Thymic stromal lymphopoietin drives the development of IL-13$^+$ Th2 cells

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T helper 2 (Th2) cells are pivotal in the development of allergy. Allergen exposure primes IL-4$^+$ Th2 cells in lymph node, but production of effector cytokines including IL-5 and IL-13 is thought to require additional signals from antigen and the environment. Here we report that a substantial proportion of naive CD4$^+$ T cells in spleen and lymph node express receptors for the epithelium-derived inflammatory cytokine thymic stromal lymphopoietin (TSLP). Culture of naive CD4$^+$ T cells in anti-(a)CD3, aCD28, and TSLP-supplemented Th2 conditions enabled the development of a unique population of IL-13-single positive (IL-13-SP) CD4$^+$ T cells; TSLP and Th2 conditions were both required for their development. Sorting experiments revealed that IL-13-SP Th2 cells originated from IL-4-negative precursors and coexpressed transcripts for the Th2 cytokines IL-5 and IL-9. In vivo, high TSLP levels acted directly on CD4$^+$ T cells to induce the development of IL-13-SP and IL-4$^+$IL-13$^+$ double-positive populations in lymph node. These cells were phenotypically similar to Th2 effector cells and were CXCR5^{low}PD1^{low} and expressed low levels of *Bcl6* and *Il21* transcripts and high levels of *Gata3*, *Il3*, and *Il5*. Our findings suggest a role of TSLP in directly promoting Th2 cell effector function and support the notion of TSLP as a key driver of Th2 inflammation.

TSLP | Th2 cells | effector T cells | IL-13 | IL-4

The cytokine-regulated differentiation of naive CD4$^+$ T cells into type 2 helper T (Th2) cells expressing IL-4, IL-13, and IL-5 represents a key event in the development of allergic responses (1, 2). Initial investigations led to the concept that CD4$^+$ T cells primed in Th2 conditions rapidly acquire the ability to produce multiple Th2 cytokines, with stochastic mechanisms driving heterogeneity in cytokine production at the effector stage (3). Contrary to that notion, recent work suggests a more complex regulation in which Th2 cytokine production is temporally and spatially compartmentalized (4–6). These studies suggest that the initial priming of Th2 cells to IL-4 production in lymph node (LN) is followed by the gain of capacity to produce additional cytokines on secondary exposure to antigen in nonlymphoid tissue (7). In line with those first observations, detailed studies using the inhaled allergen house dust mite (HDM) showed that naive CD4$^+$ T cells are primed in LN to a CXCR5$^+$PD1$^+$BCL6$^+$ T follicular helper (Tfh) phenotype, producing IL-4 and IL-21 but no IL-5 or IL-13. On secondary exposure to HDM via the airway, primed Th2 cells in lung are able to differentiate into CXCR5^{lo}PD1^{lo} Th2 effector cells that are GATA3$^+$ and produce IL-13, but express lower BCL6 and IL-21 than Tfh cells in LN (8–10).

The cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) are produced by epithelia exposed to various insults (11) and are proposed to play a key role in the acquisition of effector function by primed Th2 cells (7). However, how these tissue-derived factors mediate their effect on the evolving Th2 response is not well understood. IL-33 was recently found to promote Th2 effector activity by suppressing BCL6 expression in Tfh cells, in turn de-repressing IL-4 transcription (12). This mechanism of action is compatible with the known expression of the IL-33 receptor ST2 on activated Th2 cells (13). Interestingly, unlike ST2, expression of the TSLP receptor (TSLPR) does not require T cell activation and is already observed on naive CD4$^+$ T cells. Based on this different pattern of receptor expression, it seems reasonable to assume that the effect of TSLP, IL-25, and IL-33 on the differentiation of CD4$^+$ T cells to Th2 effectors may also differ.

TSLP is an epithelial cytokine that plays a key role in a range of conditions, including allergic responses, viral infections, and tumor progression (14–16). TSLP binds to a heterodimeric receptor formed by a TSLP-specific TSLPR subunit and the IL7R signaling chain (14, 17) and acts on several immune cell types including dendritic cells, type 2 innate lymphoid cells (ILC2), mast cells, basophils, and T cells (14, 16). The direct effects of TSLP on T cells have been studied by several groups, and both human and mouse CD4$^+$ T cells stimulated in a TCR-dependent manner in the presence of TSLP show enhanced proliferation and IL-4 secretion (18, 19). It remains unclear whether TSLP may become available in LN during T cell priming, or whether exposure is restricted to epithelia such as skin and mucosal surfaces, which are only accessed by T cells after activation.

We used the *Il4* and *Il13* transcriptional reporter 4C13R mice (20) to specifically investigate the effect of TSLP on the differentiation of Th2 cells in vitro and in vivo. We find that, in addition to enhancing IL-4 expression by CD4$^+$ T cells (19), TSLP was able to drive the development of a separate population of IL-13-DsRed single-positive (IL-13DR SP) CD4$^+$ T cells that also expressed *Il5* and *Il9* transcripts and originated from IL-4-AmCyan (IL-4AC)-negative T cell precursors in vitro. In vivo, IL-13DR SP were found in LN, but lacked expression of Tfh surface markers and expressed the low *Bcl6* and high *Gata3* characteristic of effector Th2 cells. Thus, our findings identify

Significance

T helper 2 (Th2) cells are defined by their ability to produce the hallmark cytokine IL-4. However, to mediate allergic inflammation in tissues, Th2 cells must secrete additional cytokines including IL-13 and IL-5. We used IL-4 and IL-13 dual-reporter mice to show that naive CD4$^+$ T cells cultured in the presence of IL-4 and thymic stromal lymphopoietin (TSLP) generate a population of IL-4^{neg}IL-13^{pos} Th2 cells that develop from IL-4^{neg} precursors and express the Th2 effector cytokines IL-5 and IL-9. In vivo, high TSLP levels promote the development of a similar population of IL-4^{neg}IL-13^{pos} T cells that also express *Gata3*, *Il5*, and *Il3* transcripts. Thus, TSLP drives the early differentiation of a distinct population of effector Th2 cells with pro-inflammatory properties.

