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*J Immunol* 2001; 167:6884-6892; 
doi: 10.4049/jimmunol.167.12.6884
http://www.jimmunol.org/content/167/12/6884

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Differential IL-10R1 Expression Plays a Critical Role in IL-10-Mediated Immune Regulation

Yaozhong Ding, * Lihui Qin, * Dmitriy Zamarin, * Sergei V. Kotenko, † Sidney Pestka, † Kevin W. Moore, ‡ and Jonathan S. Bromberg *

In this study, we characterized the differential receptor-binding specificity, affinity, and Janus kinase-STAT activation of cellular IL-10 (cIL-10) compared with viral IL-10 (vIL-10). Only cells expressing IL-10R1 bind human IL-10 or vIL-10. IL-10R2 does not bind to cIL-10 or vIL-10 alone and its presence does not enhance the receptor-binding affinity of cIL-10 or vIL-10, but it is essential for both cIL-10- and vIL-10-mediated signal transduction and immune regulation. Responses initiated by cIL-10 and vIL-10 were compared in B cell and mast cell lines, and demonstrated that the inability of vIL-10 to stimulate immune responses, as compared with human IL-10, is due to failure to initiate signaling. Absent signal transduction is due to low level expression of cell surface IL-10R1, since overexpressing IL-10R1 allows vIL-10 to initiate cIL-10-like signals and subsequent biological responses. These results are similar in primary cells, since splenocytes respond to both cIL-10 and vIL-10, while thymocytes respond only to cIL-10 and have very low mouse IL-10R1 but not mouse IL-10R2 expression. These data demonstrate that IL-10R1 expression plays a critical role in determining whether cells respond to IL-10. Modulation of cell surface IL-10R1 density might be an important mechanism for determining whether IL-10 leads to immunostimulation or immunosuppression in vivo. The Journal of Immunology, 2001, 167: 6884–6892.

The biological effects of cytokines are mediated through cell surface receptors. These receptors transduce the binding of their cognate cytokines into cytoplasmic signals that eventually trigger a cascade of intracellular responses. The functional receptor complex of IL-10 consists of at least two subunits, IL-10R and IL-10R2. IL-10R1 is a cell surface receptor with a single transmembrane domain and is a member of the class II cytokine receptor family (1, 2). Recently, the second chain of the IL-10R complex, IL-10R2, was identified and characterized (3), and later it was shown that mice with a disruption of the IL-10R2 (CRF2–4) gene, found in a cluster with other IFNR genes on chromosome 21 in humans and chromosome 16 in mice. IL-10R2 also belongs to the class II cytokine receptor family (3, 4). Only cells expressing both human IL-10 (hIL-10)1 R1 plus hIL-10R2 transduce hIL-10-mediated signals in Chinese hamster ovary (CHO) cells, indicated by STAT activation, and the ternary complex of ligand plus two heterologous receptor chains can be coprecipitated (3). CRF2–4 (IL-10R2) knockout mice have characteristics similar to IL-10 knockout mice and developed chronic colitis (7, 8), indicating that IL-10R2 is essential for IL-10-mediated immune responses (3, 4).

hIL-10R1 is species specific and does not bind the mouse IL-10 (mIL-10), whereas mIL-10R1 binds both mIL-10 and hIL-10 (1, 2). Parenthetically, the homologue of the EBV BCRF1 gene product, known as viral IL-10 (vIL-10), is active on both murine and human cells (9–13). Despite the evidence that vIL-10 shares many immunosuppressive activities with cellular IL-10 (cIL-10) (9–13), vIL-10 lacks several cIL-10 activities on certain cell types. vIL-10 neither up-regulated MHC class II expression on mouse B cells, nor stimulated mouse thymocyte or mast cell proliferation (11, 14, 15). The findings in vivo that vIL-10, but not cIL-10, prolongs allograft transplant survival while cIL-10, but not vIL-10, accelerates tumor rejection confirmed their differential activities (16–18). Further analysis of IL-10R1 function showed that vIL-10 binds to IL-10R1 with at least 1000-fold lower affinity than cIL-10 (18, 19). Despite its poorer receptor-binding ability, vIL-10 has similar specific activities on certain cells (9–13, 18, 19). The existence of additional IL-10R component(s) besides IL-10R1 that may complement the deficiency in binding and/or mediate enhanced IL-10R activation by vIL-10 has been proposed (1, 2, 19). The discovery of IL-10R2 has made it possible to investigate whether IL-10R2 differentially regulates vIL-10 binding and/or signal transduction, and whether differentially regulated receptor binding and/or signal transduction lead to changes in the spectrum of biological activities.

