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Regulation and Function of T1/ST2 Expression on CD4$^+$ T Cells: Induction of Type 2 Cytokine Production by T1/ST2 Cross-Linking[1]

Christian Meisel,[2,3]* Kerstin Bonhagen,[2]* Max Löhning,* Anthony J. Coyle,[†] Jose-Carlos Gutierrez-Ramos,[†] Andreas Radbruch,* and Thomas Kamradt[4]*[‡]

The orphan receptor T1/ST2, a member of the IL-1R family, is preferentially expressed on the surface of murine Th2 cells. In this study, we analyzed the kinetics and function of T1/ST2 expression on Th2 cells in vitro. Whereas naive CD4$^+$ cells did not express T1/ST2, most CD4$^+$ cells became T1/ST2$^+$ upon repeated antigenic stimulation under Th2-polarizing conditions. Flow cytometric analyses revealed that the kinetics of T1/ST2 expression on Th2 cells was delayed compared with the kinetics of type 2 cytokine production. Exogenous IL-6, IL-5, IL-1, and TNF-α enhanced the expression of T1/ST2 on Th2 cells, and IL-6 was by far most effective in this regard. However, the expression of T1/ST2 did not depend on the presence of IL-6 and was also detected in IL-6-deficient mice. Most important, cross-linking of T1/ST2 provided a costimulatory signal for Th2 but not Th1 cells and directly induced proliferation and type 2 cytokine production. Thus, T1/ST2 is not only a Th2 cell marker but also plays an important role in the activation of Th2 cells. ***The Journal of Immunology,*** **2001, 166: 3143–3150.**

The discovery of functionally heterogeneous subsets of CD4$^+$ Th cells on the basis of their cytokine expression pattern (1) has provided important insights into the mechanisms of both protective and pathological immune responses. Originally, two subsets were described. Th1 cells produce mostly IFN-γ, IL-2, and TNF-β and mediate delayed-type hypersensitivity and protection against intracellular pathogens, whereas Th2 cells produce mainly IL-4 and IL-5 and are implicated in humoral and allergic immune responses (2, 3). Although there are many well-documented Th1 and Th2 immune responses, it is now widely accepted that Th cells can produce cytokine patterns different from the canonical Th1/Th2 pattern and that the classical Th1 and Th2 subsets represent the end points of a continuous differentiation process (2–5).

Th phenotype differentiation is influenced by a number of factors including different APC, MHC genes, non-MHC "background genes," costimulatory molecules, Ag dose or structure, and cytokines present during Th priming. The major factors for the induction of a Th1 or Th2 response seem to be IL-12 and IL-4, respectively (6, 7).

The transient nature of cytokine expression and the methodological limits of its detection for functional analysis of different Th cell subsets have stimulated an intensive search for further differences between Th1 and Th2 cells. The Th2-specific expression of two transcription factors, GATA3 and c-maf, and the Th1-specific expression of another transcription factor, T-bet (8), appear to represent, at least in part, the transcriptional basis for differential cytokine expression (9). In addition, some chemokine receptors such as CCR3, CCR4, and CCR8 have been reported to be predominantly expressed on Th2 cells, whereas others, such as CXCR 3 or CCR5, were found to be preferentially expressed on Th1 cells (10).

Recently, several groups, including ours, reported that T1/ST2, an orphan receptor with sequence homology to the IL-1R, is preferentially expressed on murine Th2 cells (11–14) and also on mast cells (15). T1/ST2 is important for Th2 effector functions since treatment with Abs against T1/ST2 decreased Th2 effector functions in vivo (11, 12, 16). Here, we analyzed the kinetics and functional importance of T1/ST2 expression on Th2 cells in vitro. We found that the kinetics of type 2 cytokine expression in Th2 cells is faster than that of T1/ST2 expression. In vitro, T1/ST2 expression is enhanced by proinflammatory cytokines. Importantly, cross-linking of T1/ST2 induces proliferation and cytokine production in Th2 but not Th1 cells. Our results suggest that T1/ST2 expression is a late event during Th cell commitment to the Th2 phenotype and that signaling through T1/ST2 specifically enhances Th2 effector functions.

Materials and Methods

Reagents

The OVA peptide $OVA_{323–339}$ was synthesized according to standard Fmoc machine protocols with a multiple peptide synthesizer (Abimed) and was kindly provided by A. Kramer (Humboldt University, Berlin). Mouse recombinant IL-1α and IL-1β, TNF-α, and IL-6 were purchased from TEBU (Frankfurt am Main, Germany). Recombinant murine IL-13, anti-CD28 (37.51), and rat IgG1 isotype control mAb were purchased from PharMingen (Hamburg, Germany). Recombinant IL-5 (X63IL5) at a specific activity of 158 U/μg and the hamster anti-CD3 (145-2C11) mAb were obtained from institutional facilities.

The recombinant baculovirus GP67T1S-Flag driving the synthesis of soluble murine T1/ST2 in insect cells was a gift from A. K. Werenskiold (Technische Universität München). Flag-tagged soluble T1/ST2 was purified from supernatants of GP67T1S-Flag baculovirus-infected Sf9 insect cells as described (17).

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Animals

Mice transgenic for the OVA-specific DO11.10 T cell Ag receptor (TCR) (18) were from D. Loh (Washington University, St. Louis, MO) and were maintained on the BALB/c background. IL-6-deficient C57BL/6 mice (19) and their heterozygous littermates were from T. Blankenstein (Max Delbrück Centrum, Berlin, Germany). Mice were kept under pathogen-free conditions in accordance with institutional and state guidelines.

