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A Role for STAT5 in the Pathogenesis of IL-2-Induced Glucocorticoid Resistance

Elena Goleva,* Kevin O. Kisich,* and Donald Y. M. Leung2‡†

Glucocorticoids (GCs) are highly effective in the control of diseases associated with T cell activation. However, a subset of individuals is GC insensitive. Previous studies have demonstrated that IL-2 can induce steroid resistance in mouse T cells. However, the mechanism for this phenomenon is unknown. In the current study we found that the murine cell line (HT-2) is steroid resistant when incubated with IL-2, but steroid sensitive when grown in IL-4. Furthermore, when HT-2 cells are treated with IL-2, the glucocorticoid receptor (GCR) does not translocate to the cell nucleus after dexamethasone treatment. In contrast, the GCR in IL-4-stimulated HT-2 cells does translocate into the cell nucleus after dexamethasone treatment. IL-2-induced steroid insensitivity in HT-2 cells appears to be a signaling event as the effects of IL-2 on nuclear translocation of the GCR occurred within 30 min even in the presence of cycloheximide. Indeed, preincubation of HT-2 cells with a Janus-associated kinase 3 inhibitor restored nuclear translocation of the GCR even in the presence of IL-2. Immunoprecipitation experiments revealed that phosphorylated STAT5 and GCR formed immune complexes. This association may lead to retardation of GCR nuclear translocation because IL-2 was not able to induce steroid insensitivity in splenocytes from STAT5 knockout mice. This study demonstrates a novel role for STAT5 in IL-2-induced steroid resistance. The Journal of Immunology, 2002, 169: 5934–5940.

Glucocorticoid (GC) resistance has been widely recognized as complicating the management of chronic inflammatory diseases such as asthma, inflammatory bowel disease, autoimmune diseases, and transplantation rejection (1–6). It poses a major clinical challenge as corticosteroid therapy is the cornerstone of anti-inflammatory therapy. Delineation of the molecular basis for GC insensitivity is critical for the development of new treatment approaches for this group of refractory patients and may provide new insights into the pathogenesis of chronic inflammation.

GC action is mediated through the GC receptor (GCR), which is found in the cytosol of most human cells. As the result of alternative splicing of the GCR pre-mRNA, there are two homologous mRNAs and protein isoforms, termed GCRA and GCRB (6). GCRB differs from GCRA only in its C terminus, with replacement of the last 50 aa of GCRA with a unique 15-aa sequence lacking a steroid binding domain. These differences render GCRB unable to bind GCs, reduce its binding affinity for DNA recognition sites, abolish its ability to trans-activate GC-sensitive genes, and make it function as a dominant inhibitor of GCRA. Although GCRB has been reported to be increased in steroid-resistant diseases, its role in steroid resistance has been controversial (7, 8).

In steroid-resistant asthmatics, GCs fail to inhibit T cell proliferation and to reduce secretion of proinflammatory cytokines (5). Their airways have abnormally high levels of IL-2 and IL-4 expression (5, 9). Recent studies indicate that the combination of IL-2 and IL-4 induces T cell resistance to GCs and increased GCRB expression in the T cells of normal subjects (10). However, in contrast to neutrophils (11), the increased GCRB expression in T cells is not sufficiently high to solely account for steroid resistance. Interestingly, in mice IL-2 alone can induce T cell resistance. The mouse genome does not contain the GCRB gene (12). Therefore, the study of mouse T cells provides an opportunity to examine mechanisms of steroid resistance in the absence of GCRB. The aim of this work was to characterize the molecular basis for the functional interaction between IL-2 and the GCR signal transduction pathways to establish the mechanisms by which IL-2 can induce steroid insensitivity in lymphocytes.

