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Mast Cell FcεRI-Induced Early Growth Response 2 Regulates CC Chemokine Ligand 1–Dependent CD4⁺ T Cell Migration

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Mast cells are well positioned in host tissue for detecting environmental signals, including allergens, leading to activation of the high-affinity IgE receptor FcεRI, and initiating a signaling cascade that perpetuates the production of biologically potent mediators, including chemokines. We have identified a novel target of mast cell FcεRI activity in the transcription factor early growth response 2 (Egr2) and sought to characterize its function therein. Egr2 was transiently activated following FcεRI-mediated signaling, targeted the promoter of the chemokine *CCL1*, and was critical for allergen-induced mast cell CCL1 production. Egr2-deficient mast cells were incapable of directing CD4⁺ T cell migration via the CCL1–CCR8 axis. In a model of allergic asthma, reconstitution of mast cell-deficient mice with Egr2-deficient mast cells demonstrated that mast cell Egr2 was essential for migration of CD4⁺ T cells to the inflamed lung. These findings position Egr2 as a critical regulator of mast cell-directed CD4⁺ T cell migration. *The Journal of Immunology*, 2013, 190: 000–000.

Mast cells are tissue sentinels enriched at host–environment interfaces that detect and direct the host response to encounters with environmental cues such as allergens. Upon allergen-induced activation, mast cells secrete a panel of preformed granule-bound mediators that initiate the immediate local inflammatory response and also begin to produce and secrete a variety of receptor signaling-dependent chemokines and cytokines (1). Mast cell–produced chemokines and cytokines shape the late-phase immune response at the site by signaling to other cells of the immune system and activating and recruiting competent cell types, such as T cells, eosinophils, macrophages, and neutrophils to the site of inflammation.

CD4⁺ T cells are critical effectors and regulators in immunological homeostasis, but are also active participants in the pathology of a variety of diseases, including allergy and asthma, by driving IL-4–dependent IgE production (2, 3), type I diabetes (4), and neurodegenerative disorders, including Parkinson's disease and multiple sclerosis (5, 6). CD4⁺ T cell recruitment and activation is a major theme in the study of these diseases and, as such,

understanding the mechanisms governing CD4⁺ T cell recruitment and function represents an area of intensive study toward therapeutic approaches for each.

Specific immune cell recruitment is dependent on the production of particular chemokines. In the lung mucosa, local mast cells activated through IgE have been shown to be critical in mediating the recruitment of CD4⁺ T cells and directing the subsequent allergic inflammatory pathology (7). Communication between locally activated mast cells and peripheral CD4⁺ T cells is regulated through mast cell–produced CCL1 and its receptor, CCR8, expressed on the surface of CD4⁺ T cells, an interaction termed the CCL1–CCR8 axis (8).

Allergen-induced mast cell activation is mediated by aggregation of the high-affinity IgE receptor FcεRI on the surface of sensitized mast cells (9), resulting in the activation of critical transcription factors that drive chemokine and cytokine gene expression (10). Among these activated transcription factors critical in mast cell function are several of the early growth response (Egr) family genes, including Egr1 and Egr2, also known as Krox20 (11, 12). The Egr family of zinc finger transcription factors is activated by a broad array of stimuli and is implicated in diverse cell type–specific biological processes depending on the context of activation-related signals (13). For example, Egr2 is critical in the development and maturation of NKT cells as well as B and T cells (14, 15) and is also an essential transcription factor in the nervous system, regulating peripheral nerve myelination as well as segmentation in hindbrain development (16, 17). We have previously described the role of family member Egr1 in regulating the full responsiveness of cytokine production in FcεRI-activated mast cells (18–20); however, the role of Egr2 in mast cell activation has not been described.

In this study, we have dissected a novel role for inducible Egr2 in FcεRI-activated mast cells. Mast cells lacking Egr2 expression had a specific defect in CCL1 production due to impaired Egr2 binding at the *CCL1* promoter. Consequently, Egr2-deficient mast cells were unable to direct chemotactic migration of CD4⁺ T cells in vitro or in an in vivo model of allergic asthma. These findings indicate that allergen-induced mast cell direction of peripheral

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; Egr, early growth response; LMC, liver-derived mast cell; SSH, suppression subtractive hybridization; TNP, trinitrophenyl.

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CD4⁺ T cells to local sites of allergic inflammation is dependent on mast cell Egr2.

Materials and Methods

Animals

Egr2-deficient mice were generated as previously described (21). C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mast cell-deficient W-sh mice were obtained from The Jackson Laboratory (B6Cg-kit W-sh/HNihJacBsmJ NistltF4). Protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care.

Abs and reagents

Abs to Egr1, Egr2, NF- κ B p65, NF- κ B p50, NFATc1, and NFATc2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PerCP-Cy5.5-conjugated rat anti-mouse c-kit/CD117 mAb (IgG2b, κ), PerCP-Cy5.5 rat IgG2b, κ , FITC-conjugated rat anti-mouse IgE (IgG1), FITC-rat IgG1, allophycocyanin-CD4, FITC-CD25, allophycocyanin-rat IgG2a, κ , and FITC-rat IgM, κ were purchased from BD Biosciences (San Jose, CA). Abs to PE-Foxp3 and PE-rat IgG2b, κ were purchased from eBioscience (San Diego, CA). Alexa Fluor 594 goat and rabbit secondary Abs were from Invitrogen (Eugene, OR). Abs to CCL1, CCL3, CCL9, and TNF, as well as mouse rCCL1 and standards (DuoSet) for ELISA were from R&D Systems (Minneapolis, MN). Recombinant murine stem cell factor was purchased from PeproTech (Rocky Hill, NJ).

Mast cell culture, activation, degranulation, and calcium mobilization

Mouse liver-derived mast cells (LMCs) were obtained by culturing liver cells from newborn mice from Egr2^{+/-} breeders. Egr2 deficiency results in neonatal or perinatal death (22). Briefly, liver tissue was removed to a sterile environment and was ground to produce a single-cell suspension in RPMI 1640 medium. Cells were collected, centrifuged at 500 $\times$ g for 5 min at 4°C, and resuspended at a density of 0.5 $\times$ 10⁶ cells/ml in complete medium (RPMI 1640 medium containing 10% FBS, 10% WEHI-3B-conditioned medium, 30 ng/ml stem cell factor, 50 U/ml each of penicillin and streptomycin, 50 μ M 2-ME, and 200 nM PGE₂). An aliquot of cells from each mouse was used for genotyping. Nonadherent cells were resuspended in complete medium twice per week and transferred to a fresh flask once per week. After 5–6 wk, mast cell purity of >98% was achieved, as assessed by toluidine blue staining of fixed cytocentrifuged preparations. Sensitization, degranulation, and calcium mobilization were as previously described (20).

Subtractive hybridization, virtual Northern analysis, and real-time quantitative PCR

Subtractive hybridization was carried out as described previously (19). The GAPDH probe was produced by PCR from cDNA with primers 5'-CGATGCCCATGTTTGTGAT-3' and 5'-CTCCTTGGAGGCCATGTAGG-3'. The mouse Egr2 probe was prepared by PCR from the subtractive hybridization plasmid clone pCR4-TOPO 1F7 with M13 forward (-20) and M13 reverse primers. Real-time quantitative PCR for Egr2 and Egr1 was performed and analyzed as previously described (18).

Immunofluorescence

Immunofluorescence was as described previously (18), except samples were incubated overnight with anti-Egr2 Ab (16 μ g/ml) or rabbit serum (Cedarlane) at 4°C.

Electrophoretic mobility shift assay

Nuclear protein extracts were isolated using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer's protocol. EMSA was performed as previously described (18). The following synthesized double-stranded oligonucleotides (Sigma-Aldrich) were used: NF- κ B-binding consensus sequences on mouse IL-6 promoter, 5'-TTATCAAATGTGGGATTTTCCCAT-3' and the mutant sequence, 5'-TTATCAAATGTGTTACCTTCCCAT-3'; NFAT-binding consensus sequence on mouse IL-13 promoter, 5'-AAGGTGTTTCCCAAGCCTTCCC-3' and the mutant sequence, 5'-AAGGTGTCCATCCAAGCCTCCA7C-3'; and Egr2-binding consensus sequence on CCL1 promoter, 5'-TCAGCCAGGACAGCTCCACGTATTTTG-3' and the mutant sequence, 5'-TCAGCCAGGACATATCCATATTTTG-3' (italics indicate mutated bases). For competition assays, 1 μ l nonradiolabeled wild-type or mutant oligonucleotides (50-fold excess compared with radiolabeled probe) were added and incubated for 30 min

before the addition of the radiolabeled probe. For blocking assays, 4 μ g anti-NFATc1, anti-NFATc2, anti-p50, anti-p65, anti-Egr1, or serially diluted anti-Egr2 Abs were added and incubated at 4°C for 30 min before the addition of the ³²P radiolabeled probe. For quantifications, densitometry was conducted using Image J software.