Generation of Th1 and Th2 cells in vitro

CD4$^+$CD62L$^+$ spleen cells (SC)[5] from DO11.10 TCR-transgenic mice were isolated by high-gradient magnetic cell separation with MACS Multisort (Miltenyi Biotec, Bergisch Gladbach, Germany) as described elsewhere (12). SC from BALB/c depleted of T cells with CD4, CD8, and Thy1.2 microbeads (Miltenyi Biotech) were used as APC. Cells were cultured in complete RPMI 1640 containing 100 U/ml penicillin, 0,1 mg/ml streptomycin, 2 mM glutamine (Biochrom, Berlin, Germany), 25 μM 2-ME, and 10% FCS (Sigma, Deisenhofen, Germany). T cells were stimulated either with $OVA_{323–339}$ (0, 3 μM) and APC or with immobilized anti-CD3 (1 μg) and anti-CD28 (5 μg/ml). For Th1 phenotype development, recombinant murine IL-12 (gift from M. Gately, Hoffman-LaRoche, Nutley, NJ) and neutralizing anti-IL-4 mAb 11B11 (20) were added, and for Th2 development recombinant murine IL-4 (BioSource International, Camarillo, CA) and neutralizing anti-murine IL-12 polyclonal Ab (M. Gately) or, when indicated, recombinant murine IL-4 and neutralizing anti-IFN-γ mAb (RA-6A2 5 μg/ml) were added as described previously (12). In some experiments, mouse recombinant IL-1α and IL-β, TNF-α, IL-5, IL-6, and IL-13 at concentrations between 0.5 and 50 ng/ml were added as indicated. Three days after Ag stimulation, recombinant human IL-2 (100 U/ml) was added. Cultures were maintained for 7 days. In some experiments, several rounds of Ag stimulation and rest were performed as indicated. In the experiments of IL-6$^{-/-}$ mice and their IL-6$^{+/-}$ littermates, CD4$^+$ T cells were MACS isolated from peripheral lymph nodes and Thy1.2-depleted, irradiated SC were used as APC. Soluble anti-CD3 mAb (3 μg/ml) and anti-CD28 (2.5 μg/ml) were added to the culture. For polarization of Th2 cells, recombinant IL-4 (R&D Systems, Wiesbaden, Germany) and anti-IFN-γ mAb (5 μg/ml) and anti-IL-12 mAb (C17.15, 5 μg/ml) were added.

Abs and flow cytometry

For surface staining, the following rat anti-mouse mAbs were used: CD4 (GK1.5), CD8 (53-6.7), CD62L (L-selectin; MEL-14), DO11.10 TCR (KJ.26.1) (21), and T1/ST2 (3E10) (12). To prevent unspecific binding of the T1/ST2-specific mAb, all samples were preincubated with blocking anti-FcγR mAb 2.4G2/75 (100 μg/ml) and purified rat IgG (200 μg/ml; Dianova, Hamburg, Germany) 10 min before and during staining with digoxigenized 3E10 (1.5 μg/ml), FITC-conjugated 3E10 (3 μg/ml), or biotinylated 3E10 (3 μg/ml). Digoxigenized 3E10-labeled cells were detected by anti-digoxigenin (DIG) Fab fragments (Boehringer Mannheim, Mannheim, Germany) conjugated to PE or Cy5; biotinylated 3E10 was detected with streptavidin-PE (PharMingen). Staining of 3E10 was blocked by preincubating the cells with a 100-fold excess of unconjugated 3E10. Samples were analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA). Gates were set on viable cells according to forward and sideward scatter and exclusion of propidium iodide-binding particles (0.3 μg/ml).

Analysis of cytokine production by flow cytometry and ELISA

Analysis of intracellular cytokines and surface markers were performed as described elsewhere (12). Cells (10^6/ml) were stimulated with PMA (5 ng/ml) and ionomycin (0.5 μg/ml; Sigma) for 5 h. At 2 h, brefeldin A (Sigma) was added at 5 μg/ml. Before fixation, cells were stained with biotinylated mAb against DO11.10 TCR (KJ26.1) and digoxigenized mAb 3E10 against T1/ST2. Cells from IL-6$^{-/-}$ or IL-6$^{+/-}$ mice were stained before fixation with anti-CD4 FITC. Stained cells were fixed with 2% formaldehyde. DO11.10 TCR and T1/ST2 were detected on the surface of fixed cells by streptavidin coupled to PerCP (PharMingen) and anti-DIG Fab fragments (Boehringer Mannheim) conjugated to Cy5, respectively. Cells were permeabilized and incubated with two of the following mAbs (PharMingen): anti-IL-4-PE (1D11; 3 μg/ml), anti-IL-4-FITC (1D11; 5 μg/ml), anti-IL-4 DIG (11B11, 1.3 μg/ml), anti-IL-5-PE (TRFK5; 3 μg/ml), anti-IL-6-PE (MP5-20F3, 2 μg/ml; PharMingen), anti-IL-10-FITC (5 μg/ml), or anti-IFN-γ-FITC (XMG1.2, 5 μg/ml), followed where indicated by incubation with Cy5-conjugated anti-DIG Fab fragments. FITC- or PE-labeled isotype control mAbs (PharMingen) were used at 5 and 3 μg/ml, respectively. Samples were analyzed by four-color flow cytometry. Concentrations of IL-4, IL-5, and IL-10 in culture supernatants were determined by ELISA as described previously (12). IFN-γ was determined with a commercially available kit according to the manufacturer's instructions (Genzyme, Cambridge, MA). Lower detection limits for each ELISA were as follows: IL-4 and IFN-γ, 40 pg/ml; IL-5, 10 U/ml; and IL-10, 0.3 ng/ml.

Measurement of T cell proliferation and cytokine expression

mAbs were immobilized to 96-well round-bottom cell culture plates (Costar, Bodenheim, Germany) in a final volume of 0.15 ml PBS for 2 h at 37°C. Ten to 12 days after the last Ag stimulation, 1×10^5 T cells/well were cultured in complete medium at 37°C in 5% CO_2. Supernatants were collected at 48 h for analysis by sandwich ELISA. Proliferation was measured by 16-h incorporation of 1 μCi [^{3}H]thymidine on day 3. Results were calculated from triplicate cultures and expressed as the mean ± SEM.

