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The role of TRB3 in mast cells sensitized with monomeric IgE

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ABSTRACT

Mast cells play a key role in immunoglobulin E (IgE)-associated allergic disorders; however, the cellular effects of sensitization remain poorly understood. Using gene microarrays and the multiplexing ELISA method, we examined the effects of sensitization on RBL-CCR1 cell transcription and chemokine/cytokine secretion. Sensitization most prominently increased transcription of *Trb3*, the gene for tribbles homolog 3 (TRB3), and also increased the release of most of the cytokines and chemokines tested. Knockdown of TRB3 via RNAi significantly induced the production of tumor necrosis factor- α (TNF- α), interleukin-4 (IL-4), interleukin-6 (IL-6), and the chemokine mast cell protease-1 (MCP-1). TRB3 deficiency also induced I κ B α phosphorylation. TRB3 may therefore serve as a negative regulator of pro-inflammatory cytokines and chemokines, controlling the extent of the inflammatory response.

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Introduction

Mast cells, which originate in the bone marrow, play key roles in immunity and inflammation (Morse et al., 2010; Taylor and Metcalfe, 2001). Mast cell activation occurs via immunoglobulin E (IgE), a class of antibodies that binds with high affinity to the receptor Fc ϵ RI. Initial exposure to an allergen results in mast cell sensitization. In this process, antigen-specific IgE binds to Fc ϵ RI on the mast cell surface. Subsequent exposure to allergen clusters the IgE–Fc ϵ RI complexes, resulting in mast cell degranulation and secretion of inflammatory mediators

(Jeong et al., 2002; Kinet, 1999; Morohoshi et al., 2009; Prussin and Metcalfe, 2003). Sensitization is therefore a critical component of allergic responses; however, the specific cellular events that occur in response to sensitization are still poorly understood.

Recent studies suggest that monomeric IgE, even in the absence of antigen, increases mast cell activity. IgE enhanced functionality of mast cells isolated from human umbilical cord blood (Yamaguchi et al., 1999; Yano et al., 1997). IgE also activated mouse bone marrow-derived mast cells (BMMC), resulting in upregulation of cell survival-enhancing kinases such as protein kinase B (PKB) and extracellular signal-regulated kinases (ERKs), phosphorylation of signaling molecules, and release of mediators (Kalesnikoff et al., 2001; Kawakami and Kitaara, 2005; Kitaara et al., 2003). IgE concentrations as low as 0.5 μ g/ml prevent cell death by increasing levels of interleukin (IL) 13 or stem cell factor, two well-documented mast cell survival factors (Kawakami and Galli, 2002). Monomeric IgE also has a strong influence on the levels of Fc ϵ RI (Yamaguchi et al., 1997).

The effects of monomeric IgE on mast cell activity may have important implications for allergic diseases and inflammation. Mast cell accumulation was induced *in vivo* by epicutaneous application of highly cytokinergic IgE without prior inflammation (Kawakami and Kitaara, 2005). The ability of IgE to attract mast cells suggests that the amplification of inflammation can last as long as local IgE synthesis continues, even after the elimination of antigen (Kawakami and Kitaara, 2005). A positive feedback-loop, in which monomeric IgE stimulates mast cell release of inflammatory cytokines that in turn recruit additional mast cells and increase IgE production, has been proposed to explain inflammatory situations often found in atopic diseases associated with high serum IgE levels (Kawakami and Kitaara, 2005; Wu et al., 2003).

Abbreviations: ANOVA, analysis of variance; ATF, activating transcription factor; BCA, bicinchoninic acid; BMMC, bone marrow-derived mast cells; CCR, chemokine receptor; C/EBP, CCAAT-enhancer binding protein; CHOP, C/EBP homologous protein; DNP, 2,4-dinitrophenol; ECL, enhanced luminol-based chemiluminescent; ELISA, enzyme-linked immunosorbent assay; ERKs, extracellular signal-regulated kinases; Fc ϵ RI, high affinity IgE receptor; GCRMA, genechip robust multi-array analysis; IFN- γ , interferon gamma; Ig, immunoglobulin; IL, interleukin; I κ B α , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; MAPK, mitogen-activating kinase proteins kinase; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP-1 α , macrophage inflammatory protein 1 alpha; MIP-2, macrophage inflammatory protein 2-alpha; NF- κ B, nuclear factor kappa light chain enhancer of activated B cells; PVDF, polyvinylidene fluoride; RANTES, regulated and normal T cell expressed and secreted; RBL, rat basophilic leukemia; PKB, protein kinase B; RNAi, RNA interference; RT, reverse transcription; SE, standard error; siRNA, small interfering RNA; TNP, 2,4,6-trinitrophenol; TNF- α , tumor necrosis factor alpha; TRB3, tribbles homolog 3.

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We predicted that mast cell sensitization would affect transcription and secretion of protein mediators, independently of allergen-mediated FcεRI clustering. Rat basophilic leukemia (RBL)-2H3 cells expressing human CCR1 (RBL-CCR1 cells) (Richardson et al., 2000) were used in this study, as they have previously been shown to display greater mast cell activity than the parental cell line (Beer et al., 2007; Toda et al., 2004). Chemokine receptor 1 (CCR1) is expressed by mast cells, (Miyazaki et al., 2005) and its ligand CC chemokine ligand 3 (CCL3), also known as macrophage inflammatory protein 1α (MIP-1α) stimulates mast cell degranulation (Alam et al., 1992; Miyazaki et al., 2005). To identify the specific downstream effects of sensitization in mast cells at a molecular level, we utilized gene chip arrays and the multiplex ELISA method to determine levels of gene transcription and chemokine/cytokine secretion. The role of the candidate gene *Trb3* was then determined using RNA interference to disrupt protein function.

