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TSLP Signaling in CD4⁺ T cells is Required for Th2 Memory

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Abstract

Background—Thymic stromal lymphopoietin (TSLP) is a key factor in the development of allergic asthma. Th2 memory cells gradually increase in allergic patients with the progression of disease and persist in the lungs during remission although the mechanism is not clear.

Objective—To define the role of TSLP in Th2 memory generation and maintenance in vivo.

Methods—Adoptive transfer of wild type (WT) and TSLP receptor (TSLPR)-deficient ovalbumin (OVA)-specific CD4⁺ T cells before Th2 sensitization was used to define T cell specific TSLP effects. Atopic dermatitis and elevated serum TSLP concentration was induced by topical application of the vitamin D3 analog MC903. Memory cells in peripheral blood were monitored weekly with flow cytometry. Memory recall was tested following intranasal OVA challenge.

Results—TSLP signaling in CD4⁺ T cells is required for the generation/maintenance of memory cells following in vivo priming. TSLPR-deficient CD4⁺ T cells have no defects in proliferation but fail to survive one week after sensitization, and elevated TSLP expression during sensitization significantly increased the frequency of memory cells. Although in vitro differentiated TSLPR-deficient Th2 cells develop into memory cells with equal efficiency to wild type cells, the recall response to airway antigen challenge is impaired. Moreover, after antigen challenge of mice with established Th2 memory, TSLP signaling in CD4⁺ T cells significantly affects memory cell generation/maintenance from secondary effector cells.

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Conclusion—TSLP signaling in CD4⁺ T cells is required for not only Th2 memory formation in vivo, but also the recall response of the memory cells to local antigen challenge.

Keywords

TSLP; Th2 memory; Memory recall; Allergic airway inflammation; Eosinophilia

INTRODUCTION

One defining feature of adaptive immunity is the generation of immunological memory wherein the persistence of memory T cells arising from the primary immune response provides prolonged protective immunity for the host. However, robust memory T cell responses present unique challenges for attenuating deleterious immune responses such as autoimmunity and allergic diseases. Evidence from patients and experimental models indicates that memory T helper 2 (Th2) cells reside in the lungs during disease remission and, upon allergen exposure, become activated effectors involved in disease exacerbation.^{1–6} Thus targeting memory Th2 cells might provide a valuable therapeutic strategy for allergic asthma.^{7, 8}

During priming of T cells, T-cell receptor (TCR) signals, secondary signals provided by costimulatory molecules and cytokines determine the differentiation pathway to form memory T cells.^{9, 10} Among other cytokines, interleukin (IL)-7 plays an essential role in committing effector CD4 T cells into memory T cells and promoting survival of memory T cells via induction of the anti-apoptotic Bcl-2 gene expression.^{11, 12}

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine and shares IL-7R α as a receptor component.^{13, 14} TSLP is critical for the pathogenesis of allergic inflammation in mice.^{15–18} In humans, elevated TSLP expression is associated with atopic dermatitis (AD) and asthma.^{19, 20} It has also been identified as a susceptibility gene for asthma in various populations by genome wide association studies.^{21–26} The actions of TSLP in allergic inflammation are multifold,²⁷ including promotion of Th2 differentiation either directly^{28, 29} or through dendritic cells (DCs),^{16, 19} promotion of Th9 differentiation and function,³⁰ and inhibition of antigen-specific regulatory T cells (Treg).³¹ Furthermore, Wang et al.³² in an in vitro study demonstrated that human TSLP-activated DCs are more efficient than IL-7 and IL-15 for inducing the expansion of Th2 memory cells, and maintaining the central memory phenotype and Th2 commitment in the absence of antigen. In mice, TSLP induces expression of the anti-apoptotic genes Bcl-2 and cFLIP in Th2 cells,^{33, 34} one of the mechanisms by which IL-2 and IL-7 promote T cell survival, suggesting TSLP might play a role in Th2 memory. However, the requirement of TSLP for Th2 memory generation and maintenance, especially in vivo, is yet to be defined.

In this report we demonstrate that TSLPR-deficient CD4⁺ T cells proliferate normally but undergo massive cell death 7 days after OVA+Alum sensitization and fail to form a long-lasting memory population. Although in vitro differentiated Th2 cells bypass the requirement of TSLP to form long-term memory in recipient mice, TSLPR-deficient memory Th2 cells induce significantly weaker airway inflammation and eosinophilia upon

memory recall and are maintained less efficiently after antigen re-exposure. Thus TSLP signaling in CD4⁺ T cells is important for Th2 memory formation and recall.

METHODS

Mice strain

Wild type Balb/C mice and DO11.10 TCR transgenic mice were purchased from Jackson laboratory. TSLPR^{-/-} mice were described previously.¹⁸ Breeding and maintenance of the mice were performed under institutional guidelines, and all experiments involving animals were approved by the IACUC of Indiana University School of Medicine.