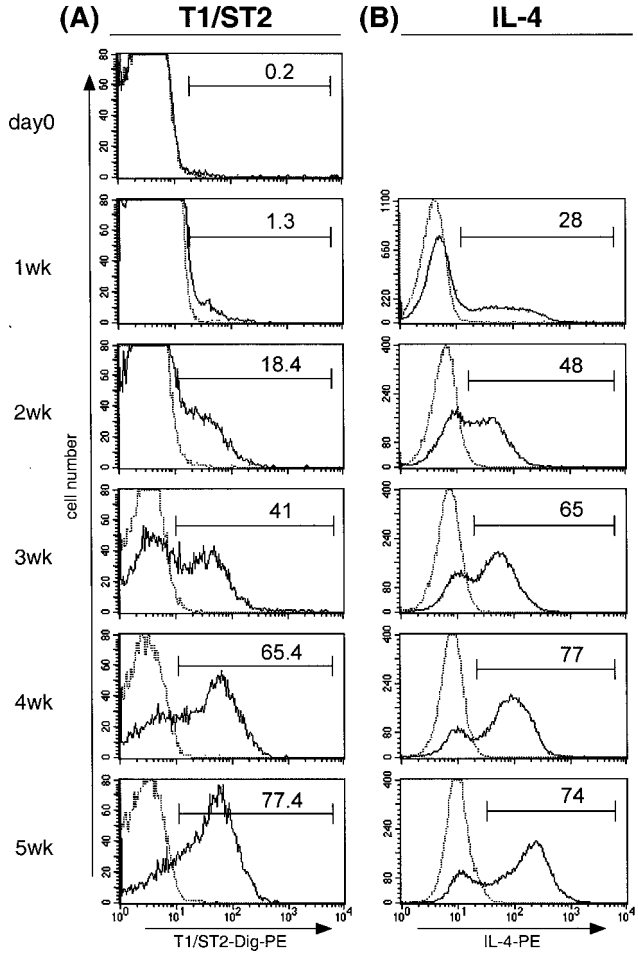

FIGURE 1. Kinetics of T1/ST2 expression is delayed compared with IL-4 expression in vitro. Purified naive CD4$^+$CD62L^{high} T cells from DO11.10 TCR-transgenic mice were stimulated in vitro with peptide and T cell-depleted BALB/c SC in the presence of IL-4 and anti-IL-12 for several rounds. Seven days after each Ag stimulation, the percentage of T1/ST2$^+$- and IL-4-producing DO11.10 TCR$^+$ T cells were determined. *A*, Aliquots of cells were stained with mAbs against DO11.10 TCR and T1/ST2 (solid line) or isotype control mAb (dotted line). Gates were set on viable cells as described in *Material and Methods*. *B*, Aliquots of cells were stimulated with PMA/ionomycin, stained with biotinylated clonotypic anti-DO11.10 TCR mAb (KJ26.1), and then fixed. KJ26.1$^+$ T cells were detected with streptavidin coupled to PerCP. Subsequently, intracellular cytokine staining was performed with anti-IL-4-PE mAb (solid line) or isotype control mAb (dotted line). The numbers given in *A* and *B* indicate the percentage of T1/ST2$^+$- and the percentage of IL-4-producing DO11.10 TCR$^+$ T cells, respectively. Data are representative of three independent experiments.

[5] Abbreviations used in this paper: SC, spleen cell; DIG, digoxigenin.

Table I. *Kinetics of T1/ST2 and cytokine expression in Th2 cells in vitro*[a]

Week	T1/ST2[b]	IL-4	IL-10	IL-5
1	1.5 ± 0.8	28.1 ± 5.7	4.9 ± 1.4	0.5 ± 0.4
2	25.2 ± 5.4	51.9 ± 8.7	22.9 ± 1.2	0.7 ± 0.2
3	44.1 ± 4.3	67.1 ± 7.6	42.4 ± 5	2.9 ± 1.7
4	69.3 ± 5.1	79.4 ± 3.4	48.2 ± 6.8	4.2 ± 1
5	81.6 ± 5.9	76.6 ± 3.7	31 ± 2.8	6.8 ± 1.1

[a] Naive DO11.10 TCR$^+$ CD4$^+$ T cells were primed under Th2-polarizing conditions for several rounds and analyzed for T1/ST2 and cytokine expression 7 days after each Ag stimulation as described for Fig. 1.

[b] The numbers given are the percentages of T1/ST2-positive and cytokine-producing DO11.10 TCR$^+$ T cells expressed as mean ± SEM of three independent experiments at the indicated time points after priming.

Statistical analysis of intracellular cytokine coexpression and surface T1/ST2

The observed value for cytokine-coexpressing cells in percent was compared with the expected value calculated for random coincidence of two independent variables. Correlations of cytokine coexpression in the total DO11.10 TCR$^+$ CD4$^+$, T1/ST2$^+$, and T1/ST2$^-$ DO11.10 TCR$^+$ CD4$^+$ T cells were calculated using the test for ϕ correlation coefficients (22) as described elsewhere (13). Coefficients of $\phi \leq -0.1$ or ≥ 0.1 were considered to be significant in this analysis.

Results

Kinetics of T1/ST2 expression on Th2 cells in vitro is delayed compared with IL-4 production

To determine the kinetics of T1/ST2 expression on Th2 cells naive (CD62L^{high}) CD4$^+$ spleen cells from DO11.10 TCR-transgenic mice were subjected to several rounds of in vitro Ag stimulation and rest. T cells were stimulated with OVA peptide ($OVA_{323–339}$) and APC in the presence of IL-4 plus anti-IL-12. Seven days after each Ag-stimulation, T1/ST2 expression on DO11.10-TCR$^+$ cells and intracellular cytokine expression upon restimulation with PMA/ionomycin was analyzed by flow cytometry. As shown in Fig. 1*A*, the number of T1/ST2$^+$ cells increased during continual Ag stimulation under Th2-polarizing conditions. In contrast, neither Th1-polarized T cells (IL-12 plus anti-IL-4) nor T cells primed with peptide alone expressed T1/ST2 (Ref. 16 and data not shown). Following priming under Th2-polarizing conditions, the expression of T1/ST2 was not dependent on further addition of