Material and methods

Cell culture

RBL-CCR1 cells and rat basophilic leukemia cell line (RBL-2H3) (CRL-2256, American Type Culture Collection) (Richardson et al., 2000) were maintained as monolayers in Dulbecco's modified Eagle medium (DMEM; +4500 mg/L glucose, +GlutaMAX, +pyruvate) supplemented with 15% fetal bovine serum, and 100 units/ml penicillin and streptomycin. The cells were maintained at 3.5×10^5 cells/ml and cultured at 37 °C in 5% CO₂. Culture medium was refreshed every 3 days, and 1 mg/mL of Geneticin (G-418 sulfate) was added to the medium of RBL-CCR1 cells as described previously (Toda et al., 2004; Fifadara et al., 2009). To generate primary BMMCs, low-density mononuclear cells (LDMNCs) were isolated from BALB/c mice (Charles Rivers) Laboratories, and cultured in RPMI1640 containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential amino acids, and 50 µM 2-mercaptoethanol in the presence of 5 ng/ml of recombinant murine IL-3 (PeproTech) for 4–6 weeks (Toda et al., 2004; Fifadara et al., 2009). The purity of BMMCs exceeded 80%, which was determined by flow cytometric analysis after staining c-Kit and FcεRI on the cell surface. Tissue culture media and cell culture supplements were from Gibco, Invitrogen (UK and USA).

Preparation of IgEs and mast cell sensitization

Anti-2,4-dinitrophenol (DNP) IgE (clone SPE7, Sigma, St. Louis, MO) and anti-2,4,6-trinitrophenyl (TNP) IgE (clones c38-2 and c48-2, BD Biosciences, San Diego, CA) were centrifuged for 2 h at 100,000 g and 4 °C to remove the aggregated form. Except where indicated otherwise, RBL-CCR1 cells were passively sensitized for 16 h (overnight sensitization) with or without anti-DNP IgE or anti-TNP IgE at 25 ng/ml.

RNA extraction for GeneChip analysis

RBL-CCR1 cells were sensitized with 25 ng/ml monoclonal anti-DNP IgE (clone SPE-7, Sigma) in the culture medium overnight and washed 3 times in DMEM containing 0.1% bovine serum albumin to remove unbound IgE. The cells (6×10^5 /sample) were then suspended in 1 ml of TRIzol (Invitrogen, Gibco Life Technologies), and total RNA was extracted according to the manufacturer's protocol. Replicate samples were taken every 7 days for 3 weeks.

Target preparation for GeneChip experiments

The concentration, purity, and integrity of the extracted RNA were determined using the Agilent 2100 Bioanalyzer Nano assay (Agilent Technologies, Palo Alto, CA). Double-stranded cDNA was synthesized

by reverse transcription of twice-purified mRNA using T7–T24 primers (Genset Corp., La Jolla, CA) and the Superscript II cDNA Synthesis System (Invitrogen Life Technologies). The cDNA was used as a template for the generation of biotin-labeled *in vitro* transcription products using the Ambion T7 Megascript System (Ambion, Austin, TX) and biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics Inc., Farmingdale, NY). After fragmenting biotinylated *in vitro* transcription products (cRNA) in 40 mM Tris acetate, pH 8.1, 10 mM potassium acetate, and 30 mM magnesium acetate at 94 °C for 35 min, targets for chip hybridization were prepared by combining 10 µg of fragmented cRNA with sonicated herring sperm DNA (0.1 mg/ml; Sigma, St. Louis, MO) plus a mixture of four control bacterial and phage cRNA (1.5 pM BioB, 5 pM BioC, 25 pM BioD, and 100 pM Cre) and 5 nM of control oligonucleotide (3 nM oligo B2) as internal controls for hybridization efficiency. The quality of the biotin-labeled cRNA transcripts was determined using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Purified cRNA (15 µg) was fragmented and hybridized to Affymetrix GeneChip® Rat Genome 230 2.0 Arrays according to Affymetrix standard protocols (<http://www.affymetrix.com>). Labeled GeneChips were scanned using a confocal argon ion laser (Agilent Technologies).

GeneChip data analysis

GeneChip data files were scaled to 100 and normalized to the median prior to analysis with GeneSpring 7.3 software (Agilent). Genes were excluded if the signal strength did not significantly exceed background values and if expression did not reach a threshold value for reliable detection based on the Affymetrix GCRMA (GeneChip Robust Multi-Array Analysis) probability of detection. The GCRMA-normalized intensity values (log 2) were used for the data analysis.

Gene selection

GeneChip data were filtered on flags to remove genes that were not expressed at detectable levels in any condition. Present or marginal genes (the genes with a “present” or “marginal” flag), whose value appeared in at least one sample, were chosen based on the relative signal intensity of the transcripts on the GeneChip. Differential expression was filtered on fold change by volcano plots. Replicates were used as the mode of interpretation, log of ratio was chosen to calculate a measure of central tendency from the sets of replicate measurements, and the means were compared using the fold change filter. Differential expression across multiple conditions was tested by analysis of variance (ANOVA) using the pre-filtered present or marginal gene list to reduce the number of false-positive genes. The Tukey's post-hoc test was used to compare pairs of means to identify pairs of conditions between which gene expression differed significantly. The log of ratio mode was applied. The Cross-Gene Error Model was kept active to use model-derived variances in the statistical test. Triplicates of each treatment type were compared as groups of samples. The parametric test was chosen, and the variances were assumed as equal. The Benjamini and Hochberg False Discovery Rate (FDR) was used as a multiple testing correction function to reduce the number of false positives. The FDR cut-off was set at 0.05.

