



Identification of genes and proteins specifically regulated by costimulation of mast cell Fcε Receptor I and chemokine receptor 1

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ABSTRACT

Mast cell function is a critical component of allergic reactions. Mast cell responses mediated by the high-affinity immunoglobulin E receptor FcεRI can be enhanced by co-activation of additional receptors such as CC chemokine receptor 1 (CCR1). To examine the downstream effects of FcεRI-CCR1 costimulation, rat basophilic leukemia cells stably transfected with CCR1 (RBL-CCR1 cells) were sensitized and activated with antigen and/or the CCR1 ligand CC chemokine ligand (CCL) 3. Gene and protein expression were determined at 3 h and 24 h post-activation, respectively, using GeneChip and Luminex bead assays. Gene microarray analysis demonstrated that 32 genes were differentially regulated in response to costimulation, as opposed to stimulation with antigen or CCL3 alone. The genes most significantly up-regulated by FcεRI-CCR1 costimulation were *Ccl7*, *Rgs1*, *Emp1* and *RT1-S3*. CCL7 protein was also expressed at higher levels 24 h after dual receptor activation, although RGS1, EMP1 and RT1-S3 were not. Of the panel of chemokines and cytokines tested, only CCL2, CCL7 and interleukin (IL)-6 were expressed at higher levels following costimulation. IL-6 expression was seen only after FcεRI-CCR1 costimulation, although the amount expressed was very low. CCL7, CCL2 and IL-6 might play roles in mast cell regulation of late-phase allergic responses.

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Introduction

Allergic diseases are a major public health concern affecting one fifth of the world population (Manzouri et al., 2006). The incidence of allergies in developed countries has been increasing in recent years (Bielory et al., 2007) and anaphylaxis requiring hospitalization is increasing in the age group younger than 20 years (Lin et al., 2008). In the United States, studies have shown incidences greater than 50% for skin responses to injected allergens (Arbes et al., 2005) and symptoms of allergic rhinitis (Lamb et al., 2006). In the presence of asthma and nasal symptoms, ocular allergy is often overlooked although it is common in adults and children worldwide (Bielory et al., 2007). Ocular allergy is not only irritating but can also cause visual loss in case of severe forms such as atopic keratoconjunctivitis. New research into the pathological basis of ocular allergy is needed for

development of more-effective therapeutic agents and delivery methods.

Mast cells play a critical role in mediating allergic responses. Upon initial exposure to an allergen, antigen-specific IgE binds to the high-affinity receptor FcεRI on mast cells. Subsequent allergen exposure results in IgE-FcεRI clustering and initiation of mast cell signaling cascades (Rivera and Olivera, 2008). FcεRI activity can be significantly modified via activation of other receptors expressed on mast cells, including the A₃ adenosine receptor (Church et al., 1983; Laffargue et al., 2002; Ramkumar et al., 1993) and CC chemokine receptor 1 (CCR1) (Toda et al., 2004).

CCR1 and one of its ligands, CC chemokine ligand 3 (CCL3), have been shown to participate in mast cell regulation in ocular allergy (Miyazaki et al., 2005). CCL3 is rapidly upregulated in specific mononuclear cells after allergen challenge, and this expression is required for optimal mast cell degranulation during immediate hypersensitivity in the conjunctiva (Miyazaki et al., 2005). *In vitro*, costimulation of FcεRI and CCR1 results in greater mast cell degranulation than does stimulation of either receptor alone (Toda et al., 2004). To better understand the effects of FcεRI-CCR1 costimulation on mast cells, we examined transcriptional profiles and protein expression in singly stimulated- and costimulated mast cells. We hypothesized that certain mediators would be upregulated specifically when the cells were costimulated. Such mediators might be targets for novel therapies for allergic diseases.

Abbreviations: BSA, bovine serum albumin; CCL, chemokine ligand; CCR, chemokine receptor; DMEM, Dulbecco's modified Eagle medium; DNP, dinitrophenol; EMP1, epithelial membrane protein 1; FDR, false discovery rate; IL, interleukin; IFN, interferon; MMP, matrix metalloproteinase; PE, phycoerythrin; RBL, rat basophilic leukemia 2H3 cells stably transfected with human CCR1; RGS1, regulator of G-protein signaling 1; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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Materials and methods

RBL-CCR1 cell culture

Rat basophilic leukemia 2H3 cells stably transfected with human CCR1 (RBL-CCR1 cells) (Richardson et al., 2000) were cultured in Dulbecco's modified Eagle medium (DMEM; +4500 mg/L glucose, + GlutaMAX, + pyruvate) supplemented with 15% foetal bovine serum and 100 U/ml penicillin-streptomycin (all reagents from GIBCO life technologies, Paisley, UK). The cells were maintained as monolayers at 3.5×10^5 cells/ml and cultured at 37 °C in 5% CO₂. Fresh culture medium was added every 3 days, and Geneticin (G-418 sulfate, Gibco, Carlsbad, CA, USA) was added to the medium at 1 mg/ml once every 2 weeks. All products were purchased from Gibco, Invitrogen (UK and USA).

