

# TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand

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**We recently showed that dendritic cells (DCs) activated by thymic stromal lymphopoietin (TSLP) prime naive CD4<sup>+</sup> T cells to differentiate into T helper type 2 (Th2) cells that produced high amounts of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), but no interleukin (IL)-10. Here we report that TSLP induced human DCs to express OX40 ligand (OX40L) but not IL-12. TSLP-induced OX40L on DCs was required for triggering naive CD4<sup>+</sup> T cells to produce IL-4, -5, and -13. We further revealed the following three novel functional properties of OX40L: (a) OX40L selectively promoted TNF- $\alpha$ , but inhibited IL-10 production in developing Th2 cells; (b) OX40L lost the ability to polarize Th2 cells in the presence of IL-12; and (c) OX40L exacerbated IL-12-induced Th1 cell inflammation by promoting TNF- $\alpha$ , while inhibiting IL-10. We conclude that OX40L on TSLP-activated DCs triggers Th2 cell polarization in the absence of IL-12, and propose that OX40L can switch IL-10-producing regulatory Th cell responses into TNF- $\alpha$ -producing inflammatory Th cell responses.**

CD4<sup>+</sup> Th2 cells are historically defined as effector T cells with the capacity to produce IL-4, -5, -10, and -13 (1, 2). Th2 cells are critical for the development of antibody responses against extracellular parasitic infection and of allergic immune responses to allergens. However, there are characteristics of conventional Th2 cells that seem to preclude their involvement in allergic inflammation. First, IL-10 does not appear to contribute to allergic inflammation in either humans or mice (3–5); in fact, many studies have demonstrated that IL-10 suppresses allergic inflammation (6–9). Second, in animal models, low affinity–altered peptides that have been used to prime Th2 cell responses do not cause allergic inflammation, but rather induce tolerance (10, 11). Third, historically, although IFN- $\gamma$ -producing Th1 cells are defined as inflammatory, Th2 cells that produce IL-4, -5, -10, and -13 are defined as antiinflammatory (12). Thus, it is difficult conceptually and experimen-

tally to comprehend how antiinflammatory Th2 cell is involved in the development of allergic inflammation.

We recently described a new type of human Th2 cell that produces the classic Th2 cytokines IL-4, -5, and -13, but not IL-10 (13). Remarkably, these Th2 cells produce very high levels of TNF- $\alpha$ . These TNF- $\alpha$ -highly positive (TNF- $\alpha$ <sup>2+</sup>), IL-10–negative (IL-10<sup>-</sup>) inflammatory Th2 cells were originally generated from human CD4<sup>+</sup> naive T cells cultured with allogeneic myeloid DCs (mDCs) activated by human thymic stromal lymphopoietin (TSLP), an IL-7–like cytokine (13). We believe, therefore, these TNF- $\alpha$ <sup>2+</sup>IL-10<sup>-</sup> Th2 cells most likely represent the pathogenic Th2 cells that cause allergic inflammation, in contrast to the conventional IL-10–producing Th2 cells.

We found that TSLP is expressed by keratinocytes of atopic dermatitis (13). TSLP expression is also associated with Langerhans cell migration and activation in situ (13). These findings suggest that allergic insults from chemicals, microbes, or allergens initially cause

## CORRESPONDENCE

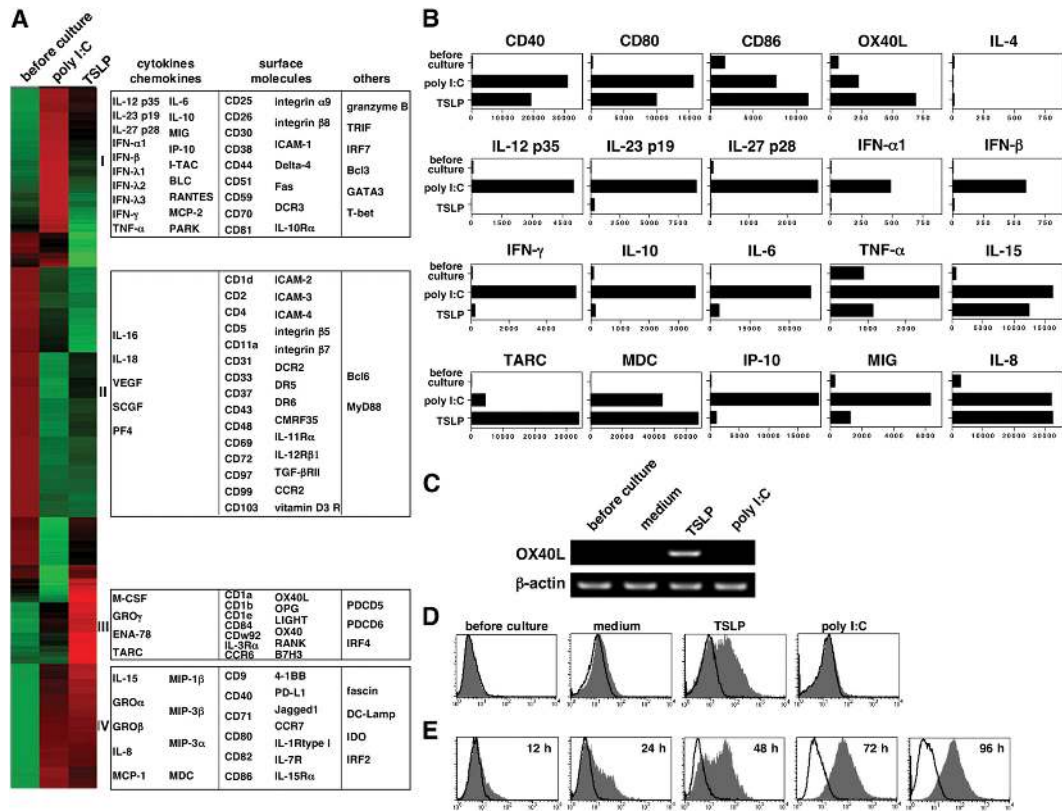
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Abbreviations used in this paper: APC, allophycocyanin; CD40L, CD40 ligand; mDC, myeloid DC; OX40L, OX40 ligand; TLR, Toll-like receptor; TSLP, thymic stromal lymphopoietin.

T. Ito and Y.-H. Wang contributed equally to this work.

The online version of this article contains supplemental material.



**Figure 1. TSLP-DCs express OX40L.** (A) Each column represents data from human blood CD11c<sup>+</sup> immature mDCs either resting or activated by poly I:C or TSLP. The selected 2,166 genes were grouped, based on similarity in expression patterns, by hierarchical clustering as described in Materials and Methods. Each row represents relative hybridization intensities of a particular gene across different samples. Colors reflect the magnitude of relative expression of a particular gene across samples. Cluster I, II, and III include genes highly expressed in poly I:C-activated DCs, resting DCs, and TSLP-activated DCs, respectively. Cluster IV includes genes highly expressed in both poly I:C-activated and TSLP-activated DCs. Under culture with different stimuli, expression profiles of the indi-

cated genes (B) and OX40L mRNA expression (C) in DCs were analyzed at the 24-h time point by microarray and RT-PCR, respectively, and surface expression of OX40L on DCs were analyzed at the 48-h time point by flow cytometry (D). OX40L expression on TSLP-DCs were monitored at different time points (E). The staining profile of anti-OX40L mAb and isotype-matched control are shown by the shaded and open areas, respectively. The results of the gene expression profiles (B) are shown as the relative hybridization intensity level by microarray analysis. Accession number of each microarray dataset are available at <http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene>.

mucosal epithelial cells or skin keratinocytes to produce TSLP. TSLP then activates epidermal-dermal DCs to migrate into the draining lymph nodes and to prime allergen-specific naive T cells to expand and differentiate into inflammatory TNF- $\alpha$ <sup>+</sup>IL-10<sup>-</sup> Th2 cells, which ultimately contribute to the induction of allergic inflammation.

