Inhibition of T1/ST2 during Respiratory Syncytial Virus Infection Prevents T Helper Cell Type 2 (Th2)- but not Th1-driven Immunopathology

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Abstract

T cells secreting interleukin (IL)-4 and IL-5 (T helper cell type 2 [Th2] cells) play a detrimental role in a variety of diseases, but specific methods of regulating their activity remain elusive. T1/ST2 is a surface ligand of the IL-1 receptor family, expressed on Th2- but not on interferon (IFN)-γ-producing Th1 cells. Prior exposure of BALB/c mice to the attachment (G) or fusion (F) protein of respiratory syncytial virus (RSV) increases illness severity during intranasal RSV challenge, due to Th2-driven lung eosinophilia and exuberant Th1-driven pulmonary infiltration, respectively. We used these polar models of viral illness to study the recruitment of T1/ST2 cells to the lung and to test the effects of anti-T1/ST2 treatment in vivo. T1/ST2 was present on a subset of CD4+ cells from mice with eosinophilic lung disease. Monoclonal anti-T1/ST2 treatment reduced lung inflammation and the severity of illness in mice with Th2 (but not Th1) immunopathology. These results show that inhibition of T1/ST2 has a specific effect on virally induced Th2 responses and suggests that therapy targeted at this receptor might be of value in treating Th2-driven illness.

Key words: bronchiolitis, viral • immunity, mucosal • immunity, cellular • pulmonary infection • eosinophil

Introduction

In several common diseases, some CD4+ T cells cause enhanced pathology, while others are protective. Th1 cells produce IFN-γ, activate macrophages, and are associated with inflammatory disorders, whereas Th2 cells secrete predominantly IL-4 and IL-5 and are pivotal in asthma and atopy (1–3). Selective depletion of Th1 or Th2 cells would provide a novel therapeutic strategy but requires the identification of reliable markers and targets against which therapy could be directed.

To date, the Th1 phenotype is associated with chemokine receptor 1, 3, and 5 (references 4 and 5) and IL-18 receptor expression (6). The Th2 phenotype, on the other hand, preferentially upregulates the transcription factors c-maf (7) and GATA-3 (8), chemokine receptors 3 and 4 (references 4, 9, and 10), and the orphan receptor T1/ST2 (references 11–15). Resting murine Th2 cells constitutively express the multidrug resistance protein transmembrane pump, whereas expression on Th1 and Th2 cells is equal after antigenic stimulation (16–17). Recently, a novel leukocyte chemoattractant receptor expressed on activated human Th2 but not Th1 cells has been reported (designated CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells). Allergen-induced proliferation of peripheral blood mononuclear cells is significantly reduced by antibody-mediated depletion of CRTH2+ cells (18).

T1/ST2 is an IL-1 receptor family member originally identified in murine fibroblasts (19–20). Alternate 3′ processing generates a long and short mRNA. The short mRNA encodes a secreted glycoprotein that is constitutively expressed in embryonic tissues and mammary tumors.
and is inducible in fibroblasts. The long mRNA encodes a membrane-spanning protein expressed only in the lung and hematopoietic tissue (21–23). Studies of T1/ST2 knockout mice provide conflicting evidence regarding its functional significance (24–26). Anti-T1/ST2 treatment of immunocompetent mice partially inhibits Th2 differentiation and allergic airway inflammation (13), but the effects of anti-T1/ST2 treatment have not been evaluated in virus-infected animals.

Respiratory syncytial virus (RSV) is a common cold virus that causes bronchiolitis in infants, killing up to one million children per year worldwide (27). Children hospitalized with bronchiolitis often suffer recurrent wheezing in later childhood and are frequently diagnosed as asthmatic (28). There is strong evidence that viral bronchiolitis is a T cell–mediated immunopathological condition (29, 30). In the BALB/c mouse, primary intranasal infection with RSV causes mild self-limiting illness characterized by transient T cell infiltration. However, prior sensitization to the attachment protein (G; expressed by recombinant vaccinia virus) results in extensive lung eosinophilia and enhanced weight loss after RSV challenge. CD4+ T cells secreting IL-4 and IL-5 are necessary and sufficient for this response (31). This phenomenon is reminiscent of human vaccine trials using formalin–inactivated RSV that caused disease enhancement and peripheral and lung eosinophilia and lead to some deaths in RSV-infected vaccinees (29). After sensitization with the fusion (F) protein, an exuberant T cell infiltrate causes enhanced weight loss, but eosinophil recruitment is not seen (32). A major advantage of the BALB/c model is that immune responses can be manipulated in parallel groups of mice undergoing Th1- or Th2-mediated pathology to the same pathogen.

