Preferential Signaling and Induction of Allergy-promoting Lymphokines Upon Weak Stimulation of the High Affinity IgE Receptor on Mast Cells

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Abstract

Mast cell degranulation and de novo cytokine production is a consequence of antigen-aggregation of the immunoglobulin E (IgE)-occupied high affinity receptor for IgE (FceRI). Herein, we report that lymphokines that promote allergic inflammation, like MCP-1, were potently induced at low antigen (Ag) concentrations or at low receptor occupancy with IgE whereas some that down-regulate this response, like interleukin (IL)-10, required high receptor occupancy. Weak stimulation of mast cells caused minimal degranulation whereas a half-maximal secretory response was observed for chemokines and, with the exception of TNF-α, a weaker cytokine secretory response was observed. The medium from weakly stimulated mast cells elicited a monocyte/macrophage chemotactic response similar to that observed at high receptor occupancy. Weak stimulation also favored the phosphorylation of Gab2 and p38MAPK, while LAT and ERK2 phosphorylation was induced by a stronger stimulus. Gab2-deficient mast cells were severely impaired in chemokine mRNA induction whereas LAT-deficient mast cells showed a more pronounced defect in cytokines. These findings demonstrate that perturbation of small numbers of IgE receptors on mast cells favors certain signals that contribute to a lymphokine response that can mediate allergic inflammation.

Key words: cytokines • Gab2 • IgE • LAT • mast cells

Introduction

Mast cells function in innate and acquired immunity (1, 2) and likely through evolutionary adaptation have acquired a role in the allergies (3). These cells perform their physiological role by responding to a diverse array of challenges through various receptors expressed on their surface and by releasing a variety of compounds that enhance an immune response. Central to allergic disease is the expression of the high affinity Fc receptor for IgE (FceRI) on mast cells (4). This receptor becomes occupied by allergen-specific IgE primarily produced in response to T cell-dependent activation of B cells. Aggregation of IgE-occupied FceRI by a specific allergen causes the production of mRNA or protein of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, TGF-β, GM-CSF, IFN-γ, monocyte chemoattractant protein 1 (MCP-1),* macrophage inflammatory protein 1α (MIP1α), MIP1β, TCA3, LIF, ET-1, MARC, and others (5, 6).

*Abbreviations used in this paper: BMMC, bone marrow–derived mast cell; MCP-1, monocyte chemoattractant protein 1; MIP1α, macrophage inflammatory protein 1α; RPA, ribonuclease protection assay.
Heterogeneity in cytokines produced by different mast cell populations is a well-documented phenomenon. Purified mouse serosal mast cells were shown to produce IL-3, IL-6, TGF-B1, and TNF-α (7–9) but a similar population of rat mast cells produced only IL-6 and TNF-α (10, 11). These differences in the profile of produced cytokines can also be seen in vitro by modifying culture conditions. For example, addition of stem cell factor (c-KIT ligand) to a bone marrow–derived mast cell (BMMC) causes selective expression of IL-12 but not IL-4. In contrast, BMMC cultivated in the presence of only IL-3 were reported to contain mRNA for IL-4 but not for IL-12 (12). This heterogeneity of cytokine gene expression seemingly extends to human tryptase+ chymase− mast cells (MC negatively regulated) and tryptase+ chymase+ mast cells (MC positively regulated) as IL-5 and IL-6 were restricted to MC negatively regulated from allergic rhinitis patients whereas MC positively regulated preferentially expressed IL-4 and minimal IL-5 or IL-6 (13, 14). Furthermore, whereas 90% of purified human foreskin mast cells are positive for IL-4, only a small number of these cells showed immunoreactivity for IL-5 and IL-6 (14).

This plasticity in mast cell phenotype makes the mast cell a particularly useful effector cell, capable of responding appropriately to changes in its microenvironment, in both innate and acquired immunity (1, 2). However, this characteristic also makes it difficult to gain an understanding of the underlying mechanisms governing a mast cells lymphokine response to a given stimulus, as generalization of the observations is not possible given that the influence of the microenvironment may not be known. In the current study we sought to address whether an environmentally homogeneous population of mast cells can differentially express cytokine and/or chemokine genes as a result of an IgE-mediated challenge. Specifically, we sought to answer whether differences in the Ag concentrations or the extent of FcεRI occupancy with antigen-specific IgE could cause differences in mast cell responses. The present study shows that mast cells’ chemokine and cytokine responses, to a given Ag, can differ depending on the antigen concentration or occupancy of FcεRI. This reveals a new regulatory mechanism where a weak mast cell stimulus elicits a Th2-like lymphokine response that supports allergic inflammation.

