

Thymic stromal lymphopoietin signaling in CD4⁺ T cells is required for T_H2 memory

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Background: Thymic stromal lymphopoietin (TSLP) is a key factor in the development of allergic asthma. Numbers of T_H2 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: We sought to define the role of TSLP in T_H2 memory cell generation and maintenance *in vivo*.

Methods: Adoptive transfer of wild-type and thymic stromal lymphopoietin receptor (TSLPR)-deficient ovalbumin-specific CD4⁺ T cells before T_H2 sensitization was used to define T cell-specific TSLP effects. Atopic dermatitis and increased serum TSLP concentrations were 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 after intranasal ovalbumin challenge.

Results: TSLP signaling in CD4⁺ T cells is required for the generation/maintenance of memory cells after *in vivo* priming. TSLPR-deficient CD4⁺ T cells have no defects in proliferation but do not survive 1 week after sensitization, and increased TSLP expression during sensitization significantly increased the frequency of memory cells. Although *in vitro*-differentiated TSLPR-deficient T_H2 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 T_H2 memory, TSLP signaling in CD4⁺ T cells significantly affects memory cell generation/maintenance from secondary effector cells.

Conclusion: TSLP signaling in CD4⁺ T cells is required for not only T_H2 memory cell formation *in vivo* but also the recall response of the memory cells to local antigen challenge. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Thymic stromal lymphopoietin, T_H2 memory, memory recall, allergic airway inflammation, eosinophilia

Abbreviations used

7-AAD:	7-Aminoactinomycin D
APC:	Antigen-presenting cell
BAL:	Bronchoalveolar lavage
CD62L:	CD62 ligand
CFSE:	Carboxyfluorescein succinimidyl ester
DC:	Dendritic cell
IL-7Rα:	IL-7 receptor α
medLN:	Mediastinal lymph node
OVA:	Ovalbumin
TCR:	T-cell receptor
Treg:	Regulatory T
TSLP:	Thymic stromal lymphopoietin
TSLPR:	Thymic stromal lymphopoietin receptor
WT:	Wild-type

One defining feature of adaptive immunity is the generation of immunologic 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_H2 cells reside in the lungs during disease remission and, on allergen exposure, become activated effectors involved in disease exacerbation.¹⁻⁶ Thus targeting memory T_H2 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, IL-7 plays an essential role in committing effector CD4 T cells into memory T cells and promoting survival of memory T cells through induction of anti-apoptotic Bcl-2 gene expression.^{11,12}

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine and shares IL-7 receptor α (IL-7Rα) as a receptor component.^{13,14} TSLP is critical for the pathogenesis of allergic inflammation in mice.¹⁵⁻¹⁸ In human subjects increased TSLP expression is associated with atopic dermatitis and asthma.^{19,20} It has also been identified as a susceptibility gene for asthma in various populations by using genome-wide association studies.²¹⁻²⁶ The actions of TSLP in allergic inflammation are multifold,²⁷ including promotion of T_H2 differentiation either directly^{28,29} or through dendritic cells (DCs),^{16,19} promotion of T_H9 differentiation and function,³⁰ and inhibition of antigen-specific regulatory T (Treg) cells.³¹ Furthermore, in an *in vitro* study Wang et al³² demonstrated that human TSLP-activated DCs are more efficient than IL-7 and IL-15 for inducing the

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expansion of T_H2 memory cells and maintaining the central memory phenotype and T_H2 commitment in the absence of antigen. In mice TSLP induces expression of the antiapoptotic genes Bcl-2 and cFLIP in T_H2 cells,^{33,34} one of the mechanisms through which IL-2 and IL-7 promote T-cell survival, suggesting TSLP might play a role in T_H2 memory. However, the requirement of TSLP for T_H2 memory cell generation and maintenance, especially *in vivo*, is yet to be defined.

In this report we demonstrate that thymic stromal lymphopoietin receptor (TSLPR)-deficient CD4⁺ T cells proliferate normally but undergo massive cell death 7 days after ovalbumin (OVA) plus aluminum hydroxide gel (Alum; Sigma, St Louis, Mo) sensitization and do not form a long-lasting memory population. Although *in vitro*-differentiated T_H2 cells bypass the requirement of TSLP to form long-term memory cells in recipient mice, TSLPR-deficient memory T_H2 cells induce significantly weaker airway inflammation and eosinophilia on memory recall and are maintained less efficiently after antigen re-exposure. Thus TSLP signaling in CD4⁺ T cells is important for T_H2 memory formation and recall.

METHODS

Mouse strain

Wild-type (WT) BALB/c mice and DO11.10 TCR transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Me). 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 Institutional Animal Care and Use Committee of Indiana University School of Medicine.

Naive DO11.10 CD4 T-cell transfer and OVA sensitization

Naive CD4⁺ CD62L ligand (CD62L)-positive T cells were isolated from the spleens and lymph nodes of 6- to 12-week-old female DO11.10 or TSLPR^{-/-} DO11.10 TCR transgenic mice by using the MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Naive CD4⁺ T cells (2×10^6) were adoptively transferred to recipient mice through tail intravenous injection. Twenty-four hours after cell transfer, mice were sensitized by means of intraperitoneal injection of 100 μ g of OVA (Worthington Biochemical, Lakewood, NJ) emulsified in 1.3 mg of Alum and then boosted with OVA plus Alum a week later. Except in the experiment involving 2 rounds of MC903 treatment, the boost immunization was given 2 weeks later to allow recovery of skin inflammation from the first round of MC903 treatment.

Induction of TSLP by MC903 topical treatment

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

Differentiation and transfer of T_H2 cells and intranasal challenge

Antigen-presenting cells (APCs) were prepared from CD4 T cell-depleted splenocytes and lymphocytes and pretreated with mitomycin C (Calbiochem, San Diego, Calif) for 20 minutes at 37°C. RPMI 1640 medium was supplemented with 10% FBS, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES buffer, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 50 μ mol/L 2-mercaptoethanol. To induce T_H2 T cells, naive DO11.10 T cells were stimulated with 5 μ g/mL OVA peptide (323-339; GenScript, Piscataway, NJ) plus APCs (naive CD4⁺CD62L⁺ T cell/APC ratio = 1:5.5) under T_H2

conditions (20 ng/mL rmIL-4, 1 μ g/mL anti-mouse IFN- γ , 2 μ g/mL anti-mouse IL-12, and 0.5 μ g/mL anti-mouse CD28) for 5 consecutive days *in vitro*.

In vitro-differentiated T_H2 cells (20×10^6) were adoptively transferred to recipient mice through tail intravenous injection. The presence of donor cells in peripheral blood was monitored weekly by using CD4 and KJ1.26 staining and flow cytometry. At the end of the monitoring period, the recipient mice were challenged intranasally with 100 μ g of OVA in 40 μ L of PBS for 3 days. Mice were killed 24 hours after the last challenge for further analysis.

Bronchoalveolar lavage fluid harvest, differential cell counts, and lung histology

Mice were killed by means of intraperitoneal injection of an overdose of ketamine-xylazine. Bronchoalveolar lavage (BAL) fluid was collected, as previously described.¹⁸ The lungs were rinsed 4 times with ice-cold PBS, each time using 1 mL of PBS. The first 1 mL of BAL fluid was centrifuged at 1400g for 5 minutes, and the supernatant was used to measure the cytokine profile. The pellet was pooled with the subsequent lavage fluid. BAL fluid cells were resuspended in RPMI complete medium and counted with a hemocytometer after red blood cell lysis. After cyto-spin and staining with a modified Wright-Giemsa stain on a Hema-Tek 2000 slide stainer (Bayer Corp, Diagnostics Division, Leverkusen, Germany), differential counting of BAL cells was 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 and periodic acid-Schiff. Slides were semiquantitatively scored for inflammatory severity (hematoxylin and eosin stained) and goblet cell metaplasia (periodic acid-Schiff 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 XMGI.2), anti-Bcl2 (BCL/10C4), and anti-mouse/rat Ki-67 (solA15) were used for antibody staining, and rat IgG₁ (clones MOPC-21 and RTK2071), rat IgG_{2a} (clone RTK2758), and rat IgG_{2b} (clone RTK4530) were used as isotype controls. Staining with 7-aminoactinomycin D (7-AAD) and Annexin V was used for analysis of cell viability. All surface staining was carried out according to the manufacturer's protocol. Cells were restimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin for 3 hours to detect intracellular cytokine production of IL-4 and IFN- γ . Monensin (BioLegend, San Diego, Calif) was added to the culture for the last 3 hours of incubation. Cells were then processed to stain for anti-IL-4 and IFN- γ production. Cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; eBioscience, San Diego, Calif), as described in the protocol.