Naive DO11.10 CD4 T cell transfer and ovalbumin sensitization

Naive CD4⁺CD62L⁺ T cells were isolated from spleens and lymph nodes of 6–12 week-old female DO11.10 or TSLPR^{-/-} DO11.10 TCR transgenic mice using MACS isolation kit (Miltenyi Biotec). 2×10^6 naïve CD4⁺ T cells were adoptively transferred to recipient mice via tail intravenous (iv) injection. 24 hr after cell transfer, mice were sensitized by intraperitoneal (IP) injection of 100 µg OVA (Worthington) emulsified in 1.3 mg aluminum hydroxide gel (Alum, Sigma), and then boosted with OVA+Alum a week later. Except in the experiment involving two rounds of MC903 treatment, the boost immunization was given two weeks later to allow the recovery of the skin inflammation from first round of MC903 treatment.

Induction of TSLP by MC903 topical treatment

To induce TSLP expression in the keratinocytes, we used vitamin D analogue MC903 (calcipotriol, Sigma).^{35, 36} MC903 was dissolved in 100% ethanol and topically applied on mouse ears (2 nmol in 25 µl per ear) for 7 days, the same amount of ethanol was used as vehicle control.

Differentiation and transfer of Th2 cells, and intranasal challenge

Antigen presenting cells (APC) were prepared from CD4 T cell-depleted splenocytes and lymphocytes and pretreated with mitomycin C (Calbiochem) for 20 mins at 37°C. RPMI 1640 medium was supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-ME. To induce Th2 T cells, the naïve DO11.10 T cells were stimulated with 5 µg/ml OVA peptide (323–339) (GenScript) plus APCs (naïve CD4⁺CD62L⁺T cell: APC=1:5.5) under Th2 conditions (20 ng/ml rmIL-4, 1 ug/ml anti-mouse IFN-γ, 2 ug/ml anti-mouse IL-12, 0.5 ug/ml anti-mouse CD28) for 5 consecutive days in vitro.

20×10^6 in vitro differentiated Th2 cells were adoptively transferred to recipient mice via tail intravenous (iv) injection. The presence of donor cells in peripheral blood was monitored weekly by CD4 and KJ1.26 staining and flow cytometry. At the end of monitoring period, the recipient mice were challenged intranasally with 100 µg OVA in 40 µl PBS for 3 days. Mice were euthanized 24 h after the last challenge for further analysis.

BAL fluid harvest, differential cell counts and lung histology

Mice were sacrificed by intraperitoneal injection of an overdose of ketamine-xylazine. BAL was collected as previously described.¹⁸ The lungs were rinsed 4 times with ice-cold PBS, each time using 1ml PBS. The first 1ml BAL was centrifuged at 1,400g for 5 min and the supernatant was used to measure cytokine profile. The pellet was pooled with the subsequent lavages. BAL fluid cells were resuspended in RPMI complete medium and were counted with a hemocytometer after red blood cell lysis. After cyto spin and stained with a modified Wright-Giemsa stain on a Hema-Tek 2000 slide stainer (Bayer Corp, Diagnostics Division), differential counting of BAL cells were performed under a microscope. At least 200 cells on random fields were counted for each sample.

After lavage, lungs were excised and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) and Periodic acid–Schiff (PAS). Slides were semiquantitatively scored for inflammatory severity (H&E stained) and goblet cell metaplasia (PAS stained) as absent (0), minimal (1), slight (2), moderate (3), marked (4) or strong (5).

Antibodies and cell staining

Anti-mouse/human CD44 (clone IM7), anti-mouse CD62L (clone MEL-14), anti-mouse CD4 (clone GK1.5), anti-mouse DO11.10 TCR (clone KJ1-26), anti-mouse IL-4 (clone 11B11), anti-mouse-IFN- γ (clone XMG1.2), anti-Bcl2 (BCL/10C4) and anti-mouse/rat Ki-67 (solA15) were used for antibody staining, rat IgG1 (clone MOPC-21), rat IgG1 (clone RTK2071), rat IgG2a (clone RTK2758), and rat IgG2b (RTK4530) were used as isotype controls. 7-AAD and annexin V staining were used for analysis of cell viability. All surface staining was carried out according to manufacturer's protocol. To detect intracellular cytokine production of IL-4 and IFN- γ , cells were restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 3 hours. Monensin (Biolegend) was added to the culture for the last 3 hours incubation. Cells were then processed to stain for anti-IL-4 and IFN- γ production. Cells were labeled with CFSE (eBioscience) as described in protocol.

OVA-specific IgE in serum

Relative titers of OVA-specific IgE in serum were determined by ELISA. Plates were coated with 2 mg/ml ovalbumin overnight at 4°C. After blocking with 1% BSA, serial dilutions of serum samples were added and incubated for 4 h at room temperature. OVA-specific IgE was detected with the detecting antibody and substrate according to manufacturer's instruction (Biolegend).