Materials and Methods

Reagents and Antibodies and Radiolabeling. Dinitrophenyl (DNP)-specific mouse IgE was produced as described previously (15). Rabbit antibodies to ERK2, JNK1, and p38MAPK were from Santa Cruz Biotechnology, Inc., mouse anti-phospho-ERK2, rabbit anti-phospho-JNK, and rabbit anti-phospho-p38MAPK were from Cell Signaling Technologies. Rabbit anti-phospho-Akt (Ser472/473/474) was from BD Biosciences. Secondary antibodies used for immunoblotting were: sheep anti–mouse IgG horseradish peroxidase (HRP) and donkey anti–rabbit IgG-HRP (Amersham Biosciences). DNP-human serum albumin (DNP-HSA, Ag), monovalent hapten DNP-Iysine (Hap), murine thrombin, and ATP were from Sigma-Aldrich. The MEK1 inhibitor, PD 98059, and the p38MAPK inhibitor, SB202190, were from Calbiochem. Iodination of IgE was as described with the exception that an Iodo-Gen precoated tube (Pierce Chemical Co.) was used. The concentration and binding efficiency of the iodinated IgE was determined as described previously (16).

Bone Marrow Isolation and BMMC Culture. Animals were maintained and used in accordance with National Institutes of Health (NIH) guidelines. Bone marrow was isolated from 8-wk-old SV129 × C57/b6 wild-type and gene–disrupted mice as described (17, 18). LAT-null mice were kindly provided by L.E. Samelson (NCI, NIH). Cell responses of the genetically-altered mice were compared with age and sex matched wild type litter mates. BMMCs were grown in RPMI media supplemented with FBS and IL-3 as described (17, 18). Because IL-3 induces cytokine mRNA responses, cells were removed from IL-3 for 4 h before stimulation.

Receptor Occupancy and Hapten Experiments. BMMCs (1 × 10⁶ cells/ml) were incubated in IL-3 free media containing 0.5 μg of IgE/ml (sensitization) during 4 h at 37°C; conditions that result in 100% receptor occupancy. To achieve varying degrees of receptor occupancy the iodinated-DNP-specific mouse IgE was mixed at varying ratios with unlabeled rat IgE of unknown specificity. The degree of FcεRI occupancy with DNP-specific IgE was determined by direct calculation of the cell surface bound [125I]-DNP-specific IgE after removal of the unbound iodinated-IgE by centrifugation through phthalate oils as described (19). The remaining cells were washed twice by centrifugation (300 g, 10 min) to remove the unbound IgE, stimulated with Ag as described below and the extent of degranulation, cytokine mRNA levels and protein secretion, and MAP kinase activation was determined.

To analyze the effect of hapten on cytokine and chemokine mRNA expression, BMMCs were sensitized as above with saturating amounts of unlabeled DNP–specific IgE and washed twice in Tyrodes/BSA (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, and 5.6 mM glucose containing 0.05% BSA) at 37°C. Cells were resuspended in the same buffer at 2 × 10⁶/ml and three aliquots (1 ml) were transferred to tubes containing: (a) 20 μl Tyrodes/BSA, or (b) 20 μl of Hap (2.5 mM) or (c) 20 μl of Hap (2.5 mM) + 30 ng Ag and incubated at 37°C. The remaining cells were also stimulated with 30 ng/ml of Ag at 37°C and 1 ml aliquots were placed in three tubes (one at 37°C, one on ice, and other at 37°C with 10 μl of Hap, 5 μM) at the indicated times. Incubation continued for 60 min and hexosaminidase release was then determined. Cell pellets were used for total RNA isolation.