OVA-specific IgE in serum

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

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). The Student *t* test was used for comparison between 2 groups, and ANOVA was used when there were more than 2 groups of data.

RESULTS

TSLP signaling in CD4⁺ T cells is required for memory formation *in vivo* after T_H2 sensitization

TSLP is capable of acting on multiple cell lineages to induce allergic inflammation.²⁷ Despite the importance of T_H2 memory in asthma exacerbation,¹⁻⁵ whether TSLP plays a role in long-term T_H2 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 T_H2 sensitization and inflammation.¹⁶ To examine whether TSLP signaling in CD4⁺ T cells is necessary for the generation, maintenance, or both of T_H2 memory cells, we isolated naive CD4⁺ T cells from WT DO11.10 or TSLPR-deficient (knockout) DO11.10 mice and adoptively transferred them to WT BALB/c recipient mice by means of tail vein injection. The mice were sensitized by immunizing intraperitoneally with OVA plus Alum twice 7 days apart. The generation and maintenance of T_H2 memory cells was monitored weekly in the following 6 weeks by using fluorescence-activated cell sorting analysis of the PBMCs for the presence of donor CD4⁺ T cells recognized by the anti-DO11.10 TCR mAb clone KJ1.26. Although the percentages of WT and knockout DO11.10 T cells in PBMCs were similar 1 week after the second 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 1, A). The difference between the 2 populations was further confirmed by examining the donor cells in peripheral (inguinal) lymph nodes, mediastinal lymph nodes (medLN), and spleens after 6 weeks (Fig 1, B).

In naive mice TSLPR deficiency had no significant effect on T- and B-cell development.^{37,38} Similar to these findings, we also found that TSLPR-deficient CD4⁺ T cells in naive mice had similar levels of TCR (CD3 and TCRβ) and costimulatory molecule (CD28) expression and normal development of memory phenotype cells with CD44 and CD62L expression comparable with that seen in the WT CD4⁺ T cells. More importantly, TSLPR-deficient CD4⁺ T cells in naive mice were not more susceptible to apoptosis (see Fig E1 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). To further determine whether TSLPR deficiency affects homeostatic proliferation and survival of DO11.10 CD4⁺ T cells in recipient mice, we transferred 2 × 10⁶ naive WT and TSLPR-deficient DO11.10 T cells into WT BALB/c mice and immunized the recipient mice, as described in Fig 1, A, except the nonspecific antigen BSA is used. In contrast to OVA plus Alum immunization (Fig 1, A and B), WT and knockout CD4⁺KJ1.26⁺ donor cells in BSA plus Alum-immunized recipient mice demonstrated a similar pattern over time in PBMCs and similar frequencies in medLNs and peripheral lymph nodes (see Fig E2, A and B, in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). Furthermore, the majority of WT and knockout CD4⁺KJ1.26⁺ donor cells exhibited the CD44⁺CD62L⁺ naive phenotype in medLNs 6 weeks after BSA plus Alum immunization compared with the CD44⁺CD62L⁺ and CD44⁺CD62L⁺ memory phenotypes after OVA plus Alum immunization (see Fig E2, C).

To determine the effect of TSLP signaling in CD4⁺ T cells on their transition to memory cells after immunization, we followed the expression of 2 commonly used memory markers, CD44 and CD62L, on DO11.10 T cells in the

medLNs. Mirroring the changes of the donor populations in peripheral blood (Fig 1, A), there was no difference between the percentages of WT and TSLPR-deficient DO11.10 T cells in the medLNs until 1 week after the second immunization, when TSLPR-deficient cells started to collapse (Fig 1, C). Both WT and KO DO11.10 T cells acquired an effector phenotype by upregulating CD44. Although the percentage of CD62L⁺ WT cells gradually increased from day 4 after immunization to approximately 20% at week 2 and maintained, CD62L expression on TSLPR-deficient DO11.10 T cells reached a peak at week 1 but decreased as the knockout population decreased (Fig 1, C). Taken together, these data indicate TSLP signaling is dispensable for normal CD4⁺ T-cell development but necessary for T_H2 memory cell generation/maintenance in OVA plus Alum-primed T_H2 sensitization.

TSLP signaling in CD4⁺ T cells promotes effector cell survival but not proliferation after *in vivo* T_H2 priming

The fact that WT and TSLPR-deficient DO11.10 T-cell populations showed no difference until 1 week after the second immunization (Fig 1, A 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 × 10⁶ WT and TSLPR-deficient DO11.10 T cells into BALB/c mice and immunized the recipients with OVA plus Alum. Apoptosis of these OVA-specific effector cells in the medLNs was analyzed with 7-AAD/Annexin V staining 7 and 10 days after immunization. One week after the second immunization, numbers of TSLPR-deficient DO11.10 T cells were only slightly less than those of WT cells and showed a slightly increased early apoptotic cell population (Annexin V⁺7-AAD⁻; Fig 2, A). However, compared with WT cells, the TSLPR-deficient DO11.10 T-cell population in medLNs was significantly lower, with a drastic increase in the frequency of late apoptotic cells (Annexin V⁺7-AAD⁺) at 10 days after immunization (Fig 2, B).

The IL-7-dependent induction of the antiapoptotic gene Bcl-2 was critical for CD4⁺ T memory development.^{11,12} We thus examined CD127 (IL-7Rα) expression on DO11.10 T cells. Compared with control CD4⁺ T cells isolated from naive mice, CD127 expression on both WT and TSLPR-deficient DO11.10 T cells was downregulated to a similar level 4 days after the second immunization and then gradually increased afterward (see Fig E3 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). 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 day 4 (Fig 2, C), which is consistent with *in vitro* studies showing induction of Bcl-2 by TSLP.^{33,34}

Further characterization showed that after being transferred into WT recipient mice, TSLPR-deficient DO11.10 T cells had no defects in activation and differentiation, upregulating CD28 and inducible costimulator and expressing Gata3, T-bet, IL-4, and IL-13 at levels similar to those in WT cells 4 days after the second immunization (see Fig E3).

