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TSLP Expression: Analysis with a ZsGreen TSLP Reporter Mouse

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Thymic stromal lymphopoietin (TSLP) is a type I cytokine that together with IL-7 plays an important role in T cell development (1) in mice and in T cell development in humans (2). TSLP is a critical inducer of allergic inflammatory responses (3). It shares with IL-7 the use of IL-7Rα as a receptor component, but uses the TSLPR rather than the γc chain to form a signaling complex (4). It has been reported that TSLP activates Jak1 and Jak2 to cause STAT5 phosphorylation, whereas IL-7 activates STAT5 phosphorylation by activating Jak1 and Jak3 (5).

A large body of research has implicated TSLP as playing a major role in the induction of Th2-type immune responses and in the mediation of allergic inflammation in the skin, lung, and intestine (3). There is much evidence that TSLP acts on dendritic cells (DCs) that, in turn, favor Th2 differentiation when they present Ag to naïve CD4 T cells in draining lymph nodes (6, 7). In particular, TSLP-treated DCs, rather than producing proinflammatory cytokines, express OX-40 ligand, which in turn plays a role in induction of Th2 differentiation by CD4 T cells (8). Such OX-40 ligand–stimulated Th2 cells have been reported to produce substantial amounts of TNF-α and little IL-10 (6).

TSLP can also act directly on naïve CD4 T cells (9) and may aid their differentiation to Th2 cells by providing the STAT5 signals that have been shown to be essential for in vitro Th2 differentiation (10). Furthermore, TSLP can synergize with IL-33 in inducing both cytokine-dependent IL-13 and IL-5 production by Th2 cells and in driving Th2 cell proliferation (11). TSLP may also enhance IL-33–mediated expansion and IL-13 production by type 2 innate lymphoid (ILC2) cells (12), potentially contributing to allergic inflammation. The relative contribution of TSLP– activated DCs, of direct action of TSLP on differentiation of naïve CD4 T cells to the Th2 phenotype, and of TSLP action on differentiated Th2/ILC2 cells to sustain allergic inflammation remains to be determined.

The study of the regulation of TSLP production has been somewhat enigmatic as direct visualization of cytosolic TSLP has been difficult. In general, TSLP has been shown to be a product of epithelial cells such as skin keratinocytes (13). There is some controversy as to whether mast cells and/or basophils are a rich source of TSLP (14). It has been proposed that papain and other cysteine proteases act as allergens because they stimulate basophils to produce TSLP (15), although it is also plausible that papain acts directly on keratinocytes and other epithelial cells to induce expression of the cytokine. Strikingly, activation of PAR2 receptors has also been implicated in TSLP induction (16), although in this work it is serine proteases rather than cysteine proteases that are responsible for TSLP induction.

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Abbreviations used in this article: AIRE, autoimmune regulator; BAC, bacterial artificial chromosome; cTEC, cortical TEC; DC, dendritic cell; EpCAM, epithelial cell adhesion molecule; ILC2, type 2 innate lymphoid; mTEC, medullary TEC; TEC, thymic epithelial cell; TSLP, thymic stromal lymphopoietin; UEA-1, *Ulex europaeus* agglutinin-1; ZsG, ZsGreen.

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proteases that are inducers. Equally interesting is the concept that TSLP may be part of a feedback loop in which it both induces/sustains IL-4/IL-13–producing Th2 cells and in which its production is stimulated by cytokines produced by inflammatory Th2 cells.

To examine these issues in greater detail, we prepared a surrogate for TSLP expression in which a ZsGreen (ZsG) construct was introduced by recombineering at the translation-initiating ATG in bacterial artificial chromosome (BAC) clone RP23-256L23. Substantial amounts of 5’ and 3’ DNA flanked the TSLP gene in this 183-kb BAC, suggesting that many of the regulatory elements controlling TSLP expression may be present in the introduced genetic material, and thus that the reporter would reflect physiologic expression of TSLP.