We now report that CD4+ T cells expressing T1/ST2 occur exclusively during the Th2-driven eosinophilic RSV disease and are reduced in number by treatment with IL-12 (which antagonizes the development of eosinophilia). Furthermore, treatment of mice with anti-T1/ST2 antibody decreases inflammatory cell recruitment and weight loss only in mice with eosinophilic immunopathology. These results indicate that T1/ST2+ cells are specifically associated with RSV-induced eosinophilia in this model and that blockade of this cell surface receptor may be of therapeutic use in Th2-driven illnesses.

Materials and Methods

Generation of mAb 3E10. The generation and characterization of rat anti-T1/ST2 mAb (clone 3E10) is described elsewhere (15). In brief, a DNA sequence containing the extracellular domain of T1/ST2 was amplified by PCR and cloned into a vector containing the CDS signal sequence and the human IgG1 constant region. COS cells were transiently transfected by using the LipofectAMINE™ (GIBCO BRL) protocol and cultured in Ultra Low IgG Fetal Bovine Serum™ (GIBCO BRL) for 1 wk.

Recombinant protein was purified from culture supernatants using a protein A column. Lou/M rats were immunized with 0.5 mg of purified recombinant T1/ST2 subcutaneously, followed by intraperitoneal boosting twice at 2-wk intervals. Sera were analyzed for reactivity to the fusion protein by ELISA 10 d after the final boost. Animals with serum antibody to T1/ST2 were boosted again 4 wk later and killed after a further 3 d. Splenocytes were fused with SP/2 myeloma cells, and resulting clones were screened for selective binding to murine Th2 clones. The hybridoma 3E10 produced a blocking rat IgG1 anti-T1/ST2 mAb. Serum from unimmunized Lou/M rats was used as negative control.

Mice and Virus Stocks. 8–10-wk-old female BALB/c mice were purchased from Harlan Olac Ltd. and kept in pathogen-free conditions. RSV and recombinant vaccinia virus expressing the attachment protein (Gvac) or the fusion protein (Fvac) of RSV or control β-galactosidase (β gal-vac) were grown in HEp-2 cells and assayed for infectivity as previously described (33). All stocks were mycoplasma free as assessed by DNA hybridization (Gen-Probe Inc.).

Mouse Infection and Treatment. Anesthetized mice were scarified on the rump on day 0 with 3 × 10^6 pfu Gvac, Fvac, or β gal-vac in a final volume of 10 μl (four or five mice per group). On day 14, mice were challenged intranasally with 3 × 10^6 pfu of human RSV (A2 strain). Some mice were injected intravenously with 100 μg of anti-T1/ST2 (rat IgG) or isotype-matched control antibody each day, starting 1 d before RSV challenge. In addition, some mice were treated with recombinant IL-12 (300 ng in PBS) daily from day −2 to day +2 relative to scarification as described previously (34). The appearance of illness was scored by a blinded observer, and the weights of mice were monitored daily after RSV challenge. Illness was scored using the following scale: 0 = healthy; 1 = barely ruffled fur; 2 = ruffled fur but active; 3 = ruffled fur and inactive; 4 = ruffled fur, inactive, and hunched; and 5 = dead. Mice were killed on day 21 (7 d after intranasal RSV challenge) by injection of 3 mg of pentobarbital and exanguinated via the femoral vessels.

Lung Virus Titer. Clearance of RSV was assessed in lung homogenates on days 2 and 4 after virus challenge. Lungs were removed from four mice per group and homogenized. After centrifugation at 4,000 rpm for 4 min, the supernatant was titrated in doubling dilutions on HEp-2 cell monolayers in 96-well flat-bottomed plates. 24 h later, monolayers were washed and incubated with peroxidase-conjugated goat anti-RSV antibody (Biogenesis). Infected cells were detected using 3-amino-9-ethylcarbazole (AEC), and infectious units were enumerated by light microscopy.

