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Thymic Stromal Lymphopoietin Is a Key Mediator of Breast Cancer Progression

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BM, bone marrow; CD44+, CD44+ lymphocytes; FOXO1, forkhead box protein O1; GALE, galactose-α-1,4-glucosyltransferase; hMSC, human mesenchymal stem cell; hTSLP, human thymic stromal lymphopoietin; IL, interleukin; LCM, lymphocytic choriomeningitis virus; MDSC, myeloid-derived suppressor cell; MSC, myeloid suppressor cell; OVA, ovalbumin; TSLP, thymic stromal lymphopoietin; TSLP receptor; TSLPR, thymic stromal lymphopoietin receptor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin.

Inflammation is a double-edged sword that can promote or suppress cancer progression. In this study, we report that thymic stromal lymphopoietin (TSLP), an IL-7–like type 1 inflammatory cytokine that is often associated with the induction of Th2-type allergic responses in the lungs, is also expressed in human and murine cancers. Our studies with murine cancer cells indicate that TSLP plays an essential role in cancer escape, as its inactivation in cancer cells alone was sufficient to almost completely abrogate cancer progression and lung metastasis. The cancer-promoting activity of TSLP primarily required signaling through the TSLP receptor on CD4+ T cells, promoting Th2-skewed immune responses and production of immunosuppressive factors such as IL-10 and IL-13. Expression of TSLP therefore may be a useful prognostic marker, and its targeting could have therapeutic potential. The Journal of Immunology, 2011, 186: 5656–5662.

Cancer progression and metastasis is a multifaceted process that involves inflammation and the active participation of immune cells, such as myeloid suppressive cells (MSCs) and regulatory T cells (Tregs) (1–3). For example, in the mouse mammary adenocarcinoma 4T1 cancer model, which represents a highly aggressive model of human breast carcinoma (4), cancer-produced factors promote the generation of MSCs to impair antitumor immune responses (5). In this model, Tregs play an essential role in lung metastasis, as in their absence, 4T1 cancer cells progressed at the primary site (mammary gland) but could not metastasize. The role of Tregs is to protect metastasizing 4T1 cells from NK cells using β-galactoside binding protein (3). Consistent with a recent report that mice with OVA-induced pulmonary allergy had an enhanced growth of i.v.-injected B16 melanoma in the lungs (6), metastasis in 4T1 tumor-bearing mice appears to require an allergic response in the lungs to mediate production of CCL17 and CCL22 and thereby recruit CCR4+ 4T1 cancer cells and Tregs (3).

Airway hypersensitivity and production of Th2-type chemokines are associated with the expression of thymic stromal lymphopoietin (TSLP) (7, 8), a type I cytokine that signals via a receptor consisting of TSLP receptor (TSLPR) and IL-7Rα to activate JAK1, JAK2, STAT5A, and STAT5B (9). It is produced by epithelial cells, keratinocytes, mast cells, and basophils in response to a range of stimuli (10) and affects mouse T cell expansion, survival, and differentiation (11–13). TSLP is a key factor for initiating allergic responses (11, 12), for example, to OVA in the lungs of mice (12, 14). It promotes Th2 responses acting directly on T cells (12), mast cells (15), and NK cells (16) or indirectly through the education of dendritic cells (DCs) (14, 17). Despite its importance in allergy, the role of TSLP in oncogenesis is not known. In this study, using two cancer models coupled to inhibiting TSLP expression in tumor cells and TSLPR-deficient (Tslpr−/−) mice, we report that TSLP also plays an essential role in tumor progression.

Materials and Methods

Cells and mice

Female BALB/c and C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME), and Tslpr−/− mice were described previously (13). The use of 4T1 cells and the generation of their nonmetastatic 4T1-PE clones were described elsewhere (3). B16F10, MCF-7, MDA-MB-231, OVCAR433, 2008, HOSEB, and BG1 cells were from American Type Culture Collection. UACC1237 and 938 melan were from Dr. Ashani Weeraratna (National Institute on Aging/National Institutes of Health, Bethesda, MD). CD4+ T cells were isolated from the mouse T cell CD4+ column kit and separated from CD25+ cells using the CD25 subset column kit and separated from CD25+ cells using the CD25 Microbead kit (Miltenyi Biotec, Auburn, CA). The generation of bone marrow (BM)-derived immature DCs was described elsewhere (18).