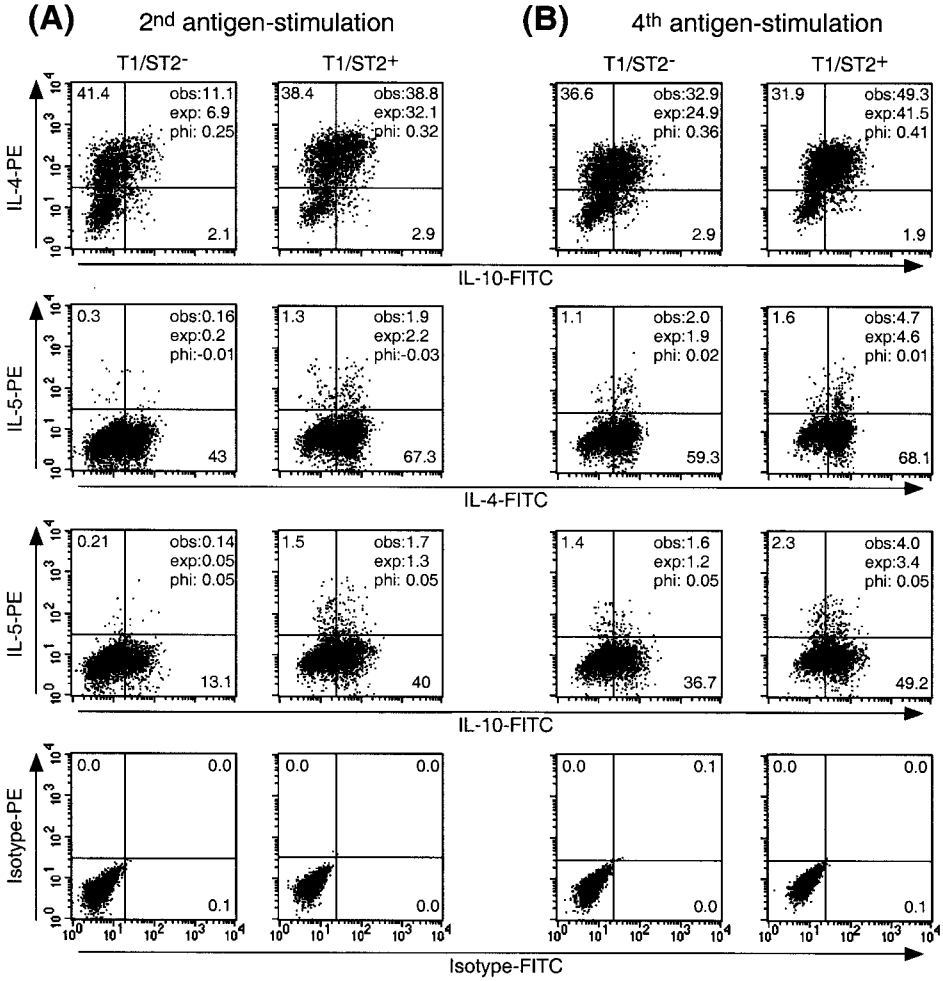

FIGURE 2. Cytokine coexpression in T1/ST2$^+$ and T1/ST2$^-$ in vitro-polarized Th2 cells. Purified naive CD4$^+$CD62L^{high} T cells from DO11.10 TCR-transgenic mice were stimulated as described in Fig. 1. After the second and fourth Ag stimulation, cells were restimulated with PMA/ionomycin, stained with biotinylated clonotypic mAb (KJ26.1), digoxigenized mAb against T1/ST2 (3E10), and then fixed. DO11.10 TCR and T1/ST2 were detected by streptavidin coupled to PerCP and anti-DIG Fab fragments conjugated with Cy5, respectively. Subsequently, intracellular cytokine staining was performed simultaneously using FITC- or PE-conjugated anti-cytokine mAbs or isotype control mAbs. Gates were set on 5% of DO11.10 TCR$^+$ cells which stained most brightly or dim for T1/ST2. The frequency of cytokine-producing cells is indicated in the quadrants in percent. In the *upper right quadrants*, the observed frequency (obs) of cytokine-coexpressing cells, the expected frequency (exp), and the ϕ (phi) correlation coefficient are given. Data are representative of three independent experiments.

exogenous IL-4 during the subsequent rounds of restimulation and rest as restimulation with peptide alone resulted in the same kinetics of T1/ST2 expression. Even when endogenous IL-4 was neutralized with mAbs during restimulation, a delayed yet steady increase of T1/ST2 expression was observed (data not shown).

The kinetics of T1/ST2 expression on in vitro Th2-polarized T cells was delayed compared with type 2 cytokine expression. One week after priming, 1.5 ± 0.8% (mean ± SD of three independent experiments) of the T cells were T1/ST2$^+$, whereas 28.1 ± 5.7% produced IL-4 upon PMA/ionomycin stimulation at that time point (Fig. 1 and Table I). Thus, 1 wk after priming most IL-4 producers were T1/ST2 negative. After the second round of Ag stimulation and rest IL-4 was produced by 51.9 ± 8.7% of T cells, whereas 25.2 ± 5.4% of the T cells expressed T1/ST2 (Table I). Over the course of repeated antigenic stimulation, the percentage of T1/ST2$^+$ Th cells gradually approached the percentage of IL-4 producers. After four to five rounds of in vitro Ag stimulation and rest, the percentages of OVA-TCR$^+$ cells expressing T1/ST2 or IL-4 were approximately equal (79.4 ± 3.4 IL-4 producers and 69.3 ± 5.1 T1/ST2$^+$ cells after 4 wk; 76.6 ± 3.7 IL-4 producers and 81.6 ± 5.9 T1/ST2$^+$ cells after 5 wk). The frequency of IL-10-producing cells (4.9 ± 1.4) 1 wk after priming was higher than that of T1/ST2$^+$ cells (Table I). However, already after the second Ag stimulation, the percentages of OVA-TCR$^+$ cells expressing T1/ST2 or IL-10 were approximately equal. IL-5 expression was low throughout the experiment and ranged from 0.1 to 7.9% positive cells. The percentage of cytokine-producing cells was always higher in the T1/ST2$^+$ population than in the T1/ST2$^-$ population (Fig. 2), although the percentage of T1/ST2$^+$ cells was initially low (Table I and Fig. 2).

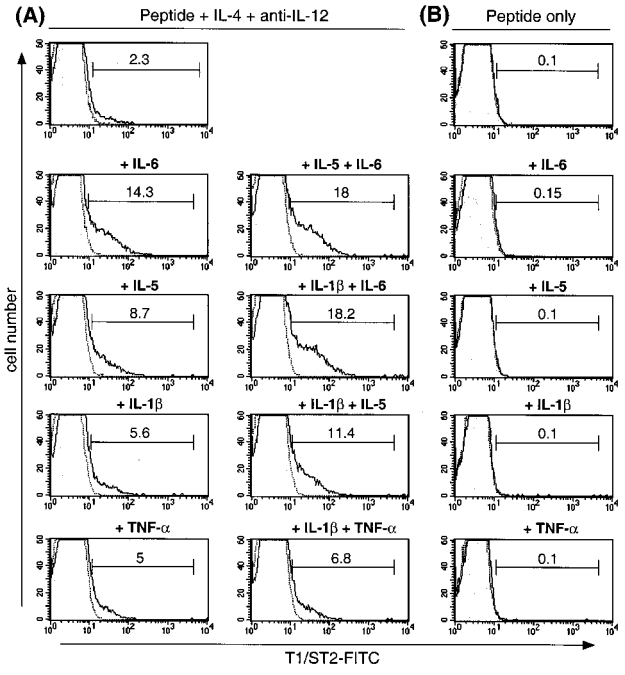

FIGURE 3. Effects of cytokines on T1/ST2 expression in vitro. Purified naive CD4$^+$CD62L^{high} T cells from DO11.10 TCR-transgenic mice were primed under Th2-polarizing conditions (*A*; plus IL-4 and anti-IL-12) or under neutral conditions with peptide only (*B*) in the presence or absence of mouse recombinant IL-1β, TNF-α, IL-5, and IL-6 (20 ng/ml) as indicated. Seven days after Ag stimulation, cells were stained with FITC-conjugated mAb against T1/ST2 (solid line) or isotype control mAb (dotted line). Gates were set on live cells as described in *Materials and Methods*. The results are representative of at least three independent experiments.