Statistical analysis

Statistical analysis was performed with GraphPad Prism. Student t-test was used for comparison between two groups and analysis of variance (ANOVA) was used when there were more than two groups of data.

RESULTS

TSLP-signaling in CD4⁺ T cells is required for memory formation in vivo after Th2 sensitization

TSLP is capable of acting on multiple cell lineages to induce allergic inflammation.²⁷ Despite the importance of Th2 memory in asthma exacerbation,^{1–5} whether TSLP plays a role in long-term Th2 memory and is thus critical for the development of chronic allergic disease has not been clearly defined. Recent studies showed that interruption of TSLP signaling in DCs only is sufficient to abrogate TSLP-dependent Th2 sensitization and inflammation.¹⁶ To examine whether TSLP signaling in CD4⁺ T cells is necessary for generation and/or maintenance of Th2 memory, we isolated naïve CD4⁺ T cells from wild type (WT) DO11.10 or TSLPR-deficient (KO) DO11.10 mice and adoptively transferred to WT BALB/c recipient mice by tail vein injection. The mice were sensitized by immunizing intraperitoneally (i.p.) with OVA+Alum twice, 7 days apart. The generation and maintenance of Th2 memory was monitored weekly in the following 6 weeks by FACS analysis of the peripheral blood mononuclear cells (PBMC) for the presence of donor CD4⁺ T cells, recognized by the anti-DO11.10 TCR monoclonal antibody clone KJ1.26. Although the percent of WT and KO DO11.10 T cells in PBMC were similar one week post 2nd immunization, significantly fewer TSLPR-deficient DO11.10 T cells were observed than WT DO11.10 T cells thereafter in the peripheral blood of recipient mice (Fig. 1A). The difference between the two populations was further confirmed by examining the donor cells in peripheral (inguinal) lymph nodes (pLN), mediastinal lymph nodes (medLN) and spleens after 6 weeks (Fig. 1B).

In naïve mice, TSLPR-deficiency had no significant impact on T and B cell development.^{37, 38} Similarly to these findings, we also found that TSLPR-deficient CD4⁺ T in naïve mice had similar levels of TCR (CD3 and TCR β) and costimulatory molecule (CD28) expression, and normal development of memory-phenotype cells with comparable CD44 and CD62L expression to the WT CD4⁺ T cells. More importantly, TSLPR-deficient CD4⁺ T cells in naïve mice were not more susceptible to apoptosis (see Figure E1 in the Online Repository). To further determine whether TSLPR-deficiency affects homeostatic proliferation and survival of DO11.10 CD4⁺ T cells in recipient mice, we transferred 2×10^6 naïve WT and TSLPR-deficient DO11.10 T cells into wild type BALB/c mice and immunized the recipient mice as described in Figure 1A except a non-specific antigen bovine serum albumin (BSA) is used. In contrast to OVA+Alum immunization (Fig. 1A and B), WT and KO CD4⁺KJ1.26⁺ donor cells in BSA+Alum immunized recipient mice demonstrated a similar pattern over time in PBMC and similar frequencies in medLN and pLN (see Figure E2 A and B in the Online Repository). Furthermore, the majority of WT and KO CD4⁺KJ1.26⁺ donor cells exhibited CD44⁺ CD62L⁺ naïve phenotype in medLN 6 weeks after BSA+Alum immunization compared to the CD44⁺CD62L⁺ and CD44⁺CD62L⁺ memory phenotypes after OVA+Alum immunization (see Figure E2 C in the Online Repository).

To determine the effect of TSLP signaling in CD4⁺ T cell on their transition to memory cells after immunization, we followed the expression of two commonly used memory markers

CD44 and CD62L on DO11.10 T cells in the medLN. Mirroring the changes of the donor populations in the peripheral blood (Fig. 1A), there was no difference between the percentages of WT and TSLPR-deficient DO11.10 T cells in the medLN until 1 week after the 2nd immunization when TSLPR-deficient cells started to collapse (Fig. 1C). Both WT and KO DO11.10 T cells acquired an effector phenotype by up-regulating CD44. While the percentage of CD62L⁺ WT cells gradually increased from D4 post immunization to ~20% at week 2 and maintained, CD62L expression on TSLPR-deficient DO11.10 T cells reached peak at week 1 but decreased as the KO population decreased (Fig. 1C). Taken together, these data indicate TSLP signaling is dispensable for normal CD4⁺ T development but necessary for Th2 memory generation/maintenance in OVA+Alum primed Th2 sensitization.

