Modulation of Interleukin (IL-)13 Binding and Signaling by the γc Chain of the IL-2 Receptor*

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Interleukin (IL-)13 and IL-4 are cytokine products of T helper 2 cells which exert similar effects in a variety of cell types. We recently described IL-13R expression on human renal cell and colon carcinoma cells and demonstrated that γc is not a component of IL-13R or IL-4R systems in these cells. In lymphoid cells such as B cells and monocytes, which respond to IL-13, γc is a component of IL-4R but does not appear to be a component of IL-13R. Furthermore, while significant IL-13 binding is observed on carcinoma cells, IL-13 barely binds these lymphoid cells and the binding characteristics are different. To better understand the role of γc in IL-13 binding and signaling, we have transfected a renal cell carcinoma cell line with γc and examined IL-13 and IL-4 binding and signaling. IL-13 binding as well as IL-13 and IL-4 signaling through the JAK-STAT signaling pathway were severely inhibited. This inhibition was paralleled by a loss of expression of one of the IL-13R chains and intercellular cell adhesion molecule-1. Thus, although γc has been shown to enhance IL-4 binding and function in some cell types, its influence on IL-13R function in tumor cells appears to be largely negative.

Interleukin-13 (IL-13) and IL-4 are pleiotropic immune regulatory cytokines, which are predominantly produced by activated lymphocytes. Both cytokines mediate similar effects on B cells and monocytes; however, IL-13 has not been shown to have direct effects on T cells (1–3). The effects of both cytokines are mediated by cell surface receptors (R) that are specific for each of these ligands (4, 5). The receptor for IL-4 has been extensively investigated and its structure appears to vary with cell types. We have proposed three models for the structure of the IL-4R complex (6). However, despite their structural differences, all of the identified forms of the IL-4R complex are quite effective in transducing signals in response to IL-4 binding.

Unlike the IL-4 receptor system, the receptor for IL-13 has not been well characterized. We have demonstrated that RCC cells express a large number of IL-13 binding sites and that these cells respond to IL-13 (4, 7). Cross-linking studies indicate that the IL-13R is predominately composed of a ~65–70-kDa protein (4). RCC cells also express IL-4R, and 125I-IL-4 cross-links to IL-4Rβ (the p140 chain of IL-4R) and a 65–70-kDa IL-4-binding protein. The inhibition of IL-4 binding to both proteins by IL-13 supports the notion that IL-4 and IL-13 receptors are structurally and functionally interrelated (4, 7–10). We recently proposed several models to account for the complex interactions among IL-13, IL-4, and the receptors for these ligands as they are expressed in different cell types. We proposed that IL-13R shares IL-4Rβ (p140) and IL-4Rα (p70) with the IL-13R system in certain cell types (11). We had also predicted that IL-13 binds to a heterodimer consisting of two ~70-kDa proteins, one of which binds IL-13 alone (we termed this IL-13Rα) while the other binds IL-13 as well as IL-4 (we termed this IL-13Rα’) (7, 11). The genes for a murine IL-13-binding protein, its human homologue, and a second human IL-13-binding protein were subsequently identified and characterized (12–14). The two human IL-13R genes encode 65–70-kDa proteins with different IL-13 binding characteristics. One of these (corresponding to our IL-13Rα’) requires IL-4Rβ to bind and transduce IL-13 signal (14), while the other (corresponding to our IL-13Rα) can bind IL-13 in the absence of IL-4Rβ, but its role in IL-13 signaling remains unclear (13). Whether other as yet unidentified proteins are required to constitute a functional high affinity IL-13R remains to be resolved.

The IL-2Rγ chain termed γc is shared by receptors for IL-4, IL-7, IL-9, and IL-15 on immune cells (15–18). It was hypothesized that IL-2Rγc is also a component of the IL-13R system (15). However, we have reported that an anti-γc antibody did not immunoprecipitate any 125I-IL-13-bound protein from RCC (4) or colon carcinoma (6) cell lysates, indicating that the γc protein may not be directly involved in IL-13 binding. The γc protein was not expressed in these cells. However, whether γc affects IL-13 binding in cells normally express it is not known. It is also not known whether γc affects IL-13R structure and signal transduction.

