The Absence of Interleukin 1 Receptor–related T1/ST2 Does Not Affect T Helper Cell Type 2 Development and Its Effector Function

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Summary

T1/ST2, an orphan receptor with homology with the interleukin (IL)-1 receptor family, is expressed constitutively and stably on the surface of T helper type 2 (Th2) cells, but not on Th1 cells. T1/ST2 is also expressed on mast cells, which are critical for Th2-mediated effector responses. To evaluate whether T1/ST2 is required for Th2 responses and mast cell function, we have generated T1/ST2-deficient (T1/ST2−/−) mice and examined the roles of T1/ST2. Naive CD4+ T cells isolated from T1/ST2−/− mice developed to Th2 cells in response to IL-4 in vitro. T1/ST2−/− mice showed normal Th2 responses after infection with the helminthic parasite Nippostrongylus brasiliensis as well as in the mouse model of allergen-induced airway inflammation. In addition, differentiation and function of bone marrow–derived cultured mast cells were unaffected. These findings demonstrate that T1/ST2 does not play an essential role in development and function of Th2 cells and mast cells.

Key words: gene targeting • interleukin 1 receptor family • mast cell • asthma • T helper cells types 1 and 2

The orphan receptor T1/ST2 belongs to the IL-1R family. The T1/ST2 gene was originally identified as a late response gene that was induced by either serum or overexpression of v-mos and Ha-ras oncogenes in murine fibroblasts (1–3). Alternative 3' processing of the primary transcript generates two messengers of 2.7 and 5 kb long. The short mRNA encodes a 38.5-kD peptide with similarity to the extracellular portion of the IL-1Rs (4), and is secreted from the cell as a heavily glycosylated protein of 50–60 kD. The short mRNA is found in embryonic tissues and mammary tumors, and is induced in fibroblasts upon stimulation with proliferation-inducing agents such as serum, platelet-derived growth factor, fibroblast growth factor, 12-O-tetradecanoylphorbol-13-acetate, and lysophosphatidic acid (2, 5, 6). On the other hand, the long mRNA encodes a single transmembrane-spanning protein with significant homology to the type 1 IL-1R. Expression of the long mRNA was restricted to hematopoietic organs and the lung (5, 7). The gene for ST2 was mapped to mouse chromosome 1 closely linked to the il-1r locus containing both type I and type II receptor genes, in support of their common ancestry (8). Despite their similarity to the IL-1R, the T1/ST2 proteins do not bind either IL-1α or IL-1β (9, 10). Two different groups have reported the identification of putative T1/ST2 ligands, but it is still unclear whether these proteins indeed represent the physiological T1/ST2 ligands (9, 11).

Th cells are categorized into two subsets, Th1 and Th2 cells, based on their cytokine production. Th1 cells secrete IL-2 and IFN-γ, which promote mainly cellular immunity. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, and promote mainly humoral immunity (12, 13). The Th1 to Th2 balance determines the outcome of a wide variety of immune responses involving infectious, autoimmune, and allergic diseases. Acquired resistance against intracellular bacteria is associated with the Th1 response, whereas resistance against helminths is achieved by the Th2 response (14). Autoimmune diseases such as multiple sclerosis and Crohn’s disease are associated with an abnormally strong Th1 response, whereas allergic diseases seem to involve an abnormally strong Th2 response (15–18). Both Th1 and Th2 cells are derived from a common precursor cell named naive Th cells. IL-12 stimulates the differentiation of naïve Th to Th1 cells (19), whereas IL-4 regulates Th2 differentiation (20, 21). These cytokines are produced in the early phase of in-
 recently it has been demonstrated that Th1/ST2 is preferentially expressed on murine Th2 cells and is important for Th2 effector functions. Using differential display PCR comparing a panel of Th1 and Th2 clones, Xu et al. (26) and Löhning et al. (27) independently identified T1/ST2 as a gene expressed on Th2 but not Th1 cells. T1/ST2 is constitutively and stably expressed on the surface of murine Th2 cells. Furthermore, Th1/Th2 cytokine coexpression is observed in the T1/ST2-negative population but not in the T1/ST2-positive population, indicating that surface expression of T1/ST2 is an advanced commitment to the Th2 phenotype. In vivo administration of anti-T1/ST2 Ab enhanced Th1 responses by increasing IFN-γ production and decreasing IL-4 and IL-5 synthesis, and thereby induced resistance to Leishmania major infection in BALB/c mice and exacerbated collagen-induced arthritis in DBA/1 mice. In addition, pretreatment with anti-T1/ST2 Ab or T1/ST2 fusion protein abrogated both the accumulation of eosinophils in the airways and the secretion of Th2 cytokines in a murine model of Th2-dependent allergic airway inflammation. Mast cells are critical effectors in Th2 cell–dependent immune responses and in the pathogenesis of Th2 cell–dependent disorders. Mast cells are also found to express T1/ST2 at high levels (28, 29).