Lysates, Immunoblots, Akt, MAP Kinase Analysis. For preparation of cell lysates, IgE-sensitized BMMC (7 × 10⁶) were stimulated for various times or with different concentrations of Ag in a final volume of 250 μl. The reaction was stopped by the addition of 250 μl of boiling SDS sample buffer. For MAP kinase experiments using hapten competition, 100 ng/ml of DNP–HSA was added at t = 0, hapten (50 μM final) was added at the indicated times and incubated for 10 min at 37°C before stopping the reaction with SDS sample buffer. Immunoblotting conditions were previously described (20, 21). Akt or MAP kinase phosphorylation was determined by phoshospecific antibodies. Relative quantitation of immunoblots was performed by densitometry. To determine the effect of MAP kinase inhibitors, PD98058 (10 μM, MEK1 and thus ERK2 selective) or SB202190 (5 μM, p38MAPK selective) was added to 5 × 10⁶ cells/ml for 15 min at 37°C before Ag stimulation (100 ng/ml) for 1 h at 37°C. Cells were centrifuged and the pellet was used to isolate total RNA.
Hexosaminidase, Chemotaxis, and Cytokine Assays. Degranulation was measured by assaying B-hexosaminidase release as described (18). To measure chemotaxis, mast cells were loaded with different amounts of [125]Iodinated-DNP-specific IgE as described above. Thirty million cells were resuspended in 1 ml of Tyrodes/BSA buffer at 37°C and stimulated with Ag (100 ng/ml) or with an equal volume of buffer for controls. Cells were centrifuged and supernatants collected after 1, 2, or 4 h of stimulation. Chemotaxis was measured by Boyden’s blind well chamber technique using 48-well microchambers (Neuroprobe) essentially as described (22). The number of cells migrating through the filter was determined microscopically. To measure lymphokin secretion, BMMCs (5.0 × 10^6 cells) were incubated with IgE as described above to achieve known receptor occupancies. Cells were stimulated with Ag (10 or 100 ng/ml) at 37°C for varying times (1–24 h) and centrifuged at 4°C for collection of the supernatant. Lymphokine concentration was determined by ELISA (BioSource International) as previously described (23).

RNA Extraction, RT-PCR, and Ribonuclease Protection Assay. Pellets from stimulated cells (2 × 10^6 per sample) were solubilized in 1 ml of TRI-Reagent (Molecular Research Center) for 15 min at room temperature. Low abundance mRNA was measured by RT-PCR. First strand cDNA synthesis was with the Life Technologies SuperScript first-strand synthesis system. Primers for amplification of IL-2, IL-3, IL-4, IL-10, and IFN-γ were obtained from CLONTECH Laboratories, Inc., and PCR conditions were: 96°C for 1 min; 2 cycles of 96°C for 1 min and 60°C for 4 min; 28 cycles (IL-4) or 35 cycles (IL-2, IL-3, IL-10, IFN-γ) of 94°C for 1 min, 60°C for 2.5 min, and 72°C for 4 min; additional extension at 72°C for 10 min; and holding at 4°C. Cytokine mRNA detection was linear. Fragments were separated on 2% TBE-agarose gels and a negative exposure was used for densitometric quantitation.

For ribonuclease protection assay (RPA), either commercially available multiprobe template sets or two custom-made templates were used (BD Biosciences). Cytokine genes included were TNF-α, IL-13, M-CSF, LIF, and IL-6. Chemokine genes were MIP1β, MIP1α, MIP-2, LIF, and MCP-1. L32 and GAPDH were control genes. Probe synthesis was with RiboQuant™ In vitro Transcription Kit (BD Biosciences) and 100 µCi of [α-33P]-UTP (ICN Biomedicals), following the provided protocol. Hybridization used 10 µg of total RNA and 10^6 cpm of probe in 13 µl of hybridization buffer at 56°C overnight. RNaseI digestion and subsequent steps were per manufacturer’s instruction. Precipitation of protected fragments was in the presence of 1.5 µg of carrier, glycolblue (Ambion). Fragment resolution was on a denaturing 6% polyacrylamide gel, which was autoradiographed and developed in Kodak Biomax Transcreen-LE using Kodak BioMax MS (Eastman Kodak Co.) film. Quantitation of the autoradiograph was by densitometry using ImageQuant™ software version 3.3 from Molecular Dynamics. Data was normalized to the control genes L32 or GAPDH.

Results

Mast Cell Cytokine mRNA Profiles Are Similar in Response to Different Stimuli. We initially tested the hypothesis that differential expression of mast cell cytokine mRNA could occur as a consequence of the stimulus used to activate the cell. Cells were stimulated with IgE/Ag, thrombin, and ATP as the receptors (FcεRI, proteinase-activated receptor-1 (PAR-1), and purinergic receptors, respectively) responding to each stimulus utilize a different functional coupling mechanism; namely, nonreceptor tyrosine kinases (24), the Gq subclass (25), and the Gi subclass (26) of heterotrimeric GTP binding proteins, respectively. As shown in Fig. 1, all three stimuli were capable of inducing degranulation. Additionally, all three stimuli induced the same cytokine mRNA profile (Table I). However, ATP showed a tendency to induce a stronger degranulation and cytokine response than IgE/Ag whereas thrombin was only able to induce between 20 to 33% of the IgE/Ag or ATP re-
Table I. Stimulated BMMC Lymphokine mRNA Response (% of Resting Levels ± SEM)