Cell stimulation and RNA extraction

RBL-CCR1 cells were sensitized with 20 ng/ml monoclonal anti-dinitrophenol (DNP) IgE (clone SPE-7, D8406, Sigma-Aldrich, Dorset, UK and St. Louis, MO, USA) in culture medium overnight, washed three times in DMEM containing 0.1% bovine serum albumin (BSA) to remove unbound IgE, and then activated with 10 ng/ml DNP-human serum albumin (A6661, Sigma-Aldrich, Dorset, UK and St. Louis, MO, USA) and/or 50 ng/ml recombinant human CCL3/MIP-1 α (Isoform LD78 α , 270-LD, R&D systems, Abingdon, UK and Minneapolis, MN, USA) in DMEM/0.1% BSA for 20 min. The cells (6×10^5 /sample) were then suspended in 1 ml of TRIzol (Invitrogen Ltd., Gibco Life Technologies, Paisley, UK), and total RNA was extracted according to the manufacturer's protocol. Total RNA was extracted three hours after activation. Replicate samples were taken every seven days for three weeks.

GeneChip target preparation

Concentration, purity and integrity of RNA were determined using the Agilent 2100 Bioanalyzer Nano assay (Agilent Technologies, Palo Alto, CA, USA). Double-stranded cDNA was synthesized by reverse transcription of twice-purified mRNA using T7-(T24) primers (GenSet Corp., La Jolla, CA, USA) and the Superscript II cDNA Synthesis System (Invitrogen Life Technologies, San Diego, CA, USA). This 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, USA) and biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics Inc., Farmingdale, NY, USA). 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 μ g of fragmented cRNA with sonicated herring sperm DNA (0.1 mg/ml; Sigma, St. Louis, MO, USA), 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-labelled cRNA transcripts was determined using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Purified cRNA (15 μ g) was fragmented and hybridised to Affymetrix GeneChip® Rat Genome 230 2.0 Arrays according to the manufacturer's standard protocols. Labelled GeneChips were scanned using a confocal argon ion laser (Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA).

Gene chip data analysis

GeneChip data files were scaled to 100 and normalised to the median prior to analysis with GeneSpring GX 7.3 software (Silicon Genetics, Agilent Technologies, Santa Clara, CA, USA). 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-normalised intensity values (log₂) were used for the data analysis.

GeneChip data were filtered on flags to remove genes that were not expressed at significant 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 ANOVA using the prefiltered present or marginal gene list to reduce the number of false positive genes. The Tukey post-hoc test was used to identify pairs of conditions between which gene expression differed significantly by comparing pairs of means. 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. Benjamini and Hochberg False Discovery Rate (FDR) was used as a multiple testing correction function to reduce the number of false positives in our analysis results. The FDR cutoff was set at 0.05.

Real time quantitative PCR

QuantiTect SYBR green real time PCR (Qiagen, MD, USA) was performed to measure the expression levels of the seven most significantly up-regulated synergy-related genes using rat sequence-based primers on an ABI Prism 7500 Sequence detection system (Applied Biosystems, Austin, TX, USA) with the same RNA used for the microarray analyses. Two micrograms of each total RNA sample were used to generate cDNA using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's protocol. Primers were designed to hybridize across exon junctions, and their sequences were as follows: Ccl17, tctctctctccaccatgc (sense) and ttctcttgggaatcttttg (antisense); Rgs1, agtgggaatggacatgaagc (sense) and gcaaggagttttccagaga (antisense); Emp1, tgccattatgctgtttgtct (sense) and gtgctcagagacgcgtca (antisense); Nr4a2, gggctgcaaggtttcttta (sense) and tctttaaccatccaacagc (antisense); RT1-Aw2 primers (Qiagen); RT1-S3, tacctctgcctattgtctc (sense) and gtgccaacgacatgaacc (antisense) and Map2k3, ccttctatggtgccctttt (sense) and aatctccccagatgtctt (antisense). Gapdh expression levels were used for normalization.

For each target gene, cDNA corresponding to 20 ng RNA was used for a 25 μ l reaction volume. Each reaction was performed in triplicate on white Thermo-Fast 96 detection plates (Abgene) using the standard conditions determined by the company. According to the manufacturer's guidelines, the level of expression was calculated based upon the PCR cycle number (Ct) at which the exponential growth in fluorescence from the probe passes a certain threshold value. Relative expression was determined by the difference in the Ct values for the target genes after normalization to RNA input level, using GAPDH Ct values (Δ Ct = Ct of the target gene - Ct of the internal control GAPDH). Relative quantification between different samples (e.g. treated vs non-treated) was determined according to the $2^{-\Delta\Delta$ Ct method (Livak and Schmittgen, 2001) ($\Delta\Delta$ Ct = Δ Ct treated sample - Δ Ct non-treated sample) and examined for concordance with microarray data.

Flow cytometry

RBL-CCR1 cells were stimulated for 6, 8, 12 and 24 h, blocked and incubated with rabbit Emp-1 antiserum (courtesy of Professor Hsuan-Shu Lee, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan), rabbit polyclonal antibody against RGS1 (H-

70, Santa Cruz Biotechnology, Inc.) or rabbit antiserum against RT.BM1 (courtesy of Professor Geoff Butcher, Babraham Institute, Cambridge, UK). Phycoerythrin (PE)-conjugated, donkey anti-rabbit IgG (Abcam ab7007, Cambridge, MA, USA) was used as the secondary antibody; and normal rabbit serum (R & D Systems) was the isotype control. The events were acquired using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed using Flow Jo Software (Tree star, San Carlos, CA, USA).

