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Thymic Stromal Lymphopoietin: A Cytokine That Promotes the Development of IgM⁺ B Cells In Vitro and Signals Via a Novel Mechanism

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A novel cytokine from a thymic stromal cell line (thymic stromal lymphopoietin (TSLP)) promotes the development of B220⁺/IgM⁺ immature B cells when added to fetal liver cultures, long term bone marrow cultures, or bone marrow cells plated in semisolid medium. Because the activities of TSLP overlap with those of IL-7 in some in vitro assays, we compared the signaling mechanisms employed by TSLP and IL-7. Proliferation of a factor-dependent pre-B cell line (NAG8/7) in response to either TSLP or IL-7 was inhibited by anti-IL-7Rα mAbs, suggesting that the functional TSLP receptor complex uses IL-7Rα. In contrast, three different Abs to the common cytokine receptor γ-chain had no effect on the response of these cells to TSLP, indicating that the functional TSLP receptor complex does not use the common cytokine receptor γ-chain. Both cytokines induced activation of Stat5, but only IL-7 induced activation of the Janus family kinases Jak1 and Jak3. In fact, TSLP failed to activate any of the four known Janus family kinases, suggesting that Stat5 phosphorylation is mediated by a novel mechanism. Taken together, these data support the idea that TSLP can make unique contributions to B lymphopoiesis and indicate that it does so by mechanisms distinct from IL-7.


Cytokines play pivotal roles in the regulation of immune responses and can influence lymphocyte development. In particular, IL-7 contributes significantly to the development and expansion of both B and T cells in a number of experimental systems. IL-7 stimulates the development and expansion of pre-B lymphocytes in in vitro culture systems (1–3), and mice deficient in IL-7 fail to support normal pre-B lymphocyte maturation (4). Neutralizing Abs to the IL-7Rα-chain (IL-7Rα) also interfere with B cell development both in vitro and in vivo (5). Importantly, disruption of the IL-7Rα gene (6) or treatment of mice with anti-IL-7Rα Ab (5) arrests B cell development before the pro-B cell stage. Hence, interference with IL-7Rα function has more severe consequences than interference with IL-7 function. These results have been interpreted to suggest the existence of another cytokine that uses the IL-7Rα as part of its receptor complex (6). Sharing of cytokine receptor subunits by different cytokines has been well documented, including coutilization of the IL-2Rβ and γ subunits by IL-2 and IL-15 (7) and of the IL-4R by IL-4 and IL-13 (reviewed in Ref. 8), and the sharing of the IL-2Rγ-chain (also known as the common γ-chain (γc)) by IL-4, IL-9, and IL-7 in addition to IL-2 and IL-15 (reviewed in Ref. 9). Cytokine signaling mechanisms have been investigated extensively in recent years, and perhaps the best studied is that of the IL-2/IL-2R complex. Here, ligand binding induces the tyrosine phosphorylation of the receptor (reviewed in Refs. 10 and 11) and the activation and association of the Stat family of transcription factors (specifically Stat5 and Stat3 for IL-2 signaling), which dimerize and migrate to the nucleus where they influence transcription of numerous genes (15). The IL-7R complex includes the IL-7Rα-chain and γc. Activation of these kinases leads to tyrosine phosphorylation and activation of the STAT family of transcription factors (specifically Stat5 and Stat3 for IL-2 signaling), which dimerize and migrate to the nucleus where they influence transcription of numerous genes (15).

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4 Abbreviations used in this paper: γc, common γ-chain; TSLP, thymic stromal lymphopoietin; LTBMC, long term bone marrow cultures; CM, conditioned medium; TSLP-R, thymic stromal lymphopoietin receptor; RAG, recombinase-activating gene.
therefore sought to define the mechanisms by which IL-7 and TSLP exert their distinctive effects by examining the signaling responses that each cytokine evokes.

We have undertaken a series of experiments to characterize the biochemical signals induced by TSLP and to distinguish them from responses to IL-7. Our studies demonstrate that both cytokine receptors employ the IL-7Rα-chain as part of their signaling complexes. Nonetheless, whereas TSLP upon binding to its receptor complex activates both Stat5a and Stat5b, this activation event does not involve any of the known Janus family kinases. Taken together, these results suggest that although TSLP-generated signals require a receptor unit also used by IL-7, the biochemical changes induced in responding cells are dramatically different. Aside from providing insight into the physiological differences that attend IL-7 as opposed to TSLP treatment, our results demonstrates that there exist alternative mechanisms for STAT activation in hemopoietic cells.