| Gene       | RPA          | RT-PCR        |
|------------|--------------|---------------|
|            | DNP | Thrombin | ATP | DNP | Thrombin | ATP | n* |
| IL-1α      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| IL-1β      | 460 ± 35 | 130 ± 12 | 996 ± 46 | nt  | nt       | nt  | 2,0 |
| IL-1R      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| IL-2       | nd  | nd       | nd  | 1,732 ± 120 | 1,400 ± 136 | 2,500 ± 219 | 1,3 |
| IL-3       | nd  | nd       | nd  | 776 ± 128  | 125 ± 16  | 929 ± 115  | 1,3 |
| IL-4       | nd  | nd       | nd  | 1,760 ± 217 | 218 ± 74  | 3,280 ± 195 | 1,3 |
| IL-5       | nd  | nd       | nd  | nt  | nt       | nt  | 1,0 |
| IL-6       | 1,636 ± 162 | 322 ± 93   | 2,248 ± 131 | 1,589 ± 216 | 398 ± 91  | 2,179 ± 154 | 2,2 |
| IL-7       | nd  | nd       | nd  | nt  | nt       | nt  | 1,0 |
| IL-9       | nd  | nd       | nd  | nt  | nt       | nt  | 1,0 |
| IL-10      | nd  | nd       | nd  | 738 ± 214  | 198 ± 22  | 917 ± 254  | 2,3 |
| IL-11      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| IL-12 p35  | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| IL-12 p40  | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| IL-13      | 1,239 ± 265 | 150 ± 23   | 2,569 ± 194 | nt  | nt       | nt  | 3,0 |
| IL-15      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| IL-16      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| IFN-γ      | nd  | nd       | nd  | 750 ± 136  | 182 ± 16  | 1,200 ± 176 | 2,3 |
| IFN-β      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| MIF        | 120 ± 29 | 100 ± 23  | 435 ± 49  | nt  | nt       | nt  | 3,0 |
| TNFα       | 1,065 ± 168 | 220 ± 31  | 2,225 ± 241 | 987 ± 165 | 200 ± 29  | 2,161 ± 264 | 3,3 |
| TNFβ       | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| LIF        | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| TGFβ1      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| TGFβ2      | 350 ± 106 | 114 ± 8   | 810 ± 103 | nt  | nt       | nt  | 2,0 |
| TGFβ3      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| GM-CSF     | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| M-CSF      | 720 ± 109 | 670 ± 83  | 1,150 ± 179 | nt  | nt       | nt  | 3,0 |
| G-CSF      | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| LIF        | 1,118 ± 206 | 207 ± 28  | 1,886 ± 103 | nt  | nt       | nt  | 3,0 |
| SCF        | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| Ltn        | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| Rantes     | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| Eotaxin    | nd  | nd       | nd  | nt  | nt       | nt  | 2,0 |
| MCP-1      | 906 ± 218 | 563 ± 149 | 1,578 ± 189 | nt  | nt       | nt  | 3,0 |
| MCP-1β     | 1,724 ± 297 | 869 ± 201 | 2,100 ± 297 | nt  | nt       | nt  | 3,0 |
| MCP-2      | 724 ± 297 | 382 ± 71  | 1,079 ± 128 | nt  | nt       | nt  | 3,0 |
| TCA-3      | nd  | nd       | nd  | 1,197 ± 172 | 975 ± 171 | 1,956 ± 347 | 3,3 |
| L32        | 100  | 100      | 100   | nt  | nt       | nt  | 3,0 |
| GAPDH      | 100  | 100      | 100   | 100  | nt       | nt  | 3,0 |

BMMCs were loaded with DNP-specific IgE in media without IL-3 for approximately 4 h. After washing with Tyrodes/BSA, cells were stimulated with different concentrations of DNP, thrombin, or ATP and degranulation (hexosaminidase release) was determined as described in Materials and Methods. Pellets were used for total RNA extraction and RPA or RT-PCR was performed. Data in the table is the maximal response obtained for each gene after 1 h of stimulation with each agent under conditions where accumulation of the mRNA was linear.

*nt, the number of experiments done; RPA, RT-PCR.