Materials and Methods

Mice

C57BL/6 mice were purchased from Taconic Farms. BAC transgenic mice were bred, and all animals were housed, in the National Institute of Allergy and Infectious Diseases pathogen-free animal facility and used between 8 and 20 wk of age. All experiments were done under a protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Generation of TSLP/ZsG mice by recombineering

The murine BAC clone RP23-256L23, containing the TSLP locus, was obtained from the Children’s Hospital Research Institute BAC library (http://bacpac.chori.org). BAC DNA was purified using the Nucleobond BAC kit (Clontech) and characterized by PCR (5’-CGACAGCGT-GGTGACTATG-3’; 5’-GCTGTCAGAGGTTACACCC-3’). Generation of a modified BAC containing the ZsG reporter at the ATG site of the TSLP gene was done using the galk recombineering technique (17) (http://recombineering.ucsf.edu/protocol/Protocol2_CK0_vectors.pdf). Briefly, ZsG was cloned with BAC arms corresponding to the target BAC sequences surrounding the ATG at the beginning of the TSLP gene and recombineered into the BAC. The stop codon and internal polyA from ZsG were retained in the construct to avoid any translation of the additional copies of ZSLP or of a fusion protein. Mice were generated in the SAIC/National Cancer Institute/LASP facility (Laboratory Animal Sciences Program, Frederick, MD) by pronuclear microinjection of fertilized C57BL/6 mouse eggs and verified by fertility (Laboratory Animal Sciences Program, Frederick, MD) by pronuclear cell sorting using a FACSAria III (BD Biosciences). After sorting, ZsG+ mTEC cells were collected using a PAFSaria flow cytometer (BD Biosciences) and analyzed using FlowJo Software (Tree Star, San Carlos, CA).

Preparation of thymic stromal cells for flow cytometric analysis and sorting

Thymic stromal cells were prepared using methods modified from those reported by Gray et al. (19). Following release of thymocytes by gentle teasing of the thymus, thymic fragments were digested with collagenase/dispase at 0.25% w/v plus Dnase 1 at 0.125% w/v (Roche) in four sequential incubations at 37°C. Reactions were stopped by addition of FCS to 20%. For thymic epithelial cell (TEC) analysis, single-cell suspensions were stained with anti–CD45.2-Pacific Blue (104; BioLegend), anti–epithelial cell adhesion molecule (EpCAM)-PE (G8.8; eBioscience), and the MHC class II–allophycocyanin (M5-114; eBioscience), and Ulex europaeus agglutinin-1 (UEA-1) biotin (Vector). Dead cells were excluded with propidium iodide staining. For medullary TEC (mTEC) sorting, enriched TEC preparations were made by discontinuous density gradient fractionation (20). Enriched TECs were stained with anti-CD45.2, anti-MHC class II, anti-EpCAM, and UEA-1. CD45.2-negative, MHC II+, UEA− (cortical TEC (cTEC)), MHC II− UEA+ (medullary TEC (mTEC)), and ZsG+ cells were collected using a FACSaria flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star) FACS analysis software.

RNA extraction and DNA microarray target preparation

Flow-sorted mouse cells were lysed in 600 μL RNAsay Lysis Buffer (Qiagen, Valencia, CA). Due to low number of ZsG+ mTEC cells (<1000), genome copy number was measured by quantitative PCR before RNA lysis was processed. Briefly, DNA was extracted from a 50-μL aliquot of cell lysate according to the AlpPrep DNA/RNA 96-well kit protocol. Absolute quantitation analysis of DNA used universal expression quantitative PCR supermix universal with premixed ROX, mouse DNA standard, and ready-made 20× mouse actB primer and probe mix according to manufacturer’s instructions (Life Technologies, Carlsbad, CA). The lowest cell copy number in the sample set was 654. Due to low number of cells, RNA lysate was combined with 12 μg linear acrylamide (Life Technologies, Carlsbad, CA) to increase RNA yield from low cell count samples. RNAs were extracted using Qiagen RNasey 96-well system, according to manufacturer’s recommendations, except each RNA sample was treated with 27 U DNase I (Qiagen, Valencia, CA) for 15 min at room temperature during extraction to remove genomic DNA. RNA quality was determined using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the Agilent RNA Pico kit. RNA quantitation was performed by quantitative RT-PCR in triplicate by grey et al. in Mackey-Lawrence et al. (21). The lowest RNA yield of 0.9 ng was from 654 cells. DNA microarray targets were prepared from extracted RNAs using WT-Ovation Pico RNA amplification system and FL-Ovation cDNA biotin module, according to manufacturer’s instruction (Nugen, San Carlos, CA).

