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# T1/ST2 Expression Is Enhanced on CD4<sup>+</sup> T Cells from Schistosome Egg-Induced Granulomas: Analysis of Th Cell Cytokine Coexpression Ex Vivo<sup>1</sup>

Max Löhning,<sup>2\*</sup> Jane L. Grogan,<sup>2\*</sup> Anthony J. Coyle,<sup>†</sup> Maria Yazdanbakhsh,<sup>‡</sup> Christian Meisel,<sup>\*§</sup> Jose-Carlos Gutierrez-Ramos,<sup>†</sup> Andreas Radbruch,<sup>\* and</sup> Thomas Kamradt<sup>3\*§</sup>

Th cells are categorized into subsets based on the cytokine production of in vitro-differentiated Th populations. For in vivo-differentiated Th subsets, little is known about the heterogeneity of cytokine production in single cells. We recently described a molecule, T1/ST2, that is preferentially expressed on the surface of Th2 cells. Here we combined high-gradient magnetic cell separation with four-color single-cell cytometry to analyze simultaneously three intracellular cytokines and T1/ST2 surface expression on CD4<sup>+</sup> cells from lungs containing granulomas induced by *Schistosoma mansoni* eggs. T1/ST2 was highly up-regulated on CD4<sup>+</sup> T cells from hepatic granulomas and granulomatous lungs. T1/ST2<sup>+</sup> cells from granulomatous lungs preferentially produced type 2 cytokines ex vivo. In the total CD4<sup>+</sup> population, coexpression of type 1 and type 2 cytokines occurred frequently. However, such coproduction was drastically reduced in T1/ST2<sup>+</sup> cells compared with T1/ST2<sup>-</sup> cells. Coexpression of type 1 and type 2 cytokines was also rare in cells simultaneously producing two cytokines of one type. These findings indicate that individual CD4<sup>+</sup> T cells in vivo have different levels of commitment to a certain Th phenotype. Coexpression of two type 2 cytokines or production of one type 2 cytokine together with surface expression of T1/ST2 indicate advanced commitment to the Th2 phenotype. *The Journal of Immunology*, 1999, 162: 3882–3889.

Th cells can be categorized into subpopulations that differ in several characteristics, including cytokine production (1–3). Depending on a variety of factors, most importantly cytokines present during priming, an uncommitted precursor cell has the potential to acquire either a Th1 or a Th2 phenotype (1). The polarization observed after repeated antigenic stimulations in vitro or in vivo is at least partly due to the mutually antagonizing effects of Th1 and Th2 cytokines (4–6). Thus, Th1 or Th2 populations represent the endpoints of differentiation pathways, and Th cells that produce cytokine patterns different from the canonical Th1 or Th2 sets have been described and named Th0 (7), Th3 (8), or Tr1 (9), respectively. Th0 cells are able to produce both type 1 and type 2 cytokines (1, 7, 10, 11), and it is currently unclear if they represent a developmental stage during Th1/Th2 differentiation, a stably differentiated population, or consist of different subpopulations.

Most studies on cytokine expression examined populations of Th cells rather than single cells. Consequently, it could not be determined whether individual cells coordinately express certain cytokines or heterogenous combinations of cytokines. Some of the pioneering studies of cytokine expression of single cells (11–13) suffer from the fact that the methods employed allowed the analysis of only limited numbers of cells. We (6, 14, 15) and others (16–18) have investigated coexpression of cytokines in large numbers of individual Th cells using flow cytometry. Many of these studies were performed to determine the stability or plasticity of Th subsets and thus analyzed the (co)expression of IFN- $\gamma$  and IL-4 in transgenic T cells primed and restimulated in vitro. Collectively, these studies indicated that type 1 and type 2 cytokines can be coexpressed by an individual T cell, either simultaneously (6, 11–14, 16–18) or sequentially (15).

To investigate whether cytokine coexpression in single Th cells during an in vivo immune response is stochastic or follows the typical Th1/Th2 patterns observed in polarized T cell populations, it is necessary to examine individual in vivo-activated T cells. Such experiments have been difficult to perform, partly due to the low number of Ag-specific T cells obtainable directly ex vivo and partly for lack of reliable markers for Th subsets. We describe here a detailed analysis of CD4<sup>+</sup> T cells isolated from the lungs of mice containing *Schistosoma mansoni* egg-induced granulomas. The egg-induced granuloma formation is dependent on CD4<sup>+</sup> T cells (19). Granuloma formation occurs in the liver of naturally infected mice and can be induced in the lungs after i.v. injection of parasite eggs into mice (20). Schistosome egg-induced lung inflammation is a valuable experimental system in which to study granuloma development and immune regulation. During granuloma development, the dominant T cell response changes from an ephemeral Th1 response to a sustained Th2 response that is most prominent

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at the height of granulomatous activity (21–24). Because the Th cell response in schistosome egg-induced granulomas is Th2-dominated rather than exclusively Th2, these granulomas are very suitable to study the coexpression of type 1 and type 2 cytokines in CD4<sup>+</sup> T cells.

Recently, we (25) and others (26, 27) reported that T1/ST2, an orphan receptor with sequence homology to the IL-1RI, is expressed preferentially on murine Th2 cells. Here we examined whether during an *in vivo* immune response against *S. mansoni* eggs T1/ST2 was up-regulated and correlated with type 2 cytokine production. In addition, we analyzed individual Th cells, directly *ex vivo*, for coexpression of up to three type 1 and type 2 cytokines.

## Materials and Methods

### Mice and parasites

*S. mansoni* eggs were recovered from the livers of infected mice as described (28), washed, and frozen. Six-week-old BALB/c mice were immunized twice with 5000 *S. mansoni* eggs (frozen and thawed) in PBS *i.p.* at day 0 and week 3. At week 6, mice received 5000 eggs in 200  $\mu$ l PBS *i.v.* and were sacrificed 8–11 days later. Age- and sex-matched control BALB/c mice were injected with PBS only. Eight-week-old C57BL/6 mice were infected by exposure to 65 *S. mansoni* cercariae percutaneously and sacrificed 11 wk later. Age- and sex-matched untreated C57BL/6 mice were used as controls. All animal experiments were performed according to institutional and state guidelines.

