Interleukin-4 Down-regulates Both Forms of Tumor Necrosis Factor Receptor and Receptor-mediated Apoptosis, NF-κB, AP-1, and c-Jun N-Terminal Kinase

COMPARISON WITH INTERLEUKIN-13*

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The activity of tumor necrosis factor (TNF), a proinflammatory cytokine, is regulated by a number of other cytokines, including interleukin (IL)-4. How IL-4 regulates various activities of TNF is not fully understood. In the present report, we investigated the effect of IL-4 on the cell surface TNF receptors in human histiocytic lymphoma U-937 cells. Pretreatment of cells with IL-4 downregulated TNF receptors in a dose- and time-dependent manner; an almost 90% decrease occurred with 10 ng/ml IL-4 treatment for 24 h. Scatchard analysis revealed that the decrease was due to receptor number and not affinity. IL-13, which shares a common receptor subunit and various biological activities with IL-4, had no effect on TNF receptors. IL-4’s effect on TNF receptors was not cell type-specific, since decreases also occurred on various epithelial and T cells. Both the p60 and p80 forms of the TNF receptor were down-regulated to the same extent. Western blot showed that IL-4 induced shedding of the TNF receptors. The decrease of TNF receptors by IL-4 was accompanied by down-regulation of TNF-induced activities, including cytotoxicity, caspase-3 activation, NF-κB and AP-1 activation, and c-Jun N-terminal kinase induction. Wortmannin reversed the IL-4-induced TNF receptor down-regulation and all other measured cellular responses, indicating a critical role of phosphatidylinositol 3-kinase. Rapamycin also blocked the effect of IL-4-induced regulation, thus suggesting the role of p70 S6 kinase. Overall, our results suggest that TNF receptor down-regulation by IL-4 plays a critical role in the antagonistic effects of IL-4 on TNF-induced cellular responses and that this mechanism differs from that of IL-13.

Extensive research within the last few years has revealed that the immune system is regulated by soluble mediators, referred to as cytokines, produced in part by T-helper-1 and -2 (Th1 and Th2) cell types. The central dogma of immunology is that the cytokines produced by Th1 cells exhibit immunostimulatory function, and those produced by Th2 display immunosuppressive activities (for references see Ref. 1). Examples of the Th1 type of cytokines include tumor necrosis factor (TNF), lymphoxygen, and interferon-γ; those of the Th2 type include interleukin (IL)-4, IL-10, and IL-13. Several studies have also suggested that cytokines produced by Th2 cells regulate the activities of those produced by Th1 cells and vice versa. The exact mechanism of this regulation is less clear, but cytokine production and signal transduction may both be involved.

TNF, one of the Th1 cytokines, is also produced by macrophages and plays an important role in growth modulation, inflammation, viral replication, septic shock, and immune modulation (2). TNF transduces its effects through two distinct receptors, viz. p60 and p80, which have homologous extracellular domains but distinct cytoplasmic domains. The p60 receptors are expressed on all cell types, whereas p80 is expressed only on myeloid, lymphoid, and endothelial cells. Most of the signals are mediated through the p60 receptors. The growth-modulatory effects of TNF are in part mediated through the activation of caspases, and TNF-induced gene expression is partially mediated through the activation of the nuclear transcription factors NF-κB and AP-1. In addition, TNF is a potent activator of the serine/threonine protein kinases of the mitogen-activated protein kinase family, including c-Jun N-terminal kinase (JNK)/stress-activated protein kinase.

