

Cytokine up-regulation of IL13R- 2 in GBM cells leads to an increased potency of recombinant IL13 Cytotoxin

Research Article

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Abbreviations: activating protein-1, (AP-1); protein kinase B (AKT); American Type Culture Collection (ATCC); cancer/testes tumor antigen, (CTA); Dulbecco's Modified Eagle's Minimal Essential Medium, (DMEM); epidermal growth factor (EGF); enhanced chemiluminescent substrate, (ECL); extracellular signal-regulated kinase (ERK); Fetal Bovine Serum, (FBS); Fos-related antigen 1, (Fra-1); glioblastoma multiforme, (GBM); human endothelial cells, (HUVEC-C); interleukin 13, (IL13); c-Jun N-terminal kinase (JNK); mitogen-activated protein kinase (MAPK); non-essential amino acids, (NEAA); normal horse serum, (NHS); phosphatidylinositol-3-kinase (PI3-K); phenazine methosulfate, (PMS); tumor necrosis factor (TNF); tyrosine kinase, (TK)

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Summary

We documented that a receptor for interleukin13, IL13R- 2, is an attractive target for glioma (brain tumor) molecular therapies. Little is known whether and how the expression of this receptor is regulated in glioma cells. Recent studies suggested that this receptor might be under AP-1¹ and STAT-6 control. Thus, IL13R- 2 was examined in a variety of cancer and normal cells that had been activated by AP-1 and STAT-6 stimulatory cytokines. We found that serum-starved glioma cell lines had the levels of IL13R- 2 significantly diminished. The addition of EGF, TNF or IL4 increased IL13R- 2, the extent of which was cytokine-, cell line- and time-dependent. We also found that primarily EGF could further increase the levels of the receptor in glioma cells under normal conditions. As expected, IL13R- 2 was found low in normal cells when compared with brain tumor cells and neither AP-1 nor STAT-6 activation produced any large increase in the receptor levels in those cells. Blocking the PI3-K and ERK pathways, but not that of P38 and JNK MAPKs, resulted in the neutralization of EGF effects on IL13R- 2. Furthermore, EGF pre-treatment caused higher sensitivity of glioma cells to cell killing with very low doses of IL13-based recombinant cytotoxin, which was not found in cells with either low levels or transgene-induced expression of IL13R- 2. EGF alone did not have any significant effect on GBM cells proliferation. Thus, the expression of IL13R- 2 appears to be non-constitutive. PI3-K and ERK play important roles in activating mechanisms responsible for the EGF-dependent expression of the receptor. Also, up-regulating the levels of IL13R- 2 produces higher susceptibility to the killing by a recombinant cytotoxin molecularly targeted to IL13R- 2.

I. Introduction

We have demonstrated that a vast majority of patients with glioblastoma multiforme (GBM), high-grade glioma (grade IV), over-express binding sites for interleukin 13 (IL13) (Debinski et al, 1999a, 2000). In principle, the measurable binding of IL13 to GBM is independent of its homologue, IL4 and thus, more

restricted from the one found in many vital organs; the latter is shared between IL13 and IL4. A monomeric plasma membrane protein, IL13R- 2, has been identified as a molecular entity responsible for the restricted binding of IL13 to GBM (Debinski et al, 1995c; Debinski and Gibo, 2000; Mintz and Debinski, 2000; Mintz et al, 2002). On the other hand, the physiological form of the IL13R is

a 140-kDa chain of the IL4R, termed IL4R α , which dimerizes with a 45-50 kDa IL13-responsive subunit (Zurawski et al, 1993, 1995; Vita et al, 1995; Hilton et al, 1996; Miloux et al, 1997; Kelly-Welch et al, 2003). The IL13 binding protein of the IL13/4R, termed IL13R- 1, binds IL13 at a low affinity (Hilton et al, 1996). Only in the presence of IL4R α does the 140-kDa IL4 binding protein site become one of high affinity ($K_d \sim 30$ pM) (Aman et al, 1996; Hilton et al, 1996; Miloux et al, 1997).

We have characterized IL13R- 2 as a cancer/testes tumor antigen (CTA)-like factor (Debinski and Gibo, 2000). This restricted receptor is one of the first three factors ever documented to be expressed in a majority of patients with GBM and not in normal brain (Murphy et al, 1995; Rich et al, 1996). This finding has marked the start of uncovering molecular denominators in a disease as heterogeneous as GBM.

Our laboratory conceived and generated IL13-based, bacterial toxin-containing recombinant cytotoxic chimera fusion proteins that have been shown to be very potent anti-glioma cytotoxins *in vitro* and *in vivo* (Debinski et al, 1999a, c). The first generation of IL13-based cytotoxin, hIL13-PE38QQR that was produced more than a decade ago, entered Phase III clinical trials in 2004. hIL13-PE38QQR was generated as a counterpart to an IL4-based cytotoxin without intent of targeting brain tumors (Debinski et al, 1995b) and it does not have the inherent specificity for the targeting of IL13R- 2 while sparing the normal tissue receptor shared with IL4. However, several therapeutic approaches targeting specifically IL13R- 2 are being developed: vaccines (Mintz et al, 2002; Okano et al, 2002), targeted viruses (Zhou et al, 2002), re-targeted cytotoxic T cells (Kahlon et al, 2004) and new IL13-based cytotoxins (Mintz et al, 2003). Thus, IL13R- 2 is a truly attractive molecular target in the treatment of brain cancer.

We have been intrigued by the fact that the IL13R- 2 gene resides on Chromosome X and the fact that numerous cancer/testes tumor antigen have their genes on the same chromosome. We have discussed hypothetical implications for the presence of IL13R- 2 in malignancy in the context of X-linkage and suggested that a significant component responsible for the restricted IL13 receptor over-expression in GBM might be the DNA methylation status and subsequent aberrant gene expression regulation (Mintz and Debinski, 2000). Epigenetic mechanisms have been implicated previously in the appearance of other CTAs (Lethe et al, 1998). In support of this contention, other investigators demonstrated an 8-fold increase in the expression of IL13R- 2 in bladder cancer cells in response to a DNA de-methylating agent, 5-aza-2'-deoxycytidine; this was not seen in the corresponding normal cells (Liang et al, 2002). We treated GBM cells with 5-aza-2'-deoxycytidine and found a 3-fold increase in IL13R- 2 gene expression against the background of already elevated levels of the receptor in these cells (Debinski et al, 2003). Thus, in two independent studies the IL13R- 2 expression has been increased in response to a DNA de-methylating agent. Our study in GBM cells also suggested that the expression of IL13R- 2 might not be constitutive.

We have outlined several lines of evidence suggestive of epigenetic involvement in the up-regulation of activating protein-1 (AP-1) activity (Debinski et al,

2003) and we have concomitantly documented an increase in AP-1 in gliomas (Debinski et al, 2001; Debinski and Gibo, 2005). For example, a Fos-related antigen 1 (Fra-1), an AP-1 factor is highly up-regulated in GBM (Debinski et al, 2001; Debinski and Gibo, 2005). Accordingly, recent data demonstrate that the IL13R- 2 gene promoter region contains an AP-1 binding site that might be important for receptor expression (Wu et al, 2003). The promoter region of the IL13R- 2 gene, besides containing an AP-1 binding site, has putative binding sites for the STAT6, NF κ B and C/EBP β transcription factors (David et al, 2003). IL4 and IL13 increased IL13R- 2 at both gene and protein levels (David et al, 2003). Tumor necrosis factor (TNF α), when combined with either IL13 or IL4, synergized up-regulation of IL13R- 2 in one cell type (David et al, 2003).

Initially, we have demonstrated the restricted binding of IL13 to the HGG in *in vitro* and *in situ* by using autoradiography with iodo-labeled IL13 as a ligand for the receptor (Debinski et al, 1999a, 2000). The first commercially available antibody against IL13R- 2 works only in cell sorting assays (Mintz et al, 2002). We were able to produce limited amounts of a polyclonal antibody against IL13R- 2 that showed positive staining of GBM tissue specimens (Mintz et al, 2002). Recently, antibodies have become available that perform in Western blots and immunocytochemistry. The results by others confirmed the presence and prevalence of IL13R- 2 in GBM specimens to a degree similar to that seen with autoradiography (Liu et al, 2003). Being that AP-1 and STAT-6 appear to be involved in IL13R- 2 regulation in some cells (David et al, 2003), we examined the regulation of immunoreactive IL13R- 2 expression in brain tumor cells.