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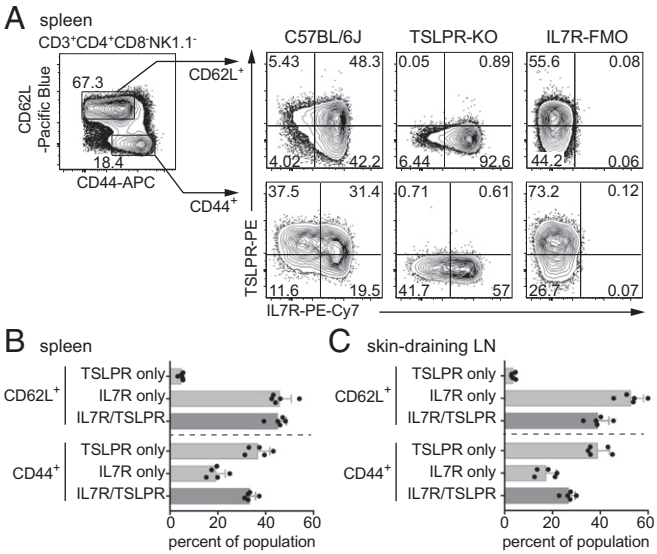

Fig. 1. Naive and antigen-experienced CD4$^+$ T cells express both subunits of the TSLP receptor. CD4$^+$ T cells from spleen and pooled skin-dLN of C57BL/6 or TSLPR-KO mice were examined for coexpression of TSLPR and IL7R by flow cytometry. (*A*) Gating strategy for splenic CD4$^+$ T cells. (*B*) Expression of TSLPR and IL7R in splenic CD4$^+$ T cell subsets. (*C*) Expression of TSLPR and IL7R in skin-dLN CD4$^+$ T cell subsets. Bar graphs show mean and SD for five mice/group; each dot corresponds to one mouse. Data refer to one of three repeat experiments that gave similar results. FMO, fluorescence minus one control.

TSLP as a key factor supporting the early differentiation of effector Th2 cells both in vitro and in vivo.

Results

CD4$^+$ T Cells in Spleen and LN Coexpress TSLPR and IL7R. To investigate whether T cells can directly respond to TSLP, we examined the expression of IL7R and TSLPR on CD4$^+$ T cells by flow cytometry. In spleen, more than 40% of the CD62L$^+$ naive CD4$^+$ T cells coexpressed IL7R and TSLPR, and a similar proportion was IL7R$^+$TSLPR$^-$ (Fig. 1 *A* and *B*). In the CD44$^+$ antigen-experienced CD4$^+$ population, 35–40% of cells coexpressed both receptor subunits, about 20% were IL7R$^+$, and 35–40% expressed TSLPR only (Fig. 1). No TSLPR expression was detected on T cells from TSLPR-KO mice (Fig. 1*A*). An analysis of TSLPR and IL7R expression on CD4$^+$ T cells in skin-draining LN (dLN) revealed a pattern of expression similar to spleen (Fig. 1*C*). In the skin, only about 10% of CD4$^+$ T cells and dendritic epithelial T cells coexpressed IL7R and TSLPR, as well as less than 5% of the CD8$^+$ and TCRγδ$^+$ T cells (Fig. S1). However, most skin T cells were IL7R$^+$. These results suggest that both naive and antigen-experienced T cells in lymphoid tissues have the capacity to respond to TSLP, whereas most T cells in skin would be expected to be TSLP unresponsive.

T Cells Directly Respond to TSLP in Vitro to Increase Expression of IL-4 and IL-13. TSLP is known to enhance Th2 cytokine secretion by human and mouse CD4$^+$ T cells stimulated in vitro (18, 19, 21). To investigate the role of TSLP in Th2 cell differentiation, we used 4C13R dual-reporter mice that express AC as a reporter for *Il4* and DR as a reporter for *Il13* (20). Sorted naive CD4$^+$ T cells from 4C13R mice were cultured on aCD3 and aCD28 in Th2 or Th0 conditions with or without TSLP, and reporter expression was examined over time. Peak expression of IL-4AC in Th2 cultures was on day 2, and this response was significantly increased by TSLP (Fig. 2). IL-13DR SP cells appeared later, on days 4 and 5, but only in Th2 cultures supplemented with TSLP. Double-positive (DP) cells remained very few, regardless of TSLP. Very few reporter-expressing cells were observed in Th0 cultures, whether supplemented with TSLP or not. The effect of TSLP was not a result of increased T cell division in culture (Fig. S2*A*), although the frequencies of IL-4AC SP, and especially IL-13DR SP, cells within each division were significantly increased by TSLP (Fig. S2*B*). The key role of TSLP in promoting IL-13DR expression was confirmed using 4C13R mice on a TSLPR-KO background (Fig. S3).