IL-4 is a cytokine produced by Th2 cells. It promotes growth of preactivated B cells, induces germ line e transcripts, directs naive B cells to switch to IgE and IgG4 synthesis, and induces expression of low affinity IgE receptors (3). It down-regulates Fcy receptor expression and inhibits the synthesis of proinflammatory cytokines including TNF (4–8). IL-4 also increases the expression of vascular cell adhesion molecule 1 on endothelial cells (9). IL-4 interacts with cells through a receptor consisting of a distinct α-chain and of a γ-chain that is shared by several other interleukins. The interaction of IL-4 with the α-chain and γ-chain of the receptor activates protein tyrosine kinases JAK1 and JAK3, respectively, leading to the activation of the transcription factor STAT6 (3, 10). IL-13 is another cytokine produced by the Th2 cells. It shares 30% amino acid identity with IL-4. IL-13 has been assigned most of the biological effects characteristic of IL-4 and shares certain receptor components (11, 12). In addition to common α-chain and γ-chain, IL-13 binds to two additional components, which include IL-13Rα1 (low affinity; 2-10 nM) and IL-13Rα2 (high affinity; 0.25 nM) subunits. The latter subunits do not bind IL-4 and thus form the basis for differences between the two cytokines in certain systems.

Several reports indicate that IL-4, as well as IL-13, can

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down-regulate a wide variety of TNF-induced effects (13–20). There are also reports demonstrating that IL-4 and TNF are synergistic in their action (21, 22). Just how IL-4 and IL-13 modulate the function of TNF is not understood. In this report, we investigated the effect of IL-4 and IL-13 on TNF receptors and the receptor-mediated early and late cellular responses. The results indicate that IL-4, but not IL-13, induces down-regulation and shedding of TNF receptors. This is accompanied by suppression of TNF-induced NF-κB and AP-1 activation, inhibition of TNF-induced cytotoxicity and activation of caspase-3, and abrogation of TNF-induced JNK activation. These suppressive effects of IL-4 on TNF appear to be mediated through activation of phosphatidylinositol 3-kinase and of p70 S6 kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Penicillin, streptomycin, neomycin, RPMI 1640 medium, and fetal calf serum were obtained from Life Technologies, Inc. Glycine, 1-(5-isooquinolinylsulfonyl)-2-methyl piperezine (H7), 3-(4,5-dihydro-6-(4-3,4-dimethoxybenzyl)-1-piperazinyl)-2H-1quinoline (MTT), and wortmannin were obtained from Sigma. Rapamycin and PD098059 were kindly provided by Dr. Bernhard Ryffel (then from the Institute for Toxicology, University of Zurich, Switzerland) and Dr. Alan R. Saltiel (Parke Davis Research Division, Warner Lambert Company, Ann Arbor, MI), respectively. Bacteria-derived recombinant highly purified human IL-4, IL-13, and TNF were kindly provided by Dr. T. L. Nagabhushan, then of Schering-Plow Inc. (Bloomfield, NJ), by Dr. Rene de Waal Malefyt of DNAX Inc. (Palo Alto, CA), and by Genentech, Inc. (South San Francisco, CA), respectively. Double-stranded NF-κB and AP-1 oligonucleotides and single-stranded NF-κB oligonucleotide were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against TNF p60 and p80 receptors raised in rabbit were polyclonal but affinity-purified. Anti-poly(ADP)-ribose polymerase antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mobility shift assay (EMSA) following the method described by Chaturvedi et al., with radiolabeled TNF (23). The cytotoxic effect of TNF on cells was determined by filtration through 10,000 M cutoff membrane. A 50-μg aliquot of cell extract protein and 60 μg of concentrated supernatant were solved on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with a rabbit polyclonal antibody against p80 TNF receptor, and then detected by chemiluminescence (ECI, Amersham Pharmacia Biotech) (24).

**Immunoblot Analysis of Poly(ADP) Ribose Polymerase (PARP) Degradation**—Radiation—TNF apoptosis was examined by proteolytic cleavage of PARP (23). Briefly, cells (2 × 10⁶/mL) were treated with cycloheximide (2 μg/mL) and TNF (1 ng/mL) for 2 h at 37 °C. After treatment, cell extracts were prepared by lysing the cells for 30 min on ice. The supernatant was concentrated by filtration through 10,000 M cutoff membrane. A 50-μg aliquot of cell extract protein and 60 μg of concentrated supernatant were resolved on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membrane, blotted with mouse anti-PARP antibody, and then detected by chemiluminescence (ECI, Amersham Pharmacia Biotech). Apoptosis was represented by the cleavage of 116-kDa PARP into an 85-kDa peptide product.