Materials and Methods

Reagents

Medium (RPMI 1640; Life Technologies, Gaithersburg, MD) was supplemented with l-glutamine, nonessential amino acids, 2-ME, and FBS (10% for long term bone marrow cultures (LTBMC) and 15% for colony assays). Agar was purchased from Difco (Detroit, MI), and methylcellulose (1500 centipasto) was purchased from Sigma (St. Louis, MO). Recombinant TSLP was provided by Dr. D. Williams, Immunex (Seattle, WA), and recombinant murine IL-3, IL-10, and IL-7 were purchased from Genzyme (Cambridge, MA). Cytokines and media were tested for the presence of endotoxin with the Limulus lysate assay and were found to be negative. Anti-IL-7Rα Ab A7R34 has been previously described (5) and was provided by Dr. S. Nishikawa. The anti-γ, Ab TGVm2 has also been described (22) and was purchased from PharMingen (San Diego, CA). Other anti-γ, Abs (4G3 and 3E12) were characterized by He and co-workers (23, 24) and were obtained from Dr. T. Malek (University of Miami). Anti-Stat5a, Stat5b, Jak1, Jak2, Jak3, and Tyk2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated 4G10 anti-phosphotyrosine Ab was from Upstate Biotechnology (Santa Cruz, CA). Biotinylated 4G10 anti-phosphotyrosine Ab and rabbit anti-Jak3 antisera were obtained from Upstate Biotechnology (Lake Placid, NY). Splelevin and anti-rabbit Ig conjugated to hors eradish peroxidase were purchased from Amersham (Arlington Heights, IL). Abs for flow cytometry were obtained from Caltag (Burlingame, CA; anti-B220-phycoerythrin), Southern Biotechnology (Birmingham, AL; anti-IgM-FTTC and anti-IgG2c-FTTC), or Becton Dickinson (San Jose, CA; anti-IgG-phycoerythrin).

Colony assays

Cleaned femurs and tibiae were ground in a mortar and pestle to recover bone marrow cells. After floating on a cushion of 50% (v/v) FBS/complete medium to remove bone chips and debris, the cells were added to agar (0.3%) or methylcellulose (1.8%) and medium containing cytokines at concentrations previously determined to be maximal (2.5 ng/ml IL-7 or 5 ng/ml TSLP). Replicate 1 ml cultures (106 cells/culture) were harvested after 1 wk in culture (37°C, 5% CO2). Agar disks containing colonies were transferred to glass slides, covered with strips of filter paper, and dried overnight at room temperature. The filter paper was then flooded with 10% neutral buffered formalin, the paper strips were removed, and after washing in PBS, the slides were stained with hematoxylin and eosin. Colonies were counted microscopically. Replicate methylcellulose colonies were diluted with medium, transferred to test tubes, and washed repeatedly by centrifugation to remove the methylcellulose. Washed cells were then processed for flow cytometry.

Long term bone marrow cultures

These cultures were prepared as originally described (25). Cytokines were added at the initiation of the cultures. Half the medium in the cultures was replaced at weekly intervals with fresh medium containing the appropriate cytokine. Cultures were harvested 5 wk after initiation.

Fetal liver cultures

Day 14/15 fetal livers were harvested aseptically and mechanically dissociated by pressing them between the frosted ends of sterile glass microscope slides. After repeated washing, cells were plated in methylcellulose at 1.5 × 106 cells/ml (1 ml cultures) as described above for colony assays. Cytokines at the same concentrations as those used in the colony assays were added at the start of the cultures. Cultures were harvested as described for bone marrow colony assays 8 days after initiation.

Proliferation assays

Proliferation assays using NAG8/7, HT-2, and BA/F3 cells were essentially as previously described (21). Specifically, assays were initiated by washing cells four times in medium and plating at 5 × 104 cells/well in 96-well flat-bottom plates with the indicated quantities of cytokine and Ab. Replicate cultures were set up in at least three wells for all experiments. Cells were cultured for 2 days, then pulsed with 1 μCi/well [3H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA), and cultured overnight. Plates were harvested using a Tomtec cell harvester (Tomtec, Orange, CT) and counted using a Wallac beta plate scintillation counter (Wallac, Gaithersburg, MD). Data are expressed as the mean counts per minute incorporated in replicate cultures. Primary cell proliferation assays were set up and run as described above, except that cells were plated at a density of 5 × 103 cells/well.