Cytokine coexpression in Th2 cells

Naive DO11.10 TCR$^+$ CD4$^+$ T cells were primed under Th2-polarizing conditions for several rounds. Seven days after each Ag stimulation, an aliquot of the cells was restimulated with PMA and ionomycin and stained for T1/ST2 and intracellular cytokines. To determine whether coexpression of type 2 cytokines was stochastic or coordinate, the observed frequencies of cytokine-coexpressing cells were compared with those calculated for expected values (i.e., random coincidence). The observed values for cytokine expression and the correlation coefficients for cytokine coexpression in T1/ST2$^-$ and T1/ST2$^+$ DO11.10 TCR$^+$ T cells are shown in Fig. 2. After two rounds of Ag stimulation, the production of IL-4 was strongly associated with IL-10 production in both the T1/ST2$^+$ and T1/ST2$^-$ fractions (Fig. 2*A*: ϕ 0.32 in T1/ST2$^+$ cells and ϕ 0.25 in T1/ST2$^-$ cells). Similar values were found after the fourth Ag stimulation (Fig. 2*B*). In contrast, although IL-5 producers were enriched in the T1/ST2$^+$ fraction (3.2% in T1/ST2$^+$ cells and 0.46% in T1/ST2$^-$ cells after 2 wk and 6.3% in T1/ST2$^+$ cells and 3.1% in T1/ST2$^-$ cells after 4 wk), coexpression of IL-5 with either IL-4 or IL-10 was random in both T1/ST2$^+$ and T1/ST2$^-$ cells (Fig. 2). The coexpression of type 1 and type 2 cytokines was not determined since there were very few if any IFN-γ- or IL-2-producing Th cells in these in vitro-generated Th2 lines.

IL-6, IL-1, TNF-α, and IL-5 increase T1/ST2 expression

We next asked whether cytokines produced by either APC or T cells influence T1/ST2 expression. Naive (CD62L^{high}) CD4$^+$ splenic T cells from DO11.10-TCR$^+$ mice were stimulated with peptide and T cell-depleted BALB/c SC under Th2-polarizing conditions in the presence or absence of recombinant TNF-α, IL-1β, IL-5, IL-6, or IL-13. In preliminary experiments, the optimal concentrations for each cytokine were determined. After 7 days, cells were counted and stained for T1/ST2. Expression of T1/ST2 was enhanced most effectively by IL-6 and to a lesser extent by IL-5, IL-1β, and TNF-α. The number of T1/ST2$^+$ cells was increased ~6-fold (6.1 ± 0.8, mean ± SEM, data from at least three independent experiments) by IL-6; 3-fold (3 ± 0.4) by IL-5; and 2-fold

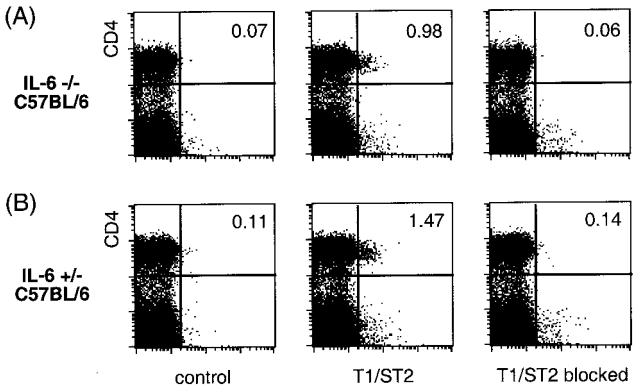

FIGURE 4. Expression of T1/ST2 in IL-6$^{-/-}$ mice. Spleen cells from IL-6$^{-/-}$ C57BL/6 mice and their heterozygous littermates were stained with digoxigenized mAbs against T1/ST2 (3E10), followed by Cy5-conjugated anti-DIG and PE-conjugated mAb against CD4. Gates were set on viable cells as described in *Materials and Methods*. All samples were incubated with blocking anti-FcγR mAb and purified rat IgG before and during staining with 3E10. The percentages shown indicate the frequency of T1/TS2$^+$ cells within the CD4$^+$ population. The control panel shows the fluorescence signal of anti-DIG-Cy5 alone. To verify its specificity, the staining of 3E10 was blocked by preincubation of the cells with a 100-fold excess of unconjugated 3E10, which reduced the subsequent 3E10 staining on CD4$^+$ cells to control levels in both IL-6$^{-/-}$ and IL-6$^{+/-}$ mice.

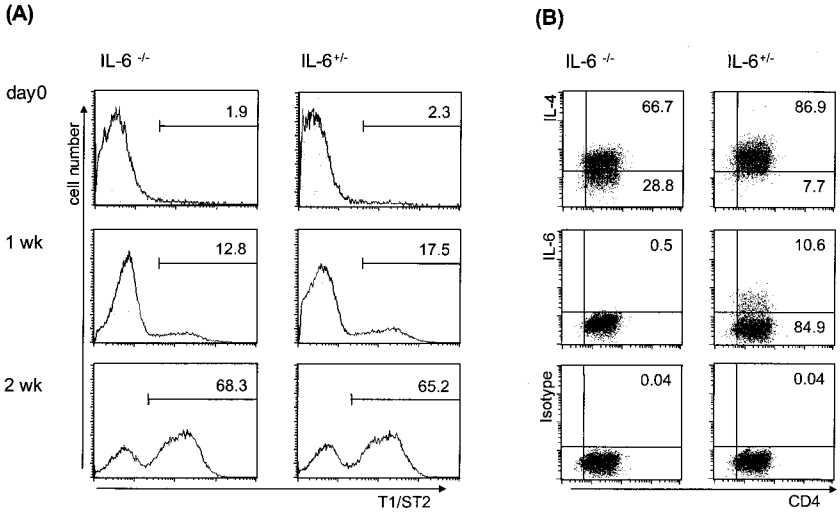

FIGURE 5. Kinetics of T1/ST2 expression and IL-4 production is identical in IL-6$^{-/-}$ and IL-6$^{+/-}$ mice. Purified CD4$^+$ T cells from peripheral lymph nodes of IL-6$^{-/-}$ mice or their IL-6$^{+/-}$ littermates were polyclonally stimulated with anti-CD3 and anti-CD28 and Thy 1.2-depleted SC in the presence of IL-4, anti-Il-12, and anti-IFN-γ for two rounds. T1/ST2 surface expression was determined at day 0 and after the first and second restimulations, respectively. *A,* Aliquots of cells were stained with mAbs against CD4 and T1/ST2 (solid line). The filled histograms show the T1/ST2 staining after preincubation with a 100-fold excess of unlabeled 3E10 mAb. Gates were set on viable cells. The numbers indicate the percentage of T1/ST2$^+$ CD4$^+$ T cells. *B,* Aliquots of cells were stimulated with PMA/ionomycin after 2 wk of culture. Cells were stained with anti-CD4 mAb before fixation. Intracellular cytokine staining was performed with anti-IL-4 DIG followed by an incubation with Cy5-conjugated anti-DIG Fab fragments and with anti-IL-6 PE. Data are representative of two independent experiments.