The fact that there was no difference in expression of the proliferation marker Ki-67 between WT and TSLPR-deficient

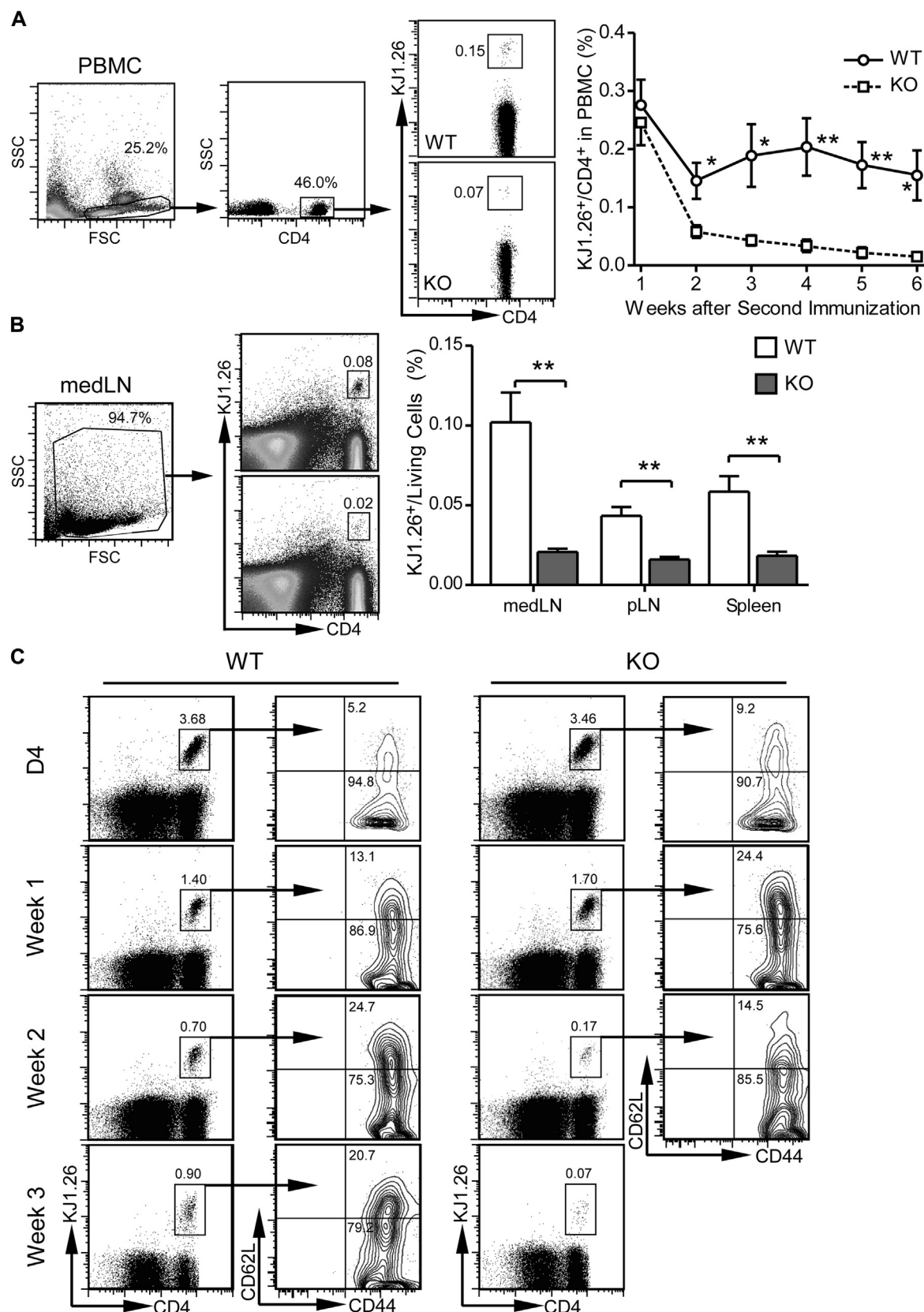


FIG 1. TSLP signaling in CD4⁺ T cells is required for T_H2 memory *in vivo*. **A**, Gating strategy and frequency of KJ1.26⁺ donor cells in gated CD4⁺ T cells in PBMCs. **B**, Gating strategy and frequency of KJ1.26⁺ donor cells in the lymph nodes and spleen 6 weeks after the second immunization. **C**, Frequency and memory phenotypes of KJ1.26⁺ DO11.10 T cells in the medLNs 4 days (D4) and 1 to 3 weeks after the second immunization. FSC, Forward scatter; KO, TSLPR-deficient; pLN, peripheral lymph nodes (inguinal lymph nodes); SSC, side scatter. **P* < .05 and ***P* < .01, repeated-measures 2-way ANOVA with the Bonferroni *post hoc* test for Fig 1, A, and the 2-tailed *t* test for Fig 1, B. Data represent means ± SEMs (*n* = 5 mice per group).

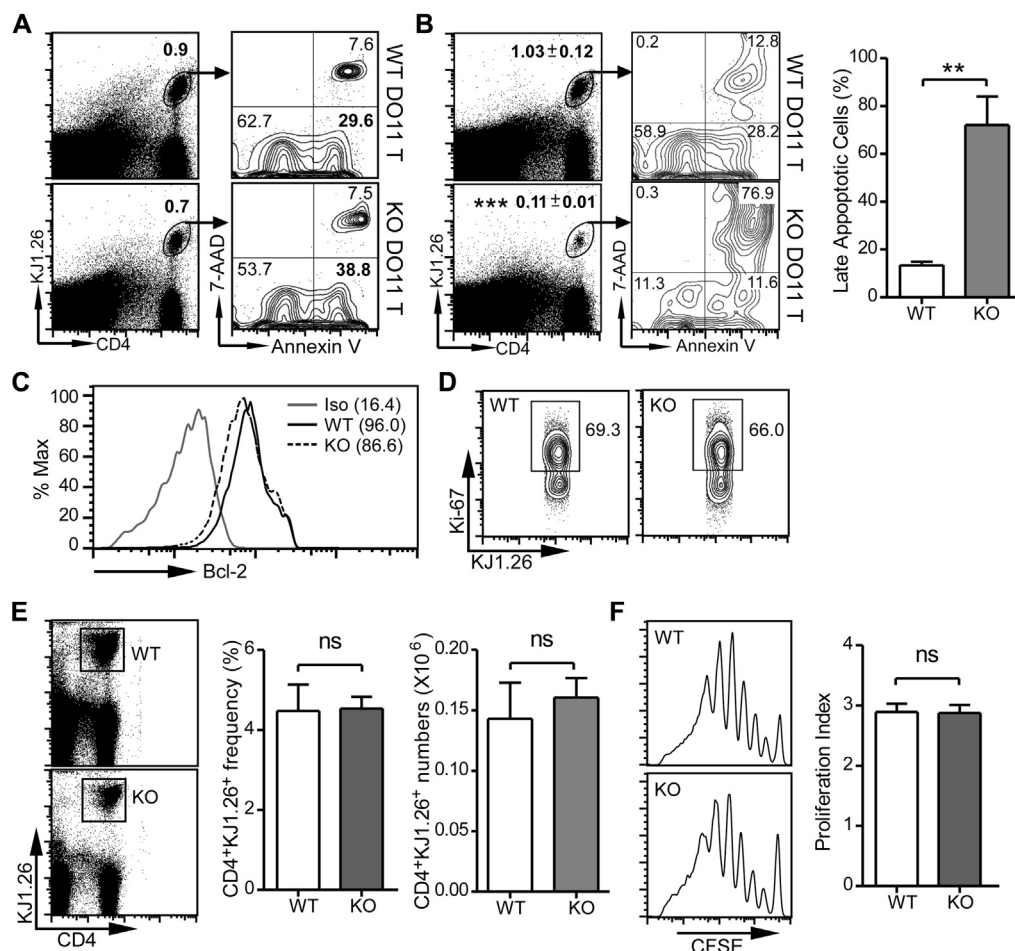


FIG 2. TSLP signaling in CD4⁺ T cells is required for cell survival but not for cell proliferation *in vivo*. **A**, Apoptosis of DO11.10 T cells in the medLNs 7 days after the second immunization. **B**, Apoptosis of DO11.10 T cells in medLNs 10 days after the second immunization. Late apoptotic cells were defined as Annexin V⁺ 7-AAD⁺. **C**, Bcl-2 expression in CD4⁺KJ1.26⁺ DO11.10 T cells in medLNs 4 days after the second immunization. *Iso*, Isotype control; *KO*, TSLPR-deficient. Numbers in parentheses represent the mean fluorescence index. **D**, Proliferation marker Ki-67 expression in CD4⁺KJ1.26⁺ cells in medLNs 4 days after the second immunization. **E**, Frequency and numbers of CD4⁺KJ1.26⁺ cells in medLNs 4 days after immunization. **F**, Proliferation of CD4⁺KJ1.26⁺ cells in medLNs 4 days after immunization. *ns*, Nonsignificant. ***P* < .01, 2-tailed *t* test. Data represent means ± SEMs (*n* = 5 mice per group).