The binding of IL-10 to its cell surface receptors activates the Janus kinase (JAK)-STAT signal transduction pathway. Following the ligand-receptor interaction, Jak1 (associated with IL-10R1) and Tyk2 (associated with IL-10R2), members of the receptor-associated Janus tyrosine kinases (JAK) family, are phosphorylated. A family of latent cytoplasmic transcription factors, STATs, are then activated (20–25). Among the seven mammalian family members,
Stat1, Stat3, and, in some cells, Stat5 are activated by IL-10 (3, 23). Further studies proved that Stat3 activation is essential for all known IL-10-mediated immune responses, although the activation of Stat3 alone is not sufficient for IL-10-mediated anti-inflammatory response (26, 27). However, signal transduction initiated by vIL-10 has not been fully investigated.

To address these issues, we compared the roles of IL-10R2 in receptor binding and activation for hIL-10 and vIL-10. Consistent with previous findings (3, 18, 19), we demonstrate here that although IL-10R2 does not bind either hIL-10 or vIL-10, its presence is essential for both hIL-10- and vIL-10-mediated signal transduction. Furthermore, we found that hIL-10 and vIL-10, despite having different binding affinities, mediate identical signal transduction in cells in which they induce similar biological responses. However, the inability to stimulate immune responses in MC/9 cells by vIL-10, compared with hIL-10, is largely due to the complete inability to initiate any signal activation in these cells. The inability to initiate signaling by vIL-10 is mainly due not wholly due to low level expression of cell surface IL-10R1. Higher level IL-10R1 expression on stably transfected MC/9 cells allows vIL-10 to have hIL-10-like activity in terms of signal transduction and biological responses. This suggests that cell surface IL-10R1 expression is a determining factor in cellular responses to vIL-10 and implies that limited activity on certain cell types may account for the differential immune regulation profile between cIL-10 and vIL-10. These results also indicate that through modulation of cell surface IL-10R1, IL-10 could have immunostimulatory or immunosuppressive profiles in vivo.

Materials and Methods

Cell lines

The 16–9 CHO cells transfected with an HLA-B7 reporter construct and expressing hIL-10R1/hIFN-γR1 chimera, hIL-10R2, or hIL-10R1/hIFN-γR1 plus hIL-10R2 (3) were maintained in complete F12 medium containing 450 μg/ml G418. The mouse pro-B cell line Ba/F3 expressing recombinant mIL-10R1 was maintained in RPMI 1640 medium containing 10% FCS, 5 mg/ml murine IL-3 (PeproTech, Rocky Hill, NJ), and 1 mg/ml G418 (Life Technologies, Grand Island, NY) (1). The mouse mast cell line MC/9 was purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM medium containing 10% FCS and 5% Con A supernatant prepared from CBA mouse spleen cells. Thymocytes and splenocytes were prepared from adult CBA mouse thymus and spleen as previously described (18).

Proteins and Abs

hIL-10, hIL-10A87A, vIL-10, and vIL-10A87I were prepared as previously described (18). The 1B1.2 rat anti-mouse IL-10R1 hybridoma (26) was grown and culture supernatants were purified by MabTrap G H columns (Amersham, Uppsala, Sweden). Biotin-labeled anti-mIL-10R1 was purchased from BD Pharmingen (San Diego, CA). Anti-phosphorylated Stat1, anti-phosphorylated Stat3, anti-phosphorylated Stat5, anti-Stat1 and anti-Stat3 (New England Biolabs, Beverly, MA), and anti-Stat5 (BD Pharmingen) were purchased.

125I-labeled hIL-10 binding/competitive displacement

125I-labeled hIL-10 (75–140 μCi/μg) was purchased from NEN Life Science Products (Boston, MA). 125I-labeled hIL-10 binding and competitive displacement was assessed as described elsewhere (18, 28, 29). CHO cells (1 × 10^6) expressing various IL-10R component(s) were incubated in 200 μl as duplicate samples in various concentrations of 125I-labeled hIL-10 (binding) or 100 pM 125I-labeled hIL-10 in the presence of between 0.6 pM and 200 nM hIL-10 or vIL-10 (competitive) for 4 h at 4°C in RPMI 1640, 2% BSA, and 0.02% sodium azide (binding buffer), with shaking. Reaction mixtures were overlaid onto 150 μl of dibutyl phosphate/dioctyl phosphate (3:2) in conical bottom tubes and centrifuged for 1 min (Eppendorf 5413). The pellets were cut off with a razor blade and analyzed in a COBRA II gamma counter (Packard Instrument, Downers Grove, IL).