Hybridization, fluidics, and scanning were performed according to standard Affymetrix protocols (http://www.affymetrix.com). Command Console (CC v3.1, http://www.affymetrix.com) and GeneChip Fluidics and Scan Control Software (GFC, Affymetrix) were used to analyze image files to cell intensity data (cel files). All cel files, representing individual samples, were normalized by using the trimmed mean scaling method within expression console (EC v1.2, http://www.affymetrix.com) to produce the analyzed cel files (cph files) along with the report files. The cel files were input into Partek Genomics Suite software (Partek, St. Louis, MO; v.6.6-6.12.0907), and quantile was normalized to produce the principal components analysis graph and dendrogram. ANOVA was performed on each chip to obtain multiple test corrected p values using the false discovery rate method (22) at the 0.05 significance level and was combined with fold change values, signal confidence (above background), and call consistency (as a percentage).
calculated using custom Excel templates for each comparison of interest. IPA (IngenuityPathway Systems, www.ingenuity.com) was used to generate a custom pathway with selected array data overlay. The array data discussed in this publication have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (23) and are accessible through GEO series accession number GSE54343 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54343).

Confocal, two-photon intravital skin microscopy and image analysis

All tissue samples were fixed in 1% paraformaldehyde overnight at 4˚C, and then transferred into 30% sucrose overnight at 4˚C. Tissue was embedded in OCT medium (Sakura 4583), frozen on dry ice, cut with a cryostat, and kept at −80˚C until staining. All confocal images were acquired with a Leica TCS-NT/SP confocal microscope (Leica Microsystems, Exton, PA) using a 40× oil immersion objective. Fluorochromes were excited using an argon laser at 488 nm for FITC, a krypton laser at 568 nm for Alexa 568, and a Helium-Neon laser (HeNe) at 633 nm for far-red Ab detection. DAPI was excited using an Argon laser (Enterprise model 651; Coherent) at 364 nm. Detector slits were configured to minimize any crosstalk between the channels. Imaging of the ears by intravital multiphoton microscopy was acquired using an inverted LSM 510 NLO multiphoton microscope (Carl Zeiss Microimaging), as described previously (24). Images were processed using Leica TCS-NT/SP software (version 1.6.587), Imaris 7.5.1

**FIGURE 1.** TSLP/ZsG reporter mice. (A) Strategy for preparation of a BAC into which a ZsG construct with a polyA site and a stop codon has been introduced at the ATG of TSLP by recombineering. (B) Analysis of TSLP mRNA in ears of mice treated with ethanol or MC-903 for 1, 3, 4, and 5 d and a comparison of induction TSLP and CYP243A1, a known vitamin D–dependent gene, after 4 d of application of ethanol or MC-903 to the ear. This experiment was performed three times with similar results. p = 0.0055 for TSLP/18S, p = 0.006 for CYP24A1/18S. (C) Tile scans of ears of BAC transgenic mice treated with ethanol or MC-903. ZsG is shown in green, and staining with anti-cytokeratin 1 in red. (D) Ethanol was applied to the left ears and MC-903 to the right ears of ZsG BAC transgenic mice for 4 or 7 d. One day later, mice were sacrificed, single-cell suspensions from ear epidermis were prepared, and mRNA for TSLP and ZsG was determined. Mean and SEM are shown. This experiment was performed twice with similar results. (E) Regression analysis of TSLP and ZsG mRNA expression in right ears of individual mice treated with MC-903 for 4 or 7 d.
Vitamin D3 induces TSLP mRNA and ZsG expression

As an initial test of the expression of ZsG as a surrogate for TSLP, we used the capacity of 1,25 dihydroxy-vitamin D3 and its non-calcemic analog MC-903 to induce keratinocyte expression of TSLP. As described in Materials and Methods, a construct encoding ZsG was inserted at the translation-initiating ATG for TSLP using the techniques of recombineering (Fig. 1A). After purification, the recombinant BAC construct was introduced by pronuclear injection into fertilized C57BL/6 mothers. Mice were screened by Southern blotting for expression of the BAC construct, and expression was verified by Northern blotting for ZsG mRNA.

