Interleukin-9 (IL-9) Induces Cell Growth Arrest Associated with Sustained Signal Transducer and Activator of Transcription Activation in Lymphoma Cells Overexpressing the IL-9 Receptor

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Abstract
Murine interleukin (IL)-9 inhibits apoptosis in murine T lymphomas via signal transducer and activator of transcription (STAT) factors. After transfection of the human IL-9 receptor, human IL-9 had a similar antiapoptotic activity, but, unlike the mouse protein, inhibited proliferation. This effect was correlated with the level of receptor expression and the extent of STAT phosphorylation. Expression of a moderate level of suppressor of cytokine signaling 3 (SOCS3) reduced STAT activation by human IL-9 and prevented inhibition of growth but not of apoptosis. Using mutated IL-9 receptors, we showed that inhibition of proliferation was correlated with STAT1 and STAT3 activation by IL-9 and induction of the cell cycle inhibitor p19/ink4d, a STAT3 target gene. Activation of STAT1 by IFN-γ did not result in cell growth arrest. In this model, cell growth inhibition is therefore associated with a higher number of receptors, a more robust STAT activation, and a greater sensitivity to SOCS3 expression, compared to apoptosis inhibition.

Introduction
IL-94 was initially characterized as a growth factor for T-cell lines, mast cell lines, and hematopoietic tumor cells (1). However, it is a relatively weak growth factor for normal cells in vitro. In vivo, IL-9 is involved in immune reactions against parasites, in asthma, and in lymphoma development (2–4). IL-9 binds to a complex composed of the specific IL-9R and γc, a subunit shared with IL-2, IL-4, IL-7, and IL-15 (5).

IL-9 signaling involves the activation of JAK1 and JAK3 tyrosine kinases and STAT1, STAT3, and STAT5 transcription factors (5, 6). These factors play a key role in cytokine intracellular signaling, regulating apoptosis, cell growth, and differentiation (7). The mechanism that leads to their activation has been studied extensively. STATs are recruited via their SH2 domain to phosphorylated sites of activated cytokine receptors and phosphorylated on a conserved tyrosine by JAK kinases associated with the receptor. STAT phosphorylation induces their dimerization, migration to the nucleus, and binding to specific DNA sites in the promoter of cytokine-regulated genes (8). The STAT family comprises seven distinct members that are implicated in signaling by a large number of cytokines. STAT6 is preferentially activated by IL-4 and IL-13, whereas STAT4 is activated by IL-12. In contrast, STAT1, STAT3, STAT5A, and STAT5B contribute to the signaling pathways of many factors, including IL-9. In addition, STAT3 and STAT5 are constitutively activated in various human lymphomas, leukemias, and breast cancers (9).

Both STAT3 and STAT5 are important mediators of apoptosis inhibition by cytokines, such as IL-6 and IL-9 (10, 11). STAT3 and STAT5 are activated by a large number of onco-genes and are required for transformation by SRC and BCR/ABL, respectively (12, 13). Constitutive activation of these factors in tumors prevents apoptosis (14, 15). A mutated STAT3 variant that is constitutively activated has been shown to transform murine cells in vitro (16). Development of murine melanoma B16, which presents a constitutive activation of STAT3, could be blocked in vivo by introducing DNA coding for a dominant negative form of STAT3 into the tumor (14).

As opposed to these tumor-promoting activities, STATs have also been implicated in cell growth inhibition by cytokines. IL-6 decreases the growth of prostate cancer cells, melanoma cell lines, and M1 leukemia via STAT3 (17–19). STAT1 or STAT5 is a mediator of growth inhibition by epidermal growth factor, IFN-γ, and thrombopoietin (20, 21). Up-regulation of cell cycle inhibitors such as p21/waf1/cip1, p27/kip1, or p19/ink4d may explain these effects (17, 20, 22). It is critical to understand how STAT proteins can mediate survival and proliferation in some situations and growth arrest in others.

We had shown that IL-9 and IL-6 protect BW5147 T lymphoma cells from corticoid-induced apoptosis via the activation of either STAT3 or STAT5 (6, 10). In the present study,
we observed that IL-9 inhibited the growth of BW5147 cells that expressed high numbers of transfected human IL-9Rs. This effect was correlated with the number of IL-9Rs present at the cell surface and the kinetics of STAT1 and STAT3 activation.

