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Level of Expression of IL-13Rα2 Impacts Receptor Distribution and IL-13 Signaling

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IL-13, a critical cytokine for allergic inflammation, exerts its effects through a complex receptor system including IL-4Rα, IL-13Rα1, and IL-13Rα2. IL-4Rα and IL-13Rα1 form a heterodimeric signaling receptor for IL-13. In contrast, IL-13Rα2 binds IL-13 with high affinity but does not signal. IL-13Rα2 exists on the cell surface, intracellularly, and in soluble form, but no information is available regarding the relative distributions of IL-13Rα2 among these compartments, whether the compartments communicate, and how the relative expression levels impact IL-13 responses. Herein, we investigated the distribution of IL-13Rα2 in transfected and primary cells, and we evaluated how the total level of IL-13Rα2 expression impacted its distribution. Our results demonstrate that the distribution of IL-13Rα2 is independent of the overall level of expression. The majority of the IL-13Rα2 protein existed in intracellular pools. Surface IL-13Rα2 was continually released into the medium in a soluble form, yet surface expression remained constant supporting receptor trafficking to the cell surface. IL-13Rα2 inhibited IL-13 signaling proportionally to its level of expression, and this inhibition could be overcome with high concentrations of IL-13.


Interleukin-13 is a Th2 cytokine that is a central mediator of allergic inflammation and asthma (1–3). IL-13 mediates its effects via a complex receptor system that includes IL-4Rα (IL-4 chain) and two other cell surface proteins, IL-13Rα1 and IL-13Rα2 (4–9). IL-13Rα1 binds IL-13 with low affinity by itself, but when paired with IL-4Rα it binds IL-13 with high affinity and forms a functional IL-13R that signals and results in activation of the Jak-Stat pathway (7). IL-13Rα1 is widely expressed and has been demonstrated on nearly every cell tested except human and mouse T cells and mouse B cells, consistent with the known functions of IL-13 (10–13). In contrast, expression of IL-13Rα2 in vitro was insufficient to render cells responsive to IL-13, despite high affinity binding even in the presence of IL-4Rα (14). IL-13Rα2 has a short cytoplasmic tail (17 aa in the human), which contains no box 1 or box 2 signaling motifs, supporting the theory that it has no signaling function. The inability of IL-13Rα2 expression to confer IL-13 responsiveness despite high affinity binding, along with the finding of soluble IL-13Rα2 in vivo (9), has led to speculation that IL-13Rα2 is a decoy receptor. Expression of IL-13Rα2 varies across cell types and can be induced by inflammation and cytokines (15–20). A role for IL-13Rα2 as a potential modulator of inflammation in asthma is suggested by the IL-13- and IL-4-dependent up-regulation of IL-13Rα2 in primary bronchial epithelial cells and the demonstration that overexpression of IL-13Rα2 in bronchial derived cells decreases IL-13-dependent Stat6 phosphorylation (21). IL-13Rα2 exists largely intracellularly and can be mobilized to the cell surface following cytokine exposure (22). A tri-leucine motif in the transmembrane domain was shown to be a critical regulator of internalization of IL-13Rα2 (23). IL-13Rα2 has also been found to exist in a soluble form (9). Thus, IL-13Rα2 exists in three compartments, cytoplasmic, surface membrane, and soluble, but little is known about the relative distribution of IL-13Rα2 among these compartments in cells or how this distribution is regulated. Modulation of the relative ratios of IL-13Rα2 in these three compartments, as well as the total level of IL-13Rα2, is likely important in determining the overall effect of IL-13 on a given cell.

Herein, we examined the relative distributions of IL-13Rα2 in primary and transfected cells. To determine the relationship between total IL-13Rα2 expression and the relative distributions among the different compartments (surface, intracellular, and soluble), we developed stable transfected clones with relatively lower or higher levels of IL-13Rα2 surface expression. We then determined how the relative amount of expression influenced the distribution of IL-13Rα2 in the soluble, surface, and cytosolic compartments. We compared the distribution of IL-13Rα2 in these transfected cells with the distribution of IL-13Rα2 in murine splenocytes to confirm that the expression of IL-13Rα2 in primary cells has a distribution similar to that seen in the transfecants. We also examined the inhibitory effect of IL-13Rα2 on IL-13 signaling. The level of IL-13Rα2 expression has been shown to be regulated in vivo by inflammation and cytokines (15–20). Our in vitro model enabled the examination of varying levels of IL-13Rα2 and their effects on IL-13 signaling and IL-13Rα2 distribution, thus mirroring the variations in expression of IL-13Rα2 that may be expected in vivo.