Results

Preparation of TSLP-ZSG mouse

BAC clone RP23-256L23 was obtained from the Children’s Hospital & Research Center (Oakland, CA). The BAC contains TSLP flanked 5’ by ∼115 kb and 3’ by ∼65 kb, suggesting that it may have all of the cis-regulatory elements necessary for physiologic expression of TSLP. As described in Materials and Methods, a construct encoding ZsG was inserted at the translation-initiating ATG for TSLP using the techniques of recombineering (Fig. 1A). After purification, the recombinant BAC construct was introduced by pronuclear injection into fertilized C57BL/6 oocytes that were then transferred to pseudopregnant foster mothers. Mice were screened by Southern blotting for expression of the BAC construct, and expression was verified by Northern blotting for ZsG mRNA.

Vitamin D3 induces TSLP mRNA and ZsG expression

As an initial test of the expression of ZsG as a surrogate for TSLP, we used the capacity of 1,25 dihydroxy-vitamin D3 and its non-calcemic analog MC-903 to induce keratinocyte expression of TSLP. As shown in Fig. 1B, TSLP mRNA increases over the first 3–4 days of application of MC-903 to the ear, reaching ~60-fold above levels in ears treated with ethanol.

ZsG fluorescence in the epidermal layer of the ear could be detected 2 d after application of MC-903 and reached a maximum on day 5. Examining an assemblage of a large portion of the ear sample by confocal microscopy by a montage of contiguous fields of view (a “tile” scan) revealed that ZsG expression was patchily detected 2 d after application of MC-903 and reached a maximum on day 5. Examining an assemblage of a large portion of the ear sample by confocal microscopy by a montage of contiguous fields of view (a “tile” scan) revealed that ZsG expression was patchily expressed in the keratinocyte layer (Fig. 1C). In some areas, keratinocytes were intensely fluorescent, whereas in adjacent areas, there was little or no ZsG expression in response to vitamin D3 application.

To confirm that ZsG was faithfully reporting TSLP production, we painted the left ears of transgenic mice with ethanol and the right ears with MC-903 for 4 d (animals 1–3) or 7 d (animals 4–6), and 1 d later isolated cells from the epidermis with trypsin. Single-cell suspensions were analyzed by RT-PCR for TSLP and ZsG mRNA. There was striking induction of both TSLP and ZsG mRNA by MC903 in the 4- and 7-d groups (Fig. 1D). Furthermore, a regression analysis of TSLP and ZsG mRNA for individual animals showed a positive slope with an $r^2$ value of 0.36 (Fig. 1E), implying that ZsG mRNA expression tracked TSLP mRNA levels.

These results indicate that ZsG is a good surrogate for TSLP and that keratinocytes are the major TSLP-expressing cells in vitamin D3–treated skin.

TSLP/ZsG expression in mTECs

TSLP was initially described as a factor expressed in supernatants of a thymic stromal cell line that could support the growth of a pre-B cell line (25). Subsequently, it was reported that, in the human thymus, TSLP was largely expressed in Hassall’s corpuscles (26). Although Hassall’s corpuscles are said to be poorly developed in the mouse, because we had the capacity to isolate TSLP-expressing thymocytes based on ZsG expression, we undertook to study these cells. We identified a small population of ZsG+ cells in the thymic medulla by confocal microscopy that were UAE+ (Fig. 2A). Flow cytometric analysis indicated that these ZsG+ cells constituted ∼1% of mTECs, defined by their coexpression of EPCAM, UAE, and MHC class II (27, 28) (Fig. 2B). We purified cTECs (EPCAM+, UAE- cells) and mTECs (EPCAM+, UAE+ cells); the latter were separated into ZsG+ and ZsG- populations.

cTECs expressed >40 times more β5T mRNA than the ZsG- or ZsG+ mTECs (Fig. 3). β5T is a thymoproteasome subunit reported to be exclusively expressed in cTECs (29). ZsG- mTECs expressed >14 times as much autoimmune regulator (AIRE) mRNA than the cTECs. These relative expression results confirm our purification procedure. The ZsG- and negative mTECs expressed similar amounts of AIRE mRNA. Essentially all ZsG mRNA was expressed in the ZsG+ mTECs, and TSLP mRNA was expressed to a substantially greater degree in the ZsG+ mTECs than the ZsG- mTECs. In a subsequent analysis of mRNA expression, we observed the involucrin, a marker for terminal epidermal differentiation (30), was ~4-fold overexpressed in ZsG+ mTECs compared with ZsG- cells (Fig. 3).