Chromatin immunoprecipitation

Assays were performed as previously described (20). PCR consisted of 42 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C. Primers for amplification of the CCL1 promoter region were 5'-GGCCATTGGGTATGAGTTCCTT-3' (forward) and 5'-ATCCACACGGCAGCCAGCAGCA-3' (reverse).

IgE-mediated passive cutaneous anaphylaxis

Mast cell-deficient W-sh mice were reconstituted with Egr2^{+/-} (right ear) and Egr2^{-/-} (left ear) LMCs. Eight months later, passive cutaneous anaphylaxis was conducted as previously described (20).

Transwell migration assays

Mast cell-conditioned cell-free supernatants were prepared using sensitized Egr2^{+/-} and Egr2^{-/-} LMCs (1 $\times$ 10⁶/ml) stimulated with trinitrophenyl (TNP)-BSA for 24 h in RPMI 1640 (Invitrogen) supplemented with 10% FBS and antibiotics. Splenic T cells were isolated from 8-wk-old C57BL/6 mice using a CD4⁺CD25⁺ isolation kit (Miltenyi Biotec) allowing for purification of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells. Isolated T cells were tested by flow cytometry with anti-CD4 and anti-CD25 or isotype control Abs. Assays were conducted using 5 μ m polycarbonate filters in Costar 24-well Transwell chambers (Corning, Corning, NY) with 600 μ l conditioned media in the lower chamber and 2 $\times$ 10⁵ T cells in 100 μ l in the upper chamber. Assays were conducted for 70 min at 37°C, and cells that had migrated into the lower chamber were then counted. Net migration equals migrated cell number minus control migration cell number. Blocking Abs were used at a final concentration of 1 μ g/ml, and murine rCCL1 protein was used at 100 ng/ml.

OVA-induced asthma model

Eight-week-old W-sh mice were reconstituted with either Egr2^{+/-} or Egr2^{-/-} LMCs (5 $\times$ 10⁶/mouse in 200 μ l RPMI 1640) by i.v. injection. Five to 6 wk later, reconstituted mice were sensitized by i.p. injection with 10 μ g OVA with 1 mg alum (Sigma-Aldrich) in 100 μ l saline on days 1, 5, and 10, followed by intranasal challenge with 20 μ g OVA in 20 μ l saline on days 21, 25, 27, and 29. On day 30, lung tissue was collected for histological staining. Draining lymph nodes, lungs, and spleen were isolated, and cells were blocked with 1 μ g/ml rat IgG and 1 μ g/ml mouse IgG for 30 min prior to staining for surface CD4 and CD25 and subsequent permeabilization with Cytofix/Cytoperm (BD Biosciences) and intracellular staining for Foxp3 for 30 min each at room temperature. FACS analysis was conducted using WinMDI 2.9 or WinList 5.0 software.

Bronchoalveolar lavage and lung processing

Bronchoalveolar lavage fluid (BALF) was obtained by lavaging the lung with 1 ml phosphate buffer solution containing soybean trypsin inhibitor (100 μ g/ml). Cells in the BALF were counted, and slides were prepared by cytospin (Cytospin 4; ThermoShandon) and stained with Diff-Quik (Siemens Healthcare Diagnostics, Newark, DE), according to the manufacturer's protocol. The right lung was homogenized at maximum speed for 20 s (PowerGen 125; Fisher Scientific) in 50 mmol/L HEPES buffer (4 μ l/mg lung) containing soybean trypsin inhibitor (100 μ g/ml) and cleared by centrifugation at 14,000 rpm.

Eosinophil peroxidase and myeloperoxidase assays

For eosinophil peroxidase assays, 75 μ l substrate solution (3 mmol/L o-phenylenediamine dihydrochloride, 50 mmol/L chloride-free HEPES [pH 8.0], 6 mmol/L KBr, and 8.8 mmol/L H₂O₂) was added to 75 μ l of each sample. After 30 s, the reaction was stopped with 150 μ l 2 mol/L H₂SO₄, and the absorbance was read at 490 nm. For myeloperoxidase, 75 μ l substrate solution (3 mmol/L fresh 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate [Sigma-Aldrich], 0.12 mmol/L resorcinol [Sigma-Aldrich], and 2.2 mmol/L H₂O₂) was added to 75 μ l of each sample. After 2 min, the reaction was stopped with 150 μ l 2 mol/L H₂SO₄, and the absorbance was read at 450 nm within 10 min.

Statistical analysis

The paired Student *t* test was used for statistical evaluation of data. Results were considered significant when *p* < 0.05. Data are expressed as means $\pm$ SEM.

Results

Mast cell activation via FcεRI induces transient Egr2 expression and activity

To identify genes with novel expression in mast cells activated through FcεRI, we employed an unbiased method termed suppression subtractive hybridization (SSH). For this assay, wild-type mast cells cultured from murine bone marrow were activated by sensitizing the cells with IgE specific for TNP-BSA and subsequently challenging these cells with this Ag to achieve mast cell activation through FcεRI. The *Egr2* gene was among those clones identified by SSH, and a virtual Northern blot was performed on duplicate membranes containing cDNA from untreated and TNP-BSA-treated samples to confirm the enhanced expression of *Egr2* in stimulated mast cells (Fig. 1A).

To characterize the kinetics of *Egr2* mRNA expression in activated mast cells, we conducted real-time quantitative PCR studies. Following TNP-BSA treatment, *Egr2* expression was induced by 15 min and reached its highest level by 30 min. By 60 min, *Egr2* expression dropped off considerably and returned to near basal levels by 180 min post-TNP-BSA stimulation (Fig. 1B). *Egr2* transcription was similar to that of *Egr1*, but slightly delayed by comparison. PCR amplicons were also analyzed on agarose gel by ethidium bromide staining, and a representative gel is shown (Fig. 1C).

We further examined FcεRI-induced *Egr2* expression at the protein level by immunofluorescence in IgE-activated wild-type LMCs. Following TNP-BSA stimulation, strong and primarily nuclear, expression of *Egr2* protein is detected by 15 min and persisted until 60 min, before dropping off to low levels and returning to near basal levels by 180 min through 360 min (Fig. 1D). As a control, immunofluorescence was also conducted using nonspecific rabbit serum as a primary Ab as well as in *Egr2*-deficient LMCs (described below) to evaluate the specificity of the anti-*Egr2* Ab (Fig. 1D). Finally, we characterized the specific DNA-binding activity of *Egr2* in our model (further information in Supplemental Fig. 1A, 1B). Collectively, these results indicate that active *Egr2* is transiently expressed de novo in mast cells from 15–60 min following FcεRI-mediated activation.

Egr2 is not required for mast cell development or degranulation

To determine the role of *Egr2* in mast cell function, wild-type and *Egr2*-deficient primary LMC cultures were established from fetal liver cells cultured in media conditioned with stem cell factor, IL-3, and PGE₂ to promote differentiation and proliferation of mast cells from liver-derived progenitor cells. PCR-based genotyping was carried out to confirm each culture (Supplemental Fig. 1C). Assessment of mast cell development was carried out using a toluidine blue stain that showed similar metachromatic staining and morphology between wild-type and *Egr2*-deficient LMCs (Supplemental Fig. 1D). To further examine the development of LMCs in vitro in the absence of *Egr2*, cultures were analyzed by flow cytometry for the presence of c-kit and IgE receptor expression. No deficit in expression of these mast cell markers was detected in *Egr2*-deficient LMCs (Supplemental Fig. 1E). These results indicate that mast cells develop normally in the absence of *Egr2*.