In this study, we have generated mice deficient in the T1/ST2 gene, and using the mutant mice we examined the roles of T1/ST2 in helminth infection and a mouse model of asthma, as well as in mast cell differentiation and function.

Materials and Methods

Reagents. Recombinant murine IL-12 was provided by Haya-shibara Biochemical Laboratories, Inc. (Okayama, Japan). FITC-conjugated anti–mouse T1/ST2 Ab (clone D8J) was purchased from Mornwell Diagnostics GmbH. Levels of IL-4, IL-5, IL-10, and IFN-γ were determined by ELISA (Genzyme).

Cell Culture. Lymphoid cell growth medium consisted of RPMI 1640 medium (Life Technologies) supplemented with 10% (vol/vol) fetal bovine serum, 100 μg/ml 2-ME, 5 U/ml penicillin, and 50 μg/ml streptomycin. Bone marrow cell culture medium consisted of αMEM (Life Technologies) supplemented with 10% (vol/vol) fetal bovine serum, 5 U/ml penicillin, and 50 μg/ml streptomycin.

Generation of T1/ST2−/− Mice. The T1/ST2 locus on the mouse genome has been submitted to the EMBL/GenBank/DBJ database (available under accession no. X60184). A targeting vector was designed to replace a 1,280-bp genomic fragment encoding amino acids 1–96 of the mature T1/ST2 with neomycin resistance gene (neo) from pMC1-neo (Stratagene). The targeting vector was flanked by the 5.8-kbp fragment at the 3′ end and the 1.0-kbp fragment at the 5′ end. A herpes simplex virus-thymidine kinase gene (HSV-TK) was inserted into the 3′ end of the vector. The targeting vector was linearized with SalI and electroporated into E14.1 embryonic stem (ES) cells. The clones resistant to 4G18 and ganciclovir were screened by PCR and confirmed by Southern blot analysis with the probe shown in Fig. 1A for homologous recombination. An external primer (5′-AGGTG-GATTATGACGTGGTGCATGG-3′) specific for the T1/ST2 gene upstream of the targeting construct and a neo primer (5′-ATCCGCTCTTACGCCCTTTGACGAG-3′) specific for neo were used in the PCR screening. Generation of chimeric mice and mutant mice was essentially as described previously (30).

In Vitro Induction of Th1/Th2 Cell Differentiation. Splenocytes were incubated with biotinylated anti-1Aβ Ab (clone 25-9-17; Pharmingen), biotinylated anti-CD8 Ab (clone 53-6-7; Pharmingen), biotinylated anti–pan-NK cell Ab (clone DX5; Pharmingen), biotinylated anti-CD45R Ab (clone RA3-6B2; Pharmingen), and streptavidin microbeads (MACS; Miltenyi Biotec) followed by magnetic field separation to remove MHC class II, CD8, and B220-expressing cells and NK cells. Cell purity was analyzed by flow cytometry using FITC-conjugated anti-CD3 Ab (clone 145-2C11; Pharmingen) and PE-conjugated anti-CD4 Ab (clone L3T4; Pharmingen). Purified cells were cultured on 10 μg/ml anti-CD3 Ab–coated plates (clone 145-2C11; Pharmingen) in the presence of exogenous cytokines and anticytokine Ab as indicated. Th2 differentiation was promoted in the presence of 1,000 U/ml IL-4 (Pharmingen) and 10 μg/ml anti–IFN-γ Ab (clone XMG1.2; Endogen), whereas Th1 differentiation was promoted in the presence of 2 ng/ml IL-12. After 6 d culture, cells were harvested and washed with HBSS. Cells (106/ml) were stimulated on an anti-CD3 Ab–coated 96-well plate for 24 h. Supernatants were analyzed by cytokine ELISA.