(2.1 $\pm$ 0.3) by IL-1β or TNF-α (Fig. 3). Studies were also performed with IL-1α and virtually identical results were obtained as with IL-1β (data not shown). The effect of IL-6 on T1/ST2 expression was detectable even at a concentration as low as 500 pg/ml. IL-13 had no effect on T1/ST2 expression at any of the tested concentrations (0.5–50 ng/ml, data not shown). Combinations of different cytokines had additive but not synergistic effects on T1/ST2 expression (Fig. 3). The cytokine-induced increase in T1/ST2 expression was not due to enhanced T cell proliferation or survival, since similar numbers of viable T cells were recovered from cytokine-treated and control cultures.

Although IL-6 strongly enhanced T1/ST2 expression on Th2 cells, it was not required for T1/ST2 expression: as shown in Fig. 4, the frequency of CD4$^+$ T1/ST2$^+$ cells in IL-6$^{-/-}$ mice was similar compared with their heterozygous littermates (~0.9 and 1.3%, respectively, Fig. 4). Moreover, when lymph node cells from IL-6$^{-/-}$ mice or IL-6$^{+/-}$ heterozygous littermates were polyclonally stimulated in vitro, the kinetics of both IL-4 production and surface expression of T1/ST2 were identical (Fig. 5).

In contrast, when naive CD4$^+$ T cells were primed with Ag in the presence of anti-IL-12 but in the absence of exogenous IL-4, Th2 phenotype development did not occur: only 0.2% of OVA-TCR$^+$ cells produced IL-4 and 7% produced IFN-γ. Under those conditions, none of the investigated cytokines induced Th2 phenotype development or T1/ST2 expression (Fig. 3 and data not shown).

To test directly the importance of APC for T1/ST2 induction, naive splenic CD4$^+$ T cells from DO11.10-TCR$^+$ mice were stimulated with immobilized anti-CD3 plus anti-CD28 mAb in the presence of IL-4 and anti-IFN-γ mAb for three rounds of stimulation and rest. The differentiation of T cells under these conditions resulted in similar kinetics of T1/ST2 expression as observed with Ag stimulation in the presence of APC (Fig. 6). Thus, T1/ST2 expression is neither dependent on the presence of APC-derived cytokines, nor on cell-cell contact between T cells and APC.

Cross-linking of T1/ST2 costimulates Th2 activation by suboptimal concentrations of anti-CD3 and induces Th2 proliferation and cytokine production in the absence of any further stimuli

In a murine model of Th2-dependent allergic airway inflammation, administration of either anti-T1/ST2 mAb or T1/ST2-Ig fusion protein inhibited the secretion of Th2 cytokines and infiltration of eosinophils into the airways (12, 16). We therefore hypothesized

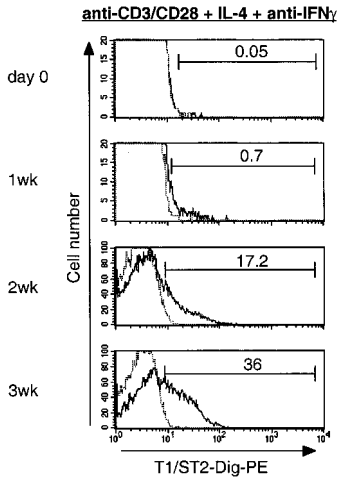

FIGURE 6. T1/ST2 expression is independent of APC. MACS-purified naive CD4$^+$CD62L^{high} T cells from DO11.10 TCR-transgenic mice were stimulated with immobilized anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) in the presence of IL-4 and anti-IFN-γ (5 μg/ml, R4-6A2) once weekly over a period of 3 wk. Seven days after each stimulation, the percentage of T1/ST2$^+$ DO11.10 TCR$^+$ T cells was determined. Cells were stained with mAbs against DO11.10 TCR and T1/ST2 (solid line) or isotype control mAb (dotted line). Gates were set on viable cells as described in *Materials and Methods*. The results shown are from one of two similar experiments.

that T1/ST2 could act as a costimulatory molecule for Th2 responses. To test whether T1/ST2 influences T cell proliferation and cytokine production, we examined the effects of T1/ST2 cross-linking on APC-independent and Ag-specific T cell activation in vitro. In vitro-polarized DO11.10 TCR$^+$ Th2 cells were used for proliferation and cytokine assays. Cross-linking of T1/ST2 enhanced proliferation (3- to 6-fold) as well as IL-4 and IL-5 production (4- to 10-fold) of Th2 cells that were stimulated with suboptimal concentrations of anti-CD3 mAb (<100 ng/ml, Fig. 7*A*) alone or in combination with anti-CD28 mAb (data not shown).

At optimal anti-CD3 mAb concentrations (≥100 ng/ml), T1/ST2 cross-linking did not further increase the proliferation or type 2 cytokine production induced by anti-CD3 (Fig. 7*A*) or anti-CD3/anti-CD28 (data not shown). In contrast, immobilized anti-T1/ST2 mAb alone or in combination with anti-CD3 had no effect on proliferation and cytokine production by Th1 cells (Fig. 7*B*).

When in vitro-polarized DO11.10 TCR$^+$ Th2 cells were restimulated with APC and OVA, cross-linking of T1/ST2 enhanced IL-4 production of Th2 cells that were stimulated with suboptimal concentrations of OVA. Furthermore, cross-linking T1/ST2 strongly induced Th2 cell proliferation that was not further enhanced by the addition of OVA (Fig. 7*C*).

Immobilized but not soluble anti-T1/ST2 mAb 3E10 alone induced proliferation and IL-4 and IL-5 production in a dose-dependent manner (Fig. 8). Both proliferation and cytokine production were significantly inhibited by a 4-fold molar excess of soluble recombinant T1/ST2. Proliferation was not inhibited in the presence of neutralizing anti-IL-4 mAb or anti-IL-2 mAb (data not shown).

