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IgE-Induced Mast Cell Survival Requires the Prolonged Generation of Reactive Oxygen Species

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We show in this study that the ability of five different monomeric IgEs to enhance murine bone marrow-derived mast cell (BMMC) survival correlates with their ability to stimulate extracellular calcium (Ca^{2+}) entry. However, whereas IgE+Ag more potently stimulates Ca^{2+} entry, it does not enhance survival under our conditions. Exploring this further, we found that whereas all five monomeric IgEs stimulate a less robust Ca^{2+} entry than IgE+Ag initially, they all trigger a more prolonged Ca^{2+} influx, generation of reactive oxygen species (ROS), and ERK phosphorylation. These prolonged signaling events correlate with their survival-enhancing ability and positively feedback on each other to generate the prosurvival cytokine, IL-3. Interestingly, the prolonged ERK phosphorylation induced by IgE appears to be regulated by a MAPK phosphatase rather than MEK. IgE-induced ROS generation, unlike that triggered by IgE+Ag, is not mediated by 5-lipoxygenase. Moreover, ROS inhibitors, which block both IgE-induced ROS production and Ca^{2+} influx, convert the prolonged ERK phosphorylation induced by IgE into the abbreviated phosphorylation pattern observed with IgE+Ag and prevent IL-3 generation. In support of the essential role that IgE-induced ROS plays in IgE-enhanced BMMC survival, we found the addition of H_{2}O_{2} to IgE+Ag-stimulated BMMCs leads to IL-3 secretion.


Mast cells are responsible for immediate hypersensitivity and chronic allergic reactions through the binding of extracellular IgE to their high affinity IgE receptors (FcεRIs) and the subsequent cross-linking of these IgE/FcεRI complexes by multivalent allergens. This cross-linking activates multiple signaling pathways that lead to degranulation, PG, and leukotriene synthesis and production of various cytokines and chemokines (1). Within this traditional scenario, it was thought until quite recently that IgE binding by itself was simply a passive pre-sensitization step that awaited receptor aggregation via multivalent Ags to induce intracellular changes. However, there is now substantial evidence that monomeric IgE (mIgE) alone is not only capable of up-regulating the cell surface expression of FceRI (2), but of initiating cell signaling events (3). Specifically, with regard to the latter, Kawakami and colleagues (5) and we (4) showed that the binding of mIgE alone, in the absence of Ag, was capable of enhancing bone marrow-derived mast cell (BMMC) survival, whereas IgE followed by Ag cross-linking (IgE+Ag) was not. Moreover, we found, using SPE-7 anti-DNP IgE, that mIgE binding stimulated multiple phosphorylation events in these cells and led to a more potent production of cytokines than IgE+Ag. As well, we provided evidence that mIgE prevented the apoptosis of cytokine-deprived BMMCs, at least in part, by maintaining Bcl-xL levels and producing autocrine-acting cytokines.

A number of groups have subsequently confirmed these findings and shown that mIgE alone can also lead to enhanced degranulation, leukotriene release, histidine decarboxylase expression (6), increased adhesion to fibronectin (7), FcεRI internalization, migration, and DNA synthesis (3), to varying degrees, depending on the mast cell type studied. As well, several groups have substantially increased our understanding of how mIgE enhances mast cell survival by showing that it requires the tyrosine kinase Syk (8) and the ITAM motif within the FcεRI chain of the FcεRI (9) (i.e., the same ITAM required for IgE+Ag-induced degranulation). As well, a weak, but sustained signal via this γ-chain was shown to be sufficient for mast cell survival (10), and, using IL-3-dependent BMMCs, the IgE-induced autocrine production of IL-3 was responsible for mast cell survival, in part via a Jak2/STAT5-induced maintenance of Bcl-xL and Bcl-2 (11). Importantly, Kawakami and colleagues (8) found that some mIgEs, defined as highly cytokinergic, were far more capable of stimulating intracellular signaling and survival than others (defined as poorly cytokinergic), and this appeared to correlate with their ability to trigger FcεRI aggregation.

To further elucidate the mechanisms underlying the ability of some IgEs to promote BMMC survival better than others, we have compared in this study the intracellular signaling of five different
mlgEs with markedly different abilities to enhance BMMC survival. As well, we have compared the signaling of IgE with IgE+Ag to further elucidate why IgE+Ag does not enhance survival under the conditions used in our laboratory. Our results suggest that the ability of an IgE to promote IL-3 production, and thereby survival, depends on its ability to trigger a prolonged generation of reactive oxygen species (ROS). Interestingly, this IgE-induced ROS is not generated via S-lipoxygenase (5-LO), distinguishing it from IgE+Ag-induced ROS, and is markedly dependent upon extracellular calcium (Ca\(^{2+}\)) entry and MEK, suggesting that positive feedback loops from these two signaling intermediates are involved.