Results
IL-9 Inhibits the Growth of T Lymphomas Overexpressing the IL-9R. Murine IL-9 inhibits apoptosis induced by corticoids in various T lymphomas such as BW5147 (23). IL-9 also up-regulates various genes, including Ly-6A/E, granzyme A, Bcl-3, and IL-TIF/IL-22, in the same cell line (10, 24–26). hIL-9, which does not bind to the murine IL-9R, has the same effects provided that the hIL-9R is transfected into BW5147 cells (BW-hIL9R). We observed that hIL-9 inhibited BW-hIL9R growth (Ref. 6 and Fig. 1A). Similar results were obtained by transfecting the hIL-9R into another T lymphoma, TH1821 (Fig. 1B).

To determine whether this effect was linked to the level of receptor expression, we selected 11 clones expressing the transfected IL-9R at various levels, as assessed by fluorescence-activated cell-sorting analysis with a specific anti-hIL-9R monoclonal antibody. The extent of growth inhibition by hIL-9 was also measured for each clone. Fig. 2A shows that there was a significant correlation between the IL-9R level and the reduction of proliferation by hIL-9 (r = 0.83; P = 0.0015). To compare the expression of the transfected hIL-9R and the endogenous murine IL-9R, we performed a flow cytometry experiment with fusion proteins consisting of a constant immunoglobulin γ3 domain and either human (solid line) or murine (dashed line) IL-9 and with antimouse immunoglobulin antibodies coupled to FITC. Cells were analyzed by flow cytometry.

Stronger STAT Activation in Cells Overexpressing the IL-9R. Because murine IL-9 and hIL-9 activate the same set of STAT proteins in parental and transfected BW5147 cells, respectively (6), we asked whether hIL-9R overexpression could result in a stronger STAT activation, which was tested by Western blotting with antibodies recognizing the tyrosine-phosphorylated form of STAT1, STAT3, or STAT5. Fig. 3 shows that murine IL-9 induced a transient STAT1 and STAT5 phosphorylation and a more sustained activation of the three STAT proteins in response to hIL-9R overexpression (clone 6; Fig. 3, right panel). This was confirmed by electrophoretic mobility shift assays (data not shown). After 24 h, STAT3 phosphorylation
was 3-fold more intense, as determined by quantification of the blot presented in Fig. 3. STAT activation level was intermediate in clone 45, which expressed hIL-9R to a lesser extent and whose proliferation was only weakly affected by hIL-9. Interestingly, IFN-γ induced a sustained STAT1 phosphorylation in BW5147 cells, similar to that induced by hIL-9 in BW5147-hIL9R-clone 45 (Fig. 3) but did not affect BW5147 cell growth or enhance growth inhibition by hIL-9 (data not shown). We did not observe phosphorylation of ERK1, ERK2, or protein kinase B kinases in response to murine IL-9 or hIL-9 in these cells, in contrast to other cell lines (28).

These observations raise the possibility that growth inhibition was elicited by a sustained STAT signaling by IL-9. To test this hypothesis further, we transfected SOCS3, an inhibitor of the JAK/STAT pathway (29). When strongly overexpressed, SOCS3 abrogates murine IL-9 signaling and activities, including STAT activation and inhibition of corticoid-induced apoptosis (30). Here, we used clones expressing an intermediate level of SOCS3 (30). STAT activation by hIL-9 was transient in these cells, unlike control clones (Fig. 4A). As expected, IL-9 did not affect cell growth in the presence of SOCS3 (Fig. 4B). In contrast, it still promoted cell survival in the presence of dexamethasone (Fig. 4C), supporting the hypothesis that growth inhibition in our model required a more robust STAT signaling than protection against apoptosis.

Growth Inhibition Is Correlated with STAT1 and STAT3 Activation and with Induction of p19/ink4d Expression.

We have shown previously that BW5147 growth inhibition depends on a single phosphorylated tyrosine of IL-9R (tyrosine 367 in the mature protein). Mutation of this amino acid into phenylalanine (mut1) also abolishes apoptosis inhibition and activation of STAT transcription factors by IL-9 (6). Recently, we described two other mutated IL-9Rs that more specifically activated either STAT5 (mut7) or both STAT1 and
Cell Growth Arrest and STAT Activation Kinetics

After 3 days, proliferation was evaluated by measuring [3H]thymidine incorporation. Clones expressing a similar level of receptor were stimulated with hIL-9. The cell cycle is controlled by cyclin-dependent kinases, which can be inhibited by three types of proteins: (a) p21/ waf1/cip1; (b) the kip family; and (c) the ink4 family. Several of these proteins have been implicated in cell growth inhibition by cytokines (17, 20, 22). To test whether IL-9 could induce the expression of such cell cycle inhibitors, we performed reverse transcription-PCR experiments with oligonucleotides specific for each family member (data not shown). We found that hIL-9 up-regulated ink4d expression in BW5147 cells, which was confirmed at the protein level by Western blotting (Fig. 6A). p19/ink4d expression was not significantly affected by murine IL-9 treatment, suggesting that this protein could be one of the mediators of hIL-9-induced cell growth arrest. Moreover, IL-9R mutations that blocked growth inhibition by hIL-9 (mut1 and mut7) also abolished ink4d induction, whereas the mut6 receptor behaved as the wild type (Fig. 6B), supporting the role of ink4d as a potential mediator of BW-hIL9R cell growth inhibition by IL-9.