Immunoblotting

Whole cell lysates from stimulated RBL-CCR1 were harvested and the protein content measured using the DC Protein Assay Kit I (Bio-Rad, Hercules, CA, USA). Thirty μ g of protein per sample was electrophoresed through 10–20% polyacrylamide gels (Ready Gel, Bio-Rad, Hercules, CA, USA) under denaturing conditions and then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). After blocking with 0.5% non-fat dry milk, the blots were probed using the Emp-1-antiserum and Rgs-1 antibody used for the FACS analysis and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Abcam ab16284, Cambridge, MA, USA). Bands were visualized by incubation with HRP ECL substrate (Pierce, Rockford, IL, USA) and subsequent exposure to Hyperfilm ECL (Amersham Biosciences UK Ltd, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

Whole cell ELISA for EMP-1

RBL-CCR1 cells were cultured for 72 h as described above. The cells (1×10^4 cells/well) were then cultured in serum free medium for 6 h in 96-well flat bottom microtiter plates before sensitization and stimulation as described above. The cell were then washed twice in PBS, fixed with 1% paraformaldehyde in PBS for 15 min at room temperature, washed with 0.1% BSA in PBS, and incubated in EMP-1 anti-serum (1:500, 1:1000 and 1:3000 dilutions) for 1 h. Cells were next incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed three times with PBS/0.1% BSA, and incubated in TMB solution. The reaction was terminated using 1 M sulfuric acid, then the absorbance of each well was measured at a wavelength of 405 nm. Normal rabbit serum (Biomed Corporation, CA, USA) was used as the control.

Detection of secreted proteins

RBL-CCR1 cells were sensitized, then stimulated for 24 hr as described above. Triplicate supernatant samples were collected and kept frozen at -20°C . The release of cytokines interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-11, IL-12p70, IL-17, IL-18, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , vascular endothelial growth factor (VEGF), chemokines CCL2, CCL3, CCL4, CCL5, CCL7, CCL9, CCL12, CCL19, CXCL2 and matrix metalloproteinase (MMP)-9 was measured by Rules-Based Medicine (TX, USA) using the Luminex Beads Assay.

Multi-Analyte Profiles were performed using 96-well-plates in which a microsphere set labeled with a unique fluorescence signature was used for each analyte-specific assay, together with 10 μ L of the supernatant sample. After 1 h of incubation at room temperature, multiplexed cocktails of biotinylated reporter antibodies were then added. After an additional hour of incubation at room temperature, multiplexes were developed by incubating with an excess of streptavidin-PE solution for 1 h at room temperature. The volume of each multiplexed reaction was reduced by vacuum filtration and the volume increased by dilution into matrix buffer for analysis. Analysis was performed in a Luminex 100 instrument and the resulting data stream was interpreted using proprietary data analysis software. For each multiplex, both calibrators and controls were included on the

microtiter plate. Testing results were determined first for the high, medium and low controls for each multiplex to ensure proper assay performance. Unknown values for each of the analytes localized in a specific multiplex were determined using 4 and 5 parameter, weighted and non-weighted curve fitting algorithms included in the data analysis package. The fold change ratio was calculated using the formula $\text{FCi} = x_{ij}/y_{ij}$ where x_{ij} and y_{ij} are the raw expression levels of gene i in replicate j in the treatment and control, respectively (Tusher et al., 2001).

Results

To better define the effects of Fc ϵ R1-CCR1 costimulation on mast cell function, gene expression profiles were compared for various sensitization and stimulation conditions using RBL-CCR1 cells. To ensure that the cells analyzed displayed functional responses to costimulation, the same culture was used for both degranulation assays and for gene chip analysis. At three hours after activation, RNA was harvested and the remaining cells were examined for β -hexosaminidase release. As expected based on our previous work (Toda et al., 2004), degranulation was markedly increased following treatment with both antigen and CCL3 compared to stimulation of Fc ϵ R1 or CCR1 alone (Fig. 1). Indeed, costimulation-mediated degranulation was increased by approximately 8-fold, 2.5-fold and 4.4-fold compared to sensitized, cross-linked and CCL3-activated conditions, respectively. Similar results were observed 20 min after stimulation (data not shown).

Genes differentially regulated by costimulation of Fc ϵ R1 and CCR1

GeneChip data were filtered as described in Fig. 2A. Expression levels for present or marginal genes whose value appeared in at least one of the 15 samples, based on the relative signal intensity of the transcripts on the GeneChip, were compared between the antigen cross-linked, CCL3-treated and costimulated groups to identify differentially expressed genes. Genes whose expression was significantly different after costimulation, but not after stimulation with antigen or chemokine treatment alone, were considered possible synergy-related genes. Comparisons of the various sensitized samples generated a list of 212 genes differentially expressed genes after costimulation compared to sensitization. Clear differences in the overall

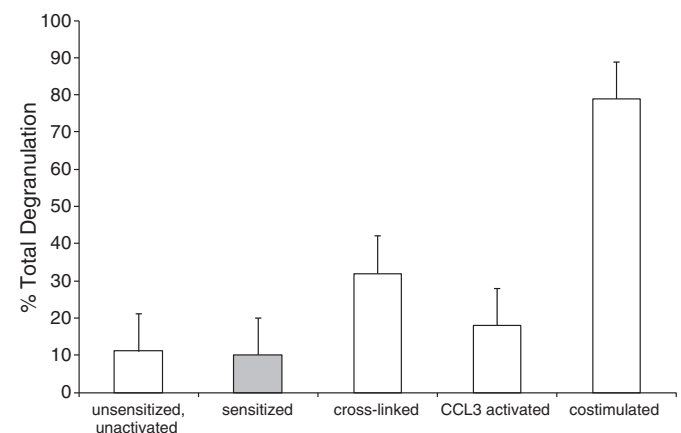


Fig. 1. Degranulation of RBL-CCR1 cells following stimulation of Fc ϵ R1 and CCR1. β -hexosaminidase assays were used to assess mast cell degranulation following treatment with IgE (sensitized), antigen (cross-linked), CCL3 or both antigen and CCL3 (costimulated). This experiment was repeated more than three times, with similar results.