After 8 d of culture, cells were also analyzed for intracellular cytokines. Staining was performed with Cytofix/CytoPerm Plus kit (Pharmingen). The following Abs were used: Cy-Chrome-conjugated anti–CD4 Ab (clone H129.19; Pharmingen), PE-conjugated anti–IL-4 Ab (clone BVD4.1D11; Pharmingen), FITC-conjugated anti–IFN-γ Ab (clone XMG1.2; Pharmingen). Samples were analyzed by three-color flow cytometry, and gates were set on CD4+ lymphocytes.

Nippostrongylus Infection. 800 third-stage Nippostrongylus brasi- liensis larvae were injected subcutaneously into 10-wk-old T1/ST2−/− and wild-type control mice. Mice were killed 8 d after infection, and CD4+ T cells were enriched from the spleen by positive selection on LS+ columns (MACS), according to the manufacturer's instructions (Miltenyi Biotec). CD4+ T cells were cultured at 106/ml in 96-well plates coated with 10 μg/ml anti-CD3 Ab for 24 h. Supernatants were analyzed by cytokine ELISA. Nippostrongylus infection in BALB/c mice was assayed by measuring the IgE levels of the infected animals.

Bone Marrow–derived Mast Cell Preparation and Stimulation. Bone marrow was aseptically flushed from femora of 8-wk-old mice. The cells were cultured at 106/ml in bone marrow cell culture medium supplemented with 10% (vol/vol) PWM-stimulated spleen cell conditioned medium (31) for 3 wk. The bone marrow cultures were enriched for mast cells by repetitively transferring the suspension cell fraction into fresh culture flasks. After 3 wk of culture, culture medium was changed to bone marrow cell culture medium containing 10 ng/ml recombinant mouse IL-3 (Genzyme/Techne). After 1 wk of culture, mast cells were analyzed for T1/ST2 expression by incubating with IgEκ, (clone IgE3; Pharmingen), PE-conjugated anti–c-Kit (clone 3C1; Immunotech), and 0.8 μg/ml FITC-conjugated anti-T1/ST2 Ab, followed by biotinylated anti–mouse IgE (clone R35-92; Pharmingen) and Cy-Chrome-conjugated streptavidin (PharMingen).

After washing, cells were resuspended at 106/ml and stimulated for 24 h with the polyclonal activator PMA at 50 ng/ml and ionomycin. Supernatants were analyzed by cytokine ELISA. IgE cross-linking was performed by first incubating the cells for 30 min at 37°C in the presence of 3 μg/ml of monoclonal mouse
IgE anti-dinitrophenyl group (DNP) (clone SPE-7; Sigma Chemical Co.). The mast cells were washed three times and then incubated with 10 ng/ml DNP 30-40 HSA (Sigma Chemical Co.) for 30 min at 37°C. After the treatment with antigens, supernatants were measured for histamine concentration by RIA (SR L, Inc.).

Sensitization Procedure and Allergen Exposure. Immunization and exposure of mice were carried out according to the method of Foster et al. (32). In brief, 8- or 10-wk-old mice were sensitized by intraperitoneal injection with 50 μg OVA/1 ml aluminum hydroxide in 0.9% saline on days 0 and 12. Nonsensitized mice received 1 mg of aluminum hydroxide in 0.9% saline. On day 24, mice were placed individually in a chamber and inhaled aerosolized OVA (10 mg/ml) in 0.9% saline (sensitized, OVA-challenged group) for 30 min three times at 1-h intervals, and then every second day thereafter, for 8 d. Nonsensitized mice received saline only (nonsensitized, nonchallenged group). The aerosols were generated by ultrasonic nebulization. 24 h after the last allergen challenge, the mice were anesthetized by intraperitoneal injection of pentobarbital (80 mg/kg body wt) and used for the following experiments. The blood was collected, and serum Ig levels were measured by ELISA.