### Cell suspensions

Single-cell suspensions were prepared from spleens, and paratracheal, mesenteric, inguinal, axillary, and mandibular lymph nodes (LN)<sup>4</sup> in RPMI 1640 supplemented with serum and antibiotics as described (25). Spleen cells ( $5 \times 10^6$ /ml) or LN cells ( $3 \times 10^6$ /ml) were cultured with medium alone or soluble egg antigen (SEA; 20  $\mu$ g/ml) prepared as described (29). Supernatants were collected at 72 h for detection of IL-4, IL-5, IL-10, IL-2, and IFN- $\gamma$  by sandwich ELISA as described (25).

### Isolation of cells from hepatic granulomas and granulomatous lungs

BALB/c mice immunized with *S. mansoni* eggs, and control mice, were sacrificed and the lungs immediately perfused with 10 ml PBS through the right ventricle of the heart. Single-cell suspensions were prepared by forcing the lung tissue through fine wire mesh. Such cell suspensions contain cells from granulomas, bronchial-associated lymphoid tissue, and other inflammatory cells. The isolation of hepatic granulomas from *S. mansoni*-infected C57BL/6 mice was performed as described (30).

### Abs and flow cytometry

The T1/ST2-specific mAb 3E10 (25) was used together with phycoerythrin (PE)- or FITC-conjugated mAbs against CD4 (GK1.5), CD8 (53-6.7), CD11b (Mac-1; M1/70), CD11c (N418), CD45R (B220; RA3-6B2), CD62L (L-selectin; MEL-14), and  $\gamma\delta$ -TCR (GL3; PharMingen, San Diego, CA) as described (25). To prevent unspecific binding of 3E10, all samples were incubated with blocking anti-Fc $\gamma$ R/II/III mAb 2.4G2/75 (100  $\mu$ g/ml) and purified rat IgG (200  $\mu$ g/ml; Nordic, Tilburg, The Netherlands) 10 min before and during staining of T1/ST2. T1/ST2<sup>+</sup> cells were detected using digoxigeninized 3E10 (1.5  $\mu$ g/ml), followed by anti-digoxigenin (Dig) Fab (Boehringer Mannheim, Mannheim, Germany) conjugated to Cy5. To control for specificity, staining of 3E10 was blocked by preincubating cells with a 100-fold excess of unconjugated 3E10 as described (25). Samples were analyzed on a FACS-Calibur using CellQuest software (Becton Dickinson, Mountain View, CA). Gates were set on viable cells according to forward and side scatter and exclusion of propidium iodide-binding particles (0.3  $\mu$ g/ml).

### Magnetic separation of CD4<sup>+</sup> cells

CD4<sup>+</sup> cells were isolated from lungs of BALB/c mice with *S. mansoni* egg-induced granulomas by high-gradient magnetic cell separation

(MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany). Lung cells were incubated with CD4 mAb coupled to magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions and sorted using VS<sup>+</sup> MACS columns and the MidiMACS system (Miltenyi Biotec). Aliquots of the unsorted and the CD4-selected cell fractions were stained with CD4-PE and analyzed by flow cytometry. The CD4<sup>+</sup> population was sorted to a purity of >98%.

### Four-color single-cell analysis of surface T1/ST2 and three intracellular cytokines in CD4<sup>+</sup> cells by flow cytometry

Separated CD4<sup>+</sup> cells ( $10^6$ /ml) from lungs of BALB/c mice with *S. mansoni* egg-induced granulomas were stimulated with PMA (50 ng/ml) and ionomycin (1  $\mu$ g/ml; Sigma, St. Louis, MO) for 5 h. At 2 h, brefeldin A (Sigma) was added at 5  $\mu$ g/ml. Before fixation, cells were washed, incubated with blocking anti-Fc $\gamma$ R mAb and rat IgG as described above, and stained for T1/ST2 with biotinylated 3E10 (5  $\mu$ g/ml). Cells were washed and fixed with 2% formaldehyde for 15 min as described (14). T1/ST2 was detected on the surface of the fixed CD4<sup>+</sup> cells using streptavidin coupled to peridinin chlorophyll protein (PerCP; 1  $\mu$ g/ml; Becton Dickinson). Cells were washed and then permeabilized with saponin (0.5%; Sigma) for intracellular staining simultaneously with three anti-cytokine mAbs conjugated to either FITC, PE, or Dig. The following mAbs were used: FITC-coupled anti-IFN- $\gamma$  (XMG1.2), anti-IL-5 (TRFK5), and anti-IL-10 (JES5-16E3) at 5  $\mu$ g/ml (PharMingen); PE-coupled anti-IL-4 (11B11), anti-IL-5 (TRFK5), anti-IL-10 (JES5-16E3), and anti-TNF- $\alpha$  (MP6-XT22) at 3  $\mu$ g/ml (PharMingen); digoxigeninized anti-IL-2 (S4B6), anti-IL-4 (11B11), and anti-TNF- $\alpha$  (MP6-XT22) were prepared in our lab, used at 3  $\mu$ g/ml, and subsequently detected with anti-Dig conjugated to Cy5. Anti-Dig-Cy5, FITC-, or PE-labeled isotype control mAbs (PharMingen) were used at the same concentrations as the respective anti-cytokine mAbs. Samples were analyzed by four-color flow cytometry on a FACS-Calibur, and ~150,000 cells were acquired for each sample.