Materials and Methods

Chemicals and reagents

Recombinant murine IL-2 and IL-4 were purchased from R&D (Minneapolis, MN), Janus-associated kinase 3 (JAK3) inhibitor 1 (IC50 = 78 μM) and p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB203580 (IC50 = 39 nM) were purchased from Calbiochem (San Diego, CA); dexamethasone (DEX) and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Murine IL-2-dependent HT-2 cell line was cultured in complete medium, IMDM (Sigma-Aldrich) containing 5.7% heat-inactivated FCS (Gemini Bio Products, Calabasas, CA) with supplements as previously described (13) in the presence of 50 U/ml murine IL-2. For the studies of GCR nuclear translocation in response to steroids, charcoal-filtered GC-free FCS (Gemini Bio Products, Calabasas, CA) was used in the growth medium. Murine spleen cells from wild-type and knockout mice were grown at a concentration of 106 cells/ml IMDM (Cellgro; Mediatech, Herndon, VA) containing 10% heat-inactivated FCS, 400 μM l-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 20 mM HEPES (Mediatech) with 5 μg/ml PHA (Sigma-Aldrich) for 48 h.
Mice
C57BL/6J mice were purchased from Harlan Sprague Dawley (Indianapolis, IN), and STAT5a/b knockout mice were provided by Dr. Ihle (St. Jude Medical and Research Hospital, Memphis, TN).

Lymphocyte proliferation assays
HT-2 cells were plated in 96-well round-bottom culture plates (Nalge Nunc International, Rochester, NY) at 1 × 10^4 cells/well in growth medium in the absence or presence of 10^{-8}–10^{-5} M DEX. HT-2 cells were stimulated with different concentrations of murine IL-2 (1, 5, 10, and 50 U/ml) or IL-4 (50, 100, 250, and 500 U/ml). Cultures were incubated for 24 h at 37°C in humidified, 7% carbon dioxide-containing incubator. IC_{50} was determined as the concentration of DEX that inhibits the proliferation of cytokine-stimulated lymphocytes by 50% of the level seen in the absence of DEX, was used as the parameter to measure the steroid sensitivity of the cells.

Similar proliferation assays were performed with primary murine splenocytes. Murine spleen cells were aseptically isolated and put into culture as described above. After 48 h the cells were washed twice, resuspended in fresh medium, plated in 96-well, round-bottom culture plates at 1 × 10^5 cells/well, and stimulated by different concentrations of murine IL-2 or IL-4. DEX (10^{-8}–10^{-5} M) was used to examine the steroid sensitivity of the cells. Cultures were incubated for 24 h at 37°C in humidified, 7% carbon dioxide-containing incubator. IC_{50} was defined as the concentration of DEX that inhibits the proliferation of cytokine-stimulated lymphocytes by 50% of the level seen in the absence of DEX, was used as the parameter to measure the steroid sensitivity of the cells.

Immunofluorescence staining of HT-2 cells for GCR
Number 1 coverslips (22 × 22 mm; thickness range, 0.13–0.17 mm; Fisher Scientific, Pittsburgh, PA) were precoated by soaking with poly-l-lysine (Sigma-Aldrich) at a concentration of 1 mg/ml for 10 min, then washed twice in distilled water. Cells were resuspended in growth medium in 10× 10^6 cells/ml, and stimulated by different concentrations of murine IL-2 or IL-4. DEX (10^{-8}–10^{-5} M) was used to examine the steroid sensitivity of the cells. Cultures were incubated for 24 h at 37°C in humidified, 7% carbon dioxide-containing incubator. IC_{50} was defined as the concentration of DEX that inhibits the proliferation of cytokine-stimulated lymphocytes by 50% of the level seen in the absence of DEX, was used as the parameter to measure the steroid sensitivity of the cells.

Statistical methods
Immunofluorescence and cell proliferation data were analyzed using Student’s t test. A value of p < 0.05 was considered significant. Data are expressed as the mean ± SEM.

Results
IL-2-induced proliferation of HT-2 cells is steroid insensitive
Proliferation of the murine T cell line HT-2 can be stimulated with either IL-2 or IL-4. IL-4 induced proliferation of the murine T cell line CTLL-2 has previously been shown to be sensitive to inhibition by DEX, while IL-2-induced proliferation was insensitive to DEX (14). We have extended these observations by determining the effects of DEX on IL-2- and IL-4-stimulated proliferation of the HT-2 cell line. The amounts of IL-4 (500 U/ml) and IL-2 (1 U/ml) used were titrated to stimulate similar levels of proliferation in the HT-2 cultures. Fig. 1 shows the results of a representative dose-response experiment (of six total experiments performed) demonstrating that IL-4-induced proliferation was inhibited by >50% at 10^{-8} M DEX, while IL-2-induced proliferation was unaffected even at 10^{-5} M DEX (p < 0.05).