Materials and Methods

Mast cell isolation

SHIP\(^{+/+}\) and SHIP\(^{-/-}\), Lyn\(^{+/+}\) and Lyn\(^{-/-}\), and linker for activation of T cells (LAT\(^{+/+}\)) and LAT\(^{-/-}\) bone marrow cells, aspirated from 4- to 8-wk-old C57BL6 mice, were cultured in IMDM, 15% FCS, and 150 μM monothioglycerol containing 50 ng/ml murine stem cell factor (SCF), 10 ng/ml murine IL-3, and 10 ng/ml human IL-6 for 1 wk, and then these cytokines were replaced with 30 ng/ml IL-3. By 6–8 wk, greater than 99% of the cells were c-kit and FcεR1 positive (12, 13).

Preparation of mIgEs

Five distinct mouse monoclonal IgEs were used in this study: clone SPE-7 anti-DNP (S; Sigma-Aldrich), clone 91.58 anti-nitrophenol (A; provided by K. Hayglass, University of Manitoba, Winnipeg, Canada), anti-DNP clone DNP48 (48; derived in R Siragianian’s laboratory), anti-Epo26 (E; Stem-Cell Technologies), and the H1 26.82 Liu anti-DNP (L) (14). mIgEs were prepared by fractionation using a Waters HPLC system with a BioSep SEC S3000 gel filtration column (300 x 7.8 mm; Phenomenex), as described previously (4), and stored at -4°C. IgE concentrations were determined using an IgE ELISA (BD Pharmingen).

Survival studies

BMMCs were washed with IMDM and incubated at 5 x 10\(^5\) cells/ml in IMDM plus 10% FCS, 0.1% BSA ± IgE, or IgE+Ag, as indicated, in Falcon 3047 24-well, flat-bottom plates (total volume 0.2 ml/well) ± neutralizing Abs to SCF (R&D Systems), IL-3 (15), or isotype control Ab (15). Viability was assessed by trypan blue exclusion.

BMMC stimulation and Western blotting

BMMCs were incubated without IL-3 for 4 h or overnight at 37°C in IMDM, 10% FCS, 150 μM MTG, and P/S, and then washed three times with, and resuspended in, IMDM, 0.1% BSA, monothioglycerol (MTG), and penicillin/streptomycin (P/S) or in Tyrode’s buffer ± 1.8 mM Ca\(^{2+}\), Cl\(^{-}\).

Calcium measurements

Ca\(^{2+}\) fluxes were measured, as described previously (12). For stimulation with the mlgEs, BMMCs were incubated with 2 μM fura 2-AM (Molecular Probes) in Tyrode’s buffer at 23°C for 45 min, washed twice, resuspended in Tyrode’s buffer at 5 x 10\(^5\) cells/ml in a stirring cuvette, and stimulated with 10 μg/ml IgE. To stimulate with IgE+Ag, BMMCs were preloaded with 0.1 μg/ml S IgE overnight in IMDM, 10% FCS, 150 μM MTG, and P/S, washed three times to remove unbound IgE; resuspended in Tyrode’s buffer and 2 μM fura 2-AM for 45 min at 23°C; washed twice; resuspended in Tyrodes at 5 x 10\(^5\) cells/ml, as above; and stimulated with 20 ng/ml DNP-HSA (30–40 mol DNP/mol human serum albumin (HSA); Sigma-Aldrich). Cytosolic Ca\(^{2+}\) was measured by monitoring fluorescence intensity at 510 nm by exciting with the sample with two different wavelengths (340 and 380 nm) with a Thermo Spectronic Aminco Bowman Series 2 Luminescence Spectrometer.

ROS measurements

BMMCs were washed twice in IMDM without IL-3 (starve medium) and resuspended at 0.5 x 10\(^5\) cells/ml overnight. Cells were stimulated with Ag were preloaded overnight with 0.1 μg/ml S IgE, and the cells were then washed twice in starve medium and resuspended at 1 x 10\(^5\) cells/70–80 μl of Tyrode’s buffer. The 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFDA; Molecular Probes) was prepared fresh each day, by dissolving 50 μg in 8 μl of DMSO and diluting immediately to 100 μM in Tyrode’s buffer, and stored in the dark. A total of 10 μl of CM-DCFDA stock was added per 10\(^6\) cells 20 min before stimulation in a black (small-well) 96-well plate at 37°C, 5% CO\(_2\), in the dark. Inhibitors (10x stocks prepared by dilution in Tyrode’s) were added 15 min before stimulation in a volume of 10 μl used in the assay. Cells were stimulated with 10 μl of IgE or Ag prepared by dilution of stock to a 10x concentration in Tyrode’s buffer. The fluorescence of the plate was read immediately before stimulating the cells, immediately after stimulation, every 1 min for 5 min and every 5 min thereafter up to 30 min. Relative fluorescence was detected with an excitation wavelength of 485 nm and an emission wavelength of 527 nm in a 96-well plate fluorometer (Fluoroskan Accent FL; ThermoLabsystems). Fluorescence values reported have been corrected by subtracting the background fluorescence of similarly treated, but unstimulated cells.

ELISAs

IgE, IL-3, and IL-6 ELISAs (BD Pharmingen) were performed, according to the manufacturer’s instructions.