Materials and Methods

Cells

The coding sequence cDNA of mature human IL-13Rα2 (GenBank NM_000640 without signal peptide) was tagged with a Flag epitope at the N terminus and cloned into pEF-Neo-PPL-SP vector, kindly provided by Dr. Yutaka Tagaya (National Cancer Institute, Metabolism Branch, Bethesda, MD) (24), which uses bovine preprolactin signal peptide to direct
the surface expression of IL-13Rα2. The construct produces a fusion protein of bovine preprolactin signal peptide followed by the Flag epitope tag and IL-13Rα2 without its innate signal peptide. A total of $5 \times 10^5$ U937 cells (American Type Culture Collection) were washed, resuspended in RPMI 1640 containing 20 μg of uncut plasmid and pulsed with a Gene-pulser II electroporation device (Bio-Rad) set at 960 μJ and 200 V. After electroporation, the cells were grown for 24 h in 10 ml of complete RPMI 1640 and then selected for resistance to G-418 (Invitrogen Life Technologies) at 400 μg/ml for 2–3 wk. Cell populations were screened by flow cytometry for surface expression by staining with FITC-conjugated anti-Flag Ab. Positive transfectant pools were cloned by limiting dilution.

Murine splenocytes were prepared from C57 mice obtained from The Jackson Laboratory. After RBC lysis, the cells were washed three times in RPMI 1640 and resuspended in PBS. Cells were then treated with trypsin, and lysates were prepared as described below.

**PCR for IL-13Rα2**

cDNA was prepared, using TRIZol reagent (Invitrogen Life Technologies), from cells stimulated overnight with the indicated cytokines. Message for IL-13Rα2 was detected by PCR using the following primers: forward, TTCCCTATTTGGGACATCGAC; reverse, CACCCTCCCCAGGTATACAC; and PCR conditions, 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, yielding an expected fragment of 400 bp.

**Flow cytometry**

Flag-IL-13Rα2 expression was assessed by flow cytometry. Briefly, cells were incubated with 5 μg/ml anti-Flag (Sigma-Aldrich) or anti-IL-13Rα2 (R&D Systems) for 30 min at 4°C. Bound Abs were detected with Alexa Fluor 488 goat anti-mouse or chicken anti-goat (Invitrogen Life Technologies) Ab. IL-13 binding was determined by incubating cells with 200 ng/ml murine IL-13 (mIL-13; Peprotech) for 30 min at 4°C. Cells were washed twice in cold PBS and incubated with 4 μg/ml anti-mI13-13 (R&D Systems) for 30 min and washed twice in cold PBS. Bound Ab was detected by Alexa Fluor 488 chicken anti-goat Ab.

Murine splenocytes were assessed for IL-13Rα2 expression by isolating single cell suspensions from spleens obtained from FVB/N mice. These splenocytes were either fixed with 2% paraformaldehyde in PBS and permeabilized with 0.2% saponin or were allowed to remain intact. They were then incubated with anti-murine IL-13Rα2 goat polyclonal Ab and biotinylated anti-goat IgG (Vector Laboratories) for 2 h at room temperature. This was followed by streptavidin-HRP conjugate and substrate solution (R&D Systems) at room temperature for 20 min each. Optical density was read at 450 nm. Background (PBS only) OD450 was subtracted from the OD450 obtained from the samples. IL-13Rα2 levels were expressed as mean optical density ± SD of three independent samples.

**Tryptin treatment of cells**

Cells ($5 \times 10^5$; for flow cytometry), $5 \times 10^6$ cells (lysates for ELISA), or $10^5$ splenocytes for ELISA were resuspended in 500 μl of trypsin-EDTA at 4°C. Cells were then warmed to 37°C for the indicated times. Tryptin was inactivated with 1 ml of PBS, and the cells were washed once in RPMI 1640 and twice in PBS. Cells were stained for surface Flag-IL-13Rα2 and analyzed by flow cytometry or lysates were prepared for ELISA as previously described.