To determine how TSLP-activated DCs (TSLP-DCs) induce TNF- $\alpha$ <sup>+</sup>IL-10<sup>-</sup> inflammatory Th2 cell differentiation, we performed microarray global gene expression analyses of freshly isolated peripheral blood mDCs and mDCs activated by TSLP and poly I:C. We found that TSLP stimulated mDCs to express the OX40 ligand (OX40L), which is a member of the TNF superfamily that has been implicated in the B cell-T cell interaction (14), the DC-T cell interaction (15, 16), and the initiation of Th2 cell responses (15–18).

In this study, we showed that the OX40L expressed by TSLP-DCs induces naive CD4<sup>+</sup> T cells to differentiate into

TNF- $\alpha$ <sup>+</sup>IL-10<sup>-</sup> inflammatory Th2 cells in the absence of IL-12. OX40L also converted an IL-10-producing regulatory Th1 cell response induced by IL-12 into a TNF- $\alpha$ -producing inflammatory Th1 cell response. This therefore indicated that OX40L serves as the TSLP-DC-derived original Th2 cell-polarizing signal that operates in an IL-12 default fashion. We also demonstrated that OX40L acts as a switch that inhibits IL-10 but promotes TNF- $\alpha$  in both Th1 and Th2 cell responses. We propose that Th1 and Th2 cell responses be divided into inflammatory and regulatory subtypes.

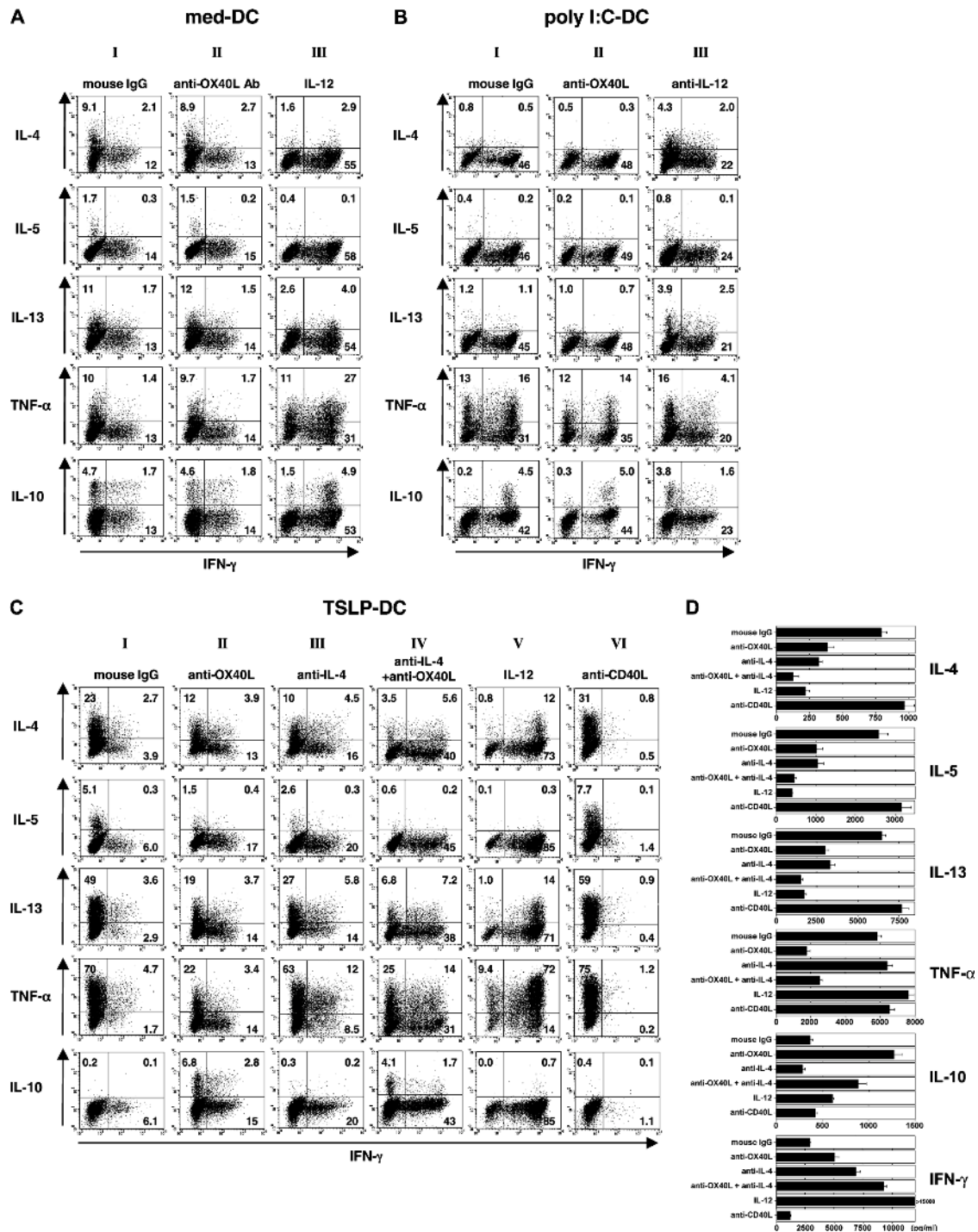
## RESULTS

### TSLP activates DCs to express OX40L but not proinflammatory cytokines

To search for the molecular mechanism by which TSLP-DCs induce naive CD4<sup>+</sup> T cells to differentiate into TNF- $\alpha$ <sup>+</sup>IL-10<sup>-</sup> inflammatory Th2 cells, we performed human

Affymetrix microarray gene expression analyses in human peripheral blood CD11c<sup>+</sup> immature mDCs either resting or activated by TSLP or poly I:C. The resulting gene expres-

sion data were organized on the basis of the overall similarity in the gene expression patterns by using an unsupervised hierarchical clustering algorithm of 2,166 out of 38,500 well-



**Figure 2.** TSLP-DC-mediated inflammatory Th2 cell response requires OX40L as a positive Th2 cell-polarizing signal and default of a lack of IL-12. CD4<sup>+</sup> naive T cells were cocultured with med-DCs (A), poly I:C-DCs (B), or TSLP-DCs (C and D) for 7 d in the presence of the indicated neutralizing Abs or recombinant IL-12. Cytokine production by T cells was analyzed intracellu-

larly by flow cytometry (A–C) and measured in supernatants after restimulation with anti-CD3 and anti-CD28 mAbs for 24 h by ELISA (D). Percentages of the respective cytokine-producing T cells are indicated in each dot blot profile in A–C. Error bars in D represent standard deviations of triplicate cultures. Data are from one of four independent experiments.

characterized genes (Fig. 1 A). Distinct clusters were identified: Cluster I included genes highly expressed by DCs activated by poly I:C (poly I:C-DCs); cluster II included genes highly expressed in resting DCs and down-regulated during activation; cluster III included genes highly expressed in DCs activated by TSLP; and cluster IV included genes highly expressed in DCs activated by both poly I:C and TSLP. The relative expression levels of the genes involved in T cell activation, polarization, and chemotaxis are shown in Fig. 1 B.