T cells in Th2 cultures up-regulated CD69 and CD44, whereas IL7R was quickly down-regulated (Fig. S4). RT-qPCR confirmed that *Il7r* and *Crlf2* (encoding TSLPR) were down-regulated in culture (Fig. S5*B*) and were also expressed by BALB/cByJ naive CD4$^+$ T cells (Fig. S5*A*). Anti-CD4 positive selection did not affect expression of *Il7r*, *Crlf2*, or *Il4* (Fig. S5*B*).

Thus, TSLP supplementation increases the percentage of IL-4AC SP cells and is necessary for the expression of IL-13DR by naive CD4$^+$ T cells cultured in Th2 conditions.

IL-13DR$^+$ Cells Develop from IL-4AC-Negative Precursors. To determine whether IL-13DR SP cells arise from cells that were previously IL-4AC SP, naive CD4$^+$ T cells were sorted and stimulated under Th2 conditions with or without TSLP. On day 2, at the peak of IL-4AC expression, cells were sorted into AC-positive and AC-negative cells (Fig. 3*A*). Cells were returned to culture in the same cytokine conditions and analyzed daily for reporter expression. Sorted IL-4AC SP cells continued to express the same cytokines regardless of TSLP supplementation. In clear contrast, IL-13DR SP cells developed only in TSLP-supplemented cultures of IL-4AC-negative cells (Fig. 3 *B* and *C*). No IL-13DR SP cells were found in cultures of IL-4AC SP cells, suggesting that IL-4AC SP and IL-13DR SP cells originated from distinct CD4$^+$ naive precursors.

IL-13DR-SP Cells from TSLP Cultures Express Inflammatory Th2 Cytokines. To assess production of other cytokines, naive CD4$^+$ T cells cultured in different conditions for 5 d were sorted into double-negative (DN), IL-4AC SP, and IL-13DR SP (if present) populations for RT-qPCR analysis. As shown in Fig. 4*A*, IL-4AC SP T cells from Th2 cultures without TSLP expressed slightly higher transcript levels of *Il4* and other cytokines compared with DN cells in the same cultures; however, none of these differences was statistically significant. The low levels of *Il4* transcripts in these cultures were likely a result of the cultures being assessed on day 5, 2–3 d after IL-4AC expression had peaked. T cells cultured in Th2 conditions + TSLP expressed variably higher levels of *Il13*, *Il5*, and *Il9* transcripts compared with control, whereas *Il10* and *Ifng* transcripts were similar. This pattern was most evident in the IL-13DR SP population. None of these cytokine transcripts except *Ifng* was detectable in Th0 cultures, with or without TSLP. The expression of *Gata3* and *Bcl6* transcripts was also examined but did not reveal statistically significant differences, except for *Gata3* being lower in Th0 cultures.

To confirm RT-qPCR results, we performed intracellular cytokine staining for IL-13 together with IL-5 or IL-9 (Fig. 4 *B* and *C*, respectively). As observed with the IL-13DR reporter, IL-13-SP cells first appeared on day 4 in TSLP-supplemented cultures. Interestingly, IL-9$^+$ and IL-5$^+$ cells also started to emerge on day 4 and required TSLP. Notably, a substantial proportion of both

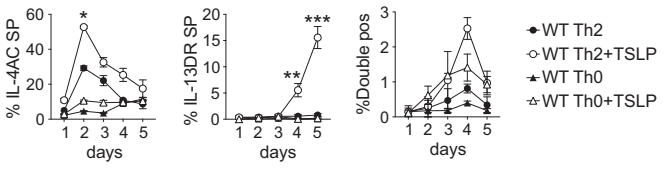

Fig. 2. TSLP elicits IL-13DR expression by naive CD4$^+$ T cells cultured in Th2 conditions. Splenic naive CD4$^+$ T cells from 4C13R reporter mice were sorted and cultured in Th2 or Th0 conditions in the presence or absence of TSLP. Cells were harvested after 1–5 d in culture and analyzed for expression of IL-4-AC only (IL-4AC SP), IL-13-DR only (IL-13DR SP), or both. Data refer to one of at least three repeat experiments that gave similar results. ***P < 0.001; **P < 0.01; *P < 0.05.