Discussion

In this study, we show that the level of expression of the hIL-9R is correlated with the intensity of STAT signaling and the ability of hIL-9 to inhibit cell growth. These results point to the number of receptor molecules as a limiting factor for signal transduction. Accordingly, regulation of cytokine receptor expression levels, which is frequently observed, is a potent way of regulating cytokine responses.

Higher expression of the hIL-9R probably explains the difference between murine IL-9 and hIL-9 in regulation of BW5147 cell growth. Overexpression of the IL-9R affected activation of STAT1, STAT3, and STAT5 differentially. STAT1 and STAT5 activation was much stronger and lasted for a longer time after hIL-9R transfection. STAT3 activation was also increased significantly, although it was affected to a lesser extent. STAT3 could have a higher affinity for the single STAT docking site of the IL-9R and may compete for binding of other STATs in the presence of limiting numbers of receptors. Alternatively, there might be some differences between the affinities of the murine IL-9R and the hIL-9R for STATs, although the IL-9R STAT docking site region is highly conserved. Activation of phosphatases by these receptors may also be different, but there are no available data about activation of such enzymes by IL-9.

Using mutated IL-9Rs, we showed that growth inhibition was correlated with activation of STAT1 and STAT3 but not STAT5. STAT1 is a good candidate because its activation is more dramatically affected by transfection of the hIL-9R. However, because IFN-γ induced phosphorylation of STAT1 to a similar extent as hIL-9 in transfected BW5147 cells but did not inhibit cell proliferation, this activity is unlikely to be mediated by STAT1 alone. In addition, expression of a dominant negative variant of STAT1 failed to affect the growth inhibition effect of IL-9 (data not shown). We observed that this effect was associated with expression of the cyclin-dependent kinase inhibitor p19/ink4d, a well known inhibitor of cell cycle progression. This gene has also been implicated in growth inhibition by IL-6 and IL-10 (22, 31). Both studies demonstrated that ink4d induction was mediated by STAT3. Binding sites for this factor were found in the ink4d promoter (31). Interestingly, in our model, sustained ink4d expression was only observed when STAT3 was activated to the highest level. In contrast, a lower STAT3 activation by murine IL-6 or IL-9 is sufficient to induce the expression of various genes, such as Ly-6AE and Bcl-3 (24, 25). The molecular basis for this difference in sensitivity to STAT3 activation is not clear. Although ink4d up-regulation via STAT3 most likely plays a role in BW-hIL9R growth inhibition, other genes may also be involved. In this respect, we also observed a modest induction of p21/waf1/cip1 gene expression by hIL-9 (data not shown).

In BW5147 cells, STAT3 activation is also involved in apoptosis inhibition in response to IL-6 and IL-9 (10, 30). Thus, protection against apoptosis may require a lower level of STAT3 activation than cell growth inhibition. Our results could explain why STAT3 has been shown in some studies to favor tumorigenesis by inhibiting apoptosis and in other reports to block cell growth in similar cell lines (14, 17, 19, 32, 33). It is possible that a relatively weak constitutive or in-
duced STAT3 activation is enough to block apoptosis in these cells, whereas a stronger activation affects cell growth. In line with this hypothesis, tumor cell growth arrest in response to IL-6 often requires the addition of soluble IL-6Rα, which enhances STAT3 activation by this cytokine (17, 33). Recently, Nosaka et al. (34) observed that transfection of a constitutively activated STAT5 promoted the growth of Ba/F3 cells, whereas hyperactivation of STAT5 by IL-3 in these cells induced differentiation and apoptosis. Similarly, we suggest that the extent of STAT3 activation may critically affect the outcome for cell proliferation and survival.

Overexpression of SOCS proteins has been shown to completely block JAK/STAT signaling by various cytokines, including IL-9 (29, 30). Here, we show that a moderate level of SOCS3 expression, which is closer to the level that can be achieved in response to cytokines, has a differential effect on growth inhibition and apoptosis. This might suggest a more subtle role for SOCS proteins in specifically inhibiting some cytokine effects that require a more sustained signaling intensity.