Activation of Stat5 and JAK kinases

NAG8/7 cells were washed four times in medium, cytokine starved for 4–5 h, and then stimulated at 105 cells/ml with either 25 ng/ml IL-7 or 100 ng/ml TSLP. After the indicated times, cells were harvested by centrifugation, washed once in cold PBS, and lysed in 25 mM Tris (pH 8.0)/150 mM NaCl/1% Triton X-100/1 mM Na2VO4 plus protease inhibitors TNT buffer at 106 cells/ml as previously described (26). Insoluble material was pelleted in a microfuge at 4°C, and supernatants were transferred to fresh microfuge tubes. Stimulated cell lysates were prepared in the same way for BA/F3 cells, except that cells were stimulated with 25 ng/ml murine IL-3. Total mouse spleenocyte preparations were stimulated with 25 ng/ml murine IL-10 as described above.

Lysates from 1–2 × 107 cells (2 × 105 cells were used for primary mouse spleenocyte lysates) were then incubated overnight at 4°C with 0.5 μg of the indicated Ab plus 25 μl of a 30% slurry of protein G-Sepharose (Pharmacia, Piscataway, NJ). Pellets were washed twice in 1 ml of TNT buffer and resuspended in SDS loading buffer. Samples were split, subjected to SDS-PAGE, and electroblotted to nitrocellulose membranes (Bio-Rad, Hercules, CA). Blots were then blocked in 5% BSA in Tris-buffered saline (TBS) plus 0.1% tween-20 (TBST) for anti-phosphotyrosine blots or in 5% milk in TBST for other Abs. For phosphotyrosine blots, biotinylated 4G10 was used at a 1/5000 dilution in 1% BSA/TBST and streptavidin conjugated to horseradish peroxidase at 1/5000 in the same buffer. Abs to other proteins were also used at 1/5000 dilutions in 1% milk/TBST with either anti-rabbit Ig (1/3000) or anti-goat Ig (1/2000) conjugated to hors eradish peroxidase. After extensive washing in TBST, blots were developed using chemiluminescent detection (New England Nuclear, Boston, MA).

Results

Effect of TSLP in fetal liver cultures

TSLP was initially identified as an activity in conditioned medium (CM) from a thymic stromal cell line (Z210R.1) that supported the development of IgM+ B cells in long term fetal liver cultures (21). This activity was distinct from IL-7 by two criteria: 1) IL-7 did not promote the development of IgM+ B cells under those conditions; and 2) the NAG8/7 pre-B cell line derived from fetal liver lymphoid precursors proliferated in response to Z210R.1 CM even in the presence of neutralizing Abs to IL-7 (21). Since the NAG8/7 cell line that responded to TSLP is a pre-B cell line and because the original characterization of TSLP activity had used fetal liver as a source of lymphocyte precursors in long term cultures that produced B-lineage lymphocytes (21), B cell development was chosen as a model system to compare the biological effects of TSLP and IL-7.

Methylcellulose cultures with fetal liver lymphocyte precursors were established including either purified IL-7 or TSLP, and the resultant cells were recovered and analyzed by flow cytometry. While IL-7 induced the development of B220+/IgM- pre-B lymphocytes, TSLP addition promoted the development of substantial numbers of B220+/IgM+ cells that also expressed κ light chains on their surfaces (Fig. 1). This result confirms our previous observation that CM from Z210R.1 cells promoted the development of B220+/IgM+ cells in an IL-7-independent manner (21) and supports the conclusion that TSLP was responsible for the production of these cells. Moreover, Fig. 1 indicates that while both IL-7 and
TSLP can promote B lymphopoiesis in fetal liver cultures, the phenotypes of the cells that develop are dramatically different. Our results differ from those of Ray et al. (27), who reported that in 4-day cultures, cells that developed in IL-7 and TSLP were indistinguishable. The reason for the discrepancy in these results is not known, but may relate to the additional period in culture we have employed (8 days) or the enrichment for “bipotential precursors” employed by Ray and co-workers (27).