Detection of secreted proteins

Untransfected RBL-CCR1 cells and cells transfected with *Trb3* siRNA or control siRNA for 48 h were incubated overnight (16 h) with anti-DNP IgE (25 ng/ml or 5 µg/ml) at 37 °C. Supernatants were then collected in triplicate. These samples were sent to Scientific SearchLight (Aushon Biosystems Inc., Billerica, MA), where the multiplex ELISA method was used to measure levels of interferon α (IFN-α), tumor necrosis factor α (TNF-α), IL-4, IL-6, IL-10, monocyte chemoattractant protein 1 (MCP-1), macrophage-derived

chemokine (MDC), MIP-1 α , regulated and normal T cell expressed and secreted (RANTES), fractalkine, macrophage inflammatory protein 2- α (MIP-2) and eotaxin.

Measurement of gene expression by quantitative PCR

IL-6 primers and QuantiTect Primer Assay kits were purchased from Qiagen (Valencia, CA, USA). All other primers were designed using Primer 3 (<http://fokker.wi.mit.edu/primer3/>) developed by Steve Rozen and Helen Skaletsky, Whitehead Institute Center for Genome Research, Cambridge, MA). Primer sequences were as follows: *Trb3*, 5'-ACTCCGAGATAGGCTCAGTG-3' (forward) and 5'-TCAAATC CAACGGTTTCTT-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5'-CTCATAGACAAGATGGTAAGG-3' (forward) and 5'-ATGAAGGGGTCGTTGATG-3' (reverse). These primers were synthesized by MWG-Biotech AG. Total RNA was purified using the Qiagen RNeasy Purification kit and diluted to a concentration of 10 ng/ml. Real-time reverse transcriptase (RT) PCR was conducted using the QuantiTect SYBR Green RT-PCR Kit (Qiagen), with a reaction mix containing 2 $\times$ QuantiTect SYBR Green, 1 $\times$ QuantiTect RT Mix, RNase-free water, 10 μ M primers, and 40 ng/ml RNA. PCR results were obtained and analyzed using the Applied Biosystems 5500 real-time PCR machine, with a PCR protocol consisting of 40 cycles of the following: 96 °C denaturation for 15 s, 52 °C primer annealing for 30 s, and 72 °C extension for 40 s.

Western blotting

Total and cytoplasmic extracts of RBL-CCR1 cells were prepared in mammalian protein extraction reagent (M-PER and NE-PER; Pierce Chemical Co. Rockford, IL) containing protease inhibitors (Complete, Roche Diagnostic). Protein concentrations were determined using the bicinchoninic acid (BCA) Protein Assay kit (Pierce Chemical Co. Rockford, IL). Equal amounts of protein were separated using a 12% polyacrylamide SDS gel (BioRad) and transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad). After 1 h of blocking in 5% milk/TBS-Tween (0.1%), the membrane was incubated overnight at 4 °C with rabbit polyclonal anti-TRB3 (M-165, Santa Cruz Biotechnology), rabbit polyclonal anti- β Actin (ab8227, Abcam, Cambridge, MA), mouse monoclonal anti-phospho-I κ B α or rabbit monoclonal I κ B α antibody (Cell Signaling Technology). The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology). Protein expression was visualized using enhanced luminol-based chemiluminescent (ECL) Chemiluminescence Substrate (Pierce, Rockford, IL, USA).

RNA interference

RNA interference was conducted using double-stranded *Silencer Select* Pre-designed siRNA duplexes (Ambion, Austin, TX). Duplexes corresponding to the rat *Trb3* coding region (5'-CCGUUGAGUUU GAUGACATT-3' and 5'-UGUCAUCAAACUCCAACGGTT-3') as well as scrambled RNAi duplexes (negative control) were transfected into RBL-CCR1 cells using lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA). After a 48-hour incubation at 37 °C, the cells were collected using Versene (Invitrogen), replated, and sensitized as indicated for assays.

ELISA for I κ B α

siRNA-transfected RBL-CCR1 cells were sensitized with IgE for different lengths of time. Cell lysates were prepared as described in the manufacturer's instructions (eBioscience). Using the NF κ B Pathway Activation InstantOne™ ELISA kit (eBioscience), the untreated and treated cell lysates were analyzed for levels of phosphorylated I κ B α (Ser32/36).

Statistics

The Student's *T*-test or ANOVA (SPSS version 17.0 for Windows software) was used to determine whether group means differed at a significance level of $p < 0.05$.

Results

Sensitization alters gene expression in RBL-CCR1 cells

Genes differentially expressed in sensitized RBL-CCR1 cells compared to the unsensitized cells were identified using the limitation process shown in Fig. 1A. Of the 31,099 genes present in the rat genome, 18,731 were expressed in the triplicates of the unsensitized and sensitized samples. Among the 18,731 genes expressed, 206 genes were differentially expressed between the two conditions. The most dramatically upregulated gene (7.7 fold) was *Trb3* (Fig. 1 and Table 1).