**EMSA**

EMSA was performed as previously described (25). Briefly, after cytokine treatment for 30 min, $5 \times 10^6$ cells were lysed in 20 μl of MLE 40 ± soluble buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% Nonidet P-40, 1.0 mM DTT, 0.5 mM PMSF) for 5 min on ice and centrifuged for 5 min at 10,000 × g. Pellets were resuspended in 20 μl of EMSA extraction buffer (20 μM HEPES (pH 7.9), 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 25% glycerol, 1.0 mM DTT, 0.5 mM PMSF) for 30 min on ice and centrifuged for 20 min at 20,000 × g. The resulting nuclear extracts were incubated with 32P-labeled Stat6 probe (Santa Cruz Biotechnology), resolved by gel electrophoresis, and visualized by autoradiography. Stat6 activity was quantified by densitometry.

**Functional assessment of conditioned medium**

Functional assessment of the conditioned medium was performed by obtaining conditioned medium (medium cultured with $2 \times 10^7$ cells/ml for 1 h (MDH or untransfected)). This conditioned medium was then mixed with IL-4 or IL-13 at the indicated concentrations, incubated for 10 min on ice, and then added to U937 cells, and Stat6 activation was determined by EMSA as previously described (25).

**CD23 induction**

Induction of CD23 surface expression was assessed by stimulating $5 \times 10^5$ cells with the indicated concentration of cytokine in medium for 48 h. Cells were then washed and stained with FITC-conjugated anti-CD23 Abs (Beckman Coulter). Cells were washed twice, and bound anti-CD23 was detected by flow cytometry.

**Results**

**Characterization of Flag-IL-13Rα2 expression and ligand binding**

To study the role of IL-13Rα2 in vitro, U937 cells were stably transfected with Flag-human IL-13Rα2. The parent U937 line was selected because it is IL-13 responsive and expresses negligible endogenous IL-13Rα2. Stable transfectants were assessed for IL-13Rα2 mRNA, surface expression, and IL-13 binding. The relative amount of surface and intracellular staining for IL-13Rα2 were determined, and clones with different levels of surface expression were isolated. Untransfected U937 cells demonstrated negligible mRNA by RT-PCR for IL-13Rα2 in contrast to cells transfected with Flag-IL-13Rα2 (Fig. 1A). Transfected cells demonstrated surface expression of Flag-IL-13Rα2 by flow cytometry, and similar staining was observed with both anti-Flag and anti-IL-13Rα2 Abs (Fig. 1B). To verify that the presence of the Flag epitope did not interfere with the ability of the Flag-IL-13Rα2 to bind IL-13, we used a flow cytometry sandwich assay. Cells were first incubated with IL-13, and the amount of surface-bound IL-13 was then detected with Abs directed against IL-13. IL-13 binding in the transfectants correlated with the Flag and IL-13Rα2 expression. Furthermore, IL-13 binding was unaffected by Abs to Flag. Untransfected U937 cells demonstrated no appreciable staining for Flag, IL-13Rα2, or IL-13 binding.
Evidence of surface and intracellular IL-13Rα2 compartments

We next determined the localization of Flag-IL-13Rα2 by confocal microscopy. Transfected cells were stained with anti-Flag Abs either intact or after permeabilization. As shown in Fig. 1C, intact cells demonstrated membrane-associated receptor. Permeabilized cells show intense cytoplasmic staining of Flag-IL-13Rα2, demonstrating that the majority of IL-13Rα2 is located intracellularly. Negligible staining was observed in untransfected cells or with isotype control Abs. Because the regulation of IL-13Rα2 and its ultimate effects on IL-13 signaling may be determined by its relative level of expression, limited dilution cloning was used to isolate clones with relatively lower (MDL1, MDL2, MDL3, MDL4) or higher (MDH1, MDH2, MDH3, MDH4) expression of surface Flag-IL-13Rα2. Representative histograms of MDH and MDL clones are shown in Fig. 1D. We compared the expression of IL-13Rα2 in the transfectants to primary keratinocytes to determine whether the expression of IL-13Rα2 in our transfected cells was similar to an untransfected primary cell. We determined that MDH and MDL clones approximated the expression of IL-13Rα2 in primary keratinocytes within 2- to 4-fold (data not shown).