EMSA of STATs

CHO cells (2 × 10^6) expressing different IL-10R component(s) were treated with either hIL-10 or vIL-10 for 15 min. The cells were lysed and nuclear extracts obtained for binding to 32P-labeled 22-bp IFN-γ-activation sequence element in the promoter region of the human IFN-γ gene (3, 18). For supershift assays, 1 μl of anti-Stat1 or anti-Stat3 Abs was added to the incubation mixture, incubated for 20 min at 22°C, and then 4 μl of the reaction mixture was electrophoresed at 200 V for 4 h at 4°C on a 16 × 16-cm 5% polyacrylamide (19:1 acrylamide: bisacrylamide) gel. The dried gel was exposed to Kodak XAR-5 film (Kodak, Rochester, NY) with an intensifying screen for 1 day at −80°C. When Ba/F3-mIL-10R1 or MC/9 cell lines were used, 5 × 10^4 cells were plated in complete RPMI 1640 medium, cultured for 4 h, and then stimulated with hIL-10 or vIL-10 at various times. Nuclear extracts and EMSAs were performed as previously described by using the 32P-labeled 22-bp probe based on the IFN-γ response region (GRR) within the promoter of the FcγR1 gene (25).

Northern blot hybridization analysis

Total RNA was isolated from Ba/F3, Ba/F3 transfected with mIL-10R1, and MC/9 cell lines by an acid guanidium thiocyanate-pheno1-chloroform extraction method, and poly(A)^+ RNA was purified using a RiboSep mRNA isolation kit (Collaborative Research, Bedford, MA). Northern blot hybridization was performed as recommended by protocol with the PerfectHyb Plus hybridization buffer system (Sigma–Aldrich, St. Louis, MO). Full-length mIL-10R1 and mIL-10R2 probes were generated by RT-PCR.

Flow cytometric analysis

Cells were washed three times in PBS supplemented with 1% BSA (PBS/BSA) and then stained with 5 μg/ml purified or biotin-labeled rat anti-mIL-10R1 mAb in PBS/BSA at 4°C for 45 min. After washing three times with PBS/BSA, cells were then stained with goat anti-rat IgG conjugated with FITC or streptavidin-PE for 45 min at 4°C (1:30 dilution; BD Biosciences, Mountain View, CA). The cell surface expression of the mIL-10R1 was analyzed in a BD Biosciences FACScan using CellQuest software. Controls included unstained cells or secondary reagent alone.

Generation of stable MC9-mIL-10R1 cell lines

mIL-10R1 cDNA was cloned into the pcdNA3.1 vector (Invitrogen, Carlsbad, CA) by RT-PCR from Ba/F3-mIL-10R1 cells and sequenced to confirm fidelity. pcdNA3.1-mIL-10R1 plasmid was linearized by SspI enzyme digestion. DNA constructs were then transfected into 1 × 10^6 MC9 cells at 600 V in a cuvette with a 0.2-cm electrode gap in 200 μl of DMEM medium by Gene Pulser apparatus (Bio-Rad, Hercules, CA). Two days later, cells were cultured with selective medium containing 1.2 mg/ml G418 and 2 wk later flow cytometry was performed to verify mIL-10R1 expression. Cell sorting was conducted on single-cell suspensions stained with mIL-10R1 held on ice during sorting. The selected populations expressing different levels of mIL-10R1 were sorted directly into tubes with DMEM medium with 5% Con A supernatants. All cells were routinely monitored by flow cytometry to ensure stable expression of mIL-10R1.

Proliferative responses

MC9 cells were rested in RPMI 1640 with 10% FCS overnight, then 5 × 10^4 cells were plated in 96-well round-bottom plates (Corning, Corning, NY) at a density of 5 × 10^3 cells/well in 200 μl hIL-10 or vIL-10, with or without 5 μg/ml anti-mIL-10R1 mAbs, was added as indicated at a range of concentrations. Cells were incubated with stimuli for 24 h, and proliferation was assessed 6 h after adding 1 μCi/well [3H]thymidine (Amersham Life Sciences, Arlington Heights, IL) by measuring radioactivity using a MicroBeta TriLux β counter (Wallac, Turku, Finland). Thymocyte proliferation assay was performed as previously described (18).