TSLP-signaling in CD4⁺ T cells promotes effector cell survival but not proliferation after in vivo Th2 priming

The fact that WT and TSLPR-deficient DO11.10 T cell populations showed no difference until 1 week after 2nd immunization (Fig. 1A and C) suggests that TSLP-signaling in CD4⁺ T cells enhanced cell survival rather than increased proliferation. To test our hypothesis, we similarly transferred 2×10^6 WT and TSLPR-deficient DO11.10 T cells into BALB/c mice and immunized the recipients with OVA+Alum. Apoptosis of these OVA-specific effector cells in the mediastinal lymph nodes (medLN) were analyzed with 7-AAD/Annexin V staining 7 days and 10 days after the immunization. One week after the 2nd immunization, TSLPR-deficient DO11.10 T cells were only slightly lower than WT cells and showed slightly increased early apoptotic cell population (Annexin V⁺7-AAD⁻) (Fig. 2A). However, compared with WT cells, TSLPR-deficient DO11.10 T cell population in medLN was significantly lower with a drastic increase in the frequency of late apoptotic cells (Annexin V⁺7-AAD⁺) at 10 days after the immunization (Fig. 2B).

The IL-7-dependent induction of the anti-apoptotic gene Bcl-2 was critical for CD4⁺ T memory development.^{11, 12} We thus examined CD127 (IL-7R α) expression on DO11.10 T cells. Compared with control CD4⁺ T cells isolated from naïve mice, CD127 expression on both WT and TSLPR-deficient DO11.10 T cells was down-regulated to a similar level 4 days (D4) after the 2nd immunization and then gradually increased afterwards (see Figure E2 in the Online Repository). Despite similar CD127 expression profiles, we found that Bcl-2 expression in WT cells was slightly higher than that in TSLPR-deficient DO11.10 T cells on D4 (Fig. 2C), which is consistent with in vitro studies showing induction of Bcl-2 by TSLP.^{33, 34}

Further characterization showed that, after transferred into wild type recipient mice, TSLPR-deficient DO11.10 T cells had no defects in activation and differentiation, up-regulating CD28 and ICOS, and expressing Gata3, T-bet, IL-4 and IL-13 at levels similar to WT cells 4 days after the 2nd immunization (see Figure E3 in the Online Repository).

The fact that there was no difference in the expression of proliferation marker Ki-67 between WT and TSLPR-deficient DO11.10 T cells 4 days after the OVA-Alum sensitization (Fig. 2D) indicated that TSLPR-deficient DO11.10 T cells could proliferate normally upon activation. To confirm this, we labeled naïve WT or TSLPR-deficient

DO11.10 T cells with CFSE and transferred into WT BALB/c recipient mice at 2×10^6 cells per mouse. The recipient mice were immunized i.p. with OVA+Alum on the next day and sacrificed 4 days after sensitization for analysis. The percentage and number of WT and TSLPR-deficient DO11.10 cells in medLN remained identical at day 4 post immunization (Fig. 2E). More importantly, no significant difference was observed in their proliferation based upon CFSE dilution (Fig. 2F).

Taken together, these results indicate that TSLPR-deficient DO11.10 T cells in WT recipients do not have defects in activation, proliferation or differentiation in response to antigen in the presence of the adjuvant Alum, but do have lower Bcl-2 expression and a higher apoptotic rate at contraction phase leading to defects in memory formation.

Elevated systemic TSLP during sensitization promotes Th2 memory generation in vivo

Having established that TSLP signaling in CD4⁺ T cells is required for Th2 memory generation, we next examined whether systemic elevation of TSLP concentration during Th2 sensitization affects memory generation. We used a well-established experimental murine model of atopic dermatitis in which topical treatment with the vitamin D analog calcipotriol (MC903) results in skin inflammation, TSLP expression in keratinocytes, and a systemic increase in TSLP concentration in serum.^{35, 36, 39} While not detectable in ethanol treated control mice, serum TSLP reached 639 ± 164 pg/ml ($n = 5$) on day 4 of MC903 treatment. 2×10^6 naïve WT DO11.10 CD4 T cells were adoptively transferred into WT BALB/c mice, which were subjected to topical MC903 or ethanol treatment. The mice were immunized i.p. with OVA+Alum as depicted in Figure 3A. We observed that, starting from 2 weeks post 2nd immunization, CD4⁺KJ1.26⁺ frequency was significantly higher in the peripheral blood of mice treated with MC903 than that in the mice treated with ethanol (Fig. 3B). Examination of the transferred cells in spleens and peripheral lymph nodes further confirmed the existence of increased Th2 memory cells 5 weeks after the 2nd immunization in the mice treated with MC903 (Fig. 3C).

To confirm that it was MC903-induced TSLP that acted on CD4⁺ T cells to enhance Th2 memory generation, rather than the effect of MC903 itself, we repeated the above experiment with TSLPR-deficient DO11.10 T cells. TSLPR-deficient DO11.10 T cells in MC903 treated mice behaved identical to those in ethanol treated mice. No differences were observed in peripheral blood over experimental time or in the spleens and peripheral lymph nodes 5 weeks after the 2nd immunization (Fig. 3 D, E). Therefore, elevated systemic TSLP concentration during sensitization promotes Th2 memory generation. Likely, this effect is directly exerted on CD4⁺ T cells as cells in the wild type recipients, except the TSLPR-deficient donor cells, are capable of response to TSLP.