II. Materials and Methods

A-172 MG, SNB-19, U-251 MG, normal human endothelial cells (HUVEC-C) and SVGp12 transformed normal glial cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD), G48a cell line was isolated in our laboratory (Debinski and Gibo, 2005), HaCat cells were kindly provided by Dr. Abby Maizel. Media were obtained from Invitrogen. Tissue culture ware was from Corning Glass (Corning, NY). SDS-PAGE supplies were from Bio-Rad. Antibodies, including anti-IL13R- 2 antibody, EGF and TNF α were purchased from the R & D System, Inc (Minneapolis, MN). Antibodies against ERK, phosphorylated ERK, JNK, phosphorylated JNK, p38, phosphorylated p38, AKT and phosphorylated AKT, were purchased from Cell Signaling (Beverly, MA). IL4 was produced by our laboratory. EGFR tyrosine kinase inhibitor (AG1478), MAPK inhibitor (SB203580), and PI-3K inhibitor (Wortmannin) were purchased from Calbiochem (La Jolla, CA). The inhibitors of ERK (PD 98059) and PI3-K (LY 294002) were obtained from Sigma (St. Louis, MO). SP 600125 inhibitor of JNK was purchased from A.G. Scientific, Inc. (San Diego, CA). SuperSignal Substrate ECL for chemiluminescent detection was purchased from Amersham Biotech. Chamber slides were purchased from Nalge Nunc (Naperville, IL) and Alexa Fluor 488 donkey anti-goat IgG was from Molecular Probes Inc (Eugene, OR). DAPI was from Sigma. Recombinant IL13.E13K-PE38QQR was produced and purified in our laboratory (Mintz et al, 2003).

A. Cell culture

Several GBM cell lines, such as A-172 MG, G48a, Snb19 and U-251 MG were grown in the appropriate media. Normal

glial (SVGp12) and human umbilical vein endothelial cells (HUVEC-C) were used as normal cell controls. The A-172 MG and HaCat cells were cultured in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (Sigma); G48a cells were grown in RPMI 1640, 100 µg/ml L-Cystine (Sigma), 20 µg/ml L-Proline (Sigma), 100 µg/ml Sodium Pyruvate (GIBCO), 1X HT Supplement (GIBCO), 10% FBS; SNB19 cells were cultured in RPMI 1640, 0.1 mM non-essential amino acids (NEAA) (Invitrogen), 100 µg/ml Sodium Pyruvate, 10% FBS; U-251 MG cells were cultured in DMEM, 0.1mM NEAA, 10% FBS; SVGp12 cells were cultured in Eagle's MEM, 0.1 mM NEAA, 100 µg/ml Sodium Pyruvate, 10% FBS; HUVEC cells were cultured in F-12 Kaighn's, 100µg/ml Heparin (Sigma), 30 µg/ml Endothelial Cell Growth Supplement (ECGS) (Sigma), 10% FBS. Cells were cultured in regular or serum-free medium supplemented with EGF, TNF , IL4 and TNF + IL4. Serum-free groups started treatment after 24h serum starvation.

B. Western blot analyses

For Western blot, cells were seeded onto 100-mm cell culture dishes and allowed to reach 80% confluence. Cultures either in normal or in 24-h serum-starved media were stimulated with EGF, TNF , IL4 and TNF +IL4 for 8, 24 and 48 h, then the cells were harvested in RIPA buffer. The cell lysates were electrophoresed on 12 or 15% SDS-PAGE and transferred to polyvinylidene difluoride membrane, blocked with 5% blotto in phosphate-buffered saline (PBS/0.05% Tween) for 1 h at room temperature. The polyclonal goat anti-human IL13R- 2 antibody was diluted into blotto and incubated at 4 °C overnight. The membrane was washed three times for 5 min each in 0.05% Tween 20/PBS and was incubated for 1h in 5% blotto containing anti-goat conjugated to horseradish peroxidase. Subsequently, the membrane was washed three times with 0.05% Tween 20/PBS.. The membrane was stripped by 0.5 N NaOH for 15 min, then re-probed for -Actin as internal standard. The proteins were detected on the film by enhanced chemiluminescent substrate (ECL) detection system. The density of the immunoreactive bands was measured using "Scion Image Software" from Scion Corporation (Frederick, Maryland).

C. EGFR pathway block assay

The G48a and U251-MG cells were seeded onto 100-mm dishes and allowed to reach 80% confluence. The cells were pre-treated with AG1478 (20 µM), SB203580 (10 µM), Wortmannin (100 ng/ml), PD 98059 (20 µM), SP 600125 (40 µM), and LY 294002 (10 µM) for 1 h, then 100 ng/ml of EGF was added for 5 or 15 min, or 24 h, dependent on the target for the inhibitors. Cell lysates were harvested and analyzed for IL13R- 2 expression by Western blot.

D. Immunofluorescence

Expression of IL13R- 2 in GBM cells or control cells was assessed by immunofluorescence. Cells were seeded onto 8-well chamber slides and grown to 80% confluency. Following washing twice for 10 min with PBS, cells were fixed in acetone for 2 min at -20 °C. The slides were washed twice 10 min each in PBS and either used immediately or air dried and stored at -80 °C until assayed. The slides were blocked in PBS with 10% (V/V) normal horse serum (NHS) for 30 min. Primary antibody diluted with PBS/1.5% NHS was added to cells for 1 h at room temperature. Cells were washed three times in PBS for 5 min and incubated with the secondary antibody (1:200) and DAPI diluted with PBS/1.5% NHS for 45 min in the dark. After washing the cells three times in PBS for 5 min, a cover-slip was mounted using gel mount (Biomed). Fluorescent staining was digitally captured with a Zeiss Axiocam using a FITC filter set. Images were obtained from the same experiment at the same exposure. Non-specific binding of the secondary antibody was examined by

following the outlined protocol, without the addition of primary antibodies.

E. Cell proliferation assay

Glioblastoma cells (U-251 MG and G48a) were plated into 96-well culture plates and incubated overnight. Then, 20 or 100 ng/ml of EGF were added to cells and incubated for 24 h at 37 °C. After the incubation, increasing concentrations of hIL13. E13K-PE38QQR were added (0.01-100 ng/ml final concentration) and the cells were incubated for 2 days. The rate of proliferation of the cells was determined by a colorimetric MTS [3-(4, 5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium, inner salt]/PMS (phenazine methosulfate) cell proliferation assay (Promega). The cell samples were incubated with the dye for 2 h and then their absorbance at 490 nm was recorded for each well using a micro-plate reader. The wells with cells treated with high concentrations of cycloheximide served as background for the assay.

III. Results

Using gene expression analysis, radio-receptor binding assays and autoradiography, we found IL13R- 2 to be highly over-expressed in a vast majority of GBM cell lines and tissue specimens (Debinski et al, 1999a, c; Debinski and Gibo, 2000; Mintz et al, 2002). We now demonstrate an immunoreactive IL13R- 2 using Western blotting (**Figure 1**). The A-172 MG, G48a, SNB-19, U-87 MG and U-251 MG GBM cells demonstrate highly up-regulated receptor protein (**Figure 1**). The only exception among the high-grade glioma cell lines was the T-98G cells, which contain less than 500 IL13 binding sites per cell (Debinski et al, 1995c). In sharp contrast, normal human cells, such as HaCat and HUVEC and the transformed human glial cells, SVGp12 exhibited faint immunoreactive bands of IL13R- 2 (**Figure 1**).

We thus began analyzing the regulation of IL13R- 2 in glioma cells by monitoring immunoreactive protein. The serum-starved G48a GBM cells were treated with either 5 or 20 ng/ml of EGF and the addition of EGF caused a time- and dose-dependent increase of IL13R- 2 (**Figure 2A**). Of interest, the levels of IL13R- 2 in cells treated with EGF were up to five-fold higher than the background levels of the receptor, irrelevant of the serum content in the media (**Figure 2A**). TNF , IL4 or their combination also increased the intensity of the IL13R- 2 immunoreactive band in cells deprived of serum; however, contrary to the EGF effect, they were unable to elevate IL13R- 2 to its background level detected at normal culture conditions (data not shown).