Results

The ability of an IgE to enhance BMMC survival correlates with its ability to trigger a prolonged calcium influx and ERK phosphorylation

To further investigate the mechanism by which IgE enhances BMMC survival, we first tested a panel of 10 different mouse monoclonal IgEs, quantitated using an IgE ELISA, and found that they were all capable of enhancing BMMC survival to some extent (data not shown). Because the most potent IgE, i.e., S IgE, possessed a λ chain, we obtained another λ chain IgE, i.e., λ, to see whether this exceptional potency was characteristic of λ-containing IgEs. The λ IgE, however, proved to be the least potent. Based on these results, we chose a subset of five different mlgEs with very different survival-enhancing abilities for further study. As shown in the left panel of Fig. 1A, the order of survival-enhancing ability of these five IgEs was S > 48 ≥ λ > E > λ. The effect of IgE+Ag was also tested (Fig. 1A, right panel), using 0.01, 0.05, and 0.1 μg/ml S IgE, followed by 20 ng/ml DNP-HSA, and was found to be incapable of enhancing BMMC survival. Thus, we could not simulate the survival-enhancing ability of mIgE by simply activating a very small number of IgE receptors on BMMCs without Ag, which contrasts somewhat with a report showing that weak to moderate stimulation of BMMCs with IgE+Ag can enhance survival (17).

We then examined the ability of the five mlgEs to stimulate Ca\(^{2+}\) fluxes and found that this correlated precisely with their ability to enhance survival (Fig. 1B, left panel). The Ca\(^{2+}\) response stimulated by IgE+Ag occurred faster than any mlgE alone, peaking at 100 s after Ag addition and then declining. Ca\(^{2+}\) mobilization stimulated by the five mlgEs, in contrast, steadily increased over the time course of the experiment. Carrying out these studies in the presence and absence of EGTA established that the bulk of the IgE-induced Ca\(^{2+}\) mobilization was due to extracellular Ca\(^{2+}\) entry (Fig. 1B, right panel).

We then tested the ability of the five mlgEs and IgE+Ag to stimulate the phosphorylation of ERK1/2 (to monitor Ras pathway activation). As can be seen in Fig. 1C, IgE+Ag-induced phosphorylation of ERK peaked early and returned to baseline levels within
FIGURE 1. The ability of an IgE to induce survival correlates with its ability to trigger a prolonged calcium entry and ERK phosphorylation. 

A. BMMCs were cultured at 37°C with 10% FCS alone (C) (●) or + 5 μg/ml S (●), L (■), 48 (▲), E (○), or λ (□) IgE (left panel), or with 10% FCS alone (C) (●) or + 5 μg/ml S, 1 μg/ml (▲) S IgE, or 0.1 (○), 0.05 (□), or 0.01 (△) μg/ml S IgE overnight at 37°C, and washed; and 20 ng/ml DNP-HSA was added (right panel), and viable cells were counted on the days indicated. Values shown are the mean ± SEM of duplicate determinations.

B. BMMCs were incubated with fura 2-AM at 23°C for 45 min, washed twice, and stimulated with (left panel) 10 μg/ml S, L, 48, E, or λ IgE (arrow indicates time of addition). C = unstimulated cells and S+Ag (dark line) = BMMCs pretreated with 0.1 μg/ml S overnight, washed, and stimulated with 20 ng/ml DNP-HSA at the time indicated by the arrow or (right panel) 10 μg/ml S (arrow indicates time of addition) ± pretreatment for 1 min with 5 mM EGTA. C, BMMCs were treated with 5 μg/ml indicated IgE or with 0.1 μg/ml S overnight, washed, and stimulated with 20 ng/ml DNP-HSA (IgE+Ag) at 37°C for the times shown, and the SDS-solubilized total cell lysates were subjected to Western analysis with phospho-specific Abs to ERK1/2. The blot was reprobed with anti-ERK1/2 to confirm equal loading. The numbers between the two panels indicate relative densitometry values normalized to ERK1/2. D. BMMCs were cultured with 10% FCS alone (C) or + 0.1 μg/ml S IgE overnight at 37°C, washed, and exposed to 20 ng/ml DNP-HSA (S+Ag) or not preloaded and stimulated + 5 μg/ml S, L, E, 48, or λ IgE for 3 h, and the conditioned medium was subjected to an IL-6 ELISA. Results are the mean ± SEM of triplicate determinations. *, p < 0.0001; **, p < 0.001; ***, p < 0.006. Similar results were obtained in five (left panel of A) or three (right panel of A, B, C, and D) separate experiments. For the left panel of B and D, only two separate experiments were conducted with IgE+Ag present.
60 min, in keeping with earlier reports (4, 10). The ERK phosphorylation induced by the five mIgEs, in contrast, was prolonged, with peak levels visible out to 60 min. Importantly, the level of ERK phosphorylation correlated with the survival-enhancing ability of the five mIgEs, with S displaying the highest and L the lowest.

We also compared the ability of the five different mIgEs to stimulate IL-6 production and found that the rank order of production correlated with their survival-enhancing ability (Fig. 1D). However, IgE+Ag also stimulated the production of IL-6 at levels only slightly below that triggered by S IgE, in keeping with our previous findings (4) and substantially above that stimulated by the other mIgEs. Thus, IL-6 production did not correlate with BMMC survival.