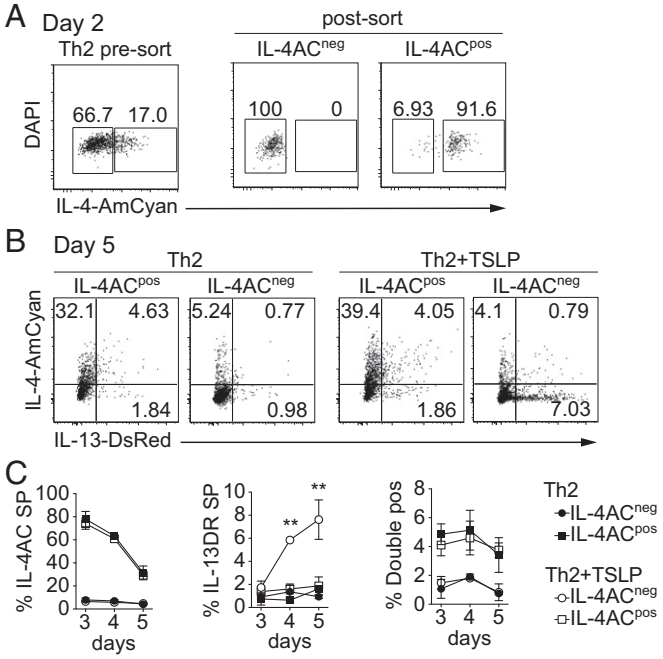

Fig. 3. IL-13DR-expressing Th2 cells arise from an IL-4AC-negative population in the presence of TSLP. Naive CD4$^+$ T cells were purified and cultured as in Fig. 2. On day 2, cells were harvested, sorted into IL-4AC$^+$ or IL-4AC$^-$, and cultured in the same media for up to 3 d before analysis of reporter expression. (*A*) Expression of IL-4AC in representative presort and sorted populations on day 2. (*B*) Expression of IL-4AC and IL-13DR in recultured populations on day 5. (*C*) Time-course analysis of IL-4AC and/or IL-13DR reporter expression in culture. Graphs show mean ± SD for three independent cultures/group. Data refer to one of three repeat experiments that gave similar results. $^{**}P < 0.01$.

IL-9$^+$ and IL-5$^+$ cells also expressed IL-13. Thus, IL-13$^+$ cells generated in Th2+TSLP cultures are heterogeneous and include subsets that also produce IL-9 and/or IL-5.

High Levels of TSLP in Vivo Drive Expression of IL-13DR and an Effector Phenotype in dLN CD4$^+$ T Cells. To investigate the effects of TSLP in vivo, C57BL/6 mice were either treated with MC903 on ear skin for up to 4 consecutive days or injected intradermally with HDM once into the ear pinna. The levels of *Tslp* transcripts in the epidermal layer were quantified by RT-qPCR at different times after treatment (Fig. 5*A*). MC903 induced strong *Tslp* transcription, which peaked on day 4. HDM also induced *Tslp*, but the peak levels were 10- to 100-fold lower than for MC903. The serum levels of TSLP, which are undetectable in untreated C57BL/6 mice, were strongly elevated for several days in MC903-treated mice (Fig. 5*B*), but remained below the level of detection in HDM-treated mice at all times tested (12, 24, and 48 h).

The phenotype of cytokine reporter-expressing CD4$^+$CD44^{hi} T cells in vivo was examined at the peak of total LN cellularity on day 7. In HDM-sensitized mice, most of the IL-4AC SP cells in LN also expressed high levels of the Tfh markers PD-1 and CXCR5 (Fig. 5*C*). As previously reported (8–10), no DP or IL-13DR SP cells were detected in these mice. In contrast, IL-4AC SP, IL-4AC IL-13DR DP, and about 1% IL-13DR SP cells were all detected in dLN after MC903 treatment (Fig. 5*E*). The expression of Tfh markers on these T cell populations was variable and was highest on IL-4AC SP cells and lowest on IL-13DR SP cells. In each case, the larger proportion of cells expressed low PD-1 and CXCR5, suggesting a trend to an inflammatory Th2 effector (Th2eff) phenotype. Similar differences in pattern of cytokine and Tfh marker expression between the HDM and MC903 models were also observed, although less prominently, on days 4 and 10 (Fig. 5 *E* and *F*). Loss of TSLP responsiveness in TSLPR-KO mice did not affect the IL-4AC response to HDM, but reduced the percentages of reporter-positive cells responding to MC903 to background levels (Fig. 6*A*).

To determine whether TSLP was acting directly on CD4$^+$ T cells or via other cell populations including dendritic cells, we carried out adoptive transfer experiments in which 4C13R WT and TSLPR-KO CD4$^+$ T cells were coinjected into TSLPR-WT hosts treated with MC903 (Fig. S6*A*). These experiments showed that only WT T cells were able to express IL-13DR after MC903 immunization (Fig. 6 *B* and *C*). In contrast, IL-4AC was expressed on both TSLPR-WT and TSLPR-KO CD4$^+$ T cells.

To further define the phenotype of cytokine reporter-expressing CD4$^+$ T cells in HDM and MC903 immunized mice, IL-4AC SP, IL-13DR SP, and IL-13DR DP cells were sorted from skin-dLN on day 7 and analyzed by RT-qPCR without further restimulation. IL-4AC SP cells from HDM or MC903-treated mice expressed the high *Bcl6* and low *Gata3* characteristic of Tfh cells (Fig. 7). In contrast, IL-13DR SP and DP cells were *Gata3* high and *Bcl6* low, consistent with a Th2eff phenotype. Reporter-negative CD4$^+$ T cells from naive or immunized mice expressed low levels of both transcripts.

Cytokine transcripts were also examined: *Il4* transcripts were detected in IL-4AC SP cells from HDM and MC903 mice, and also in IL-13DR-DP and, to a lesser extent, IL-13DR SP cells from MC903-immunized mice (Fig. 7). *Il21* transcripts showed a similar pattern of expression (Fig. 7), which is consistent with these populations including high proportions of Tfh cells. Expression of *Il13*, *Il5*, and *Il3* transcripts was also examined and was highest in IL-13DR SP and IL-13DR DP cells from MC903 mice, but low in IL-4AC SP cells (Fig. 7). This cytokine

