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A Novel IL-25 Signaling Pathway through STAT5

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IL-25 is a member of the IL-17 family of cytokines that promotes Th2 cell–mediated inflammatory responses. IL-25 signals through a heterodimeric receptor (IL-25R) composed of IL-17RA and IL-17RB, which recruits the adaptor molecule Act1 for downstream signaling. Although the role of IL-25 in potentiating type 2 inflammation is well characterized by its ability to activate the epithelium as well as T cells, the components of its signaling cascade remain largely unknown. In this study, we found that IL-25 can directly activate STAT5 independently of Act1. Furthermore, conditional STAT5 deletion in T cells or epithelial cells led to a defective IL-25–initiated Th2 polarization as well as defective IL-25 enhancement of Th2 responses. Finally, we found that STAT5 is recruited to the IL-25R in a ligand-dependent manner through unique tyrosine residues on IL-17RB. Together, these findings reveal a novel Act1-independent IL-25 signaling pathway through STAT5 activation. The Journal of Immunology, 2015, 194: 4528–4534.

Chronic pulmonary inflammation associated with allergic asthma is characterized by the recruitment of CD4-positive Th2 cells, eosinophils, and mast cells. These events are orchestrated systemically via the production of Th2-derived cytokines (IL-4, IL-5, IL-9, and IL-13) and locally with elevated IgE in the serum. IL-17E (also known as IL-25) is a member of the IL-17 cytokine family. Unlike the other prototypic IL-17 family cytokines, IL-25 distinctly promotes Th2 cell–mediated inflammatory responses (1–4). IL-25 is produced by the epithelium in response to allergen exposure and can promote the recruitment of Th2 cells, eosinophils, innate lymphoid cells, and mast cells to the site of inflammation. Overexpression of IL-25 can result in enhanced Th2 responses that promote allergic inflammation that manifests into airway eosinophilia and IgE production (1). Intriguingly, epithelial-derived IL-25 acting via an autocrine feed-forward mechanism can induce the epithelium to produce more IL-25 as well as other potent Th2-driving cytokines like IL-33 and thymic stromal lymphopoietin, thus amplifying the allergic response (4–6). In murine models, IL-25 deficiency results in a defective Th2 response, rendering mice susceptible to helminth infections, yet protected against allergic diseases (4, 6, 7).

IL-25 signals through a heterodimeric receptor complex (IL-25R) composed of two subunits, IL-17RA and IL-17RB, both of which contain a conserved SEFIR domain at the cytoplasmic region (8, 9). Upon ligand binding, IL-25R recruits the adaptor molecule Act1 through the homotypic interactions of the SEFIR domains and this domain also exists on Act1 (10, 11). Act1 deficiency results in a loss of IL-25–dependent gene expression and renders mice resistant to IL-25–mediated allergic airway inflammation (10, 12, 13). Using cell-specific deletion of Act1, we have shown that Act1 in the epithelium and in T cells plays a critical role in IL-25–dependent type 2 responses for allergic lung inflammation (10, 13). Aside from signaling through Act1, IL-25 has been shown to activate MAPKs like P38 and JNK as well as NF-κB (14). Although the function of IL-25 in inducing type 2 allergic inflammatory responses is well recognized, the components of its signaling cascade remain largely unknown.

In this article, we identified a novel IL-25 signaling pathway through the activation of STAT5. STAT5 activation is critical for T cell development as well as during Th2 cell differentiation via its cooperation with GATA-3 to prime T cells to the Th2 phenotype (15, 16). We found that IL-25–dependent STAT5 activation was independent of Act1. IL-25–mediated STAT5 activation in T cells and epithelial cells is required for the induction of Th2 cytokines. Moreover, STAT5 is recruited to the IL-25R in a ligand-dependent manner that relies on the phosphorylation of tyrosine 444 and 454 of IL-17RB. These findings demonstrate that IL-25 signaling through STAT5 can potentiate type 2 responses and initiate T cell polarization toward a Th2-like phenotype.

Materials and Methods

Mice

The ERT2Cre line (B6.129-Gt(Rosa)26Sortm1(cre/ERT2)Tyj/J; purchased from The Jackson Laboratory) was crossed with STAT5fl (generated by Lothar Hennighausen, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) (17). The Il-17rb−/− mice were generated by Dr. Wenjun Ouyang. All animal procedures were ap-
proved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation.