Next, we examined whether under normal cell culture conditions the levels of IL13R- 2 could be further increased in response to various cytokines in G48a cells. We used higher concentrations of EGF, TNF and IL4 (100, 50 and 100 ng/ml, respectively), in the presence of serum. We found that EGF was able to further up-regulate IL13R- 2 from its basal levels, while TNF was deprived of such an effect (data not shown) and IL4 was less potent than EGF in elevating the receptor. The combination of TNF and IL4 did not produce any larger effect from that of TNF or IL4 alone in G48a cells.

We further analyzed the localization of immunoreactive IL13R- 2 when up-regulated by the cytokines in glioma cells. We performed

immunofluorescence in G48a cells under normal serum conditions after treating with either 20 or 100 ng/ml of EGF for various periods of time. We found that 100 ng/ml of EGF produced a large increase in immunoreactive

IL13R- 2 that localized mainly to the plasma membrane of G48a cells (**Figure 2B**).

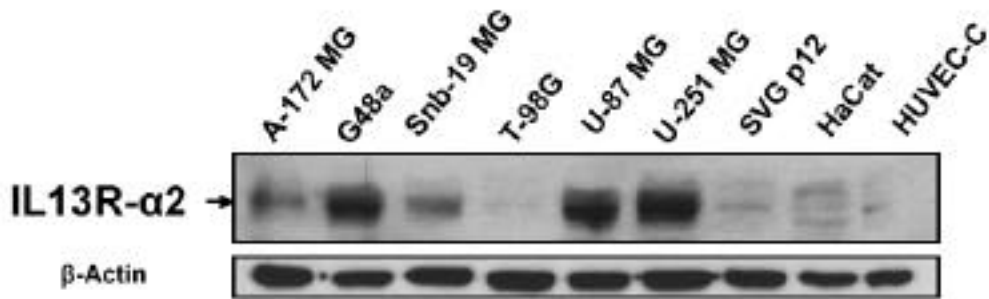


Figure 1. Western blot of IL13R- 2 in various human GBM and normal (SVGp12, HaCat and HUVEC-C) cell.

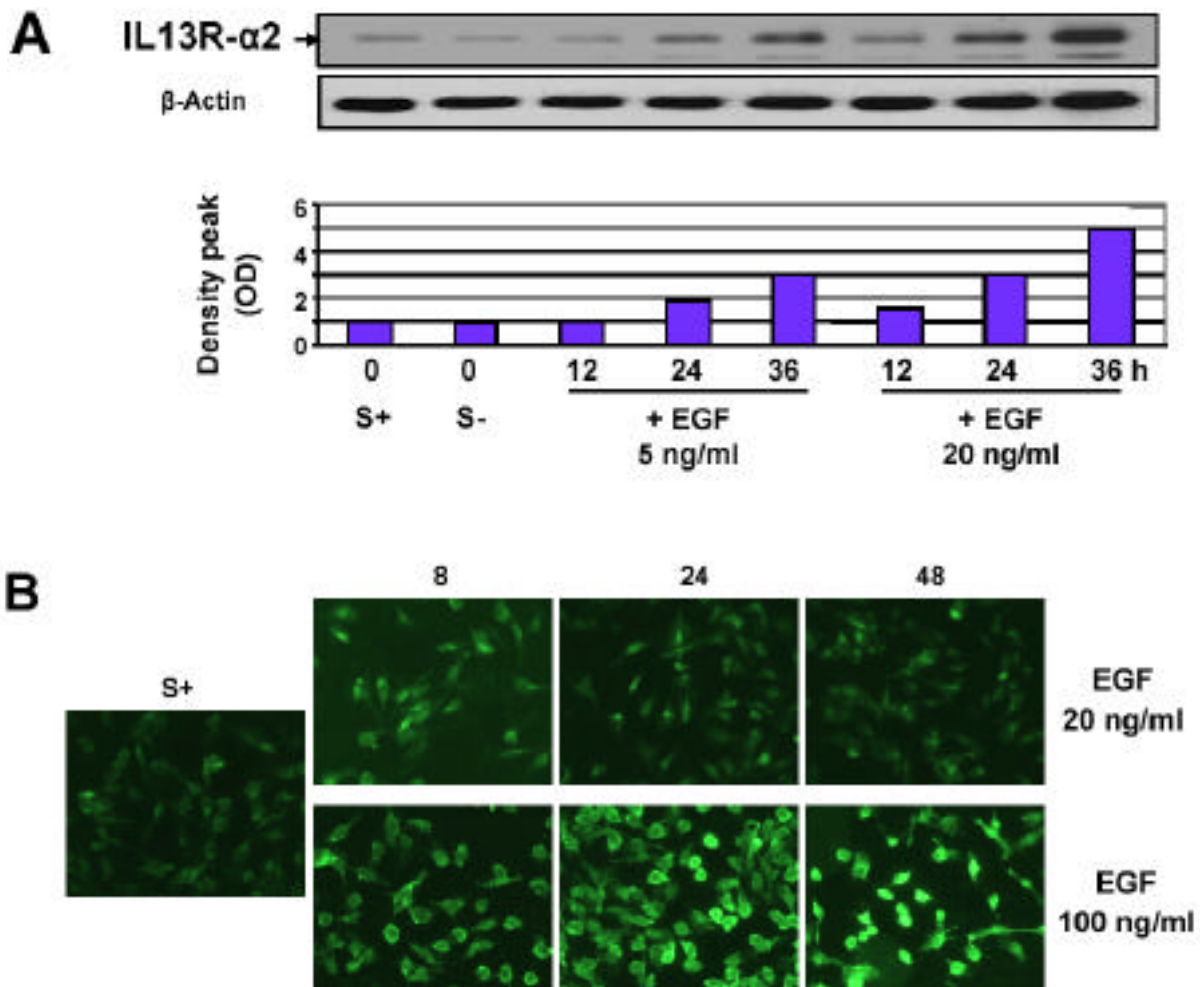


Figure 2. A. Immunoreactive IL13R- 2 in G48a GBM cells maintained under serum-free conditions. The cells were treated with EGF for 12, 24 and 36 hr. **B.** Immunofluorescent staining of IL13R- 2 of human G48a GBM cells in serum-containing media treated with either 20 ng/ml or 100 ng/ml of EGF for 8, 24 and 48 hr.

We subsequently monitored the immunoreactive IL13R- 2 in three other GBM cell lines in order to verify whether the potential regulation of this receptor by various cytokines is a general phenomenon in glioma cells. A-172

MG, SNB-19 and U-251 MG GBM cells were examined under serum-free and normal culture conditions and treated the same way as the G48a cells with EGF, TNF , IL4 or the combination of the latter. In the A-172 MG

cells, serum starvation caused a substantial decrease in IL13R- 2, but EGF raised the levels of the receptor even above its basal levels, as found in G48a cells (**Figure 3A**). Both TNF and IL4 had a more prominent effect on IL13R- 2 in the A-172 MG cells than that seen in serum-free G48a cells (data not shown). EGF upregulated IL13R 2 in A-172 MG cells, in serum containing media (**Figure 3B**). TNF and IL4 and their combination were as effective as EGF alone, or more effective, in these glioma cells, but at different time intervals (**Figure 3C**). The newly induced IL13R 2 was detected readily by

immunofluorescence and localized to the cell membranes (data not shown).

Similar responses to the cytokines were seen in the U-251 MG cells, which were good responders to EGF, but not the other cytokines (**Figure 4A**). EGF caused IL13R- 2 to increase under both serum-free and normal serum conditions. Immunofluorescent IL13R- 2 also prominently increased in response to the EGF treatment in the U-251 MG cells under normal serum conditions. The biggest increase was found 24 hr post EGF addition and IL13R- 2 localized primarily to the plasma membrane of these cells, as was seen with G48a cells (**Figure 4B**).