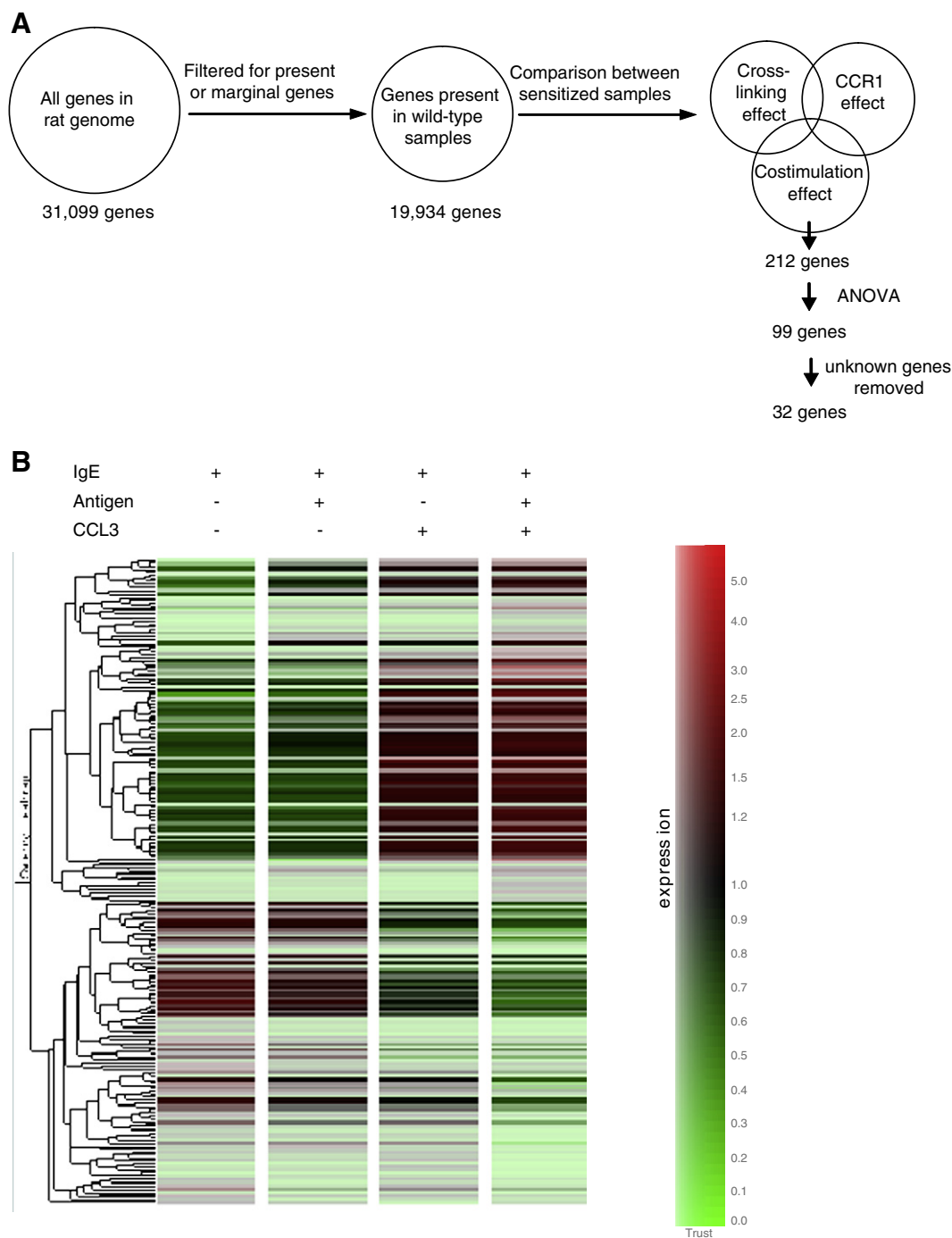


Fig. 2. Selection of possible FcεRI-CCR1 synergy-related genes. (A) Filtering strategy. All genes in the rat genome were filtered to remove the genes that were not present, and expression levels for the present or marginal genes were compared between the samples. The genes that were at least two-fold up- or down-regulated in cross-linked, CCL3-activated and costimulated conditions relative to the sensitized condition were chosen. The common genes from these three comparisons were excluded and the genes that were differentially expressed at significant levels after costimulation were designated as possible synergy-related genes. (B) Heatmap of the 212 possible synergy-related genes. Each bar represents one gene. Green color shows down-regulation and red color shows up-regulation of the genes. Stronger color represents stronger up- or down-regulation.

expression pattern between these four treatment conditions were visible in the GeneChip heat map profiles (Fig. 2B).