We additionally confirmed and extended our previous conclusion that unlike Toll-like receptor (TLR) ligands and the CD40 ligand (CD40L), TSLP does not stimulate DCs to produce Th1 cell-polarizing cytokines, such as IL-12, IL-23, IL-27, IFN- $\gamma$ , IFN- $\alpha$ , and IFN- $\beta$ , or proinflammatory cytokines, such as IL-6 and TNF- $\alpha$  as well as IFN-inducible protein 10 (IP-10/CXCL10) and monokine induced by IFN- $\gamma$  (MIG/CXCL9) (Fig. 1 B) (13). We found that TSLP did not stimulate DCs to produce the Th2-polarizing cytokine IL-4 and antiinflammatory cytokine IL-10. However, TSLP induced DCs to produce high levels of Th2 cell-attracting chemokines thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22), as well as IL-8 and IL-15. In contrast, triggering of TLR3 by poly I:C strongly induced mDCs to produce all the Th1 cell-polarizing cytokines and proinflammatory cytokines (Fig. 1 B) but few Th2 cell-attracting chemokines. Although both TSLP and poly I:C induced mDCs to up-regulate the expression of costimulatory molecules, including CD40, CD80 and CD86, TSLP preferentially induced mDC to express the mRNA for OX40L (Fig. 1, A and B), a member of the TNF superfamily that has been implicated in the initiation of Th2 cell responses (15–18). This expression of OX40L by TSLP-DCs was confirmed by RT-PCR (Fig. 1 C) and flow cytometry analyses using anti-OX40L mAbs (Fig. 1 D). The induction of OX40L on mDC surface by TSLP was detectable by 12 h and reached a maximal level during 48–72 h after TSLP activation (Fig. 1 E).

### TSLP-DCs use OX40L to generate TNF- $\alpha$ <sup>+</sup>IL-10<sup>-</sup> inflammatory Th2 cells

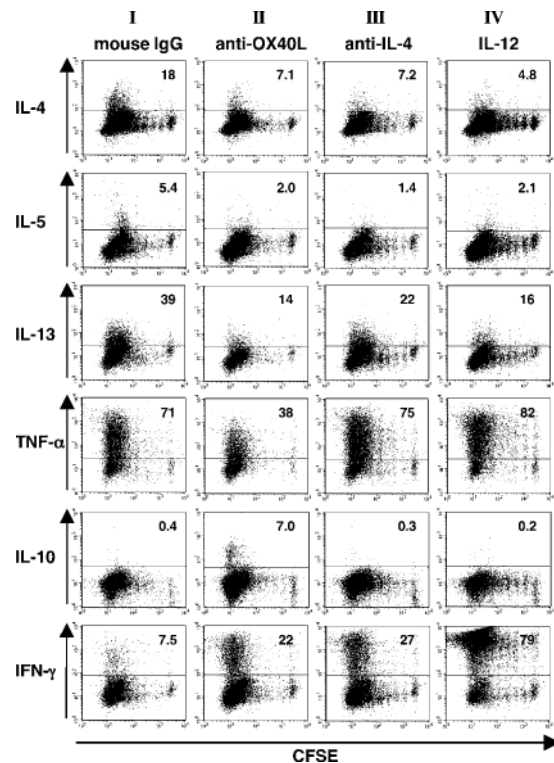
Because an OX40–OX40L interaction has been implicated in the triggering of Th2 cell immune responses in both human and mice (15–18), we investigated whether OX40L is the Th2 cell-polarizing signal from TSLP-DCs. Although naive CD4<sup>+</sup> T cells primed by DCs cultured in the absence of TSLP (med-DCs) produced low levels of both Th1 cytokine (IFN- $\gamma$ ) and Th2 cytokines (IL-4, -5, and -13) (Fig. 2 A, column I) and CD4<sup>+</sup> T cells primed by poly I:C-DCs produced less Th2 cytokines and a large amount of the Th1 cytokine IFN- $\gamma$  (Fig. 2 B, column I), CD4<sup>+</sup> T cells primed by TSLP-DCs produced large amounts of Th2 cytokines IL-4, IL-5, and IL-13, but lower Th1 cytokine IFN- $\gamma$  (Fig. 2 C, column I). Furthermore, TSLP-DCs induced naive CD4<sup>+</sup> T cells to produce large amount of TNF- $\alpha$  but no IL-10 (Fig. 2 C, column I). A neutralizing anti-OX40L mAb did not have sub-

stantial effects on the cytokine production by CD4<sup>+</sup> T cells primed by med-DCs (Fig. 2 A, column II) or by poly I:C-DCs (Fig. 2 B, column II). In contrast, anti-OX40L mAb strongly inhibited IL-4, -5, and -13 production and promoted IFN- $\gamma$  production by CD4<sup>+</sup> T cells primed by TSLP-DCs (Fig. 2, C [column II] and D). This demonstrates that OX40L represents the Th2 cell-polarizing signal from TSLP-DCs.

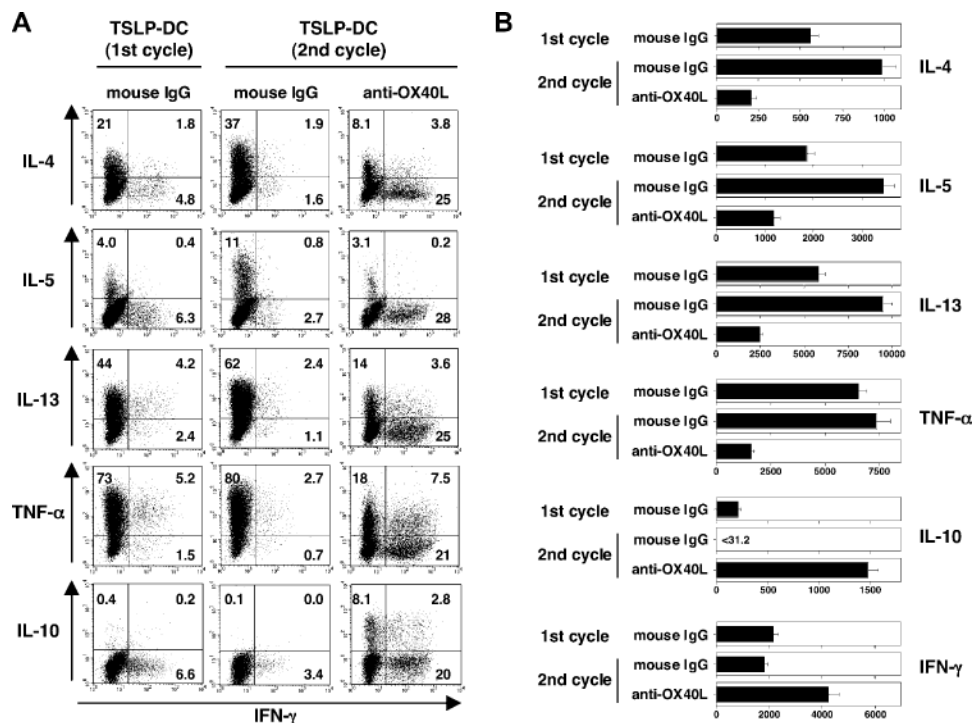
Most strikingly, the anti-OX40L mAb dramatically inhibited TNF- $\alpha$  production, but promoted IL-10 production by CD4<sup>+</sup> T cells primed by TSLP-DCs (Fig. 2, C [column II] and D). These data suggest that OX40L expressed by TSLP-DCs is responsible for not only polarizing naive CD4<sup>+</sup> T cells into Th2 cells, but also endowing the Th2 cells with a TNF- $\alpha$ <sup>+</sup>IL-10<sup>-</sup> inflammatory phenotype.