IgE-induced survival of BMMCs is dependent on the production of autocrine-acting IL-3

Although the above studies demonstrated that the ability of the five mIgEs to trigger extracellular Ca\(^{2+}\) entry and ERK phosphorylation correlated with their survival-enhancing abilities, they did not establish which signaling events were actually required for enhancing survival. In an attempt to address this, we first compared the IgE-induced survival of Lyn\(^{+/+}\) and Lyn\(^{-/-}\) BMMCs using the S mIgE. As shown in the left panel of Fig. 2A, the IgE-mediated enhancement of BMMC survival was significantly reduced in Lyn\(^{-/-}\) BMMCs, in keeping with results obtained by Kitaura et al. (8). Similar results were obtained with these cells using the L IgE (data not shown). We also tested the ability of both S and L mIgEs to enhance the survival of LAT\(^{-/-}\) BMMCs (18, 19), and found that the absence of LAT reduced the ability of the S (Fig. 2A, right panel) and L (data not shown) mIgE to enhance survival as well.

Because previous studies in our laboratory suggested that IgE was enhancing survival, at least in part, by producing autocrine-acting cytokines (4), we also attempted to identify the cytokines involved. From our earlier studies using RNase protection assays and ELISAs, we found that S mIgE alone stimulated the production of IL-2, IL-3, IL-4, IL-6, IL-13, and TNF-\(\alpha\), and that a combination of these cytokines, at the levels produced in response to mIgE, enhanced survival almost as well as mIgE (4). We therefore tested these cytokines, as well as GM-CSF, M-CSF, IL-10, and SCF because these latter proteins have been shown to be produced by mast cells and/or affect mast cell proliferation (20–23). We found that only SCF and IL-3 supported the proliferation and survival of BMMCs (data not shown). We then used neutralizing Abs to SCF and IL-3 to see whether they could block IgE-induced BMMC survival and found that anti-SCF had no detectable effect (Fig. 2B, left panel), whereas anti-IL-3 substantially reduced the survival of IgE-treated BMMCs (Fig. 2B, right panel), in keeping with elegant studies from Saito and colleagues (11) using IL-3\(^{-/-}\) BMMCs. We then compared the IL-3 production from BMMCs stimulated with the five mIgEs. Not surprisingly, the pattern was very similar to that obtained with IgE-triggered IL-6 production (Fig. 1D), with S IgE generating the most IL-3 and L the least (Fig. 2C, left panel).

Importantly, however, IgE+Ag was incapable of triggering any detectable IL-3 production, in keeping with IL-3 playing a critical role in IgE-induced BMMC survival. This result, although in agreement with recent data from Saito’s laboratory (11), contrasts with early studies showing that IgE+Ag can trigger IL-3 production (24–26) and may reflect differences in the type of mast cells used or the nature of the Ag. To determine whether the levels of IL-3 produced in response to the different mIgEs could account for the observed survival, we conducted IL-3 dose-response studies and found that the level of IL-3 secreted in response to the five mIgEs was within the range required to maintain BMMC survival (Fig. 2C, right panel), in keeping with prior results (11). Thus, autocrine-acting IL-3 alone might indeed explain the survival effects, but to explore this further we asked whether the IgE-induced maintenance of Bcl-x\(_L\) (4, 11) was dependent on IL-3 production. To test this, we examined the effect of a neutralizing anti-IL-3 Ab on IgE-induced Bcl-x\(_L\) levels following 30 h of incubation, at which time there is no loss as yet of cell viability. Preliminary studies established that the level of anti-IL-3 Ab that we used was sufficient to block exogenously added IL-3 (at 10 times the level of IL-3 produced in response to 5 \(\mu\)g/ml S IgE) from promoting BMMC proliferation or survival (data not shown).

As can be seen in Fig. 2D, we found that anti-IL-3 Ab substantially, but not completely, abrogated the maintenance of Bcl-x\(_L\),
with the use of inhibitors in the longer-term survival studies. Bcommented... inhibitors. This avoided toxicity concerns associated... (Ag) or stimulated with 5... (IgE) with 25 μM UO126 before IgE stimulation or 5 min after IgE stimulation (IgE5+/UO). SDS-solubilized total cell lysates were then subjected to Western analysis with phospho-specific Abs to ERK1/2 and with Abs to ERK1/2. The numbers between the two panels indicate relative densitometry values normalized to ERK1/2. B, BMMCs were stimulated with 0.1 μg/ml S overnight at 37°C, washed, and given 20 ng/ml DNP-HSA for 3 h (IgE+Ag) or stimulated with 5 μg/ml S IgE in 0.1% BSA for 3 h (IgE) in the presence of 25 μM UO126 added 5 min before IgE (UO+IgE) or 5 min after IgE (IgE+UO), and the conditioned medium was subjected to IL-3 ELISAs. A is representative of five separate experiments. For B, results are the mean ± SEM of three independent experiments, and the levels of IL-3 obtained from unstimulated cells have been subtracted.