Colony assays in IL-7 vs TSLP

Although the liver is the primary site of fetal B lymphopoiesis, postnatal B cell development takes place primarily in the bone marrow. To investigate the differentiative capacity of bone marrow cells in the presence of TSLP, unfractionated bone marrow cells were cultured in methylcellulose medium without cytokines or in medium supplemented with either TSLP or, as a positive control, IL-7. Cultures without cytokine failed to produce lymphoid colonies (data not shown), whereas addition of IL-7 supported the development of numerous lymphoid colonies (Fig. 2A). Phenotypic analysis of the cells in these IL-7-induced colonies showed them to be primarily B220⁺/IgM⁺ pre-B cells (Fig. 2C) with forward light scatter profiles typical of cycling cells (Fig. 2B). In contrast, colony growth in response to TSLP was about half that observed in IL-7 (Fig. 2A) with a generally smaller colony size (data not shown). Analysis of the composition of these colonies revealed a strikingly high percentage of IgM⁺ cells (Fig. 2C) that were predominantly IgD⁻ (data not shown) and displayed reduced forward scatter profiles, suggesting they were primarily immature B cells that were no longer cycling (Fig. 2B). Consistent with this interpretation, we found that these cells failed to respond to restimulation with either IL-7 or TSLP, whereas cells from IL-7 colonies proliferated in response to IL-7 in secondary cultures (data not shown). Hence, IL-7 and TSLP appear to influence the development of B lymphoid cells from adult bone marrow in a manner that parallels the way they affected B lymphopoiesis in fetal liver cultures.

Effect of TSLP on LTBM C

We also examined the effects of exogenous TSLP in LTBM C and compared these results to those obtained with addition of IL-7. In the absence of added cytokine these LTBM C produced primarily B220⁺/IgM⁺ pre-B cells (Fig. 3). Consistent with previous reports (1–3), bone marrow cultures supplemented with exogenous IL-7 produced large numbers of B220⁺/IgM⁺ IL-7-dependent cells (Fig. 3). These cells showed increased forward light scatter profiles, suggesting that they were a population of rapidly cycling pre-B lymphoblasts (data not shown). Addition of exogenous TSLP to bone marrow cultures yielded IgM⁺ B cells that phenotypically resembled immature B lymphocytes (Fig. 3). Many of these cells also expressed κ light chain, but the cells were produced in fewer numbers than was observed with added IL-7 (Fig. 3) and showed reduced forward light scatter profiles (data not shown). While the total number of cells produced in cultures supplemented with TSLP was considerably lower than that in cultures supplemented with IL-7, a determination of the absolute number of cells revealed that this was primarily at the expense of the B220⁺/IgM⁺ population (Fig. 3). Moreover, the increased percentage of IgM⁺ cells in these cultures actually reflected a minimal increase in the absolute number of IgM⁺ cells produced. These data correlate well with information obtained from colony forming assays (Fig. 2) and give the same phenotypic readout as that of fetal liver cultures. However, the production of IgM⁺ cells in fetal liver cultures can all be attributed to de novo development, since there were no IgM⁺ cells at their initiation (data not shown). Thus, it appears that addition of IL-7 and TSLP to three different culture systems designed to examine B lymphopoiesis produced the same effects: IL-7 favored the development of large numbers of B220⁺/IgM⁺ cells.

FIGURE 1. Purified TSLP induces the development of IgM⁺ B cells from fetal liver lymphocyte precursors. Lymphoid cells were isolated from fetal liver and cultured for 8 days in methylcellulose in the presence of IL-7 or TSLP as described in Materials and Methods. Single parameter histograms show surface expression of the indicated markers on cells isolated from cultures containing TSLP (solid lines) or IL-7 (dashed lines).

FIGURE 2. TSLP promotes the development of IgM⁺ B cell colonies from bone marrow precursors in methylcellulose. A, Lymphoid colonies obtained in methylcellulose cultures with added IL-7 or TSLP were counted and are presented as the mean colony number per 10⁵ input lymphoid cells ± SD. B, The forward scatter profiles for cells contained in methylcellulose colonies grown in TSLP (solid line) or IL-7 (dashed line) demonstrate that cells grown in IL-7 are larger blasts. C, Phenotypic analysis of cells from methylcellulose colonies grown in IL-7 (left panel) or TSLP (right panel) shows that there are substantially more B220⁺/IgM⁺ cells after culture in TSLP than IL-7.
obtained from cultures.