### IL-4 has no effect on TNF- $\alpha$ and IL-10

IL-4 has been suggested as the classic Th2 cell polarizing signal (1, 15, 19). However, because IL-4 is the major cytokine produced by Th2 cells themselves and human antigen-presenting cells including DCs activated by TSLP and other stimuli do not produce IL-4 (Fig. 1 B) (13), it has been suggested that IL-4 is not the original trigger of Th2 cell responses. To determine the function of IL-4 in the genera-



**Figure 3. Relationship between cytokine production and cell division in T cells primed with TSLP-DCs.** CFSE-labeled CD4<sup>+</sup> naive T cells were primed with TSLP-DCs in the presence of an anti-OX40L mAb, an anti-IL-4 mAb, or recombinant IL-12 for 7 d, and then intracellular cytokines were analyzed by flow cytometry. Numbers in each dot blot profile indicate the percentages of the respective cytokine-producing T cells. Data are from one of three independent experiments.



**Figure 4. TSLP-DCs further promote the generation of Th2 cells without inducing IL-10 production after two rounds of stimulation.** CD4<sup>+</sup> naive T cells were stimulated by TSLP-DCs for 7 d (one cycle of stimulation), and then these differentiated CD4<sup>+</sup> T cells were restimulated by TSLP-DCs from the same allogeneic donor for further 7 d (two cycles of stimulation) in the presence or absence of anti-OX40L mAbs. Cytokine

production by these two types of CD4<sup>+</sup> T cells was analyzed intracellularly by flow cytometry (A) and measured in supernatants after restimulation with anti-CD3 and anti-CD28 mAbs for 24 h by ELISA (B). Percentages of the respective cytokine-producing T cells are indicated in each dot blot profile in A. Error bars in B represent standard deviations of triplicate cultures. Data are from one of three independent experiments.

tion of inflammatory TNF- $\alpha$ <sup>2</sup>IL-10<sup>-</sup> Th2 cells induced by TSLP-DCs, we added a neutralizing anti-IL-4 mAb at the beginning of the coculture of naive CD4<sup>+</sup> T cells and allogeneic TSLP-DCs. We found that, as with anti-OX40L mAb, anti-IL-4 mAb decreased the frequency with which Th2 cells produced IL-4, -5, and -13, but concomitantly increased the frequency with which cells produced IFN- $\gamma$  (Fig. 2 C, column III). However, unlike anti-OX40L mAb, anti-IL-4 mAb had no effect on TNF- $\alpha$  or IL-10 production by T cells cultured with TSLP-DCs (Fig. 2, C [column III] and D).

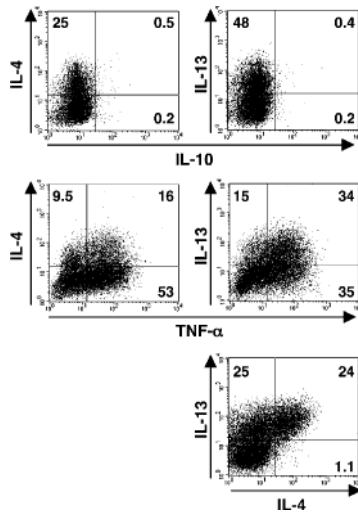
#### Synergy between DC-derived OX40L and T cell-derived IL-4

Because anti-OX40L mAb or anti-IL-4 mAb alone only partially blocked the generation of Th2 cells that produced IL-4, -5 and -13, we further investigated whether the combination of anti-OX40L and anti-IL-4 mAbs would completely block the generation of Th2 cells induced by TSLP-DCs. Indeed, we found a synergistic effect of anti-OX40L and anti-IL-4 mAbs: the combination of both almost completely switched a Th2 cell response to a Th1 cell response (Fig. 2, C [column IV] and D). Because TSLP-DCs do not produce IL-4, this experiment suggested that whereas OX40L is the original Th2 cell-polarizing signal from TSLP-DCs, IL-4 is a critical autocrine stabilizer and enhancer of the developing Th2 cells. It appears that OX40L

and IL-4 may work synergistically and sequentially in driving Th2 cell responses in T cell and TSLP-DC coculture.

#### IL-12 is dominant over OX40L

Because TSLP does not induce human mDCs to produce Th1 cell-polarizing signal IL-12 (13) (Fig. 1 B), we further investigated whether the ability of OX40L expressed by mDCs to induce Th2 cell responses depends on a default mechanism of no IL-12. We performed two experiments. First, we added exogenous IL-12 into the coculture of naive CD4<sup>+</sup> T cells and TSLP-DCs and found that exogenous IL-12 markedly inhibited the generation of Th2 cells, but strongly polarized T cell differentiation toward to Th1 cell (Fig. 2, C [column V] and D). Exogenous IL-12 also induced a strong Th1 cell polarization of naive CD4<sup>+</sup> T cells primed with DCs nonactivated by TSLP (med-DCs) (Fig. 2 A, column III), and neutralizing anti-IL-12 Ab inhibited the generation of Th1 cells induced by poly I:C-DCs (Fig. 2 B, column III). Second, we added neutralizing anti-CD40L mAb to block the ability of activated T cells to induce mDCs to produce endogenous IL-12 through CD40L during the cognate T cell and TSLP-DC interaction. We found that anti-CD40L mAb completely blocked the generation of residual Th1 cells and further promoted Th2 cell differentiation in the coculture of CD4<sup>+</sup> T cells and TSLP-DCs (Fig. 2, C [column VI] and D). The results of these two experiments sug-



**Figure 5. Th2 cells induced by TSLP-DCs coexpress TNF- $\alpha$  but not IL-10, as analyzed by three-color intracellular cytokine staining.** Naive CD4<sup>+</sup> T cells were primed with TSLP-DCs for 7 d, and then cytokine production by the CD4<sup>+</sup> T cells was analyzed intracellularly by flow cytometry using three patterns of triple-color staining as follows: (a) FITC-labeled anti-TNF- $\alpha$  + PE-labeled anti-IL-4 + APC-labeled anti-IL-10; (b) FITC-labeled anti-TNF- $\alpha$  + PE-labeled anti-IL-13 + APC-labeled anti-IL-10; and (c) FITC-labeled anti-TNF- $\alpha$  + PE-labeled anti-IL-13 + Alexa Fluor 647-labeled anti-IL-4. Percentages of the respective cytokine-producing T cells are indicated in each dot plot profile. Data are from one of three independent experiments.

gest that the ability of TSLP-DCs to induce Th2 cell differentiation depends on a positive Th2 cell–polarizing signal, namely, OX40L, and a default mechanism of low or absent IL-12.

#### IL-10–producing T cells are not generated by default T cell activation

Because OX40L has been shown to be critical for the proliferation and survival of activated T cells (20, 21), it is possible that anti-OX40L mAb allows the generation of IL-10–producing T cells by arresting T cell proliferation induced by TSLP-DCs. To test this possibility, naive CD4<sup>+</sup> T cells were labeled with CFSE, and then cultured with allogeneic TSLP-DCs in the presence of anti-OX40L mAb, anti-IL-4 mAb, or isotype control mAb and IL-12 for 7 d. Unlike the TNF- $\alpha$ - and IFN- $\gamma$ -producing T cells, the IL-10–producing T cells generated in the presence of anti-OX40L mAb all underwent at least seven divisions (Fig. 3), suggesting that the generation of IL-10–producing T cells is not the result of T cell proliferation arrest caused by the blockade of OX40L.