### Statistical analysis of intracellular cytokine coexpression and surface T1/ST2

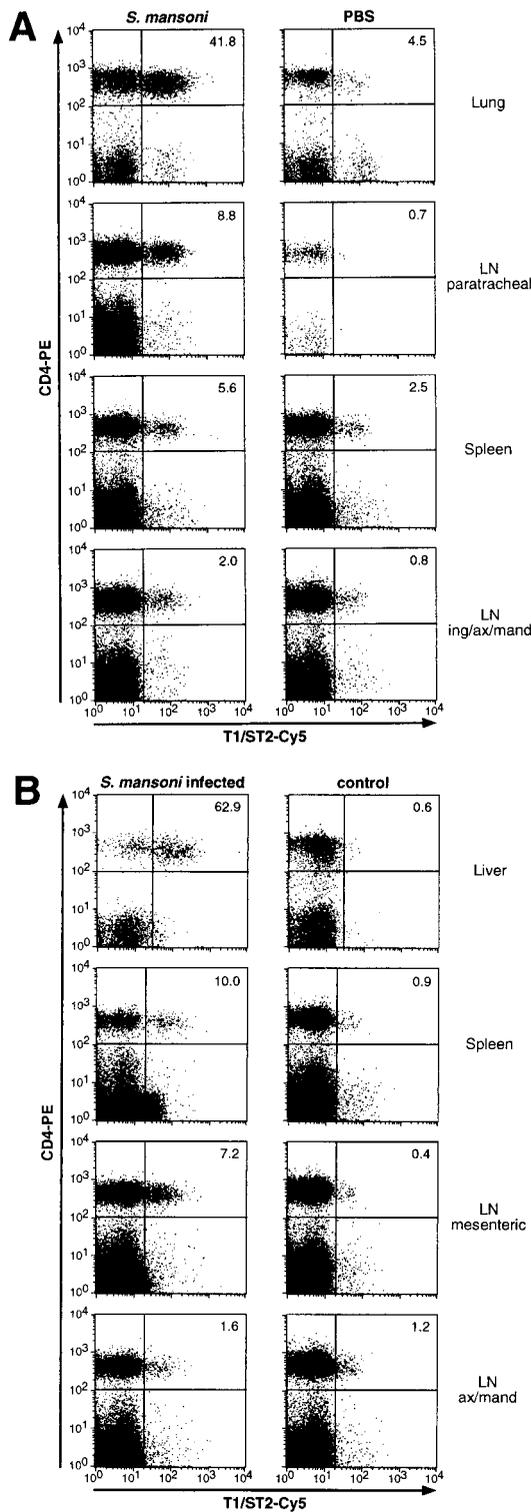
The observed value for cytokine-coexpressing cells in percent was compared with the expected value calculated for random coincidence of two independent variables. Correlations of cytokine coexpression in total CD4<sup>+</sup>, T1/ST2<sup>+</sup>, and T1/ST2<sup>+</sup> CD4<sup>+</sup> cells were calculated using the test for  $\phi$ -correlation coefficients (31). Coefficients of  $\phi \leq -0.1$  or  $\geq 0.1$  were considered significant in this analysis.

## Results

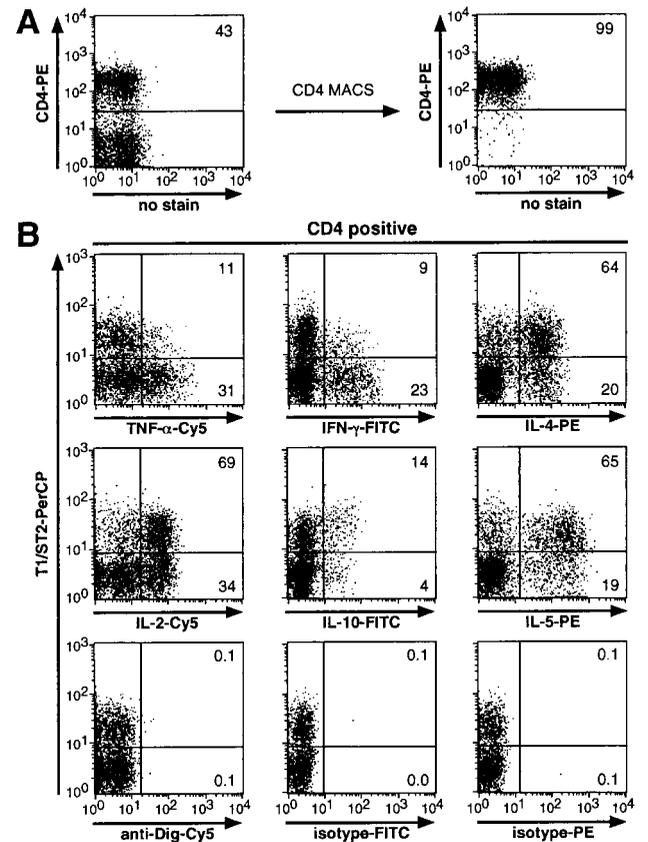
### Expression of T1/ST2 colocalizes with the immune response to *S. mansoni*

We investigated T1/ST2 expression and cytokine production in CD4<sup>+</sup> T cells obtained from lungs of mice with *S. mansoni* egg-induced granulomas. BALB/c mice were immunized with *S. mansoni* eggs as described in *Materials and Methods*. The frequency of T1/ST2-expressing CD4<sup>+</sup> cells was highest in the granulomatous lungs:  $42.9 \pm 1.7\%$  (mean  $\pm$  SD) of CD4<sup>+</sup> cells vs  $4.4 \pm 1.4\%$  in PBS-injected control mice. This decreased to  $8.7 \pm 0.3\%$  in the paratracheal draining LNs ( $0.7 \pm 0.1\%$  in controls),  $5.6 \pm 0.3\%$  of splenic CD4<sup>+</sup> cells ( $2.0 \pm 0.9\%$  in control mice), and  $2.0 \pm 0.2\%$  of the CD4<sup>+</sup> cells in nondraining LNs ( $0.8 \pm 0.2\%$  in controls, Fig. 1A). Determination of cytokine secretion in *in vitro* cultures of spleen and LN cells in response to SEA showed that IL-4, IL-5, and IL-10 were preferentially produced in the paratracheal LN (data not shown). In naturally infected C57BL/6 mice, the frequency of T1/ST2-expressing CD4<sup>+</sup> cells was highest in the liver granulomas ( $61.9 \pm 1.4\%$  of CD4<sup>+</sup> cells vs  $0.6 \pm 0.1\%$  in control livers) and gradually decreased to control values in nondraining LNs (Fig. 1B). More than 90% of the CD4<sup>+</sup> T1/ST2<sup>+</sup> cells from lung or liver granulomas were Ag-experienced CD62L<sup>low</sup> cells, which complements our earlier findings in unimmunized mice (25). Additionally, in the lung T1/ST2 was not expressed on CD8<sup>+</sup> T cells,  $\gamma\delta$  T cells, CD45R<sup>+</sup> B cells, CD11b<sup>high</sup> macrophages, or CD11c<sup>high</sup> dendritic cells (data not shown).

<sup>4</sup> Abbreviations used in this paper: LN, lymph node; Dig, digoxigenin; MACS, high-gradient magnetic cell separation; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SEA, soluble egg antigen.