The absolute numbers of cells of each phenotype was calculated from the percentage of cells in each region multiplied by the total number of cells

were analyzed by flow cytometry for expression of surface IgM and B220

were proliferated in response to both cytokines, but that murine IL-7 is a somewhat more potent

FIGURE 4. Proliferative responses of NAG8/7 cells. A, NAG8/7 cells proliferate in response to both IL-7 and TSLP. Proliferation assays on NAG8/7 cells were set up with varying concentrations of IL-7 or TSLP, and responses were measured by [3H]thymidine incorporation. B and C, Abs to the IL-7R block proliferative responses to both IL-7 and TSLP in NAG8/7 cells, but Abs to γc only inhibit IL-7-mediated proliferation. Shown here are results from one set of proliferation assays measuring [3H]thymidine incorporation in NAG8/7 cells in the presence of 0.5 ng/ml murine IL-7 (B) or 5 ng/ml TSLP (C; both shown to induce maximal proliferation of NAG8/7 cells) with varying concentrations of normal rat Ig, anti-IL-7 receptor Ab, or the anti-γc Ab TUGm2. Similar results were obtained with the anti-γc Abs 4G3 and 3E12 (23, 24), in that neither inhibited TSLP responses although 3E12 also did not affect IL-7 responses. These data are representative of at least five separate experiments. Assays were conducted as described in Materials and Methods.

As a further test of this, we also performed proliferation assays in the presence of Abs to IL-7R. Administration of this Ab to mice in vivo or inclusion in LTBMC inhibits IL-7-dependent responses (5), and hence, we reasoned that this would be an effective way to specifically affect the IL-7 response by this cell line. As expected, we found that high concentrations of Ab inhibited IL-7-mediated proliferation of NAG8/7 cells (Fig. 4B). Lower concentrations of anti-IL-7Rα caused a significant augmentation of proliferation that may reflect enhanced receptor aggregation. In any case, this effect was dependent on IL-7, since the Ab alone did not induce proliferation at any concentration tested (data not shown). Importantly, we also saw dramatic reductions in TSLP-induced proliferation by treatment with anti-IL-7Rα Ab (Fig. 4C). This Ab did not affect the proliferation of the IL-2-dependent cell line, HT-2, or of the IL-3-dependent cell line, BA/F3, indicating that this was not a general toxic effect of the Ab (data not shown). Taken together these results suggest that the TSLP receptor (TSLP-R) requires the IL-7Rα-chain as part of its functional signaling complex.

Proliferation of pre-B cells, while TSLP produced relatively fewer B220+/IgM+ immature B cells. Taken together these data support the idea that TSLP uniquely supports the development of immature B cells.

Proliferation of NAG8/7 cells in response to TSLP Requires the IL-7R α-chain, but not the γc.

Because of the observed differences in biologic activity of IL-7 and TSLP, we initiated studies to examine signaling pathways activated by the two cytokines. As previously noted, NAG8/7 is a factor-dependent cell line that proliferates in response to both IL-7 and TSLP (21). Fig. 4A demonstrates that NAG8/7 cells proliferate in response to both cytokines, but that murine IL-7 is a somewhat more potent mitogen. Earlier indications that NAG8/7 cells responded weakly to IL-7 reflected the human IL-7 used in those assays (21). To distinguish TSLP from IL-7, we performed proliferation assays in the presence of neutralizing Abs to IL-7 and found proliferation induced by TSLP to be unaffected (data not shown) (21). As a further test of this, we also performed proliferation assays in the presence of Abs to IL-7Rα. Administration of this Ab to mice in vivo or inclusion in LTBMC inhibits IL-7-dependent responses (5), and hence, we reasoned that this would be an effective way to specifically affect the IL-7 response by this cell line. As expected, we found that high concentrations of Ab inhibited IL-7-mediated proliferation of NAG8/7 cells (Fig. 4B). Lower concentrations of anti-IL-7Rα caused a significant augmentation of proliferation that may reflect enhanced receptor aggregation. In any case, this effect was dependent on IL-7, since the Ab alone did not induce proliferation at any concentration tested (data not shown). Importantly, we also saw dramatic reductions in TSLP-induced proliferation by treatment with anti-IL-7Rα Ab (Fig. 4C). This Ab did not affect the proliferation of the IL-2-dependent cell line, HT-2, or of the IL-3-dependent cell line, BA/F3, indicating that this was not a general toxic effect of the Ab (data not shown). Taken together these results suggest that the TSLP receptor (TSLP-R) requires the IL-7Rα-chain as part of its functional signaling complex.