Cell Recovery. After sacrifice, bronchoalveolar lavage (BAL) fluid, lung tissue, and serum were harvested as previously described (35). In brief, the lungs of each mouse were inflated six times with 1 ml of 12 mM lidocaine in Eagle's MEM and placed undiluted on ice in sterile tubes. 100 μl of BAL fluid from each mouse was cyt centrifuged onto glass slides, and eosinophils were enumerated using hematoxylin and eosin staining based on cell morphology and presence of red granules. The remainder of the BAL fluid was centrifuged, and the supernatant was removed and stored at −70°C in 200-μl aliquots for analysis of cytokines by ELISA. The pellet cells were resuspended in RPMI containing 10% FCS, 2 mM/ml l-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (R10F). Viable cells were counted by trypan blue exclusion.

Flow Cytometric Analysis. T1/ST2 antibody (2 μg) or control antibody was added to 10^6 pelletted cells on ice for 30 min; cells were then washed and treated with goat anti–rat FITC (Sigma-
Aldrich). After blocking with rat serum, Quantum Red™-conjugated CD8 or B220 and PE-conjugated CD4 or DX5 (anti-NK cell antibody) (Sigma-Aldrich) were added for 30 min. After washing again, cells were fixed for 20 min at room temperature with 2% formaldehyde. Samples were analyzed on a Beckman Coulter EPICS Elite™ flow cytometer collecting data on at least 40,000 lymphocytes. Eosinophils were enumerated as granulocytes by flow cytometry using their distinctive forward and side scatter properties.

Cytokine ELISAs. IL-4, IL-5, IFN-γ, and TNF-α in BAL fluid were quantified using OptEIA™ kits from Pharmingen. In brief, microtiter plates were coated with 100 µl of capture antibody diluted in the recommended buffer overnight at 4°C. After five washes with PBS containing 0.5% Tween-20, plates were blocked with 200 µl of PBS containing 10% FCS and left for 1 h at room temperature. Samples and standards (diluted in PBS plus 10% FCS) were then incubated for a further 2 h at room temperature. After five washes, bound cytokine was detected using biotinylated antibodies premixed with avidin–horseradish peroxidase followed by tetramethylbenzidine and hydrogen peroxide. Optical densities were read at 450 nm. The mean optical density of wells containing no cytokine was subtracted from the results obtained for samples and standards. The concentration of cytokines was determined by subtracting the optical density obtained by incubation of wells containing no cytokine from the results obtained for samples and standards. The concentration of cytokines was determined using a standard curve range for IL-4 lies between 31.3 and 2,000 pg/ml, for TNF between 15.6 and 1,000 pg/ml, and for IFN-γ between 7.8 and 500 pg/ml, for IL-5 between 15.6 and 1,000 pg/ml.

Optical densities were read at 490 nm. The amount of RSV-specific antibody was determined by subtracting the optical density obtained by incubating serum on HEP-2–coated plates from the same sample incubated on RSV–coated plates.

Statistical Analysis. One-way ANOVA (analysis of variance) assuming unequal variance was used and significance assumed at P < 0.05.

Results
To simplify the description in Results and Discussion, we will refer to Gvac-primed, RSV-challenged mice as G/RSV, Fvac-primed, RSV-challenged mice as F/RSV, and control β gal-vac–primed, RSV-challenged mice as β gal/RSV.

T1/ST2+ Cells Are Present in the BAL during Eosinophilic Illness. No T1/ST2+ cells were observed in the lungs of uninfected mice (data not shown). T1/ST2+ cells were apparent 3 d after RSV challenge in G/RSV (which showed lung eosinophilia) but not F/RSV mice (which did not have lung eosinophilia). T1/ST2+ cells were CD4+ (4.4% of CD4+ T cells at day 3; Fig. 1 A) and did not express CD8, B220 (CD45R), or DX5 (data not shown). The number of T1/ST2+ cells in the lungs peaked at day 5 after RSV challenge in G/RSV mice (13.3% of CD4+ T cells; Fig. 1 B) and were mostly CD45RB low (>90%), indicating recent activation. A small population of T1/ST2+ cells were CD4+ but did not express any of the other lymphoid markers described above. It is possible that these cells have downregulated CD4 expression after antigen activation. These data show that T1/ST2+ cells are present only in the lungs of mice undergoing eosinophilic inflammatory responses.