**T cell polarization**

Naïve T cells were isolated from the spleen and lymph nodes of mice with a Miltenyi Biotec CD4^+CD62L^+ isolation kit and were cultured for 4 d on plate-bound anti-CD3 (3 μg/ml) and anti-CD28 (3 μg/ml) (BD Pharmingen catalog no. 553057 and no. 553294). Th cells were treated as follows: Th0: anti-IL-4 (10 μg/ml) (BD Pharmingen catalog no. 554432), anti-IFN-γ (10 μg/ml) (BD Pharmingen catalog no. 554408); Th1: anti-IL-4 (10 μg/ml), IL-12 (10 ng/ml); Th17: anti-IL-4 (10 μg/ml), anti-IFN-γ (10 μg/ml), IL-6 (20 ng/ml), TG-β (5 ng/ml); Th2: anti-IFN-γ (10 μg/ml), IL-4 (10 ng/ml), IL-2 (10 ng/ml); Th25: anti-IFN-γ (10 μg/ml), IL-25 (100 ng/ml). For intracellular staining, cells were stimulated with PMA and ionomycin for 5 h, and then GolgiStop was added during the last 2 h of stimulation.

**Cell culture and immunoprecipitation**

HEK293 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. HEK293 cells were transfected with 1 μg DNA, using Lipofectamine 2000. Primary kidney epithelial cells isolated from mice were infected with adenovirus encoding Cre-recombinase or control to mediate deletion for 2 d, followed by stimulation with IL-25. Adenoviruses expressing Cre-recombinase or empty (GFP) control were purchased from Vector Biolabs and used at 1 × 10^7 PFU/ml. IL-17RB wild-type (WT) and tyrosine mutants were subcloned into pAdCMV/V5 adenovirus vectors, and viral generation and titering were done according to the manufacturer’s instructions (Invitrogen). Cells were lysed in lysis buffer (0.5% Triton X-100, 20 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM sodium orthovanadate, 20 mM aprotinin, and 1 mM PMSF) and incubated with Ab conjugated to agarose beads as described previously (11).

**Results**

**IL-25 activates STAT5 independently of Act1.** IL-17RB is basally expressed on naïve T cells but is highly induced upon Th2 polarization (4). We polarized T cells to the Th2 phenotype followed by IL-25 stimulation to elucidate components activated upon IL-25 stimulation. IL-25 stimulation in polarized Th2 cells led to an Act1-dependent activation of NF-κB (indicated by the phosphorylation of IκBα), as well as the activation of MAPKs such as P38 and JNK (Fig. 1A). It is well established that STAT proteins play critical roles in T cell polarization. Therefore, we screened whether IL-25 stimulation had any effect on the phosphorylation of STAT proteins. Although IL-25 had no effect on the activation of STAT6 and STAT3 (data not shown), IL-25 stimulation led to the activation of STAT5, as indicated by the phosphorylation of STAT5 (Fig. 1A). Furthermore, IL-25 treatment led to the phosphorylation of JAK2. In contrast to the other pathways, for which Act1 was required for activation, the phosphorylation of STAT5 was Act1 independent. Act1-deficient Th2 cells exhibited no defect in the ability to phosphorylate STAT5 in response to IL-25 stimulation. In fact, IL-25–induced STAT5 activation was stronger in the absence of Act1 than in its presence. These findings suggest for the first time, to our knowledge, that STAT5 plays a role in the IL-25 signaling cascade.

**STAT5 is recruited to the IL-25R in a ligand-dependent manner**

To further study IL-25–dependent STAT5 activation, we used WT and Act1^−/− mouse embryonic fibroblasts (MEFs) that stably express a Myc-tagged IL-25R (11). IL-25 stimulation led to the recruitment of P-STAT5 to the IL-25R (Fig. 1B). Pulldown of the Myc-tagged IL-25R resulted in the communoprecipitation of Act1, P-STAT5, and P-JAK2 in a ligand-dependent manner, suggesting that the association of JAK2 and STAT5 with the receptor complex can be induced upon IL-25 stimulation. Moreover, in Act1^−/− MEFs, immunoprecipitation of IL-25R also resulted in the communoprecipitation of P-STAT5 and P-JAK2, indicating that the interaction of JAK2 and STAT5 with the IL-25R is independent of Act1.