IL-2 stimulation prevents nuclear translocation of GCR in HT-2 cells
To investigate the mechanism by which IL-2 induced steroid insensitivity in HT-2 cells, we initially compared the ability of GCR...
to translocate into the nuclei of IL-2- or IL-4-stimulated cells after treatment with 10^{-6} M DEX for 30 min at 37°C. In these experiments the nuclei were defined by staining with the blue fluorescent DNA dye DAPI. GCR localization was examined by indirect immunofluorescence using anti-GCR rabbit polyclonal antiserum detected by Cy3-conjugated secondary Ab (red). In the absence of DEX stimulation GCR was primarily in the cell cytoplasm (Fig. 2, A and B). The Cy3 MFI values for the nuclear region of the cells were 355.27 ± 20.50 (IL-2 stimulated cells) and 451.46 ± 18.80 (IL-4 stimulated cells). After DEX treatment GCR translocated to the cell nucleus in IL-4-stimulated cells, but failed to translocate in IL-2-stimulated cells (Fig. 2). The Cy3 MFI values for the nuclear region of the cells were 449.55 ± 30.58 (IL-2-stimulated cells treated with 10^{-6} M DEX) and 857.60 ± 66.20 (IL-4-stimulated cells treated with 10^{-6} M DEX; p < 0.01 compared with all other groups). These data suggest that the resistance of IL-2-stimulated HT-2 cells to DEX is due to the failure of GCR to translocate from cytoplasm to nucleus.

IL-2-inhibited GCR nuclear translocation is a signaling event

Since HT-2 cells treated with IL-4 were steroid sensitive and effectively translocated their GCR to the cell nucleus, we chose them as the model comparison for studies of IL-2-induced steroid resistance. HT-2 cells were grown in medium with 500 U/ml of IL-4 for 18–20 h, and then the medium was changed to medium containing 50 U/ml of IL-2 for 30 min. Cells were then treated with 10^{-6} M DEX for 30 min. The cells were fixed and stained, and GCR localization was examined by confocal microscopy.

HT-2 cells stimulated with IL-4 and treated with DEX for 30 min revealed effective nuclear translocation of GCR (GCR staining nuclear/cytoplasm ratio, 1.63 ± 0.09; Fig. 3A), which was blocked by 30 min of IL-2 exposure (GCR staining nuclear/cytoplasm ratio, 1.07 ± 0.07; Fig. 3C; p < 0.01 between groups A and C). Fig. 3, B and D, show that when protein synthesis was blocked with 50 μg/ml of cycloheximide, neither nuclear translocation nor IL-2-mediated inhibition of translocation was affected (GCR staining nuclear/cytoplasm ratio, 1.45 ± 0.10 and 1.18 ± 0.06, respectively; p < 0.01 between groups A and D). The rapidity with which IL-2 induced steroid resistance coupled with the insensitivity of this effect to cycloheximide suggested that the synthesis of new proteins was not required for IL-2 to induce steroid resistance.

Inhibition of JAK3 prevents induction of steroid insensitivity by IL-2

Since IL-2-induced steroid insensitivity of HT-2 cells was not associated with the synthesis of new proteins, we sought to determine whether the prevention of signaling through IL-2R γ-chain could block the induction of steroid insensitivity by IL-2. Indeed, JAK3 inhibitor 1, which blocks JAK3 kinase activity (15, 16), was able to prevent the induction of steroid insensitivity of HT-2 cells at a concentration of 7.8 μM in a dose-dependent manner after 24 h of incubation (Fig. 4A). Incubation of HT-2 cells with 312 μM JAK3 inhibitor 1 (IC_{50} = 78 μM) for 1 h before treatment with IL-2 also restored DEX-induced nuclear translocation of GCR (Fig. 4B). Incubation with JAK3 inhibitor for this period of time was not toxic to the cells, as determined by exclusion of trypan blue.