To investigate the role of *Egr2* in early mast cell FcεRI activation-related functions, we first assessed mast cell degranulation in vitro by measuring the release of β-hexosaminidase. *Egr2* deficiency did not alter LMC degranulation from wild-type levels, nor did it alter calcium influx (Fig. 2A, 2B). Finally, an in vivo model of passive cutaneous anaphylaxis was conducted in mast cell-deficient (W-sh) mice that had been reconstituted with wild-type

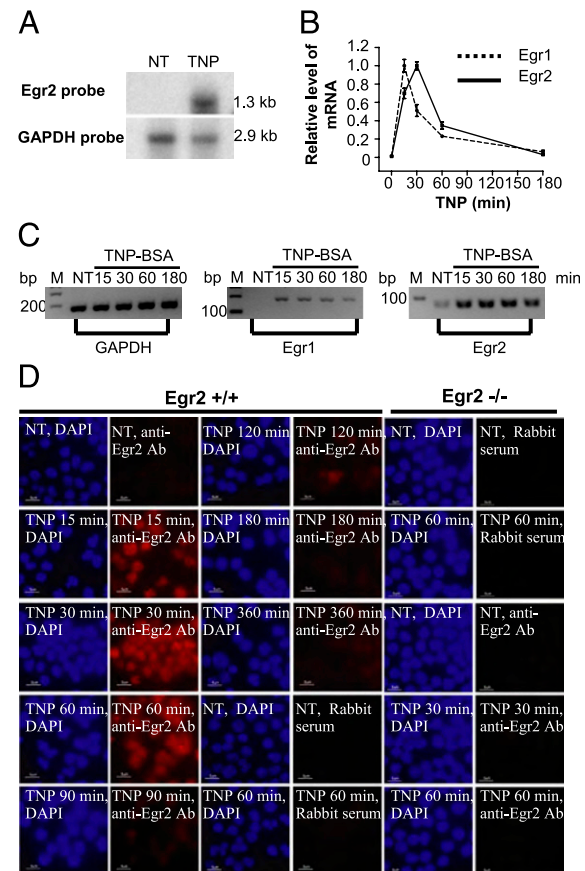


FIGURE 1. Mast cell activation via FcεRI induces transient *Egr2* expression and activity. **(A)** RNAs from untreated and FcεRI-stimulated bone marrow-derived mast cells were reverse transcribed, creating cDNAs that were blotted and hybridized with either a GAPDH probe or an *Egr2* probe. A representative Northern blot is shown. **(B and C)** RNA from murine mast cells activated via FcεRI (NT or 0, 15, 30, 60, 180 min) was used in real-time quantitative PCR. *Egr2* and *Egr1* mRNA expression was normalized to GAPDH and shown with SE ($n = 3$). PCR products were also separated on agarose gel and stained with ethidium bromide, and a representative gel is shown. **(D)** LMCs were stimulated through FcεRI by sensitizing with anti-TNP IgE and subsequently stimulating with TNP-BSA for 15, 30, 60, 90, 120, 180, and 360 min. *Egr2* protein expression was detected by immunofluorescence using an anti-*Egr2* Ab and DAPI as a nuclear stain. Immunofluorescence was also carried out using rabbit serum as the primary Ab control as well as in *Egr2*-deficient LMCs to demonstrate the specificity of the *Egr2* Ab. Cells were examined using a fluorescence microscope (Nikon E600; Nikon, Tokyo, Japan) equipped with a DMX 1200 camera; original magnification $\times 100$.

and *Egr2*-deficient LMCs. No significant difference in dye leakage into tissue was observed when mice with wild-type LMCs were compared with *Egr2*-deficient LMCs (Fig. 2C). These results indicate *Egr2* does not play a role in the early activation signals leading to mast cell degranulation in vitro or in vivo.

CCL1 production by FcεRI-activated mast cells is regulated by Egr2

Following evidence from protein array data suggesting a deficiency in CCL1 production in *Egr2*-deficient LMCs following IgE-mediated TNP-BSA stimulation, we conducted an ELISA analysis on cell-free supernatants. To examine the kinetics of cytokine and chemokine production, wild-type and *Egr2*-deficient LMCs were stimulated with TNP-BSA over a time course. *Egr2*-deficient LMCs displayed a significant defect in CCL1 production following FcεRI-mediated activation, whereas CCL3 and CCL9 remained

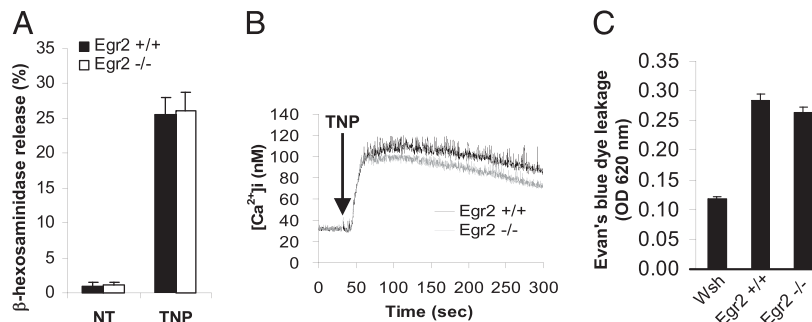


FIGURE 2. Mast cells degranulate normally in the absence of Egr2. **(A)** Wild-type and Egr2-deficient LMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA. Degranulation was assessed *in vitro* by the release of β -hexosaminidase. No difference was detected due to Egr2 deficiency in $n = 4$ experiments. **(B)** Wild-type (Egr2^{+/+}) and Egr2-deficient (Egr2^{-/-}) LMCs were stimulated with TNP-BSA for the assessment of intracellular calcium flux following activation. A representative of $n = 3$ independent experiments is shown. **(C)** Mast cell-deficient (W-sh) mice were reconstituted locally with wild-type (right) or Egr2-deficient (left) LMCs in the ear tissue by intradermal injection. Reconstituted mice were then sensitized locally with anti-dinitrophenyl IgE, and the following day were challenged by i.v. injection of dinitrophenyl-BSA in a 1% solution of Evan's blue dye for 30 min. Passive cutaneous anaphylaxis was determined by measuring dye leakage into the collected ear tissue using a spectrophotometer at 620 nm. Unreconstituted control (Wsh) data are also shown to demonstrate the inducible reaction. Data are expressed as $\pm$ SEM for $n = 6$ mice.

unaffected (Fig. 3). IL-6 and TNF production were reduced at later time points, but the impairment did not reach significance (Fig. 3). It is also notable that mast cell CCL1 production displays delayed kinetics in comparison with the other cytokines examined, consistent with a requirement for *de novo* gene expression preceding CCL1 production. These results indicate that mast cell CCL1 production is preferentially regulated by Egr2.

Mast cell activation through Fc ϵ RI results in the activation of a host of transcription factors and signaling pathways, including NF- κ B, NFAT, and MAPK, which cooperate to deliver the full mast cell response to IgE Ags (10, 23, 24); however, these pathways were not significantly affected by Egr2 deficiency in LMCs (Supplemental Fig. 2).

Egr2 binds to the promoter of the CCL1 gene following Fc ϵ RI-mediated mast cell activation

To examine the direct interaction of Egr2 on CCL1 gene transcription, a DNA probe was created using an Egr consensus-binding sequence from the promoter of CCL1 (Fig. 4A). Specific Egr2 binding to this sequence was demonstrated by EMSA using nuclear proteins from wild-type LMCs treated with TNP-BSA (Fig. 4B). Additional dsDNA EMSA probes specific to pu-

tative binding sites in the CCL1 promoter were also created and tested, but no Egr2 binding was detected at these positions (Supplemental Fig. 3A, 3C). An anti-Egr2 Ab significantly blocked the interaction of the CCL1 promoter probe with Egr2 nuclear protein in activated mast cells, and unlabeled and mutant competitor probes demonstrated the specificity of Egr binding to the probe (Fig. 4B). Finally, the Egr2-binding EMSA probe was used to characterize the kinetics of Egr2 activity on the CCL1 promoter sequence (Supplemental Fig. 3D), demonstrating a peak binding at 1 h, followed by a persistent interaction lasting at least to 6 h.

To demonstrate Egr2 binding to the endogenous CCL1 promoter in Fc ϵ RI-activated mast cells, chromatin immunoprecipitation was performed in untreated and TNP-BSA-stimulated LMCs using an Egr2 Ab and primers specific to the CCL1 promoter. Following TNP-BSA treatment, a strong induction of Egr2 binding to the

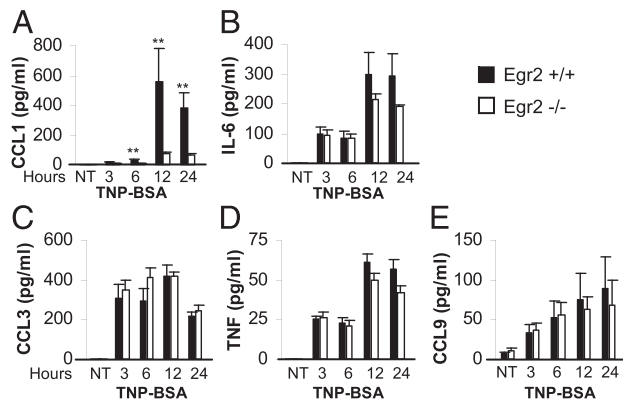


FIGURE 3. Egr2 specifically regulates CCL1 expression following mast cell activation via Fc ϵ RI. Wild-type (Egr2^{+/+}) and Egr2-deficient (Egr2^{-/-}) LMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA for various times. Cell-free supernatants were analyzed for CCL1 **(A)**, IL-6 **(B)**, CCL3 **(C)**, TNF **(D)**, and CCL9 **(E)** cytokine and chemokine content by ELISA. CCL1 production was significantly impaired in activated Egr2-deficient LMCs. Data are expressed as $\pm$ SEM for $n = 4$ experiments, $**p < 0.01$.