**FIGURE 1.** T1/ST2 expression on lung or liver granuloma cells, spleen, and LN cells ex vivo. **A**, BALB/c mice were immunized with *S. mansoni* eggs as described in *Materials and Methods*. Control mice received PBS only. Cells from perfused granulomatous lungs, paratracheal draining LNs, spleens, and pooled inguinal, axillary, and mandibular LNs were stained with mAbs against CD4, CD62L, and T1/ST2. Gates were set on viable cells as described in *Materials and Methods*. Data are representative of three separate experiments. **B**, C57BL/6 mice were naturally infected with *S. mansoni* cercariae as described in *Materials and Methods*. FACS analysis was performed as described in **A**. For isolation of liver lymphocytes from untreated control mice, livers were perfused via the hepatportal vein. Data are representative of two experiments. Upper right corner shows percentage T1/ST2<sup>+</sup> of CD4<sup>+</sup> cells.



**FIGURE 2.** Cytokine production of T1/ST2<sup>-</sup> and T1/ST2<sup>+</sup> CD4<sup>+</sup> lung cells ex vivo. **A**, CD4<sup>+</sup> lung cells were purified by MACS 8 days after *S. mansoni* egg-induced granuloma formation. **B**, CD4<sup>+</sup> cells were stimulated with PMA/ionomycin, stained with biotinylated mAb against T1/ST2 (3E10), and then fixed. T1/ST2 was detected on the surface of the fixed cells by streptavidin coupled to PerCP. Subsequently, three-color intracellular cytokine staining was performed simultaneously using digoxigenated, FITC-, or PE-conjugated anti-cytokine mAbs or isotype control mAbs. The numbers given in the right quadrants indicate the percentage of cytokine-producing cells within the T1/ST2-positive or -negative population, respectively. Data are representative of two separate experiments.

#### Cytokine production of T1/ST2<sup>-</sup> and T1/ST2<sup>+</sup> Th cells from granulomatous lungs

CD4<sup>+</sup> cells from granulomatous lungs were sorted by MACS to 99% purity (Fig. 2A), stimulated with PMA/ionomycin, and stained for surface T1/ST2 and intracellular cytokines (Fig. 2B). IFN- $\gamma$  was produced by 23% of the T1/ST2<sup>-</sup> CD4<sup>+</sup> cells compared with 9% of the T1/ST2<sup>+</sup> cells. In the T1/ST2<sup>+</sup> IFN- $\gamma$ <sup>+</sup> population, the brightness of staining for T1/ST2 and IFN- $\gamma$  was inversely correlated such that very few cells that stained brightly for T1/ST2 produced IFN- $\gamma$  (2% IFN- $\gamma$ <sup>+</sup> cells among the 5% T1/ST2 brightest cells). Similarly, TNF- $\alpha$  producers were found mostly in the T1/ST2<sup>-</sup> fraction of Th cells. On the other hand, IL-4, IL-5, and IL-10 producers were all enriched more than three-fold in the T1/ST2<sup>+</sup> fraction. IL-10-producing cells were particularly frequent among the cells staining brightest for T1/ST2 (37% IL-10<sup>+</sup> cells among the 5% T1/ST2 brightest cells). Of note, the frequency of IL-2-expressing cells was higher in the T1/ST2<sup>+</sup> subset (69%) compared with the T1/ST2<sup>-</sup> cells (34%). Thus, T1/ST2 expression was not only positively correlated with type 2 cytokines but also with IL-2 production.

Table I. Coexpression of cytokines in total CD4<sup>+</sup>, T1/ST2<sup>-</sup> or T1/ST2<sup>+</sup> CD4<sup>+</sup> lung cells<sup>a</sup>

Cell Population	$\phi$ obs/exp <sup>b</sup>	IL-4	IL-5	IL-10	IL-2	IFN- $\gamma$
Total CD4 <sup>+</sup>	IL-5	<b>0.33</b> 23.7/16.0	–	–	–	–
	IL-10	<b>0.10</b> 6.4/4.9	<b>–0.06</b> 3.8/4.7	–	–	–
	IL-2	<b>0.36</b> 41.0/32.1	<b>0.41</b> 39.0/28.9	<b>–0.14</b> 3.1/5.1	–	–
	IFN- $\gamma$	<b>0.09</b> 15.6/13.7	<b>0.04</b> 13.8/12.9	<b>–0.02</b> 2.2/2.5	<b>0.18</b> 18.8/15.0	–
	TNF- $\alpha$	<b>0.01</b> 16.1/15.9	<b>–0.12</b> 9.4/12.0	<b>–0.10</b> 1.3/2.7	<b>0.28</b> 17.5/11.3	<b>0.32</b> 12.7/6.7
	T1/ST2 <sup>-</sup> CD4 <sup>+</sup>	IL-5	<b>0.41</b> 19.9/11.0	–	–	–
IL-10	<b>0.02</b> 2.3/2.0	<b>–0.04</b> 1.5/1.9	–	–	–	
IL-2	<b>0.40</b> 33.7/23.9	<b>0.43</b> 32.9/22.3	<b>–0.03</b> 2.1/2.4	–	–	
IFN- $\gamma$	<b>0.22</b> 20.9/15.6	<b>0.12</b> 17.1/14.3	<b>0.09</b> 3.1/2.1	<b>0.26</b> 25.5/19.3	–	–
TNF- $\alpha$	<b>0.09</b> 19.2/17.0	<b>–0.06</b> 9.4/10.8	<b>–0.03</b> 1.9/2.1	<b>0.34</b> 23.0/15.0	<b>0.29</b> 19.5/12.9	–
T1/ST2 <sup>+</sup> CD4 <sup>+</sup>	IL-5	<b>0.19</b> 28.9/24.4	–	–	–	–
	IL-10	<b>0.10</b> 11.9/10.2	<b>–0.17</b> 7.0/9.9	–	–	–
	IL-2	<b>0.27</b> 50.8/44.8	<b>0.37</b> 47.3/38.6	<b>–0.26</b> 4.4/8.9	–	–
	IFN- $\gamma$	<b>0.04</b> 8.5/7.8	<b>0.06</b> 9.2/8.2	<b>–0.06</b> 1.0/1.8	<b>0.14</b> 9.8/7.6	–
	TNF- $\alpha$	<b>0.04</b> 11.9/11.3	<b>0.03</b> 9.3/8.9	<b>–0.12</b> 0.6/2.1	<b>0.24</b> 10.0/5.9	<b>0.20</b> 3.8/1.6

<sup>a</sup> CD4<sup>+</sup> lung cells were analyzed for T1/ST2 and cytokine expression as described in Fig. 2.

<sup>b</sup> Observed (obs) and expected (exp) frequencies of cytokine-coexpressing cells are given in percent. The  $\phi$ -correlation coefficient ( $\phi$ , printed in bold, range –1 to 1) was calculated from ~150,000 cells. Coefficients of  $\phi \leq -0.1$  or  $\phi \geq 0.1$  were considered significant. Data shown is representative of two separate experiments.