To take further advantage of the ZsG marker, we purified both cTECs and ZsG+ and negative mTECs and carried out a microarray analysis. Strikingly, principal component analysis revealed that ZsG+ mTECs were substantially different from ZsG- mTECs and from cTECs (Supplemental Fig. 1). Interestingly, ATG13, BCN1, and Eph receptor A1 were overexpressed in ZsG+ mTECs;
ATG13 has 6.8-fold ratio in ZsG+ compared with ZsG− mTECs, BCN1, 14.9, and Eph receptor A1 3.2. All three are part of an autophagy pathway that is regulated by mTOR and that, in other situations, may be involved with autophagic cell death. Indeed, as shown in Supplemental Fig. 2, ATG13, BCN1, and EPH A1 are part of a pathway in which several of the members are overexpressed and in which other members of the pathway that are normally inhibited when ATG13 and related genes are overexpressed are, in fact, downregulated (31). In prostatic epithelial cells and endothelial cells, but not fibroblasts, treatment with ephrin-A1 inhibits cell proliferation (32).

TNF-α and IL-4/13 induce ZsG expression in keratinocytes

We then turned to the analysis of ZsG expression in the back skin or ears of mice in response to injection of various cytokines. PBS, TNF-α (1 μg), IL-13 (1 μg), or IL-4 (1 μg) was injected into the shaved back skin of TSLP-ZsG transgenic mice on successive 2 d. Confocal microscopy carried out on skin biopsies taken 24 h later revealed that TNF-α, IL-4, and IL-13 each caused induction of ZsG, with TNF-α being the most striking inducer (Fig. 4A). A particularly graphic example of the degree of induction is shown by injection of TNF-α or PBS into the ear of TSLP-ZsG mice twice and examination by multiphoton microscopy 24 h later (Fig. 4B). In this case, the keratinocytes are being seen from directly above, whereas in the confocal views, they are being seen transversely.

We also evaluated ZsG expression in response to TNF-α by flow cytometry. Staining epidermal preparations from injected skin with anti-CD3, anti-CD11c, and anti-cytokeratins 10 or 14 revealed three major cell populations. Because staining with anti-cytokeratins required fixation, which diminishes ZsG fluorescence, we repeated the staining on nonfixed cells with anti-CD3 and anti-CD11c only. Only the cells negative for both these markers expressed ZsG, implying that only the anti-cytokeratin–positive cells expressed ZsG (Fig. 5). Because cytokeratins are mainly restricted to epidermal cells (33), we conclude in mice stimulated with TNF-α, ZsG expression in the skin was confined to the keratinocyte population.

In addition to TNF-α, IL-4 and IL-13, IL-1, and LPS each induced ZsG expression upon injection into back skin when evaluated by confocal microscopy (data not shown).

Subcutaneous activation of Th2 and Th1 cells induces ZsG expression

The finding that TNF-α and IL-4/13 induce TSLP/ZsG expression when injected into the skin implies that CD4 T cells capable of making these cytokines, if stimulated in the skin, should induce keratinocytes to express ZsG. Accordingly, we primed OT-2 cells in vitro under Th1- or Th2-inducing conditions through one or two rounds of priming. We verified that the cells had attained either a Th1 or a Th2 phenotype based on their production of IFN-γ or IL-4/13 (Fig. 6A). Both the Th1 and Th2 cells were robust producers of TNF-α. After culturing the primed cells in IL-2 (Th1) or IL-2 and IL-7 (Th2), we transferred 3–5 × 10⁶ cells i.v. Twenty-four hours later, we challenged the mice with 100–250 μg OVA or, as a control, with PBS, in the back on successive 2 d and evaluated keratinocyte expression of ZsG 24 h later by confocal microscopy. Recipients of both Th1 and Th2 cells showed strong induction of ZsG expression in the keratinocyte layer in response to challenge with OVA, but not hen egg lysozyme (Fig. 6B). PBS-injected controls showed little or no induction.