Determination of relative distribution of soluble, membrane, and intracellular IL-13Rα2

Mean surface expression of Flag-IL-13Rα2 was 2.24-fold higher in MDH clones than MDL clones as determined by flow cytometry (Fig. 2A). To determine whether the surface expression of Flag-IL-13Rα2 correlated with the total Flag-IL-13Rα2, the amount of Flag-IL-13Rα2 in whole cell lysate was quantified by ELISA. Total Flag-IL-13Rα2 was 2.16-fold greater in MDH than in MDL clones based on OD450 ratios. A similar ratio (2.42) was obtained by quantitative ELISA (data not shown). This ratio was similar to the differences in their levels of surface expression (Fig. 2B). Because IL-13Rα2 has been shown to exist in a soluble form in vivo (26), and an inhibitory role for soluble IL-13Rα2 is suggested by the use of IL-13Rα2 fusion proteins in vivo to block IL-13 signaling (2, 3), we next determined whether IL-13Rα2 was spontaneously released into the medium by MDH and MDL clones. IL-13Rα2 was released into the medium spontaneously by the transfectants. MDH clones released more Flag-IL-13Rα2 into the medium than MDL clones (Fig. 2C), and receptor release was proportional to the surface expression of Flag-IL-13Rα2. Levels of soluble IL-13Rα2 in the medium yielded OD450 values that were 2.03-fold higher in MDL clones than in MDH clones. Comparisons of IL-13Rα2 concentrations in lysates vs supernatants revealed that both MDH and MDL clones had greater IL-13Rα2 that remained cell associated vs that which was spontaneously released into the medium. Although soluble IL-13Rα2 was detected in the medium by the more sensitive anti-Flag capture ELISA, it was below the threshold of the quantitative ELISA using anti-IL-13Rα2 capture (<100 pg/ml).
Our data thus far revealed that the level of intracellular IL-13Ra2 is much greater than the level of surface IL-13Ra2 or soluble IL-13Ra2. To validate this observation and confirm that the distribution of IL-13Ra2 seen in the transfected cells is similar to what is seen in primary cells in vivo, we compared the relative amounts of surface and cytoplasmic IL-13Ra2 in transfected and primary murine splenocytes. Trypsin, a nonspecific protease that cleaves IL-13Ra2, was used to remove surface IL-13Ra2. MDH clones were treated with trypsin at 37°C for the indicated times, and residual surface IL-13Ra2 was assessed by flow cytometry (Fig. 3). Trypsin efficiently cleaved IL-13Ra2 from the surface of the cells, removing 50% of the surface receptor in 10 min and virtually all of the receptor in 1 h (Fig. 3A). Cells remained >99% viable following trypsin treatment for 1 h. We then examined whole cell lysates obtained from IL-13Ra2-transfected cells before and after trypsin treatment to determine the relative proportion of IL-13Ra2 that is intracellular and not accessible to trypsin. This allowed determination of the relative proportions of membrane vs total cellular IL-13Ra2. Although trypsin efficiently removed surface IL-13Ra2, whole cell lysates showed a slower and incomplete decrease in IL-13Ra2 consistent with the fact that the intracellular pool is resistant to trypsin (Fig. 3B). Decrease in total IL-13Ra2 after trypsin treatment was ~77% at 60 min, the time at which we observed nearly complete removal of surface receptor by trypsin. This demonstration of residual IL-13Ra2 after removal of surface IL-13Ra2 agrees with our findings of a pool of cytoplasmic IL-13Ra2 by confocal microscopy. Although surface IL-13Ra2 is completely removed at 1 h, the amount of IL-13Ra2 in whole cell lysate continues to decrease during trypsin treatment at 2 h to 86% removal. This likely represents mobilization of cytoplasmic stores of IL-13Ra2 to the surface where they are accessible to trypsin cleavage, demonstrating that the cytoplasmic and membrane compartments communicate, trafficking IL-13Ra2 to the cell surface. This is further supported by the fact that despite the ongoing release of soluble IL-13Ra2 from cells, the surface level of IL-13Ra2 remains constant at the same steady state.