In vivo manipulations

Animal care was provided in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 86-23, 1985). Experiments were performed using 4–8-wk-old female mice in a pathogen-free environment. Syngeneic mice were s.c. challenged with 4T1 cancer cells or their subsets (1 × 107 cells, in the fourth mammary gland) or B16F10 melanoma cells (1 × 105) at day 0. To test the role of T cells and DCs, congenic 1P.B.O., Y.R., and M.B. are equal contributors to this work. Accepted for publication March 16, 2011.

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Cytokines shown in Table I were measured using Bio-Plex cytokine assay array by following the manufacturer’s instructions (Bio-Rad). TSLP, IL-5, and IL-13 (eBioscience, San Diego, CA), IL-10, and TARC/CCL17 (R&D Systems) in the blood (plasma) and bronchoalveolar lavage (BAL) fluid were measured using ELISA in s.c.-injected mammary glands of BALB/c mice treated with 20 μl anti-TSLP Ab (clone 152614; R&D Systems) or control IgG via the intranasal (i.n.) route (total of three times starting on days 2, 7, and 17).

Detection of cytokine expression

Cytokines shown in Table I were measured using Bio-Plex cytokine assay array by following the manufacturer’s instructions (Bio-Rad). TSLP, IL-5, and IL-13 (eBioscience, San Diego, CA), IL-10, and TARC/CCL17 (R&D Systems) in the blood (plasma) and bronchoalveolar lavage (BAL) fluid were measured using ELISA in s.c.-injected mammary glands of BALB/c mice with 0.2 ml serum-free tumor CM daily for 4 d, and cytokines were measured by ELISA 24 h after last treatment. A separate group of similarly treated mice were used to assess cytokine production by ELISA in CD4+ T cells [isolated as described elsewhere (3)] after 2 d of stimulation with anti-CD3/CD28 beads (Invitrogen). Immunohistochemistry staining was performed as described (3) using paraffined lung sections with mouse 4T1.2 cells or patients with breast cancer (Experimental Transplantation and Immunology Branch, National Cancer Institute, Bethesda, MD). Anti-mouse TSLP Ab (BAF555; R&D Systems), anti-human TSLP Ab (ab47943; Abcam, Cambridge, MA), biotinylated anti-rabbit IgG (BA1000, Fisher Scientific), and immunohistochemistry reagents were from Thermo Scientific (Fremont, CA).

Statistical analysis

Results are presented as the mean of triplicates ± SEMs of at least three experiments. Differences were tested using the Student t test, and a two-sided p value <0.05 was considered statistically significant.

Results

Mouse and human tumors express TSLP

We previously reported that lung metastasis of 4T1 adenocarcinoma growing in the mammary gland requires activation of the lungs, with production of TARC/CCL17 and MDC/CCL22 (3). To identify cancer-produced factors responsible for the activation, we compared cytokine expression profiles of metastatic 4T1 and 4T1.2 cells with their nonmetastatic clones 4T1-PE and 4T1-NM (3). The cells differentially expressed variety of cytokines and chemokines; in particular, metastatic cells secreted a number of chemokines, such as CCL5, CCL17, and CXCL1 (Table I) previously associated with metastasis of cancer cells (3, 19). Surprisingly, we also found that metastatic cells also produced TSLP (Table I). In particular, its expression was highest in 4T1.2 cells (Fig. 1A, Table I), a clone of 4T1 cells that was selected for its enhanced lung metastasis (Fig. 1B) (4). TSLP was also found expressed in various human cancer lines, such as breast cancer MCF-7 and MDA-MB-231 cells and melanoma 938 mel cells (Fig 1C), and in human metastatic breast cancer biopsy (Fig. 1D), indicating that this is not solely a mouse cancer cell-associated phenomenon.

Cancer-produced TSLP promotes tumor progression

Although the role of TSLP in cancers is not known, TSLP is mostly shown to be a key factor that initiates Th2-type responses (11, 12).
To clarify the abundant expression of TSLP by cancer cells, we hypothesized that TSLP is produced to mediate Th2 responses to promote cancer escape. To test this idea, we first tested the ability of short hairpin RNA (shRNA)-generated 4T1 cell clones expressing low (clone B7; $<150 \pm 15 \, \text{pg/ml}$) or moderate levels (clone C7; $>380 \pm 30 \, \text{pg/ml}$) of TSLP to progress and metastasize.