#### TSLP-DCs fail to induce Th2 cells to produce IL-10 after two rounds of stimulation

Complete differentiation of Th1 and Th2 cells requires two cycles of stimulation, and the differentiation of IL-10- and IFN- $\gamma$ -double producing T cells also requires extensive expansion induced by IL-12 for several weeks (22, 23). To exclude the possibility that TSLP-DCs could induce IL-10–producing conventional Th2 cells after extended clonal

expansion, we analyzed the cytokine production by CD4<sup>+</sup> T cells after two rounds of stimulation by TSLP-DCs. We found that, when compared with the single cycle stimulation by TSLP-DCs, two cycles of stimulation further promoted the CD4<sup>+</sup> T cells to produce IL-4, IL-5, IL-13, and TNF- $\alpha$  without inducing IL-10 production (Fig. 4, A and B). The addition of an anti-OX40L mAb during the two rounds of stimulation strongly inhibited CD4<sup>+</sup> T cells to produce IL-4, IL-5, IL-13, and TNF- $\alpha$ , but concomitantly promoted CD4<sup>+</sup> T cells to produce IL-10 and IFN- $\gamma$ . These results suggest that the ability of TSLP-DCs to induce the generation of TNF- $\alpha$ <sup>2+</sup>IL-10<sup>-</sup> inflammatory Th2 cells from naive CD4<sup>+</sup> T cells through OX40L is not caused by the differential kinetics of IL-10 production by the activated T cells.

#### The IL-4<sup>+</sup>/IL-13<sup>+</sup> Th2 cells induced by TSLP-DCs express TNF- $\alpha$ but not IL-10, as analyzed by three-color intracellular cytokine staining

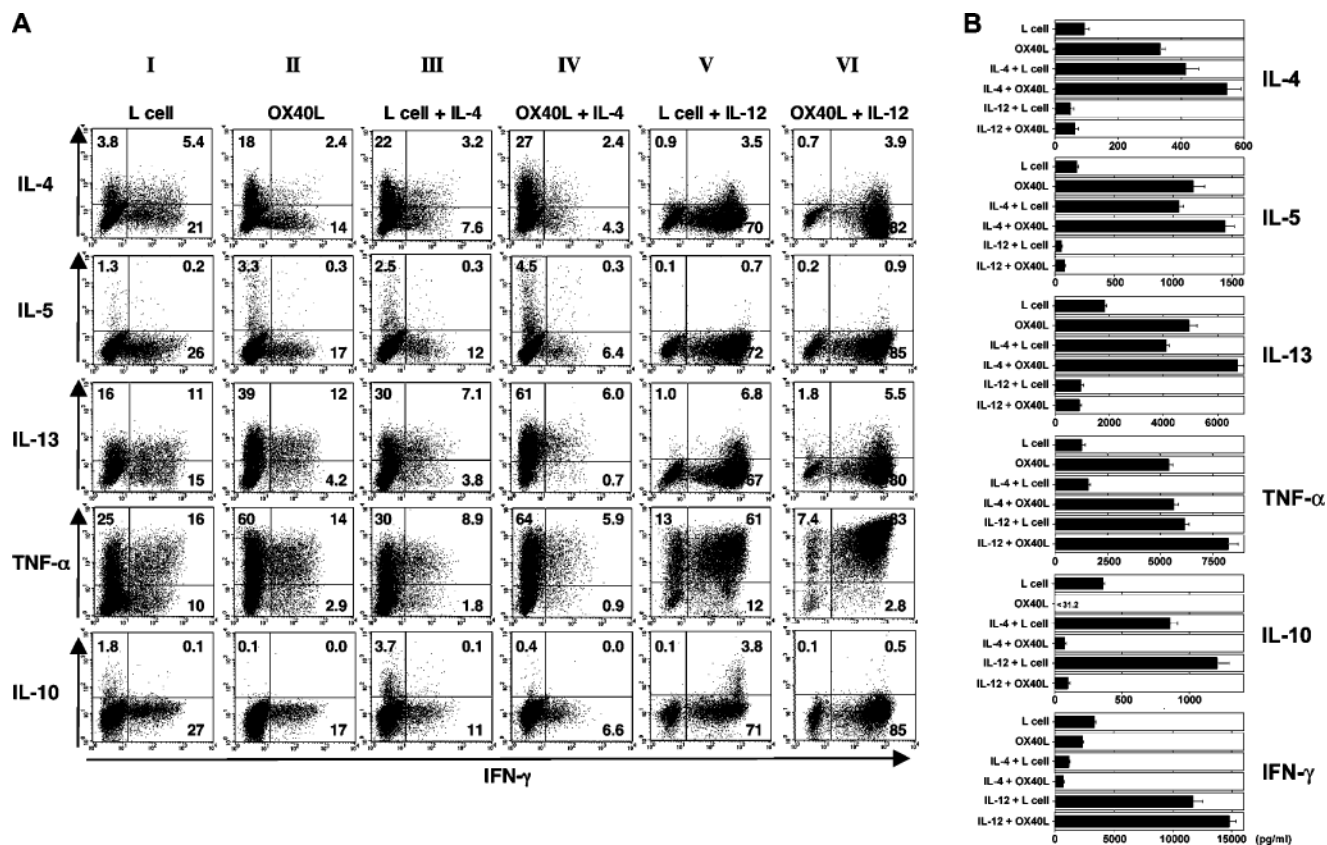
To further confirm that TSLP-DCs induced naive CD4<sup>+</sup> T cells to differentiate into TNF- $\alpha$ <sup>2+</sup>IL-10<sup>-</sup> inflammatory Th2 cells, we performed three-color intracellular cytokine staining. Fig. 5 shows that, after a 6-h restimulation of CD4<sup>+</sup> T cells primed with TSLP-DCs, ~25% of CD4<sup>+</sup> T cells expressed IL-4 and 48% of T cells expressed IL-13 (top). None of these IL-4- or IL-13–producing CD4<sup>+</sup> T cells expressed IL-10. In contrast, over two thirds of the IL-4- or IL-13–producing CD4<sup>+</sup> T cells expressed TNF- $\alpha$  (Fig. 5, middle). Interestingly, while all the IL-4-expressing CD4<sup>+</sup> T cells expressed IL-13, ~50% of the IL-13–producing T cells did not express IL-4 (Fig. 5, bottom). These data confirm that the majority of the Th2 cells induced by TSLP-DCs are TNF- $\alpha$ <sup>2+</sup>IL-10<sup>-</sup> inflammatory Th2 cells, although these Th2 cells may produce IL-13 or both IL-13 and IL-4.

#### Recombinant OX40L induces inflammatory Th2 cell responses

To further confirm the effects of the OX40L expressed by TSLP-DCs in polarizing TNF- $\alpha$ <sup>2+</sup>IL-10<sup>-</sup> inflammatory Th2 cell responses, we cultured naive CD4<sup>+</sup> T cells in the presence of anti-CD3 and anti-CD28 mAbs with parental L cells or OX40L-transfected L cells. After 7 d of culture, the primed CD4<sup>+</sup> T cells were then examined for their ability to produce cytokines by intracellular staining (Fig. 6 A) and ELISA analysis of the culture supernatants after restimulation with anti-CD3 and anti-CD28 mAbs (Fig. 6 B). OX40L strongly primed naive CD4<sup>+</sup> T cells to produce the Th2 cytokines IL-4, -5, and -13 and the inflammatory cytokine TNF- $\alpha$ , but little IFN- $\gamma$  and IL-10 (Fig. 6, A [column II] and B). These data confirm our conclusion that OX40L is the downstream molecule from TSLP-DCs that induces TNF- $\alpha$ <sup>2+</sup>IL-10<sup>-</sup> inflammatory Th2 cell responses.