In this study, we have examined the effect of the γc chain on IL-13 and IL-4 binding and signaling. We have transfected ML-1 RCC cells with the γc cDNA and examined its influence on certain biological responses of these cells to IL-13 and IL-4. We present evidence that γc severely decreased the IL-13 binding capacity of these cells and prevented the expression of the α chain of the IL-13 receptor as well as ICAM-1. Furthermore, although γc had no significant effect on the binding of IL-4 to its receptors, its presence altered IL-13 and IL-4 signaling pathways in these cells in several ways, including the inhibition of IL-13- and IL-4-induced STAT-6 activation.

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* The abbreviations used are: IL, interleukin; R, receptor; rhIL, recombinant human IL; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; NF, nuclear factor; ICAM, intercellular cell adhesion molecule; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
EXPERIMENTAL PROCEDURES

Cytokines and Reagents—Recombinant human IL-13 (rhIL-13) was expressed in Escherichia coli and purified as described previously (4). Recombinant human IL-4 was kindly provided by Dr. Michael Widmer of Immunex Corp., Seattle, WA. Antibodies to Jak1s 1 and 2 and biotin-anti-phosphotyrosine (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies to γc, Jak3, Tyk2, and STATs 3, 4, and 5 were purchased from Santa Cruz (Santa Cruz, CA). Antibodies to STAT1 and STAT5 were provided by Dr. David Finbloom (Center for Biologics Evaluation and Research, Food and Drug Administration (CBER, FDA), Bethesda, MD).

Cells—The ML-RCC (synonym: MA-RCC) renal cell carcinoma cell line was established in our laboratory as described previously (19) from primary surgical tissues and was maintained in HEPES-buffered Dulbecco’s modified Eagle’s medium with high glucose supplemented with glutamine plus 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

Transfection of ML-RCC Cells—ML-RCC cells (3 × 10^5) were plated in 12-well tissue culture plates and cultured overnight. They were then transfected with a mixture of cDNAs encoding the genes for the γc subunit of the IL-2 receptor or neomycin transrepressor or both. Transfection was effected by the calcium phosphate method using the PROFECTANT kit (Promega) according to the manufacturer’s instructions. Neomycin-resistant transfecants were selected by incubating the transfected cells in 0.8 mg/ml active G418 for 10 days. Selected cells were examined by Northern blot analysis to identify clones containing the γc gene in addition to the neomycin resistance gene. Two such clones isolated from the ML-RCC cell line were identified as ML-γc. Clones transfected with the neomycin resistance but not the γc gene were used as negative controls for the double transfecants. Additional negative control transfecants (i.e. neomycin-resistant, γc (-)) were generated by using a recombination deficient retroviral vector to introduce the neomycin resistance gene into the target ML-RCC cells. This was achieved by infecting target cells with supernatant generated by the amphotropic retroviral vector-producing PA317 cell line (20), kindly provided by Dr. Carolyn Wilson (CBER, FDA). Identical results were achieved by infecting target cells with supernatant generated by the calcium phosphate method using the PROFECTANT kit (Promega) according to the manufacturer’s instructions.

RESULTS

Radioreceptor Binding Assay—γc mRNA-positive (+) clones of ML-RCC (MLγc) were selected from five neomycin-resistant (MLneo) clones. As shown in Fig. 1A, alternatively spliced γc mRNA (16) was detected in MLγc but not in MLneo cells. Both of the MLγc clones were tested in all experiments shown, and both yielded identical results. Similarly, all the negative control clones (MLneo) yielded identical results in tests performed. To confirm that the γc mRNA being transcribed was also being properly translated into its protein in MLγc cell lines, we tested the cell lysate of each γc mRNA+ cell lines for the presence of γc protein by immunoprecipitation and Western blot analysis. The results indicate that MLγc cells expressed the γc protein as detected by anti-γc antibody, whereas the control and untransfected parental cell lines were negative (Fig. 1B). We have also demonstrated γc protein expression on the surface of these γc mRNA + cells by
flow cytometry (22). Following γc transfection, the MLγc clones took on a different morphology, appearing smaller and more elongated than the neomycin-resistant control cell lines (data not shown).