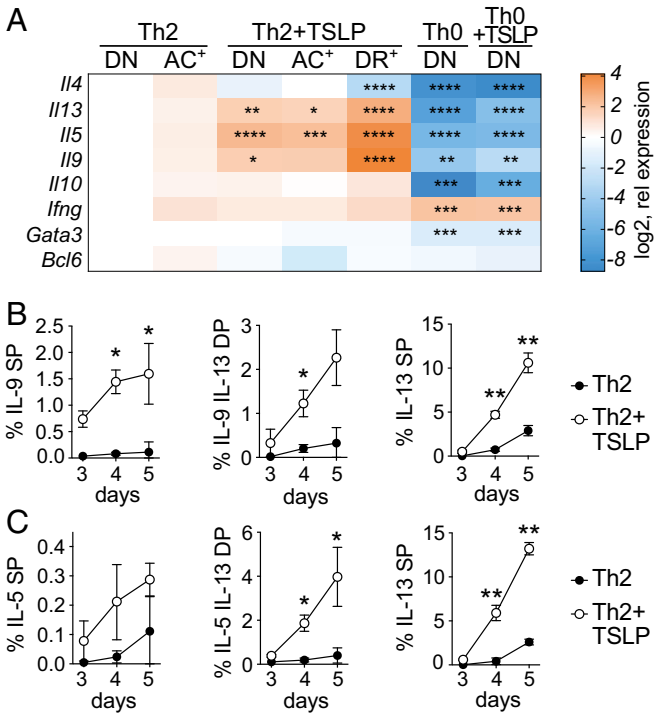

Fig. 4. Culture in Th2 conditions and TSLP generates a population of Th2 cells that express IL-13, IL-5, and IL-9. Naive CD4$^+$ T cells were purified and cultured as in Fig. 2. (*A*) Cultured cells were harvested on day 5 and sorted into DN, IL-4AC SP (AC$^+$), or where applicable, IL-13DR SP (DR$^+$) for RT-qPCR analysis. Cytokine expression data are normalized to *Gapdh* and relative to Th2 DN cells (left column). (*B* and *C*) On day 3–5, T cells were harvested, restimulated with aCD3/aCD28, and stained for intracellular IL-13 and IL-9 (*B*), or IL-13 and IL-5 (*C*). Graphs show mean ± SD for three independent cultures/group. Data refer to one of two repeat experiments that gave similar results. $^{**}P < 0.01$; $^{*}P < 0.05$.

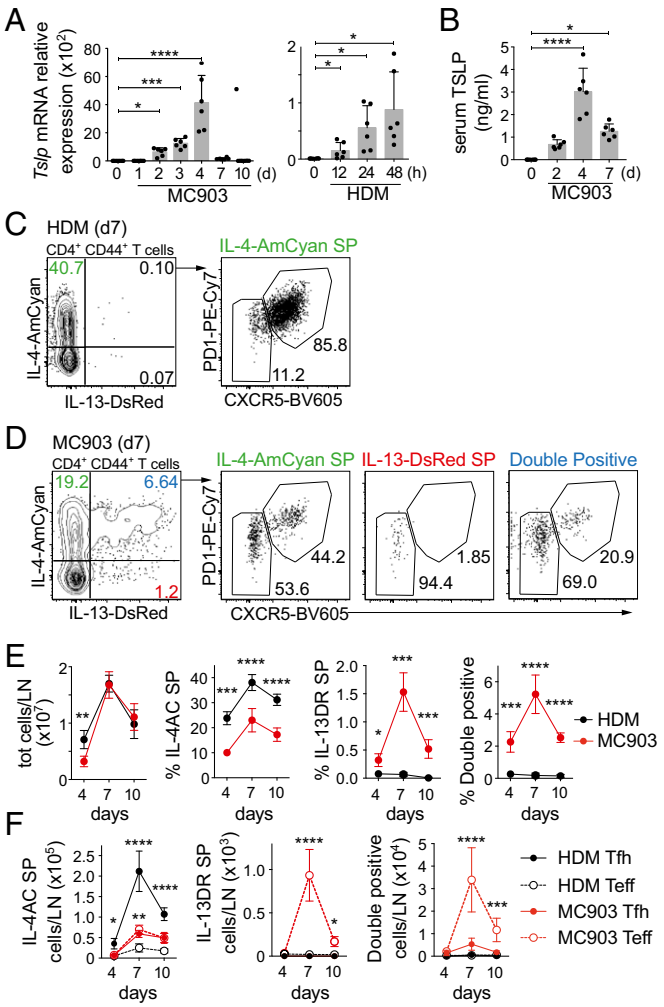

Fig. 5. High TSLP levels promote the development of CD4$^+$ IL-13DR$^+$ cells with a PD1^{low}CXCR5^{low} phenotype in vivo. C57BL/6 and 4C13R mice were treated with HDM or MC903. Tissues were collected for analysis at the indicated points. (*A*) Expression of *Tslp* transcripts in the epidermal layers of C57BL/6 mice. Expression is normalized to 18S RNA and relative to day 0. (*B*) Serum TSLP in MC903-treated C57BL/6 mice. Data are from one of two repeat experiments that gave similar results; each dot corresponds to one mouse. (*C* and *D*) Phenotype of reporter-expressing CD4$^+$CD44^{high} T cells in the dLN of HDM-treated (*C*) or MC903-treated (*D*) 4C13R mice on day 7. No reporter expression was detected in the CD44-low population. (*E*) Total dLN cellularity and percentage of reporter-expressing CD4$^+$CD44^{high} T cells in 4C13R mice treated with HDM or MC903. (*F*) Numbers of reporter-expressing CD4$^+$CD44^{high} T cells with a "Tfh" or "Th2eff" phenotype in the dLN of 4C13R mice treated with HDM or MC903. Phenotype was determined as in *C* and *D*. Line graphs show mean ± SD for six mice/group from one of two repeat experiments that gave similar results. *P* values in *F* refer to the comparisons of HDM to MC903. ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

expression pattern is consistent with the Th2eff phenotype of these cells. Together, these results suggest that TSLP acts directly on CD4$^+$ T cells to promote their development into Th2eff in LN.