Prolonged ERK phosphorylation is required for IL-3 production

To determine the IgE-induced signaling pathways that were required for BMMC survival, we measured IL-3 production (as a surrogate marker for survival) from BMMCs after 3 h. We found it completely ablated S IgE-induced ERK phosphorylation (Fig. 3A, top panel). This complete ablation was also observed when BMMCs were pretreated for either 15 or 5 min before IgE treatment. This showed that U0126 rapidly enters BMMCs and inhibits MEK activity (Fig. 3A, lower panel). Interestingly, however, when we added U0126 5 min after IgE exposure, in an attempt to convert the prolonged IgE-induced activation of ERK into an acute activation (to simulate IgE+Ag stimulation), it did not inhibit ERK phosphorylation (i.e., phosphorylation of ERK persisted with the same kinetics as in the absence of U0126) (Fig. 3A, lower panel). This was consistent with the prolonged IgE-induced phosphorylation of ERK being due to a lack of induction or activation of one or more MAPK phosphatases (MKPs) (also known as dual specificity MAPK phosphates (DUSP) or dual specificity phosphatases (DSP)) rather than to a prolonged activation of MEK.

Looking downstream at the effect of the MEK inhibitor on IL-3 production, we found that if it was added 5 min before IgE stimulation, it dramatically inhibited IL-3 production, but had no effect if added 5 min after IgE (Fig. 3B). Taken together, this suggested that ERK activation was important for IgE-induced IL-3 production (because IL-3 levels were markedly reduced with U0126) and that it was prolonged ERK activation that was important because IgE+Ag, which only triggers an acute ERK activation, could not stimulate IL-3 production.

Prolonged calcium influx is required for IL-3 production

To determine why IgE results in a prolonged ERK phosphorylation, whereas IgE+Ag does not, we asked whether the delayed, but sustained Ca2+ influx observed with IgE alone (see Fig. 1B) played a role. Specifically, we examined IgE-induced ERK phosphorylation in the presence and absence of extracellular Ca2+. As shown in the top panel of Fig. 4A, addition of IgE to BMMCs in the presence of Ca2+ displayed a more prolonged ERK phosphorylation (peaking at 30 min) than in its absence (peaking at 5 min). However, no difference was observed with IgE+Ag-induced phosphorylation of ERK (Fig. 4A, bottom panel), suggesting that IgE+Ag-induced ERK phosphorylation is not dependent on an influx of extracellular Ca2+. Importantly, IgE-induced IL-3 production was dramatically reduced in the absence of extracellular Ca2+ (Fig. 4B), consistent with IgE inducing a prolonged Ca2+ influx, which leads to a prolonged ERK phosphorylation and subsequent IL-3 production.

Because a recent study suggested that BCR activation leads to both a rapid influx of Ca2+ and the generation of ROS and that they engage in a cooperative interaction that acts in a positive feedback loop to amplify signaling (29), we asked whether IgE alone generated ROS and, if so, what effect extracellular Ca2+ would have on this generation. As shown in Fig. 4C, we found that IgE+Ag induced a rapid generation of ROS that plateaued after 5 min of exposure to Ag. Of note, whereas no additional ROS is generated in response to IgE+Ag after 5–10 min, high fluorescence is still seen at 25 min because it is not degraded in this in vitro system. Interestingly, S IgE alone also induced ROS, but at a much slower rate, and the level continued to climb for at least 30 min (we could not follow it longer because of photobleaching of the fluorescent substrate). Of note, in the absence of extracellular Ca2+, IgE-induced ROS was markedly inhibited. However, this inhibition only started 5 min after IgE stimulation, consistent with IgE-induced ROS generation preceding extracellular Ca2+ entry. Also worthy of note, IgE+Ag-induced ROS was also inhibited in the absence of extracellular Ca2+, albeit far less than IgE-induced ROS (Fig. 4C).