Sensitization alters cytokine and chemokine secretion by RBL-CCR1 cells

To determine the baseline effects of monomeric IgE on mast cell secretion, protein concentrations for several cytokines and chemokines were measured in supernatants collected from unsensitized and IgE-sensitized RBL-CCR1 cells. Cells were sensitized using two anti-DNP IgE concentrations, 25 ng/ml and 5 μ g/ml, and concentrations for twelve chemokines and cytokines were measured using multiplex assay. Following exposure to IgE at 25 ng/ml, IL-6, TNF- α , MCP-1, and IL-4 concentrations were all significantly increased compared to unsensitized controls (Fig. 2). The higher concentration of IgE resulted in further increases for some proteins. MCP-1, for example, was expressed at 3-fold higher levels following treatment with 5 μ g/ml IgE (512 pg/ml MCP-1) than with 25 ng/ml IgE (181.4 pg/ml MCP-1, $p < 0.01$, Fig. 2). MDC, MIP-1 α , RANTES, fractalkine, IL-10, MIP-2, and eotaxin showed no significant changes after IgE treatment as compared with unsensitized cells. IFN- γ was not detected in any of the supernatants.

Trb3 expression is increased in response to sensitization

To verify the upregulated *Trb3* expression observed with the gene chip microarray, RNA was isolated from unsensitized and sensitized RBL-CCR1 cells, RBL-2H3 cells, and BMMCs. *Trb3* mRNA expression was 6.96-fold and 18.51-fold higher in RBL-CCR1 cells sensitized with 25 ng/ml and 5 μ g/ml anti-DNP IgE, respectively, than in unsensitized cells ($p < 0.01$, Fig. 3A). Inductions in *Trb3* expression were also found in sensitized RBL-2H3 and BMMCs (data not shown). *Trb3* was also expressed at significantly higher levels in the anti-TNP IgE-sensitized RBL-CCR1 cells as compared to unsensitized cells, with 5.91-fold and 7.11-fold increases for clones C48-2 and C38-2, respectively, as determined by real-time RT-PCR ($p < 0.01$, Fig. 3B). Immunoblotting demonstrated that TRB3 protein expression was also increased in anti-DNP IgE or anti-TNP IgE-sensitized cells (Figs. 3C, D).

TRB3 knockdown alters sensitization-induced changes in chemokine and cytokine secretion

To test the effect of disrupting *Trb3* gene transcription, *Trb3* siRNA was transfected into RBL-CCR1 cells. *Trb3* gene expression was significantly decreased in the unsensitized, *Trb3* siRNA-transfected cells to 38.9% of the level observed in unsensitized, control (scrambled) siRNA-transfected cells (Supplementary Fig. S1). The *Trb3* siRNA treatment also inhibited the upregulation of *Trb3* expression following IgE sensitization. Sensitization of control siRNA-transfected cells with anti-DNP IgE resulted in a 1.8-fold increase in the levels of *Trb3* transcripts compared to unsensitized, control siRNA-transfected cells. No increase in *Trb3* expression was observed when the *Trb3* siRNA-transfected cells were sensitized.