Ninety-nine genes were expressed at significantly different levels following costimulation. After unknown genes such as transcribed loci and hypothetical genes were removed, this list was reduced to 32 synergy-related genes. Of these, 27 genes were up-regulated and 5 genes were down-regulated (Table 1). The chemokine gene *Ccl7* was the most strongly up-regulated (23.6-fold, $p < 0.05$), and the Casitas B-lineage lymphoma b gene *Cblb* was the most strongly down-regulated (−6.0-fold, $p < 0.05$).

Validation of the gene chip data

Real-time PCR analysis was used to validate the expression data for the seven genes that were more than 2.5-fold up-regulated in the GeneChip analysis. Of these genes, *Ccl7*, *Rgs1*, *Emp1* and *RT1-S3* showed synergistic upregulation, with *Ccl7* showing the most pronounced costimulation effect (Fig. 3). The remaining genes – *RT1-Aw2*, *Nr4a2* and *Map2k3* – were not expressed at significantly higher levels following costimulation.

Table 1

Genes differentially regulated by FcεRI-CCR1 costimulation of RBL-CCR1 cells.

Affymetrix gene ID	P-value	Fold change	Gene Symbol	Gene Title
Up-regulated synergy-related genes				
1379935_at	0.03	23.60	Ccl7	chemokine (C-C motif) ligand 7
1398540_at	0.05	6.09	Rgs1	regulator of G-protein signaling 1
1371527_at	0.01	4.21	Emp1	epithelial membrane protein 1
1369007_at	0.03	3.57	Nr4a2	nuclear receptor subfamily 4, group A, member 2
1393108_at	0.01	2.96	RT1-Aw2	RT1 class Ib, locus Aw2
1388164_at	0.01	2.70	RT1-S3	RT1 class Ib, locus S3
1388858_at	0.01	2.53	Map2k3	mitogen activated protein kinase kinase 3
1371123_x_at	0.01	2.49	RT1-S3	RT1 class Ib, locus S3
1389263_at	0.03	2.42	Rai14	retinoic acid induced 14
1369146_a_at	0.05	2.41	Ahr	aryl hydrocarbon receptor
1396275_at	0.04	2.37	Dusp18	dual specificity phosphatase 18
1379563_at	0.01	2.29	Atp6v1d	ATPase, H + transporting, V1 subunit D
1369788_s_at	0.02	2.28	Jun	Jun oncogene
1371684_at	0.01	2.25	Pelo	pelota homolog
1377944_at	0.03	2.25	Upp1	Uridine phosphorylase 1
1369738_s_at	0.01	2.24	Crem	cAMP responsive element modulator
1371943_at	0.00	2.20	Lyk4	Ser/Thr-like protein kinase lyk4
1370571_at	0.00	2.18	Slco3a1	solute carrier organic anion transporter family, member 3a1
1380195_at	0.01	2.17	Atp2b1	ATPase, Ca++ transporting, plasma membrane 1
1388212_a_at	0.01	2.15	RT1-S3	RT1 class Ib, locus S3
1389368_at	0.03	2.14	Cnksr3	Cnksr family member 3
1387394_at	0.04	2.13	Il2rb	interleukin 2 receptor, beta chain
1386426_at	0.01	2.12	Atp2b1	ATPase, Ca++ transporting, plasma membrane 1
1368301_at	0.01	2.12	Adora2a	adenosine A2a receptor
1387060_at	0.04	2.08	Klf6	Kruppel-like factor 6
1377869_at	0.01	2.06	Ccrn4l /// LOC310395	CCR4 carbon catabolite repression 4-like (<i>S. cerevisiae</i>), similar to Nocturnin (CCR4 protein homolog) ADP-ribosylation factor 2
1368946_at	0.01	2.04	Arf2	
Down-regulated synergy-related genes				
1390826_at	0.04	−5.95	Cblb	Casitas B-lineage lymphoma b
1370196_at	0.01	−2.19	Pias3	protein inhibitor of activated STAT 3
1389999_at	0.01	−2.11	Eef2k	Eukaryotic elongation factor-2 kinase
1370336_at	0.01	−2.06	Ok138	pregnancy-induced growth inhibitor
1367602_at	0.05	−2.00	Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2

To further characterize the effects of CCR1-FcεRI costimulation on expression patterns, we examined the protein levels for CCL7, RGS1, EMP1, and RT1-S3. Since CCL7 is a secreted protein, we analyzed its levels in the supernatant of unsensitized and sensitized RBL-CCR1 cell cultures. Conditioned medium was collected 24 h after receptor activation with antigen and/or CCL3, and then the Luminex bead assay was used to determine CCL7 levels. A synergistic increase in CCL7 concentration was observed following costimulation in three replicate experiments (Fig. 4A). RGS1 is an intracellular protein, so its expression was determined by immunoblotting of total cell lysates. Expression was similar for all conditions tested (Fig. 4B). Expression of EMP-1, a cell surface protein, was examined using flow cytometry at 6, 8, 12 and 24 h after receptor activation. No difference in expression was seen at any of the time points tested (Fig. 4C). Whole cell ELISA was also used to examine EMP-1 expression. This analysis, too, failed to demonstrate an increase in expression upon costimulation (data not shown). Expression of RT1-S3, another cell surface protein, was examined by flow cytometry at 6, 8, 12 and 24 h after receptor activation. The expression upon costimulation was no higher than that induced by antigen alone (Fig. 4D).