#### Recombinant OX40L enhances TNF- $\alpha$ production and inhibits IL-10 production by the conventional Th2 cells induced by IL-4

Previous studies have demonstrated that IL-4 plus anti-CD3 and anti-CD28 mAbs can induce naive CD4<sup>+</sup> T cells to dif-



**Figure 6. OX40L-transfected L cells enhance TNF- $\alpha$  production and inhibit IL-10 production in T cells.** CD4<sup>+</sup> naive T cells were cultured with anti-CD3 and anti-CD28 mAbs on OX40L-transfected L cells and/or the indicated recombinant cytokines for 7 d. Cytokine production by CD4<sup>+</sup> T cells was analyzed intracellularly by flow cytometry (A) and measured in

supernatants after restimulation with anti-CD3 and anti-CD28 mAbs for 24 h by ELISA (B). Percentages of the respective cytokine-producing T cells are indicated in each dot blot profile in A. Error bars in B represent standard deviations of triplicate cultures. Data are from one of four independent experiments.

ferentiate into the conventional Th2 cells producing IL-4, -5, -13, and -10 (24, 25). We confirmed these published data by culturing naive CD4<sup>+</sup> T cells with IL-4 and anti-CD3 and anti-CD28 mAbs (Fig. 6, A [column III] and B). OX40L strongly promoted the conventional Th2 cells induced by IL-4 to produce more IL-4, -5, and -13, but less IFN- $\gamma$  (Fig. 6, A [column IV] and B). Most importantly, OX40L promoted TNF- $\alpha$  production while shutting down IL-10 production by the conventional Th2 cells induced by IL-4. This experiment further demonstrated that OX40L is a master switch that converts a conventional IL-10-producing Th2 cell response induced by IL-4 into a TNF- $\alpha$ -producing inflammatory Th2 cell response.

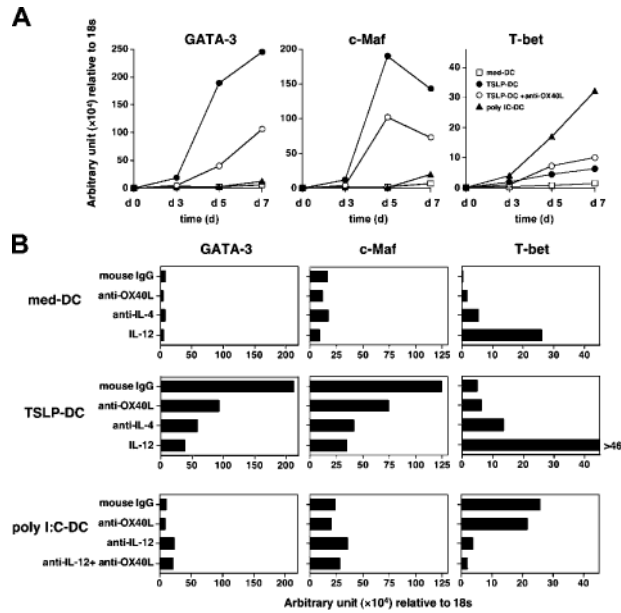
#### Recombinant OX40L enhances TNF- $\alpha$ production and inhibits IL-10 production by Th1 cells

Although OX40L expressed by TSLP-DCs failed to induce a Th2 cell response in the presence of IL-12, the question raised here is whether OX40L can change the quality of Th1 cell response induced by IL-12. We therefore cultured naive CD4<sup>+</sup> T cells in the presence of anti-CD3 and anti-CD28 mAbs and IL-12 in the presence or absence of OX40L. Na-

ive CD4<sup>+</sup> T cells cultured with anti-CD3 and anti-CD28 mAbs and with IL-12 differentiated into conventional Th1 cells that produce IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 (Fig. 6, A [column V] and B), which is consistent with previous studies (24, 26). The addition of OX40L to the above Th1 cell culture did not induce the cultured T cells to produce IL-4, -5, and -13, but it strongly promoted the production of TNF- $\alpha$  and IFN- $\gamma$  while inhibiting IL-10 production by the developing Th1 cells (Fig. 6, A [column V] and B).

#### OX40L increases GATA-3 and c-Maf in T cells cultured with TSLP-DCs

Th1 and Th2 cell differentiation is regulated by key transcriptional factors such as T-bet for Th1 and GATA-3 and c-Maf for Th2 (19). Because Th1 cells express high T-bet but low GATA-3 and c-Maf and Th2 cells express low T-bet but high GATA-3 and c-Maf, these transcriptional factors can be used as a molecular markers for Th1 or Th2 cell differentiation. We therefore examined the kinetics of GATA-3, c-Maf, and T-bet expression by quantitative PCR in CD4<sup>+</sup> T cells primed by med-DCs, TSLP-DCs, or poly I:C-DCs, respectively. Although CD4<sup>+</sup> T cells that



**Figure 7. OX40L expressed by TSLP-DCs induces the expression of GATA-3 and c-Maf in T cells.** Expression levels of transcriptional factors involved in Th1 and Th2 cell differentiation—T-bet, GATA-3, and c-Maf—were quantitatively measured by real-time PCR in CD4<sup>+</sup> T cells primed with med-DCs, TSLP-DCs, and poly I:C-DCs in the presence of the indicated neutralizing mAb or recombinant IL-12 at different time points (A) or after a 7 d-culture (B). The amounts of mRNA were shown by arbitrary units relative to the amount of 18s mRNA. Representative data of three independent experiments are shown.

were primed by inactivated DCs (med-DCs) expressed low levels of GATA-3, c-Maf, and T-bet at any time points after priming (Fig. 7 A), the addition of IL-12 strongly up-regulated T-bet expression in the primed CD4<sup>+</sup> T cells (Fig. 7 B). In contrast, CD4<sup>+</sup> T cells primed by TSLP-DCs expressed high levels of GATA-3 and c-Maf, but low T-bet (Fig. 7, A and B). Adding anti-OX40L mAb or anti-IL-4 mAb greatly reduced GATA-3 and c-Maf expression (Fig. 7 B), confirming that the Th2 cell polarization induced by TSLP-DCs is mediated by OX40L and IL-4. However, adding IL-12 into the CD4<sup>+</sup> T cell that was primed with TSLP-DCs strongly inhibited GATA-3 and c-Maf, while strongly up-regulating T-bet expression (Fig. 7 B). This confirms that the ability of IL-12 to induce Th1 cells is dominant over the ability of OX40L to induce Th2 cells. In contrast to TSLP-DCs, poly I:C-DCs induced naive CD4<sup>+</sup> T cells to express T-bet, but neither GATA-3 nor c-Maf (Fig. 7, A and B). The ability of poly I:C-DCs to induce T-bet in naive CD4<sup>+</sup> T cells was inhibited by anti-IL-12 mAb (Fig. 7 B).

These data suggest that the expression of T-bet or GATA-3 and c-Maf can be used as molecular markers for Th1 or Th2 cell differentiation. OX40L expressed by TSLP-DCs induced the expression of two transcriptional factors GATA-3 and c-Maf in T cells, further supporting their critical roles in Th2 cell polarization.