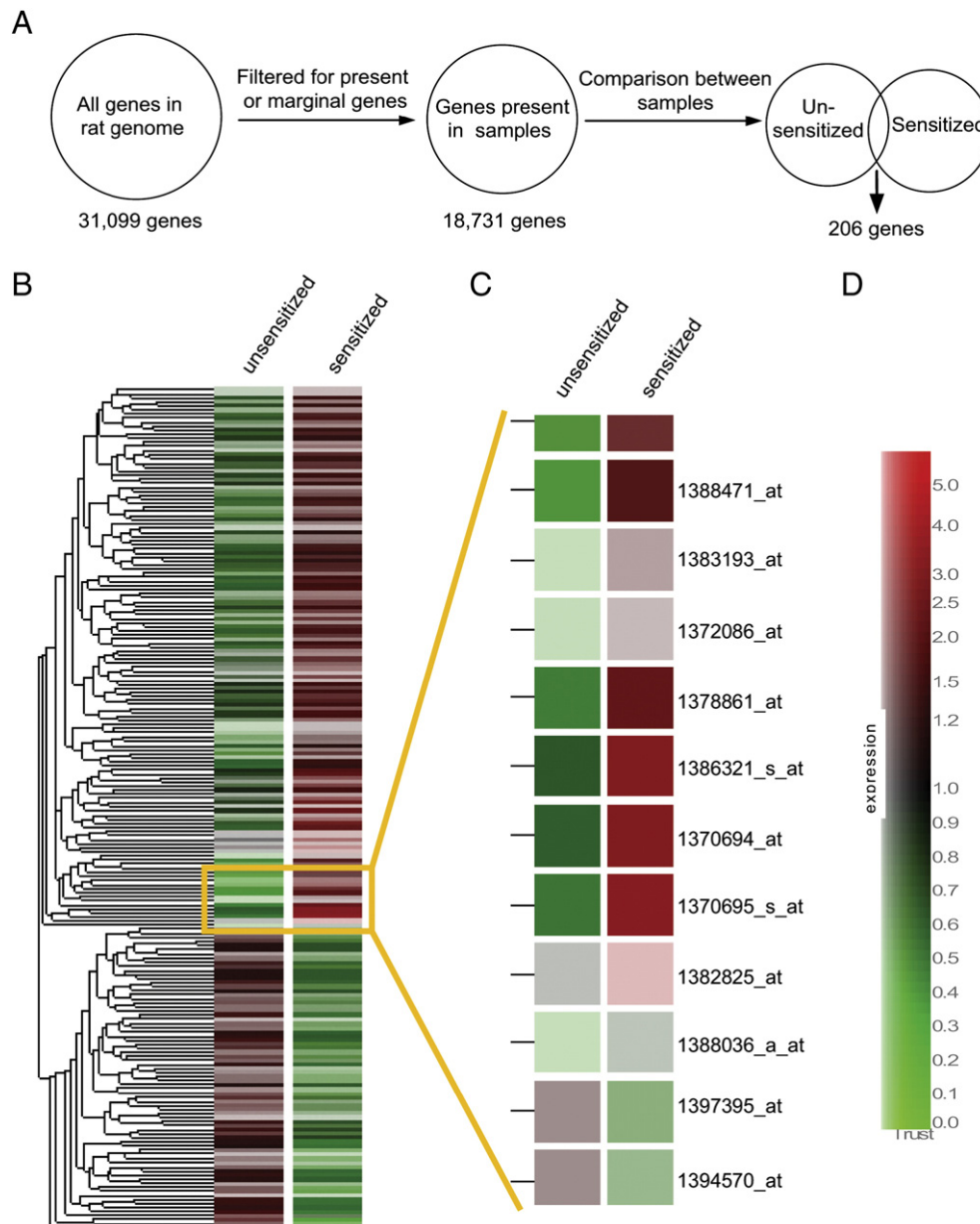


Fig. 1. Identification of genes differentially expressed by IgE-sensitized RBL-CCR1 cells. (A) The procedure to identify the genes differentially expressed after sensitization. (B) Heatmap of the 206 genes differentially expressed after overnight sensitization. Each bar represents one gene. Green color indicates downregulation, and red color indicates upregulation of gene expression, with stronger colors indicating greater upregulation or downregulation of gene expression. (C) Heatmap area showing the most strongly upregulated genes (from low expression [light green] to high expression [light red]), magnified to show the Affymetrix gene ID for identification. (D) Color gradient showing differences in gene expression levels from low expression (light green) to high expression (light red).

To investigate the role of TRB3 in secretion of chemokines and cytokines, the multiplex assay was repeated using cells transfected with *Trb3* siRNA. Following exposure to 25 ng/ml anti-DNP IgE, both IL-6 and IL-4 were expressed significantly higher, 1.3-fold and 1.6-fold increases, respectively, in the *Trb3* siRNA-transfected cells as compared to the control siRNA-transfected cells ($p < 0.05$, Fig. 4A). IL-6, MCP-1, TNF- α , and IL-4 were expressed significantly higher in the *Trb3* siRNA-transfected cells after exposure to 5 μ g/ml anti-DNP IgE, having increases of 1.3, 1.6, 1.4, and 2.8-fold, respectively ($p < 0.05$, Fig. 4B).

TRB3 siRNA increases *Il6* transcription

Since TRB3 is a negative regulator of the transcription factor NF- κ B, (Wu et al., 2003) and NF- κ B is known to stimulate transcription of

IL-6, (Libermann and Baltimore, 1990), we used real-time RT-PCR to determine whether *Il6* transcription was increased in the *Trb3* siRNA-transfected RBL-CCR1 cells. Levels of *Il6* transcripts were nearly 8-fold greater in the *Trb3* siRNA-transfected cells than in the control siRNA-transfected cells following sensitization with anti-DNP IgE ($p < 0.01$, Fig. 5).

Trb3 siRNA induces activation of NF- κ B pathway

To define the molecular mechanisms responsible for the induction of cytokine gene expression in our *Trb3* knockdown RBL-CCR1 cells, we analyzed the signaling pathway downstream of Fc ϵ RI sensitization. As the expression of TNF- α and IL-6 critically depends on the activation of NF- κ B and NF- κ B activity is regulated by I κ B, we analyzed I κ B- α phosphorylation in the *Trb3* siRNA-transfected RBL-CCR1 cells

Table 1

Genes most strongly regulated by sensitization in RBL-CCR1 cells. Genes differentially expressed by sensitized cells as compared to unsensitized cells were identified using Affymetrix GeneChip® arrays.

Affymetrix gene ID	Fold change	Gene symbol	Gene title
<i>Most strongly upregulated:</i>			
1370695_s_at	7.7	<i>Trb3</i>	Tribbles homolog 3
1370694_at	6.4	<i>Trb3</i>	Tribbles homolog 3
1386321_s_at	5.4	<i>Trb3</i>	Tribbles homolog 3
1386889_at	3.4	<i>Scd2</i>	Stearoyl-coenzyme A desaturase 2
1367838_at	3.4	<i>Cth</i>	CTL target antigen
1387127_at	3.2	<i>Atrn</i>	Attractin
1376845_at	2.9	<i>Isg12(b)</i>	Putative ISG12(b) protein
1387270_at	2.8	<i>Hhex</i>	Hematopoietically expressed homeobox
1367668_a_at	2.8	<i>Scd2</i>	Stearoyl-coenzyme A desaturase 2
1373787_at	2.7	<i>Slc6a9</i>	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9
1370355_at	2.7	<i>Scd1</i>	Stearoyl-coenzyme A desaturase 1
1368549_at	2.7	<i>Hbp1</i>	High mobility group box transcription factor 1
1371735_at	2.7	<i>LOC497920</i>	LOC497920
1368527_at	2.5	<i>Ptgs2</i>	Prostaglandin-endoperoxide synthase 2
<i>Most strongly downregulated:</i>			
1368146_at	−4.0	<i>Dusp1</i>	Dual specificity phosphatase 1
1390036_at	−2.8	<i>Slc16a6</i>	Solute carrier family 16 (monocarboxylic acid transporters), member 6

by Western blotting and ELISA (Fig. 6). The early events of I κ B- α phosphorylation at 20 min were not affected by *Trb3* deficiency in anti-DNP IgE-sensitized cells. In contrast, I κ B- α phosphorylation was significantly enhanced in these cells at the later time point (60 min) (Figs. 6A, B). These findings suggest that TRB3 may regulate NF- κ B localization by inhibiting I κ B phosphorylation. Thus, TRB3 may be involved in inhibiting IgE-Fc ϵ R1-mediated NF- κ B activation signals.