Because a very recent study nicely showed that IgE+Ag stimulated the production of ROS via 5-LO and, to a small extent, via cyclooxygenase-1 (30), we asked whether these enzymes were also responsible for IgE-induced ROS. Specifically, we first tested various concentrations of the 5-LO inhibitor, AA861, and the cyclooxygenase-1 inhibitor, FR122047, on IgE+Ag vs IgE-induced ROS production.
FIGURE 4. A prolonged calcium influx is required for IgE-induced IL-3 production. A, BMMCs were stimulated for the indicated times with 5 μg/ml S IgE (top panel) or with 0.1 μg/ml S IgE overnight at 37°C, followed by 20 ng/ml DNP-HSA (lower panel) in Tyrode’s buffer containing 1.8 mM CaCl2 or Tyrode’s buffer without Ca2+. SDS-solubilized total cell lysates were then subjected to Western analysis with Abs to phospho-ERK1/2 and ERK1/2. The numbers under the lanes indicate relative densitometry values normalized to ERK1/2. B, BMMCs were stimulated for 3 h with 5 μg/ml S IgE in Tyrode’s buffer ± 1.8 mM CaCl2 and IL-3 ELISAs conducted on the conditioned medium. *, p < 0.0001. Results are the mean ± SEM of three independent experiments. C, BMMCs were incubated for 20 min with CM-DCFHDA, as described in Materials and Methods, and then stimulated with 5 μg/ml S IgE (IgE) or with 20 ng/ml DNP-HSA (after preloading with 0.1 μg/ml S IgE overnight) (IgE + Ag) ± Tyrode’s buffer ± 1.8 mM CaCl2 (no added Ca2+ = −Ca2+), and fluorescence levels were measured. IgE (●), IgE + Ag (■), IgE − Ca2+ (○), IgE + Ag − Ca2+ (□). Fluorescence values shown have had the background fluorescence of unstimulated cells subtracted. D, BMMCs were incubated for 20 min with CM-DCFHDA ± 0.1, 1, or 10 μM AA861 (or vehicle control, DMSO) or 3, 30, or 300 nM FR122047 (or dimethylformamide (DMF)) and then stimulated with 20 ng/ml DNP-HSA (after preloading with 0.1 μg/ml S IgE overnight) (left panel) or with 5 μg/ml S IgE (right panel), and fluorescence levels were measured. Fluorescence values shown have had the background fluorescence of unstimulated cells subtracted. The symbols for both the left and right panels are as follows: stimulus alone (●); stimulus + vehicles; DMF (■); DMSO (△); and stimulus + 0.1 (▲), 1 (△), and 10 (▲) μM AA861 or + 3 (□), 30 (□), and 300 (□) FR122047. E, BMMCs were incubated for 20 min with CM-DCFHDA ± 10 μM AA861 (or vehicle control, DMSO). 300 nM FR122047 (or DMF), or both, and then stimulated with 20 ng/ml DNP-HSA (after preloading with 0.1 μg/ml S IgE overnight) (left panel) or with 5 μg/ml S IgE (right panel), and fluorescence levels were measured. Fluorescence values shown have had the background fluorescence of unstimulated cells subtracted. The symbols for both the left and right panels are IgE (○), IgE with the vehicles, DMF (□), DMSO (△), and both (224), or with AA861 (■), FR122047 (▲), or both (●). Similar results were obtained in three separate experiments for A–D and two separate experiments for E.
We found that 10 μM AA861 markedly inhibited, and 300 nM FR122047 slightly inhibited, IgE+Ag-induced ROS production (Fig. 4D, left panel), consistent with prior results (30). However, whereas FR122047 had a similar inhibitory effect on IgE-induced ROS, AA861 had negligible effects (Fig. 4D, right panel). We then retested these two inhibitors at these concentrations, alone and together, on IgE+Ag vs IgE-induced ROS production and found that whereas AA861 plus FR122047 dramatically inhibited IgE+Ag-induced ROS (Fig. 4E, left panel), they had very little effect on IgE-induced ROS (Fig. 4E, right panel).

**Prolonged ROS generation is required for IL-3 production**

To explore the role of IgE-induced ROS further, we asked whether the level of ROS generated by the five IgEs correlated with their ability to enhance BMMC survival. Similar to our Ca^{2+} influx results, the five IgEs triggered a slow, prolonged increase in ROS, with the rate of ROS generation by each IgE correlating with survival-enhancing ability (Fig. 5A). We then asked whether this IgE-induced ROS was important to subsequent signaling events implicated in BMMC survival. For these experiments, we first conducted preliminary dose-response studies with two ROS scavengers, NAC, a glutathione precursor that alters the intracellular redox balance, and ebselen, which selectively scavenges H_2O_2 (31). We found that at 2.5 mM NAC and 1.5 mM ebselen, S IgE-induced ROS generation was completely blocked (Fig. 5B). We then used these inhibitors to determine whether they could block the prolonged phosphorylation of ERK obtained with IgE alone. As can be seen in the upper panel of Fig. 5C, the presence of ebselen or NAC had no effect on the early phosphorylation of ERK (i.e., at 5 min), but markedly inhibited phosphorylation at later times (Fig. 5C, bottom panel). These findings suggest that prolonged ROS generation is required for IL-3 production.
fluorescence levels were measured. A total of 25 ng/ml IL-4 ( ), or with 50 ng/ml IL-4 ( ), and then stimulated with 5 μg/ml S IgE (left panel) or 20 ng/ml DNP-HSA (after overnight incubation with 0.1 μg/ml S IgE) (right panel) for the indicated times, and fluorescence levels were measured. B, BMMCs were incubated for 20 min with CM-DCFHDA ( ), or with 25 ng/ml UO126 ( ), and then stimulated with 5 μg/ml S IgE (left panel) or 20 ng/ml DNP-HSA (after overnight incubation with 0.1 μg/ml S IgE) (right panel), or with 50 ng/ml IL-4 ( ), or with 50 ng/ml IL-4 ( ), and fluorescence levels were measured. A total of 25 μM UO126 alone yielded no detectable ROS. C, BMMCs were stimulated for 3 h with 5 μg/ml S IgE ( ), or with 20 ng/ml DNP-HSA (after overnight incubation with 0.1 μg/ml S IgE (IgE-Ag), or with 50 ng/ml IL-4 ( ), and fluorescence levels were measured. A total of 25 μM UO126 alone yielded no detectable ROS. C, BMMCs were stimulated for 3 h with 5 μg/ml S IgE (IgE) or with 20 ng/ml DNP-HSA (after overnight incubation with 0.1 μg/ml S IgE (IgE-Ag) in the presence of the indicated concentrations of H2O2. These levels of H2O2 did not affect the IL-3 ELISA. The conditioned medium was then subjected to an IL-3 ELISA. NS = not significantly different from IgE alone. *p < 0.01 vs IgE alone. **p < 0.002 vs IgE+Ag alone. The mean ± SEM from three independent experiments, each assayed in duplicate.