Bronchoalveolar Lavage. The trachea was cannulated, and the airway lumina were washed twice with 0.5 ml of PBS (Ca²⁺, Mg²⁺ free) supplemented with 50 μM EDTA and 0.1% BSA. The bronchoalveolar lavage (BAL) fluid was immediately centrifuged (10 min at 4°C, 700 g). The BAL cells were resuspended in 0.2 ml of rat serum and counted using a hemocytometer. For differential cell counts, 2-4 × 10⁶ cells were spun onto glass slides, air dried, fixed with ethanol, and stained with Giemsa solution. The numbers of eosinophils, neutrophils, lymphocytes, and macrophages in 200 cells were counted based on morphology and staining characteristics.

ELISA for Measurement of Serum Ig Level. Serum IgG1 level was measured as described previously (33), except that the following reagents were used: goat anti-mouse IgG1, mouse IgG1, biotinylated goat anti-mouse IgG1, and streptavidin-conjugated alkaline phosphatase (Southern Biotechnology Associates). Serum IgE level was measured according to protocol supplied by PharMingen.

Results and Discussion

Generation of T1/ST2-deficient Mice. The mouse T1/ST2 gene was disrupted by homologous recombination in E14.1 ES cells. A targeting vector was designed to delete the first and second ATG codons of T1/ST2 with the neomycin resistance gene (Fig. 1 A). 2 out of 733 clones screened were positive for homologous recombination. Two targeted ES clones were microinjected into C57BL/6 blastocysts, and successfully transmitted the disrupted T1/ST2 gene through the germline (Fig. 1 B). T1/ST2−/− mice were born at the expected Mendelian ratios (+/+ : +/− : −/− = 55: 96: 49). The mice grew healthy and showed no obvious abnormalities until 20 wk old. Homozygous mutants of both sexes were fertile, and there was no significant difference in the birth rate between T1/ST2−/− and wild-type mice. Thus, T1/ST2 was not required for mouse development or fertility. Reverse transcription (RT)-PCR analysis using total RNA from bone marrow cells of T1/ST2−/− mice confirmed the absence of expression of T1/ST2 mRNA (Fig. 1 C). T1/ST2 has been shown to be abundantly expressed on the cell surface of mast cells and Th2 cells (26–29). When bone marrow cells were cultured in the presence of IL-3, these cells differentiated into mast cells expressing both high-affinity IgE receptor, FcεRI, and c-Kit receptor. Analysis by two-color dot plot revealed that

Figure 1. Targeted disruption of the mouse T1/ST2 gene. (A) Targeting vector and restriction map of the T1/ST2 locus. The restriction map of the wild-type allele, targeting vector, and mutated allele is shown. Open and filled boxes denote the noncoding and coding exons, respectively. The orientation of the neomycin resistance gene (neo) and the herpes simplex virus-thymidine kinase gene (HSV-TK) are indicated by the arrow. E, EcoRI; B, BamHI. (B) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with BamHI, electrophoresed, and hybridized with the probe as shown in A. The approximate size of the wild-type band is 6.8 kbp, and the mutated band is 2.2 kbp. H, wild-type; +/−, heterozygous mutant; −/−, homozygous mutant. (C) RT-PCR analysis of T1/ST2 mRNA. Total RNA was isolated from the bone marrow cells. PCR analysis was performed using specific primers for T1/ST2 or for β-actin (reference 38). (D) Expression of T1/ST2 on BM CMCs. BM CMCs were triple stained with FITC-anti-T1/ST2 Ab, PE-anti-c-Kit Ab, IgE/biotin-anti-IgE Ab/Streptavidin-Cy-Chrome. T1/ST2 expression was analyzed in histogram mode by gating on the IgE- c-Kit+ double positive population which was >99% of the cells analyzed.
cell differentiation was not affected in T1/ST2 mice. These results indicate that IL-4–induced in vitro Th2 cytokine composition was not altered in 6-wk-old T1/ST2 mice. Th cells from wild-type mice developed into IL-4 producers in response to IL-4 or into IFN-γ producers in response to PMA and ionomycin (Fig. 4 A). Further, both wild-type and T1/ST2−/− cells released equal levels of histamine in response to IgE and Ag (Fig. 4 B). Mast cells were classified into two subtypes: the connective tissue–type mast cell, which is found in the skin, musculature, perivascular tissues, and peritoneal cavity, and the mucosal mast cell, which is widely distributed in mucosal tissues of the respiratory tract and the intestinal lamina propria (37). The number of connective tissue–type mast cells in the skin of T1/ST2−/− mice was almost the same as that of wild-type mice. The number of mucosal mast cells in the lamina propria of the stomach was also equivalent between T1/ST2−/− and wild-type mice, indicating that mast cell development is not affected in T1/ST2−/− mice (data not shown). Thus, our data suggest that T1/ST2 expression does not contribute to development and function of mast cells.