*b, not detected.
responses. Notable exceptions were found in IL-2 and the chemokines, MIP-1β, MIP-1α, M-CSF, and MCP-1, where thrombin stimulation elicited ~50% of the response obtained with IgE/Ag. These initial experiments provided evidence of the potential for mast cells to express similar cytokine profiles to diverse stimuli, but also suggested that differential sensitivity of certain genes to a stimulus could influence the cytokine profile.

Selective IgE-mediated Induction of Mast Cell Cytokine mRNA Occurs in Response to Varying Concentrations of Ag. To test whether the strength of a stimulus affected the cytokine mRNA profile we focused solely on IgE/Ag-mediated responses because this system was readily amenable to manipulation of the number of receptor aggregates by varying the concentration of Ag or the amount of IgE-occupied FcεRI. As shown in Fig. 2 A, synthesis of IFN-γ, TNF-α, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, M-CSF, LIF, MIP-1α, MIP-1β, MIP-2, and MCP-1 was detected by RPA or by RT-PCR. Quantitation of the net response to varying concentrations of Ag revealed that the mRNA accumulation of MIP-1α, MIP-1β, MCP-1, M-CSF, IL-2, and IL-4 reached a maximum at significantly lower concentrations of Ag than IL-3, IL-6, IL-10, LIF, MIP-2, TNF-α, and IFN-γ (Fig. 2, B and C). Quantitative analysis of the effective concentration of Ag required for 50% of the maximal response (EC50), summarized in Table II, showed that genes, like MIP-1α and β, were extremely sensitive to small amounts of Ag whereas others, like IL-3 and IL-10, required almost 10-fold higher concentrations to reach their EC50. Interestingly, some chemokine and cytokine mRNA responses reached their EC50 at concentrations of Ag that elicited minimal degranulation (Fig. 1). This suggested that aggregation of small numbers of receptors was sufficient to induce increased mRNA levels of most tested chemokines and some cytokines in the absence of substantial degranulation.

The Level of Receptor Occupancy with IgE/Ag Causes Differential Expression of Cytokines and Chemokines. It has been suggested that atopic individuals may differ in their symptoms because the amounts of allergen-specific IgE produced, and thus receptor occupancy, may differ (27, 28). To test this hypothesis cells were loaded with [125I]-labeled...
Table II.  Half-maximal (EC50) Lymphokine mRNA Response of BMMCs to Ag

| Gene   | Ag concentration (ng/ml) | n   |
|--------|--------------------------|-----|
| MIP-1α | 1.1 ± 0.5                | 4   |
| IL-4   | 1.1 ± 0.3                | 3   |
| IL-2   | 1.2 ± 0.3                | 3   |
| MIP-1β | 1.3 ± 0.6                | 4   |
| M-CSF  | 1.7 ± 0.3                | 5   |
| MCP-1  | 1.9 ± 0.4                | 4   |
| IFN-γ  | 3.6 ± 0.4                | 3   |
| IL-13  | 5.4 ± 0.3                | 5   |
| TNF-α  | 5.6 ± 0.7                | 5   |
| MIP-2  | 8.9 ± 1.2                | 4   |
| LIF    | 9.8 ± 1.8                | 4   |
| IL-6   | 10.4 ± 2.7               | 5   |
| IL-10  | 11.7 ± 2.3               | 3   |
| IL-3   | 14.3 ± 2.2               | 3   |

The Ag (DNP-HSA) concentration for half-maximal response (EC50) was calculated from the data shown in Fig. 2. Data is the mean of all experiments with standard error. Data is arbitrarily grouped.

| Gene   | Ag concentration (ng/ml) | n   |
|--------|--------------------------|-----|
| MIP-1α | 1.1 ± 0.5                | 4   |
| IFN-γ  | 3.6 ± 0.4                | 3   |
| IL-13  | 5.4 ± 0.3                | 5   |
| IL-6   | 10.4 ± 2.7               | 5   |
| IL-10  | 11.7 ± 2.3               | 3   |
| IL-3   | 14.3 ± 2.2               | 3   |