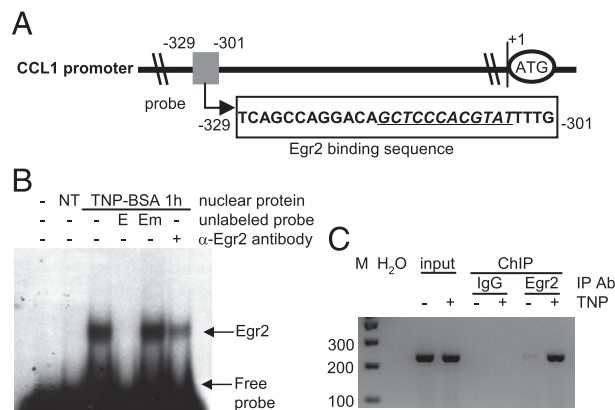


FIGURE 4. Egr2 directly interacts with the CCL1 promoter following Fc ϵ RI-mediated mast cell activation. **(A)** CCL1 gene promoter with location and sequence of Egr2-binding EMSA probe. **(B)** Nuclear proteins extracted from wild-type LMCs that had been sensitized and stimulated with TNP-BSA for 1 h or left untreated (NT) were used in EMSA with a ³²P-labeled dsDNA probe (above). Mast cell activation (TNP-BSA 1 h) induced a strong Egr2 interaction with the CCL1 promoter probe. Competition with unlabeled (E) and mutant (Em) probes was used to demonstrate specificity, and an anti-Egr2 Ab partially blocked Egr2 binding. A representative from $n = 3$ experiments is shown. **(C)** Chromatin immunoprecipitation (ChIP) was conducted using either an anti-Egr2 or IgG control Ab in LMCs left untreated or stimulated for 1 h. Immunoprecipitated (IP) chromatin was analyzed by PCR using primers specific to the CCL1 promoter. Samples from chromatin pre-IP were used as input controls. Shown is a representative gel from $n = 3$ experiments.

CCL1 promoter is detected (Fig. 4C). Additional primers with specificity in the *CCL1* promoter were also tested, but no specific *Egr2* binding at these sites was detectable (Supplemental Fig. 3A, 3B). These results indicate that *Egr2* interacts with the *CCL1* promoter following mast cell activation, suggesting that *Egr2* mediates *CCL1* production through a direct interaction.

Egr2-dependent mast cell *CCL1* production drives $CD4^+$ T cell migration in vitro

Mast cells can direct the migration of $CD4^+CD25^+$ T regulatory cells through the *CCL1*–*CCR8* axis (7, 25). To investigate the role of *Egr2*-dependent mast cell *CCL1* in T cell migration, an in vitro Transwell migration assay was conducted. Splenic $CD4^+$ T cells were isolated (Fig. 5A, 5B), and $CD4^+$ cells were then further purified into $CD25^+$ and $CD25^-$ populations. Transwell migration assays were conducted with 24-h TNP-BSA-stimulated wild-type or *Egr2*-deficient LMC-conditioned media in the lower chamber. $CD4^+CD25^+$ T cell migration was significantly impaired in response to media conditioned with *Egr2*-deficient mast cells, with 8-fold more cells migrating across the membrane in response to wild-type mast cell-conditioned media (Fig. 5C). Migration in response to mast cell-conditioned media was significantly dependent on *Egr2* until the wild-type media had been serially diluted to 12.5% (8-fold) (Fig. 5C).

To examine the contribution of mast cell-produced *CCL1* in $CD4^+CD25^+$ T cell migration, we added either specific Abs to *CCL1* or *CCL9*, or isotype control Abs to wild-type mast cell-conditioned media in our Transwell migration assay. Interestingly, *CCL1* Ab significantly reduced T cell migration to levels con-

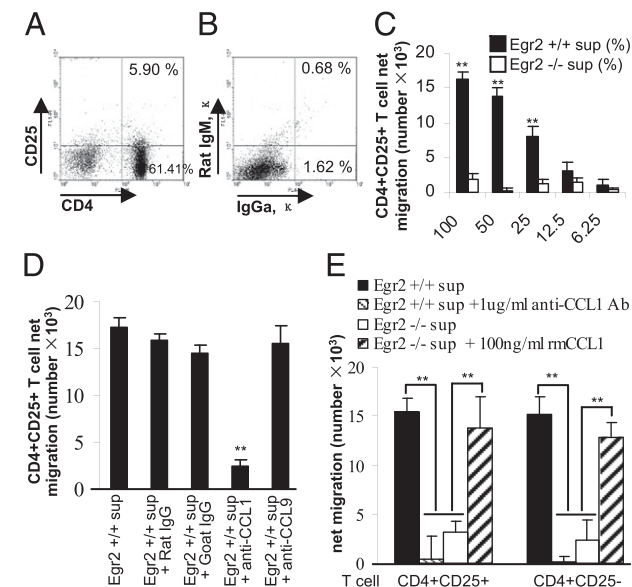


FIGURE 5. Mast cell *Egr2*-dependent *CCL1* production regulates $CD4^+$ T cell migration in vitro. **(A)** and **(B)** Flow cytometry analysis of $CD4^+$ $CD25^+$ T cells isolated from wild-type murine spleen tissue. Isotype control Abs were used to demonstrate specificity. **(C)** Supernatants from wild-type (*Egr2*^{+/+}) and *Egr2*-deficient LMCs were prepared following 24 h of TNP-BSA stimulation. Transwell migration (from upper to lower chamber) of $CD4^+CD25^+$ T cells was analyzed in response to LMC supernatants and titrated in a 2-fold serial dilution. **(D)** Transwell migration assays were conducted in response to wild-type LMC-conditioned media with either anti-*CCL1* or anti-*CCL9* Abs. Only anti-*CCL1* Ab blocked $CD4^+$ T cell migration. **(E)** Impaired $CD4^+$ T cell migration in response to *Egr2*-conditioned media is restored by adding r*CCL1* protein to *Egr2*^{-/-} LMC supernatants. Data are expressed as $\pm$ SEM for $n = 4$ independent experiments, $^{**}p < 0.01$.

sistent with that of *Egr2*-deficient mast cell media shown in Fig. 5C, whereas anti-*CCL9* and isotype control Abs had no blocking effect on T cell migration (Fig. 5D). Finally, T cell migration assays were conducted using *Egr2*-deficient mast cell-conditioned media with purified r*CCL1* protein added at a final concentration of 100 ng/ml. Reconstitution of *Egr2*-deficient mast cell media with *CCL1* restored the migration of $CD4^+CD25^+$ and $CD4^+$ $CD25^-$ T cells to that detected in response to wild-type mast cells (Fig. 5E). These results indicate that in vitro, mast cell-dependent