Results

IL-10R2 does not bind or enhance the binding to IL-10

To determine whether IL-10R2 binds or enhances the binding of IL-10 to IL-10R1, 16–9 CHO cells expressing various IL-10R components were used. IL-10-induced signaling and biological responses are cell restricted and CHO cells will not transduce IL-10R and Stat3 events, but will transduce IFN-γ-like signaling and Stat1-dependent MHC class I up-regulation (3). Therefore, cells were transfected to express hIL-10R2 alone; hIL-10R1 chimeric receptor, in which the transmembrane and intracellular domains of
the hIFN-γR1 chain are substituted for the transmembrane and intracellular domains of the hIL-10R1 chain; or both receptors. Nontransfected 16-9 CHO cells were included as a control. As shown in Fig. 1A, CHO cells and cells expressing IL-10R2 do not bind 125I-labeled hIL-10. In contrast, cells expressing IL-10R1 alone or IL-10R1 along with IL-10R2 bind 125I-labeled hIL-10. These data demonstrate that IL-10R1 but not IL-10R2 binds hIL-10. Scatchard analysis showed that the calculated $K_d$ for hIL-10 is $\sim 56$ pM on cells expressing IL-10R1/γR1 alone or both receptors (Fig. 1B). These data indicate that IL-10R2 does not enhance the binding affinity of hIL-10 for IL-10R1.

The fact that vIL-10 has the same specific activity on certain cells, despite impaired receptor binding affinity, suggested that IL-10R2 might differentially bind or increase the binding affinity of IL-10R1 for vIL-10 (19). A quantitative comparison of hIL-10 and vIL-10 binding was obtained in competitive displacement experiments in cells expressing IL-10R1 or IL-10R1 plus IL-10R2 (Fig. 1C). Cells were incubated with a constant concentration of 125I-labeled hIL-10 along with various amounts of unlabeled hIL-10 or vIL-10. As expected, hIL-10 is an effective competitor while vIL-10 is a very weak competitor. The receptor binding affinity for hIL-10 is at least 1000-fold higher than vIL-10. The data showed that there are

**FIGURE 1.** IL-10R1 but not IL-10R2 is required to bind hIL-10 or vIL-10. A, CHO cells expressing IL-10R1/γR1 bind 125I-labeled hIL-10 and 125I-labeled hIL-10 binds to CHO cells expressing IL-10R1/γR1 or IL-10R1/γR1 plus IL-10R2 with similar specific capacity. B, Scatchard analysis of ligand binding. CHO cells (1 × 10⁶) expressing various IL-10R components were incubated in 200 μl in concentrations between 0.6 pM and 200 nM of 125I-labeled hIL-10. The calculated value of $K_d$ is 56 pM, and the IL-10R number is 2300 per cell for IL-10R1/γR1 cells and 2700 per cell for IL-10R1/γR1 plus IL-10R2 cells. C, Comparison of hIL-10 and vIL-10 as competitors for hIL-10 binding to hIL-10R in CHO cells. Cells were incubated with 100 pM 125I-labeled hIL-10 plus various concentrations of unlabeled hIL-10 or vIL-10 as competitors. The assays were performed in triplicate and the data are presented as mean cpm ± SD. D, IL-10R2 is required for hIL-10 or vIL-10 signal transduction. EMSA in hamster 16-9 cells. Hamster cells were stably transfected with the receptor expression plasmids indicated in the figure. Specific anti-Stat1 Ab was used for supershift assays. The positions of Stat DNA-binding complexes are indicated by an arrow.
no binding differences for either hIL-10 or vIL-10 on cells expressing IL-10R1 alone or both receptors, indicating that IL-10R2 does not bind to or enhance binding affinity of vIL-10 to the IL-10R complex.

Previous studies revealed that only cells expressing both IL-10Rs could mediate cIL-10-induced signal transduction and immune responses (3). To determine whether the functional difference between cIL-10 and vIL-10 is due to a differential utilization of the IL-10R complex, we examine the signal transduction of vIL-10 in CHO cells expressing IL-10R2 alone, IL-10R1/IFN-γR1 chimeric receptor alone, or both receptors. We previously showed that hIL-10 stimulates IFN-γ-like responses only in those cells that express both receptors, inducing both Stat1 and MHC class I Ag reporter gene up-regulation (3, 18). Fig. 1D shows that both vIL-10 and hIL-10 induced STAT activation only in cells expressing both receptors, and the DNA-binding complex can be completely supershifted by anti-Stat1 mAb. This indicates that despite affinity differences, vIL-10 induces the same STAT protein activation as hIL-10, and this activation requires expression of both receptors. Flow cytometric analysis of MHC class I Ag expression on the same cells showed that both hIL-10 and vIL-10 induced similar class I MHC up-regulation (data not shown). This further supports the finding that vIL-10 induces signal transduction and immune responses through the same receptor complex as does cIL-10 in these cells.