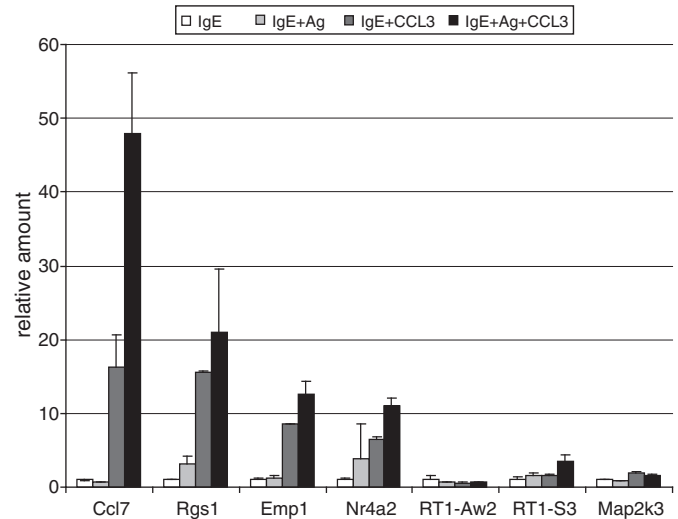


Fig. 3. Real time PCR data for possible synergy-related genes following treatment of RBL-CCR1 cells with antigen, CCL3 or both. The bars show the average relative expression based on 3 individual experiments, and the error bars show the standard deviation.

Cytokine and chemokine release from RBL-CCR1 cells after activation with IgE, antigen and/or CCL3

To further examine the downstream effects of FcεRI-CCR1 costimulation, we next examined secretion levels for various chemokines and cytokines following single- or costimulation. RBL-CCR1 cells were sensitized and activated with antigen and/or CCL3 for 24 h, then the supernatants were collected and analyzed using the Luminex Bead assay. IL-6, CCL2 and CCL7 were expressed at the highest levels following costimulation compared to stimulation of either receptor alone (Fig. 5). Expression levels for most cytokines and chemokines tested were higher following co-stimulation, but this upregulation appeared to be additive rather than synergistic. Although the costimulatory increase in IL-6 release was pronounced, it should be noted that the actual amount of release was very small (1 pg/ml for IL-6, compared to 1216 pg/ml and 576 pg/ml for CCL2 and CCL7, respectively).

Discussion

To summarize, costimulation of RBL-CCR1 cells with antigen and CCR3 resulted in up-regulation of several genes. The genes most significantly up-regulated by FcεRI-CCR1 costimulation were *Ccl7*, *Rgs1*, *Emp1* and *RT1-S3*. CCL7 protein was also expressed at higher levels 24 h after dual receptor activation, though RGS1, EMP1 and RT1-S3 were not. At the protein level, IL-6 expression was seen only after FcεRI-CCR1 costimulation, although the amount expressed was very low; CCL2 was also expressed at higher levels following costimulation.

Costimulation of CCR1 and FcεRI results in increased degranulation and arrested chemotaxis of RBL-CCR1 cells, suggesting synergistic cross-talk between FcεRI and CCR1 (Toda et al., 2004), but this is the first report of downstream effects of FcεRI-CCR1 costimulation on gene- and protein expression. Others have examined mast cell gene expression profiles following stimulation with antigen or cytokines (Babina et al., 2004; Jayapal et al., 2006; Nakajima et al., 2002; Saito et al., 2001), but none of these studies addressed the effects of costimulation on mast cells.

Compared to sensitization alone, FcεRI stimulation alone (our unpublished data) and CCR1 stimulation alone (Aye et al., submitted), FcεRI-CCR1 costimulation of RBL-CCR1 cells yielded the greatest degree of change in gene expression. Many genes displayed differential regulation 3 h after FcεRI and/or CCR1 activation. This is not