Development of Allergen-induced Airway Inflammation in T1/ST2−/− Mice. It has been shown that Th2-mediated eosinophilic lung inflammation of airways is attenuated by pretreatment with anti-T1/ST2 mAb (27). Therefore, we examined whether T1/ST2 deficiency influences inflam-

Figure 2. Cytokine production after in vitro Th cell differentiation. (A) CD4+ T cells purified from spleen from wild-type and T1/ST2−/− mice were stimulated under Th1 or Th2 culture conditions for 5 d, washed, and restimulated on anti-CD3 Ab–coated plates for 24 h. Supernatants were assayed for cytokine production by ELISA. Both wild-type and T1/ST2−/− CD4+ T cells produced the same amounts of IL-4 (Fig. 3 A). IFN-γ production from both CD4+ T cells was almost the same. IL-4 is shown to be required for Ig class switching of B cells to IgE and IgG1 producers (20, 21, 34–36). Therefore, we measured serum Ig levels before and after N. brasiliensis infection. Total serum IgE and IgG1 levels after infection were almost equivalent in both wild-type and T1/ST2−/− mice (Fig. 3 B). These data indicate that T1/ST2 is not functionally involved in Th2 response in vivo.

Normal Th2-mediated Functions in T1/ST2−/− Mice. It has recently been shown that treatment of mice with anti-T1/ST2 Ab resulted in decreased Th2 cell–mediated functions, indicating that T1/ST2 might be functionally associated with Th2 cells (26, 27). Therefore, we analyzed in vitro differentiation assays to assess the effect of T1/ST2 deficiency on cytokine production from Th cells. CD4+ Th cells were cultured for 5 d in the presence of IL-12 or IL-4 plus anti–IFN-γ Ab to develop into Th1 or Th2 cells respectively. Th1-developing cells from both wild-type and T1/ST2−/− mice produced almost equal amounts of IFN-γ (Fig. 2 A). Th2-developing cells from wild-type mice produced increased levels of Th2 cytokines such as IL-4, IL-5, and IL-10. Th cells from T1/ST2−/− mice also produced the equivalent levels of Th2 cytokines. Th cells from wild-type mice developed into IL-4 producers in response to IL-4 or into IFN-γ producers in response to IL-12 (Fig. 2 B). The cells from T1/ST2−/− mice developed into IL-4 or IFN-γ producer cells as did the cells from wild-type mice. These results indicate that IL-4–induced in vitro Th2 cell differentiation was not affected in T1/ST2−/− mice.