To delineate the nature of the TSLP-R more precisely, we also performed these proliferation assays in the presence of Abs to γc, which is essential for IL-7 responses. We tested three different mAbs against γc (TUGm2, 4G3, and 3E12) (22–24). In cultures including TUGm2 and 4G3, we consistently observed inhibition of IL-7-mediated proliferation at high Ab concentrations and in most cases observed an augmentation of the IL-7 response at lower Ab levels (Fig. 4B and data not shown). Consistent with previous reports, the 3E12 Ab did not affect IL-7-mediated proliferation. In contrast, the response of NAG8/7 cells to TSLP was unaffected by any of the three mAbs to γc (Fig. 4C and data not shown). From these results, we concluded that the TSLP-R complex includes the IL-7R α-chain, but that it does not require γc, in contrast to the IL-7R complex, which requires the IL-7R α-chain and γc. These results suggest a relationship between IL-7 and TSLP similar to that observed for IL-4 and IL-13, where IL-4 responses are mediated by the IL-4R and γc, whereas IL-13 responses depend on a unique receptor component (the IL-13R) and the IL-4R (reviewed in Ref. 8).
TSLP triggers tyrosine phosphorylation of Stat5

Because our results showed that both IL-7 and TSLP use the IL-7R α-chain, we undertook a series of experiments designed to compare the biochemical changes induced by treatment of cells with IL-7 and TSLP. We reasoned that some features of the signal transduction mechanism would be shared by virtue of a common receptor component, but that others may be different because the functional receptor complexes are distinct. Consistent with previous reports in other cell systems (20), we readily observed IL-7-induced tyrosine phosphorylation of Stat5 (Stat5a and Stat5b) in NAG8/7 cells (Fig. 5A).

We also observed Stat5 activation after treatment of these cells with TSLP (Fig. 5A), supporting the hypothesis that at least certain aspects of the signaling pathways used by these two cytokines overlap. The level of tyrosine-phosphorylated Stat5 from TSLP-treated cells was slightly lower than that in IL-7-treated cells, and the kinetics of accumulation were somewhat slower (Fig. 5A and data not shown). This suggested that tyrosine phosphorylation of Stat5 was induced less efficiently by TSLP than by IL-7, perhaps reflecting different levels of receptor expression or participation of alternative signaling pathways (see below). The antiserum used for these experiments has been reported to recognize both Stat5 isoforms (Stat5a and Stat5b, which are the protein products of closely related, but distinct, genes), although it was raised against a Stat5b peptide (see Materials and Methods). While these two isoforms have slightly different mobilities on SDS-PAGE (Stat5a migrates at about 94 kDa and Stat5b at about 92 kDa), we could not clearly distinguish this difference on the 10% gels employed for this analysis (Fig. 5A). Hence, it was formally possible that only one isoform was being activated, and the other was not, by IL-7 and/or TSLP. To examine this possibility we used an antiserum that specifically recognized Stat5a only and analyzed the immune complexes with 8% acrylamide gels (Fig. 5B). Cell extracts that had been immunodepleted of Stat5a were then subsequently immunoprecipitated with the antiserum that recognized both isoforms (see below). The antiserum used for these experiments has been reported to recognize both STAT isoforms (Stat5a and Stat5b, which are the protein products of closely related, but distinct, genes), although it was raised against a Stat5b peptide (see Materials and Methods). While these two isoforms have slightly different mobilities on SDS-PAGE (Stat5a migrates at about 94 kDa and Stat5b at about 92 kDa), we could not clearly distinguish this difference on the 10% gels employed for this analysis (Fig. 5A). Hence, it was formally possible that only one isoform was being activated, and the other was not, by IL-7 and/or TSLP. To examine this possibility we used an antiserum that specifically recognized Stat5a only and analyzed the immune complexes with 8% acrylamide gels (Fig. 5B). Cell extracts that had been immunodepleted of Stat5a were then subsequently immunoprecipitated with the antiserum that recognized both isoforms. In this way we were able to assess the phosphorylation state of each Stat5 isoform. Moreover, the use of 8% polyacrylamide gels allowed a distinction to be made between the two based on their migrations. We found that TSLP and IL-7 activated both the Stat5 isoforms to a similar extent (Fig. 5B) and hence differential activation of these transcription factors is unlikely to account for the different biological responses observed in IL-7-treated vs TSLP-treated bone marrow cultures. We also noted a mobility shift in Stat5b in extracts from cells treated with IL-7 that was less prevalent in extracts from TSLP-treated cells (Fig. 5B and data not shown). The biological importance of this observation is not yet known.