Discussion

The orphan receptor T1/ST2, a member of the IL-1R family, is preferentially expressed on the surface of murine Th2 cells and important for Th2 effector functions in vivo (11–14, 16, 23). In CD4$^+$ spleen and lymph node cells from nonimmunized healthy mice, most IL-4 and IL-10 producers and the vast majority of IL-5

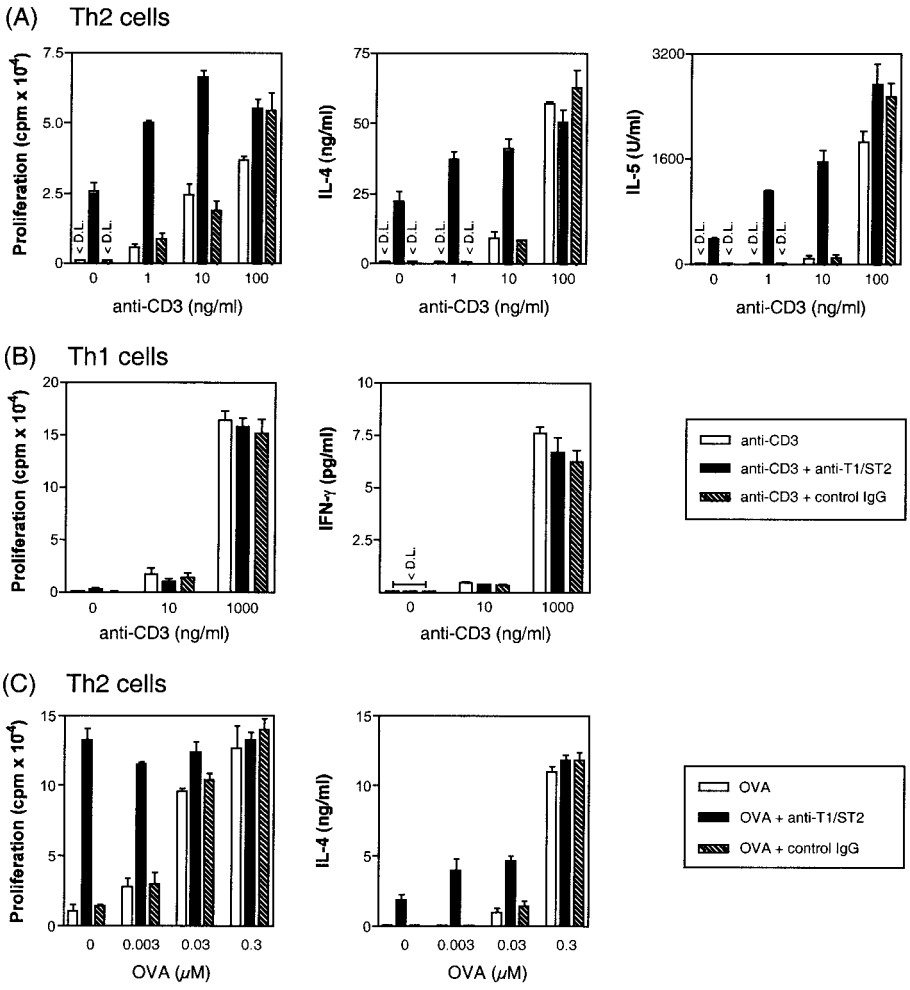

FIGURE 7. Cross-linking of T1/ST2 in vitro enhances anti-CD3-induced Th2 but not Th1 cell activation. Purified naive CD4$^+$CD62L^{high} T cells from DO11.10 TCR-transgenic mice were Ag stimulated under Th1-or Th2-polarizing conditions as described in *Materials and Methods*. For polyclonal restimulation (*A* and *B*), Th1 and Th2 cells were used 12 days after the second and third antigenic stimulation, respectively. At that time point, 72% of Th2 cells expressed T1/ST2, whereas Th1 cells were T1/ST2 negative as determined by FACS. Cells (1×10^5/well) were cultured in 96-well round-bottom microplates that had been coated with the indicated doses of anti-CD3 mAb plus 20 μg/ml anti-T1/ST2 mAb or isotype control mAb. Supernatants were collected after 48 h for cytokine analysis by ELISA and proliferation was measured by 16-h incorporation of tritiated thymidine on day 3. Data shown are the means and SEM of triplicate wells from one of four similar experiments. For Ag-specific restimulation (*C*), Th2 cells were used 10 days after the fourth antigenic stimulation. At that time point, 69% of the Th2 cells expressed T1/ST2 as determined by FACS. Th2 cells (1×10^5/well) were then cultured with irradiated T cell-depleted SC from BALB/c mice (2×10^5/well) in the absence or presence of OVA at the indicated concentrations in 96-well round-bottom microplates that had been coated with anti-T1/ST2 mAb (5 μg/ml) or isotype control mAb (5 μg/ml), respectively. Cytokine production and proliferation were determined as described above.

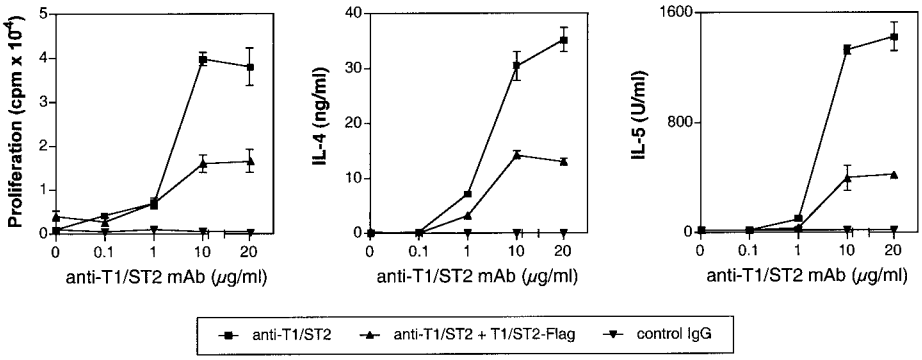

FIGURE 8. Cross-linking of T1/ST2 in vitro directly induces proliferation and cytokine production in Th2 cells. Experimental settings and Th2 cells used were as described for Fig. 7. Cells (1×10^5/well) were cultured in 96-well round-bottom microplates that had been coated with the indicated concentrations of anti-T1/ST2 (3E10) mAb or isotype control mAb in the presence or absence of 20 μg/ml recombinant T1/ST2-Flag-tagged protein. Supernatants were collected after 48 h for cytokine analysis by ELISA and proliferation was measured by 16-h incorporation of tritiated thymidine on day 3. Data shown are the means and SEM of triplicate wells from one of four similar experiments.

producers were T1/ST2$^+$ (12). In contrast, a sizable fraction of type 2 cytokine-producing CD4$^+$ T cells obtained directly ex vivo from schistosome egg-induced granulomas was T1/ST2 negative (13). Therefore, we hypothesized that during Th2 cell differentiation type 2 cytokine production precedes surface expression of T1/ST2 and that T1/ST2 expression is indicative of advanced Th2 commitment. Our current data support this hypothesis. One week after in vitro priming under Th2-polarizing conditions, the majority of IL-4 and IL-10 producers did not express T1/ST2. Over the course of repeated cycles of Ag stimulation and rest, the percentage of T1/ST2$^+$ cells increased constantly and after 4 wk in culture almost all type 2 cytokine producers were T1/ST2. Thus, it is possible that factors in addition to those required for the induction of type 2 cytokine production are necessary for T1/ST2 expression.