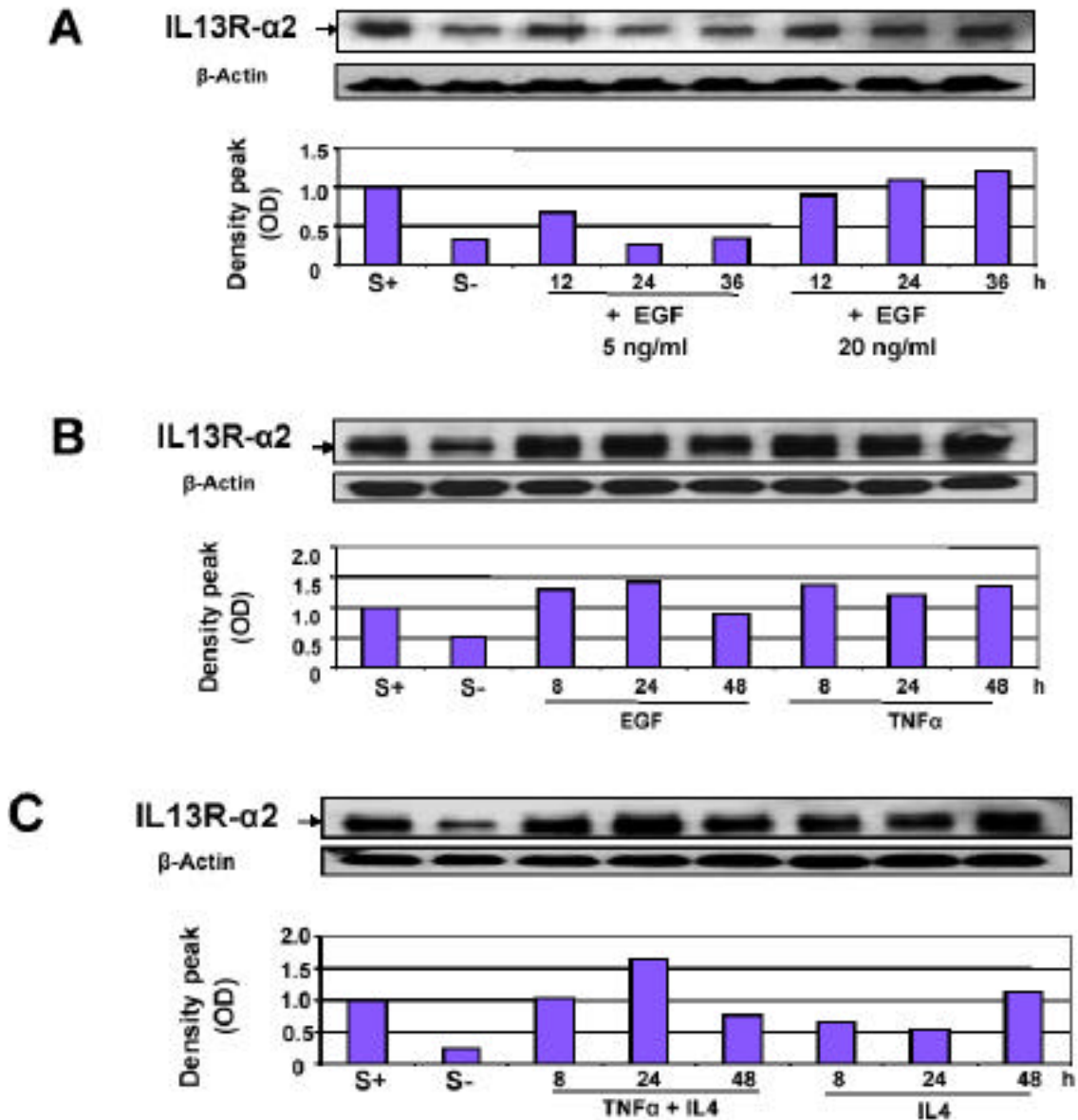


Figure 3. A. Immunoreactive IL13R- 2 in A-172 MG GBM cells maintained under serum-free conditions. The cells were treated with EGF for 12, 24 and 36 hr. B. Immunoreactive IL13R- 2 in Western blot performed on lysates from cells maintained in normal media. The A-172 MG cells were treated with EGF (100 ng/ml) or TNF (50 ng/ml). C. A-172 MG cells were treated with TNF + IL4 or IL4 (100 ng/ml). S+, serum containing media; S-, no serum.

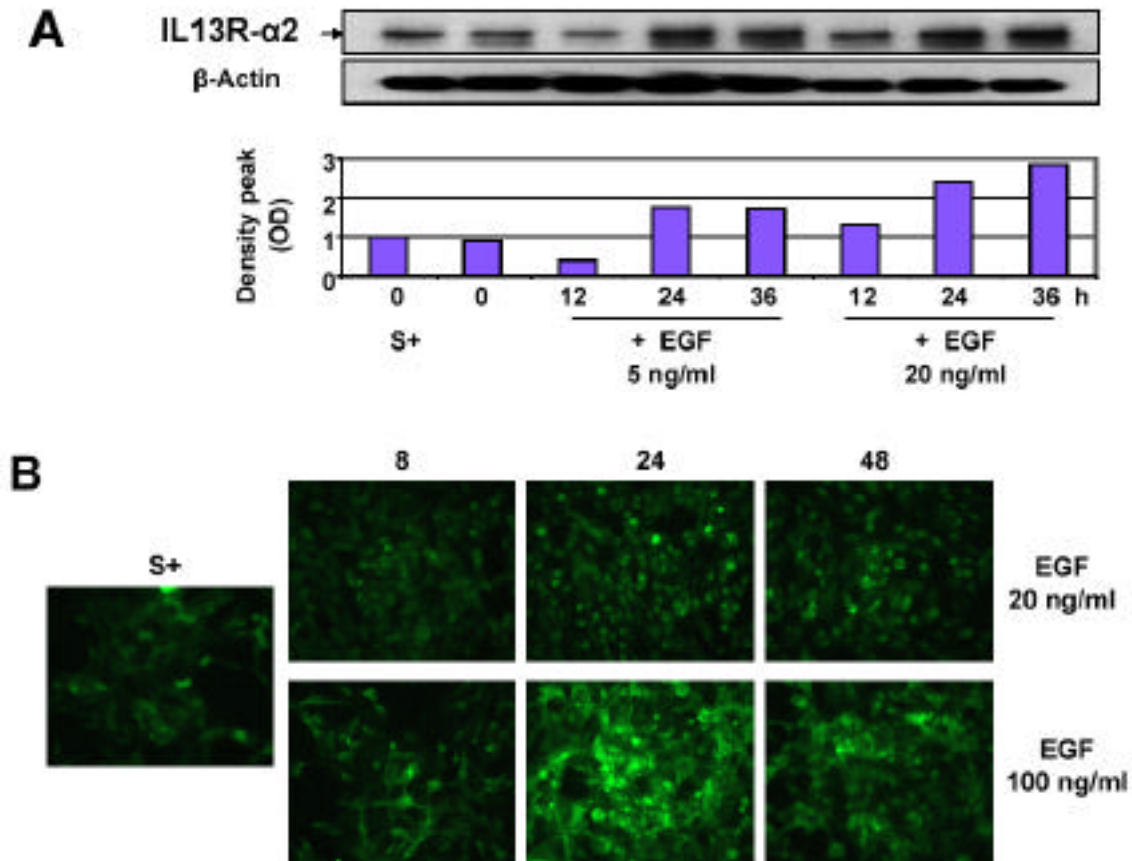


Figure 4. A. Immunoreactive IL13R- 2 in U-251 MG GBM cells maintained under serum-free conditions. The cells were treated with EGF for 12, 24 and 36 hr. **B.** Immunofluorescence of IL13R- 2 staining of human U-251 MG GBM cells in serum-containing media treated with either 20 ng/ml or 100 ng/ml of EGF.

In addition, we used a glioma cell line, T-98G expressing low number of the restricted binding sites for IL13 when compared with other GBM cells (**Figure 1**). We examined the response of T-98G cells to the cytokines similarly to the four other GBM cell lines over-expressing IL13R- 2. The levels of immunoreactive IL13R- 2 remained low independent of the cytokine added and culture conditions used (data not shown).