Both hIL-10 and vIL-10 induce STAT activation in Ba/F3 cells

hIL-10 and vIL-10 have similar specific activities despite 1000-fold differences in receptor binding affinity in Ba/F3 cells (16, 17). To investigate whether vIL-10 induces different signals compared with hIL-10, Ba/F3 cells stably transfected with mIL-10R1 were used to examine IL-10-mediated JAK-STAT activation. It was previously reported that cIL-10 treatment of these IL-10R1-bearing Ba/F3 cells led to activation of Stat1, Stat3, and Stat5 (25, 26). Fig. 2A shows that vIL-10 and hIL-10 induce similar DNA-binding complexes, and the DNA-binding complexes appeared within 5 min, were maximal by 15 min, and disappeared 90 min after stimulation (Fig. 2A). Fig. 2B shows that the dose response of STAT activation to hIL-10 and vIL-10 are also similar. These data indicate that hIL-10 and vIL-10 are concordant in their abilities to induce JAK-STAT in this Ba/F3 cell line. Fig. 3C further shows that both ligands similarly activate Stat1, Stat3, and Stat5 in this cell line.

Impaired signal transduction in discordant MC/9 cells

Since cIL-10, but not vIL-10, is a potent stimulator of murine MC/9 mast cell line proliferation (4, 6), we next examined STAT activation by hIL-10 and vIL-10 using EMSAs in this discordant cell line. In hIL-10-stimulated cells, STAT activation occurred within 10 min (Fig. 3A). However, in vIL-10-treated cells, STAT activation was not detected, even after prolonged stimulation (Fig. 3A). The supershift experiment shows that the DNA-binding complexes in MC/9 cells consist of at least Stat1 and Stat3 (Fig. 3B). Western blotting is consistent with these findings and shows that IL-10 activates only Stat1 and Stat3 in MC/9 cells (Fig. 3C). Fig. 3C also demonstrates that hIL-10 but not vIL-10 induced STAT activation in MC/9 cells; vIL-10-induced STAT phosphorylation only occurred in Ba/F3 cells (Fig. 3C). These results demonstrate that proliferative responses correlate with STAT activation, and that hIL-10 induces normal STAT activation, whereas vIL-10-induced signal transduction appears to be completely impaired in MC/9 cells.

Impaired signal transduction by vIL-10 in MC/9 cells is due to differential IL-10R1 but not IL-10R2 expression

IL-10 and IL-10Rs share many structural and functional features with IFN-γ and IFNRs (1, 2, 30–36). In addition, IFN-γ activates signal transduction in Th2 but not Th1 cells, due to an absence of IFN-γR2 in the Th1 cells (37, 38). Therefore, we determined whether the unresponsiveness of MC/9 cells to vIL-10 is due to lack of expression of IL-10R2. Northern blot analysis shows that there are similar levels of IL-10R2 mRNA expression in Ba/F3 and MC/9 cells (Fig. 4A). The result is consistent with the fact that hIL-10 can mediate normal signal transduction in these cells and suggests that differential IL-10R2 expression likely does not play a role in the differential activities between hIL-10 and vIL-10 in MC/9 cells.

On the contrary, IL-10R1 mRNA is expressed at much higher levels in Ba/F3-mIL-10R1 cells compared with MC/9 cells (Fig. 4A), consistent with the fact that Ba/F3-mIL-10R1 was established by stably transfecting mIL-10R1 into Ba/F3 cells (1, 19). Flow cytometric analysis to measure cell surface mIL-10R1 protein expression (Fig. 4B) confirms that mIL-10R1 is expressed at much higher levels on Ba/F3-mIL-10R1 than MC/9 cells. Scatchard analysis further confirms there are ~3000 receptors per Ba/F3-mIL-10R1 cell with an affinity of 50 pM (23), compared with 200 receptors per MC/9 cell with the same affinity (Fig. 4C).

High-level IL-10R1 expression can render MC/9 cells responsive to vIL-10

To determine whether the differential expression of IL-10R1 between Ba/F3-mIL-10R1 and MC/9 cells could account for the differential signal transduction and proliferative responses by hIL-10

FIGURE 2. Human IL-10 and vIL-10 induce similar STAT activation in Ba/F3-mIL-10R1 cells. Nuclear extracts were prepared from Ba/F3 cells stably transfected with mIL-10R1, treated or untreated with hIL-10 or vIL-10, and examined for (A) kinetics after treatment with 30 ng/ml hIL-10 or vIL-10 or (B) dose response of STAT activation was performed 15 min after treatment.
and vIL-10, we generated a series of MC/9 cells expressing different levels of mIL-10R1. Full-length mIL-10R1 cDNA was generated by RT-PCR from Ba/F3-mIL-10R1 cells and cloned into the pcDNA3.1 expression vector. This plasmid encoding mIL-10R1 was transfected into MC/9 cells by electroporation, and stable transfecants were then selected in the presence of G418. We were able to establish MC/9 cells expressing different levels of mIL-10R1 by FACS sorting (Fig. 5A). The higher level of expression in MC/9 cells was comparable to that in Ba/F3-mIL-10R1 cells, while medium and low-level mIL-10R1 expression were considerably less.