Next, we determined the relative proportions of surface and intracellular IL-13Ra2 in murine splenocytes. Trypsin treatment resulted in a decrease in total cellular IL-13Ra2 of 12% in splenocytes (p = 0.037; Fig. 3C), supporting the presence of IL-13Ra2 on the surface of murine splenocytes. However, the kinetics of the loss of IL-13Ra2 after trypsin treatment was slower than that seen in the transfected cells presumably because in splenocytes there was a larger percentage of IL-13Ra2 located intracellularly, and thus protected from trypsin. We verified the predominance of intracellular IL-13Ra2 in splenocytes by flow cytometry. IL-13Ra2 was detected in intact and permeabilized splenocytes to determine surface and total cellular IL-13Ra2, respectively. Fig. 3D shows histograms from intact and permeabilized cells demonstrating the significant increase in staining for IL-13Ra2 in permeabilized cells. These findings confirm that there is an intracellular pool of IL-13Ra2 in primary cells.

Effect of IL-13Ra2 expression level on IL-4 and IL-13 signaling

We next investigated whether the level of IL-13Ra2 expression affected IL-13- and IL-4-dependent Stat6 activation (Fig. 4A). Untransfected cells demonstrate significant Stat6 activation even at low concentrations of IL-13. Cells overexpressing IL-13Ra2 (MDH or MDL clones) demonstrated decreased activation of Stat6 in response to IL-13 when compared with untransfected cells at lower concentrations of IL-13 but appear to activate Stat6 normally at higher concentrations of IL-13. Densitometry was performed on four experiments to quantify the amount of Stat6 activation (Fig. 4B). MDL clones show less Stat6 activation than untransfected cells only at 10 ng/ml IL-13 (p < 0.013); MDH clones showed decreased Stat6 activation at 10, 25, and 50 ng/ml (p < 0.001, p < 0.031, p < 0.012, respectively). The inhibitory effects of the IL-13Ra2 were overcome at the highest concentrations of IL-13. These findings demonstrate that the inhibitory effects of IL-13Ra2 on IL-13 signaling are dependent on both the level of expression of the receptor and the concentration of IL-13. There was no difference in the IL-4 responses between the untransfected cells and the MDH clones as demonstrated by their similar IL-4 dependent Stat6 activation shown in the EMSA (Fig. 4A). Additionally, activation of Stat6 in response to as much as 200 ng/ml IL-4 revealed no significant differences between MDH and untransfected cells (data not shown).
As shown in Fig. 2C, soluble IL-13Rα2 is detectable in the supernatant of cultured MDH and MDL clones. We next investigated the ability of this spontaneously released receptor to inhibit Stat6 activation in response to IL-13. MDH cells or untransfected U937 cells were cultured in complete medium for 1 h, and their supernatants were isolated. These supernatants together with 10–200 ng/ml IL-13 were added to naive U937 cells, and Stat6 activation was assessed. Although IL-13Rα2 was detected in these supernatants (Fig. 2C), it was very low (<100 pg/ml) and was insufficient to inhibit IL-13 responses.

**Effect of IL-13Rα2 expression level on IL-4- and IL-13-dependent CD23 surface expression**

To further define the effect of IL-13Rα2 expression on IL-13 responses, we investigated its role in IL-13-dependent increase in CD23 surface expression. Whereas Stat6 activation in response to IL-4 or IL-13 occurs in minutes, increased CD23 surface expression in response to IL-4 or IL-13 occurs over 48–72 h. Because IL-4 responses were unaffected by IL-13Rα2 expression, data on response to IL-13 for each clone were normalized to their peak IL-4 response. Both IL-4 and IL-13 induced CD23 surface expression in untransfected cells (Fig. 5A). In contrast, the MDH transfectants revealed significantly decreased CD23 induction at all concentrations of IL-13 when compared with untransfected cells (Fig. 5B). The CD23 response of the MDH clones to IL-4 remained intact. In contrast, the MDL clones displayed decreased IL-13-dependent CD23 surface expression compared with untransfected cells only at 5 and 10 ng/ml IL-13, the two lowest concentrations assayed. MDL clones had a greater induction of CD23 than MDH clones at 25–200 ng/ml IL-13 (Fig. 5C).