We next examined how IL13R- 2 responds to the cytokines in normal human cells. IL13R- 2 gene expression is poor in normal organs and on average normal cells contain little if any of this receptor protein (Debinski and Gibo, 2000; Debinski et al, 1995c). We used normal human endothelial cells, HUVEC-C and immortalized normal human glial cells, SVGp12 that show minute amounts of immunoreactive IL13R- 2 when compared with the studied GBM cell lines (**Figure 1**). Treatment with the cytokines (EGF, TNF or IL4) caused sporadic changes in the levels of the receptor, but they were minimal and of magnitudes lower when compared with the background levels of IL13R- 2 in GBM cells, such as G48a (**Figure 5A**, HUVEC and **Figure 5B**, SVGp12 cells; EGF and TNF treatment shown).

Being that EGF was the most potent cytokine in the induction of IL13R- 2 in GBM cells, we attempted to document (i) a direct involvement of the EGFR receptor in

transducing the signal for the IL13 receptor expression and (ii) which of the major pathways of EGFR stimulation that are operational in cancer cells is responsible for this phenomenon. We thus employed three different inhibitors of the EGFR signaling pathway in GBM cell lines. The EGF tyrosine kinase inhibitor, AG1478, either had a minimal effect on the basal levels of IL13R- 2 (G48a cells) or significantly reduced the levels of the receptor under baseline conditions (U-251 MG cells) (**Figure 6A**). However, AG1478 prevented completely an up-regulation of the receptor in response to EGF in all these cells (**Figure 6A** and data not shown). This directly demonstrates that the activation of the EGFR is indeed transducing stimulatory signals for the IL13R- 2 expression in GBM cells. These stimulatory signals can be carried out by either PI3-K or MAPK in cancer cells (Cuadrado et al, 2003). SB203580, an inhibitor of P38 MAPK changed moderately the basal levels of IL13R- 2 in glioma cells, but contrary to AG1478, it did not prevent an increase in IL13R- 2 in response to EGF (**Figure 6A**). On the other hand, an inhibitor of PI3-K, Wortmannin, exerted a similar to AG1478, but to a smaller extent, effect in neutralizing EGF-induced up-regulation of IL13R- 2 in GBM cells (**Figure 6A**).

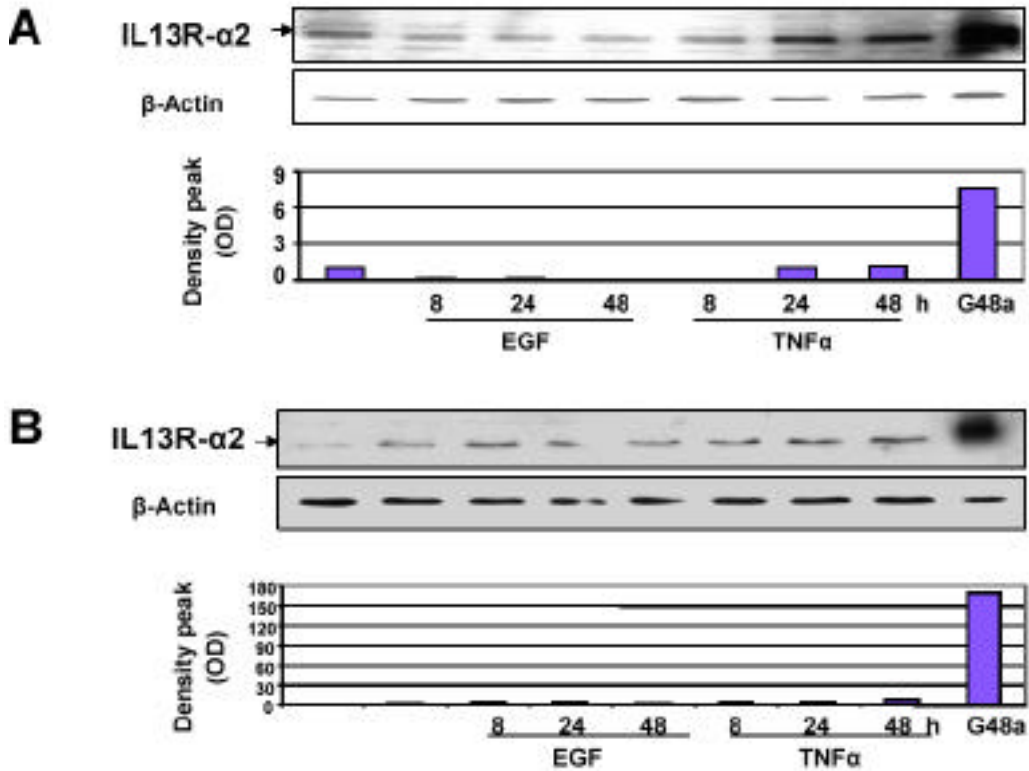


Figure 5. A. Immunoreactive IL13R- 2 in HUVEC-C cells maintained in normal media. The cells, were treated with EGF (100 ng/ml) or TNF (50 ng/ml) for 8, 24 and 48 hr. **B.** Immunoreactive IL13R- 2 in Western blot performed on lysates from SVGp12 cells maintained in serum-free media. The cells were treated with EGF (20 ng/ml) and TNF (5 ng/ml) for 8, 24 and 48 hr. S+, serum containing media; S-, no serum. The lysate of G48a GBM cells was loaded for comparison.

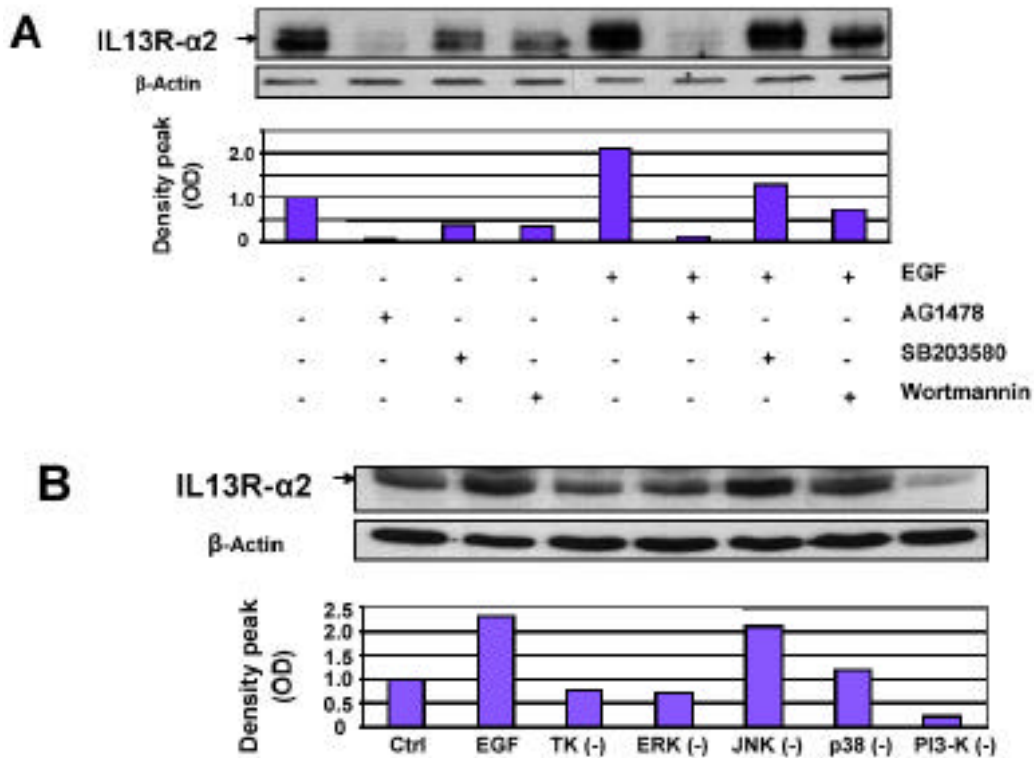


Figure 6. A. The effect of inhibitors of the EGFR and its signaling on immunoreactive IL13R- 2 in U-251 MG cells. Cells were pre-treated with AG1478 (10 μ M), SB203580 (10 μ M) and Wortmannin (100 ng/ml) 1h before the addition of EGF (100 ng/ml) for 24 hr. **B.** Cells were pre-treated with inhibitors of TK (AG 1478; 20 μ M), ERK (PD 98059; 20 μ M), JNK (SP 600125; 40 μ M), p38 (SB 203580; 10 μ M) and PI3-K (LY 294002; 10 μ M) inhibitors 1h before the addition of EGF (100 ng/ml) for 24 hr.