DO11.10 T cells 4 days after the OVA-Alum sensitization (Fig 2, D) indicated that TSLPR-deficient DO11.10 T cells could proliferate normally on activation. To confirm this, we labeled naive WT or TSLPR-deficient DO11.10 T cells with CFSE and transferred them into WT BALB/c recipient mice at 2×10^6 cells per mouse. The recipient mice were immunized intraperitoneally with OVA plus Alum on the next day and killed 4 days after sensitization for analysis. The percentage and number of WT and TSLPR-deficient DO11.10 cells in medLNs remained identical at day 4 after immunization (Fig 2, E). More importantly, no significant difference was observed in their proliferation based on CFSE dilution (Fig 2, F).

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 the contraction phase, leading to defects in memory formation.

Increased systemic TSLP concentration during sensitization promotes T_H2 memory cell generation *in vivo*

Having established that TSLP signaling in CD4⁺ T cells is required for T_H2 memory cell generation, we next examined whether a systemic increase in TSLP concentration during T_H2 sensitization affects memory cell 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} Although not detectable in ethanol-treated control mice, serum TSLP levels reached 639 ± 164 pg/mL (*n* = 5) on day 4 of MC903 treatment. Naive WT DO11.10 CD4 T cells (2×10^6) were adoptively transferred into WT BALB/c mice, which were subjected to topical MC903 or ethanol treatment. The mice were immunized intraperitoneally with OVA plus Alum, as depicted in Fig 3, A. We observed that the CD4⁺KJ1.26⁺ cell

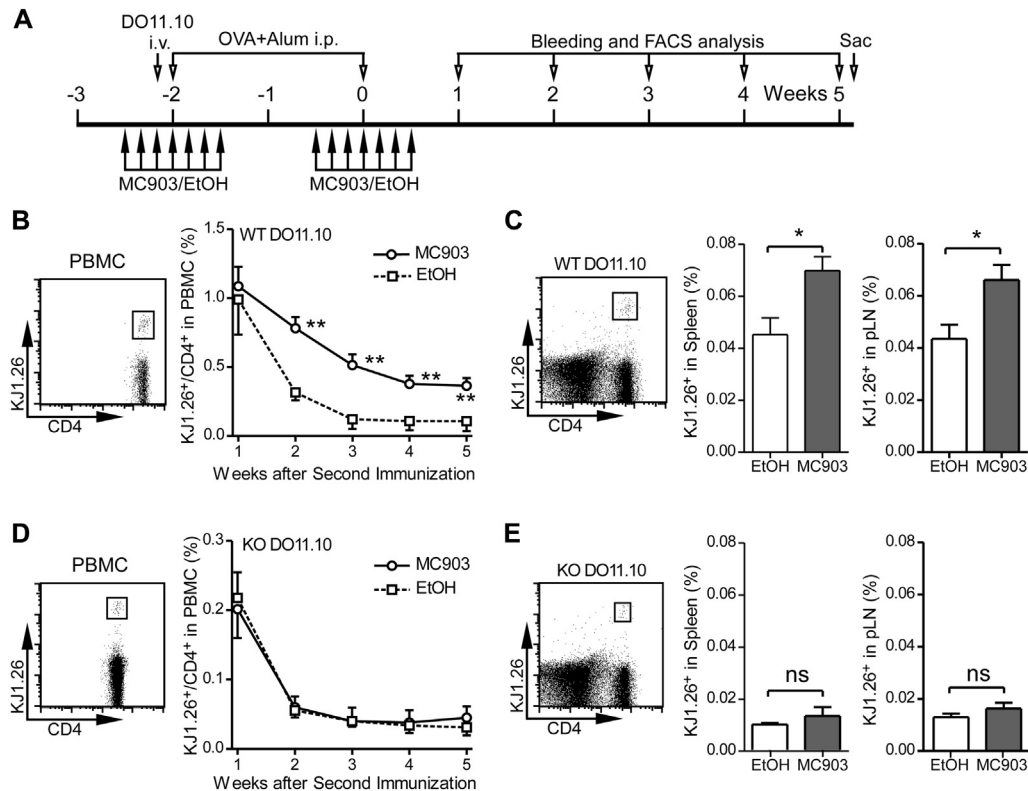


FIG 3. Increased TSLP expression promotes T_H2 memory cell generation. **A**, Diagram of experimental design. *i.p.*, Intraperitoneal. **B** and **D**, Frequency of KJ1.26⁺ cells in CD4⁺ T cells monitored in PBMCs. **C** and **E**, Frequency of CD4⁺KJ1.26⁺ cells in spleens and peripheral lymph nodes (pLN) 5 weeks after the second immunization. KO, TSLPR-deficient; ns, nonsignificant. * $P < .05$ and ** $P < .01$, repeated-measures 2-way ANOVA with the Bonferroni *post hoc* test for Fig 3, B and D, or the 2-tailed *t* test for Fig 3, C and E. Data represent means $\pm$ SEMs ($n = 5$ mice per group).

frequency was significantly higher in the peripheral blood of mice treated with MC903 than in the mice treated with ethanol starting from 2 weeks after the second immunization (Fig 3, B). Examination of the transferred cells in spleens and peripheral lymph nodes further confirmed the existence of increased T_H2 memory cell numbers 5 weeks after the second immunization in mice treated with MC903 (Fig 3, C).

To confirm that it was MC903-induced TSLP that acted on CD4⁺ T cells to enhance T_H2 memory cell 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 identically 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 second immunization (Fig 3, D and E). Therefore increased systemic TSLP concentration during sensitization promotes T_H2 memory cell generation. It is likely that this effect is directly exerted on CD4⁺ T cells because cells in the WT recipients, except 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 T_H2 cells but hinders recall response

Our *in vivo* data indicated that TSLP signaling in CD4⁺ T cells during sensitization is important for generation of T_H2 memory

cells. Next, we wanted to examine whether TSLP signaling in CD4⁺ T cells after priming affects T_H2 memory. We differentiated WT or TSLPR-deficient DO11.10 T cells into T_H2 cells *in vitro* and transferred 20×10^6 T_H2 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 T_H2 cells equally well *in vitro* (Fig 4, A). These cells also developed into memory cells at equal efficiency (Fig 4, B) with the same memory phenotypes after being transferred into recipient mice (Fig 4, C).

Because the recipient mice used in the experiment were naive mice, it is possible that there was not enough TSLP, if it was expressed, to affect the *in vitro*-differentiated T_H2 cells to commit and develop into memory cells. We again used topical MC903 (Fig 4, D, arrows) to induce TSLP expression and increase its systemic concentration. Unlike during sensitization, when T cells were activated (Fig 3, A), increased TSLP concentrations had no significant effects on the frequency of resting T_H2 memory cells in peripheral blood (Fig 4, D).