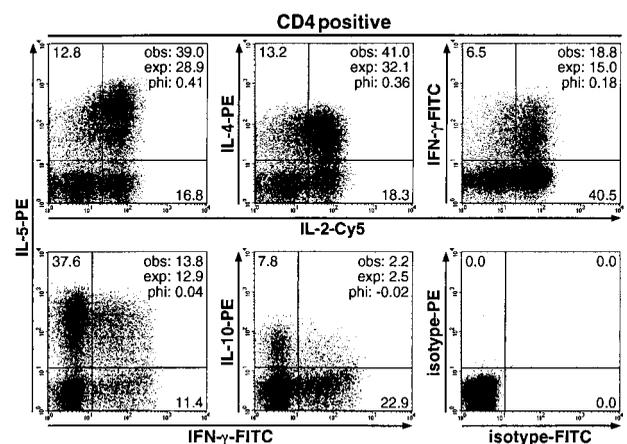
#### Coexpression of intracellular cytokines and surface T1/ST2

The cytokine coexpression capacity of the T1/ST2<sup>-</sup> vs T1/ST2<sup>+</sup> cells was determined by four-color FACS. CD4<sup>+</sup> cells from BALB/c lungs with schistosome egg-induced granulomas were purified, stimulated, and stained as described for Fig. 2. The coexpression of each possible pair of the analyzed cytokines in the total CD4<sup>+</sup> population and in T1/ST2<sup>-</sup> or T1/ST2<sup>+</sup> CD4<sup>+</sup> cells is summarized in Table I. The observed frequencies of cytokine-coexpressing cells are compared with those calculated for expected values (i.e., random coincidence) to determine whether coexpression is coordinate or independent. Correlation coefficients are shown for each cytokine pair.

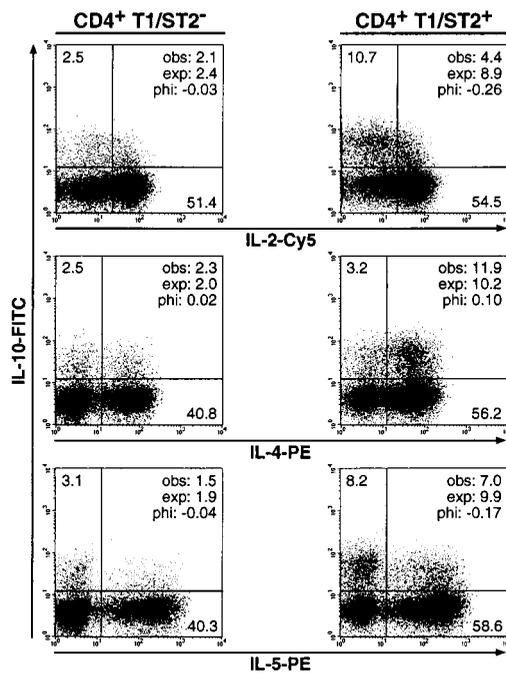
In total CD4<sup>+</sup> cells IL-2 production was positively correlated, not only with the expression of IFN- $\gamma$  and TNF- $\alpha$ , but also with the expression of IL-4 and IL-5 (Fig. 3 and Table I). The association of IL-2 with these other cytokines was observed in both the T1/ST2<sup>+</sup> and T1/ST2<sup>-</sup> subsets. However, IL-2 expression was negatively correlated with the production of IL-10 (Table I). Thus, for Th cells obtained directly ex vivo from lungs containing schistosome egg-induced granulomas, IL-2 production seems to be a marker of activated Th cells, regardless of whether the same cell coproduces type 1 cytokines (such as IFN- $\gamma$ ) or type 2 cytokines (such as IL-4 or IL-5), with the exception that these IL-2-expressing cells are unlikely to coexpress IL-10.

IL-10 production was negatively correlated with both IL-2 and TNF- $\alpha$  in the total CD4<sup>+</sup> population. Conversely, IL-10 was positively associated with IL-4 but not with IL-5. These correlations were all confined to the T1/ST2<sup>+</sup> cells and not found in the T1/ST2<sup>-</sup> cells (Fig. 4 and Table I). The coexpression of IL-10 and

IFN- $\gamma$ , which was random in the total CD4<sup>+</sup> population (Fig. 3), tended toward a positive association in the T1/ST2<sup>-</sup> cells (Table I). Furthermore, exclusively in T1/ST2<sup>-</sup> cells the production of both IL-4 and IL-5 was positively correlated with the production of IFN- $\gamma$  (Table I and Fig. 3).



**FIGURE 3.** Cytokine coexpression in CD4<sup>+</sup> cells. CD4<sup>+</sup> lung cells were purified and stained as described in Fig. 2. The frequency of cytokine-producing CD4<sup>+</sup> 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) calculated for random coincidence of two independent variables, and the  $\phi$ -correlation coefficient ( $\phi$ ) of the respective cytokine pair are indicated (see also Table I).



**FIGURE 4.** Coexpression of IL-10 with IL-2, IL-4, or IL-5 in CD4<sup>+</sup> T1/ST2<sup>-</sup> or T1/ST2<sup>+</sup> cells. CD4<sup>+</sup> lung cells were purified and stained as described in Fig. 2. Gates were set on T1/ST2<sup>-</sup> or T1/ST2<sup>+</sup> cells as shown in Fig. 2B. 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  $\phi$ -correlation coefficient (phi) are given (see also Table I).

The data given here is representative of two separate experiments. Within both the T1/ST2<sup>+</sup> and the T1/ST2<sup>-</sup> population all but one of the correlations that were found significant in one experiment ( $\phi$  coefficient  $\leq -0.1$  or  $\geq 0.1$ ) were also significant in the other. The one difference in the T1/ST2<sup>+</sup> population was the coexpression of IL-10 and IL-4. The  $\phi$  values were 0.10 (Table I) and 0.08 in the second experiment (data not shown). Within the T1/ST2<sup>-</sup> population, the exception was IL-10 and IFN- $\gamma$  coexpression, where the  $\phi$  values were 0.09 (Table I) and 0.14 in experiment 2 (data not shown).