The secreted lymphokines induced a potent monocyte/macrophage (J774.1 cells) chemotactic response that was similar to that observed at full receptor occupancy (Fig. 3 D). MCP-1 alone, at concentrations similar to those secreted (1,000–4,000 pg/10⁶ cells), caused a comparable chemotactic response. IL-4 and IL-10 were not detected at 5 h after stimulation but small amounts were detected after 24 h of stimulation. Under low receptor occupancy only IL-4 was detected whereas IL-10 was not (Table III). Quantitation of secreted IL-6 and secreted MCP-1 showed that at low receptor occupancy almost a 10-fold difference in absolute amounts of these lymphokines exists whereas this difference is strikingly narrowed (2.3-fold) at high receptor occupancy. With the exception of TNF-α, the results are consistent with a skewed lymphokine response that favors chemokines at low receptor occupancy. FceRI-mediated TNF-α mRNA responses required higher Ag concentrations or receptor occupancy (Figs. 2 and 3), however, substantial secretion of TNF-α, upon a weak stimulus, may result as a consequence of its synthesis and storage in preformed secretory granules (8).

IgE/Ag-induced Gene Responses Show Dependence or Early Independence of Receptor Aggregation. We previously demonstrated that TNF-α, which requires high receptor occupancy for complete induction, was exquisitely sensitive to disaggregation of receptors by a monovalent hapten (29). We reasoned that it was possible that genes that required a larger number of receptor aggregates could be most sensitive to disruption of receptor aggregates and vice versa. As shown in Fig. 4 A, degranulation of mast cells required continued aggregation of receptors for up to 6 min after Ag addition. At 3 min after Ag addition, hapten addition resulted in ~50% of the normal degranulation response. The inhibitory effect of hapten was mirrored by the addition of ice cold buffer indicating the interruption of an active process.

Analysis of the sensitivity of the cytokine and chemokine mRNA response to hapten revealed three distinct responses: first, mRNA accumulation of MIP-1α, MIP-1β, MCP-1, and M-CSF became rapidly independent (between 1–4 min after Ag stimulation) of continued receptor aggregation (Fig. 4 B). Second, mRNA accumulation of LIF, IL-3, IL-4, IL-6, and IL-10 became independent of continued receptor aggregation between 4 and 8 min after Ag stimulation (Fig. 4 C). Third, mRNA accumulation of IL-2, IL-13, IFN-γ, and TNF-α required more than 15 min to show considerable independence of receptor aggregation (Fig. 4 D). Non-linear regression analysis of the mRNA response of IL-2, IL-13, IFN-γ, and TNF-α showed two distinct groups (IL-2, IFN-γ and IL-13, TNF-α). However, the cytokines in each group showed a poor fit suggesting additional regulatory complexity in their response. With some notable exceptions, like IL-2, most mRNA responses induced at low Ag concentrations or low receptor occupancy became rapidly independent of continued receptor aggregation. Vice versa, those genes requiring high Ag concentrations or high receptor occupancy for induction were more dependent on continued receptor aggregation. Despite the noted exception, this suggests that most genes the respond to low Ag or low receptor occupancy conditions can generate intermediates that rapidly become independent of continued receptor aggregation.
Low Ag or Low Receptor Occupancy Favors Fyn/Gab2-initiated Signals and Activation of p38MAPK. Fyn and Lyn kinases are FcɛRI-proximal Src family kinases that propagate signals through the adapters Gab2 and LAT, respectively (30, 31). Gab2 is required for the activation of PI3K and Akt in mast cells whereas LAT is essential for calcium responses (30, 32). We now tested if low Ag concentrations or low receptor occupancy could induce the phosphorylation of the adapters Gab2 or LAT and other proteins downstream of these adapters. As shown in the upper panel of Fig. 5 A, low Ag concentrations were substantially effective in stimulating the phosphorylation of Fyn, Gab2, and the p85 subunit of PI3K. Because the available antibodies to Gab2 are of relatively low affinity, phosphorylated Gab2 was difficult to detect below stimulation with 1 ng/ml of Ag. LAT phosphorylation was detected at 10 ng/ml of Ag. Low receptor occupancy also showed a preference for the Gab2/PI3K pathway as the downstream kinase, Akt, was phosphorylated to 80% of that seen at high receptor occupancy. In contrast, ERK2 phosphorylation, which depends primarily on LAT (18), was at best 15% of that seen at high receptor occupancy when films were overexposed. This suggested that the Gab2/PI3K pathway was more sensitive in its response to low Ag or low receptor occupancy. Given our previous finding of a potent MCP-1 response under conditions of low Ag concentrations or low receptor occupancy, this suggested that Gab2 might be more important to MCP-1 mRNA responses than LAT. As shown in Fig. 5 B, Gab2-deficient mast cells were defective in the mRNA response of all the lymphokines. Curves shown are representative of the distinct mRNA responses observed. (D) The medium from Ag-stimulated BMMCs having low (17.0% ± 1.5) or high receptor occupancy (100.0%) with IgE was collected at the indicated times after Ag stimulation. Chemotaxis of the monocyte/macrophage cell line J774.1 induced by the collected medium was measured using a Boyden chamber assay. MCP-1 (1 ng/ml) or 4 ng/10^6 cells) was added for 4 h as a positive control. Five randomly selected high power fields were counted per filter. Data shown is the net average of two experiments with triplicate samples. Migration toward medium from unstimulated cells (41 ± 7 cells per field) is subtracted.