FIGURE 2. Unlike shRNA control K5 clone or C7 clone (high-level TSLP expressers) of 4T1 cells or K3 of 4T1.2 cells, a TSLP-low clone B7 of 4T1 cells (A, B) and A6 and B5 clones of 4T1.2 cells (C, D) poorly progressed (A, C) and metastasized (B, D) in BALB/c mice. Shown are the cross-sectional area ($\text{mm}^3$) $\pm$ SEM of s.c. injected tumors cells (A) or tumor weight/mouse body weight $\pm$ SEM at day 28 post-tumor challenge (C) and mean of lung metastatic foci (B, D) $\pm$ SEM of four BALB/c mice/group experiment. The data were reproduced at least three times. $^*p < 0.05$.

FIGURE 3. Tslpr$^{-/-}$ mice do not efficiently support cancer progression (A, 4T1.2 cells; C, B16 melanoma) and metastasis (B, 4T1.2 cells). Serum TSLP levels positively correlate with a cancer burden (D, $r^2 = 0.996$). Shown are data of individual mice plotted on the basis of low (close to background) and high levels of serum TSLP levels (D), the cross-sectional area ($\text{mm}^3$) $\pm$ SEM of tumors (A, C), and mean of lung metastatic foci (B) $\pm$ SEM of four Tslpr$^{-/-}$ mice and congenic BALB/c (A, B) and C57BL/6 (C, D) mice/group experiment. The data were reproduced at least three times. $^*p < 0.05$. 

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tastasize after s.c. implantation in the mammary gland of female BALB/C mice. Whereas control mice injected with irrelevant shRNA-transduced 4T1 cells (K5) or with clone C7 generated large tumors (Fig. 2A) and many lung metastases (Fig. 2B), a low TSLP producer clone, B7, grew poorly and had few metastases (Fig. 2A, 2B). To rule out potential cell cloning-associated problems, we have also generated additional shRNA-mediated TSLP nonexpresser clones in 4T1.2 cells (clones A6 and B5). Unlike the control shRNA-transduced K3 clone (Fig. 2C, 2D), the TSLP nonexpresser clones A6 and B5 failed to induce tumor growth (Fig. 2C) and metastasis (Fig. 2D). Because TSLP knockdown did not affect in vitro proliferation and viability of any of the clones used (data not shown), these data indicate that cancer cell-produced TSLP is required for cancer progression and metastasis.

To confirm this, we challenged congenic \( Tslpr^{-/-} \) mice with wild-type (WT) 4T1.2 cancer cells and found that cancer progression (Fig. 3A) and metastasis (Fig. 3B) were also significantly decreased in these mice as compared with WT mice. This observation was not restricted to breast cancer cells, as s.c.-injected B16 melanoma progressed significantly slower in congenic \( Tslpr^{-/-} \) mice than in WT C57BL/6 mice (Fig. 3C). Interestingly, serum levels of TSLP were also positively correlated with tumor growth in WT mice (Fig. 3D), further indicating the importance of TSLP in malignant cell growth. Taken together, our data indicate that TSLP promotes cancer progression and metastasis through the activation of the host’s responses, as cancers cannot progress when the host is deficient in TSLPR.

The importance of CD4+ T cells as targets of cancer-produced TSLP

TSLP is known to induce airway allergic inflammation by activating CD4+ T cells either directly (12) or indirectly through DCs (14, 17), suggesting that cancers may also use TSLP to promote cancer progression by targeting CD4+ T cells. To test this idea, non-Treg CD4+ T cells (CD4+CD25- T cells depleted of CD25+ Tregs) and BM-derived DCs isolated from WT BALB/c mice were adoptively transferred into congenic 4T1 tumor-bearing \( Tslpr^{-/-} \) mice. In concordance with the importance of Tregs in metastasis (3), the transfer of non-Treg WT CD4+ T cells did not reverse the inability of 4T1 cancer cells to metastasize in \( Tslpr^{-/-} \) mice (Fig. 4A). However, we detected significantly enhanced primary tumor growth in \( Tslpr^{-/-} \) mice transferred with non-Treg WT CD4+ T cells (Fig. 4B), suggesting the importance of WT CD4+ cells in cancer progression. Consistent with this, the depletion of CD4+ T cells in 4T1 tumor-bearing WT mice also reduced cancer progression (Fig. 4C). To our surprise, the transfer of the WT DCs did not increase and in fact markedly reduced the already poor ability of \( Tslpr^{-/-} \) mice to support tumor growth (Fig. 4D). As expected, there was no effect when the DCs were added to WT mice (Fig. 4D). Because the DC transfer results in \( Tslpr^{-/-} \) mice may also