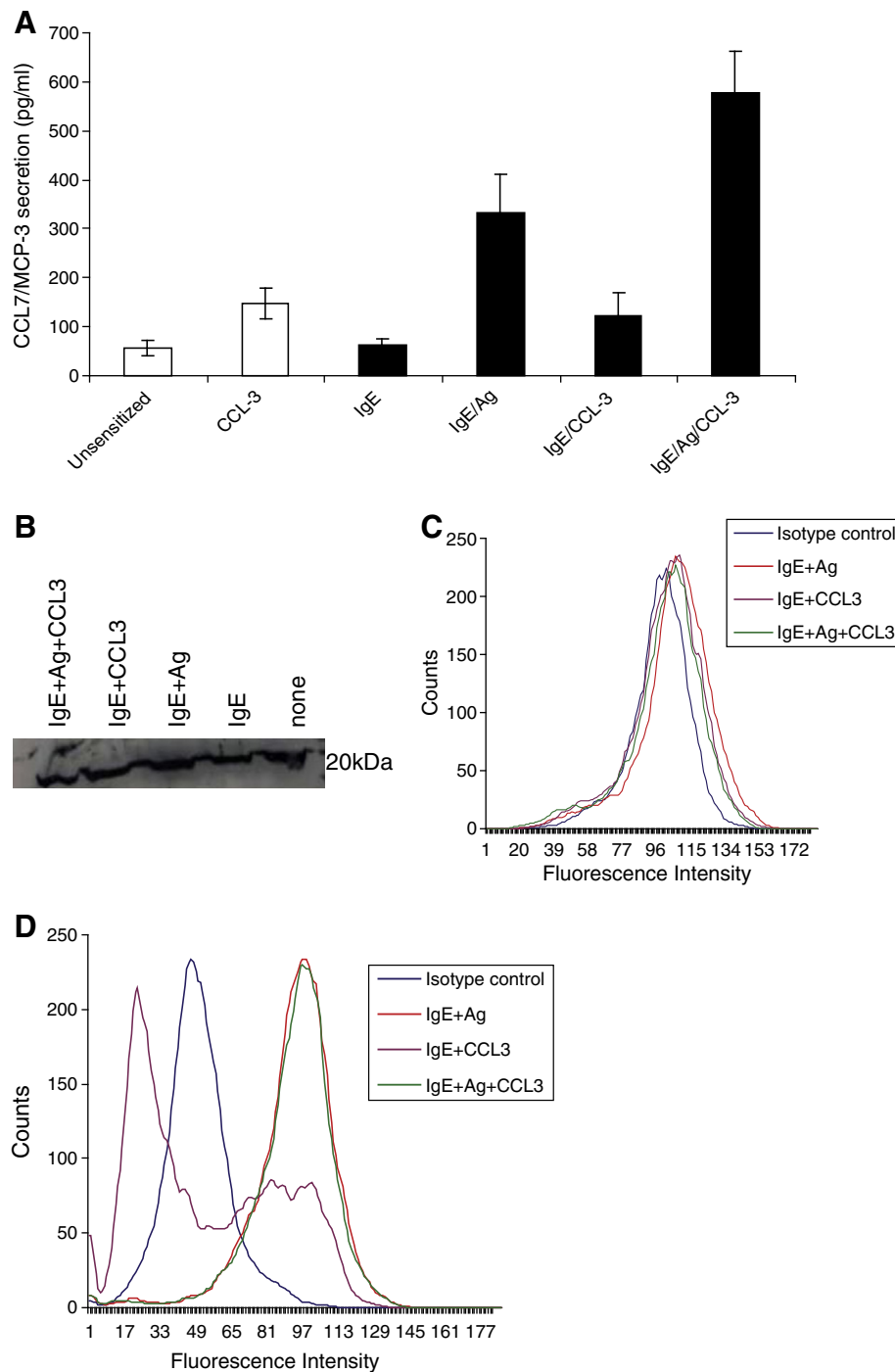


Fig. 4. Protein expression levels for synergy gene products following CCR1- and/or FcεR1 stimulation of RBL-CCR1 cells. All protein samples were collected 24 hr after cell stimulation. (A) Release of CCL7 in the cell culture supernatant, as measured using Luminex beads. The graph shows the average of 3 independent experiments. Error bars indicate standard deviation. (B) Expression of RGS1 in cell lysates, as assessed by immunoblotting. This experiment was conducted four times, and one representative experiment is shown. (C) Cell surface expression of EMP-1, as assessed by flow cytometry. (D) Cell surface expression of RT1-S3, as assessed by flow cytometry.

surprising, given that mast cells are known to synthesize and release a variety of chemokines and cytokines at around 6 h after stimulation (Izuhara and Saito, 2006). This suggests that the stage following the early phase of activation (less than 2 h) but preceding the late phase (more than 24 h) is crucial for transcriptional regulation of mast cell effector genes.

CCL7, previously known as monocyte chemotactic protein 3, is a particularly promising mediator of mast cell activity. It is expressed at multiple sites of inflammation (Jacobsson et al., 2005; Schenk et al., 2007), and is produced by monocytes, fibroblasts, endothelial

cells and mast cells (Wakahara et al., 2001). CCL7 acts as a chemoattractant for monocytes, memory T lymphocytes, eosinophils, basophils, dendritic cells and natural killer cells, though its strongest response is to activate monocytes (Dahinden et al., 1994). Production of this chemokine by costimulated mast cells may help to coordinate allergic responses.

Ccl2 and *Ccl7* displayed similar gene expression patterns in response to FcεRI and/or CCR1 activation on RBL-CCR1 cells, and chemokines were found at higher levels in the supernatant following costimulation. This finding is not surprising, as the receptors and

