trem12) であることを同定した。さらに、B7-H3 と Tlt2 の会合は T 細胞応答を亢進し、また接触性アレルギーを亢進することを明らかにした。

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Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses

Masaaki Hashiguchi*, Hiroko Kobori*, Patcharee Ritprajak*, Yosuke Kamimura*, Haruo Kozono[†], and Miyuki Azuma**

*Department of Molecular Immunology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan; and [†]Division of Biosignaling, Research Institute for Biological Sciences, Tokyo University of Science, Noda, Chiba 278-0022, Japan

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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.

allergy | cosignal | CD8 | T cell activation | contact hypersensitivity

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- κ B, and AP-1 (6, 7, 22). In B7-H3-deficient mice Th1-mediated hypersensitivity 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 CD28 family members, were transduced into J558L cells that lacked B7-H3 by using the IRES-eGFP bicistronic retroviral vector. B7-H3Ig 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-H3Ig 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 (K_d) was determined by Scatchard plot analysis. The K_d value for B7-H3Ig binding to cell surface TLT-2 was 90 ± 44 nM (Fig. 1B), which was comparable to K_d 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 rat IgG2a, κ) and TLT-2 (MIH47, rat IgG2a, κ and MIH49, rat IgM, κ). MIH32 showed higher reactivity than MIH35 toward B7-H3/eGFP-transduced J558L cells [supporting information (SI) Fig. S1A], whereas pretreatment of B7-H3Ig with MIH35 (Fig. 1C Upper), but not MIH32 (data not shown) inhibited B7-H3Ig binding to TLT-2 on the surfaces of DO11.10 transfectants. Therefore, we used MIH32 and MIH35 for flow cytometric staining and the functional blocking analyses, respectively. Although MIH47 and MIH49 reacted similarly to TLT-

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[†]To whom correspondence should be addressed. E-mail: miyuki.mim@tmd.ac.jp.

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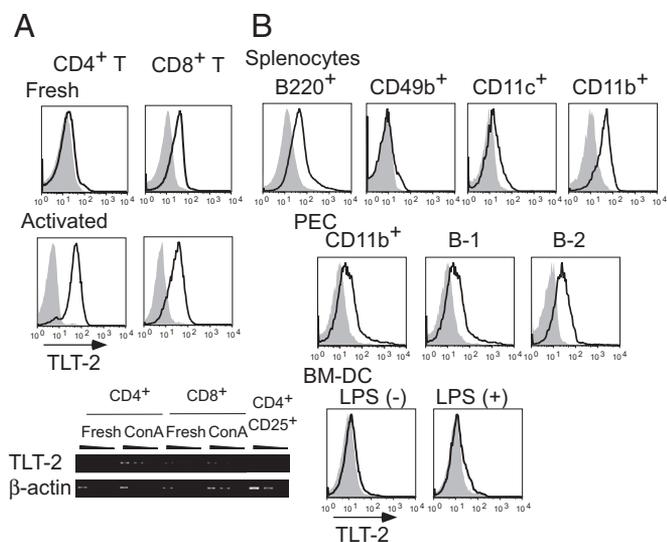


Fig. 2. TLT-2 expression on immune cells. (A) Freshly isolated and activated CD4⁺ and CD8⁺ T cells stimulated with anti-CD3 mAb for 2 days were stained with FITC-anti-CD4 or anti-CD8 mAb and biotinylated anti-TLT-2 mAb, followed by streptavidin-APC or the appropriate fluorochrome-conjugated control mAbs. (Top and Middle) The data for TLT-2 expression gated on CD4⁺ or CD8⁺ cells are displayed as histograms with the control histograms nearest the ordinate (shaded histograms). (Bottom) RT-PCR analysis of TLT-2. Serially (4-fold) diluted cDNAs from freshly isolated or Con A-activated CD4⁺ and CD8⁺ T cells, and freshly isolated CD4⁺CD25⁺ T cells were analyzed. (B) TLT-2 expression in splenocytes, peritoneal exudate cells (PEC), and BM-DC. Freshly isolated splenocytes and PEC, and either unstimulated or LPS-stimulated BM-DCs were stained with FITC-anti-B220, anti-CD49b (DX5), anti-CD11c or anti-CD11b and biotinylated anti-TLT-2 (MIH47) mAb, followed by streptavidin-APC or the appropriate fluorochrome-conjugated control mAbs. The data for TLT-2 expression gated on the respective antigen are displayed as histograms with the control histograms nearest the ordinate (shaded).