TSLP signaling in CD4⁺ T cells does not affect memory development from in vitro primed Th2 cells but hinders recall response

Our in vivo data indicated that TSLP signaling in CD4⁺ T cells during sensitization is important for generation of Th2 memory. Next, we wanted to examine whether TSLP signaling in CD4⁺ T cells after priming affects Th2 memory. We differentiated WT or TSLPR-deficient DO11.10 T cells into Th2 cells in vitro and transferred 20×10^6 Th2 cells

into BALB/c recipient mice to track memory cells derived from these cells over time. In the presence of 20 ng/ml IL-4 and IFN- γ neutralization antibody, both WT and TSLPR-deficient DO11.10 T cells developed into Th2 cells equally well in vitro (Fig. 4A). These cells also developed into memory cells at equal efficiency (Fig. 4B) with same memory phenotypes after being transferred into recipient mice. (Fig. 4C).

Since the recipient mice used in the experiment were naïve mice, it is possible that there was not enough TSLP, even if it is expressed, to affect the in vitro differentiated Th2 cells to commit and develop into memory cells. We again used topical MC903 (arrows in Fig. 4D) to induce TSLP expression and increase its systemic concentration. Unlike during sensitization when T cells were activated (Fig. 3A), elevated TSLP concentration had no significant effects on the frequency of resting Th2 memory cells in peripheral blood (Fig. 4D).

To define the role of TSLP on memory cell function, we challenged recipient mice intranasally with ovalbumin for three days, seven weeks after transfer of in vitro Th2-differentiated DO11.10 T cells. Despite similar Th2 memory frequencies and phenotypes before challenge (Fig. 4B), mice that received TSLPR-deficient Th2 cells had significantly less total cell count (Fig. 5A) and eosinophil count (Fig. 5B) in BAL fluid after OVA challenge than those received wild type Th2 cells. There was also significantly lower OVA-specific serum IgE in these mice (Fig. 5C). Lung histopathology showed reduced eosinophilic peribronchial/perivascular infiltration and goblet cell metaplasia in recipients of TSLPR-deficient Th2 cells as compared with recipients received WT Th2 cells (Fig. 5D). However, consistent with our finding that TSLP signaling in CD4⁺ T cells is not required for antigen-driven cell activation and proliferation (Fig. 2D-F), the frequency of CD4⁺KJ1.26⁺ cells in the inguinal lymph nodes and mediastinal lymph nodes remained identical between these two groups 24 hr after OVA challenges (Fig. 5E).

These data demonstrated that in vitro differentiated Th2 cells bypass the requirement of TSLP for generation and maintenance of resting Th2 memory cells. However, TSLPR-deficient Th2 memory cells are impaired in recall responses in response to local antigen challenge.

TSLP signaling in CD4⁺ T cells is required for secondary Th2 memory generation after antigen exposure in vivo

Allergic asthma is characterized by remissions and exacerbations, and the memory cells are reactivated to become secondary effectors during an exacerbation. Therefore, it is of interest and clinical relevance to examine whether TSLP affects memory populations upon antigen re-exposure after Th2 memory had established. We took advantage of our finding that in vitro primed WT or TSLPR-deficient Th2 cells developed into memory cells in WT hosts with equal efficiency (Fig. 4B – C) to circumvent the effects of TSLP on Th2 differentiation and memory formation during sensitization. 20×10^6 in vitro primed WT or TSLPR-deficient Th2 cells were transferred into BALB/c recipient mice and memory cells were monitored weekly. Three weeks later, recipient mice were exposed to OVA intranasally for three days (arrows in Fig. 6A). Similar to previous experiments, no difference between TSLPR-deficient cells and WT cells was seen before antigen exposure. However, starting

from one week after antigen challenge, the frequency of TSLPR-deficient donor cells in PBMC was significantly lower than that of WT cells (Fig. 6A). The TSLPR-deficient DO11.10 T cells continued to decline over the following three weeks. When we challenged the recipient mice in week 8, mice that received TSLPR-deficient Th2 cells showed significantly less total cell count (Fig. 6B) and eosinophil count (Fig. 6C) in BAL fluid. Lung histopathology demonstrated that mice received TSLPR-deficient Th2 cells had reduced inflammatory infiltration and goblet cell metaplasia in the lungs comparing with mice received WT Th2 cells (Fig. 6D). Furthermore, in contrast to the results in Figure 5E, significantly lower frequencies of TSLPR-deficient donor cells in both inguinal lymph nodes and mediastinal lymph nodes than WT donor cells were observed (Fig. 6E). These data indicates that TSLP signaling in CD4⁺ T cells is required for re-generation of Th2 memory from secondary effectors derived from resting memory cells re-exposed to allergen.