To define the role of TSLP on memory cell function, we challenged recipient mice intranasally with OVA for 3 days, 7 weeks after transfer of *in vitro* T_H2 -differentiated DO11.10 T cells. Despite similar T_H2 memory cell frequencies and phenotypes before challenge (Fig 4, B), mice that received TSLPR-deficient T_H2 cells had significantly lower total cell counts (Fig 5, A) and eosinophil counts (Fig 5, B) in BAL fluid after

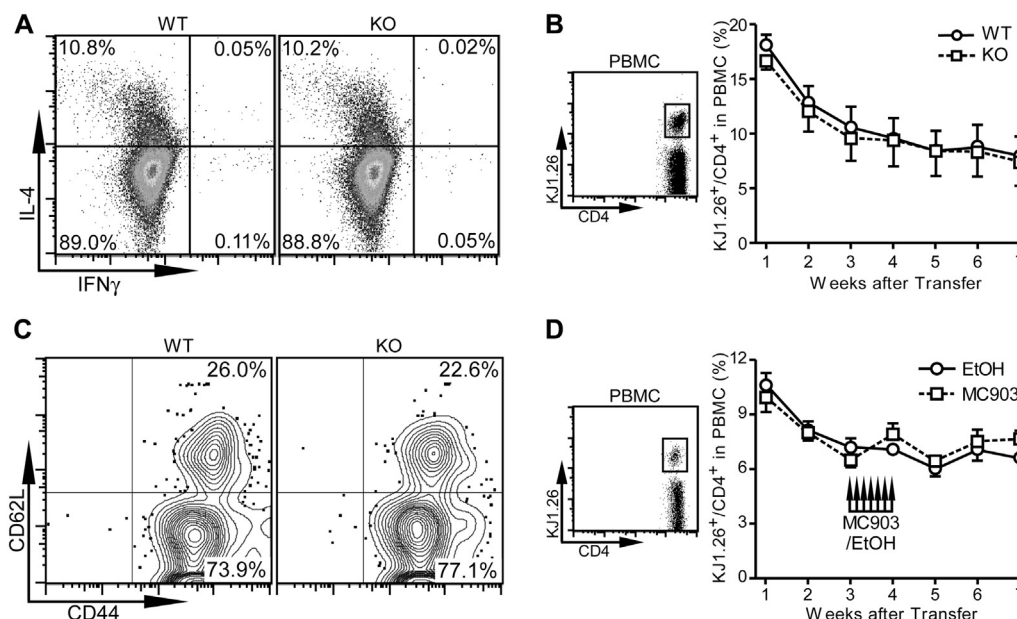


FIG 4. TSLP signaling in CD4⁺ T cells is not required for the generation and maintenance of TH2 memory cells from *in vitro*-differentiated TH2 cells. WT or TSLPR-deficient (KO) DO11.10 T cells were differentiated into TH2 cells *in vitro* for 5 days and transferred into BALB/c recipient mice. **A**, Intracellular staining of *in vitro*-differentiated TH2 cells. **B**, Frequency of KJ1.26⁺ cells in CD4⁺ T cells monitored in PBMCs. **C**, Phenotypes of CD4⁺KJ1.26⁺ TH2 memory cells 5 weeks after transfer. **D**, WT 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 a control for 7 days (arrows). Frequency of KJ1.26⁺ cells in CD4⁺ T cells was monitored in PBMCs. Data represent means $\pm$ SEMs (n = 5 mice per group).

OVA challenge than those that received WT TH2 cells. There were also significantly lower OVA-specific serum IgE levels in these mice (Fig 5, C). Lung histopathology showed reduced eosinophilic peribronchial/perivascular infiltration and goblet cell metaplasia in recipients of TSLPR-deficient TH2 cells compared with those seen in recipients of WT TH2 cells (Fig 5, D). However, consistent with our finding that TSLP signaling in CD4⁺ T cells is not required for antigen-driven cell activation and proliferation (Fig 2, D-F), the frequency of CD4⁺KJ1.26⁺ cells in the inguinal lymph nodes and medLNs remained identical between these 2 groups 24 hours after OVA challenges (Fig 5, E).

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 cell 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 on antigen re-exposure after TH2 memory has been 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 4, B and C) to circumvent the effects of TSLP on TH2 differentiation and memory formation during sensitization. *In vitro*-primed WT or TSLPR-deficient

TH2 cells (20×10^6) were transferred into BALB/c recipient mice, and memory cells were monitored weekly. Three weeks later, recipient mice were exposed intranasally to OVA for 3 days (Fig 6, A, arrows). Similar to previous experiments, no difference between TSLPR-deficient cells and WT cells was seen before antigen exposure. However, starting from 1 week after antigen challenge, the frequency of TSLPR-deficient donor cells in PBMCs was significantly lower than that of WT cells (Fig 6, A). Numbers of TSLPR-deficient DO11.10 T cells continued to decrease over the following 3 weeks. When we challenged the recipient mice in week 8, mice that received TSLPR-deficient TH2 cells showed significantly less total cell counts (Fig 6, B) and eosinophil counts (Fig 6, C) in BAL fluid. Lung histopathology demonstrated that mice that received TSLPR-deficient TH2 cells had reduced inflammatory infiltration and goblet cell metaplasia in the lungs compared with mice that received WT TH2 cells (Fig 6, D). Furthermore, in contrast to the results in Fig 5, E, significantly lower frequencies of TSLPR-deficient donor cells in both the inguinal lymph nodes and medLNs than WT donor cells were observed (Fig 6, E). These data indicate that TSLP signaling in CD4⁺ T cells is required for regeneration of TH2 memory cells 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, as reviewed by Zhang and Zhou.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} and