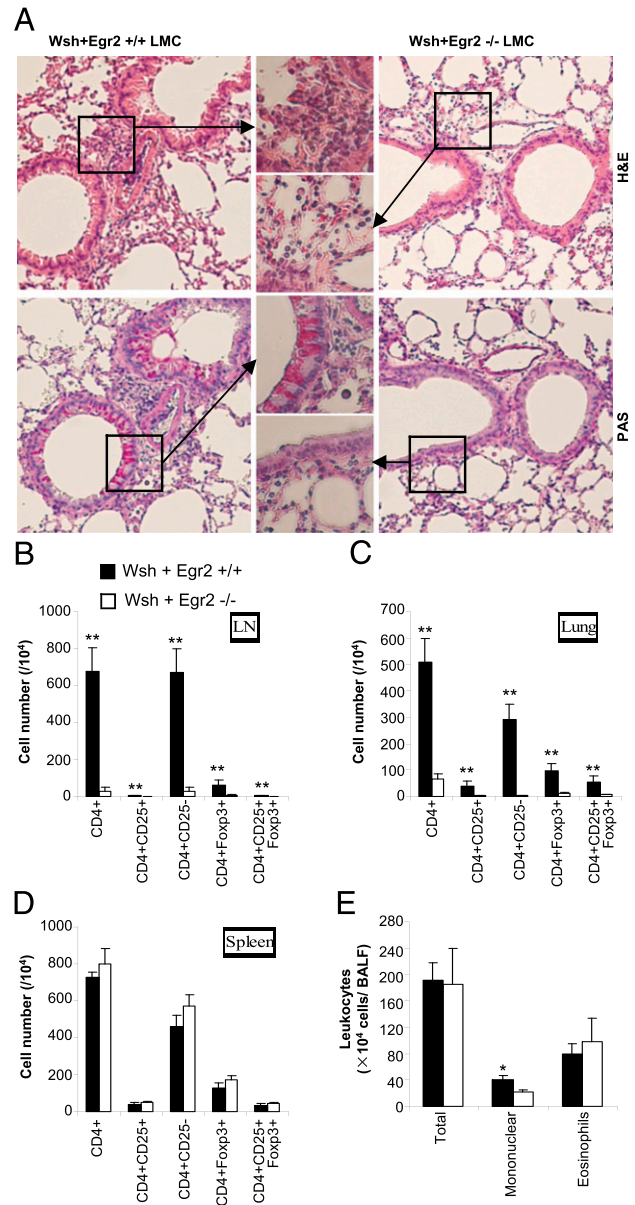


FIGURE 6. $CD4^+$ T cell migration is regulated by *Egr2*-dependent mast cell *CCL1* in an in vivo model of asthma. **(A)** Six weeks following LMC reconstitution, W-sh mice were sensitized and later challenged with intranasal OVA. Lung tissue was collected and stained with H&E and periodic acid–Schiff stain to examine OVA-induced cellular infiltration. **(B–D)** Draining lymph nodes, lung, and spleen tissue were collected and analyzed by flow cytometry for subpopulations of $CD4^+$ T cells. Data are expressed as $\pm$ SEM for $n = 7$ mice, $^{**}p < 0.01$. **(E)** The total number of cells from BALF was counted, and slides were prepared and stained with Diff-Quik to assess the number of mononuclear cells and eosinophils as a percentage of total cells in 10 fields for $n = 4$ wild-type and $n = 6$ *Egr2*^{-/-} reconstituted mice; data are expressed as $\pm$ SEM. $^{*}p < 0.05$.

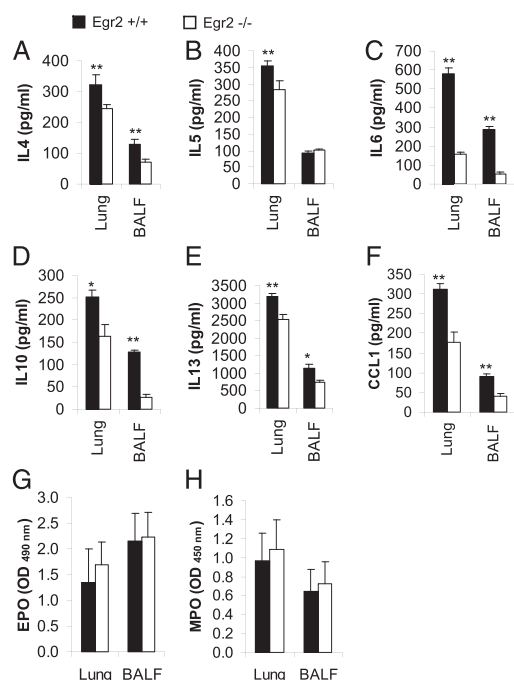


FIGURE 7. Mast cell Egr2 is critical in directing Th2 cytokine production in the lung during allergic asthma, but is not required for eosinophilia. (A–F) W-sh mice reconstituted with either wild-type or Egr2-deficient LMCs were used in a model of asthma. The production of Th2 cytokines IL-4, IL-5, IL-6, IL-10, IL-13, and CCL1 in the lung and BALF was assessed by ELISA. * $p < 0.05$, ** $p < 0.01$. (G and H) Lung tissue from Egr2^{+/+} and Egr2^{-/-} mast cell-reconstituted mice from the same asthma model was used to measure the activity of eosinophil peroxidase (EPO), a marker of eosinophil infiltration, and myeloperoxidase (MPO) activity, a marker of neutrophil infiltration. Data are expressed as $\pm$ SEM, $n = 6$.

CD4⁺ T cell migration through the CCL1–CCR8 axis is specifically controlled by Egr2-dependent mast cell production of CCL1.

Egr2-dependent mast cell CCL1 regulates CD4⁺ T cell migration in allergic asthma

To evaluate the significance of Egr2-dependent mast cell CCL1 production on T cell migration in vivo, an OVA-induced model of asthma was conducted in mast cell-deficient W-sh mice that had been reconstituted with either wild-type or Egr2-deficient LMCs. Mast cell-deficient mice reconstituted with Egr2-deficient LMCs displayed a deficit in immune cell infiltration into the lung and restricted goblet cell hyperplasia compared with mice reconstituted with wild-type control LMCs (Fig. 6A). To evaluate the degree of mast cell reconstitution by wild-type and Egr2-deficient LMCs, we stained sectioned lung tissues and evaluated skin and lung tissues by flow cytometry for mast cell content and found no discrepancy between groups (Supplemental Fig. 4). These mice were then evaluated for CD4⁺ T cell subsets, including the T

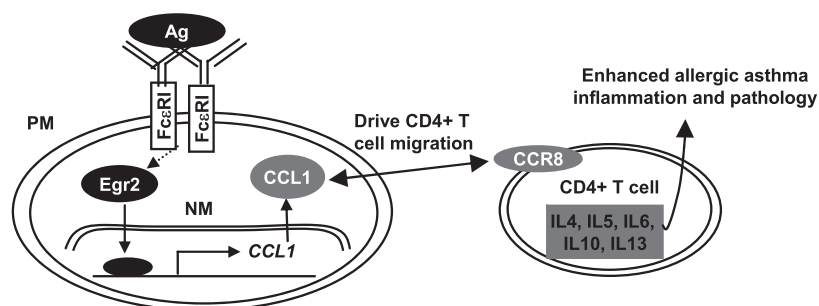
regulatory-dependent transcription factor Foxp3, at the draining lymph node, lung, and spleen by flow cytometry. A significant deficit in all CD4⁺ T cell subsets analyzed (CD25⁺–Foxp3⁺) was detected at the draining lymph node and in the lung, whereas cell numbers at the spleen remained unaffected (Fig. 6B–D). The cellular content of the BALF was also assessed revealing an Egr2-dependent defect in mononuclear cells, whereas eosinophil numbers were not impaired (Fig. 6E). Collectively, these data suggest a defective CCL1–CCR8 migratory axis in vivo. Lung tissue was then further analyzed for Th2 cytokine levels, and significant defects in IL-4, IL-6, IL-10, IL-13, and CCL1 were detected in lung and BALF as well as a defect in IL-5 levels in the lung (Fig. 7A–F), consistent with reduced CD4⁺ T cells in the lung. Airway hyperresponsiveness was also assessed in these OVA-challenged mice, but no significant difference was detected with airway hyperresponsiveness reaching 5.01 ± 0.48 - and 6.09 ± 0.54 -fold change over baseline at 50 mg/ml methacholine challenge for mice reconstituted with wild-type and Egr2-deficient LMCs, respectively ($n = 6$). Finally, eosinophil and neutrophil infiltration was evaluated by measuring the activity of eosinophil peroxidase and myeloperoxidase, respectively, in the lung tissue of these mice, and no significant defect was detected (Fig. 7G, 7H). Collectively, these results suggest that Egr2 specifically directs mast cell production of CCL1 and that this mechanism is critical for CD4⁺ T cell homing in the cellular response to allergic inflammation (Fig. 8).

Discussion

Previous work has demonstrated the fundamental importance of de novo gene expression as a mechanism for mast cell responses to IgE-mediated FcεRI aggregation. When mast cells are treated with the general protein synthesis inhibitor, cyclohexamide, prior to activation, related mast cell secretory responses are significantly impaired, indicating that newly synthesized proteins are critical in the production of mast cell mediators (26). More recently, we have described the significance of de novo expressed Egr1 in the production of several key cytokines (18). In the study presented in this work, we demonstrate a novel role for the de novo expression of the transcription factor Egr2 in specifically directing the production of the critically important chemokine CCL1 following FcεRI-mediated mast cell activation. We further demonstrate that CD4⁺ T cell migration to sites of allergen-activated mast cells through the CCL1–CCR8 axis is significantly dependent on mast cell Egr2 expression (Fig. 8).

The Egr family of transcription factors functions in directing transcriptional events for a broad array of cell type- and signaling context-specific events, including differentiation, proliferation, and response to extracellular signals. Target gene promoters include the vascular endothelial and platelet-derived growth factors (27, 28), as well as regulators of hematopoietic differentiation (29, 30) and neuronal development (31). In this work, we demonstrate

FIGURE 8. Mast cell FcεRI-induced Egr2 regulates CCL1 production in the asthmatic lung, driving CD4⁺ T cell migration, and enhancing Th2 cytokines and inflammatory pathology. Egr2 represents a novel FcεRI-induced transcription factor in mast cells and specifically regulates mast cell CCL1-dependent CD4⁺ T cell migration via the CCL1–CCR8 axis, resulting in enhanced Th2 cytokine production and allergic pathology in the inflamed lung tissue. NM, Nuclear membrane; PM, plasma membrane.



a novel finding, that *CCL1* is a target of Egr2 in FcεRI-activated mast cells, and, upon induction, Egr2 can directly bind to the promoter of *CCL1* and drive gene expression. Egr2 deficiency results in a specific and significant defect in mast cell CCL1 production with functional consequences for chemotactic responses to allergic inflammation. Notably, a previous microarray analysis of TCR-induced genes supporting T cell anergy listed both *Egr2* and *CCL1* as upregulated, although no direct association was identified (32). Although further study is required, this finding may suggest that Egr2 can regulate CCL1 expression in other cell type-specific contexts, including promotion of T cell anergy, raising the possibility of Egr2 as a critical regulator of CCL1 expression in a broader scope of cellular functions.