**TNF Cytotoxicity Assay**—The cytotoxic effect of TNF on cells was determined by the modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (27). Briefly, cells (10,000 cells/well) were incubated in the presence or absence of the indicated test sample in a final volume of 0.1 ml for 24 h at 37 °C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well. After a 2-h incubation at 37 °C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethyl formamide) was added. After an overnight incubation at 37 °C, the optical densities at 590 nm were measured using a 96-well multispecimen autoreader (Dy- natech MR 5000), with the extraction buffer as a blank. Percentage of cytotoxicity was determined as follows: % Cytotoxicity = (1 – A(test)/A(control)) × 100%

**c-Jun Kinase Assay**—The c-Jun kinase assay was performed by a modification of the method described earlier (23). Briefly, after treatment of cells (3 × 10⁶/mL) with TNF for 10 min, cell extracts were prepared by lysing cells in buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM phenylmethylsulfonil fluoride, 0.5 μg/mL benzamidine, and 1 mM diithiothreitol. The proteins were lysed and the supernatant was collected. Cell extract protein (50 μg) was resolved in 7.5% SDS-polyacrylamide gel electrophoresis, electrotransferred onto a nitrocellulose membrane, blotted with mouse anti-JNK antibody, and then detected by chemiluminescence (ECI, Amersham Pharmacia Biotech). Apoptosis was represented by the cleavage of 116-kDa PARP into an 85-kDa peptide product.
IL-4 Down-regulates TNF Receptors and TNF Cellular Responses

RESULTS

The aim of this study was to examine the effect of IL-4 on the TNF receptors and receptor-mediated cellular responses of U-937 cells. Treatment of these cells with either cytokine under the experimental conditions had no effect on the viability of these cells.

IL-4 but Not IL-13 Inhibits TNF Binding—U-937 cells (2 x 10^6/ml) were treated with different concentrations of either IL-4 or IL-13 for 24 h at 37°C in a CO_2 incubator. Then cells were suspended in fresh medium and examined for specific binding to TNF. The results in Fig. 1A indicate that IL-4 inhibited TNF binding in a dose-response fashion. A greater than 80% decrease in TNF binding occurred with 10 ng/ml of IL-4. IL-13, however, had no effect on TNF binding. The lack of effect of IL-13 was not due to either lack of receptors or lack of biological activity, because it was quite active in down-regulating TNF-induced NF-kB activation in U-937 cells (data not shown). Because IL-13 lacked effect, it was not tested any further.

To determine the time needed to down-regulate TNF receptors by IL-4, U-937 (2 x 10^6/ml) cells were pretreated with 10 ng/ml IL-4 for 1, 2, 4, 6, 9, 12, 18, and 24 h at 37°C and then washed with fresh ice-cold medium, cooled on ice, and then examined for TNF binding. The results in Fig. 1B indicate that TNF binding was inhibited with increase in time of exposure to IL-4; an almost 80% decrease in binding occurred with a 24-h pretreatment.

IL-4 Decreases TNF Receptor Number but Not the Affinity—To determine whether IL-4 down-regulates TNF receptor number or its affinity or both, ligand-competitive displacement experiments were performed. U-937 cells (1 x 10^6/ml) were treated with 0, 2, and 10 ng/ml IL-4 for 24 h at 37°C in a CO_2 incubator. Then cells (1.2 x 10^6/ml) were washed, suspended in fresh cold medium, and examined for TNF binding; 1.2 x 10^6 cells/ml were incubated at 4°C with different concentrations of [125I]-TNF in the presence and absence of unlabeled TNF (50-fold excess). The results in Fig. 2A indicate that specific TNF binding increased with increases in labeled TNF. Pretreatment of cells with IL-4, however, decreased the binding. Scatchard analysis of the data (Fig. 2B) showed that treatment of cells with 2 and 10 ng/ml IL-4 decreased TNF receptor number from 7142/cell to 4761/cell and 1039/cell, respectively. These results suggest that IL-4 decreased the TNF receptor number and not the affinity.