## DISCUSSION

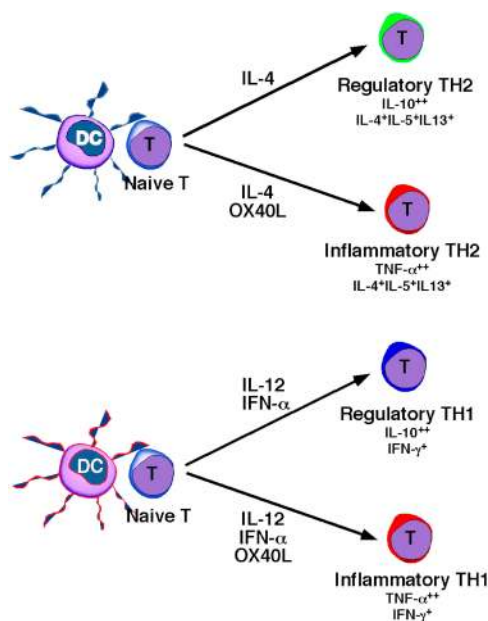
We previously demonstrated that TSLP strongly activates human mDCs to up-regulate major histocompatibility classes I and II and costimulatory molecules CD40, CD80, CD83, and CD86 (13). However, unlike conventional DC activation signals such as ligands for different TLRs and CD40L, TSLP does not induce mDCs to secrete proinflammatory/Th1-inducing cytokines such as IL-1 $\alpha/\beta$ , IL-6, IL-12, IL-23, IL-27, or TNF- $\alpha$ . These findings suggest that TSLP activates mDCs through a pathway that is independent of TLR-IL-1R-MyD88 or CD40-TRAF-6 signaling pathways, which is consistent with recent findings regarding the signaling pathways underlying Th2 cell development (27–29). Our findings also suggest that the absence of IL-12 production by TSLP-DCs creates a permissive condition in which a positive Th2 cell-polarizing signal from TSLP-DCs can induce Th2 cell responses.

By using a neutralizing monoclonal antibody to OX40L and recombinant OX40L, we demonstrated that OX40L is the molecule downstream from TSLP-DCs that induces naive CD4<sup>+</sup> T cells to differentiate into TNF- $\alpha$ <sup>2+</sup>IL-10<sup>-</sup> inflammatory Th2 cells. Because TSLP-DCs do not produce detectable IL-4 at both mRNA and protein levels, our study suggests that OX40L represent the initial signal from DCs for polarizing naive CD4<sup>+</sup> T cells to Th2 cells. Although many previous studies suggest a critical role of OX40-OX40L interaction in driving Th2 cell responses (15–18), the concept of OX40L being a Th2 cell-polarizing factor has been questioned by many *in vivo* studies showing that blocking OX40-OX40L interaction inhibits the development or decreases the severity of Th1 cell-mediated autoimmune diseases (30–33). This can now be explained by our study showing that OX40L is unable to induce Th2 cell response in the presence of IL-12. In contrast, OX40L has the ability to promote IL-12-mediated Th1 autoimmunity by enhancing TNF- $\alpha$  and IFN- $\gamma$  production while inhibiting IL-10 production. OX40L may also contribute to promote Th1 cell-mediated autoimmunity by prolonging the survival and proliferation of Th1 memory and effector cells and by enhancing the effector functions of CD8<sup>+</sup> T cells (34–36).

It has been suggested that Th2 cell differentiation is a simple default fate, which occurs in the absence of Th1 cell-polarizing signals (37). However, IL-12 deficiency does not result in a spontaneous Th2 cell response, suggesting that the development of a Th2 cell response is not a simple default fate (27). Our current study suggests that the development of a Th2 cell response depends on a Th2 cell-polarizing signal OX40L, as well as a default mechanism of no IL-12. Only TSLP, but not CD40L or poly I:C, can stimulate DCs to provide both. The current finding that a Th1 cell-polarizing factor IL-12 is dominant over a Th2 cell-polarizing factor OX40L may also provide an explanation at the molecular level for the “hygiene theory” of atopy.

Historically, Th2 cells have been defined as CD4<sup>+</sup> T cells that have the ability to produce IL-4, IL-5, IL-10, and IL-13, and Th1 cells have been defined as CD4<sup>+</sup> T cells that





**Figure 8.** Schematic illustration of Th1 and Th2 cell responses classified into inflammatory versus regulatory subtypes according to IL-10 and TNF- $\alpha$  expression. The figure depicts the hypothesis from our study. IL-4 and IL-12 are classic Th2 cell- and Th1 cell-polarizing factors, respectively. IL-4 and IL-12/IFN- $\alpha/\beta$  induce conventional Th2 and Th1 cells, respectively, which produce IL-10. In contrast, OX40L from DCs promotes TNF- $\alpha$ , but inhibits IL-10 production by the developing Th2 cells induced by IL-4 or Th1 cells induced by IL-12. These inflammatory Th2 and Th1 cells may contribute to the induction of allergic and autoimmune diseases, respectively.

have the ability to produce IFN- $\gamma$  and sometimes TNF- $\alpha$  (1, 2). However, many studies have demonstrated that IL-10 is not involved in Th2 cell-mediated allergic inflammation (3–5), by contrast it inhibits allergic diseases in animal models (6–9). In addition, TNF- $\alpha$  has been implicated in Th2 cell-mediated allergic diseases such as asthma and atopic dermatitis (38–41). Therefore, IL-10 and TNF- $\alpha$  should not be classified as either a Th2 or a Th1 cytokine.

Similarly a recent study by Umetsu and colleagues (42) defined a new type of Th1 cell-like regulatory T cells that expressed both IFN- $\gamma$  and IL-10 and had the ability to inhibit airway hyper-reactivity. We therefore propose on the basis of these findings that Th1 cells or Th2 cells be further classified into a TNF- $\alpha^{2+}$ IL-10 $^{-}$  inflammatory subtype and a TNF- $\alpha^{+/-}$ IL-10 $^{+}$  regulatory subtype (Fig. 8). OX40L expressed by DCs may convert TNF- $\alpha^{+/-}$ IL-10 $^{+}$  regulatory T helper cells into TNF- $\alpha^{2+}$ IL-10 $^{-}$  inflammatory T helper cells during both Th1 cell development (in the presence of IL-12) or Th2 cell development (in the absence of IL-12) (Fig. 8).

In summary, we demonstrated that OX40L is the positive Th2 cell-polarizing signal from TSLP-DCs that induces inflammatory Th2 cell responses, in conjunction with an IL-12 default mechanism. We further showed that OX40L acts as a switch that inhibits IL-10, but promotes TNF- $\alpha$  in both

Th1 and Th2 cell responses. These results may shed new light on the unsolved paradoxes of Th2 cell biology and lead us to propose that Th1 and Th2 cell responses can be divided into inflammatory and regulatory subtypes.

## MATERIALS AND METHODS

**Isolation and culture of blood myeloid DCs.** CD11c $^{+}$  DCs were isolated from the buffy coat of blood from healthy adult volunteers, as described previously (13, 43). In brief, the DC-enriched population (lineage $^{-}$  cells) was obtained from PBMCs by negative immunoselection using a mixture of mAbs against the lineage markers, CD3 (OKT3), CD14 (M5E2), CD15 (HB78), CD20 (L27), CD56 (B159), and CD235a (10F7MN), followed by using goat anti-mouse IgG-coated magnetic beads (M-450; Dynal and Miltenyi Biotec). The CD11c $^{+}$ , lineage $^{-}$ , and CD4 $^{+}$  cells were isolated by a FACS Aria (BD Biosciences) by using allophycocyanin (APC)-labeled anti-CD11c (B-ly6), a mixture of FITC-labeled mAbs against lineage markers, CD3 (HIT3a), CD14 (MOP9), CD15 (HI98), CD16 (3G8), CD19 (HIB19) and CD56 (NCAM16.2); and APC-Cy7-labeled CD4 (RPA-T4) to reach >99% purity. CD11c $^{+}$  DCs were cultured in Yssel's medium (Gemini Bio-Products) containing 2% human AB serum. Cells were seeded at a density of  $2 \times 10^5$  cells/200  $\mu$ l medium in flat-bottomed 96-well plate in the presence of 15 ng/ml TSLP (recombinant human TSLP had been prepared in-house using an adenovirus vector system as described previously [reference 13]), 25  $\mu$ g/ml of poly I:C (InvivoGen), or culture medium alone.