DISCUSSION

TSLP has been shown to exert its functions on a broad range of tissues and cell types (Reviewed in ²⁷). Its effect on DCs in the pathogenesis of allergic diseases is probably most clearly demonstrated.^{16, 19} TSLP also directly acts on CD4⁺ T cells to promote Th2 and Th9 differentiation^{28–30, 33, 40} while suppress regulatory T cell development.³¹ In this report, we demonstrate that TSLP signaling in CD4⁺ T cells is required for the effector cells that have been primed in vivo to antigen in the presence of Th2 adjuvant Alum to develop into resting memory cells. Furthermore, Th2 memory cells also require TSLP signaling to fully establish airway eosinophilic inflammation in response to local antigen challenge.

IL-7 signaling has been shown to be important for the transition from effector to memory and long-term survival of the memory cells predominantly through induction of anti-apoptotic gene Bcl-2 expression.^{11, 12, 41} We found that TSLPR-deficient CD4⁺ T cells, despite expressing similar levels of IL-7R α (Fig. E2 and ³⁸) and being capable of responding to IL-7, fail to develop into memory cells after in vivo Th2 priming, indicating that TSLP non-redundantly regulates Th2 memory development. TSLPR-deficient CD4⁺ T cells show no defects in proliferation, but most of the cells undergo apoptosis 7 days after OVA+Alum sensitization (Fig. 2). However, unlike IL-7 which is absolutely required not only for the generation but also survival of CD4⁺ memory cells, we found that enhanced memory generation is only achieved when TSLP expression is induced during sensitization, when T cells are activated (Fig. 3), but not in the absence of antigen when T cells are resting (Fig. 4D). Furthermore, in vitro differentiated TSLPR-deficient Th2 cell survived equally well compared to its WT counterpart (Fig. 4B). Therefore, the requirement of TSLP in Th2 memory formation might be confined at the time of antigen exposure, but not for the long term survival of already formed resting memory cells.

Our study indicates that TSLPR-deficient DO11.10 T cells in WT recipients have no defects in proliferation after OVA+Alum i.p. immunization. Until the first week after immunization, no differences between TSLPR-deficient and WT donor cell frequencies were observed in peripheral blood (Fig. 1A and Fig. 3B) and mediastinal lymph nodes (medLN, Fig. 1C, and Fig. 2A & E). Indeed, 4 days after immunization, TSLPR-deficient DO11.10 T cells in medLN showed similar proliferation marker Ki-67 expression (Fig. 2D) and CFSE dilution

(Fig. 2F) to WT DO11.10 T cells. Kool et al demonstrated that OVA+Alum i.p. immunization mainly drains to and induces robust antigen-specific CD4⁺ T activation and proliferation in medLN, but not in peripheral lymph nodes and spleen as would be expected in a systemic response.⁴² Limited DO11.10 T proliferation in the inguinal lymph node was observed depending on the location of injection due to needle stick injury of the skin. The proliferation difference in an earlier report¹⁵ between TSLPR-deficient and WT DO11.10 T cells observed in lymph nodes likely resulted from the location of injection relative to the inguinal lymph node collected for analysis.

It is not clear currently how TSLP signaling in CD4⁺ T cells at the time of antigen exposure affects Th2 memory formation. CD4⁺ T cells in naïve TSLPR deficient mice have no overt defects and surface molecule expression, including TCR/co-stimulatory molecules, IL-7R α , and memory marker CD44/CD62L (Fig. E1A&B and ³⁸), neither are they prone to apoptosis (Fig. E1C). In vitro studies demonstrate that TSLP at 50 ng/ml induces anti-apoptotic gene Bcl-2 expression in Th2 cells.³⁴ Upon activation in vivo by OVA+Alum immunization, TSLPR-deficient DO11.10 T cells show slight lower expression of Bcl-2 than WT DO11.10 T cells (Fig. 2C). Whether this slight deficiency in Bcl-2 expression renders the TSLPR-deficient donor cells inferior to, and possibly outcompeted by, the WT memory cells from the recipients needs to be further studied.

While presenting the antigen at the time of their interaction with T cells, the DCs provide multiple signals to determine the outcome and fate of activated T cells.⁴³ TSLP signaling in DCs is critical to prime Th2 differentiation,^{16, 19} and DC-specific deletion of Stat5 abolished Th2 allergic response but not Th1 response.¹⁶ In the wild type recipients with competent DCs and other innate cells, TSLPR-deficient DO11.10 T cells showed no defects in activation and differentiation, and all cells take on an effector phenotype by up-regulation of CD44 surface expression 4 days after 2nd OVA+Alum sensitization (Fig. 1C and Fig. E2). It remains to be explored whether TSLPR-deficient CD4⁺ T cells are deficient in their interaction with DCs leading to defects in cell survival.