IL-4 Decreases TNF Receptors on Myeloid, Epithelial, and T Cells—We examined the effect of IL-4 on other myeloid (ML1a), epithelial (HeLa and MCF-7), T cell (Jurkat), and glioma (H4) cells. For this, 2 x 10^6 cells/ml were pretreated with IL-4 (10 ng/ml) for 24 h at 37°C in a CO_2 incubator and then examined for specific TNF binding at 4°C. IL-4 decreased the cell surface expression of TNF receptors on all different cell types (Fig. 3), thus suggesting that the effects of IL-4 are not cell type-specific.

IL-4 Down-regulates both p60 and p80 Forms of TNF Receptors—Previously, it has been shown that myeloid and T cells express both the p60 and p80 forms of the TNF receptor, whereas all other cell types express only the p60 form (2). Since the effects of IL-4 are not cell type-specific, most likely both types of TNF receptors are down-regulated. To ascertain more directly the effect of IL-4 on each type of receptor, we used receptor-specific antibodies. U-937 cells were pretreated with IL-4 (10 ng/ml) for 24 h at 37°C in a CO_2 incubator and then incubated with antibodies to either the p60 or p80 receptor for 1 h at 37°C and thereafter examined for TNF receptors as described earlier. In untreated cells, IL-4 down-regulated TNF receptor by almost 80% (Fig. 4A). Anti-p80 antibody incubation led to approximately 70% inhibition of TNF binding, indicating that the remaining 30% of surface TNF receptors were p60. In contrast, anti-p60 antibody incubation led to about 30% inhibition of TNF binding, indicating that about 70% of surface TNF receptors were p80. IL-4 down-regulated both p60 and p80 receptors to the same extent. In contrast, IL-13 (10 ng/ml for 24 h) had no effect either on total, p60, or p80 forms of the receptor. Thus, these results affirm the conclusion that IL-4...
down-regulates both types of TNF receptor.

**IL-4 Induces Shedding of TNF Receptors**—To determine if the decrease in receptor number is due to the shedding of TNF receptors, we examined the supernatants from IL-4-treated cells by Western blot analysis. The number of TNF receptors expressed on most cells were too low to be detected by the Western blot analysis. Therefore, we used HeLa cells that were stably transfected with a plasmid containing the human p80 TNF receptor gene. These cells have been previously characterized for the expression of the TNF receptor (23). HeLa p80-transfected cells were treated with IL-4 (2 and 10 ng/ml) for 24 h at 37 °C in a CO₂ incubator, and then the cell extract was prepared and the supernatants were concentrated and analyzed by Western blot for p80 receptor by using polyclonal anti-p80 receptor antibodies. The band for p80 receptor disappears from the cell extract fraction with increasing concentrations of IL-4 and reappears in the supernatant fraction (Fig. 4B). The band in the cell pellet fraction was at the 70–80-kDa location, whereas that in the supernatant fraction was at 35–40 kDa. This location is consistent with published reports, which indicate that shedding of TNF receptors involves only the extracellular domain (28). Thus, it is clear that IL-4 induces the shedding of the TNF receptors.

**IL-4 Inhibits TNF-induced Apoptosis**—How down-regulation of TNF receptors by IL-4 affects TNF-induced cellular responses was also investigated. TNF is cytotoxic to a wide variety of cells, including U-937 cells. To test the effect of IL-4 on TNF-induced cytotoxicity, these cells were pretreated with IL-4 (10 ng/ml) for 24 h, stimulated with different concentrations of TNF for 2 h in the presence of cycloheximide (2 μg/ml); then the extracts were analyzed for PARP cleavage by Western blot. The results in Fig. 5B indicate that 1000 pM TNF induced complete cleavage of PARP in untreated cells, but in IL-4-treated cells TNF-induced PARP cleavage was completely inhibited. These results suggest that IL-4 also blocks TNF-induced apoptosis.