In a further attempt to dissect more precisely which intracellular signaling pathways take part in the IL13R- 2 up-regulation in response to EGF, we treated the U-251 MG cells with 100 ng/ml EGF in the presence of TK, ERK, JNK, p38 and PI3-K inhibitors, and measured immunoreactive IL13R- 2 by Western blot. In these experiments, we used more specific inhibitors of PI3-K (LY 294002), JNK (SP 600125) and ERK (PD 98059). We found that an increase in immunoreactive IL13R- 2 due to the treatment with EGF was best prevented by the TK, PI3-K and ERK inhibitors (Figure 6B). In order to demonstrate that PD 98059 blocked its target activation, we measured phosphorylated ERK in the presence or absence of the inhibitor (Figure 7A). Phosphorylated ERK increased by more than forty times after 5 and 15 min of

treatment with EGF and PD 98059 prevented the rise in activated ERK almost completely in the U-251 MG cells (Figure 7A). Furthermore, in order to document that the PI3-K inhibitor, LY 294002, exerted its specific function in GBM cells, we measured phosphorylated AKT and observed a potent inhibition of both baseline and EGF-induced levels of the activated AKT (Figure 7B).

IL13R- 2 is a molecular target for IL13-based recombinant cytotoxin candidate drugs (Debinski et al, 1999a). Even though it is over-expressed in GBM cells, its levels can be further increased by PI3-K or ERK pathways activation (Figure 6A and 6B). We have thus tested whether this increase in IL13R- 2 may lead to further sensitization of GBM cells toward IL13-based cytotoxins.

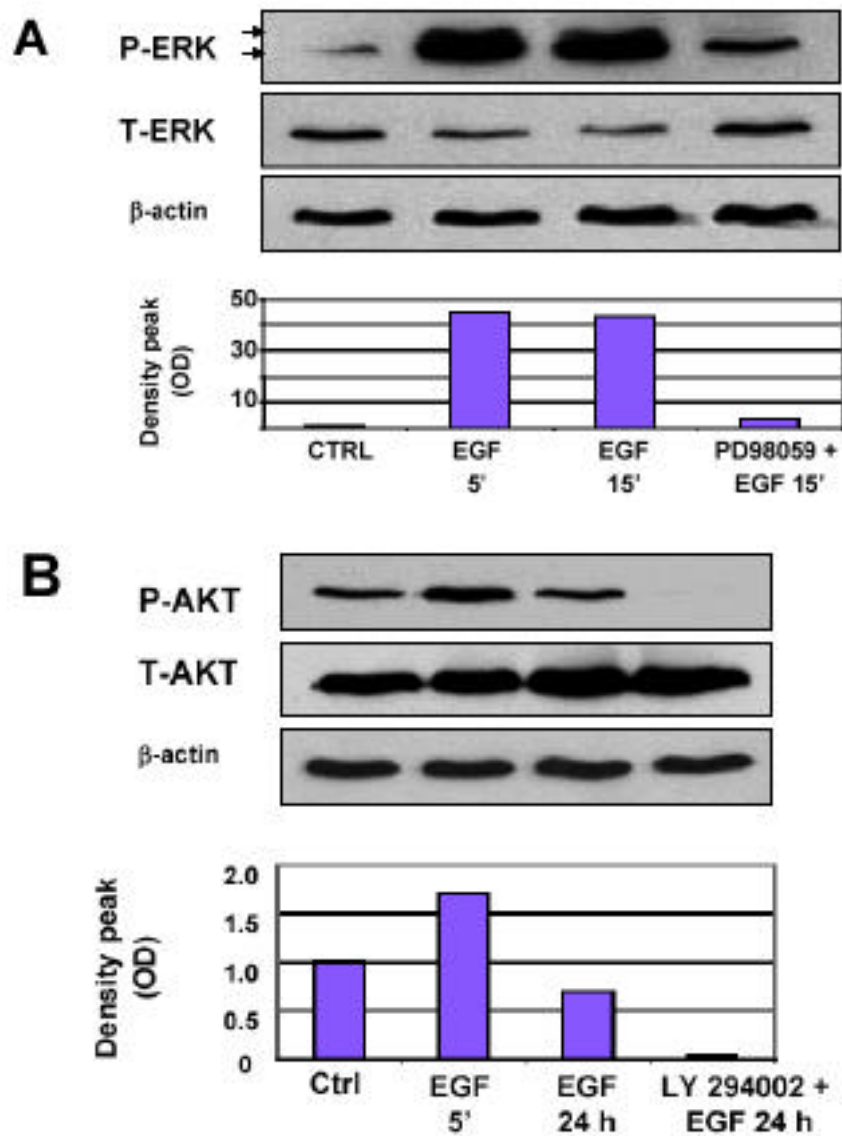


Figure 7. A. The effect of an ERK inhibitor (PD98059) on EGF-induced phosphorylated ERK (P-ERK) and immunoreactive ERK proteins (T-ERK) in U-251 MG cells. Cells were pre-treated with PD98059 (10 μM) for 1 h before the addition of EGF (100 ng/ml) for 5 or 15 min. A quantitation of the density of the immunoreactive bands is shown for P-ERK. **B.** The effect of PI3-K inhibitor () on EGF-induced immunoreactive phosphorylated AKT (P-AKT) and immunoreactive AKT protein (T-AKT) in U-251 MG cells. Cells were pre-treated with LY 294002 (10 μM) for 1 h before the addition of EGF (100 ng/ml) for 5 min or 24 h.

Thus, the G48a and U-251 MG glioma cells that over-express IL13R- 2 were pre-treated with EGF (20 or 100

ng/ml) for 24 h before the addition of IL13.E13K-PE38QQR. The pre-treatment with EGF made the G48a and U-251 MG cells more susceptible to the cytotoxin; which was seen more pronounced at a higher concentration of EGF (**Figure 8A**). The cytotoxin could achieve its IC₅₀ even at two logs lower concentration when cells were pre-treated with EGF compared to not pre-treated cells. However, when we used T-98G GBM cells that do not over-express IL13R- 2 and are non-responders to IL13.E13K-PE38QQR, pre-treatment with EGF did not result in making these cells any more susceptible to the cytotoxin (**Figure 8B**). The same was observed with mouse G-26 V2 glioma cells transfected with an empty vector (**Figure 8B**) (Mintz et al, 2003). Also, mouse G-26 H2 glioma cells that carry the IL13R- 2 transgene (Mintz

et al, 2003) did not become more responsive to the cytotoxin when pre-treated with EGF (**Figure 8B**). In a final set of experiments, we examined whether EGF alone has any influence on the glioma cells proliferation under the conditions shown in Figure 8A and B. We found no significant effect of EGF (up to 100 ng/ml) on either human or mouse glioma cells (**Figure 8C**).