Discussion

In this study, we report that culture in TSLP and Th2 conditions promotes the differentiation of naive CD4$^+$ T cells into a population of IL-13-SP Th2 cells. The development of these cells in culture does not involve an IL-4$^+$ stage and is accompanied by the up-regulation of *Il5*, *Il9*, and *Il13* transcripts, but not *Il10* or *Ifng*. In vivo, high TSLP is associated with the acquisition of a Th2eff phenotype in dLN, with down-regulation of *Bcl6* and *Il21* transcripts and up-regulation of *Gata3, Il3, Il5*, and *Il13*. These data suggest TSLP can act early during Th2 cell differentiation to support the development of a Th2eff phenotype.

We used purified naive CD4$^+$ T cells from 4C13R mice, which express fluorescent reporters for IL-4 and IL-13, and a defined in vitro culture system to show that expression of the Th2 cytokines IL-4 and IL-13 in cultured naive CD4$^+$ T cells follows distinctly different kinetics. Expression of IL-4AC peaked on day 2 and then declined regardless of the presence of TSLP in culture, whereas expression of IL-13DR peaked on day 5, but only in the presence of TSLP. This was not the result of an effect of TSLP on the division rate of cultured T cells (19), possibly because of the use of optimal culture conditions in our experiments. Instead, the different kinetics of IL-4AC and IL-13DR expression appeared to reflect the development of two separate functional T cell subsets, as indicated by sorting experiments showing that IL-13DR SP T cells did not originate from the IL-4AC$^+$ population. The cytokine profiles of cells cultured with or without TSLP also differed substantially, with T cells from TSLP cultures expressing higher levels of *Il13*, *Il5*, and *Il9* transcripts; similar *Il10* and *Ifng*; and lower *Il4* compared with T cells cultured without TSLP. The expression of multiple Th2 cytokines including IL-5 has also been reported for highly differentiated effector memory Th2 cells in human peripheral blood (22).

An interesting observation was that IL-4AC expression was not maintained in our cultured T cells, despite the continued availability of aCD3-aCD28 stimulation and cytokines in the medium. This decline in IL-4AC may be a result of limitations of the in vitro culture system; for example, it may suggest that IL-4$^+$ T cells require additional stimuli that are not provided in these cultures. The full differentiation of Tfh cells in vivo has been

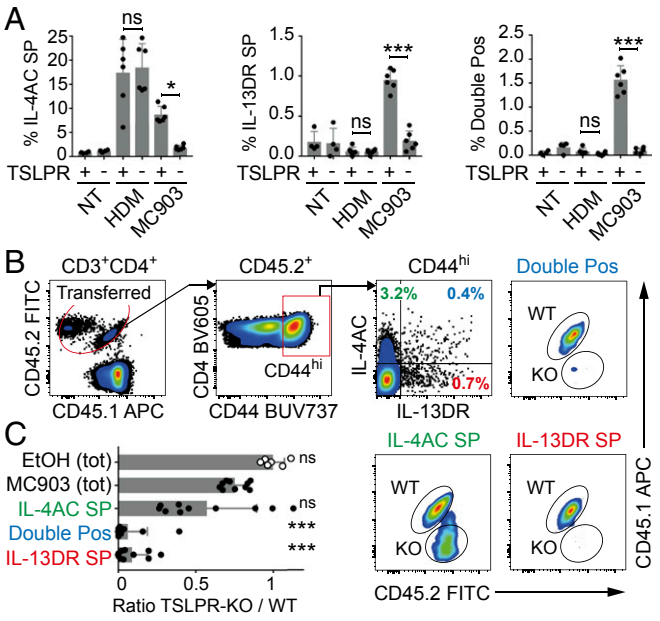

Fig. 6. The development of CD4$^+$ IL-13DR cells in vivo requires expression of TSLPR. (*A*) 4C13R reporter mice bred onto a C57BL/6 or TSLPR-KO background were treated with HDM or MC903. The dLN were harvested on day 7, and reporter expression was evaluated by flow cytometry as in Fig. 5 *C* and *D*. (*B*) CD4-enriched cells from CD45.1$^+$2$^+$ WT mice and CD45.2$^+$ TSLPR-KO mice, both expressing the 4C13R double reporter, were transferred into WT CD45.1$^+$ recipient mice at a ~1:1 ratio. Recipient mice were treated with EtOH or MC903, and dLN were harvested on day 7 for analysis of reporter expression. Dot plots show concatenated data from five mice. (*C*) Ratios of adoptively transferred TSLPR-KO to WT cells in the indicated populations; *P* values refer to the comparison with the MC903-tot group. Bar graphs show mean and SD from one of two to three repeat experiments that gave similar results; each dot represents one mouse. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns: not significant.