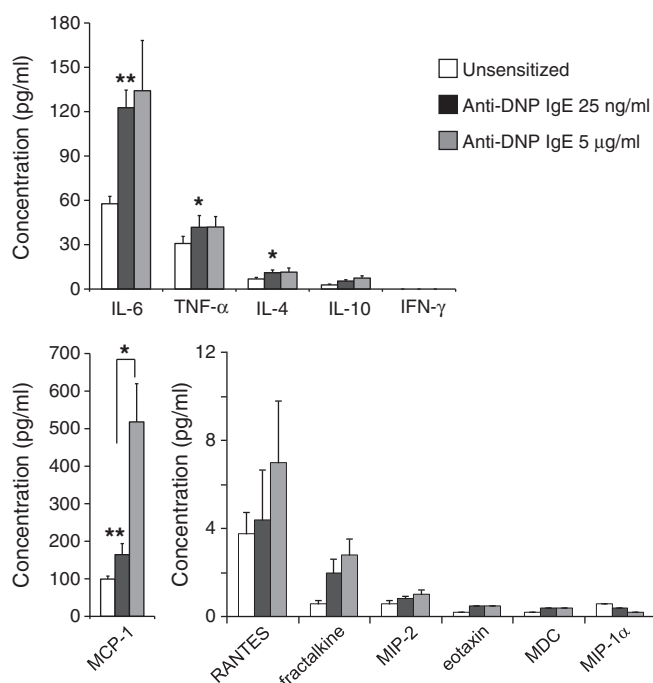


Fig. 2. IgE-induced secretion of cytokines and chemokines. Levels of cytokines and chemokines secreted by RBL-CCR1 cells were determined using a multiplex assay. Sensitized cells were sensitized with IgE at 25 ng/ml or 5 μ g/ml, as indicated. Values are mean $\pm$ SE. *, $p < 0.05$; **, $p < 0.01$; ANOVA.

Discussion

Identification of mediators of IgE-dependent mast cell activation may allow development of better therapies for immunological disorders. This study is the first report of sensitization effects on RBL-CCR1 cell transcription. Jayapal et al. (2006) identified upregulated expression of 58 genes following sensitization of human mast cells with monomeric IgE; interestingly, there was no overlap between the genes upregulated by at least 2-fold by sensitization in that study and in our study. The reasons for these differences are unclear. Although the cell types used in the two studies differ with respect to species, derivation, and culture conditions, they do display similar changes in chemokine/cytokine secretion following sensitization as discussed below.

We have shown that monomeric IgE sensitization of RBL-CCR1 cells results in upregulation of the gene *Trb3*. On the basis of our similar *Trb3* induction in BMMCs and RBL-2H3 cells, this TRB3-dependent regulation is likely a shared mechanism found in different populations of mast cells and mast cell models. Notably, BMMCs and RBL-2H3 cells have lower or no expression of CCR1, suggesting that this mechanism does not occur via CCR1. The results from our *Trb3* knockdown experiments suggest that TRB3 suppresses secretion of cytokines and chemokines and enhanced I κ B- α phosphorylation. TRB3 interacts with several members of the mitogen-activating protein kinase (MAPK) family, activating transcription factor (ATF)-4, C/EBP homologous protein (CHOP), and NF- κ B; TRB3 has also been shown to be an important regulator of glucose metabolism during fasting (Bowers et al., 2003; Gu et al., 2003; Kiss-Toth et al., 2004; Ohoka et al., 2005; Ord and Ord, 2003).

We have demonstrated that IgE sensitization increases mast cell secretion of IL-6, TNF- α , MCP-1, and IL-4, which is consistent with results observed for murine BMMCs (Kalesnikoff et al., 2001) and with the increase in MCP-1 secretion following sensitization that has been observed for human cord blood-derived mast cells (Sawada et al., 2005). Our *Trb3* knockdown study suggests that secretion of these proteins may be held in check by TRB3. This regulation may be mediated by suppression of the transcription factor NF- κ B, since NF- κ B stimulates production of IL-6 and TNF- α and is inhibited by TRB3 (Wu et al., 2003). As I κ B- α phosphorylation increased in *Trb3*-deficient cells, TRB3 may regulate the NF- κ B pathway through I κ B.