We therefore assessed the potential of several APC- or T cell-derived cytokines to increase T1/ST2 expression during Th2 effector differentiation. The strongest effect (up to 8-fold increase of T1/ST2 expression) was seen with IL-6. Other cytokines, e.g., IL-1, TNF-α, and IL-5, had reproducible but smaller effects on T1/ST2 expression. Nevertheless, the induction of T1/ST2 on Th2 cells is independent of IL-6 since the frequency of T1/ST2$^+$ CD4$^+$ cells is similar in IL-6$^{-/-}$ mice and their heterozygous littermates (Fig. 4). Our data also show that APC are not necessary for T1/ST2 induction in vitro since virtually the same T1/ST2 expression kinetics were observed in T cells stimulated either with peptide/APC or with anti-CD3/anti-CD28 under Th2-polarizing conditions.

What then, could be the significance of the enhanced T1/ST2 expression by APC-derived cytokines such as IL-1, TNF-α, and IL-6? We have shown recently that T1/ST2-Ig fusion protein inhibited Th2 phenotype development in vitro. In contrast, inhibition of T1/ST2 failed to modify IFN-γ production when cells were primed in the presence of IL-12 (16). These results suggest, that inhibition of T1/ST2 signaling results in skewing of the immune response from a Th2 to a Th1 phenotype. It is, therefore, tempting to speculate that in vivo depending on the pathogen-induced polarizing cytokine environment, the up-regulation of T1/ST2 expression on T cells by cytokines such as IL-6 favors the differentiation of naive Th cells into Th2 cells. It is clear, however, that Th2 development can occur in vivo even in the absence of T1/ST2 (23–25).

The mechanisms by which IL-6 and other cytokines increase T1/ST2 expression remain elusive. IL-1, TNF-α, and in particular IL-6 can function as growth and/or survival factors for Th2 cells in vitro and in vivo (26, 27). Therefore, the increased frequency of T1/ST2$^+$ cells observed in the presence of exogenous IL-1, TNF-α, and IL-6 could be due to enhanced T cell proliferation. However, we did not observe an increased proliferation in the presence of these cytokines, suggesting a direct effect on T1/ST2 gene expression. In vitro, but not in vivo, Th2 differentiation strictly depends on the presence of IL-4 (28). It was therefore not surprising that none of the cytokines investigated here induced T1/ST2 expression or enhanced IL-4 expression in naive Th cells primed in the absence of exogenous IL-4. In vivo, however, IL-4 is dispensable for the induction of T1/ST2 expression, which is unperturbed in IL-4 knockout mice (12, 14).

The coexpression of different type 2 cytokines in vitro differs somewhat from the coexpression patterns during a Th2-dominated immune response in vivo. In pulmonary granulomas induced by schistosome eggs, the production of IL-4 was positively correlated with IL-10 production in T1/ST2$^+$ but not in T1/ST2$^-$ CD4$^+$ cells (13). This positive association was even stronger in T1/ST2$^+$ cells which did not produce IL-5 concomitantly (our unpublished observations). In vitro, however, we found a strong positive correlation between IL-4 and IL-10 production in both T1/ST2$^+$ and T1/ST2$^-$ subsets at any time point of in vitro culture, although consistently higher ϕ correlation values were found in those cells stained most brightly for T1/ST2 (5% brightest cells, Fig. 2). In contrast, we found random coexpression of IL-4 and IL-5 as well as of IL-5 and IL-10, again in both T1/ST2$^+$ and T1/ST2$^-$ cells. Thus, under Th2-polarizing conditions in vitro, the cytokine coexpression patterns of T1/ST2$^+$ or T1/ST2$^-$ CD4$^+$ cells do not differ significantly.

Using anti-T1/ST2 mAb as a surrogate ligand, we have investigated the functional consequences of T1/ST2 receptor triggering in vitro. Plate-bound, but not soluble anti-T1/ST2 mAb 3E10 increased both proliferation and IL-4 and IL-5 production of Th2 cells stimulated with suboptimal concentrations of either anti-CD3 alone or CD3 in combination with anti-CD28, or with APC and antigenic peptide. These results are in accordance with our recent findings in a murine model of bronchial hyperreactivity where administration of either T1/ST2-Ig fusion protein or anti-T1/ST2 mAb before Ag challenge attenuated eosinophilic airway inflammation and reduced type 2 cytokine production (12, 16). It should be noted that administration of the 3E10 mAb in vivo did not result in the depletion of Ag-specific Th2 cells, suggesting that the reduction of Th2 effector function by anti-T1/ST2-treatment as well as by T1/ST2-Ig fusion protein was due to inhibition of T1/ST2 signaling by preventing its putative ligand from binding to the T1/ST2 receptor. This may explain the apparently different effects of the anti-T1/ST2 mAb observed in vivo and in vitro.

In addition to its costimulatory effects, plate-bound mAb 3E10 induced proliferation and cytokine production of Th2 cells in the absence of any further stimuli. Similar findings were reported by Cocks et al. (29), showing that ligation of the signaling lymphocytic activation molecule in the absence of TCR triggering induced proliferation and cytokine production in T cell lines and clones. The mechanisms by which T1/ST2 triggers proliferation and cytokine production of Th2 cells are currently unknown. Using a chimeric IL-1R type I expressing the intracellular domain of T1/ST2, Mitcham et al. (30) observed IL-1-like signaling including NF-κB translocation into the cell upon binding of IL-1, demonstrating not only structural but also functional homology of T1/ST2 with the IL-1RI molecule. Whether T1/ST2 like several other IL-1R family members utilizes a signaling pathway using MyD88, IRAK, TRAF6, or c-Jun N-terminal kinase (31) remains to be assessed.

In summary, we have shown that the production of type 2 cytokines precedes the expression of T1/ST2 in Th2 cells polarized in vitro. Cytokines such as IL-6 can up-regulate T1/ST2 expression. Most important, cross-linking of T1/ST2 provides a costimulatory signal for Th2 but not Th1 cells and directly induces proliferation and type 2 cytokine production. Thus, T1/ST2 is not only a Th2 cell marker but also plays an important role in the activation of Th2 cells.

Acknowledgments

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