It has been reported that p38 MAPK inhibitor is able to restore GCR binding affinity in human PBMC after preincubation of those cells with IL-2 and IL-4 (17). These data suggested that IL-2 and IL-4 preincubation induced p38 MAPK-dependent phosphorylation of GCR, which leads to reduced GCR binding activity.
According to proliferation assay results, the p38 MAPK-specific inhibitor SB203580 partially shifted the steroid sensitivity of IL-2-induced proliferation of HT-2 cells (Fig. 4B). Fig. 5, B and C, illustrate that DEX-induced nuclear translocation of the GCR occurred in the presence of JAK3 inhibitor and the p38 MAPK inhibitor SB203580 even in the presence of IL-2 (GCR staining nuclear/cytoplasm ratio, 1.38 ± 0.05 and 1.81 ± 0.26, respectively; p < 0.01 compared with GCR staining nuclear/cytoplasm ratio of 0.98 ± 0.06 for IL-2-stimulated cells). JAK3 inhibitor and p38 MAPK inhibitor did not alter the DEX sensitivity of IL-4-treated cells (data not shown). This suggested that the signal transduction pathway leading through JAK3 to STAT5 was directly involved in the genesis of IL-2-induced steroid resistance in HT-2 cells. p38 MAPK-dependent phosphorylation also restores nuclear translocation of GCR.

**STAT5-GCR heterodimer formation is associated with the steroid insensitivity of IL-2-induced proliferation in mice**

Since activation of the post-JAK3 elements of the IL-2 signal transduction pathway was required for inhibition, we examined whether there was evidence of direct interaction between GCR and IL-2-induced signal transduction factors. STAT5 was our first protein of interest, because this molecule is known to be activated by IL-2. Furthermore, our previous experiments indicated that incubation with JAK3 inhibitor 1 prevented induction of steroid insensitivity in IL-2-treated HT-2 cells.

To determine whether STAT5 is involved in induction of steroid insensitivity by IL-2, several approaches were used. Whole cell lysates were prepared from IL-2-stimulated HT-2 cells and immunoprecipitated with anti-GCR or anti-phospho-STAT5 Abs. Western blot analysis was performed using a specific anti-phospho-STAT5 antiserum to determine whether immunoprecipitation of GCR also captured phospho-STAT5. Alternatively, specific anti-GCR antiserum was used to show the association of immunoprecipitated STAT5 with GCR, revealing physical interaction between them. Fig. 6 illustrates that immunoprecipitation of STAT5 does indeed capture GCR, although it appears that only a small percentage of the total STAT5 is associated with GCR. Conversely, Fig. 6 also shows that immunoprecipitation of GCR captures STAT5. In this case, a higher percentage of the precipitable GCR is associated with STAT5. When we compared cytoplasmic
to nuclear extracts for the relative proportion of STAT5 remaining in the cytoplasm, we found a 1:1 nuclear/cytoplasmic ratio of STAT5 in IL-2-stimulated HT-2 cells. This indicates that there is no defect in STAT5 nuclear translocation in HT-2 cells.

**IL-2-induced proliferation is steroid sensitive in STAT5a/b knockout mice**

If GCR-STAT5 protein-protein interactions were important, we hypothesized that IL-2 would be unable to induce steroid insensitivity in STAT5 knockout mice. For this purpose murine splenocytes from STAT5a/b knockout mice were stimulated in culture with PHA. After 48 h cells were stimulated with different concentrations of IL-2 or IL-4 in the presence of DEX for 24 h to determine their steroid sensitivity. We found that IL-2-stimulated spleen cells from STAT5a/b knockout mice were steroid sensitive, while spleen cells from wild-type mice of the same background (C57BL6/129) were steroid insensitive after IL-2 treatment (Table 1). We observed that IL-2-induced proliferation of splenocytes from STAT5 knockout mice was up to 100-fold more sensitive to inhibition by DEX (IC50 = 10-8 to 5 x 10-8) than were control mice (IC50 = 10-7 to 5 x 10-6; p < 0.05).