We also tested the contribution of IL-4/IL-13 to the Th2 induction of ZsG expression by keratinocytes. Th2 cells were transferred into B6 mice. These animals were challenged with lysozyme or OVA. Some of the OVA-treated mice received an i.v. injection of monoclonal anti-IL-4Rα (M1) prior to challenge. This
Treatment reduced, but did not abolish ZsG induction, implying that IL-4 or IL-13 was responsible for only some of the induction of ZsG and presumably TSLP by Th2 cells (Fig. 7).

TSLP/ZsG expression in bone marrow–derived DCs, basophils, and mast cells

Although there is general agreement that epithelial cells can express TSLP when appropriately stimulated, there has been controversy about expression of TSLP by mast cells, basophils, and DCs (14). To study this point, we prepared bone marrow–derived mast cells, basophils, and DCs by culturing bone marrow cells from TSLP-ZsG and wild-type B6 mice in IL-3 (mast cells and basophils) or GM-CSF (DCs). On day 9 of the IL-3 culture, both FcεRI+ c-kit+ cells, identified as mast cells (34), and FcεRI+, c-kit−2, and CD49+ cells, identified as basophils, were present (35). When analyzed by flow cytometry, we found few, if any, mast cells or basophils to be ZsG+ after stimulation with PMA and ionomycin (Fig. 8). We analyzed DCs on day 8 of culture; no ZsG+ cells were seen by flow cytometry (data not shown).

Nonetheless, when we analyzed message expression, we did detect induction of both TSLP and ZsG mRNA in bone marrow–derived mast cells, basophils, and DCs (Fig. 8). Basophils and mast cells were stimulated with PMA and ionomycin and analyzed 3 h later; DCs were stimulated with LPS plus IL-4 for 4 h. Based on our failure to detect ZsG protein expression in mast cells, basophils, or DCs, we conclude that these three cell types, although capable of producing TSLP, do so to a much lower extent than do keratinocytes. Consistent with this, when we compared cycle number differences in the real-time PCR analysis for TSLP, ZsG, and 18S RNA, MC-903–stimulated keratinocytes showed an apparent greater expression of 100- to 1000-fold (Supplemental Fig. 3) than did the three hematopoietic cell types. Even if 18S RNA expression is somewhat different in these various cell types, this result is nonetheless consistent with a very much lower degree of expression of both TSLP and ZsG in DCs, basophils, and mast cells than in keratinocytes.

Discussion

We provide evidence that green fluorescence by cells from a BAC-transgenic mouse in which ZsG has been recombinered into the TSLP gene of BAC RP23-256L23 at the translation initiating ATG is a good reporter of TSLP expression. Vitamin D3 and MC903 were strong stimulants of ZsG expression. They modify retinoid X receptor nuclear factor regulation of the TSLP promoter (36). TNF-α was also a very strong stimulant of ZsG expression, presumably acting through TNF-RI expressed on keratinocytes and activating NF-κB and AP-1 (37). The mechanisms through which IL-4 and IL-13 induce TSLP expression are not clear.

Keratinocytes showed bright fluorescence upon stimulation by these stimulants injected into the ear or the skin of the back; LPS and IL-1β also modestly induced ZsG expression. To date, we have observed little or no fluorescence in hematopoietic cells at sites of vitamin D3 or cytokine injection.

TSLP has been reported to exert much of its function by acting on DCs in the tissues and causing them to adopt a phenotype that was most effective in stimulating responding CD4 T cells to differentiate to Th2 cells (6, 7). The TSLP-treated DCs express OX-40 ligand and fail to make cytokines such as IL-12 that are

FIGURE 4. Induction of ZsG expression by IL-13, IL-4, and TNF-α. (A) Tile scans of back skin from ZsG BAC transgenic mice treated by two daily injections of PBS, IL-13, IL-4, or TNF-α and biopsy 1 d later. This experiment was repeated at least three times with similar results. (B) Multiphoton microscopy of ear skin from ZsG BAC transgenic mice treated by two daily injections of PBS or TNF-α and analysis 1 d later. This experiment was done twice with similar results.