Anti-T1/ST2 Treatment Prevents Enhanced Illness in G/RSV but Not in F/RSV Mice. During a primary RSV infection, BALB/c mice experience mild weight loss after 5–6 d, coinciding with the peak of the inflammatory infiltrate. In G/RSV or F/RSV mice, the inflammatory infiltrate is greater and weight loss is enhanced and accelerated. However, anti-T1/ST2 treatment only prevented weight loss in G/RSV (eosinophilic) animals and had no effect on F/RSV (noneosinophilic) mice (Fig. 2). Similarly, there was no effect of anti-T1/ST2 treatment in β gal/RSV mice undergoing a primary RSV infection (data not shown).

Figure 1. T1/ST2+ cells are present in the lung during viral induced eosinophilia. Mice were scarified with Gvac and challenged intranasally with RSV 14 d later. T1/ST2+ cells were identified in BAL samples by indirect immunofluorescence. Cells were then stained with quantum red conjugated antibodies to CD4. Samples were analyzed on a Beckman Coulter EPICS Elite™ flow cytometer collecting data on at least 40,000 cells. A and B show T1/ST2+ cells in G/RSV mice at days 3 and 5 after intranasal RSV challenge. C and D show G/RSV mice treated with PBS (C) or IL-12 (D) at 4 d after intranasal RSV challenge. Representative plots for five mice per group are shown.
Anti-T1/ST2 Treatment Reduces Inflammatory Cell Influx and BAL Cytokine Levels in G/RSV but Not in F/RSV Mice. Anti-T1/ST2+ treatment significantly reduced total BAL cell recovery from G/RSV mice, affecting eosinophils, lymphocytes, CD4+ T cells, and CD8+ T cells (Fig. 3). The reduction of eosinophils was particularly prominent, as both the proportion and total number present in the sample were reduced by anti-T1/ST2 treatment. A corresponding reduction in BAL fluid TNF, IL-5, and IFN-γ (but not IL-4) was also observed (Fig. 4). Anti-T1/ST2 treatment had no significant effect on cell recruitment or phenotype in F/RSV mice (P > 0.05 for all comparisons; Fig. 5). Furthermore, no T1/ST2-expressing cells were found in the BAL of anti-T1S/T2–treated mice (data not shown).

Anti-T1/ST2 Treatment Reduces RSV-specific Antibody and Viral Clearance in G/RSV but Not F/RSV Mice. Prior scarification with Gvac or Fvac induces enhanced virus-specific antibody production after RSV challenge compared with mice infected with RSV alone. Anti-T1/ST2 treatment reduced this boost in antibody levels in G/RSV but not F/RSV mice (Table I). The reduction in inflammatory infiltrate and antibody titer was reflected by the delayed clearance of RSV from the lung in anti-T1/ST2–treated animals. On day 2 after RSV challenge, there was no difference in the titer of RSV recovered from the lungs of treated and untreated mice. However, G/RSV-infected mice treated with anti-T1/ST2 had reduced viral clearance at day 4 compared with untreated mice (P > 0.01), but treatment did not affect RSV clearance in F/RSV animals (P > 0.05) (Fig. 6). No virus was recovered from any group at day 7 or 10 after RSV infection.

G/RSV Mice Treated with Recombinant IL-12 Have Reduced Numbers of T1/ST2+ Cells. We have previously shown that IL-12 treatment reduces pulmonary eosinophilia but also enhances the weight loss and illness in animals infected with G/RSV (34). We therefore tested the effect of IL-12 on the recruitment of T1/ST2+ cells to the lung. Mice were scarified with Gvac on day 0 and treated intraperitoneally with IL-12 or PBS daily from day −2 to day +2. In this experiment, 10.5% of CD4+ BAL T cells (C) is calculated by multiplying the percent of each subset (by flow cytometry) by the number of lymphocytes present. Both CD4+ (P = 0.02) and CD8+ T cells (P = 0.03) are fewer in anti-T1/ST2–treated mice. Each point represents an individual mouse from one of two independent experiments.
IL-12 treatment reduced this figure to <1% (Fig. 1 D). Similar to previous reports (34), IL-12 treatment enhanced cellular recruitment to the lung in the G/RSV group, which was not significantly affected by anti-T1/ST2 treatment (Fig. 7 A). IL-12 treatment caused a reduction in pulmonary eosinophilia (P = 0.005), which was unaffected by the additional depletion of T1/ST2+ cells (Fig. 7 B; P = 0.07). Therefore, the proportion of recovered T1/ST2+ cells from eosinophilic mice is reduced by IL-12 treatment.