These data demonstrate that IL-13Rα2 has an inhibitory role in IL-13-dependent surface CD23 expression. Furthermore, this inhibitory effect is directly proportional to the level of expression and can be largely overcome by increased IL-13 concentrations.
Discussion

Expression of IL-13Rα2 has been demonstrated in numerous tissues, including murine spleen and brain and human liver, lung, and thymus (14). Additionally, IL-13Rα2 has been shown to be induced by cytokines (17), parasitic infection (20), and allergen sensitization and challenge (21); thus, its expression will vary in cells depending on the inflammatory state. Herein, we studied a model of IL-13Rα2 expression with higher (MDH clones) and lower (MDL clones) expressing transfectants to mirror the effects of varying IL-13Rα2 expression in cells. These transfectants were produced in U937 cells, a human monocytic cell line with negligible endogenous IL-13Rα2 expression. We characterized the effect of the overall level of expression of IL-13Rα2 on the distribution of IL-13Rα2 in soluble, surface, and cytosolic compartments. The relative ratios of IL-13Rα2 in these three compartments were independent of the overall level of expression.

Thus, even though the absolute amount of receptor was higher in MDH clones than in MDL clones, the relative ratios of IL-13Rα2 in the three compartments was similar. The level of expression correlated directly with the amount of inhibition of IL-13 responses observed.

There was communication between the soluble, membrane, and cytoplasmic compartments of the receptor. Prolonged treatment of cells with trypsin beyond that needed to remove the surface IL-13Rα2 revealed an ongoing decrease in total cell IL-13Rα2. This continued decline in IL-13Rα2 suggests mobilization of the cytoplasmic IL-13Rα2 to the surface where it is susceptible to trypsin-mediated cleavage.

Our data demonstrate that IL-13Rα2 is spontaneously released from cells into the medium. This is an important observation because soluble IL-13Rα2 has been shown to inhibit IL-13 responses (3). This represents a potential mechanism for IL-13Rα2 to have effects on cells distant from its production. It will be critical to define the mechanisms by which IL-13Rα2 is released and how these processes are regulated. It could represent a secreted receptor
or a cleaved soluble receptor. We observed that IL-13Rα2 was released at baseline into the supernatant of cells. This receptor release was roughly proportional to the level of surface expression of Flag-IL-13Rα2. This level of soluble IL-13Rα2 was unable to inhibit IL-13 signaling in untransfected cells when we transfected the supernatants, most likely because the level of soluble receptor was low. However, under condition of inflammation the levels may be higher and may be functionally relevant. Membrane, intracellular, and soluble IL-13Rα2 proportions may be altered in inflammatory states, because studies have shown that IL-13Rα2 expression is induced during inflammation. In our experiments, the total level of expression did not affect the cellular distribution, but during inflammation this may not be the case. Furthermore, there may be cellular differences in both the level and distribution of IL-13Rα2 as we observed between transfected MDH cells and splenocytes.

Overexpression of IL-13Rα2 at modest or high levels resulted in inhibition of IL-13 responses proportional to the level of expression. This was true of both experiments lasting minutes (Stat6 activation) and days (CD23 surface expression). This demonstrates that the inhibitory effects of the receptor were rapid and persistent. In addition, these inhibitory effects could largely be overcome by increasing concentrations of IL-13. Thus, the ability of IL-13Rα2 to quench IL-13 responses is dependent on both the level of expression of the receptor and the amount of IL-13 present.

The mechanism of this inhibition is most likely due to the surface receptors. We did not observe an inhibitory effect of the shed receptors in transfer experiments when conditioned medium containing released IL-13Rα2 from transfected cells was placed onto untransfected cells. Thus, the IL-13Rα2 spontaneously released by the transfected cells was insufficient to explain the inhibitory effects of IL-13Rα2, probably due to low levels. In our experiments, IL-13Rα2 receptors located at the cell surface are most likely responsible for the observed inhibition of IL-13 signaling and responses. In more complex models of inflammation, it is possible that up-regulated expression or enhanced release of IL-13Rα2 from this membrane-bound compartment creates a greater inhibitory effect.

Disclosures

The authors have no financial conflict of interest.

References