Next, these cells were stimulated with IL-10 and EMSAs were performed. As shown in Fig. 5B, the intensity of STAT activation by hIL-10 in MC/9 is dependent upon cell surface mIL-10R1 expression. In medium-level mIL-10R1-expressing cells, STAT activation by hIL-10 is stronger than in MC/9 or MC/9-neo, which express low levels of mIL-10R1, while the activation was strongest in cells expressing high levels of mIL-10R1. No vIL-10-induced STAT activation was observed in MC/9 cells or MC/9 cells expressing the empty pcDNA3.1 vector. However, in vIL-10-stimulated MC/9 cells expressing medium or high levels of mIL-10R1, activation of STATs was observed. The intensity of STAT activation is also dependent on the cell surface mIL-10R1 expression; it is much stronger in MC/9 expressing high levels of mIL-10R1 compared with cells with medium levels of mIL-10R1 expression. Supershift experiments demonstrated that the DNA-binding complexes activated by hIL-10 in MC/9 cells expressing higher level mIL-10R1 were mostly Stat3 along with some Stat1 (Fig. 5C). In vIL-10-stimulated cells, the major DNA-binding complex was also Stat3 (Fig. 5C), and Stat1 activation could be observed after longer film exposure of 72 h instead of 36 h (data not shown). The combination of anti-Stat1 and anti-Stat3 supershifted the majority of DNA-binding complexes, suggesting that Stat1 and Stat3 activation were the most dominant events in these cells, consistent with the Western blotting results of Fig. 3C. We next determined whether higher level mIL-10R1 expression could lead to proliferative responses in MC/9 cells by vIL-10. Fig. 5D demonstrates that vIL-10 could induce MC/9 proliferation only in those cells expressing higher densities of cell surface mIL-10R1. Anti-mIL-10R1 mAb completely abrogated proliferation, proving that the proliferative response requires a specific receptor-ligand interaction, rather than the overexpression of the mIL-10R1 acting as a coreceptor in a novel fashion. These data show that cell surface receptor density determines both the threshold and the magnitude of response to high- and low-affinity ligands. However, despite the three orders of magnitude differences in ligand affinity, receptor densities among the cell lines differ only 10-fold (Figs. 4C and 5A) and signaling responses also differ by far less than 1000 (Fig. 5B), once threshold is reached.

**FIGURE 3.** hIL-10 but not vIL-10 induces STAT activation in MC/9 cells. A, Kinetics of response to hIL-10 and vIL-10. Cells were treated for 5, 15, or 30 min, and EMSA was performed. B, Analysis of EMSA complex. MC/9 cells were treated with hIL-10 for 10 min, and anti-Stat1 and anti-Stat3 were used in the supershift assay. C, Phosphorylation and activation of Stat3, Stat1, and Stat5 induced by IL-10 in Ba/F3 cells or MC/9 cells. Ba/F3 transfected with mIL-10R1 and MC/9 cells were treated with PBS (Control), hIL-10, or vIL-10. Cell lysates were then probed with Abs specific for phosphorylated STATs or total STATs as indicated.

**Murine IL-10R1 expression is associated with the biologic responses of primary cells to cIL-10 and vIL-10.** cIL-10 but not vIL-10 enhances IL-2 plus IL-4-driven proliferation of thymocytes, while other cells (e.g., splenocytes) respond similarly to cIL-10 or vIL-10 (1, 14, 18, 19). To investigate whether the lack of a biological response to vIL-10 in thymocytes is also due to insufficient mIL-10R1 expression, mouse thymocytes and splenocytes were isolated and flow cytometric analysis was performed. As predicted, thymocytes express much lower levels of mIL-10R1 compared with splenocytes (Fig. 6A). Northern blotting on thymocytes and splenocytes also showed that IL-10R1 mRNA is expressed at much higher levels in splenocytes compared with thymocytes, while IL-10R2 mRNA is expressed at similar levels.
We next investigated Stat3 activation in both splenocytes and thymocytes. As shown in Fig. 6C, hIL-10 activates Stat3 phosphorylation in both splenocytes and thymocytes, and the activation is weaker in thymocytes. In contrast, vIL-10 induces Stat3 activation only in splenocytes but not thymocytes, and induces a weaker phosphorylation compared with hIL-10, probably due to its lower receptor affinity. These data support the notion that levels of IL-10R1 expression are also critical in determining IL-10-mediated immune responses of primary cells.