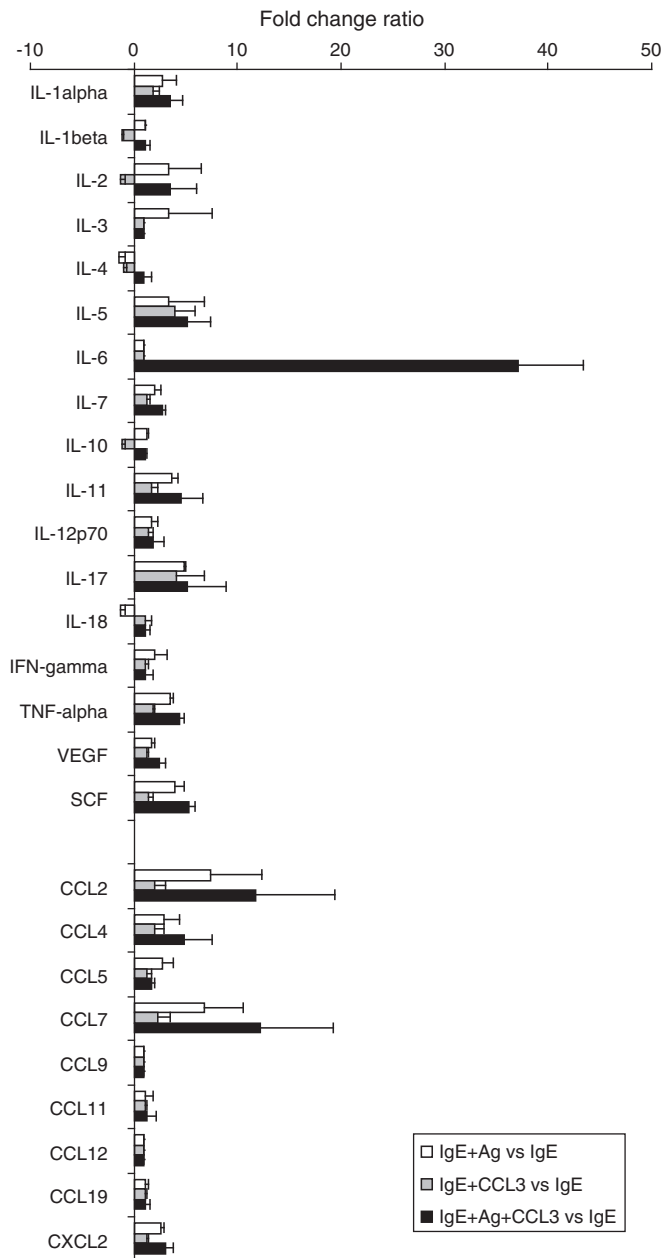


Fig. 5. Secretion of cytokines and chemokines genes following treatment of RBL-CCR1 cells with antigen, CCL3 or both. Expression was measured using Luminex beads. Fold change in supernatant levels relative to sensitized, unstimulated cells is shown. Error bars represent the standard deviation ($n = 3$).

transduction pathways for CCL7 overlap with those of CCL2 (Sozzani et al., 1994). CCL7 binds to CCR2, $+3$ and -10 ; CCL2 binds only to CCR2 and -10 (Toda et al., 2007). Of these receptors, CCR2 is the most promising as a mediator of autocrine effects. CCL7 signaling via CCR3 has a negligible effect in a mouse allergic conjunctivitis model (Miyazaki et al., 2009), and CCR10 expression on mast cells has not been described. It will be interesting to see if CCR2 is expressed at higher levels in RBL-CCR1 cells following FcεRI-CCR1 costimulation. In addition to mast cells, CCR2 is expressed by T cells, eosinophils and dendritic cells (Toda et al., 2007); mast cell-derived CCL2 and CCL7 may therefore coordinate immune responses.

IL-6, too, was secreted at markedly greater levels by RBL-CCR1 cells after FcεRI-CCR1 costimulation, though the actual amount released was very small (1 pg/ml). Increased IL-6 secretion has been

observed for IgE-exposed human lung mast cells (Cruse et al., 2008) and antigen-stimulated bone marrow-derived mast cells (Kitaoura et al., 2004). IL-6 appears to promote mast cell survival, although it is not a strong survival factor (Cruse et al., 2008). Simultaneous upregulation of CCL2 and IL-6 has been observed for a variety of cell types. In human myoblasts, IL-6 regulates expression of CCL2, inter-cellular adhesion molecule 1 and autocrine IL-6 expression (Marino et al., 2008); locally secreted IL-6 might influence monocyte chemotaxis and leukocyte trafficking through regulation of these molecules. Fibroblasts treated with soluble IL-6 receptor display an acute and transient increase in intracellular calcium concentrations, as well as increased expression of IL-6 and CCL2 (Spörri et al., 1999). In the peritoneum, where mesothelial cells, fibroblasts, granulocytes, mast cells, and macrophages are found, production of CCL2 and IL-6 occurred spontaneously during incubation with lipopolysaccharide for up to 5 h (Riese et al., 1999). Taken together, these results suggest that IL-6 and CCL2 regulation mechanisms are closely related. There is no direct relationship between mast cell degranulation and IL-6 production (Leal-Berumen et al., 1994), but upregulation of IL-6, CCL2 and CCL7 might be a response to the arrested chemotaxis resulting from costimulation.

Rgs1 and *Emp1* were transcriptionally upregulated following costimulation, though changes in protein expression were not observed. A role for *Rgs1* in mast cell biology has not yet been described, though inactivation of this gene has been shown to alter B cell migration in response to chemokines (Moratz et al., 2004). Others have also observed *Emp1* upregulation in mast cell that have been sensitized and/or stimulated with antigen (Jayapal et al., 2006). The lack of increase protein expression levels for RGS1 at 24 h and EMP1 at 6, 8, 12, and 24 h may be due to low sensitivity of the protein assays or the kinetics of the protein expression. Alternatively, the transcriptional upregulation may simply not result in protein upregulation. The co-incidence ratio between transcription level and protein expression has been estimated at less than 50% (Chuaqui et al., 2002).

This study provides a novel characterization of potential downstream mediators of FcεRI-CCR1 costimulation. Further studies will be needed to determine if chemokines such as CCL7, CCL2 and IL-6 play critical roles in mast cell effects and allergic responses *in vivo*. Mast cell secretion of any or all of these factors might be required for optimal coordination of late-phase allergic reactions.

Conflict of Interest statement

There are no conflicts of interest associated with this work.

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