#### *Influence of the production of a third cytokine on the coexpression of two other cytokines*

We analyzed the expression of cytokine triplets to determine how a third cytokine might influence the coexpression of a given cytokine pair, within the total CD4<sup>+</sup> population and its T1/ST2<sup>-</sup> and T1/ST2<sup>+</sup> subsets (Table II). In several triplets, a third cytokine drastically altered the coexpression frequency of a given pair. An example is given in Fig. 5, in which CD4<sup>+</sup> cells are gated according to T1/ST2 expression and further on their IL-2 production. The four resulting subpopulations were analyzed for coexpression of IL-4 and IFN- $\gamma$ . IL-4 and IFN- $\gamma$  production correlated significantly ( $\phi = 0.42$ ) only in the CD4<sup>+</sup> T1/ST2<sup>-</sup> cells that did not produce IL-2. In those T1/ST2<sup>-</sup> cells that produced IL-2, the association of IL-4 and IFN- $\gamma$  expression was abrogated ( $\phi = -0.05$ ). In contrast, in the T1/ST2<sup>+</sup> subset IL-4 and IFN- $\gamma$  production were independent events irrespective of the expression of IL-2. The positive correlation of IL-2 and IL-4 or IL-2 and IFN- $\gamma$  was dissociated by the additional coexpression of IFN- $\gamma$  or IL-4, respectively, in both the T1/ST2<sup>-</sup> and the T1/ST2<sup>+</sup> populations (Table II). In the T1/ST2<sup>-</sup> cells, expression of IL-5 abrogated the association of IL-4 and IFN- $\gamma$  expression. Even more drastically,

**Table II.** Triple cytokine coexpression analysis in CD4<sup>+</sup> T1/ST2<sup>-</sup> and T1/ST2<sup>+</sup> lung cells<sup>a</sup>

Cytokine Triplet	Gated Cytokine <sup>b</sup>	Cytokine Pair	T1/ST2 <sup>-</sup> ( $\phi$ ) <sup>c</sup>	T1/ST2 <sup>+</sup> ( $\phi$ ) <sup>c</sup>
IL-2, IFN- $\gamma$ , IL-4	IL-2 <sup>+</sup>	IL-4, IFN- $\gamma$	-0.05	-0.03
	IL-2 <sup>-</sup>	IL-4, IFN- $\gamma$	<b>0.42</b>	0.09
	IFN- $\gamma$ <sup>+</sup>	IL-2, IL-4	0.03	0.03
	IFN- $\gamma$ <sup>-</sup>	IL-2, IL-4	<b>0.52</b>	<b>0.29</b>
	IL-4 <sup>+</sup>	IL-2, IFN- $\gamma$	-0.09	0.09
	IL-4 <sup>-</sup>	IL-2, IFN- $\gamma$	<b>0.40</b>	<b>0.23</b>
IL-5, IFN- $\gamma$ , IL-4	IL-5 <sup>+</sup>	IL-4, IFN- $\gamma$	0.04	0.02
	IL-5 <sup>-</sup>	IL-4, IFN- $\gamma$	<b>0.23</b>	0.04
	IFN- $\gamma$ <sup>+</sup>	IL-4, IL-5	<b>0.28</b>	<b>0.14</b>
	IFN- $\gamma$ <sup>-</sup>	IL-4, IL-5	<b>0.48</b>	<b>0.20</b>
	IL-4 <sup>+</sup>	IL-5, IFN- $\gamma$	<b>-0.16</b>	0.03
	IL-4 <sup>-</sup>	IL-5, IFN- $\gamma$	<b>0.14</b>	0.08
IL-5, IL-2, TNF- $\alpha$	IL-5 <sup>+</sup>	IL-2, TNF- $\alpha$	<b>0.20</b>	<b>0.18</b>
	IL-5 <sup>-</sup>	IL-2, TNF- $\alpha$	<b>0.43</b>	<b>0.34</b>
	IL-2 <sup>+</sup>	IL-5, TNF- $\alpha$	<b>-0.27</b>	<b>-0.10</b>
	IL-2 <sup>-</sup>	IL-5, TNF- $\alpha$	-0.01	0.05
	TNF- $\alpha$ <sup>+</sup>	IL-2, IL-5	0.08	0.05
	TNF- $\alpha$ <sup>-</sup>	IL-2, IL-5	<b>0.35</b>	<b>0.25</b>
IL-5, IL-2, IL-10	IL-5 <sup>+</sup>	IL-2, IL-10	-0.02	<b>-0.19</b>
	IL-5 <sup>-</sup>	IL-2, IL-10	-0.02	<b>-0.23</b>
	IL-2 <sup>+</sup>	IL-5, IL-10	-0.03	-0.03
	IL-2 <sup>-</sup>	IL-5, IL-10	-0.02	-0.09
	IL-10 <sup>+</sup>	IL-2, IL-5	<b>0.38</b>	<b>0.33</b>
	IL-10 <sup>-</sup>	IL-2, IL-5	<b>0.43</b>	<b>0.34</b>

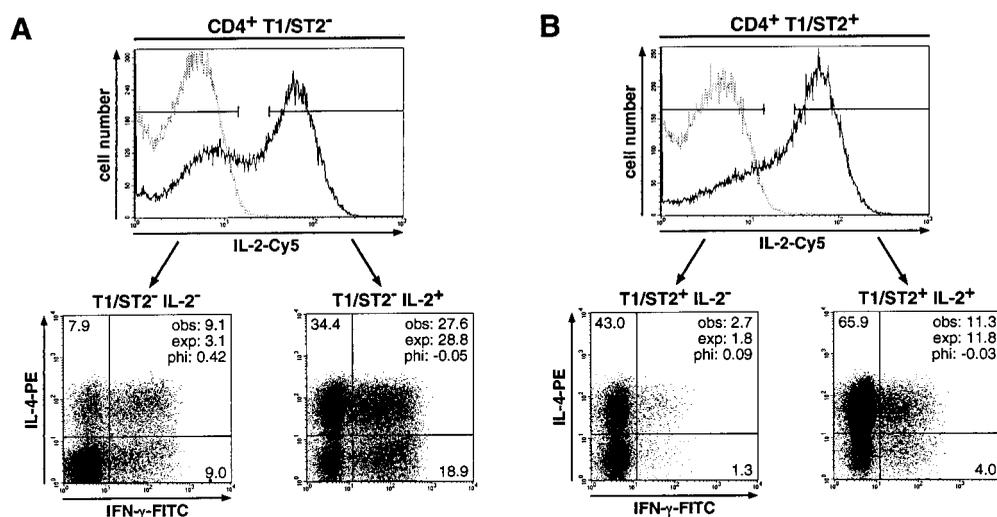
<sup>a</sup> CD4<sup>+</sup> lung cells were analyzed for T1/ST2 and cytokine expression as described in Fig. 2.