**Discussion**

The resistance of T cells to the action of GCs represents a significant clinical challenge during treatment of inflammatory diseases (6). However, the biochemical mechanisms of steroid resistance are only beginning to be understood. Undoubtedly, there will be multiple mechanisms or biochemical pathways that lead to steroid resistance. Understanding these mechanisms is important for the development of new approaches for the treatment and control of inflammation.

It has been shown that human neutrophils have high constitutive expression of GCRβ and a 73-fold greater ratio of GCRβ/GCRα compared with human PBMC (11). Moreover, proinflammatory cytokines such as IL-8 further up-regulate GCRβ levels in neutrophils, which enhances their survival in the presence of GCs during inflammation. Several additional pathways that may lead to steroid resistance have been described in PBMC. It has been established that IL-2 and IL-4 can potentiate GCRβ expression in T cells as well (18). Whether increased GCRβ can completely account for GC insensitivity in T cells, however, remains controversial. It was shown that viral transduction of GCRβ cDNA into the mouse D011.10 cell line leads to 2.5-fold GCRβ overexpression and results in GC insensitivity of these cells (19). On the other hand, Iruen et al. (17) were unable to detect increased GCRβ expression in IL-2/IL-4-stimulated human PBMC. Thus, GCRβ is unlikely to completely account for steroid resistance in PBMC (17, 20). Recent studies indicate that GCRβ does not act as a dominant negative modulator of GCRα for repression of AP-1 and NF-kB activities, and overexpression of either GCRα or GCRβ may have an anti-inflammatory effect (20). The study of mouse T cells provides an opportunity to examine mechanisms of steroid resistance in the absence of GCRβ. This makes murine T cells a good model for examining the effects of proinflammatory cytokines on the response to steroids.

GCs interact with the GCR in the cytoplasm. This causes the receptor to undergo an allosteric change that enables the hormone-receptor complex to translocate into the cell nucleus, bind to specific DNA-response elements, and modulate transcription (6). There are several signal transduction pathways and transcription factors that have been shown to interact with and modulate the function of GCR-ligand complexes, including AP-1, NF-kB, and STAT (21–23).

JAK-STAT signaling by IL-2 is critically important in the generation of immune and inflammatory responses and in T cell expansion and differentiation (24). Protein tyrosine kinases JAK1

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**Table 1. Cytokine-induced proliferation of murine splenocytes in presence of DEX**

<table>
<thead>
<tr>
<th>Cytokine Concentration (U/ml)</th>
<th>IC50 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT5a/b knockout mice</td>
<td>C57BL6/129 mice</td>
</tr>
<tr>
<td>IL-2 (1)</td>
<td>10-8</td>
</tr>
<tr>
<td>IL-2 (5)</td>
<td>3.5 x 10-8</td>
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<tr>
<td>IL-2 (10)</td>
<td>5 x 10-8</td>
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<tr>
<td>IL-2 (50)</td>
<td>4 x 10-8</td>
</tr>
<tr>
<td>IL-2 (100)</td>
<td>7 x 10-8</td>
</tr>
<tr>
<td>IL-4 (50)</td>
<td>7.5 x 10-8</td>
</tr>
<tr>
<td>IL-4 (100)</td>
<td>5 x 10-8</td>
</tr>
<tr>
<td>IL-4 (500)</td>
<td>6 x 10-8</td>
</tr>
</tbody>
</table>

IC50 was expressed as the molar concentration of DEX that inhibits proliferation of cytokine-stimulated lymphocytes to 50% of the level seen in the absence of steroid.

*p = 0.05 compared to STAT5a/b knockout mice.
and JAK3 that are associated with cytoplasmic subunits of the β- and γ-chains of IL-2R, respectively, are activated after IL-2 binding (25). The β- and γ-subunits of the IL-2 receptor become phosphorylated and provide docking sites for monomeric STAT5 proteins (26). STAT5 becomes phosphorylated, dimerizes, and translocates to the cell nucleus (26, 27).