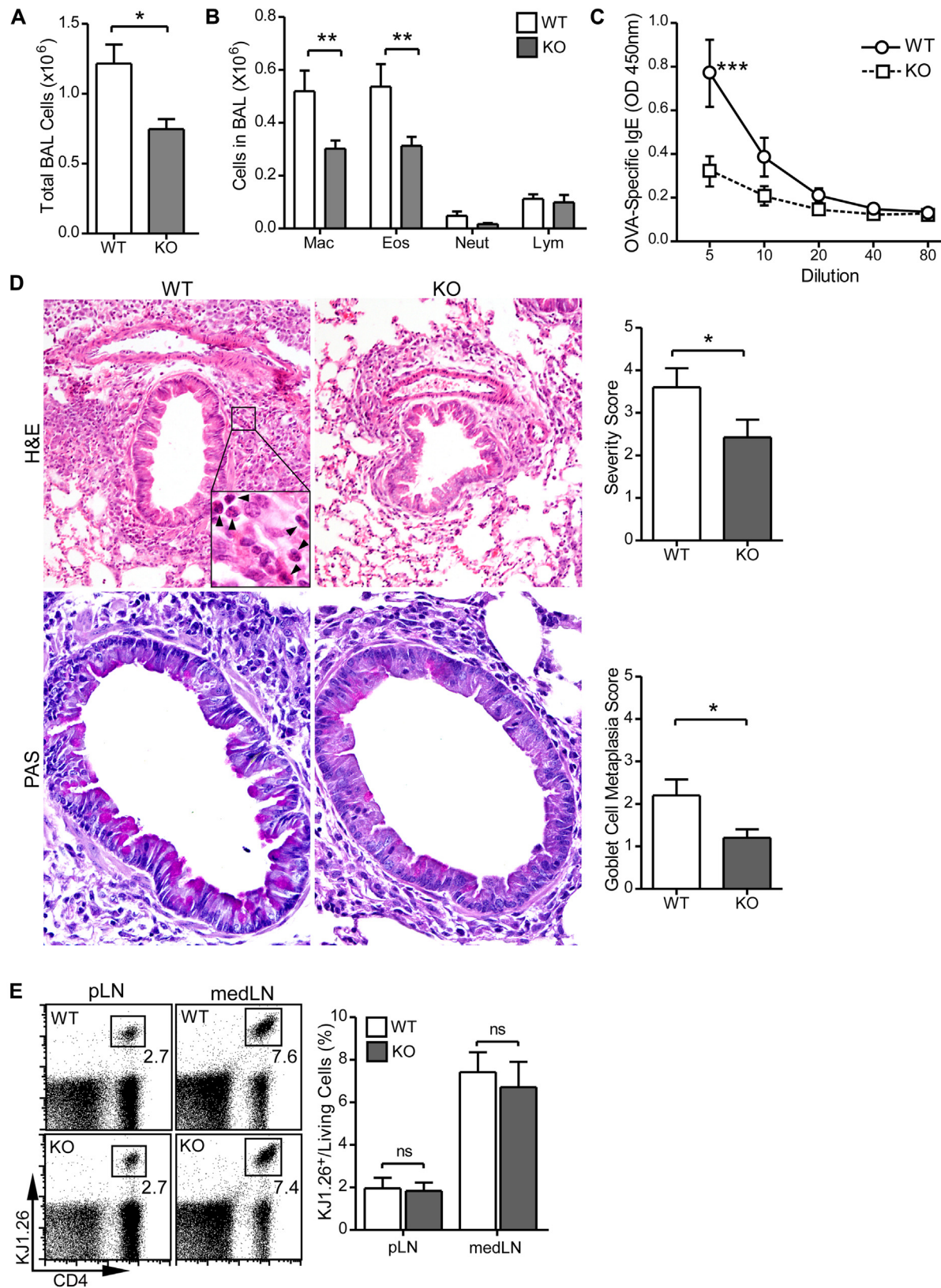


FIG 5. TSLPR-deficient T_H2 memory cells are functionally deficient at memory recall on antigen exposure. Seven weeks after transfer of *in vitro*-differentiated WT or TSLPR-deficient (KO) DO11.10 T_H2 cells, recipient mice were challenged intranasally with OVA for 3 days. **A**, Total BAL cell counts. **B**, Differential BAL cell counts. **C**, OVA-specific IgE levels in serum. **D**, Lung histopathology examined by staining with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The inset shows eosinophil (arrowhead) infiltration. Slides were scored for inflammatory severity (H&E) and goblet cell metaplasia (PAS). **E**, Frequency of CD4⁺KJ1.26⁺ cells in lymph nodes. *Eos*, Eosinophils; *KO*, TSLPR-deficient; *Lym*, lymphocytes; *Mac*, macrophages/monocytes; *Neut*, neutrophils; *ns*, nonsignificant; *pLN*, peripheral inguinal lymph nodes. * $P < .05$, ** $P < .01$, and *** $P < .001$, 2-tailed *t* test for Fig 5, A, B, D, and E, and 2-way ANOVA with the Bonferroni *post hoc* test for Fig 5, C. Data represent means $\pm$ SEMs ($n = 5$ mice per group).

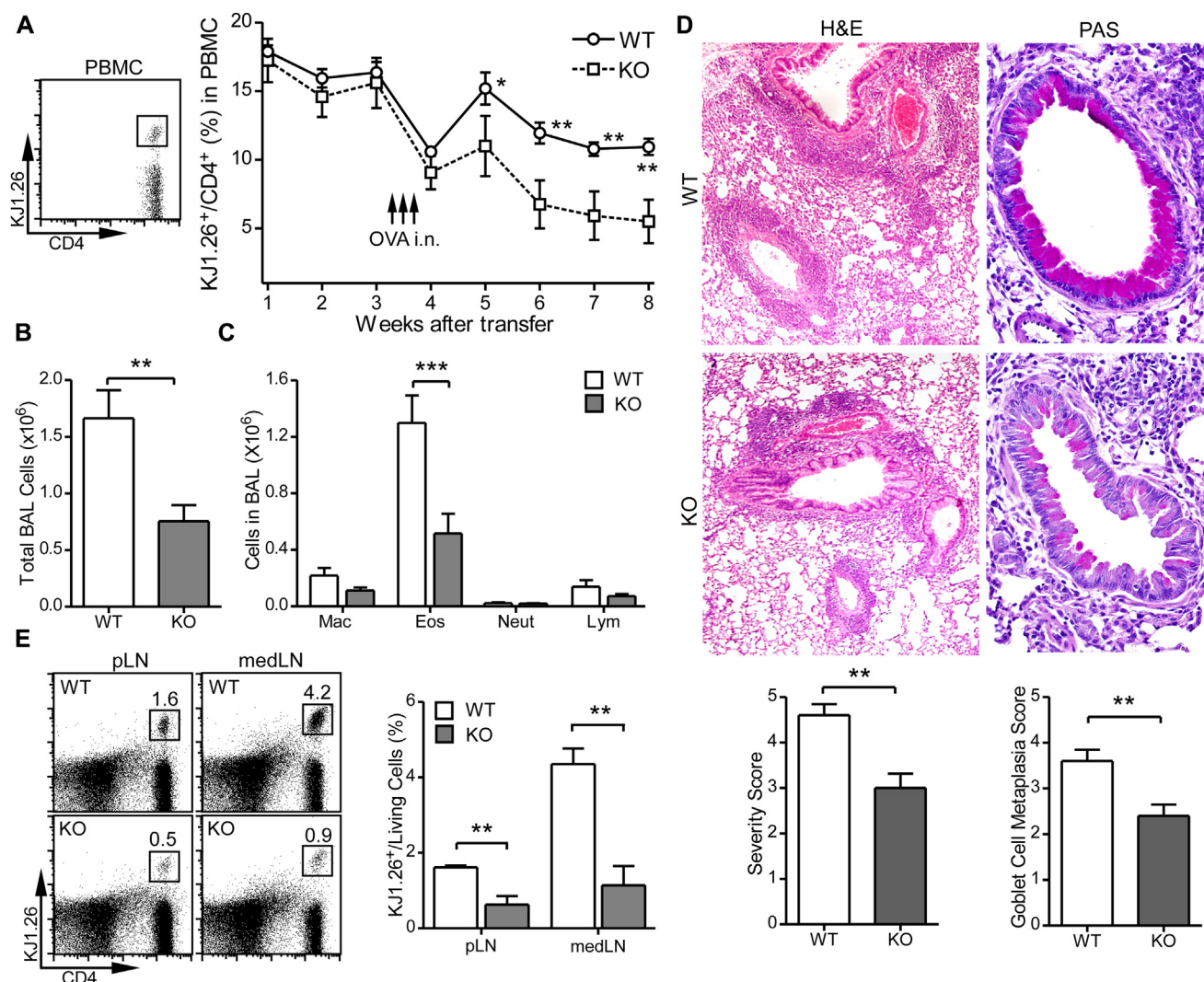


FIG 6. TSLP signaling in CD4⁺ T cells is required for subsequent memory cell development from secondary effector cells after antigen challenge *in vivo*. WT or TSLPR-deficient (KO) DO11.10 T cells were *in vitro*-differentiated into T_H2 cells and transferred into BALB/c recipient mice. Three weeks after transfer, mice were exposed to OVA intranasally (*i.n.*) for 3 days (arrows). At week 8, mice were again challenged intranasally with OVA for 3 days. **A**, Frequency of KJ1.26⁺ cells in CD4⁺ T cells monitored in PBMCs. **B** and **C**, Total and differential cell counts in BAL fluid after OVA challenge at week 8. *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages/monocytes; *Neut*, neutrophils. **D**, Lung histopathology examined by means of hematoxylin and eosin (H&E) and periodic acid-Schiff (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. KO, TSLPR-deficient; pLN, peripheral lymph nodes. **P* < .05, ***P* < .01, and ****P* < .001, repeated-measures 2-way ANOVA with the Bonferroni *post hoc* test for Fig 6, A, or the 2-tailed *t* test for Fig 6, B-E. Data represent means ± SEMs (*n* = 5 mice per group).

suppress Treg 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 the T_H2 adjuvant Alum to develop into resting memory cells. Furthermore, T_H2 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 cells and long-term survival of the memory cells predominantly through induction of antiapoptotic gene Bcl-2 expression.^{11,12,41} We found that TSLPR-deficient CD4⁺ T cells, despite expressing similar levels of IL-7Rα (see