**Association of Transfected γc Chain with IL-13R in ML-RCC Cells**—Since γc modulated the binding of IL-13 to its plasma membrane receptors, it was important to determine whether the γc protein expressed in ML-RCC cells transfected with the γc cDNA is associated with IL-13 receptor proteins. We cross-linked 125I-IL-13 to its receptors on γc-transfected and control cells. The cell lysate from each group was subsequently incubated with anti-γc antibody, and the resulting antigen-antibody complex was analyzed by SDS-PAGE. The gel was overexposed (5 days) to ensure visualization of the IL-13/γc band. The results (Fig. 1) indicate the formation of a 125I-IL-13/zIL-13R complex of 58–65 kDa in MLγc cells. Furthermore, the γc protein could be immunoprecipitated from this band in MLγc as a faint ~60-kDa band (Fig. 1) but not from the control MLneo cell line (data not shown).

**Effect of γc on IL-13 and IL-4 Binding to Their Receptors**—Since γc has previously been shown to increase the binding affinity of certain cytokine ligands to their receptor (15–18), we examined its effect on IL-13 binding to MLγc and MLneo cells by performing 125I-IL-13 equilibrium binding assays. The results (Table I, Fig. 2, A and B) show that the neoR1 control RCC cells (MLneo) significantly bound 125I-IL-13, and this binding was specific as it was displaced by increasing concentrations of the unlabeled ligand (Fig. 2). However, introduction of the γc chain into the γc-transfected cell line (MLγc) greatly reduced the number of IL-13 binding sites/cell as shown in Table I. This reduction in affinity is due to the presence of γc, which serves as a competitive ligand and reduces the affinity of IL-13 for its receptor.

![Figure 1](image_url)
expression in control (ML neo) and while a Scatchard plot of the data is shown in B. Binding data were analyzed with the LIGAND program (21). Binding data are shown in g. Binding studies were performed on ML-RCC cells by incubating 1 × 10^6 cells with 100–500 pM 125I-IL-13 for 2–5 h. Increasing concentrations (0–1000 nM) of unlabeled IL-13 were added to compete the binding of the radiolabeled ligand.

**FIG. 2.** 125I-IL-13 binding in ML γc and ML neo cells. Equilibrium binding studies were performed on γc-transfected (ML γc) and control (ML neo) ML-RCC cells by incubating 1 × 10^6 cells with 100–500 pM 125I-IL-13 for 2–5 h. Increasing concentrations (0–1000 nM) of unlabeled IL-13 were added to compete the binding of the radiolabeled ligand. Cell-bound radioactivity was determined with a γ counter and the data analyzed with the LIGAND program (21). Binding data are shown in A, while a Scatchard plot of the data is shown in B. IL-4R and IL-13R expression in control (ML neo) and γc-transfected (ML γc) cells was also investigated by direct binding to 2 nM 125I-IL-4 or 125I-IL-13. Nonspecific binding was determined by including excess unlabeled IL-4 or IL-13 in the binding tube and was subtracted from total binding values to obtain specific binding data (C).
ated in these cells (24). In the present study, we examined the impact of the presence of \( \gamma_c \) chain on the IL-13- and IL-4-induced phosphorylation of JAK tyrosine kinase in ML-RCC cell lines. As shown in Fig. 5A, IL-4 induced the phosphorylation of JAK1 and JAK2 tyrosine kinase in ML cells. JAK1 kinase was weakly phosphorylated in response to IL-13, but in contrast to our previous observation in colon carcinoma cells (24), IL-13 did not induce phosphorylation of JAK2 in ML-RCC cells. Tyk2 kinase was constitutively phosphorylated, and this level of phosphorylation was not enhanced by either interleukin. In the \( \gamma_c(1) \) ML\( \gamma_c \) cells, JAK1 was constitutively phosphorylated and the level of phosphorylation was not affected by IL-4 or IL-13 treatment. JAK2 was weakly phosphorylated in unstimulated as well as in IL-13- or IL-4-treated groups and Tyk2 kinase was not detected. As previously observed in colon carcinoma cells (24), JAK3 kinase was neither expressed nor phosphorylated in MLneo and ML\( \gamma_c \) cells, although it was expressed and phosphorylated in the EBV-B cell line used as a positive control (Fig. 5B and Ref. 24). Thus, in \( \gamma_c \)-transfected ML\( \gamma_c \) cells, Tyk2 kinase was not expressed and IL-4 and IL-13 could not enhance or induce the phosphorylation of JAK1 and JAK2 otherwise seen in control ML cells.