Discussion
Using cell lines expressing different IL-10 receptors, we first compared the binding of hIL-10 and vIL-10 to different IL-10R subunits. The data confirmed that only IL-10R1 binds to hIL-10 and vIL-10, and the affinity for vIL-10 is at least 1000-fold less than hIL-10 (Fig. 1). Expression of IL-10R2 did not change the IL-10R1-binding affinity for hIL-10 or vIL-10, showing that IL-10R2 is not involved in binding to the ligand (Fig. 1). Next, comparing signaling events, only cells expressing both IL-10R1 plus IL-10R2 can respond to vIL-10, consistent with previous findings with hIL-10 (Fig. 1D; Ref. 3). These data demonstrate that IL-10R2 is an essential receptor subunit for both cIL-10- and vIL-10-mediated signal transduction. vIL-10 and hIL-10 induce Stat1, Stat3, and Stat5 activation in the pro-B cell line Ba/F3 cells transfected with mIL-10R1 (Figs. 2 and 3). Collectively, these studies demonstrate that vIL-10 is capable of inducing the same signals and biological responses as cIL-10.

To investigate the mechanism for vIL-10 unresponsiveness in certain cells, the STAT activation mediated by hIL-10 and vIL-10 in the MC/9 mast cell line was studied. It has been previously shown that hIL-10 but not vIL-10 induces proliferation in these cells (11, 14). Here, we found that there is no detectable STAT activation after vIL-10 stimulation, indicating severely impaired signal transduction (Fig. 3). This signal transduction defect was not due to defects in JAKs or STATs, since hIL-10 can mediate normal JAK-STAT activation in these cells (Fig. 3).

Differential receptor component expression on different cell types is an important mechanism for cytokines to selectively regulate their biological responses. For example, like IL-10, the functional IFN-γR complex is also composed of two chains, IFN-γR1 and IFN-γR2 (34–36). Although IFN-γR1 binds IFN-γ with high affinity, only cells expressing both receptor components can mediate IFN-γ-exerted signal transduction and biological activity. IFN-γ inhibits the proliferation on Th2 cells but not on Th1 or Tc1 cells. Unresponsiveness to IFN-γ on Th1 cells is due to lack of IFN-γR2 expression on Th1 or Tc1 (37, 38). Down-regulation of the IFN-γR2 expression in Th1 cells may allow the immune system to selectively inhibit Th2 cells and promote Th1 cell phenotypes. Furthermore, Novelli et al. (39) showed that proliferation or apoptosis of T cells was dependent on the ratio of the IFN-γR1 and
IFN-γR2 chains on the cell surface. Because IL-10 bears close topological resemblance to IFN-γ and their receptor complexes also belong to the same cytokine receptor family, we investigated whether IL-10R2 plays the same role in determining whether cells respond to vIL-10. The results show that IL-10R2 is expressed at similar transcriptional levels in both Ba/F3 and MC/9 cells, and indicate that it is unlikely that IL-10R2 determines differential responses to vIL-10 (Fig. 4).

We next investigated whether the lack of signal transduction in MC/9 cells is due to insufficient mIL-10R1 expression. Consistent with this hypothesis, vIL-10 induced STAT activation and proliferation in MC/9 cells expressing higher levels of mIL-10R1 (Fig. 5), and an increase in intensity of DNA-binding complexes results from an increase in the density of cell surface mIL-10R1 (Fig. 5B). Thus, upon increasing cell surface IL-10R1 expression, vIL-10 mediates similar signal transduction and immune responses as does hIL-10; cell surface receptor expression is a critical factor in determining whether cells are responsive to the low-affinity ligand.

The finding that the density of IL-10R1 expression on the cell surface plays a critical role in determining whether certain cells are activated by an IL-10 molecule may explain the differential in vivo immune regulatory profiles among various IL-10 molecules (26, 27). vIL-10, which has 1000-fold lower affinity, may only activate cells with a high level of receptors. On the other hand, cIL-10 may activate cells expressing both low and high levels of IL-10R1. Depending on the method of administration (local or systemic), dose, immune mediators present, and the precise mixture of cells present, this could explain why vIL-10 is generally immunosuppressive, while cIL-10 is more pleiotropic in its effects. Our results also indicate that modulating IL-10R1 expression on certain cells might be an important mechanism for regulating IL-10-mediated immune responses. In a recent study on different types of dendritic cells, it was found that rheumatoid dendritic cells are resistant to the immunosuppressive effect of IL-10 in vitro due to insufficient expression of cell surface IL-10R1 (40).