**IL-4 Inhibits TNF-dependent NF-κB, AP-1, and JNK Activation**—One of the earliest responses induced by TNF is the activation of nuclear transcription factor NF-κB. To determine...
if IL-4 affects TNF-induced NF-κB activation, U-937 cells were preincubated for 24 h with different concentrations of IL-4, activated with TNF (100 pM) for 30 min at 37 °C. Then cells were washed with fresh medium and incubated with anti-TNF receptor antibody (both against p60 and p80) separately for 1 h at 37 °C. After the cells were washed with fresh cold medium, 125I-TNF binding was assayed as described earlier. B, effect of IL-4 on shedding of TNF receptor in p80-overexpressing cells. HeLa cells were stably transfected with human TNF p80 receptor. These cells were taken to show the shedding of TNF receptor. HeLa p80 cells were treated with IL-4 (2 and 10 ng/ml) for 24 h at 37 °C. Then cells and supernatants were collected from untreated and IL-4 (2 and 10 ng/ml)-treated cells. Supernatants were concentrated with an M_r 10,000 cut-off filter. Then both cell extract and concentrated supernatant were analyzed in 12% SDS-polyacrylamide gel electrophoresis, and Western blot was developed against polyclonal anti-p80 TNF receptor antibody raised in rabbit (1:3000).

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These results suggest that phosphatidylinositol 3-kinase and protein kinase C may play an important role in the down-regulation of TNF receptors by IL-4. Concurrently, wortmannin also reversed the suppressive effects of IL-4 on TNF-induced PARP cleavage (Fig. 7B), NF-κB activation (Fig. 7C), and AP-1 activation (Fig. 7D). These results suggest that down-regulation of TNF receptor and TFN-mediated responses by IL-4 occurs through activation of phosphatidylinositol 3-kinase.

**DISCUSSION**

The purpose of this study was to investigate the effect of IL-4 and IL-13 on TNF cellular signaling. In our experiments, IL-4 induced down-regulation of both forms of cell surface TNF receptors. In contrast, IL-13 had no effect on TNF receptors. IL-4 reduced TNF receptor number but not affinity and induced receptor-shedding, and the effect was not cell type-specific. IL-4 also blocked TNF-induced apoptosis and NF-κB, AP-1, and JNK activation. All of these effects of IL-4 could be reversed by wortmannin. IL-4-induced down-regulation of TNF receptors was also reversed by rapamycin.

Several previous reports indicate that IL-4 antagonizes the effects of TNF in vitro (13–19) and in vivo (15, 20). Among in vitro effects, IL-4 inhibits TNF-induced activation of endothelial cells (13), blocks TNF-induced expression of tissue factor and plasminogen activator inhibitor-1 from endothelial cells (17), inhibits TNF-induced E-selectin expression (19), and blocks TNF-induced proliferation of B cells (16). In vivo, IL-4 blocks TNF-induced hepatic lipogenesis (15) and cachexia and death (20). It is quite possible that all of these suppressive effects of IL-4 in vitro and in vivo on TNF-induced responses are mediated through the down-regulation of TNF receptors as described here.

Our results show that IL-4 down-regulates TNF receptors by inducing receptor shedding. These results suggest that IL-4 must activate a protease involved in the cleavage of the extracellular domain of the receptor. The soluble forms of TNF receptors have been found in the urine, synovial fluids, serum, and ovarian ascites (36). In vitro, a wide variety of agents are known to induce TNF receptor shedding, including TNF itself (2). The precise protease involved in TNF receptor cleavage is not known, but its suppression by metabolic inhibitors has suggested a metalloprotease (37). In the case of the p80 receptor, mutation of proline 211, which resides in the extracellular domain, reduces the phorbol 12-myristate 13-acetate-induced shedding of the receptor (41), suggesting that this hydrophobic residue is critical for shedding. While IL-4 down-regulated both types of TNF receptors, IL-10, which like IL-4 is known to suppress TNF signaling, has been shown to up-regulate the p80 form of the TNF receptor (40).

Although there has been a report that IL-4 can inhibit TNF-induced NF-κB activation (19), its ability to block TNF-induced apoptosis, AP-1 activation, and JNK activation has not previ-
ously been reported. Our results may explain the suppression of various other effects as indicated above induced by TNF. Other reports show that IL-4 potentiates the biological effects of TNF, including cytotoxicity, monocytic differentiation, and induction of vascular cell adhesion molecule 1 in vascular smooth muscle cells (9, 21, 22), but none of these studies examined the effect of IL-4 on TNF receptors.