Effect of \( \gamma_c \) on the IL-13- and IL-4-mediated Activation of STAT Protein—We next investigated the effect of \( \gamma_c \) expression on STAT activation by performing EMSA on lysates from these cells. As shown in Fig. 6A, in \( \gamma_c \)-negative cells (ML and MLneo), NF (nuclear factor)-IL-4 was activated and bound to the labeled GRR probe in response to IL-13 or IL-4. However, in the \( \gamma_c \)-positive ML\( \gamma_c \) cells, NF-IL-4 was not activated in response to IL-13 or IL-4. We ascertained the specificity of the assay by incubating the labeled probe with a 200-fold excess unlabeled probe before running it on the gel. No NF-IL-4/GRR bands were observed in lanes containing these samples.

To confirm the identity of the STAT protein which was activated in response to IL-4 and IL-13 in ML and MLneo RCC cells, we next performed the supershift assay. The results (Fig. 6B) show that the binding of NF-IL-4 in the IL-4-treated ML group is not affected by preincubation in normal serum or in antibodies specific for STATs 1, 3, 4, or 5, whereas the band representing the bound NF-IL-4 was not visible when the lysate was preincubated in anti-STAT6 antibody. These results indicate that NF-IL-4 corresponds to STAT6 and the other STATs were not activated in response to IL-4 in ML cells. In the \( \gamma_c \)-positive cells (ML\( \gamma_c \)), no STAT protein was activated in response to IL-4.

To further investigate the mechanism underlying the \( \gamma_c \) inhibition of STAT6 activation, we examined the phosphorylation of STAT6 protein from \( \gamma_c(1) \) and \( \gamma_c(2) \) ML-RCC cells in response to IL-13 and IL-4 by immunoprecipitation and immunoblotting. As shown in Fig. 6C, while IL-13 and IL-4 treatment induced
STAT6 phosphorylation in the parental ML and the control neoR<sup>+</sup> transfectant (ML<sub>neo</sub>) cell lines, STAT6 phosphorylation was not induced in the γ<sub>c</sub><sup>+</sup> MLγ<sub>c</sub> cell line (left panel). These results indicate that γ<sub>c</sub> inhibited the IL-4- and IL-13-mediated activation of STAT6 by inhibiting its phosphorylation.

**DISCUSSION**

In our initial characterization of IL-13 binding, we noted that while IL-13 was able to displace or inhibit IL-4 binding to RCC cells, which lack γ<sub>c</sub> expression, it did not inhibit IL-4 binding in Raji B and MLA144 T lymphoid cells in which γ<sub>c</sub> is highly expressed. Furthermore, we noted that B cells and monocytes which are highly responsive to IL-13 express very few IL-13R (4). This led us to further explore the role of γ<sub>c</sub> in IL-13 binding. In this report, we have demonstrated that the γ<sub>c</sub> protein can exert a profound influence on the binding of IL-13 to its receptors and on the signal transduction pathways of IL-13 and IL-4 following ligand binding. Expression of γ<sub>c</sub> in ML-RCC profoundly inhibited IL-13 binding, IL-13-induced signaling, and ICAM-1 expression. Although IL-4 binding was not affected by γ<sub>c</sub> expression, IL-4-induced signaling was also impaired in the γ<sub>c</sub>-transfected cell line and IL-4 failed to induce ICAM-1 expression in these cells.