Next, we examined in vivo Th2 response by infecting with the intestinal parasitic nematode N. brasiliensis. CD4+ splenic T cells were prepared 8 d after infection and stimulated with immobilized anti-CD3 Ab for 24 h. The supernatants were assayed for cytokine production by ELISA. Both wild-type and T1/ST2−/− CD4+ T cells produced the same amounts of IL-4 (Fig. 3 A). IFN-γ production from both CD4+ T cells was almost the same. IL-4 is shown to be required for Ig class switching of B cells to IgE and IgG1 producers (20, 21, 34–36). Therefore, we measured serum Ig levels before and after N. brasiliensis infection. Total serum IgE and IgG1 levels after infection were almost equivalent in both wild-type and T1/ST2−/− mice (Fig. 3 B). These data indicate that T1/ST2 is not functionally involved in Th2 response in vivo.
matory responses in the murine model of Th2-dependent allergic airway inflammation. Wild-type and T1/ST2−/− mice immunized with OVA were exposed to an aerosol of OVA. 24 h after OVA challenge, mice were bled, and serum concentrations of IgE and IgG1 were measured by ELISA. Serum concentrations of IgE and IgG1 were equally increased after OVA treatment in both wild-type and T1/ST2−/− mice (Fig. 5 A). We also analyzed the cells in BAL fluid 24 h after the exposure to OVA aerosol. The total cell number of BAL cells was dramatically increased after OVA exposure in both wild-type and T1/ST2−/− mice (Fig. 5 B). Furthermore, a dramatic increase in eosinophils was observed in both wild-type and T1/ST2−/− mice. Histological analysis of lung taken from both wild-type and T1/ST2−/− mice exposed to OVA displayed severe widespread inflammatory infiltration (data not shown). Thus, T1/ST2−/− mice exhibited normal eosinophilic response in the mouse model of allergic airway inflammation.

In this study, we generated T1/ST2−/− mice and analyzed their phenotype. T1/ST2 has been shown to be expressed on the cell surface of Th2 cells, but not Th1 cells. Furthermore, administration of anti-T1/ST2 Ab resulted in reduced Th2-mediated functions in mice (26, 27). Thus, T1/ST2 has been implicated in Th2-mediated functions. However, T1/ST2−/− mice displayed almost normal Th2 responses in both nematode infection and allergic airway inflammation. These results clearly demonstrate that T1/ST2 is not essential to Th2 cell functions. Since it has been shown that treatment of Th2 cells with anti-T1/ST2 Ab enhances complement-mediated lysis in vitro (26), the reduced Th2-mediated functions observed in the studies with anti-T1/ST2 Ab administration might be a result of complement-mediated elimination of the T1/ST2-positive cell population. In addition to Th2 cells, mast cells express high levels of T1/ST2. However, development and activity of mast cells were unaffected in T1/ST2−/− mice. Thus, our knockout study reveals that T1/ST2 does not play a critical role in development and function of Th2 cells and mast cells, although we cannot rule out the possibility that T1/ST2 may play some role in Th2 response. Alternatively, T1/ST2 may have an unknown function in these or other cell populations. At present, a ligand for T1/ST2 has not been identified. The identification of the ligand will reveal a novel function of T1/ST2 as well as its role in Th2 response.

Figure 3. Cytokine production from splenic T cells and serum Ig levels in response to parasite infection. (A) Splenic CD4+ T cells were prepared from uninfected animals (d0) or animals 8 d after infection (d8) with N. brasiliensis. Cells were stimulated with immobilized anti-CD3 Ab for 24 h. Supernatants were analyzed by ELISA. Data are representative of three experiments consisting of cohorts of two mice at each time point. (B) T1/ST2−/− mice (eight mice) and wild-type (six mice) were infected with N. brasiliensis. Serum IgE and IgG1 levels were measured at days 0, 8, 14, and 21 after infection. Serum samples were assayed by ELISA. Data are presented as mean ± SEM. N.D., not detectable.

Figure 4. Mast cell analysis. (A) BMCMCs from T1/ST2−/− and wild-type mice were stimulated with PMA and ionomycin for 24 h. Supernatants were analyzed by ELISA. (B) Histamine release from BMCMCs stimulated with IgE and specific antigen. The data shown are representative of the three experiments. Data are presented as mean ± SEM. N.D., not detectable.

Figure 5. Analysis of Th2 effector function of T1/ST2−/− mice on a mouse model of asthma. (A) Changes in serum concentrations of IgE and IgG1 induced by OVA treatment. Data are presented as mean ± SEM for six mice. (B) BAL cell number and cellular distribution of BAL fluid. BAL fluid was obtained from T1/ST2−/− and wild-type mice after treatment with or without OVA. The columns represent the absolute numbers of total BAL cells, eosinophils, neutrophils, lymphocytes and macrophages per animal. Data are presented as mean ± SEM for five mice.
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