Our results demonstrate that the FcεRI-induced transcription factor Egr2 can dictate mast cell-dependent CD4⁺ T cell migration. In a model of allergic asthma, we demonstrated that mast cell Egr2 is required for the recruitment of CD4⁺ T cells in vivo. Although splenic cell numbers were unaffected, CD4⁺ T cell numbers at the draining lymph node and local site of inflammation (lung) were dramatically impaired across all subsets examined, indicating that mast cell Egr2 is required to direct CD4⁺ T cell migration during allergic inflammatory events in the lung. Consistent with this, Th2 cytokine levels in the lungs of asthmatic mice were significantly impaired, demonstrating that this mast cell Egr2-dependent defect in CD4⁺ T cell recruitment affects the cytokine milieu in the allergically inflamed lung. Indeed, CD4⁺ T cells are significant contributors in inflammatory processes in the lung mucosa in asthma (33, 34), suggesting that mast cell Egr2 is a key regulator of the asthmatic phenotype. However, our results show a more marked impairment of CD4⁺ T cell recruitment than the detected impairment in Th2 cytokines in the lung, suggesting that cell types other than CD4⁺ T cells are contributing to the Th2 cytokine milieu in the asthmatic lung. Indeed, reports have shown that mast cells, eosinophils, and other types of T cells, including CD8⁺, NKT, and γδT cells, can contribute to the cytokine milieu in the lung during asthma (35). In addition, a series of work recently characterized a new population of innate helper cells that have been shown to significantly contribute to the cytokine milieu in Th2-driven responses (36, 37). Our results further support a role for these other cell types contributing to the total levels of Th2 cytokines in the lung, yet not to the level attainable when CD4⁺ T cells also contribute.

Mast cells are an abundant source of CCL1 following FcεRI-mediated activation and are critically important in the chemotactic recruitment of CCR8-expressing CD4⁺ T cells (25, 38), a relationship central in the development of asthmatic mucosal inflammation in the lung (7). We have detected a deficiency of CCL1 in the lungs of asthmatic mice with Egr2-deficient mast cells (Fig. 7F). It is notable, however, that, like Th2 cytokines, the defect in total CCL1 in the lung is less severe when compared with the defect in CD4⁺ T cell recruitment (Fig. 6C, 6E), suggesting a possible threshold level of CCL1 being required to initiate significant CD4⁺ T cell recruitment, and that this threshold may be represented in the difference in CCL1 levels we see between the wild-type and Egr2-deficient mast cell-reconstituted mice in our model. Interestingly, the CCL1–CCR8 axis not only regulates allergic inflammation in the lung (7), but is also critical in mast cell-dependent recruitment of T cells to sites of atopic skin inflammation (8), suggesting a likely role for Egr2 in the pathology of an additional IgE-directed disease. Notably, a recent study examined the significance of Egr2 in CD4⁺ T cell immune responses and demonstrated that Egr2 was not required for normal CD4⁺ T cell function, distinctly highlighting the cell type specificity of Egr2 in the mast cell–CD4⁺ T cell relationship (39).

The direct regulatory role of Egr2 on the CCL1–CCR8 axis identified in this work indicates that Egr2 may indeed be an attractive target for the development of novel therapeutics for the treatment of allergic inflammatory conditions such as asthma and atopic dermatitis. CCL1 expression is known to be elevated in the lung of asthmatic patients (40). Expression of the CCL1 receptor, CCR8, is elevated in asthma (41), and small molecule inhibitors of CCR8 inhibit human T cell migration (25), signifying that the reciprocal blockade of CCL1 production should impair human CD4⁺ T cell migration. The CCL1–CCR8 axis is also relevant in other diseases, including multiple sclerosis and cancer, yet a link to Egr2 remains unclear (42, 43). Interestingly, however, Egr2 is required for the expression of a myriad of myelinating genes in neurons (31), and mutations in Egr2 are associated with Charcot-Marie-Tooth disease, a condition in which impaired Egr2 DNA binding results in nerve demyelination (44), bearing phenotypic similarities to multiple sclerosis, although seemingly mechanistically distinct.

In summary, we have identified Egr2 as a novel *de novo* expressed transcription factor actively binding to the promoter of *CCL1* following FcεRI-mediated mast cell activation. Egr2 is required for CCL1 production by mast cells and directly regulates the CCL1–CCR8 mast cell–CD4⁺ T cell migratory axis in allergic inflammation. Our results define a novel function for Egr2 in the regulation of CCL1 expression that may have context beyond allergy and asthma.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure Legends

Figure S1. Egr2 activity is induced following FcεRI aggregation on mast cells, but Egr2 is not required for LMC development *in vitro*. **(A)** Nuclear proteins were isolated from wild-type LMCs that were sensitized and stimulated with TNP-BSA for 1 hour or left untreated (NT). An electrophoretic mobility shift assay (EMSA) was used to evaluate specific Egr2 activity by antibody-blocking of Egr activity using increasing quantities of anti-Egr2 antibody with or without a fixed quantity of anti-Egr1 antibody. **(B)** Densitometry analysis was used to quantify Egr activity over three independent experiments ($n = 3$) and expressed as $\pm$ SEM, * indicates $p < 0.05$ compared with the control (no antibody added). **Further description:** To evaluate the DNA-binding activity of Egr2 in LMCs activated through FcεRI a ^{32} P-labelled DNA probe with specificity for the Egr family of transcription factors was used in an electrophoretic mobility shift assay (EMSA). Following activation with TNP-BSA, a strong activation of the Egr family is detected. In mast cells, we have previously shown that Egr activity is mediated by Egr1 and Egr2. To evaluate the contribution of Egr2 in our EMSA analysis, we used antibodies to block Egr2 probe binding, either alone, or in the presence of Egr1 antibody blocking, to further enhance the specific detection of Egr2 in the assay. Nuclear proteins from wild-type LMCs stimulated with TNP-BSA for one hour were pre-treated with anti-Egr2 antibody in increasing quantity in presence or absence of a fixed quantity of Egr1 antibody prior to EMSA analysis. Egr2 antibody partially blocked the binding of nuclear Egr2 protein to the labelled DNA probe and co-incubation with Egr1 antibody enhanced Egr2 antibody blocking. A representative EMSA, including an untreated sample (NT), is shown and the results from 3 independent experiments are quantified by densitometry. These results demonstrate that Egr2 DNA-binding is specifically active in FcεRI-stimulated mast cells and that Egr2 makes a significant contribution to global Egr activity in the nucleus following mast cell activation. **(C)** PCR genotyping of LMC cultures. A representative is shown for wild-type (Egr2^{+/+}), heterozygous (+/-), and Egr2-deficient (-/-) cultures. Primers and reaction conditions were as previously described (Taillebourg et al., 2002). **(D)** Egr2^{+/+} and Egr2^{-/-} LMC cultures were analyzed by toluidine blue staining following 5 weeks of maturation in conditioned media, original magnification $\times 100$. **(E)** LMCs were sensitized with IgE and analyzed by flow cytometry for surface receptor-bound IgE and c-kit, markers of mature mast cells.

Figure S2. Egr2 is not required for NF- κ B, NFAT, or MAPK activity in Fc ϵ RI-activated mast cells. (A) Wild-type and Egr2-deficient LMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA over a time course. Nuclear proteins were extracted and analyzed for transcription factor activity by EMSA. A representative result is shown and densitometry analysis was used to quantify NF- κ B and NFAT activation for $n = 3$ independent experiments expressed as $\pm$ SEM. (B) Competition assays were performed using 50 $\times$ unlabelled and mutant probes or by pre-incubating with antibodies for NF- κ B (p50 and p65) and NFAT (c1 and c2) to block protein-probe interaction and demonstrate specificity of the probes. Shown is a representative of $n = 2$ experiments. NF- κ B probe, 5'-TTATCAAATGTGGGATTTCCCAT-3' (IL-6 promoter) and mutant probe, 5'-TTATCAAATGTGTTACCTTCCCAT-3'; NFAT probe, 5'-AAGGTGTTTCCCCAAGCCTTCCC -3' (IL-13 promoter) and mutant probe, 5'-AAGGTGTCCATCCAAGCCTCCATC-3'. (C) Egr2^{+/+} and ^{-/-} LMCs were sensitized and stimulated with TNP-BSA over a time course. LMCs were lysed in RIPA buffer supplemented with a cocktail of protease and phosphatase inhibitors. Cleared lysates (30 μ g protein) were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% non-fat milk powder, probed with primary and secondary antibodies, and detected by an enhanced chemiluminescence detection system (Western Lightning Plus-ECL; PerkinElmer) on BioMax film (Kodak). Total protein was extracted and analyzed by Western blotting for phospho- and total MAPKs (p38, JNK, and ERK) as well as I κ B α . No significant difference was detected in activation of these signalling pathways following TNP-BSA stimulation. A representative of $n = 3$ blots is shown. Antibodies to phospho-JNK (Thr 183/Tyr 185), JNK, phospho-p38 MAPK (Thr 180/Tyr 182), phospho-p44/42 MAPK (ERK1/2), p44/42 MAPK, phospho-I κ B α (Ser32), I κ B α , phospho-Gsk3 β (Ser 9), and Gsk3 β were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies to p38 MAPK, actin, and HRP-linked secondary Abs were from Santa Cruz Biotechnology.