Discussion

Because the initial observation that mIgE alone could enhance mast cell survival (4, 5), a number of groups have made considerable progress in elucidating the intracellular pathways responsible for this phenomenon (6–8, 11, 33–36). Based on their studies

times. These two inhibitors also inhibited the more abbreviated IgE+Ag-induced ERK phosphorylation at 15 min, but had less of an effect at the 5-min time point (lower panel of Fig. 5C). NAC was then tested at various concentrations for its effect on IgE-induced Ca2+ influx. As shown in Fig. 5D, 2 mM NAC completely blocked Ca2+ entry. To determine whether blocking ROS production had an impact on IgE-induced production of IL-3, we then added ebselen and NAC to IgE-stimulated BMMCs and found both inhibitors completely blocked IL-3 production (Fig. 5E).

To further explore the signaling pathways upstream of IgE- and IgE+Ag-induced ROS production, we tested various pathway inhibitors at the lowest concentration that totally ablates their target pathway in BMMCs (4). Not surprisingly, the Src family inhibitor, PP2, potently inhibited both IgE (Fig. 6A, left panel) and IgE-Ag (Fig. 6A, right panel) induced ROS production, consistent with Lyn initiating signaling in response to both stimuli and with our Lyn−/− BMMC results (Fig. 2A). Interestingly, the phospholipase C (PLC)γ inhibitor, U73122, also markedly inhibited both IgE- and IgE+Ag-induced ROS production (Fig. 6A), consistent with our earlier studies (Fig. 4C) showing the importance of extracellular Ca2+ influx to ROS generation. In contrast, the p38 and JNK inhibitors, SB203580 and SP600125, respectively, had little effect. Surprisingly, however, UO126 markedly inhibited both IgE- and IgE+Ag-induced ROS production. Given our assumption that ERK phosphorylation was downstream of ROS generation, we wanted to confirm that UO126 was specific for MEK and was not inhibiting ROS generation via off target effects. To assess this, we first asked whether IL-4 induced ROS in BMMCs, and found that it did (Fig. 6B). Because it is well established that IL-4 does not stimulate ERK phosphorylation in BMMCs (32), we then tested the effect of UO126 on IL-4-induced ROS production and found no inhibition (Fig. 6B). This was consistent with UO126 inhibiting IgE-induced ROS generation via inhibition of MEK and not via nonspecific effects on ROS generation.

Lastly, we asked whether the inability of IgE+Ag to produce IL-3 (and thus enhance BMMC survival) might be due to the abbreviated ROS that it elicited. To test this, we added various concentrations of H2O2 to IgE+Ag-stimulated vs IgE-stimulated BMMCs. As shown in Fig. 6C, addition of H2O2 slightly decreased IgE-induced IL-3 while dramatically increasing IgE-Ag-induced IL-3 production. Of note, H2O2 alone did not stimulate the production of any detectable IL-3 (data not shown). Also of note is that whereas H2O2 did not cause any detectable toxicity during the 3 h incubation with BMMCs, the fact that it reduced IgE-induced IL-3 production could suggest that the level of IL-3 that we observe when H2O2 is added to IgE+Ag-treated BMMCs is an underestimate of the level obtainable.
FIGURE 7. Proposed model for IgE-induced BMMC survival. A low, but constant mIgE-induced activation of the FceRI (1) leads to low-level Lyn activation (2) and the subsequent phosphorylation of Syk, LAT, and PLCγ (3). PLCγ-mediated intracellular Ca2+ release stimulates (4) both an initial activation of a Ca2+-responsive member of the NOX family and extracellular Ca2+ entry. The influx of extracellular Ca2+ and the initial generation of ROS act in a cooperative loop to amplify each other’s signal via (5) Ca2+-induced activation of the NOX family of NADPH oxidases and by ROS-induced inactivation of a negative protein tyrosine phosphatase (PTPase) regulator of calcium release activated calcium channel (CRAC) (6). The constant production of ROS ensures that both the PTPases that normally dephosphorylate Lyn, Syk, and LAT (and perhaps the β and γ ITAMs) and the MKPs that dephosphorylate ERK and JNK remain inactive (allowing for higher AP-1 levels) (7). Activated MEK or downstream molecules in this pathway also act in a positive feedback loop to enhance ROS production. The prolonged extracellular Ca2+ influx leads to dephosphorylation of NF-AT and its entry into the nucleus to enhance, together with AP-1, (8) the transcription of IL-3. IL-3 then acts in an autocrine fashion to (9) increase prosurvival Bcl-2 family members.