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⁺ cells by using a retroviral transduction system. Transduc-

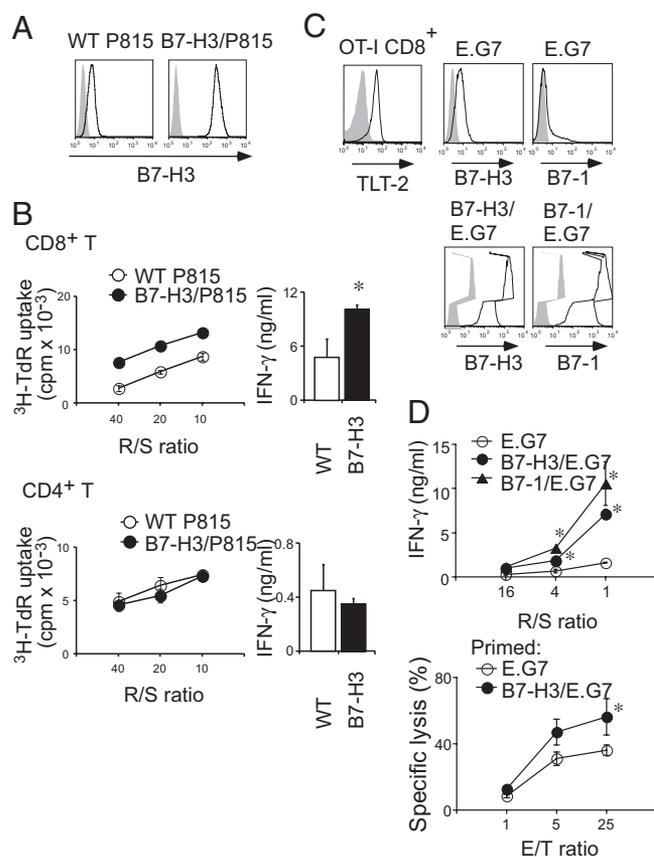


Fig. 3. B7-H3 costimulates CD8⁺ T cell responses. (A) WT P815 and B7-H3/P815 transfectants were stained with isotype control Ab or FITC-anti-B7-H3 (MIH32) mAb. Cell surface B7-H3 on WT P815 and B7-H3/P815 cells is presented as histograms with the control staining (shaded). (B) CD8⁺ or CD4⁺ T cells were cocultured with the indicated ratio of WT P815 or B7-H3/P815 in the presence of anti-CD3 mAb (0.4 μ g/ml for CD8⁺ T cells and 0.1 μ g/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 \pm SD. The data are representative of three independent experiments. (C) TLT-2 expression on OT-I CD8⁺ cells (Left). OT-I CD8⁺ cells were stained for TLT-2, as described in Fig. 2C. B7-H3 and B7-1 expression on E.G7 and E.G7 transfectants (Right). 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 (Bottom) 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 \pm SD. The data are representative of three independent experiments. *, statistically different from the WT control ($P < 0.05$).

tion 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.

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 efficacy of B7-H3-mediated costimulation was more evident for CD8⁺ T cells.

Our results suggest that the TLT-2:B7-H3 pathway costimulates the activation of T cells, especially CD8⁺ T cells. The efficient contribution 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 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 coinhibitory 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 Th1 type of CD4⁺ T cells and regulate Th1 responses. Unknown receptor:B7-H3-mediated coinhibitory 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 cells other than antigen-presenting cells, or TLT-2 expressed on myeloid lineage cells, partly contribute to the effects. The function of TLT-2 expressed on myeloid cells requires further study.

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 pCR2.1-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 [³H]thymidine for the final 18 h of culture and the incorporated 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 [³H]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 μ l 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 20 μ l 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 μ g per mouse of control rat IgG (MP Biomedicals), rat IgM (MP Biomedicals), anti-B7-H3 (MIH35) mAb or anti-TLT-2 (MIH29) 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 DNBS-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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5. 主な発表論文等

(研究代表者、研究分担者及び連携研究者には下線)

[雑誌論文] (計 1 件)

(1) Hashiguchi, M., Kobori, H., Ritprajak, P., Kamimura, Y., Kozono, H., Azuma, M. Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses. Proc. Natl. Acad. Sci. USA 105:10495-10500. 2008.

[学会発表] (計 2 件)

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6. 研究組織

(1) 研究代表者

橋口 昌章 (HASHIGUCHI, Masaaki)

東京医科歯科大学・大学院医歯学総合研究科・助教

研究者番号 : 20372443

(2) 研究分担者

東 みゆき (AZUMA, Miyuki)

東京医科歯科大学・大学院医歯学総合研究科・教授

研究者番号 : 90255654