Fig E3 and Al-Shami et al³⁸) and being capable of responding to IL-7, do not develop into memory cells after *in vivo* T_H2 priming, indicating that TSLP nonredundantly regulates T_H2 memory cell development. TSLPR-deficient CD4⁺ T cells show no defects in proliferation, but most of the cells undergo apoptosis 7 days after OVA plus Alum sensitization (Fig 2). However, unlike IL-7, which is absolutely required not only for the generation but also the survival of CD4⁺ memory cells, we found that enhanced memory cell 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 4, D). Furthermore, *in vitro*-differentiated TSLPR-deficient T_H2

cells survived equally well compared with their WT counterparts (Fig 4, B). Therefore the requirement of TSLP in T_H2 memory cell 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 plus Alum intraperitoneal immunization. Until the first week after immunization, no differences between TSLPR-deficient and WT donor cell frequencies were observed in peripheral blood (Figs 1, A, and 3, B) and medLNs (Figs 1, C, and 2, A and E). Indeed, 4 days after immunization, TSLPR-deficient DO11.10 T cells in medLNs showed proliferation marker Ki-67 expression (Fig 2, D) and CFSE dilution (Fig 2, F) similar to that seen in WT DO11.10 T cells. Kool et al⁴² demonstrated that OVA plus Alum intraperitoneal immunization mainly drains to and induces robust antigen-specific $CD4^+$ T-cell activation and proliferation in medLNs but not in the peripheral lymph nodes and spleen, as would be expected in a systemic response. Limited DO11.10 T-cell proliferation in the inguinal lymph nodes was observed depending on the location of injection because of 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 T_H2 memory formation. $CD4^+$ T cells in naive TSLPR-deficient mice have no overt defects and surface molecule expression, including TCR/costimulatory molecules, IL-7R α , and memory marker CD44/CD62L (see Fig E1, A and B, and Al-Shami et al³⁸), nor are they prone to apoptosis (see Fig E1, C). *In vitro* studies demonstrate that TSLP at 50 ng/mL induces antiapoptotic gene Bcl-2 expression in T_H2 cells.³⁴ On activation *in vivo* by OVA plus Alum immunization, TSLPR-deficient DO11.10 T cells show slightly less expression of Bcl-2 than WT DO11.10 T cells (Fig 2, C). 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.

Although presenting the antigen at the time of their interaction with T cells, DCs provide multiple signals to determine the outcome and fate of activated T cells.⁴³ TSLP signaling in DCs is critical to prime T_H2 differentiation,^{16,19} and DC-specific deletion of signal transducer and activator of transcription 5 abolished the T_H2 allergic response, although not the T_H1 response.¹⁶ In the WT 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 means of upregulation of CD44 surface expression 4 days after the second OVA plus Alum sensitization (Fig 1, C, and see 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 (Figs 1-3), *in vitro*-differentiated TSLPR-deficient T_H2 cells show no defects in the transit to lasting memory cells (Fig 4). It has also reported that, different from IL-7, supplementation of TSLP to the *in vitro* culture did not promote memory cell 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 costimulation.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 T_H2 memory cell generation *in vivo* should not be underestimated. In addition to the requirement of TSLP for T_H2 memory cell generation in the OVA plus Alum model (Figs 1-3), regeneration 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 T_H2 cells (Fig 4, B and D), the recall response of the memory cells in response to local antigen challenge is dependent on TSLP (Fig 5). Jang et al⁴⁷ reported that transfer of *in vitro*-differentiated T_H2 cells into TSLP knockout mice did not change lung pathology or T_H2 cytokine production on OVA challenge compared with transfer into WT mice. Such a discrepancy could arise from the time of OVA challenge in relating to cell transfer. Although we challenged the recipient mice 7 weeks after cell transfer, allowing the *in vitro*-primed T_H2 cells to fully differentiate into resting memory cells, Jang et al⁴⁷ challenged mice immediately after cell transfer so that the donor cells likely still remain as active effectors.

T_H2 memory cell numbers 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 T_H2 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 T_H2 memory cells but not the beneficial memory cells. The requirement of TSLP for the transition of secondary effector cells to T_H2 memory cells (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 numbers of allergen-specific Treg cells because we have shown that a small amount of TSLP could have a big effect on the differentiation of inducible Treg cells.³¹ Collectively, these findings highlight a central requirement for TSLP in supporting the generation of T_H2 memory cells *in vivo* and ultimately determining the efficiency of the T_H2 -mediated inflammatory recall response.

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Key messages

- TSLP signaling in $CD4^+$ T cells is required for memory formation after T_H2 sensitization.
- TSLPR-deficient T_H2 memory cells show impaired memory recall in response to airway antigen challenge.
- Generation of memory cells from secondary T_H2 effectors after antigen inhalation requires TSLP action on $CD4^+$ T cells.

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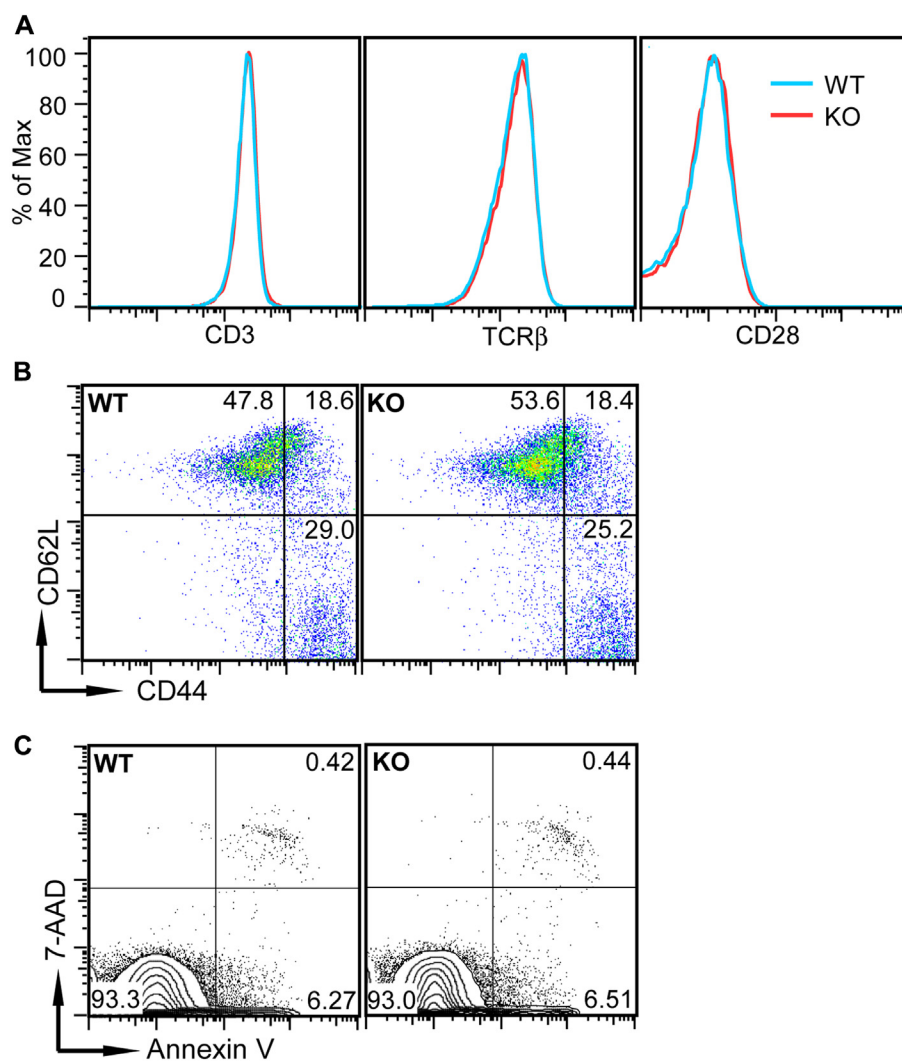


FIG E1. Phenotypic characterization of CD4⁺ T cells in WT BALB/c versus TSLPR-deficient (*KO*) mice. A single-cell suspension was prepared from peripheral lymph nodes collected from naive WT or KO mice, and cells were stained and characterized by means of flow cytometry. **A**, Expression of CD3, TCR β , and CD28 on CD4⁺ T cells. **B**, Expression of the memory markers CD44 and CD62L on CD4⁺ T cells. **C**, Apoptosis of CD4⁺ T cells shown by using Annexin V and 7-AAD staining.