FIGURE 5. Flow cytometric analysis of ZsG expression. Epidermal cells were isolated from the skin of B6 mice or ZsG BAC transgenic mice that were untreated, injected with PBS or with TNF-α. CD3-negative, CD11c-negative cells were analyzed for expression of ZsG. Percentage of ZsG+ cells and the product of percentage of ZsG+ cells and mean fluorescence intensity (MFI%+) are indicated. This experiment was performed twice with similar results.
associated with differentiation of responding CD4 T cells to a Th1 fate (8). The expression of OX-40L on DC aids their priming of normal Ag-specific naive CD4 T cells to Th2 cells. Furthermore, Liu and colleagues (6) have observed that CD4 T cells primed by OX-40L–expressing APC are more likely to produce TNF-α and less likely to secrete IL-10, suggesting that they may be regarded as inflammatory Th2 cells.

However, there are at least two other options for the function of TSLP in controlling allergic inflammation. Naive CD4 T cells express functional TSLP receptors and thus can transduce a STAT5 signal when stimulated with TSLP (5). In vitro Th2 differentiation is very dependent on STAT5 activation (10). TSLP should be capable of replacing IL-2 in such priming, and thus, production of TSLP in the vicinity of Ag-priming of naive CD4 T cells could provide the STAT5 signal needed for Th2 differentiation. However, such a scenario would require a reliable source of TSLP at or near the site of priming. Naive CD4 T cell priming occurs in the paracortex of the lymph node or in the comparable region of the spleen where little or no TSLP would be expected to be present except, possibly, as reported by Sokol et al. (15), as a result of the transient recruitment of basophils to the lymph node in papain immunization and the stimulation of these cells to produce TSLP. However, IL-3–cultured basophils stimulated with PMA and ionomycin expressed limited amounts of TSLP and ZsG mRNA, and we failed to detect ZsG expression by stimulated bone marrow–derived basophils.

An alternate possibility is that memory/effector Th2 cells would be attracted to tissues into which Ag has been introduced. If TSLP had been induced at such sites, such as might be the case in atopic dermatitis (38), then such TSLP could act on differentiated Th2 cells, possibly together with IL-33, to drive IL-13 and IL-5 production and to enhance cell proliferation (11).

An even more intriguing possibility is that Ag stimulation of Th2 cells in the tissues, particularly Th2 cells that produce TNF-α, as has been reported to be the case for CD4 cells induced by TSLP-treated DCs (6), would induce epithelial cells to produce TSLP. Indeed, we observed in this study that TNF-α, IL-4, and IL-13 induce keratinocytes from reporter mice to become ZsG+, and others have previously reported that these cytokines can cause TSLP production by keratinocytes (39–41). We also showed that Ag-mediated activation of Th2 or Th1 cells in the skin resulted in robust induction of ZsG in the keratinocytes, indicating that in situ Ag-driven CD4 T cell activation drives TSLP induction, presumably through production of TNF-α, IL-4, or IL-13 production. The mutual stimulatory properties of keratinocyte products (TSLP and IL-33) and Th2 (or possibly Th1) cell products (IL-13 in particular) could result in a chronic Ag-independent Th2-mediated allergic inflammatory response. A similar mechanism might hold for ILC2 cells. These cells have been reported to show enhanced IL-13 production when stimulated with IL-33 and TSLP, and the IL-13 they produce could contribute to a chronic allergic inflammatory response (12).
We were surprised by our failure to observe ZsG+ hematopoietic cells in skin or ear as well as in IL-3–driven bone marrow cell cultures and in GM-CSF–induced DCs. Indeed, Kashyap et al. (42) have recently reported that lung DCs from mice challenged with house dust mites expressed TSLP mRNA. The failure of IL-3–driven mast cells and basophils and of GM-CSF–induced DC to be ZsG+ on flow cytometry or confocal microscopy may reflect the presence of relatively small amounts of TSLP and ZsG in these cells compared with that in stimulated keratinocytes. However, in view of our failure to detect ZsG in stimulated basophils and/or mast cells, one may ask whether these cells are physiologically important sources of TSLP. These cells could have a different role in TSLP expression, such as through the release of proteases or proinflammatory cytokines that induce TSLP production by epithelial/stromal cells.

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Disclosures

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References