Our results regarding the expression of γ<sub>c</sub> in cells that co-express IL-4R and IL-13R demonstrate that γ<sub>c</sub> expression is disruptive to the expression of the α chain of the IL-13R while diminishing IL-13Rα expression, and may be responsible for the reduction in IL-13 binding. On a broader scale, this may explain why γ<sub>c</sub>-positive cells such as B cells, monocytes, and TF-1 cell lines, which are very responsive to IL-13, have been shown to express low levels of IL-13R. Similarly, the failure of IL-13 to compete for the binding of IL-4 on MLA 144 and Raji B lymphoblastoid cell line (4) otherwise seen in other cell types (4, 8, 10, 25) may be due to the high γ<sub>c</sub> content of these cells and the consequent deficiency in IL-13R expression.

It is of interest that the γ<sub>c</sub> protein affected IL-13R rather than IL-4R in this manner because, although previous studies have shown that γ<sub>c</sub> is a natural component of IL-4R in a number of cell types, it has not been identified as a natural component of IL-13R in any cell type examined thus far (11). In a study carried out with COS-7 cells, the inhibition of IL-13 but not IL-4 binding by γ<sub>c</sub> was demonstrated (13). However, the biologic consequences of the observation were not addressed.

Our results demonstrate that γ<sub>c</sub> has the potential to physically interact with components of native IL-13 signaling pathway and exert a downstream effect. Consistent with this concept, γ<sub>c</sub> expression altered the pattern of intracellular signaling in response to IL-13. For example, IL-13 has been shown to cause the phosphorylation and activation of JAK1 and Tyk2 tyrosine kinase in hematopoietic cells and JAK1, JAK2, and Tyk2 in non-hematopoietic cells (6, 24–31). However, in the current study, phosphorylation of JAK2 and Tyk2 was not seen in γ<sub>c</sub>-transfected MLγ<sub>c</sub> cells. Similarly, IL-13 failed to induce the phosphorylation of JAK-1 kinase in γ<sub>c</sub>-transfected cells, although IL-13 induced the phosphorylation of JAK1 in control cells. Tyk2 was constitutively phosphorylated in control cells, and IL-13 did not modulate its phosphorylation. In γ<sub>c</sub>-transfected cells, neither constitutive nor IL-13-induced phosphorylation of Tyk2 was observed. These results suggest that the expression of γ<sub>c</sub> leads to an inhibition of IL-13-mediated activation of JAK kinase in ML-RCC cell line and that IL-13Rα may play a pivotal role in these signaling events.

The activation of JAK kinase has been shown to cause the phosphorylation and activation of STAT proteins. We therefore evaluated phosphorylation and activation of STAT proteins in control and γ<sub>c</sub>-transfected MLγ<sub>c</sub> cells as well as the effects of IL-13 on these events. It is of interest to note that IL-13 phosphorylated and activated STAT6 protein in control cells but not in γ<sub>c</sub>-transfected MLγ<sub>c</sub> cells. These data suggest that γ<sub>c</sub> expression and the accompanying disruption of IL-13Rα and/or diminution of IL-13Rα expression inhibited not only the proximal events that result from IL-13 activation but the distal terminal IL-13 and IL-4 signaling events as well. The results also suggest that IL-13Rα and IL-13Rα may be essential for the transduction of certain IL-4 and IL-13 signals supporting previous suggestions that the signal pathways for both cytokines converge at one or more points.