In contrast to the in vivo primed DO11.10 cells (Fig. 1–3), in vitro differentiated TSLPR-deficient Th2 cells show no defects to transit to lasting memory cells (Fig. 4). It has also reported that, different from IL-7, supplementing TSLP to the in vitro culture did not promote memory generation after transferring the cells to recipient mice.⁴¹ The generation of memory CD4⁺ T cells can be influenced by many factors including the duration and strength of TCR stimulation,⁴⁴ cytokines,^{10, 45} and co-stimulation.⁴⁶ During in vitro priming, CD4⁺ T cells receive prolonged and strong TCR stimulation, anti-CD28 and exogenous IL-4. All these factors might provide enough signals for in vitro differentiated cells to develop into memory cells independent of TSLP. The requirement of TSLP for Th2 memory generation in vivo should not be underestimated. In addition to the requirement of TSLP for Th2 memory generation in OVA+Alum model (Fig. 1–3), re-generation of memory cells from secondary effector cells after antigen challenge also depends on TSLP (Fig. 6).

Even though TSLP does not seem to play a role in generation and maintenance of memory cells from in vitro primed Th2 cells (Fig 4B, D), the recall response of the memory cells in

response to local antigen challenge is dependent on TSLP (Fig. 5). Jang et al. reported that transfer of in vitro differentiated Th2 cells into TSLP knockout mice did not change lung pathology or Th2 cytokine production upon OVA challenge compared to transfer into WT mice.⁴⁷ Such discrepancy could arise from the time of OVA challenge in relating to cell transfer. While we challenged the recipient mice 7 weeks after cell transfer, allowing the in vitro primed Th2 cells to fully differentiate into resting memory cells, Jang et al. challenged mice immediately after cell transfer so that the donor cells likely still remain as active effectors.

Th2 memory cells have been shown to gradually increase in allergic patients with the progression of diseases and persist in the lungs during remission.^{3, 5, 6, 48} Thus targeting Th2 memory cells has been proposed and tested as a therapeutic strategy for allergic diseases.^{7, 8, 49} One difficulty is to target only the allergen-specific Th2 memory cells but not the beneficial memory cells. The requirement of TSLP for the transition of secondary effector cells to Th2 memory (Fig. 6), but not for the maintenance of resting memory cells (Fig. 4), might define a novel strategy for the immunotherapy of allergic diseases. Moreover, blocking TSLP at initial or ongoing allergen exposure might also increase the number of allergen-specific Tregs as we have shown that small amount of TSLP could have a big effect on the differentiation of inducible Tregs.³¹ Collectively these findings highlight a central requirement for TSLP in supporting the generation of Th2 memory cells in vivo and ultimately determining the efficiency of the Th2-mediated inflammatory recall response.

Supplementary Material

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Abbreviations

BAL	Bronchoalveolar lavage
CFSE	Carboxyfluorescein succinimidyl ester
DCs	dendritic cells
i.n	intranasal
i.p	intraperitoneal
KO	knock-out
medLN	Mediastinal lymph nodes
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells

pLN	Peripheral lymph nodes
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor

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Key messages

- TSLP signaling in CD4⁺ T cells is required for memory formation after Th2 sensitization.
- TSLPR-deficient Th2 memory cells show impaired memory recall in response to airway antigen challenge.
- Generation of memory cells from secondary Th2 effectors after antigen inhalation requires TSLP action on CD4⁺ T cells.

Figure 1.

TSLP-signaling in CD4⁺ T cells is required for Th2 memory in vivo. **(A)** Gating strategy, and frequency of KJ1.26⁺ donor cells in gated CD4⁺ T cells in PBMC. **(B)** Gating strategy, and frequency of KJ1.26⁺ donor cells in the lymph nodes and spleen 6 weeks after second immunization. **(C)** Frequency and memory phenotypes of KJ1.26⁺ DO11.10 T cells in the medLN 4 days (D4) and 1–3 weeks after the second immunization. WT: wild type; KO: TSLPR-deficient; PBMC: peripheral blood mononuclear cells; medLN: mediastinal lymph nodes; pLN: peripheral lymph nodes (inguinal lymph nodes). *: $p < 0.05$; **: $p < 0.01$ by repeated measures two-way ANOVA with Bonferroni post hoc test for (A) and two-tailed t -test for (B). Data represent mean $\pm$ SEM ($n = 5$ mice / group).

Figure 2.