Figure S3. Analysis of additional putative Egr2 binding sites in the promoter of *CCL1* in activated mast cells and kinetics of Egr2 promoter binding. (A) Diagram of the *CCL1* promoter with locations and sequences of three probes tested in EMSA and locations of primer tested in ChIP. (B) Representative agarose gels for negative Egr2 binding sites in the *CCL1*

promoter in FcεRI-activated (TNP-BSA) LMCs. **(C)** Representative EMSA images showing Egr2-binding ability to each of three probes. Only probe 1 was able to bind to activated Egr2 protein. **(D)** IgE-sensitized wild-type LMCs were stimulated with TNP-BSA over a time course to assess the timing of activated Egr2 binding on the promoter of *CCL1*. A representative image is shown (BL = blank). Peak Egr2-binding was reached at 1 hour and persisted at a lower level up to 6 hours.

Figure S4. Assessment of wild-type and Egr2-deficient LMC reconstitution of Wsh mice.

(A) Control (Wsh) and both Egr2^{+/+} and Egr2^{-/-} reconstituted lung tissue was fixed in Carnoy's for 24 hrs, followed 100% ethanol treatment and paraffin-embedding for sectioning. Sectioned specimens were then rehydrated, treated with 0.1 M HCl and stained with 1% Alcian blue (Sigma) overnight. The next day specimens were treated with 0.1 M HCl again and stained with 1% Safranin O (Sigma) for 20 mins followed by rinsing in tap water and mounting in DPX neutral mounting medium (Sigma). Specimens were examined on a Nikon E600 microscope equipped with a 100× objective lens and a DMX 1200 camera (Nikon, Tokyo, Japan). Mast cells are indicated with arrows. Images are representative of *n* = 5 for each group of reconstituted mice and *n* = 4 Wsh control mice. **(B)** Ear, tongue, and lung tissue was collected from Wsh mice reconstituted with either wild-type (+/+) or Egr2-deficient (-/-) LMCs and single cell suspensions were created by dissociating the tissue with Collagenase II and IV (Invitrogen) treatment. Cells were then stained with FITC-conjugated rat anti-mouse c-kit/CD117 mAb (IgG2a) or isotype control FITC-rat IgG2a (Cedarlane) and PE-conjugated Armenian hamster anti-mouse FcεRIα Ab or PE-Armenian hamster IgG (eBioscience) to quantify mast cell numbers. Data are expressed as mean cell number for *n* = 5 mice ±SEM.