IV. Discussion

We have previously found that IL13R- 2 is up-regulated in GBM cells (Debinski et al, 1999a; Mintz et al, 2002). In the current work, we have found that the level of protein significantly decreases in some serum-starved GBM cells. However, the protein can be brought back to

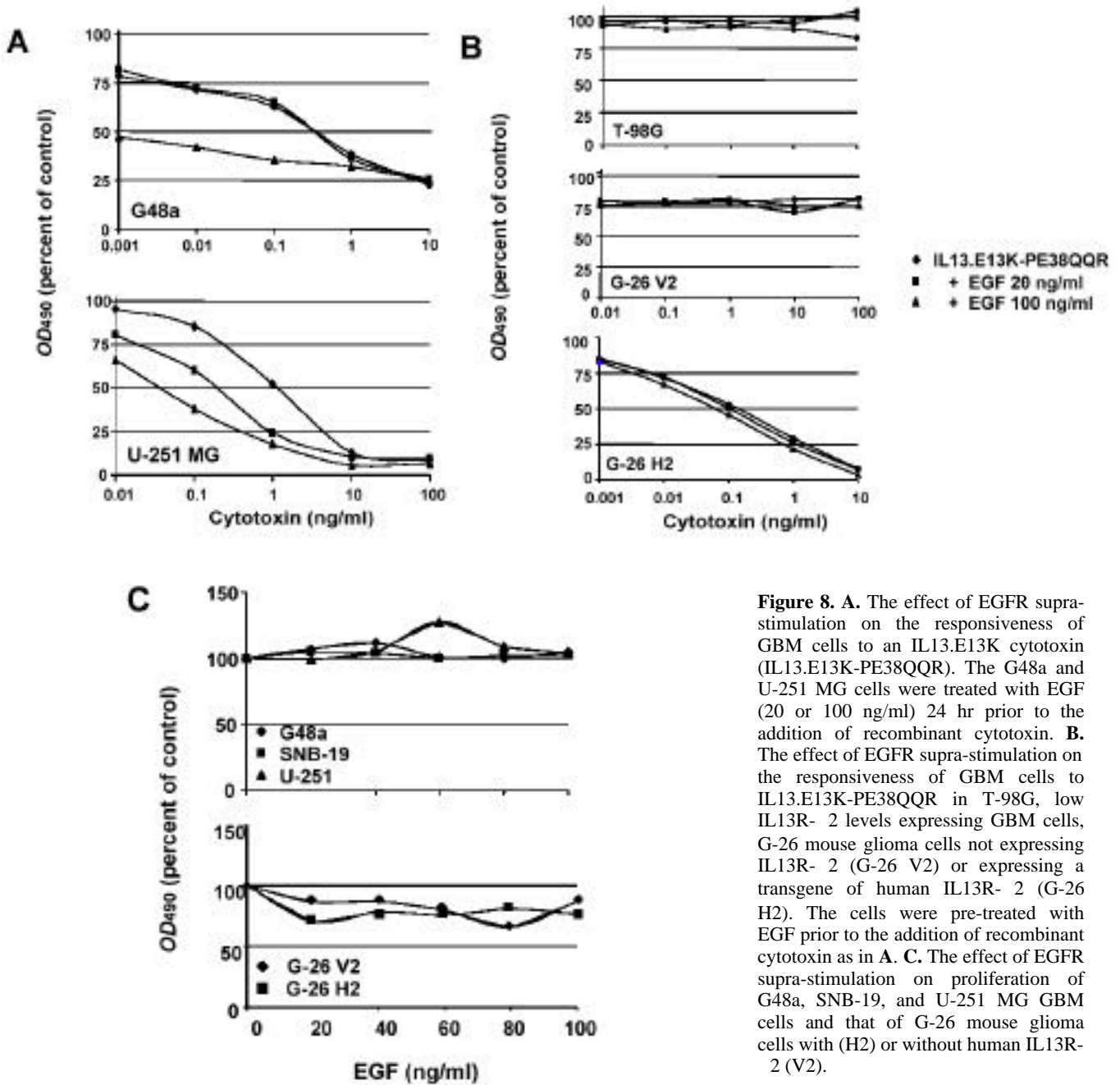


Figure 8. A. The effect of EGFR supra-stimulation on the responsiveness of GBM cells to an IL13.E13K cytotoxin (IL13.E13K-PE38QQR). The G48a and U-251 MG cells were treated with EGF (20 or 100 ng/ml) 24 hr prior to the addition of recombinant cytotoxin. **B.** The effect of EGFR supra-stimulation on the responsiveness of GBM cells to IL13.E13K-PE38QQR in T-98G, low IL13R- 2 levels expressing GBM cells, G-26 mouse glioma cells not expressing IL13R- 2 (G-26 V2) or expressing a transgene of human IL13R- 2 (G-26 H2). The cells were pre-treated with EGF prior to the addition of recombinant cytotoxin as in A. **C.** The effect of EGFR supra-stimulation on proliferation of G48a, SNB-19, and U-251 MG GBM cells and that of G-26 mouse glioma cells with (H2) or without human IL13R- 2 (V2).

the original levels, or even higher, by treating cells with

AP-1 and/or STAT-6 activators. EGF, by activating also

the AP-1 pathway, among others, was the most potent in inducing this phenomenon, while TNF and IL4 demonstrate variable ability to further up-regulate IL13R- 2 in glioma cells. Cytokine-induced IL13R- 2 immunoreactivity localizes primarily to cell membranes, i.e. the receptor is produced and placed into the plasma membrane compartment of glioma cells. Under normal cell culture conditions, supra-physiologic amounts of EGF and that of TNF /IL4 evoked even further increase in an already elevated immunoreactive IL13R- 2. This indicates that the IL13R- 2 is expressed in a non-constitutive manner in glioma tumor cells. We have also demonstrated that an increase in IL13R- 2 in response to EGF is mediated through the activation of the EGFR signaling pathways. In addition, we have documented that the PI3-K and ERK pathways, rather than P38 and JNK MAPK pathways, of EGFR activation appears to take part in regulating the restricted IL13 receptor in glioma cells. Finally, glioma cells pre-treated with EGF became more susceptible to the killing by an IL13 mutant-based recombinant anti-cancer cytotoxin. Thus, IL13R- 2 can be regulated in brain tumor cells and this can be potentially utilized to increase the responsiveness of cancer cells to anti-cancer drug candidates.

EGF was found to be the most potent among the studied cytokines in up-regulating IL13R- 2 in glioma cells. EGF activates the AP-1 pathway potently in glioma cells (Debinski et al, 2001; Debinski and Gibo, 2005). This action is mediated through the EGF receptor tyrosine kinase first and we demonstrated that blocking this step by using AG1478 EGFR kinase inhibitor prevents EGF from up-regulating IL13R- 2. There are at least two major pathways for EGFR signaling in cancer cells that involve PI3-K and MAPK (Cuadrado et al, 2003). Using respective inhibitors of these routes of signaling, we found that the PI3-K and ERK pathways appear to be more important for EGF-induced IL13R- 2 up-regulation than the MAPK pathway. In support of this contention others found that P38 and JNK are EGF signaling-independent MAPK (Dong et al, 2004). We are continuing the search for other factors related to the PI3-K and ERK that might be important for IL13R- 2 expression in glioma cells.

Recombinant anti-cancer cytotoxin killing potency is roughly proportionate to the number of targeted receptors expressed on cells (Debinski et al, 1995c; Mintz et al, 2003). It would be thus desirable to increase the expression levels of IL13R- 2 on GBM cells, in order to make them even more susceptible to the killing by the IL13 cytotoxins and/or making more GBM cells responsive to the cytotoxin in general. Our current work documents that STAT-6 and AP-1 activators influence the levels of IL13R- 2 expression in glioma cells. This increase in immunoreactive protein levels is most likely responsible for an increased sensitivity of glioma cells to the killing by the recombinant IL13-based cytotoxin that we observed. This phenomenon is specific to the presence of endogenous regulated IL13R- 2 in cells and it requires that the receptor is over-expressed to start with. This finding may offer an attractive approach in which a pre-treatment with the compounds of neutral or even anti-cancer activity on their own would lead to an up-regulation of IL13R- 2 and subsequent better efficacy of the IL13 cytotoxins. Being that normal cells which have

little if any of IL13R- 2 do not respond to various cytokines by up-regulating the receptor not even near to the levels found in GBM cells, this approach further delineated the specificity of the use of recombinant cytotoxins as anti-cancer drugs.

Recent intense interest in the inhibitors of the EGFR in cancer treatment (Yang et al, 2004) would prompt one to use such inhibitors in combination with the IL13-based cytotoxins. Unfortunately, our study suggests that by inhibiting the signaling through the EGFR the expression of IL13R- 2, a target for the recombinant cytotoxins, is significantly diminished. Thus, less of a target is available for the binding of targeted cytotoxins. However, novel anti-cancer drugs have been recently identified that appear to work through a supra-stimulation of the EGFR leading to cancer cell death (Cuadrado et al, 2003) or they are superagonist of the EGFR themselves (Monticello, 2003). It is plausible that such a drug(s) would amplify the efficacy of the IL13R- 2 targeted recombinant cytotoxins through further up-regulation of the receptor in GBM cells.