**IL-25 induces Th2 cytokines through STAT5 activation**

Previous reports have shown that T cells forced to express a constitutively active STAT5 under nonpolarizing conditions (anti-CD3 and anti-CD28 only) results in a Th2-skewed cytokine profile, implying that STAT5 activation is sufficient to generate a Th2 profile (15, 18, 19). In line with this finding, committed Th1 cells expressing a constitutively active STAT5 will acquire the capacity to produce IL-4 and IL-9, suggesting that STAT5 activation results in a bias toward a Th2 phenotype (15). Previous studies from our group and others have shown that the presence of IL-25 is sufficient to polarize naïve T cells to a Th2-like phenotype (Th25 polarization) (4, 13).

Because STAT5 is an important mediator of Th2 responses, we sought to determine the role of STAT5 in Th25 polarization. As an initial test, we examined P-STAT5 in the Th25-culture conditions at two doses of IL-25 (Fig. 2A). The IL-25–cultured cells exhibited an approximate 2-fold increase in P-STAT5 compared with unstimulated conditions (Fig. 2A). The basal activation is likely due to paracrine IL-2 signaling. Although these data clearly show that IL-25 promotes P-STAT5 activation during in vitro polarization, we next sought to determine the functional role of IL-25--
mediated STAT5 activation in these cells. To test this, we crossed tamoxifen-inducible Cre (ERT2Cre+) mice with Stat5f/f mice to generate ERT2Cre+ Stat5f/f and ERT2Cre+ Stat5f/+ mice. Naive CD4+ T cells isolated from ERT2Cre+ Stat5f/f mice or littermate controls (ERT2Cre+ Stat5f/) were treated with 10 nM of the metabolically active 4-hydroxy-tamoxifen (4-OHT) in vitro for 2 d to mediate STAT5 deletion, followed by T cell activation with plate-bound anti-CD3 and anti-CD28 under Th0, Th1, Th2, Th17, and Th25 polarizing conditions (Fig. 2B, 2C). STAT5 deletion had no effect on Th1 and Th17 polarization (Fig. 2C). Consistent with

FIGURE 2. T cell–derived STAT5 is necessary for Th25 polarization. (A) Naive CD4-positive T cells were isolated from WT mice. Cells were incubated on CD3/CD28-coated plates in the indicated conditions. On day 5, cells were fixed and P-STAT5 was analyzed by flow cytometry. (B) Naive T cells isolated from Stat5f/f ERT2Cre or Stat5f/+ ERT2Cre mice were treated with 10 nM of 4-OHT for 2 d with 10 ng/ml of IL-7 to mediate STAT5 deletion. Cell lysates were then separated on SDS-PAGE for immunoblot analysis. (C) Naive CD4+ T cells isolated from Stat5f/f ERT2Cre or Stat5f/+ ERT2Cre mice were treated with 4-OHT for 2 d, followed by T cell activation under the indicated polarizing conditions for 4 d. Cells were then collected for flow cytometry analysis for IL-4, IL-5, and IFN-γ-producing cells. Flow graphs are gated on CD4+ T cells. (D) Percentage of cytokine-producing cells from (B) in Th25 conditions. Results shown are from three experiments. (E) STAT5 WT or STAT5-deleted T cells from (B) were cultured on plate-bound anti-CD3 and anti-CD28 for 48 h with or without IL-25 (100 ng/ml). Cells were then collected for RNA isolation and RT–quantitative PCR analysis for gene expression. Gene expression is graphed as fold induction over naive CD4 T cells relative to Actin. (F) ELISA was performed from supernatants from the Th25 conditions shown in (D). Data shown are representative results from two (A) or three independently performed experiments. Error bars represent mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
what has been previously reported, STAT5-deficient Th2 cells produced less IL-4 and IL-5 than did STAT5-sufficient Th2 cells (7, 8) (Fig. 2C). Similarly, STAT5 deficiency resulted in a defect in Th25 polarization, with less expression of type 2 cytokines such as Il4, Il5, Il9, and Il13 than was found with STAT5-sufficient Th25 cells (Fig. 2C–F). These findings suggest that IL-25–dependent Th2 polarization requires STAT5. We previously demonstrated that Act1 is required for Th25 polarization (13). Because IL-25 stimulation can lead to STAT5 activation independently of Act1, our data suggest that in addition to Act1, STAT5 is required to fully mediate IL-25–dependent Th2 responses.