STAT5 was first characterized as a mammary gland transcription factor induced by prolactin (28). Most previous investigations have been focused on STAT5 inhibition of GCR trans-activation activity induced by prolactin (29, 30). In addition to prolactin it has been established that both STAT5a and STAT5b proteins, which share ~95% homology in amino acid structure, are activated by a number of cytokines (IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, GM-CSF, erythropoietin, and thrombopoietin) (26, 27). It has been suggested that GCR can act as a transcriptional coactivator for STAT5 and enhance STAT5-dependent transcription in response to prolactin. However, complex formation between STAT5 and GCR diminishes the GC responsiveness of GC response element-containing promoters (31).

Our studies confirm that heterodimers form between GCR and STAT5. However, we have further demonstrated that this leads to inhibition of the nuclear import of GCR, thus leading to the induction of steroid insensitivity. We suggest a novel role for STAT5 in IL-2-induced steroid insensitivity: the prevention of efficient nuclear translocation of GCR due to heterodimer formation between GCR and STAT5 proteins.

Recent studies indicate that GCR can interact with several STAT proteins (21, 29). Despite the high degree of homology existing between different STAT proteins, functional interaction with GCR does not necessarily imply a common mechanism. For instance, it has been demonstrated that GCR and STAT6 physically and functionally interact in CTL-2 cells (32), and that DEX was able to block the IL-4-induced proliferation of these cells (14). Transcriptional cooperation between STAT-1, the GCR, and the Ets family transcription factor PU.1 has been described in monocytes. Such interaction enhanced IFN-γ-induced expression of FcyRI (33).

Our results suggest that exposure of the murine T cell line HT-2 to IL-2 blocks both translocation of the GCR to the nucleus and steroid responsiveness. These data are consistent with previous observations obtained with CTL-2 cells, in which it has been established that IL-2 strongly inhibits GC-induced transcription from the mouse mammary tumor virus promoter due to STAT5-GCR interaction (34).

The mechanism by which IL-2 can induce these defects in steroid responsiveness is revealed by our experiments in which prevention of STAT5 phosphorylation by JAK3 inhibitor 1 prevented the effect. Therefore, phosphorylation of STAT5 must be required to enable its association with GCR.

Our studies have extended previous work from cell lines to primary murine lymphocytes, in which we observed induction of steroid insensitivity by IL-2, while IL-4-induced proliferation was steroid sensitive (Table I). The hypothesis that nuclear translocation of GCR is inhibited by interaction with STAT5 is further supported by the observation that lymphocytes from mice deficient in STAT5 do not develop steroid resistance in response to IL-2 stimulation, while lymphocytes from control mice readily develop resistance.

The precise nature of the interaction between STAT5 and GCR remains unclear. However, Wang et al. (35) recently demonstrated that phosphorylation of serine 211 of human GCR was associated with nuclear localization. In addition, nuclear localization sequences adjacent to the DNA binding domain and in the ligand binding domain of rat GCR have been shown to regulate translocation of GCR into the nucleus (36). GCR is phosphorylated on multiple serine residues in addition to serine 211, which is mediated in part by p38 MAPK (17).

We have observed that the induction of steroid resistance by IL-2 can be inhibited not only via the STAT5-dependent pathway, but also via a p38 MAPK-dependent pathway (Fig. 4). Inhibition of p38 MAPK activity also led to rescue of nuclear translocation of GCR in IL-2-treated cells (Fig. 5). This suggests that the interaction between STAT5 and GCR occurs via STAT5 binding to one of the phosphoserine residues of GCR. This may prevent translocation of GCR to the nucleus either by blocking access of importin-α to the nuclear localization-1 domain (36) or by masking other domains needed for nuclear import. Doppler et al. (37) have determined that functional interaction between GCR and STAT5 requires residues 77–262 of the human GCR, which includes serine 211, previously shown to be associated with nuclear localization.

In summary, we have shown that steroid resistance induced in T cell lines by IL-2 is associated with a defect in nuclear translocation of the GCR in response to DEX. This defect was dependent upon phosphorylation of STAT5 by JAK3 and was independent of the synthesis of new proteins. IL-2-induced steroid resistance was also inhibited by an inhibitor of p38 MAPK, suggesting that phosphorylation of the GCR was necessary in addition to STAT5 activation. This study has identified both STAT5 and JAK3 as potential targets for pharmaceutical intervention and treatment of inflammatory diseases associated with steroid insensitivity.

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References