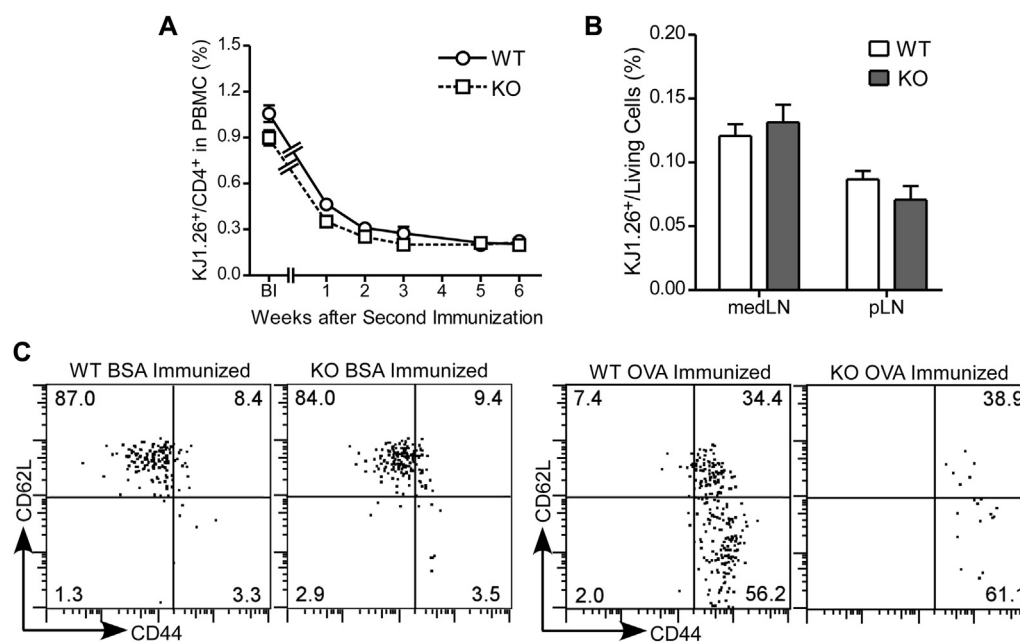


FIG E2. Homeostatic proliferation and survival of WT and TSLPR-deficient (KO) DO11.10 CD4⁺ T cells in a WT recipient. Naive WT or KO DO11.10 T cells were transferred into WT BALB/c recipients. The recipient mice were immunized twice, a week apart, with intraperitoneal injection of BSA plus Alum. **A**, Frequency of KJ1.26⁺ donor cells in gated CD4⁺ T cells in PBMCs. **BI**, Before immunization with BSA plus Alum. **B**, Frequency of KJ1.26⁺ donor cells in the lung draining medLNs and peripheral lymph nodes (pLN) 6 weeks after second immunization with BSA plus Alum. **C**, Phenotypes of KJ1.26⁺ donor cells in medLNs 6 weeks after second immunization with BSA plus Alum. As a comparison, memory phenotypes of KJ1.26⁺ donor cells 6 weeks after the second OVA plus Alum immunization are also shown.

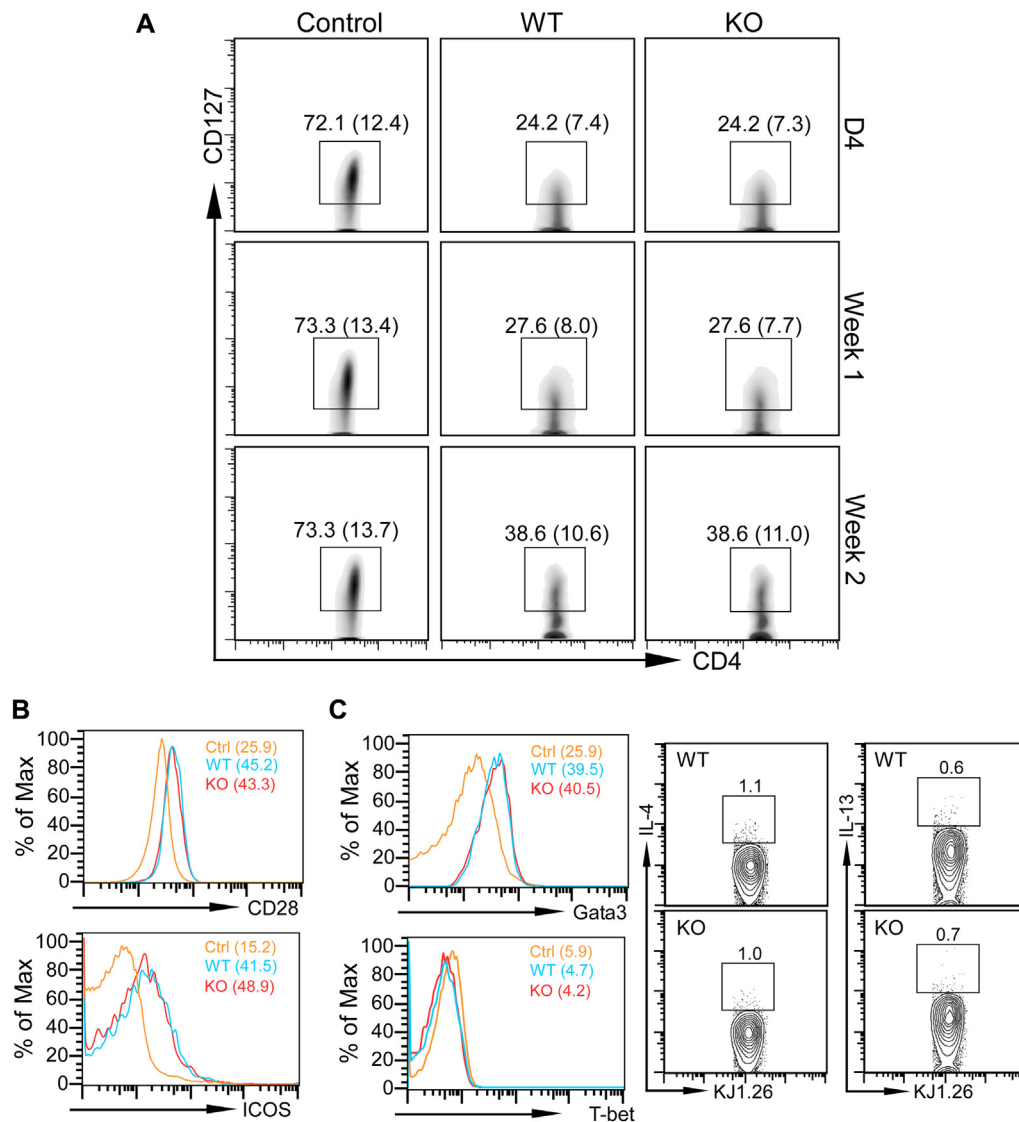


FIG E3. Activation and differentiation of DO11.10 T cells after OVA plus Alum sensitization. Naive WT or TSLPR-deficient (KO) DO11.10 T cells were transferred into WT BALB/c recipients. The recipient mice were sensitized twice, a week apart, with intraperitoneal injection of OVA plus Alum. Cells from the medLNs were stained with fluorescent-conjugated antibodies and examined by using flow cytometry. **A**, CD127 (IL-7R α) expression on CD4⁺KJ1.26⁺ donor cells. Numbers denote the percentages of CD127⁺ cells. Numbers in parentheses represent the mean fluorescence index. **D4**, Day 4. **B**, Upregulation of CD28 and inducible costimulator (ICOS) on DO11.10 T cells. **C**, Intracellular staining of Gata3, T-bet, IL-4, and IL-13 expression. Control (Ctrl) samples were CD4⁺ T cells from peripheral lymph nodes of naive BALB/c mice. Numbers in parentheses represent the mean fluorescence index.