In summary, IL13R- 2, a glioma-associated receptor is expressed non-constitutively in glioma cell; its levels can be further up-regulated by growth factors/ cytokines. The PI3-K and ERK pathways appear to be primarily involved in the regulation of IL13R- 2 in glioma cells. A possibility of regulation of the receptor levels in cancer cells may offer a therapeutic advantage while using IL13R- 2-targeted agents.

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References

- Aman MJ, Tayebi N, Obiri NI, Puri RK, Modi WS and Leonard WJ (1996) cDNA cloning and characterization of the human interleukin 13 receptor chain. **J Biol Chem** 271, 29265-29270.
- Cuadrado A, Garcia-Fenandez LF, Gozales L, Suarez Y, Losada A, Alcaide V, Martinez, T, Fernandez-Sousa JM, Sanchez-Puelles JM and Munoz A (2003) Aplidin induces apoptosis in human cancer cells via glutathione depletion and sustained activation of the epidermal growth factor receptor, Src, JNK and p38 MAPK. **J Biol Chem** 278, 241-250.
- David MD, Bertoglio J and Pierre J (2003) Functional characterization of IL13 receptor $\alpha 2$ gene promoter: a critical role of the transcription factor STAT6 for regulated expression. **Oncogene** 22, 3386-3394.
- Debinski W and Gibo DM (2000) Molecular expression analysis of restrictive receptor for interleukin 13, a brain tumor-associated cancer/testis antigen. **Mol Med** 6, 440-449.
- Debinski W and Gibo DM (2005) Fra-1 modulates malignant features of glioma cells. **Mol Cancer Res** 3, 237-249.
- Debinski W, Gibo DM and Mintz A (2003) Epigenetics in high-grade astrocytomas: Opportunities for prevention and detection of brain tumors. **Ann NY Acad Sci** 983, 232-242.
- Debinski W, Gibo DM, Hulet SW, Connor JR and Gillespie GY (1999a) Receptor for interleukin 13 is a marker and therapeutic target for human high grade gliomas. **Clin Cancer Res** 5, 985-990.
- Debinski W, Obiri NI, Pastan I and Puri RK (1995b) A novel chimeric protein composed of interleukin 13 and Pseudomonas exotoxin is highly cytotoxic to human

- carcinoma cells expressing receptors for interleukin 13 and interleukin 4. **J Biol Chem** 270, 16775-16780.
- Debinski W, Obiri NI, Powers SK, Pastan I and Puri RK (1995c) Human glioma cells overexpress receptor for interleukin 13 and are extremely sensitive to a novel chimeric protein composed of interleukin 13 and Pseudomonas exotoxin. **Clin Cancer Res** 1, 1253-1258.
- Debinski W, Slagle B, Gibo DM, Powers SK and Gillespie GY (2000) Expression of a restrictive receptor for interleukin 13 is associated with glial transformation. **J Neuro-Oncol** 48, 103-111.
- Debinski W, Slagle-Webb B, Achen MG, Stacker SA, Tulchinsky E, Gillespie GY and Gibo DM (2001) VEGF-D is an X-linked/AP-1-regulated onco-angiogen in highly vascularized tumors. **Mol Med** 7, 598-608.
- Dong J, Ramachandiran S, Tikoo K, Jia Z, Lau SS and Monks TJ (2004) EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death. *Am J Physiol Renal Physiol* 287, F1049-F1058.
- Hilton DJ, Zhang J-G, Metcalf D, Akexander WS, Nicola NA and Wilson TA (1996) Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. **Proc Natl Acad Sci USA** 93, 497-501.
- Kahlon KS, Brown C, Cooper LNJ, Raubitschek A, Forman SJ and Jensen MC (2004) Specific recognition and killing of glioblastoma multiforme by interleukin 13-zetakine redirected cytolytic T cells. **Cancer Res** 64, 9160-9167.
- Kelly-Welch AE, Hanson EM, Boothby MR and Keegan AD (2003) Interleukin-4 and Interleukin-13 signaling connections map. **Science** 300, 1527-1529.
- Lethe B, Lucas S, Michaux S, De Smet C, Godelaine D, Serrano A, DePlaen E and Boon T (1998) LAGE-1, a new gene with tumor specificity. **Int J Cancer** 76, 903-908.
- Liang G, Gonzales FA, Jones PA, Ortoft TF and Thykjaer T (2002) Analysis of gene induction in human fibroblasts and bladder cancer cells exposed to the methylation inhibitor 5-aza-2'-deoxycytidine. **Cancer Res** 62, 961-966.
- Liu TF, Tatter SB, Willingham MC, Yang M, Hu JJ and Frankel AE (2003) Growth factor receptor expression varies among high-grade gliomas and normal brain: epidermal growth factor receptor has excellent properties for interstitial fusion protein therapy. **Mol Cancer Ther** 2, 783-787.
- Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N and Ferrara P (1997) Cloning of the human IL-13R of a functional IL-4/IL-13 receptor complex. **FEBS Letters** 401, 63-166.
- Mintz A and Debinski W (2000) Cancer genetics/epigenetics and the X chromosome: Possible new links for malignant glioma pathogenesis and immune-based therapies. **Critic Rev Oncogen** 11, 77-95.
- Mintz A, Gibo DM, Madhankumar AB and Debinski W (2003) Molecular targeting with recombinant cytotoxins of interleukin-13 receptor 2-expressing glioma. **J Neuro-Oncol** 64, 117-123.
- Mintz A, Gibo DM, Madhankumar AB, Cladel NM, Christensen ND and Debinski W (2002) Effective protein and DNA active immunotherapies against interleukin 13 receptor 2-expressing gliomas. **Neuro-Oncology** 4, 334.
- Mintz A, Gibo DM, Webb (Slagle) B and Debinski W (2002) IL13R 2 is a glioma-restricted receptor for IL13. **Neoplasia** 4, 388-399.
- Monticello D (2003) Modulating epidermal growth factor receptor activity using variant EGF molecules. Strategic Research Institute Meeting on "Growth Factor Receptors", Philadelphia, PA, May 5-6.
- Murphy EV, Zhang Y, Zhu W and Biggs J (1995) The human glioma pathogenesis-related protein is structurally related to plant pathogenesis-related proteins and its gene is expressed specifically in brain tumors. **Gene** 159, 131-135.
- Okano F, Storkus WJ, Chambers WH, Pollack IF and Okada H (2002) Identification of a novel HLA-A*0201-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor 2 chain. **Clin Cancer Res** 8, 2851-2855.
- Rich T, Chen P, Furman F, Huynh N and Israel M (1996) RTVP-1, a novel human gene with sequence similarity to genes of diverse species, is expressed in tumor cell lines of glial but not neuronal origin. **Gene** 180, 125-130.
- Vita N, Lefort S, Laurent P, Caput D and Ferrara P (1995) Characterization and comparison of the interleukin 13 receptor with the interleukin 4 receptor on several cell types. **J Biol Chem** 270, 3512-3517.
- Wu AH and Low WC (2003) Molecular cloning and identification of the human interleukin 13 alpha 2 receptor (IL-13R 2) promoter. *Neuro-oncol.* 5, 179-87.
- Yang Z, Bagheri-Yarmand R, Wang RA, Adam, L, Papadimitrakopoulou VV, Clayman GL, El-Naggar A, Lotan R, Barnes CJ, Hong WK, Kumar R (2004) The epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa) suppresses c-Src and Pak1 pathways and invasiveness of human cancer cells. **Clin Cancer Res** 10, 658-667.
- Zhou G, Ye G-J, Debinski W and Roizman B (2002) Genetic engineering of a herpes virus 1 vector dependent on the IL13R 2 receptor for entry into cells: interaction of glycoprotein D with its receptors is independent of the fusion of the envelope and the plasma membrane. **Proc Natl Acad Sci** 99, 15124-15129.
- Zurawski SM, Chomarat P, Djossou O, Bidaud C, McKenzie ANJ, Miossec P, Banchereau J and Zurawski G (1995) The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor. **J Biol Chem** 270, 13869-13878.
- Zurawski SM, Vega F Jr, Huyghe B and Zurawski G (1993) Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. **EMBO J** 12, 2663-2670.



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