**FIGURE 4.** Unlike lung metastases (A), the diminished ability of \( Tslpr^{-/-} \) mice to support tumor growth was reversed by adoptive transfer of T cells (B). Depletion of CD4+ T cells inhibits 4T1 tumor progression in WT BALB/c mice (C). Compared with control IgG, GK1.5 anti-CD4–treated mice had >90% CD4+ T cell depletion in the blood at day 10 post-tumor challenge. Unlike WT mice, \( Tslpr^{-/-} \) mice transferred with BM-derived DCs had reduced ability to support 4T1.2 cancer growth (D) and metastasis (F). Shown in E is 4T1.2 tumor progression in \( Tslpr^{-/-} \) mice that were adoptively cotransferred with CD4+ T cells (i.v.) and BM-derived DCs (s.c.) from \( Tslpr^{-/-} \) mice (KO) and congenic BALB/C mice (WT). The data were reproduced at least twice. *p < 0.05.
indicate the need of having both types of the cells, CD4+ T cells and DCs isolated from WT and Tslpr−/− mice were criss-cross mixed and cotransferred into 4T1 tumor-bearing Tslpr−/− mice. The enhanced cancer progression was only detected in the mice receiving WT CD4+ T cells regardless of DCs used (WT or Tslpr−/−, Fig. 4E). In contrast and as expected, the cotransfer of CD4+ T cells and DCs from Tslpr−/− mice did not have any effect (Fig. 4E), and the transfer of Tslpr−/− CD4+ T cells and WT DCs significantly reduced tumor burden in Tslpr−/− mice (Fig. 4E).

Thus, taken together and consistent with the importance of CD4+ T cells as a target of TSLP (11, 12), cancer-produced TSLP acts on CD4+ T cells to promote cancer progression. Moreover, supporting our recent report on the importance of Tregs in metastasis (3), transfer of WT DCs did not enhance but significantly abrogated lung metastasis in Tslpr−/− mice (Fig. 4F), whether CD4+ T cells were cotransferred (data not shown). In control tumor-bearing WT mice, DC transfer did not significantly affect metastasis (Fig. 4F). Taken together, these data indicate that TSLP helps to promote tumor progression acting on CD4+ T cells.

Cancer uses TSLP to promote Th2-type immune responses

In allergic responses, TSLP conditions the lung immune environment inducing Th2-type skewed CD4+ T cell responses (20). Interestingly, we have also detected significant expression of TSLP in the lungs (airway lining cells) of 4T1 tumor-bearing mice (Fig. 5A), suggesting that cancer also induces TSLP production at distant metastasis sites, presumably enhancing Th2 responses in the lungs to regulate CD4+ T cells. To confirm this, we have s.c. injected the mammary gland of naive BALB/C mice with CM from metastatic TSLP-expresser 4T1 cancer cells (CM-4T1) and TSLP nonexpresser cells (CM-4T1PE). As a result, although TSLP was present in BAL of untreated mice (Fig. 5B), it was significantly enhanced in BAL and blood (plasma) of mice only treated with CM-4T1 cells, but not CM-4T1PE cells (Fig. 5B), supporting the possibility that cancer cells produce TSLP to favor a Th2-type response in lung cells and facilitate lung metastasis (20). In support of this hypothesis, the lungs of CM-4T1-treated mice also expressed significantly enhanced Th2-type cytokines, including IL-5, IL-13 (Fig. 5B), and IL-10 (Fig. 5C), as compared with the lungs of mice injected with TSLP nonexpresser CM-4T1PE cells. Importantly, i.n. delivery of a neutralizing Abs to TSLP, but not control Ab, abrogated the enhanced levels of both TSLP (Fig. 5D) and IL-10 (Fig. 5C) in the mice treated with CM of TSLP-expresser cells. Moreover, TSLP-expresser CM from 4T1.2 cells also enhanced CCL17 expression in the lungs (Fig. 6A), which can promote the recruitment of Tregs to protect metastasizing cells from NK cells in the lungs (3). In support of this, the lungs of mice s.c. injected with TSLP-expressing CM, but not with TSLP-expresser CM from A6 cells, had elevated amounts of Foxp3+ Tregs (Fig. 6B).