Epithelial-derived STAT5 is required for response to IL-25

One important function of IL-25 in allergic inflammation is its ability to promote type 2 inflammation in various cells. We and others have reported that epithelial cells, T cells, and innate lymphoid cells are highly responsive to IL-25 stimulation that results in the production of type 2 cytokines such as IL-4, IL-5, IL-13, and IL-9 (5–7, 10, 12, 13, 20). Given that the epithelium plays an essential role in promoting and potentially initiating allergic inflammation, we sought to determine whether STAT5 plays a role in IL-25–dependent effects in epithelial cells. We treated human airway epithelial cells (Bet1A cells) and mouse kidney epithelial cells (KECs) with IL-25 and found that although IL-25 induced modest phosphorylation of IkBa, the IL-25–STAT5 pathway is induced within 15 min of treatment in the epithelial cell compartment (Fig. 3A, 3B). Adenovirus-Cre–mediated deletion of Stat5 in Stat5f/f KECs indicated that STAT5 in epithelial cells is required for IL-25–dependent induction of type 2 cytokines (Fig. 3C–E). Although the expression of Il5, Il9, Il13, and Il25 was induced as early as 2 h in Ad-Ctrl–infected cells, STAT5-deficient KECs exhibited an attenuated or delayed response to IL-25.
Together, these data suggest a critical role for STAT5 in potentiating the IL-25 response in epithelial cells.

**IL-17RB recruits STAT5 through novel tyrosine residues**

STAT recruitment to a receptor requires the phosphorylation of specific tyrosine residues on the receptor to serve as docking sites for STAT proteins (18, 21). Within the intracellular domain of the IL-25–specific receptor subunit, IL-17RB, there are six tyrosine residues (Fig. 4A). Indeed, IL-25 stimulation led to a ligand-dependent association of IL-17RB with P-Tyr (Fig. 4B). Next, we sought to test whether the tyrosine residues within the IL-17RB were required for the STAT5 interaction. As an initial approach we mutated all of the tyrosines within the intracellular portion of IL-17RB to a structurally similar amino acid lacking the hydroxyl group, phenylalanine. Although immunoprecipitation of WT IL-17RB resulted in the coimmunoprecipitation of STAT5, mutation of all six tyrosine residues to phenylalanine (IL-17RB all Tyr) resulted in a loss of interaction between IL-17RB with STAT5, suggesting that the tyrosine residues on IL-17RB are required for STAT5 binding (Fig. 4C).

**Tyrosine residues Y444 and Y454 are critical for STAT5 recruitment**

Next, we sought to determine which of the six tyrosine residues on IL-17RB is (are) required for STAT5 interaction. To test this, individual tyrosine residues were mutated to phenylalanine. Immunoprecipitation of STAT5 resulted in the pulldown of WT IL-17RB, Y335F, Y355F, Y440F, and Y463F (Fig. 5A). However, mutating tyrosine residues 444 and 454 to phenylalanine resulted in a decreased interaction of IL-17RB with STAT5, suggesting that these tyrosine residues may be used for STAT5 binding.

Database searches revealed that IL-17RB phosphorylation has been observed in mass spectrometry analysis (PhosphoSitePlus). Thus we generated adenoviruses expressing either WT IL-17RB or IL-17RB Y444F or Y454F and infected Il-17rb 

were then stimulated with IL-25, followed by immunoprecipitation for P-Tyr. Although mutation of the Y454F attenuated the IL-25–induced IL-17RB association with tyrosine phosphorylation, the difference with Y444F was more modest (Fig. 5B). However, mutation of either tyrosine resulted in diminished IL-25–dependent STAT5 activation (Fig. 5C). Furthermore, IL-25–dependent expression of Il5 was reduced in IL-17RB Y444F- or Y454F-reconstituted KECs compared with IL-17RB WT-reconstituted cells (Fig. 5D). Together, these results indicate that STAT5 recruitment through tyrosine residues 444 and 454 of the IL-17RB subunit is essential to fully mediate IL-25–induced cytokine expression (Fig. 6).
Discussion

In this article, we demonstrated an Act1-independent pathway through STAT5 by which IL-25 can promote Th2 responses. Our observations revealed that in addition to Act1, IL-25 stimulation can lead to the recruitment of STAT5 through tyrosine 444 and 454 of IL-17RB, which are required to mediate IL-25–dependent type 2 cytokine expression. Ablation of either of these two independent pathways (by Act1 deletion or by STAT5 deletion) can result in the loss of IL-25 responses, suggesting that both pathways are required to fully mediate IL-25–dependent expression of type 2 cytokines (10, 12). Given that STAT5 activation can promote Th2 responses by increasing Th2 gene accessibility during T cell differentiation, IL-25–dependent STAT5 phosphorylation likely functions similarly to increase Th2 gene accessibility (15, 22). Thus, Act1 and STAT5 may act synergistically to mediate IL-25–dependent type 2 cytokine expression.