<sup>b</sup> Gates were set on cells producing or nonproducing a given cytokine of the triplet.

<sup>c</sup> The  $\phi$ -correlation coefficient ( $\phi$ , range -1 to 1) of the indicated cytokine pair was calculated from ~150,000 cells. Coefficients of  $\phi \leq -0.1$  or  $\geq 0.1$  are printed in bold. Data shown is representative of two separate experiments.

the positive association of IL-5 and IFN- $\gamma$  expression was reverted to a negative correlation by the additional coexpression of IL-4 in the T1/ST2<sup>-</sup> cells. In both subsets IL-2 and TNF- $\alpha$  were positively associated independently of IL-5 expression. In contrast, IL-5 and TNF- $\alpha$  were significantly inversely correlated only in the presence of IL-2 within both T1/ST2<sup>-</sup> and T1/ST2<sup>+</sup> cells. This is further demonstrated with the cytokine pair IL-5 and IL-2, which were more likely to be coexpressed in the absence of TNF- $\alpha$ . An example illustrating that a third cytokine in a given triplet does not necessarily influence the coexpression of cytokine pairs is given by the triplet IL-5, IL-2, IL-10. The correlation of any of the three possible cytokine pairs was not altered in the absence or presence of the third cytokine analyzed, in neither the T1/ST2<sup>-</sup> nor the T1/ST2<sup>+</sup> cells.

The data given here is representative of two separate experiments. Within the T1/ST2<sup>+</sup> population all but two of the correlations that were found significant in one experiment ( $\phi$  coefficient  $\leq -0.1$  or  $\geq 0.1$ ) were also significant in the other. A small difference was observed in the coexpression of IL-4 and IFN- $\gamma$  in the absence of IL-5 production between the experiments:  $\phi$  values 0.04 (Table II) and 0.10 (second experiment, data not shown). Additionally, the coexpression of IL-2 and IL-5 in TNF- $\alpha$ -producing T1/ST2<sup>+</sup> cells differed:  $\phi$  values 0.05 (Table II) and 0.21 (second experiment, data not shown). Within the T1/ST2<sup>-</sup> population, all significant correlations were reproducible except for IL-2 and IL-5 in the presence of TNF- $\alpha$ , which were randomly coexpressed in one experiment ( $\phi = 0.08$ , Table II) but positively associated in the second experiment ( $\phi = 0.13$ , data not shown).



**FIGURE 5.** Triple cytokine coexpression analysis of IL-2 with IFN- $\gamma$  and IL-4 in CD4<sup>+</sup> T1/ST2<sup>-</sup> or T1/ST2<sup>+</sup> cells. CD4<sup>+</sup> lung cells were purified and stained as described in Fig. 2. Gates were set on T1/ST2<sup>-</sup> (A) or T1/ST2<sup>+</sup> cells (B) as shown in Fig. 2B. Further gates were set on IL-2-producing or IL-2-negative cells as indicated by the bars in the histograms. The dotted line shows the staining of the anti-Dig-Cy5 control. The IL-2<sup>+</sup> or IL-2<sup>-</sup> cell subsets were analyzed for coexpression of IFN- $\gamma$  and IL-4. Isotype-control stainings are shown in Fig. 3. 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  $\phi$ -correlation coefficient (phi) are given (see also Table II).

## Discussion

We and others have recently demonstrated that T1/ST2 is preferentially expressed on murine Th2 cells and important for Th2 effector functions (25, 26). Here we used CD4<sup>+</sup> cells from lungs containing granulomas induced by *S. mansoni* eggs to study T1/ST2 expression and the coexpression of different cytokines at the single-cell level in a Th2-dominated immune response in vivo. T1/ST2 expression was highly up-regulated on granuloma CD4<sup>+</sup> T cells. With increasing distance from the egg-induced granulomas, the frequency of T1/ST2-expressing CD4<sup>+</sup> cells decreased progressively, reaching control levels in nondraining LNs (Fig. 1). Thus, T1/ST2 expression in vivo colocalized with the Th2-dominated immune response. T1/ST2<sup>+</sup> CD4<sup>+</sup> cells obtained from granulomatous lungs produced a Th2 cytokine pattern upon in vitro stimulation with PMA/ionomycin (Fig. 2), confirming and extending our earlier report (25). Importantly, there were also some differences between the T1/ST2<sup>+</sup> CD4<sup>+</sup> cells obtained from lung inflammation induced by schistosome eggs or from the spleens of unimmunized mice. Only a minority of CD4<sup>+</sup> cells from naive spleens produced any cytokine at all upon stimulation with PMA/ionomycin, and the T1/ST2<sup>+</sup> fraction contained virtually all the IL-5 producers and only very few of the IL-2 producers (25). In contrast, the vast majority of CD4<sup>+</sup> cells obtained from granulomatous lungs during the ongoing immune response against *S. mansoni* produced cytokines, and many of the T1/ST2<sup>+</sup> cells also produced IL-2, whereas a sizable fraction of the IL-5 producers was T1/ST2<sup>-</sup> (Fig. 2). Thus, it is possible that some T cells that produce type 2 cytokines never express T1/ST2 on their surface or have lost expression of T1/ST2. Alternatively, the kinetics for cytokine production may be faster than for surface expression of T1/ST2. Our current data support the latter possibility (C.M., unpublished observations).

IL-2 production was a shared characteristic of Th cells, either T1/ST2 positive or negative, obtained from the granulomas. Both IL-4 and IL-5 were frequently coproduced with IL-2 (Fig. 3). Others found IL-5 production in schistosome-infected mice dependent on IL-2 (32–34). However, IL-2 production is not characteristic of Th2 cells and we have not observed significant percentages of

IL-2-producing Th2 cells in vitro (A. Richter, M. Löhning, unpublished observations). A T cell population that coproduces IL-4 or IL-5 together with IL-2 could reasonably be called Th0. What, then, is the significance of T1/ST2 expressed on the surface of these cells? Could these cells be recently activated “en route” to becoming Th2 cells? Our coexpression data (Tables I and II and see below) would support that hypothesis. On the other hand, as almost 70% of the T1/ST2<sup>+</sup> CD4<sup>+</sup> cells produced IL-2, it is difficult to imagine that all these cells were in a transitional stage of their phenotype development. With current technology it cannot be determined if T1/ST2<sup>+</sup> IL-2/IL-4 (or IL-2/IL-5) coproducers are recently or chronically activated. Thus, we cannot answer the question if this coexpression pattern is stable or transient. In summary, the data indicate that in CD4<sup>+</sup> cells from lungs containing schistosome-induced granulomas T1/ST2 is not expressed exclusively on typical Th2 cells. Could this be because there are not too many typical Th2 cells in vivo?