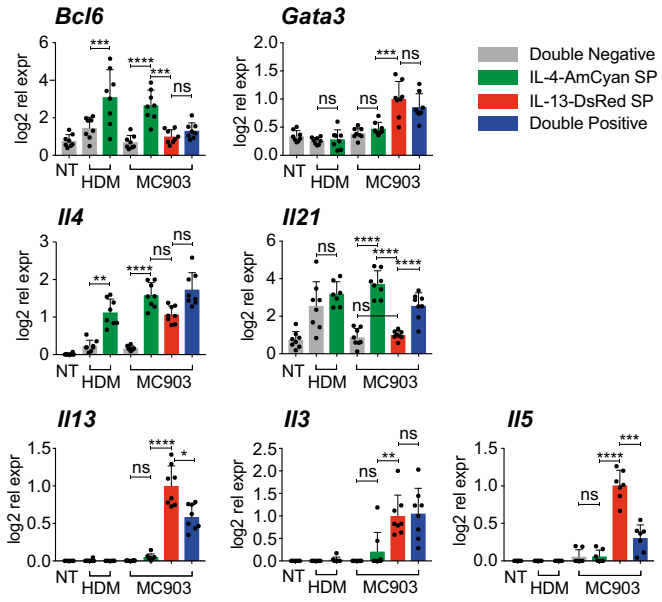

Fig. 7. CD4$^+$ IL-13DR SP LN T cells express Th2 cytokine transcripts in vivo. 4C13R mice were treated with HDM or MC903; untreated mice (NT) were used as controls. dLN were harvested on day 7, and CD4$^+$CD44^{high} T cells were sorted according to reporter expression as indicated, without in vitro restimulation. Expression of the indicated transcripts is shown relative to *Gapdh* and the IL-13DR SP population. Data are from one of three repeat experiments that gave similar results; each dot refers to one mouse. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05; ns: not significant.

shown to require a coordinated set of events that follow the initial BCL6 up-regulation, including IL-6 and IL-21 signaling and contact with B cells (23, 24). These signals were not provided in our culture conditions.

To complement our in vitro studies, we examined the role of TSLP in the differentiation of Th2eff versus Tfh in vivo. Previous studies have shown that the differentiation of Th2eff cells in vivo requires a "tissue checkpoint" phase in which Th2 cells acquire a transcriptional profile similar to ILC2 (7) and become able to produce effector cytokines and mediate protective immune responses. This "tissue checkpoint" was shown to require IL-25, IL-33, and TSLP signaling, indicating that these cytokines, either individually or as a group, are necessary for the acquisition of Th2eff function. We exploited the properties of MC903, a powerful inducer of keratinocyte TSLP production, to differentiate cytokine requirement from the requirement for entry into nonlymphoid tissues. MC903 dramatically elevates serum TSLP levels without inducing TSLP transcription in LN (25). We show that exposure to systemic TSLP in LN was sufficient for Th2 cells to acquire features of Th2eff, such as low expression of *Bcl6*, *Il21*, and Tfh surface markers, and gain of *Gata3*, *Il3*, *Il5*, and *Il13*. These changes were especially striking in the rare IL-13-DR-SP population, but were also observed, albeit with lower intensity, in the more abundant DP cells. Neither of these populations was observed in HDM-immunized mice. Therefore, low levels of TSLP, such as in the HDM model, would presumably remain restricted to the epithelia and affect only local cells such as dendritic cells, thus favoring the priming of Th2 cells with a Tfh phenotype (26–28). In contrast, high levels of TSLP acting systemically can directly support the differentiation of IL-13DR SP and IL-13DR DP cells in LN. This early effect of systemic TSLP on Th2eff differentiation is consistent with the exacerbated airway inflammation observed in mice primed with allergen in combination with TSLP or MC903 (29, 30) and the coexpression of TSLPR and IL7R on about 50% of peripheral naive CD4$^+$ T cells.

The IL-13DR SP cells from in vitro cultures or ex vivo expressed characteristics of Th2eff cells such as high *Il5* and *Il13* and low *Bcl6*. BCL6 is known to prevent up-regulation of GATA-3 and the expression of GATA3-dependent cytokines, including IL-13 (31). The down-regulation of BCL6 can also be mechanistically linked to TSLP via the known ability of TSLPR signaling to activate STAT5 (32). In CD4$^+$ T cells, activated STAT5 can antagonize BCL6 activity by competing for binding to the same genome target sequences, thereby suppressing Tfh development (33, 34) and promoting the differentiation of Th2eff expressing high levels of GATA-3 (31). In addition, enforced expression of a constitutively activated STAT5 enables CD4$^+$ T cells primed in neutral conditions to produce multiple Th2 cytokines including IL-5, IL-9, and IL-13 (35). In several of those studies, it was IL-2, not TSLP, that was proposed to act as the STAT5 activator for Th2eff differentiation. Together with the data in this paper, those studies suggest that multiple T cell cytokine receptors that signal via STAT5, including the receptors for IL-2, TSLP, and IL-25 (36), may similarly affect Th2eff differentiation. Interestingly, two recent studies on Th9 cells have also proposed overlapping roles for IL-2 and TSLP in directing the choice of CD4$^+$ T cells to differentiate into Tfh versus Th9 cells (37, 38) by enabling STAT5 to compete for BCL6 transcriptional activity (37). Similarly, in Th1 cells, IL-2-dependent STAT5 activation ultimately leads to T-bet activation, repression of BCL6 function and Tfh development (39), and Th1 effector function in vivo (40). Thus, our studies reveal a previously undocumented role of TSLP in regulating Th2eff function in vitro and in vivo via the potential activation of signaling events that are common to several different cytokines and T cell differentiation pathways.