All four of the cytokines whose expression increased with *Trb3* knockdown following IgE sensitization, IL-6, TNF- α , MCP-1, and IL-4, are known to have inflammatory functions. IL-6 is a proinflammatory cytokine that is involved in the responses that occur after infection and cellular injury. IL-6 plays a role in regulating the acute phase response by promoting release of IL-1 receptor antagonist and the soluble p55 TNF- α receptor (Schindler et al., 1990; Tilg et al., 1994). IL-6 has also been shown to affect recruitment of inflammatory cells, such as leukocytes and neutrophils, as an inflammatory response (Kaplanski et al., 2003; Kitamura et al., 1995; Romano et al., 1997; Xing et al., 1998). IL-4 promotes production of IgE, chemokines, and mucus at the site of allergic inflammation (Kelly-Welch et al., 2003; Vercelli, 2002). The multifunctional protein TNF- α is a well-known proinflammatory cytokine whose effects are mediated through the activation of NF- κ B (Jeong et al., 2002; Peng et al., 2005). NF- κ B plays a key role in immune regulation and inflammation through induction of a large set of chemokines, cytokines, adhesion molecules, and effectors (Baeuerle, 1998; Baldwin, 2001; Ghosh and Karin, 2002; Karin and Ben-Neriah, 2000; Karin and Lin, 2002). NF- κ B consists of a p50 and p65 heterodimer and is retained in the cytoplasm by binding with I κ B. NF- κ B translocation happens after serine phosphorylation of I κ B- α (Baeuerle and Baltimore, 1988). Induction of I κ B- α phosphorylation in *Trb3* knockdown cells may subsequently enhance the translocation of NF- κ B into the nucleus and further affect the expression of cytokines and chemokines. MCP-1 recruits monocytes to the site of injury or inflammation in the body and is associated with the recruitment of lymphocytes and eosinophils, though it is not a potent chemoattractant for mast cells (Taub et al., 1995). Recent report also showed that

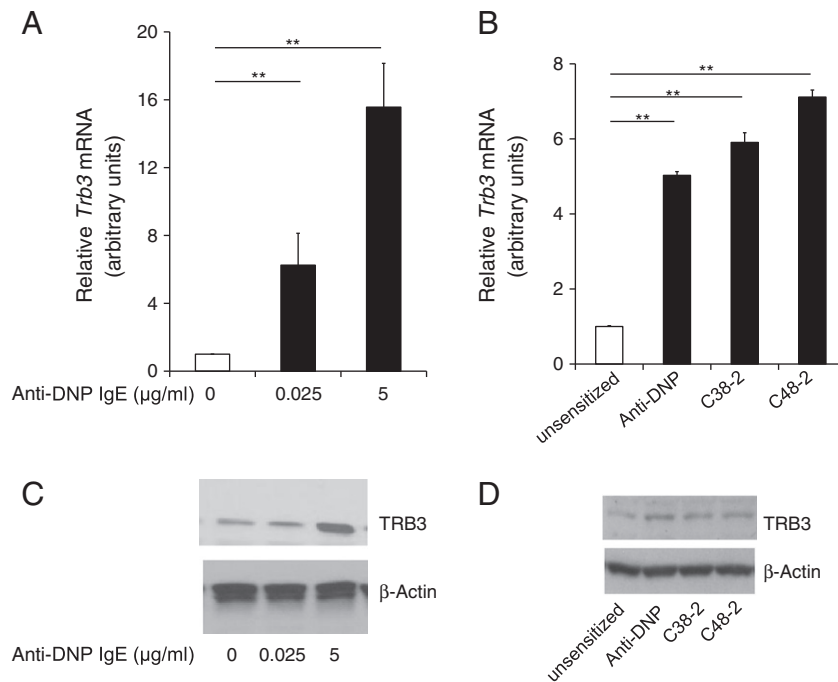


Fig. 3. TRB3 expression in unsensitized and IgE-sensitized RBL-CCR1 cells. RNA and protein isolated from lysed cells were examined by (A, B) real-time RT-PCR and (C, D) immunoblotting following treatment with anti-DNP IgE at 25 ng/ml or 5 μg/ml, anti-TNP IgE (C48-2 and C38-2) at 25 ng/ml. Data are expressed as mean ± SE from one representative experiment of three separate experiments. **, $p < 0.01$; ANOVA.

reduction of TRB3 in diabetic kidneys inhibits MCP-1 expression in podocytes (Morse et al., 2010). TRB3 may therefore serve as a negative regulator of the inflammatory response by suppressing cytokine and

chemokine secretion, perhaps as part of a self-regulating negative feedback loop. Proinflammatory cytokines and chemokines are released to mediate the inflammatory response in sensitized mast cells. *Trb3* is also induced and depresses secretion of inflammatory mediators such as IL-4 and IL-6, which are key cytokines in the proliferation of mast cells (Morse et al., 2009, 2010). In turn, this reduced secretion of inflammatory mediator decreases the relative concentration of mast cells and monocytes, returning the body to normal conditions.

Regulation of abnormal inflammation is essential to the understanding of diseases. IL-6 and TNF-α have recently been implicated in obesity, and inflammation has been shown to play a role in insulin resistance (Chacon et al., 2008). Inflammatory processes have also been identified with diseases such as cancer, atherosclerosis, and ischemic heart disease (Coussens and Werb, 2002; Virani et al., 2008). TRB3 regulation may therefore play important roles in a variety of disorders. It will be interesting to determine whether TRB3