Besides the JAK/STAT pathway, other mediators might contribute to growth inhibition by IL-9. IRS-1, IRS-2, protein kinase B, and ERK have been involved in IL-9 signaling (28). However, we failed to detect phosphorylation of these proteins in stimulated BW5147 or BW-hIL9R cells. Future studies will have to determine whether other signaling proteins play a role in this model.

In conclusion, our results suggest that kinetic parameters play a critical role in the regulation of cell growth by the JAK-STAT pathway. These parameters are influenced by the level of receptor expression and the presence of inhibitory proteins such as SOCS3.

**Materials and Methods**

**Cell Culture and Reagents.** BW5147 and TH1821 murine lymphoma cell lines were cultured in Iscove-Dulbecco’s medium supplemented with 10% FCS, 0.24 mM asparagine, 1.5 mM glutamine, 0.55 mM arginine, and 50 μM 2-mercaptoethanol. These cells were transfected by electroporation with the hIL9R inserted in the pEF-BOS-puro plasmid, as described previously (6). Transfected cell populations and clones were selected with puromycin (1.5 μg/ml; Sigma, Bornem, Belgium). BW-SOCS3 and control cells were transfected with hIL9R inserted in pEF-myc-cyto (Invitrogen, Carlsbad, CA) and selected with G418 [3 mg/ml; Life Technologies, Inc., Merelbeke, Belgium (30, 35)]. Recombinant hIL-9 and mouse IL-9 were produced in the baculovirus system and purified as described previously (6). Recombinant mouse IFN-γ was kindly provided by Dr W. Fiers (University of Gent, Gent, Belgium). Fusion proteins that consisted of a murine immunoglobulin γ3 Fc domain added at the COOH terminus of murine IL-9 or hIL-9 were produced in COS cells as described previously (27). For flow cytometry, cells were incubated with 10% COS cell supernatant and stained with FITC-conjugated antimiannobulin (Becton Dickinson, San Jose, CA). Hexoseaminidase assays, thymidine incorporations, and apoptosis assays were performed as described previously (6, 10). Each experiment was repeated three times with two to four independent clones.

**IL-9 Binding Experiments.** Mouse IL-9 and hIL-9 were labeled with Na125I using Iodobeads (Pierce Chemical Co., Rockford, IL) as described previously (36). Specific activities were 1775 and 484 cpm/fmol for murine IL-9 and hIL-9, respectively. Scatchard analysis of hIL-9 binding to BW-hIL9R indicated a Kd of 500 pm (data not shown), whereas previous determinations carried out on mouse cells gave values of ±100 pm (36). Determinations of receptor numbers were performed at concentrations equal to Kd × 5.

**Western Blots.** Stimulated cells were centrifuged and resuspended in SDS-PAGE sample buffer (BioRad) at 4 × 106 cells/ml. Proteins (25 μl) were separated on polyacrylamide gels (Novex, Groningen, the Netherlands) and transferred to nitrocellulose (Amersham, Roosendaal, the Netherlands). Western blots were performed with anti-phospho-specific antibodies recognizing STAT1 (Y701), STAT3 (Y705), or STAT5 (Y694) as described by the manufacturer (New England Biolabs, Beverly, MA). Controls for equal loading were performed with anti-actin monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed with chemiluminescence (ECL; Amersham) and quantified by a Kodak Imager charge-coupled device camera.
Northern Blots. Transfected BWS5147 cells were stimulated with murine IL-9 or hIL-9 (500 units/ml) for 24 h. Cells (10^6) were washed and lysed in 1 ml of Trizol solution (Life Technologies, Inc.). Total RNA was extracted according to the manufacturer's instructions, fractionated by electrophoresis in a 1.3% agarose gel containing 0.7 M formaldehyde (10 μg of RNA/ lane), and transferred onto Hybond-C Extra nitrocellulose membrane (Amersham) by downward capillarity (Turboblotter; Schleicher & Schuell, Keene, NH). A probe corresponding to the coding region of the (k32P)DCTP using the Rediprime kit (Amersham) and purified on Chroma Spin-10 columns (Clontech). Blots were dried, heated for 2 h at 80°C under vacuum, and hybridized with the probe overnight at 65°C in 10 ml of solution (10^6 cpm/ml; 1 M NaCl, 1% SDS, 10% dextran sulfate, and 0.2 mg/ml salmon sperm DNA). Blots were washed three times with 0.2 x SSC containing 0.1% SDS at 65°C for 10 min and exposed to Kodak Biomax MR films or quantified by PhosphorImager (Molecular Dynamics).

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References