How IL-4 can suppress the effects of TNF in one system and enhance in another is not clear. It is unlikely that this difference is due to the selective down-regulation of one TNF receptor type over another or to differences in cell type, because we found that both the p60 and p80 forms of the TNF receptor are down-regulated and that IL-4 decreases TNF receptors on a wide variety of different cell types. It is possible, however, that the cell surface TNF receptor density requirement for different signals transduced by TNF is different and thus some signals are more affected by IL-4 than others. It is also possible that the synergistic effects between TNF and IL-4 occur at the postreceptor level. For instance, TNF-induced NF-κB activation is known to synergize with STAT6 activation induced by IL-4 (39).

We found that IL-4 but not IL-13 down-regulated TNF receptors. Thus, the mechanism by which IL-13 transduces its signal must differ significantly from that of IL-4. These two cytokines induce many of the same biological responses, including class switching to IgE and induction of major histocompatibility complex class II antigens and CD23 on human B cells. IL-4 induces the tyrosine phosphorylation of a 170-kDa protein, a substrate called 4PS, and of the Janus kinase family members JAK1 and JAK3. Although both induce the tyrosine phosphorylation of 4PS and JAK1 (10), only IL-4 induces the tyrosine phosphorylation of JAK3. In addition to common α-chain and γc-chain, IL-13 binds to two additional components, which include IL-13Ra1 (low affinity; 2–10 nM) and IL-13Ra2 (high affinity; 0.25 nM) subunits. The latter subunits do not bind IL-4 and thus may explain why IL-4 and not IL-13 down-regulates TNF receptors.

Like IL-4, other cytokines are also known to down-regulate TNF-induced NF-κB activation, but their effects are not known to correlate with decreases in TNF receptors (42–44). How IL-4 transduces the signal to down-regulate TNF receptors is not clear. The effects of IL-4 were not through inhibition of glyco-
gen synthase kinase-3, since lithium chloride, an inhibitor of this kinase (38), had no effect on TNF receptors (data not shown). Our results do indicate, however, that the down-regulation of TNF receptors by IL-4 may be mediated through phosphatidylinositol 3-kinase. This is consistent with a recent report that showed the role of phosphatidylinositol 3-kinase in IL-4 signaling (32). Since phosphatidylinositol 3-kinase is known to activate p70 S6 kinase (34), we found that rapamycin, a potent inhibitor of p70 S6 kinase, blocked the IL-4-induced down-regulation of the receptors, thus suggesting that p70 S6 kinase is involved in IL-4-induced regulation of TNF receptors. Overall, our results provide the molecular basis for the suppression of TNF-mediated cellular responses by IL-4 and suggest that this mechanism of action differs from that of IL-13.

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![Fig. 8. A, effect of PD098059 and rapamycin on IL-4-mediated down-regulation of TNF receptor. U-937 cells (0.5 x 10⁶/ml) were suspended in RPMI 1640 medium supplemented with 10% FBS. Cells were pretreated with PD098059 (50 μM) and rapamycin (10 ng/ml) for 30 min and incubated with IL-4 (10 ng/ml) for 24 h at 37°C in a CO₂ incubator. Then cells were washed and suspended in fresh ice-cold medium at 3 x 10⁶/ml and examined for TNF binding as described under “Experimental Procedures.” The results indicated are as specific count in cpm ± S.D. of triplicate assays. B, effect of different concentrations of rapamycin on IL-4-mediated down-regulation of TNF receptor. U-937 cells (0.5 x 10⁶/ml) were suspended in RPMI 1640 medium supplemented with 10% FBS, pretreated with different concentrations of rapamycin for 5 min, and then treated with IL-4 (10 ng/ml) for 24 h at 37°C in a CO₂ incubator. Cells were then washed, suspended in fresh ice-cold medium at a concentration of 2 x 10⁶/ml, and examined for TNF binding. The results are mean cpm ± S.D. of duplicate assays.

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