Fig. S1

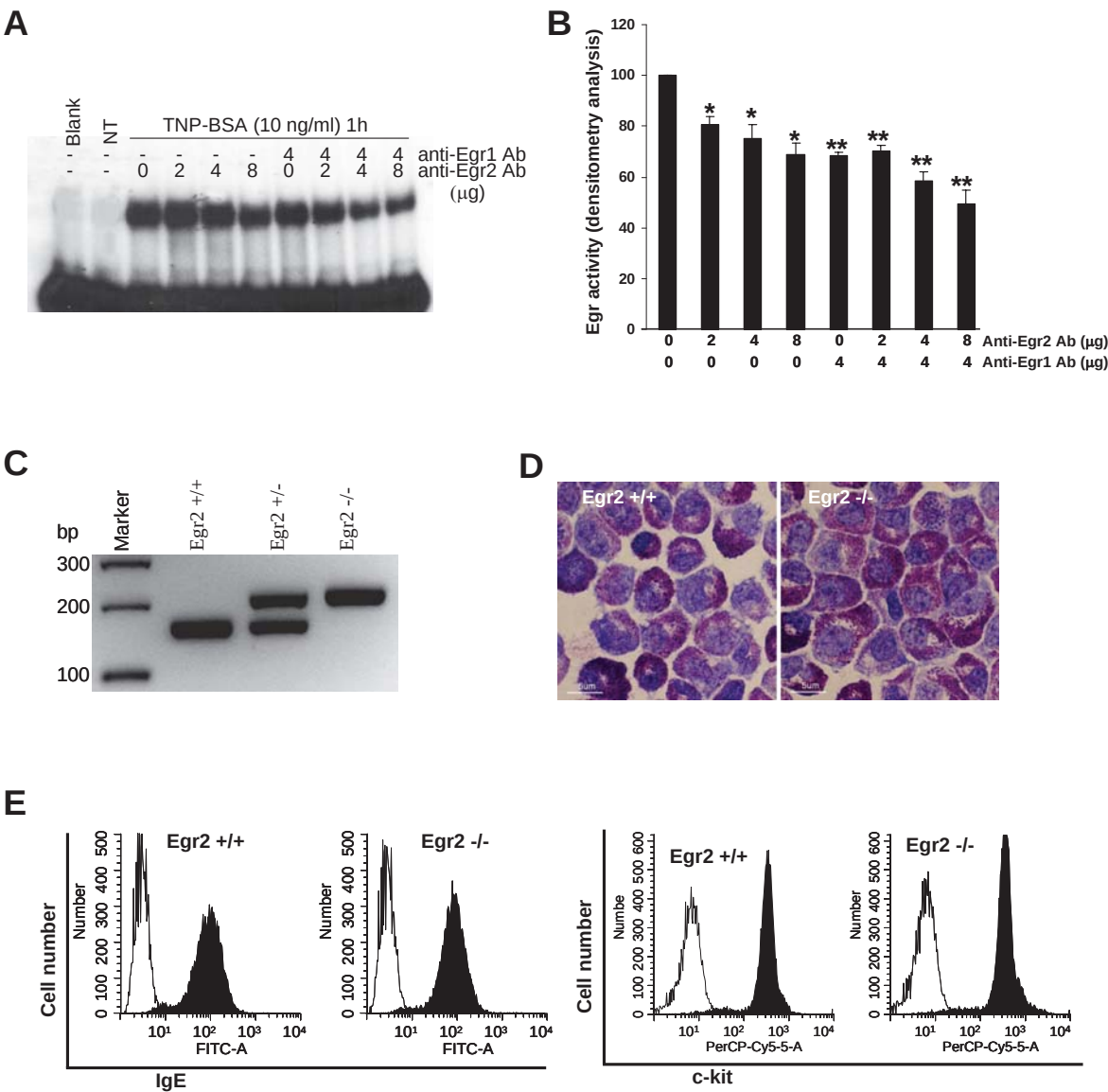


Fig. S2

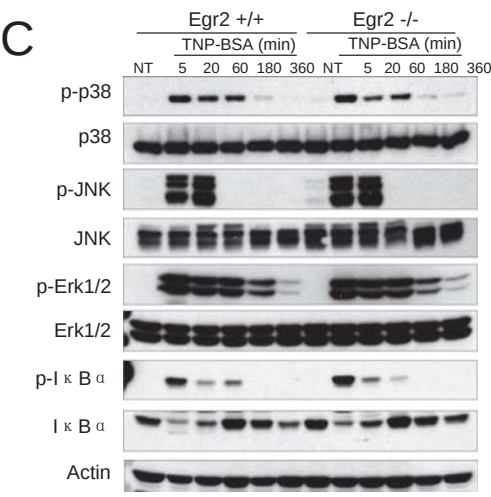
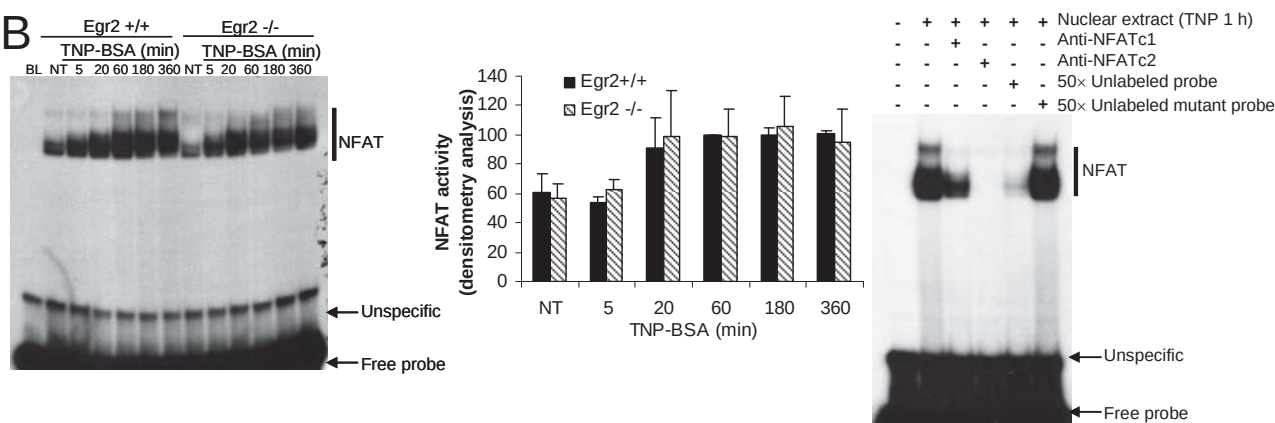
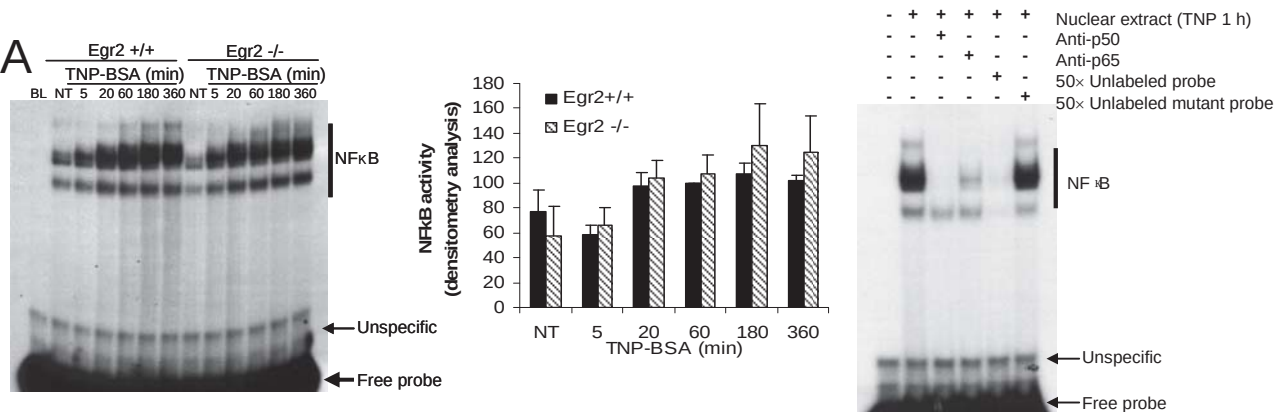


Fig. S3

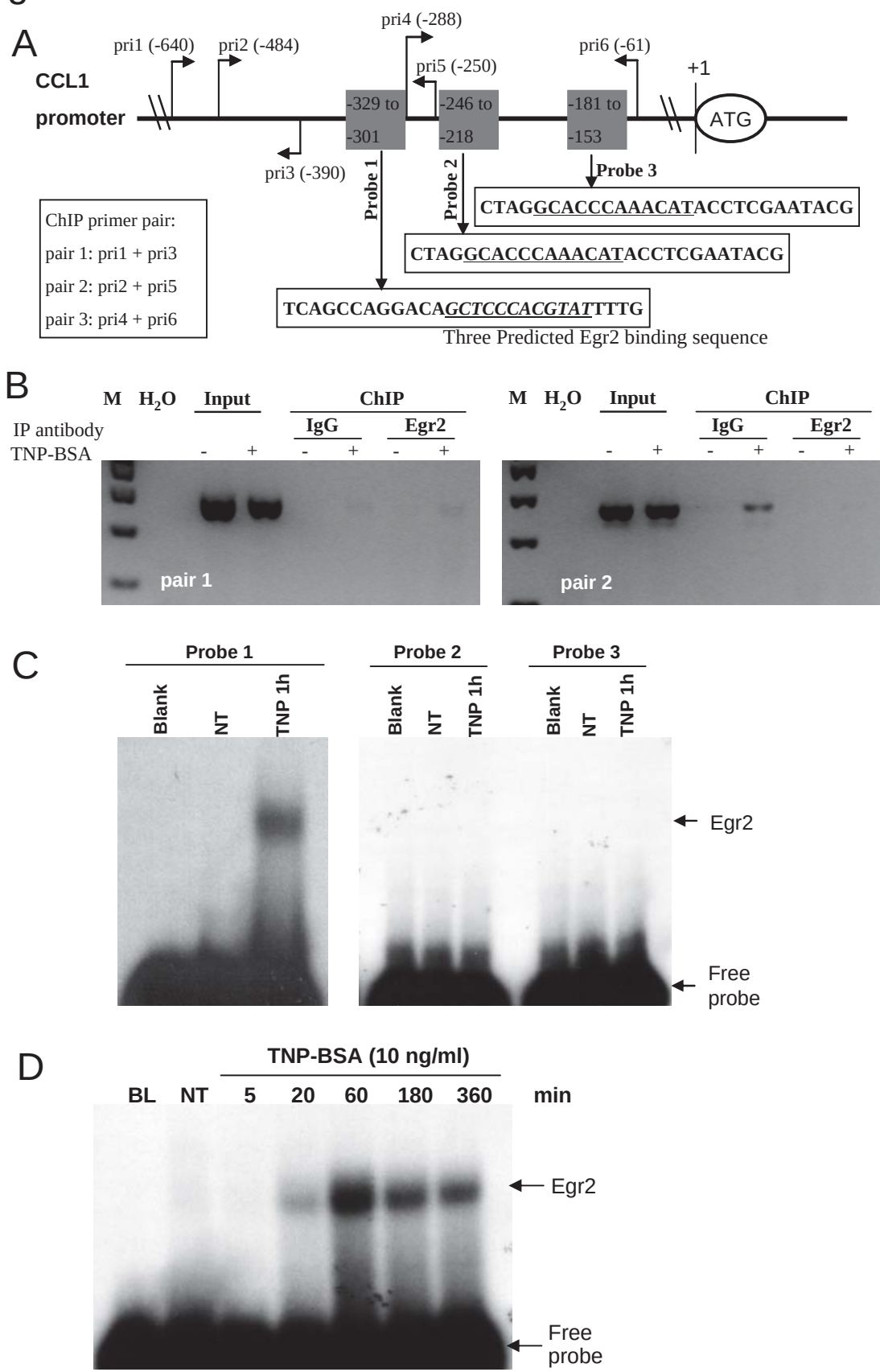
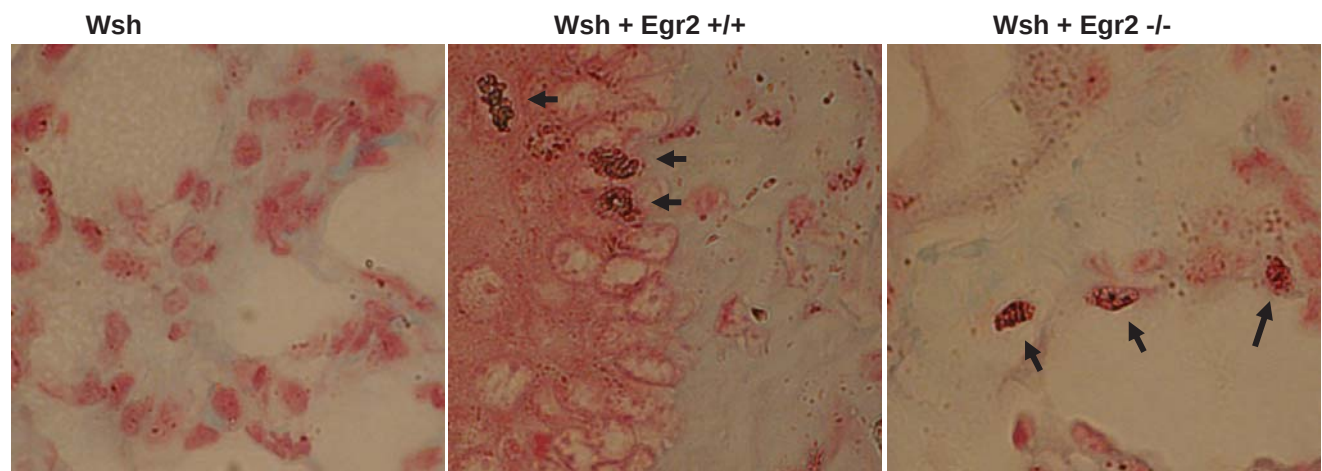


Fig. S4

A



B