Table III. BMMC Secretion of Lymphokines at Low and High Receptor Occupancies

| Lymphokine | Time (h) | pg/10^6 cells | Percentage of secretion at 100% occupancyb |
|------------|----------|---------------|------------------------------------------|
|            |          | 10% occupancya | 100% occupancya |                               |
| MCP-1      | 5        | 2,076.5       | 4,837.2        | 42.9                          |
| IL-6       | 5        | 231.4         | 2,117.3        | 10.9                          |
| MIP-1β     | 5        | 220.8         | 452.6          | 48.8                          |
| MIP-1α     | 5        | 59.8          | 124.5          | 48.0                          |
| TNF-α      | 5        | 52.4          | 153.6          | 34.1                          |
| IL-4*      | 24       | 2.3           | 4.0            | 57.5                          |
| IL-10*     | 24       | ndc           | 15.9           | –                             |

BMMCs were stimulated with 10 or 100 ng/ml of Ag (DNP-HSA) for the indicated time. After centrifugation the supernatant was recovered and the amount of lymphokine secreted was measured by ELISA as described in Materials and Methods. Data is from two independent experiments using two different BMMC cultures. Data is from one experiment as detection of these cytokines was below the sensitivity of the assay in a second experiment. Standard deviations ranged up to 18.7%.

% receptor occupancy was determined by counting the cell bound cpm of the [125I]-DNP-specific IgE for each sample and dividing by the cpm bound on cells incubated with saturating amounts of [125I]-DNP-specific IgE. Percentage of secretion at 100% occupancy was calculated by dividing the amount of lymphokine produced (pg/10^6 cells) at 10% receptor occupancy by the response observed at 100% receptor occupancy.

Table III. BMMC Secretion of Lymphokines at Low and High Receptor Occupancies
tested chemokines whereas cytokine mRNA responses were less severely affected. Strikingly, LAT-deficient mast cells showed the opposite profile with cytokines being more severely inhibited than chemokines. In Gab2-deficient mast cells, the inhibition of chemokine mRNA responses ranged from 65–90% depending on receptor occupancy. Gab2 deficiency also caused a 10–30% inhibition of cytokine mRNA responses. In contrast, LAT-deficient mast cells showed an inhibition of chemokine mRNA responses that ranged from 15–40% with MCP-1 and LIF mRNA responses being unaffected. Cytokine mRNA responses were more severely affected by LAT-deficiency with inhibition ranging from 40–80%.

To gain a further understanding of the influence of receptor occupancy on gene expression we explored the activation (phosphorylation) of MAP kinases. Fig. 6 A demonstrates that p38MAPK was most sensitive to low receptor occupancy with 50% of the maximal phosphorylation detected at ~12% receptor occupancy. In contrast, ERK2 required more than 25% and JNK1 more than 40% occupancy to reach 50% of their maximal phosphorylation levels. It should be noted that consistent with greater sensitivity of Gab2 to weak stimulation, a previous report demonstrated impaired p38MAPK activation in Gab2-deficient mast cells (32). We postulated that if p38MAPK activation is required for production of lymphokines that respond under weak stimulation, like MCP-1, its activation should become rapidly independent of continued receptor aggregation (like MCP-1). Fig. 6 B shows that p38MAPK becomes rapidly independent of continued receptor aggregation (1 min). The activation of both JNK and ERK2 showed a greater dependence on continued receptor aggregation with independence being achieved after 3 and 8 min post-Ag stimulation, respectively (graph, Fig. 6 B).

Together, with prior results on chemokine responses, these findings demonstrate a relationship between sensitivity to a weak stimulus and rapid independence of continued receptor aggregation. They also provide suggestive evidence for the importance of p38MAPK in the mRNA responses (like MCP-1) induced by low Ag concentration or low receptor occupancy.