Although the data explain why vIL-10 mediates cell proliferation in Ba/F3 cells but not in MC/9 cells, there is not a strict

**FIGURE 5.** Higher levels of IL-10R1 expression render MC/9 cells responsive to vIL-10. A, Cell surface mIL-10R1 on various MC/9 cells. MC/9 parent cells and MC/9 cells stably transfected and sorted for mIL-10R1 were stained with isotope control or anti-IL-10R1 mAb. Filled histogram, isotope control; thick line, MC/9 parent cells; dashed line, MC/9 cells expressing a medium level of mIL-10R1; and dotted line, MC/9 cells expressing the highest level of mIL-10R1. B, EMSA for various mIL-10R1 transfected MC/9 cells. C, Stat1 and Stat3 are activated in MC/9 expressing high levels of mIL-10R1. MC/9-mIL-10R1 (high expression) cells were treated with hIL-10 (30 ng/ml) or vIL-10 (30 ng/ml) for 7 min at 37°C. Anti-Stat1 and anti-Stat3 Abs were used for the supershift assay. D, Proliferative responses to vIL-10 in MC/9 cells expressing various levels of mIL-10R1. Cells were rested in complete media overnight and then treated with 10 ng/ml recombinant vIL-10, with or without 5 μg/ml anti-mIL-10R1 mAb.
correlation among affinities, receptor densities, and signaling responses, either within or between cell lines. Thus, hIL-10 and vIL-10 induce STAT activation and cell proliferation with the same specific activities in Ba/F3 cells, despite a 1000-fold difference in receptor-binding affinities. Similarly, once threshold is reached in MC/9 cells, receptor affinity, density, and signaling responses do not strictly correlate (Fig. 5). Thus, the signaling and biological responses do not represent linear correlates to ligand-receptor occupancy, indicating that additional mechanisms might regulate between Ba/F3 and MC/9 cell line responses.

First, it is possible that additional molecules are activated in one cell line but not the other. For example, beside Stat1 and Stat3 activation, Stat5 is activated in Ba/F3 cells expressing mIL-10R1 (25). This additional Stat5 activation appears to be cell restricted and recent findings suggested that Stat5 has pleiotropic functions regulating cell proliferation, differentiation, and apoptosis in the Ba/F3 cell line (41). Therefore, Stat5 might lower the threshold of proliferative signaling in Ba/F3 cells, resulting in similar responses, despite the 1000-fold difference in affinity. It is also possible there are differences in signal transduction molecules downstream of STAT activation between Ba/F3 and MC/9 cells.

A second possibility is that there is differential activity by negative regulation in Ba/F3 cells vs MC/9 cells. The suppressors of cytokine signaling (SOCS) family of molecules is characterized by an Src homology 2 domain and a carboxyl-terminal, unique conserved motif referred to as the SOCS box (42). There are at least eight SOCS proteins (SOCS1–7 and cytokine-inducible SH2-containing protein) and SOCS1 and SOCS3 appear to be the most potent inhibitors of cytokine signaling. It has been recently shown that SOCS3 plays a role in negatively regulating IL-10 signaling (43). Thus, it is possible that SOCS3 is regulated differently in Ba/F3 than in MC/9 cells. Our ongoing studies show that induction of SOCS3 expression by cIL-10 and vIL-10 is concordant in Ba/F3 cells but discordant in MC/9 cells (Y. Ding and J. S. Bromberg, unpublished observation) just as the other second messengers examined here. Thus, SOCS3 expression probably does not determine the differences between these two cell lines.

Third, there may be an additional component or components of the IL-10R complex. This component may complement the deficiency in binding and/or mediate enhanced IL-10R activation by vIL-10. Additionally, it must be noted that although we proved there are similar levels of IL-10R2 mRNA expressed in both Ba/F3 and MC/9 cells (Fig. 4C), we did not determine the levels of IL-10R2 protein due to lack of Abs against IL-10R2. Very recently, it was shown that IL-10R2 is involved in IL-22 binding and signal
transduction (44, 45). Although these reports did not provide evidence for receptor subunit competition, IL-22 and the IL-22R complex may affect IL-10-mediated signal transduction and biological responses. It must be noted that our Ba/F3 and MC9/lines do not express IL-22 transcripts (our unpublished data).

References


12. T. Decker. 1994. IL-10-induced factors belonging to the p91 family of proteins bind to IFN-γ receptor.