Janus family kinases are not activated by TSLP

The tyrosine phosphorylation of STAT transcription factors in general has been shown to be conducted by tyrosine kinases of the Janus family (JAK kinases). In particular, IL-7-induced Stat5 activation is correlated with activation of Jak1 and Jak3 (10, 19, 20). Treatment of NAG8/7 cells with IL-7 that was less prevalent in extracts from TSLP-treated cells (Fig. 5B) and data not shown). The biological importance of this observation is not yet known.

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activation of Jak2 or Tyk2 was not observed in NAG8/7 cells following TSLP stimulation, indicating that other members of this kinase family did not respond to TSLP binding to its receptor (Fig. 6C). Since we readily detected activation of these latter two kinases in other cells using cytokines known to activate them (specifically, Jak2 activation after IL-3 treatment of BA/F3 cells and Tyk2 activation after IL-10 treatment of murine splenocytes; Fig. 6C), our inability to detect activation did not reflect a failure of the Abs to immunoprecipitate the kinases. These results taken together suggest that Stat5 tyrosine phosphorylation in response to TSLP is mediated by either an unidentified JAK kinase or another mechanism altogether. Our results also demonstrate a biochemical distinction between IL-7 and TSLP signaling that may in part underlie the different responses induced by these cytokines.

**Discussion**

In the course of characterizing the novel cytokine TSLP, we have adduced evidence supporting the view that the TSLP-R complex employs the IL-7Rα-chain, but not γc. This partial receptor sharing was suggested by Pescon and co-workers (6) as the basis for the more severe lymphopoietic abnormalities observed in mice rendered genetically deficient in the IL-7Rα-chain relative to IL-7-deficient mice. Hence, we predict that the functional TSLP-R complex will include at a minimum the IL-7Rα-chain and a unique TSLP-binding receptor component. In fact, such a receptor component has recently been cloned and found to be a member of the hemopoietin receptor family (D. Williams, unpublished observation). This suggests a relationship between IL-7 and TSLP that is very similar to that seen for IL-4 and IL-13, where the functional IL-4R includes the IL-4Rα-chain and γc, while the IL-13R includes the IL-4Rα-chain and a unique IL-13R chain (8, 28). IL-4 and IL-13 show a degree of overlap in their biochemical and biological effects, but also exhibit distinct activities. We found this to be the case for IL-7 and TSLP as well.

Perhaps the most remarkable observation we made was that although tyrosine phosphorylation of Stat5a and Stat5b could be induced by treatment of NAG8/7 cells with both IL-7 and TSLP (Fig. 5), we could only detect Jak1 and Jak3 activation in IL-7-treated cells (Fig. 6). Moreover, we failed to detect activation of any of the four known Janus family kinases in response to TSLP even though we could detect their activation in other cell types under the appropriate stimulation conditions (Fig. 6). This suggests that an as yet unidentified member of the Janus family may be involved in the response to TSLP or that TSLP stimulation leads to Stat5 phosphorylation in a JAK kinase-independent manner. In fact, recent reports suggest that other PTKs can activate STAT transcription factors independent of Janus family kinases (29, 30). While we cannot formally exclude the possibility that Jak1 or any of the other JAK kinases is activated by TSLP, but with a diminished efficiency, we believe that this is an unlikely explanation. The efficiency of Stat5 phosphorylation is reduced by only two- to threefold at most, and yet we could not detect Jak1 activation in immunoprecipitates from 2 × 107 TSLP-stimulated cells, although activation was readily detected in lysates from 2.5 × 106 IL-7-stimulated cells (data not shown). Moreover, even prolonged exposures of the chemiluminescent immunoblots failed to give any indication of activation of any of the JAK kinases (data not shown). Taken together, these data strongly argue against a diminished level of Jak1 kinase activation by TSLP. It is somewhat surprising that Jak1 is not activated by TSLP treatment, since it probably interacts with and phosphorylates the IL-7Rα-chain. Jak3, on the other hand, associates with γc (12, 13), and since TSLP activity apparently does not depend on this chain, it is less surprising that Jak3 appears not to be involved in TSLP signaling. It is worth noting that while IL-13 stimulation activates Jak1 (which interacts with the IL-4Rα-chain in that system), it does not activate Jak3 (which is activated by IL-4 treatment) (31). This suggests an important distinction between these two related cytokine systems. Moreover, the demonstration here that TSLP stimulation can lead to activation of Stat5 without apparent activation of Jak3 may provide an explanation for the differential effect of Jak3 or IL-7 deficiency on B and T lymphopoiesis; in both cases B cell development is more profoundly affected than is thymocyte development (4, 32–34). These differences may reflect the bioavailability of TSLP in these lymphopoietic tissues and/or the relative contribution of a Jak3-independent action of TSLP to lymphopoiesis in the bone marrow or thymus.