The reason for the profound decrease in IL-13 binding to γ<sub>c</sub>-transfected ML cells is not completely known. However, our results suggest that IL13Rα is predominantly essential for IL-13 binding and its apparent displacement by γ<sub>c</sub> in the IL-13R complex may be related to the drastic reduction in IL-13 binding. This structural modification of the IL-13R complex by
γc may also have affected the affinity of its binding to IL-13. In this study, we evaluated IL-13 binding by 125I-IL-13 displacement assays and our results suggest that the affinity of IL-13 binding is lower in MLneo than in MLγc. However, in other studies\(^2\), we have proposed a different model for the analysis of IL-13 binding to these cells, which may be more appropriate for addressing the apparent cluster distribution of IL-13R on these cells. While γc may have favored high affinity IL-13 binding, it may also have disrupted necessary interactions between the different chains of IL-4R and IL-13R through the sequestration of IL-4Rβ by γc.

The loss of IL-4 and IL-13 signaling in MLγc may also have resulted from a disruption of necessary interactions between the intracellular domains of the receptor for IL-4 and IL-13. It was recently shown that the IL-4Rβ protein undergoes homodimerization for IL-4 signaling and that γc is not required for the phosphorylation and activation of STAT6 protein (32, 33). Although there is no direct evidence that the different intracellular domains of IL-4R and IL-13R interact, IL-13 has been shown to phosphorylate IL-4Rβ (24). Thus it is quite likely that the introduction of γc interferes with the assembly of a functional IL-4R complex, resulting in an impaired intracellular signaling by IL-4 and IL-13. It is also possible that the introduction of γc may have activated phosphatases whose activities could have resulted in the disruption of IL-13 and IL-4 signaling. Additional studies are necessary to address these possibilities.

We next examined the effect of γc on the functional response of ML-RCC cells to IL-4 and IL-13 stimulation. Surprisingly, in γc-transfected MLγc cells, there was a distinct change in morphology accompanied by a drastic diminution of ICAM-1 expression, and neither interleukin reversed these changes. The mechanism(s) underlying these changes is not clear but our results suggest that the disruption of IL-13Rα is directly or indirectly involved in a structural modification of the cytoskeletal framework of the cell to alter its shape and size. Whether this morphological effect is related to the loss of ICAM-1 expression is not clear. Alternatively, the introduction of γc into the cells may have turned off ICAM-1 gene expression and cellular responsiveness to IL-13 and IL-4. Additional studies are needed to improve our understanding of these events.

In summary, we have provided evidence for a novel role for γc protein in ML-RCC cells. Not only did this protein inhibit the binding of IL-13 to its receptor but, more importantly, it also inhibited intracellular signaling induced by IL-13 and IL-4. In addition, γc modified cellular function in these cells by inhibiting constitutive IL-13Rα and ICAM-1 expression, and this inhibition was not restored by IL-4 or IL-13. Our results suggest that an abnormal expression of γc may have significant disease implications with or without IL-13 and IL-4 involvement and that additional studies are needed to better understand the role of γc in tumor cell function.

\(^2\) V. A. Kuznetsov, N. I. Obiri, and R. K. Puri, submitted for publication.

1 ng of 32P-labeled GRR probe in 20 μl of binding buffer. A 200-fold excess of cold GRR probe was added as a competitor. For the supershift assay (B), cell lysates were preincubated with preimmune rabbit serum as a control or STAT1, 3, 4, 5, and 6 antibodies on ice for 30 min; standard EMSA was then performed as described above. C, following IL-13 and IL-4 treatment, cells were lysed in lysing buffer and STAT6 protein was immunoprecipitated (IP) with specific antibody. The complex was separated by SDS-PAGE and transferred onto PVDF membrane. STAT6 phosphorylation was detected by anti-phosphotyrosine antibody (4G10) (upper panel). STAT6 protein expression was confirmed by reprobing the stripped blot with STAT6 antibody (lower panel).
Modulation of IL-13 Signaling by $\gamma_c$

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