TSLP-signaling in CD4⁺ T cells is required for cell survival but not for cell proliferation in vivo. (A) Apoptosis of DO11.10 T cells in the mediastinal lymph nodes (medLN) 7 days after 2nd immunization. (B) Apoptosis of DO11.10 T cells in medLN 10 days after 2nd immunization. Late apoptotic cells were defined by Annexin V⁺7-AAD⁺. (C) Bcl-2 expression in CD4⁺KJ1.26⁺ DO11.10 T cells in medLN 4 days after 2nd immunization. Iso: isotype control. Numbers in parentheses represent mean fluorescence index (MFI). (D) Proliferation marker Ki-67 expression in CD4⁺KJ1.26⁺ cells in medLN 4 days after 2nd immunization. (E) Frequency and numbers of CD4⁺KJ1.26⁺ cells in medLN 4 days after immunization. (F) Proliferation of CD4⁺KJ1.26⁺ cells in medLN 4 days after immunization. WT: wild type; KO: TSLPR-deficient; ns: non-significant; **: p < 0.01; ***: p < 0.001 by two-tailed *t*-test. Data represent mean ± SEM (n = 5 mice / group).

Figure 3.

Elevated TSLP expression promotes Th2 memory generation. **(A)** Diagram of experimental design. **(B and D)** Frequency of KJ1.26⁺ cells in CD4⁺ T cells monitored in PBMC. **(C and E)** Frequency of CD4⁺KJ1.26⁺ cells in spleen and peripheral lymph nodes (pLN) 5 weeks after 2nd immunization. WT: wild type; KO: TSLPR-deficient; ns: non-significant; *: $p < 0.05$; **: $p < 0.01$ by repeated measures two-way ANOVA with Bonferroni post-hoc test for (B and D) or two-tailed t -test for (C and E). Data represent mean $\pm$ SEM ($n = 5$ mice / group).

Figure 4.

TSLP-signaling in CD4⁺ T cells is not required for the generation and maintenance of Th2 memory from in vitro differentiated Th2 cells. Wild type (WT) or TSLPR-deficient (KO) DO11.10 T cells were differentiated into Th2 cells in vitro for 5 days and transferred into BALB/c recipient mice. (A) Intracellular staining of in vitro differentiated Th2 cells. (B) Frequency of KJ1.26⁺ cells in CD4⁺ T cells monitored in PBMC. (C) Phenotypes of CD4⁺KJ1.26⁺ Th2 memory cells 5 weeks after transfer. (D) Wild type DO11.10 T cells were differentiated into Th2 cells and transferred into BALB/c recipient mice. Three weeks after cell transfer, mice received topical treatment of MC903 or ethanol as control for 7 days (arrows). Frequency of KJ1.26⁺ cells in CD4⁺ T cells was monitored in PBMC. Data represent mean $\pm$ SEM (n = 5 mice / group).

Figure 5.

TSLPR-deficient Th2 memory cells are functionally deficient at memory-recall upon antigen exposure. Seven weeks after transfer of in vitro differentiated wild type (WT) or TSLPR-deficient (KO) DO11.10 Th2 cells, recipient mice were challenged intranasally with OVA for 3 days. (A) Total BAL cell counts. (B) Differential counts of BAL cells. (C) OVA-specific IgE in serum. (D) Lung histopathology examined by staining with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS). The Inset shows eosinophil (arrow head) infiltration. Slides were scored for inflammatory severity (H&E) and goblet cell metaplasia (PAS). (E) Frequency of CD4⁺KJ1.26⁺ cells in lymph nodes. pLN: peripheral inguinal lymph nodes; medLN: mediastinal lymph nodes. ns: non-significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ by two-tailed t -test for (A-B and D-E) and by two-way ANOVA with Bonferroni post hoc test for (C). Data represent mean $\pm$ SEM ($n = 5$ mice / group).

Figure 6.

TSLP signaling in CD4⁺ T cells is required for subsequent memory development from secondary effector cells after antigen challenge in vivo. Wild type (WT) or TSLPR-deficient (KO) DO11.10 T cells were in vitro differentiated into Th2 cells and transferred into BALB/c recipient mice. Three weeks after transfer, mice were exposed to OVA intranasally (i.n.) for 3 days (arrows). At week 8, mice were again challenged i.n. with OVA for 3 days. (A) Frequency of KJ1.26⁺ cells in CD4⁺ T cells monitored in PBMC. (B and C) Total and differential cell counts in BAL after OVA challenge at week 8. Mac: macrophages/monocytes; Eos: eosinophils; Neut: neutrophils; Lym: lymphocytes. (D) Lung histopathology examined by H&E and PAS staining. Slides were scored for inflammatory severity (H&E) and goblet cell metaplasia (PAS). (E) Frequency of CD4⁺KJ1.26⁺ cells in lymph nodes after OVA challenge at week 8. pLN: peripheral lymph nodes; medLN: mediastinal lymph nodes. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ by repeated measures two-way ANOVA with Bonferroni post-hoc test for (A) or two-tailed t -test for (B-E). Data represent mean $\pm$ SEM ($n = 5$ mice / group).