Treatment with MC903 is used as a mouse model of atopic dermatitis and atopic march (25, 30, 41–43). Although induction of skin inflammation by MC903 does not require T cells, Th2 responses are generated in this model and can be demonstrated in skin-dLN and skin by assessing IL-4 production (25, 30, 44). In this article, we show that the systemic levels of TSLP induced by MC903 drive the differentiation of inflammatory Th2 cells that have already deviated away from the Tfh phenotype and have acquired the capacity to produce multiple Th2 effector cytokines in the LN. In clinical atopic dermatitis, increased TSLP serum levels have been reported in children (45), as well as in a small study in adults (46), suggesting that systemic levels of TSLP are not a peculiarity of the MC903 mouse model but are likely to be important drivers of local inflammation and the allergic march in patients. These data may provide an understanding of the complex pathogenesis of atopic dermatitis and the allergic march.

Materials and Methods

Mice. C57BL/6J, BALB/cByJ, B6.SJ-OTII, TSLPR-KO (47), and 4C13R dual-reporter mice (20) on a C57BL/6, B6.SJL-*Ptprc*^{a}*Pepc*^{b}/Boy, or TSLPR-KO background were bred on site and used when 6–10 wk old. Mice were age- and sex-matched within experiments. All experimental procedures were approved by the Victoria University of Wellington Animal Ethics Committee and performed according to institutional guidelines.

Tissue Processing. Spleen and LN cell suspensions were prepared by pressing through 70-μm nylon strainers (BD Falcon). For skin T cell preparations, ears were split into dorsal and ventral layers and digested with collagenase IV (Sigma-Aldrich) and DNase I (Roche) for 30 min at 37 °C before passing through 70-μm nylon strainers.

T Cell Sorting and Flow Cytometry. Naive CD62L$^+$CD44^{low} CD4$^+$ T cells were enriched by positive selection (Dynabeads CD4 Positive Isolation Kit; Invitrogen); labeled with fluorescent antibodies specific for CD4, CD8, CD44, and CD62L; and purified by sorting on a BD INFLUX (Becton Dickinson) with BDFACS software, version 1.2.0.142 (BD). Cells for flow cytometry were resuspended in PBS with 1% FBS, 2 mM EDTA, 0.01% azide, and then incubated in anti-(a)CD16/CD32 followed by the appropriate fluorescent antibodies and DAPI staining to exclude nonviable cells (Table S1). Data were acquired using a custom LSR-Fortessa SORP cytometer (Becton Dickinson) with BD FACSDiva software, version 6.1.1 (BD), and analyzed using FlowJo software (Tree Star).

In Vitro T Cell Cultures. Sorted naive CD4$^+$ T cells were stimulated with plate-bound aCD3 and soluble aCD28 in Th2 (hrIL-2, mrIL-4) or Th0 (hrIL-2, aIL-4, aIFN-γ) conditions with or without mouse recombinant TSLP, as in Table S2.

Cytokines were replenished every 48 h to compensate for consumption during culture. In some experiments, sorted cells were labeled with 1 μM CFSE (Invitrogen) to track cell division.

Quantitative RT-PCR. Total RNA was extracted from 1,000 (Taqman) or 50,000 (SYBR green) sorted cells, using the Quick-RNA MicroPrep (Zymo Research), and reverse-transcribed into cDNA, using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Target genes were quantitatively amplified using the Real-Time Taqman PCR assays (Applied Biosystems) in duplex (VIC and FAM probe dyes in Table S3) or PowerUp SYBR Green Master Mix (Applied Biosystems), using primers in Table S4. Each qPCR reaction was performed using the QuantStudio 7 Flex (Applied Biosytems). Cycle threshold values were converted to theoretical expressions (2^{CT} value) and normalized by GAPDH or 18S expression.

Immunizations and in Vivo Treatments. Immunization conditions were determined on the basis of prior dose–response experiments. For MC903 treatments, mice were anesthetized and 4 nM MC903 (Cayman Chemical Company) in 100% ethanol was topically applied to ear skin on up to 4 consecutive days (48). For HDM immunizations, mice were anesthetized and 200 μg HDM (Greer) in PBS was injected intradermally into the ear pinnae. Mice were killed on the indicated days for TSLP measurements or for characterization of the T cell response.

T Cell Adoptive Transfer. $CD4^+$ T cells from $CD45.1^+2^+$ TSLPR-WT or $CD45.2^+$ TSLPR-KO donors, both expressing the 4C13R double reporter, were enriched by positive selection, using either CD4 Dynabeads (Invitrogen) or CD4 microbeads with an autoMACS Pro Separator (Miltenyi Biotec), and injected i.v. into OTII mice back-crossed onto a $CD45.1^+$ background. OTII-recipient mice were used as their response to MC903 is reduced compared with C57BL/6 mice, thus allowing a more sensitive detection of transferred T cells. Similar results but lower donor cell recoveries were obtained using B6-SJ recipients. MC903 treatment was started 1 d after T cell transfer, and mice were killed on day 8.

Statistics. Statistical analyses were performed using Prism 7.0 (GraphPad). Mean and SD are shown in all graphs. Data were analyzed using ANOVA with Bonferroni posttest. *P* values lower than 0.05 were considered significant.

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