To explain the cancer-promoting role of CD4+ T cells, we hypothesized that cancer used TSLP to induce their Th2 differentiation. Indeed, CD4+ T cells isolated from spleens of BALB/C mice s.c. injected with CM from TSLP-producing 4T1.2 cells secreted significantly higher levels of IL-5, IL-10, and IL-13 (Fig. 6C) than T cells from mice treated with CM from TSLP nonproducer cells (either from nonmetastatic cells [data not shown and Fig. 5B] or from 4T1.2 cells with shRNA-mediated TSLP knockdown, Fig. 6C). Conversely, CD4+ T cells from TSLP nonexpresser CM-treated mice expressed much more IFN-γ (Fig. 6C). Considering an immunoregulatory and cancer escape-promoting role of Th2 cytokines like IL-10 and IL-13 (21–23), our

FIGURE 5. A, Primary 4T1.2 tumor induces TSLP expression in lung epithelium of BALB/c mice. Shown is a representative lung section stained with anti-TSLP and control Ab of tumor-bearing and naive mice. Original magnification ×40. Expression of IL-5, IL-13, and TSLP in BAL and blood (B) of naive BALB/c mice s.c. injected with CM-4T1 or CM-4T1PE after 24 h. Expression of IL-10 (C) and TSLP (D) are enhanced in BAL fluid of mice injected with CM-4T1, which was reversed back by i.n. neutralization of TSLP using anti-TSLP Ab, but not control IgG. The data were reproduced at least three times. *p < 0.05.
data indicate that cancer controls immune surveillance by producing and using TSLP to induce Th2-type differentiation of CD4\(^+\) T cells.

**Discussion**

We recently reported that although tumor progression was associated with lung metastasis of 4T1 cancer cells (Figs. 2, 3), they can be dissociated, as the absence of Tregs only abrogated lung metastasis without affecting the growth of a primary tumor in the mammary gland (3). The role of Tregs was to facilitate lung metastasis by protecting metastasizing cells from NK activity (3). In this study, we report that tumor progression is promoted by non-Treg Th2-differentiated CD4\(^+\) T cells in response to TSLP and that TSLP is abundantly expressed in various cancers, including human carcinomas such as breast cancer and melanoma.

Our studies in mice indicate that cancer-produced TSLP can promote cancer escape by inducing Th2 differentiation of CD4\(^+\) T cells and Th2-type skewed responses, as in allergic inflammation (7). However, our data indicate that cancer-produced TSLP may also be responsible for the lung metastasis by inducing production of CCL17 in the lungs and thereby recruiting Tregs, a key requirement for a successful lung metastasis of 4T1 cancer cells (3). Besides Tregs (24, 25), CCL17 can also recruit other immune cells with potentially regulatory activities, such as Th2-type CD4\(^+\) T cells, NK cells, invariant NKT cells, and B cells (21, 26–28). Moreover, we also detected significant infiltration of Gr1\(^+\) CD11b\(^+\) MSCs in the lungs of mice injected with CM of TSLP-expresser, but not nonexpresser, 4T1 cells, which appeared not to require CCL17 (data not shown), suggesting the induction of other chemokines. Despite the fact that cancer progression can be promoted by all of these cells, and specifically by TSLP-activated Tregs (29, 30) or DCs (14, 17), our data indicate that non-Treg subsets of CD4\(^+\) T cells are targets of TSLP, whereas DCs did not appear to be critical in this process. Our mechanistic studies indicate that, as in TSLP-mediated allergic responses (7, 8, 20), cancer-produced TSLP induced Th2 differentiation of CD4\(^+\) T cells. As a result, we detected significant production of IL-10 and IL-13, cytokines that promote cancer escape by activating NKT cells (21) and MSCs (22, 23), inducing Th2-type immune responses that suppress antitumor Th1 responses and CD8\(^+\) CTLs (31), and activating induced Tregs or generating suppressive Treg type 1 cells (32, 33). In this process, cancer appears to also use TSLP to initiate a chain of suppressive events by inducing the production of chemokines (that recruit suppressive and other immune cells) as well as the immunomodulatory cytokines IL-10 and IL-13 (31). Taken together, our data indicate that TSLP may serve as a cancer prognostic marker, which may also explain the increased lung metastases reported in asthmatic patients with breast cancer (3, 6). We propose that targeting TSLP may be a way to control cancers, as the inactivation of TSLP in tumors alone or disabling its signaling (as shown by the use of Tslpr\(^{-/-}\) mice) was sufficient to diminish both cancer progression and metastasis.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

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