**MCP-1 and TNF-α Genes Show Preferential Requirement for p38MAPK or ERK2, Respectively.** While it is well understood that multiple pathways cooperate with the activity of MAP kinases in gene expression (33, 34), we tested whether the mRNA accumulation of some cytokines and chemokines showed preferential sensitivity to inhibition of p38MAPK and ERK2 by SB202190 and PD98059, respectively. We chose to analyze the sensitivity of two genes that well-represent mRNA induction by low IgE Ag receptor occupancy (MCP-1) or high IgE Ag receptor occupancy (TNF-α; see Fig. 3, B and C). Fig. 7 shows that pretreatment of BMMC with PD98059 inhibited TNF-α mRNA accumulation by 70%, whereas SB
202190 inhibited TNF-α mRNA accumulation by ~30%. In contrast, SB202190 inhibited MCP-1 mRNA accumulation by almost 80% whereas PD98059 had no effect on the mRNA accumulation of this chemokine. However, another analyzed gene, IL-13, which responded to intermediate Ag concentrations or receptor occupancy, was not as effectively inhibited (~23–38%) by SB202190 or PD98059. The findings are consistent with
induction of p38MAPK and ERK2 at low or high IgE/Ag receptor occupancy as important in MCP-1 and TNF-α mRNA responses, respectively.

**Discussion**

Herein, we describe that weak FceRI stimulation of mast cells causes preferential cell signaling and a lymphokine response that shows potent chemokine production. Our findings demonstrate that a homogeneous population of mast cells can respond differently to the same allergen depending on its concentration or on the extent of receptor occupancy (number of receptor aggregates), or the persistence of the allergen (continued receptor aggregation).

Recently, there is increased awareness of the importance of the stability or life span of a receptor in an aggregate complex. It is thought that the life span of an activated receptor in a receptor aggregate is important for the generation of signals that lead to “productive” cellular responses (35–38). One well studied system is the TCR, where studies demonstrate that the strength of the signal can cause different cellular responses. Thus, weak TCR stimulation induces a calcium signal that is sufficient to trigger IL-4 synthesis whereas stronger TCR stimulation induces MAP kinase activation that controls IFN-γ production (37). Other studies show that the pattern of signaling events induced by stimulation of the TCR with low doses of peptides, by a partial agonists, or by CD28 costimulation can cause differential responses or anergy (39–41), and even determine Th1 or Th2 differentiation (42). Physiological concentrations of the B7 ligand, that stimulates CD28 on T cells, causes preferential activation of a subset of the signaling molecules observed when T cells are activated by antibody to CD3 or by antibody costimulation of the TCR and CD28 (42, 43). Hence, the view arising from these studies is that the overall potency of the stimulus is critical in determining what signals are generated and what cellular outcomes are observed.

T.W. McKeithan introduced a “kinetic proofreading” model to explain the ability of the TCR to discriminate between different ligands that might bind the same receptor but elicit different cellular outcomes (44). Central to this tenet is that the initiating ligand–receptor interaction be maintained for sufficient time to allow the occurrence of the required series of events leading to a particular cellular response. Torigoe and colleagues demonstrated that the
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The kinetics of FcεRI and FcεRII reside in membrane domains that exclude LAT but contain Gab2 more strongly activates signals associated with the Gab2 (32) these findings support the notion that a weak stimulus increased cyclooxygenase-2 expression in RBL cells (50). It was recently recognized that chemokines and chemokine receptors play an important role in allergic airway disease (52, 53) and antichemokine therapy represents a new and promising approach to allergy immunotherapy (54). The expression of the chemokine MCP-1, during cutaneous allergic reactions, is mast cell dependent and primarily responsible for the influx of monocytes to the reaction sites (55). Moreover, the selective production of chemokines in the absence of mast cell degranulation was reported in response to Dengue virus infection (56). We now demonstrate that conditions of low occupancy by IgE or low Ag induces a potent production of chemokines, like MCP-1 and MIP-1β, that can recruit macrophages, monocytes, and other leukocytes. Importantly, this occurs in the absence of considerable mast cell degranulation and of immunoregulatory cytokines, like IL-10, which down-regulate the Th2 response. Our findings suggest that a weak allergen or a small number of receptors occupied with an allergen-specific IgE can elicit mast cell-mediated inflammation and is consistent with the idea that production of chemokines is central to the allergic inflammation. This defines a previously unrecognized plasticity in mast cell responses that is likely to be an important component of a mast cells function in health and disease.

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