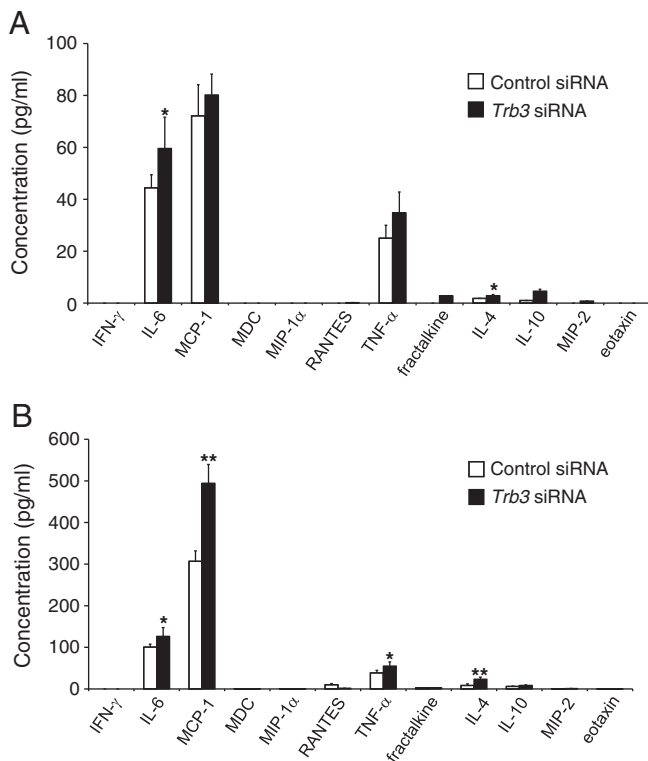


Fig. 4. Effects of *Trb3* siRNA on IgE-induced secretion of cytokines and chemokines. Secretion of cytokines and chemokines of siRNA-transfected RBL-CCR1 cells was determined using a multiplex assay. Sensitized cells were treated with anti-DNP IgE at (A) 25 ng/ml and (B) 5 μg/ml. Data are expressed as mean ± SE from one representative experiment of two separate experiments. *, $p < 0.05$; **, $p < 0.01$; Student's *T*-test.

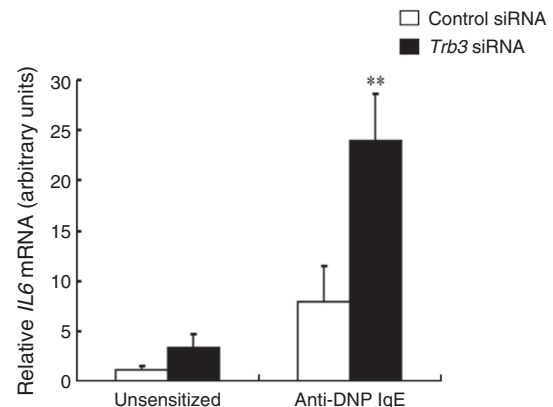


Fig. 5. Effect of *Trb3* siRNA on the transcription of *IL6*. A total of 2.5×10^5 *Trb3* siRNA- or control (scrambled) siRNA-transfected RBL-CCR1 cells were incubated for overnight in the medium containing anti-DNP IgE (25 ng/ml). Total RNA was extracted for real-time RT-PCR analyses. Data are expressed as mean ± SE from one representative experiment of three separate experiments. **, $p < 0.01$; ANOVA.

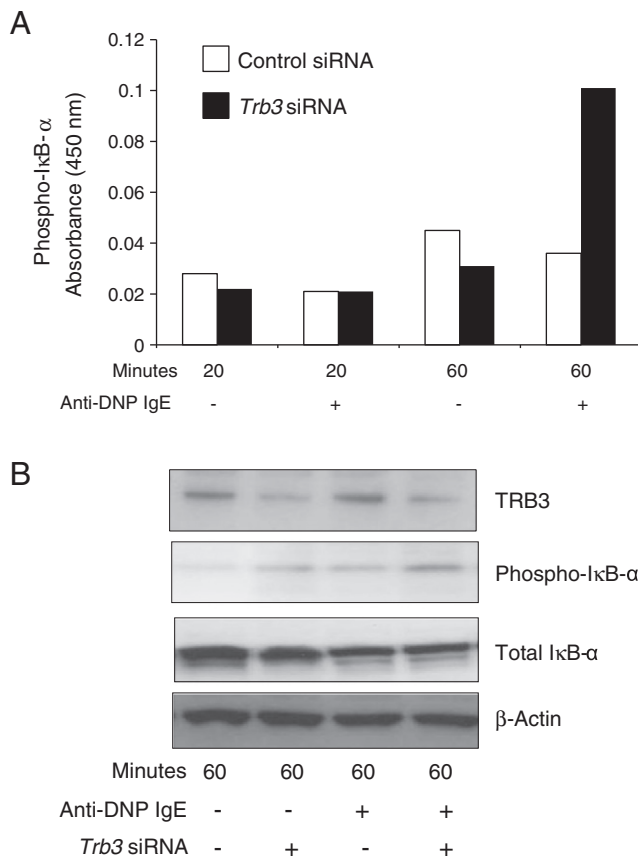


Fig. 6. Effect of *Trb3* siRNA on the IκB-α phosphorylation. A total of 5×10^5 *Trb3* siRNA- or control (scrambled) siRNA-transfected RBL-CCR1 cells were incubated for 20 or 60 min in medium containing anti-DNP IgE (25 ng/ml). (A) Cells were lysed and ELISA was performed. Data are expressed as relative light units from one representative of two separate experiments. (B) After IgE sensitization, 15 μg of total lysate protein was assayed for phospho-IκB-α, total IκB-α, TRB3, and β-actin (loading control).

suppresses inflammation *in vivo* and whether TRB3 contributes to autoregulation of allergic inflammation following exposure to allergen and other mast cell-stimulating molecules.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yexmp.2012.09.008>.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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