IL-25 is the most structurally and biologically divergent member of the IL-17 cytokine family. Compared with the other IL-17 cytokine family members, the mechanisms of the unique capacity of IL-25 to promote Th2 responses are likely attributable to two critical factors: cell type–specific expression of IL-17RB and unique molecular signaling events. Numerous studies have demonstrated that epithelial cells, T cells, monocytes, multipotent progenitor type 2 cells, and innate lymphoid cells are critical responders to IL-25 (7, 10, 12, 13, 20, 23). As demonstrated in murine models of allergic inflammatory diseases, IL-25–mediated type 2 responses are heavily reliant on the temporal and spatial orientation of the IL-17RB* cellular milieu. Despite these findings, one remaining question has been what molecular components are involved in an IL-17RB response, especially in cellular targets with diverse IL-17R-expression, namely, epithelial and T cells.

In this study, we found that IL-25 stimulation can lead to activation of STAT5 in T cells as well as in epithelial cells. It is important to note that IL-25 did not activate STAT3 or STAT6 (data not shown). Furthermore, P-STAT5–positive T cells appear to be the main IL-5–producing cells following IL-25 treatment (L. Wu, Z. Z. Zou, and X. Li, unpublished observations). Importantly, this activation event was independent of Act1 because Act1-deficient cells exhibited robust P-STAT5 expression in response to IL-25. STAT5 has two isoforms, A and B; whether one or both are sensitive to IL-25 stimulation will be an interesting line for future studies. Because Act1 has been shown to be a key component in the IL-25–signaling cascade, together these data suggest that Act1 and STAT5 are both required for an effective IL-25 response. STAT5 has been shown to be required for the expression of type 2–associated cytokines, including IL-4, IL-9, and IL-13. Mechanistically, STAT5 has been shown to facilitate GATA3 promoter access to these genes (15, 22). One possibility is that Act1 and STAT5 may also cooperate to regulate Th2-associated cytokine expression. Further experimentation would be needed to elucidate the exact mechanism or mechanisms.

Because IL-25 activates STAT5 independently of Act1, these data suggested to us that IL-25–dependent STAT5 activation may be instigated at the level of the receptor. Indeed, communoprecipitation of STAT5 with IL-17RB was observed in a ligand-dependent manner. Furthermore, six tyrosines reside within the intracellular portion of IL-17RB. With IL-25 stimulation, we observed an increased association of IL-17RB communoprecipitated with P-Tyr–specific Ab. Specific deletion of tyrosine residues Y444 and Y454 to phenylalanine abrogated the STAT5 interaction as well as IL-25–induced type 2 cytokine expression. Of interest, the IL-17RB/P-Tyr association was different between the Y444F and Y454F mutant, suggesting that the activation on each tyrosine may be temporally regulated. Whether each site has any overlapping consequences in addition to STAT5 activation is an intriguing line for future studies. It is also important to note that these two tyrosine residues are not present in the IL-17A–specific receptor subunit, IL-17RC. Moreover, we also identified JAK2 as a possible kinase linking IL-17RB to STAT5. Although these data imply that Y444 and Y454 are critical residues for IL-17R–dependent STAT5 recruitment, mass spectrometry would be needed to confirm whether these amino acids are phosphorylated following IL-25 stimulation.

Although much progress has been made in identifying signaling components activated by IL-17A, very little is known for IL-25. In a separate report, our group has also identified a negative regulatory network specific for the IL-17RB subunit by a novel P-Tyr binding protein, Dazap2. Notably, this negative regulation impairs Act1 and STAT5 recruitment and is immediately removed following ligand stimulation. Thus, unlike for IL-17A, the IL-25R is under much stricter control. Taking into consideration our present observations that IL-25 uses the STAT5 pathway, it is obvious that the molecular diversity of IL-25–activated signaling pathways imparts its Th2-promoting activity but it also offers possible therapeutic targets in Th2-elicited maladies.

Disclosures

The authors have no financial conflicts of interest.

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