As shown in Fig. 2 and Fig. 7 C, anti-T1/ST2 treatment prevented weight loss in G/RSV-infected mice. IL-12 treatment of G-primed mice enhanced weight loss during RSV challenge, as previously described (34). However, blocking of anti-T1/ST2 had no effect on IL-12–enhanced weight loss in G/RSV mice (Fig. 7 C). Therefore, although anti-T1/ST2 treatment prevented weight loss in G/RSV mice, it had no effect when the immune response to the G protein is altered by IL-12 and skewed towards a Th1 phenotype or dominated by CD8+ T cells.

### Discussion

Our results show that anti-T1/ST2 treatment reduces the severity of Th2-mediated eosinophilic immunopathology in the BALB/c mouse model of RSV infection. Treatment decreased weight loss, local recruitment of inflammatory cells (including eosinophils), and lung lavage levels of IL-5, TNF, and IFN-γ in RSV-infected mice presensitized with the attachment protein G. However, no benefit of anti-T1/ST2 treatment was found in the noneosinophilic immunopathology seen in F/RSV mice or in G/RSV mice previously treated with IL-12.

Previous studies show that T1/ST2 is preferentially expressed on T cells that predominantly produce IL-4, IL-5, or IL-10 but not IFN-γ or IL-2 (15). Expression is found in Th2-induced granulomatous lung disease of Schistosoma mansoni infection (14), and in vitro studies demonstrate up-regulation of T1/ST2 under Th2-polarizing conditions (11). Signaling through T1/ST2 after adoptive transfer of OVA-treated dendritic cells is essential for sensitization to inhaled OVA and development of Th2-dependent airway eosinophilia (37). In this study, T1/ST2 expression was only observed in G protein–sensitized (eosinophilic) mice and not in those previously sensitized with the fusion protein or those experiencing a primary RSV infection (both noneosinophilic). These results, together with the observation that IL-12 treatment reduces T1/ST2 expression in G/RSV-infected mice, support the Th2-restricted nature of this molecule.

The ability of anti-T1/ST2 antibody treatment of G/RSV-infected mice to abrogate T1/ST2 expression on BAL cells does not clarify whether T1/ST2+ cells are depleted or the receptor merely masked by the antibody. The effect on Th2-mediated responses was striking, however. Anti-T1/ST2 treatment was accompanied by a reduction in IL-5, TNF, and IFN-γ but not IL-4 levels in the lung lavage of G/RSV-infected animals. The reduction in pul-
does not necessarily enhance Th1 responses. The G protein
results also suggest that removal of T1/ST2-expressing cells
versus nonreplicating) and the infected cell type. Our re-
likely to reflect the nature of the antigen (i.e., replicating
crepancies between our results and those of others are
infected with
S. mansoni
and is similar to that reported in T1/ST2 knockout mice
egg infection with S. mansoni (24). The reduction in both
Th1 and Th2 cytokines is intriguing considering that re-
duced IL-4 and IL-5 but increased IFN-γ is observed when
OVA-specific Th2 cells are incubated with a T1/ST2 Ig
fusion protein in vitro (11). When OVA-specific cells are
adaptively transferred, however, T1/ST2 depletion reduces
Th2 cytokine levels in the lung lavage but does not signifi-
cantly alter IFN-γ. Medialinal lymph node cells from T1/
ST2 knockout mice produce less IL-4 and IL-5 and in-
creased IFN-γ in response to S. mansoni eggs (38). The dis-
crepancies between our results and those of others are
likely to reflect the nature of the antigen (i.e., replicating
versus nonreplicating) and the infected cell type. Our re-
results also suggest that removal of T1/ST2-expressing cells
does not necessarily enhance Th1 responses. The G protein
only induces CD4+ T cells and, in the absence of CD8+ T
cells, these assume a Th2 cytokine profile (36). As anti-T1/
ST2 treatment was performed during the RSV challenge
(not during recombinant vaccinia priming), the induction
of a replacement Th1 population would not be anticipated.
The lack of an effect on IL-4 in lavage fluid of anti-T1/
ST2–treated, G/RSV-infected mice may indicate IL-4
production by an alternative cell type. CD4+NK1.1+ cells
secrete IL-4 but are T1/ST2+ (15) and would therefore not
be inhibited in our experimental design.