Finally, we have noted the unique capacity of TSLP to promote the development of IgM+ immature B lymphocytes in three different B cell culture systems. It is worth noting that in bone marrow cultures, the number of IgM+ cells that develop in TSLP-supplemented cultures is relatively close to the number that develop in IL-7-supplemented cultures (Fig. 4). The elevated percentage of IgM+ cells that are produced in TSLP cultures reflect diminished numbers of B220+/IgM− cells. Hence, we cannot at this time exclude the possibility that the reason why the number of IgM+ cells produced in TSLP cultures is not higher is because of the reduced number of precursor cells available. Preliminary experiments supplementing long term cultures with both cytokines suggest this may be true, but since high levels of IL-7 may also antagonize the development of IgM− cells (35, 36), careful titration of cytokine levels may be critical to establish a ratio that facilitates maximal IgM+ cell development. This facilitation could be produced at least three ways. It is possible that TSLP simply gives the appearance of IgM+B cell development by causing input IgM+B cells to proliferate in these cultures. We believe that this is an unlikely explanation for two reasons. First of all, in fetal liver cultures there are virtually no IgM+B cells in the starting population of cells, and yet these cultures respond more rapidly than either of the adult bone marrow culture systems we employed. Second, we note the meager capacity of TSLP to promote proliferation of any primary cells tested to date, including unfractionated bone marrow, bone marrow enriched for B-lineage cells, and peripheral B cells (S. D. Levin and A. Farr, unpublished observations). TSLP might also appear to promote differentiation by serving as a "survival factor" for IgM+B lymphocytes. While this possibility has not been excluded as an explanation for enhanced IgM+ cell development in bone marrow cultures, it is an unlikely explanation for the development of IgM+B cells in fetal liver cultures because there are none at the start (data not shown), none develop in cultures without added cytokine (data not shown), and very few develop in the presence of IL-7 (Fig. 7). A third possibility is that TSLP directly promotes the differentiation of pre-B cells into IgM− immature B cells. While both pro-B cells and pre-B cells express the IL-7R (5) (our unpublished observations), only pro-B cells respond to IL-7 (37). This raises the possibility that the function of the IL-7R in pre-B cells could be to facilitate differentiative responses to TSLP rather than IL-7. The effects of TSLP could be brought about through several mechanisms. The critical event in this developmental transition is the productive rearrangement of a light chain locus, a process dependent on the recombinase-activating genes, RAG-1 and RAG-2. It should be noted that in some in vitro culture systems, IL-7 has been shown to keep expression of RAG genes low and hence prevents light chain rearrangement and developmental progression (35, 36). We suggest the possibility that TSLP may serve to antagonize this function of IL-7, perhaps directly by increasing the level of RAG expression or by competing with IL-7 for binding to its receptor complex. It is also possible that TSLP promotes accessibility of the germline light chain genes, which has been shown to be important in the initiation of rearrangement (38). Recently, it was noted that the transcription of some germline VH genes and hence their
rearrangement are diminished in pre-B cells from IL-7R-deficient mice (39). Such a result raises the possibility that this effect actually reflects a deficiency in TSLP signaling and that a similar mechanism for regulating V_{\gamma} germline transcription may influence light chain gene rearrangement. One possible way that TSLP could be affecting transcriptional events is, of course, through the activation of Stat5, as we have reported here. However, disruptions of the genes for both Stat5 isoforms, singly and when superimposed on each other, had no overt effect on B lymphopoiesis (at least as measured by the appearance of normal numbers of peripheral B cells), although the ability of bone marrow cells to form colonies in methylcellulose in response to IL-7 was compromised (40). Hence, either TSLP also influences transcription through other mechanisms as well or another STAT protein is “masking” the Stat5 defects in the knockout animals by redundant function.

We have demonstrated that TSLP has novel and intriguing properties when tested in in vitro assays of B lymphocyte development. Aside from the interesting aspects of the TSLP-invoked signaling cascade, definition of the in vivo importance of TSLP vs IL-7 in normal lymphopoiesis remains a critical challenge for future investigation.

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