The categorization of Th cells into different subsets according to their cytokine production is based on the behavior of highly differentiated Th cell populations (1, 7–9, 35). It is a matter of current debate if the cytokine production patterns of individual T cells within a highly differentiated population mirror the pattern observed for the population. Currently, there are two competing hypotheses. The first states that coexpression of type 1 or type 2 cytokines by individual cells is the cellular basis for the Th1/Th2 dichotomy observed on the population level. The competing hypothesis predicts that cytokine coexpression in individual cells is stochastic and polarized Th1 or Th2 populations are composed of selected T cells (36, 37). These hypotheses can only be tested by single-cell analysis of T cell cytokine production, preferably in vivo. There are several published reports detailing the results of studies on the coexpression of IL-4 and IFN- $\gamma$  in TCR-transgenic cells in vitro (6, 11–17). We have used an in vivo model combined with magnetic cell separation and four-color flow cytometry to analyze cytokine coexpression in Th cells. We simultaneously analyzed the expression of one cell surface molecule, T1/ST2, and three different cytokines intracellularly at the single-cell level for a

large number of CD4<sup>+</sup> cells obtained from lungs containing schistosome egg-induced granulomas. Similar to earlier findings (6, 11), we found random coexpression of IFN- $\gamma$  and IL-4 in the total CD4<sup>+</sup> population. However, analysis of the different subpopulations revealed striking differences: in the T1/ST2<sup>-</sup> population but not in the T1/ST2<sup>+</sup> population, IFN- $\gamma$  and IL-4 were frequently coexpressed. Triple analysis of intracellular cytokine staining showed that IL-4 and IFN- $\gamma$  production were positively correlated only in those T1/ST2<sup>-</sup> cells that produced neither IL-2 nor IL-5 (Table II and Fig. 5). Thus, the production of either IL-2 or IL-5 dramatically decreased the likelihood of IL-4/IFN- $\gamma$  coexpression. A similar pattern was found for the coexpression of IL-2, IL-5, and TNF- $\alpha$ . Importantly, these observations are not due to the fact that a cell is less likely to produce three different cytokines than two: our analysis of the coexpression of IL-2, IL-5, and IL-10 revealed no significant influence of either one of the cytokines on the likelihood of coproduction of the other two cytokines (Table II). Thus, a Th cell can simultaneously produce both a type 1 and a type 2 cytokine. Coproduction of two type 1 or two type 2 cytokines strongly decreases the likelihood of such a cell producing a "contrasting" cytokine.

Complementing this scenario is the finding that T1/ST2<sup>+</sup> or T1/ST2<sup>-</sup> CD4<sup>+</sup> cells displayed different patterns of cytokine coexpression. The coexpression of any type 2 cytokine (IL-4, IL-5, IL-10) with any type 1 cytokine (IFN- $\gamma$ , IL-2) was less likely in the T1/ST2<sup>+</sup> than the T1/ST2<sup>-</sup> population, albeit to varying degrees (Table I). Similarly, the  $\phi$ -correlation coefficients for coexpression of any two of the type 1 cytokines IL-2, IFN- $\gamma$ , or the proinflammatory cytokine TNF- $\alpha$  were always lower in the T1/ST2<sup>+</sup> population than in the T1/ST2<sup>-</sup> cells. On the other hand, the coexpression of IL-4 and IL-10 was more likely in the T1/ST2<sup>+</sup> than the T1/ST2<sup>-</sup> population, and this effect was further strengthened when only those CD4<sup>+</sup> cells that stained most brightly for T1/ST2 were analyzed (5% brightest cells,  $\phi = 0.16$ ). Collectively, these data are consistent with the notion that Th1 or Th2 cytokine genes are not switched on or off in mutually exclusive sets. Instead, they are compatible with the hypothesis that during the evolution of an immune response in vivo there are checkpoints for Th phenotype development. Both the coexpression of at least two type 2 cytokines or the surface expression of T1/ST2 together with the production of one type 2 cytokine indicate commitment of the cell to Th2 phenotype development, whereas expression of at least two Th1 cytokines indicates commitment to Th1 phenotype development.

Most in vitro analyses of Th phenotype development found stable expression of either IL-4 or IFN- $\gamma$  after three cycles of antigenic stimulation and rest (6, 17, 18, 38–40). This is due, at least partly, to the down-regulation of IL-4 signaling in Th1 cells (41) and the down-regulation of IL-12 signaling in Th2 cells (42). However, in the presence of IFN- $\gamma$  the IL-12 responsiveness of Th2 cells can be maintained (39, 43–45). We found that 15–25% of all Th cells from granulomatous lungs produced IFN- $\gamma$ . Similarly, others have isolated IFN- $\gamma$ -producing *Leishmania*-specific T cells from *Leishmania*-infected BALB/c mice in which a Th2 response had already been established (46, 47). Furthermore, it was recently reported that schistosome-induced liver granulomas contain Th cells capable of responding to IL-12 (48). The continuous presence of IFN- $\gamma$  could explain the greater plasticity of cytokine production that we observed in vivo as compared with the extremely skewed experimental conditions in vitro. Therefore, our in vivo findings would justify some optimism regarding the possibility of manipulating established Th1 or Th2 responses in vivo.

In summary, our data show that T1/ST2 expression colocalizes with Th2 responses in vivo. Single-cell analysis of cytokine coex-

pression revealed different levels of commitment to Th subsets. Every possible combination of one type 1 with one type 2 cytokine was observed in total CD4<sup>+</sup> cells. However, the likelihood of type1/type2 cytokine coexpression was reduced in T1/ST2<sup>+</sup> cells as compared with T1/ST2<sup>-</sup> cells, and the expression of two type 2 cytokines made the expression of a type 1 cytokine very unlikely and vice versa. Thus, coexpression of two type 2 cytokines or production of one type 2 cytokine together with surface expression of T1/ST2 are indicators of advanced commitment to the Th2 phenotype.

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