**Microarray analysis and bioinformatics.** Total RNA from DCs was immediately isolated with the RNeasy kit from QIAGEN, and used to generate cDNA according to the Expression Analysis Technical Manual (Affymetrix). cRNA samples were generated with the Bioarray High-Yield RNA Transcript Labeling kit (ENZO) and Human Genome U133 plus 2.0 array according to the manufacturer's protocol (Affymetrix). The scanned images were aligned and analyzed using the GeneChip software Microarray Suite 5.0 (Affymetrix) according to the manufacturer's instructions. The signal intensities were normalized to the mean intensity of all the genes represented on the array, and global scaling (scaling to all probe sets) was applied before performing comparison analysis. Genes with variable expression levels were selected based on the following criteria: genes should be expressed (have presence calls) in at least one of the three samples and  $\sigma/\mu$  ratio should be  $>0.65$ , where  $\sigma$  and  $\mu$  are the standard deviation and mean of the hybridization intensity values of each particular gene across all samples, respectively. An unsupervised hierarchical clustering algorithm by the software Cluster was applied to group the three samples for Fig. 1 A based on the similarity of the expression profiles of the selected genes. For genes represented by multiple probe sets, results for only one representative probe set are shown.

**Real-time PCR.** Total RNA was extracted with the Qiagen RNeasy mini protocol and was converted to cDNA using oligo-dT, random hexamers, and SuperScript RT II (Invitrogen). cDNA was diluted 1:10 and real-time PCR was performed using a sequence detector (model ABI PRISM 7500; PerkinElmer) and target mixes (TaqMan Assay-on-Demand Gene expression reagents; Applied Biosystems): T-bet (Hs00203436\_m1), GATA 3 (Hs00231122\_m1), c-Maf (Hs00286832\_m1), and 18s (Hs99999901\_s1). Threshold cycle ( $C_T$ ) values for each gene were normalized to 18s using the equation  $1.8^{(18s-GENE)}$  (10,000), where 18s was the mean  $C_T$  of triplicate 18s runs, GENE was the mean  $C_T$  of triplicate runs of the gene of interest, and 10,000 was an arbitrarily chosen factor to bring all values above zero.

**Analyses of OX40L expression.** RT reactions were performed with SuperScript RT II. The DNA resulting from each RT reaction was then subjected to PCR. The temperature profiles of the PCR were as follows: an initial denaturation step at 94°C for 5 min; followed by 36 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 30 s; and then a final elongation step at 72°C for 7 min. The sequences of primers were as follows: (for

OX40L) forward, 5'-CCCAGATTGTGAAGATGGAA-3' and reverse, 5'-GCCTGGTTTTAGATATTGCC-3'; and (for  $\beta$ -actin) forward, 5'-CTGGAACGGTGAAGGTGACA-3' and reverse, 5'-AAGGGACTTCCT-GTAACAATGCA-3'. To evaluate surface OX40L expression, CD11c<sup>+</sup> DCs freshly isolated or after being cultured at different time points were stained with PE-labeled anti-OX40L mAb (AnCell) or with an isotype-matched control mAb and then were analyzed by a FACSCalibur (BD Biosciences).

**CD4<sup>+</sup> T cell stimulation.** CD4<sup>+</sup>CD45RA<sup>+</sup> naive T cells (purity >99%) were isolated by using CD4<sup>+</sup> T cell Isolation Kit II (Miltenyi Biotec) followed by cell sorting (as a CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD25<sup>-</sup> fraction). After 24 h of culture under different conditions (medium alone, poly I:C, or TSLP), CD11c<sup>+</sup> DCs were washed twice and cocultured with 2 × 10<sup>4</sup> freshly purified allogeneic naive CD4<sup>+</sup> T cells (DC/T ratio, 1:5) in round-bottomed 96-well culture plates for 7 d. The T cells were also collected at day 3 and 5 for real-time PCR analysis. In some experiments, CD4<sup>+</sup> T cells primed with TSLP-DCs were subsequently recultured with TSLP-DCs from the same allogeneic donor (DC/T ratio, 1:5) for further 7 d. In other experiments, naive CD4<sup>+</sup> T cells were stimulated with 5  $\mu$ g/ml anti-CD3 mAb (OKT3) and 1  $\mu$ g/ml anti-CD28 mAb (CD28.2) in the presence of irradiated OX40L-transfected L cells (16) or parental L cells, at a ratio of 1:4. Yssel's medium containing 2% human AB serum was used for the T cell cultures. We used the following neutralizing Abs and recombinant human cytokines for our culture conditions: 50  $\mu$ g/ml anti-OX40L mAb (ik-5), 10  $\mu$ g/ml anti-IL-4 mAb (MP4-25D2; BD Biosciences), 1  $\mu$ g/ml anti-IL-12 Ab (AF-219-NA; R&D Systems), 10  $\mu$ g/ml anti-CD40L mAb (LL2), 10 ng/ml recombinant human IL-12 (R&D Systems), and 25 ng/ml recombinant human IL-4 (R&D Systems). Mouse IgG2a, rat IgG1, and goat IgG (R&D Systems) were used as controls. In some experiments, naive T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (Invitrogen) as described previously (43).

**Analyses of T cell cytokine production.** After one cycle of stimulation (for 7 d) or two cycles of stimulation (for total 14 d) by the DCs or anti-CD3 and anti-CD28 mAbs (for 7 d), the primed CD4<sup>+</sup> T cells were collected and washed. For detection of cytokine production in the culture supernatants, the T cells were restimulated with plate-bound anti-CD3 (OKT3, 5  $\mu$ g/ml) and soluble anti-CD28 (1  $\mu$ g/ml) at a concentration of 10<sup>6</sup> cells/ml for 24 h. The levels of IL-4, IL-5, IL-10, IL-13, TNF- $\alpha$ , and IFN- $\gamma$  were measured by ELISA (all kits from R&D Systems). For intracellular cytokine production, the primed CD4<sup>+</sup> T cells were restimulated with 50 ng/ml of PMA plus 2  $\mu$ g/ml of ionomycin for 6 h. Brefeldin A (10  $\mu$ g/ml) was added during the last 2 h. The cells were stained with the combination of PE-labeled mAbs to IL-4, IL-5, IL-10, IL-13, IFN- $\gamma$ , or TNF- $\alpha$ , FITC-labeled anti-IFN- $\gamma$  or anti-TNF- $\alpha$ , and APC-labeled anti-IL-10 or Alexa Fluor 647-labeled anti-IL-4 (all from BD Biosciences) using FIX and PERM kit (CALTAG).

**Online supplemental material.** Tables S1–S4 show the datasets from four independent experiments related to Figs. 2 (C and D) and 6 (A and B), respectively. Dataset of experiment 1 in each table was shown as Figs. 2 (C and D) and 6 (A and B). Data are shown as percentages of the respective cytokine-producing T cells (Tables S1 and S3) and as means of triplicate cultures evaluated by ELISA (Tables S2 and S4). Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051135/DC1>.

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