The reduced weight loss in the G/RSV group treated
with anti-T1/ST2 may be linked to lower TNF levels.
TNF has multiple actions and plays a critical role in inflam-
mation. TNF also plays an important role in recruitment of
cells to normal and inflamed tissues (39, 40) and may induce
early neutrophil and eosinophil recruitment (41). When re-
leased in large quantities, it enters the bloodstream and is as-
associated with weight loss and cachexia. TNF production is
seen predominantly, but not exclusively, in T1/ST2–CD4+
t cells (14), and the reduced levels of this cytokine may be
due in part to the decreased total inflammatory cell recruit-
ment seen with T1/ST2 depletion in this study. A reduc-
tion in S. mansoni–induced primary granuloma volume in
T1/ST2 knockout mice (24) is also consistent with the re-
duced inflammatory infiltrate seen in our study. No previous
reports show a decrease in TNF by T1/ST2 inhibition.
T1/ST2 depletion did not inhibit illness in G/RSV-
infected mice treated with IL-12 or those previously sensi-
tized with the F protein. As IL-12 treatment reduces T1/
ST2+ cells and such cells are scarce in F protein–sensitized
mice, anti-T1/ST2 treatment does not alter total cell re-
cruitment to the lung and weight loss remains unaltered.

An enhanced T cell–dependent antibody response occurs
in G/RSV-infected mice and promotes rapid viral clear-
ance compared with mice experiencing a primary infection
(β gal/RSV). Considering the reduced T cell recruitment
to the lung, it is not surprising that anti-T1/ST2 treatment
decreases RSV-specific antibody production and delays vi-
ral clearance. To our knowledge, there are no previous re-
ports of delayed clearance of a pathogen due to anti-T1/
ST2 treatment. As illness in this model does not depend on
uncontrolled viral replication but rather on the extent of
cell recruitment, the beneficial effects of T1/ST2 inhibition
are not offset by the delayed viral clearance. A reduction in
antibody has previously been observed in OVA-challenged
mice after T1/ST2 depletion (11) but not in T1/ST2
knockout animals infected with S. mansoni (24).

Studies of T1/ST2-knockout mice have produced con-
flicting results. One group reported that T1/ST2-deficient
animals fail to show decreased Th2 cytokine or Ig produc-
tion in response to Nippostrongylus brasiliensis infection or
allergen–induced airway inflammation (25). An indepen-
dently derived T1/ST2 knockout model, however, shows
that basal immunological functions are not affected but that
Th2 cytokine responses are impaired in a fashion similar to
that seen in depletion studies. The findings in the latter
study suggest that this molecule plays an important role in
the early events of Th2 development, as primary but not
Our results offer the prospect that selective anti-T1/ST2 T1/ST2 antibody treatment in selective Th2-directed inflammation is needed to define its role. In these studies, we driven illness in the clinical setting. T1/ST2 neutralization treatments have not been able to reliably counter Th2-inations. Th2 inhibition may be beneficial in both prevention approach to the treatment of various inflammatory condi-

but also harbor other developmental changes.

Inhibition of specific Th cell phenotypes is a promising approach to the treatment of various inflammatory conditions. Th2 inhibition may be beneficial in both prevention and management of allergic inflammation, but to date treatments have not been able to reliably counter Th2-driven illness in the clinical setting. T1/ST2 neutralization may offer a new therapeutic opportunity, but more information is needed to define its role. In these studies, we have been able to demonstrate a beneficial role for anti-

T1/ST2 antibody treatment in selective Th2-directed immunopathology in G-primed mice during RSV infection. Our results offer the prospect that selective anti-T1/ST2 treatment will be beneficial in Th2-driven conditions.

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