and the results presented in this study, we would like to propose a model (Fig. 7) in which mIgEs trigger a slow, asynchronous FceRI aggregation by an as yet unidentified mechanism (see Ref. 3). Our finding that a synchronous, rapid aggregation of very few IgE/FceRI complexes via multivalent Ag does not mimic the effects of mIgE alone (Fig. 1A, right panel) suggests that a continuous, low potency activation of IgE-bound receptors, perhaps initiated by interaction with unbound IgE in solution, is required for mediating survival. This latter idea is consistent with elegant studies from Cockcroft and colleagues (37) showing that constant exposure to unbound mIgE in the culture medium is required for a prolonged Ca2+ influx into RBL-2H3 cells, and also with studies in our laboratory (our unpublished observations) and the laboratory of Kawakami and colleagues (5) showing that IgE-induced survival is abrogated if unbound IgE is washed away within 24 h after BMMCs are bound with IgE. This slow, ongoing generation of active IgE/FceRI aggregates, which most likely localize within lipid rafts (4), leads to a low, but continual activation of FceRIβ-associated Lyn, which then tyrosine phosphorylates the FceRI β (35) and γ ITAMs, and this subsequently attracts Lyn and Syk (8), respectively. Although Fyn also associates with the tyrosine-phosphorylated FceRI ITAMs and activates PI3K (38), it is unlikely this plays a role in IgE-induced survival based on Fyn−/− BMMC studies conducted by Kohno et al. (11). PLCγ is then recruited to tyrosine-phosphorylated Syk and becomes phosphorylated/activated, generating IP3, which triggers the release of intracellular Ca2+ from the endoplasmic reticulum and mitochondria. This draining of intracellular Ca2+ leads to both an influx of extracellular Ca2+ (39) and, we propose, an early production of ROS, perhaps via an EF hand-containing member of the NOX family of NADPH oxidases. Specifically, whereas all members of this family are membrane-bound enzymes that transfer electrons from NADPH to O2 to produce superoxide anions (O2−) that are rapidly converted to H2O2 and other ROS, only NOX5, Duox1, and Duox2 contain EF hands and are Ca2+-dependent (reviewed in Ref. 40–42). In support of intracellular Ca2+ release initiating ROS production, we found that PLCγ inhibition markedly inhibits IgE-induced ROS generation (Fig. 6A) and that IgE, even in the absence of extracellular Ca2+, stimulates a low level of ROS (Fig. 4C). We then propose that this initial generation of ROS enhances extracellular Ca2+ entry (because ROS inhibitors block this entry; Fig. 5D), perhaps by inactivating PTP1B, a ROS-inhibitable tyrosine phosphatase involved in negatively regulating extracellular Ca2+ entry in HEK 293 cells (43). Similar to what has been proposed during BCR activation (29), we propose that the extracellular Ca2+ influx and ROS generated by IgE engage in a cooperative interaction to amplify both upstream and downstream signaling. Specifically, we propose that the generation of ROS, which enhances upstream signaling by inactivating receptor-coupled tyrosine phosphatases (which typically contain a redox-regulated cysteine in their catalytic site) (44), allows FceRI β and γ ITAMs to remain phosphorylated longer, and this, in turn, keeps Lyn, Syk, LAT, and non-T cell activation linker tyrosine phosphorylated, enabling the latter two adaptors to continue to activate the Ras pathway (36). In support of this, we have shown previously that the tyrosine phosphorylation of the FceRI β-chain remains at maximal levels 4 h after mIgE exposure, whereas IgE+Ag-induced phosphorylation of this β subunit peaks at 2 min and returns to baseline by 1 h (4). Related to this, Suzuki et al. (45) have reported that IgE+Ag-induced tyrosine phosphorylation of PLCγ and LAT (which is required for extracellular Ca2+ influx) is reduced by inhibiting ROS production.

Importantly, we also propose that ROS enhances downstream signaling by inactivating dual-specificity phosphatases (DUSPs, aka DSPs and MKPs) that dephosphorylate phospho-ERK and phospho-JNK (28, 46). Related to this, we not only show that
prolonged ERK phosphorylation is critical for IgE-induced IL-3 production (Fig. 3B), in agreement with previous reports (10, 36), but, more importantly, that ROS inhibition converts the prolonged ERK phosphorylation and subsequent IL-3 production observed with IgE to the abbreviated ERK phosphorylation and lack of IL-3 production normally observed with IgE + Ag (Fig. 5, C and E). This requirement for a prolonged ERK activation for survival has also been reported for other cell types (47).

In testing the effects of pharmacological inhibitors on IgE-induced ROS, we found that PP2 and the PLC inhibitor, U73122, block ROS generation, in keeping with previous studies showing that tyrosine kinase inhibitors block IgE + Ag-induced ROS (45). Unexpectedly, our studies with U0126, which dramatically suppresses IgE-induced ROS generation (Fig. 6A), and appears to be specific for MEK (Fig. 6B), suggest that activated MEK or downstream molecules in the Ras/ERK pathway also feedback activate ROS generation. As well, we propose that the positive feedback loop that exists between ROS, extracellular Ca2+ entry, and the ERK pathway results in a prolonged Ca2+ influx into mast cells that is critical for the calmodulin/cal-cineurin-mediated dephosphorylation of NFAT and its translocation into the nucleus (37, 48, 49), where it stimulates, together with the Fox/Jun complex (AP-1), the transcription of IL-3 (50, 51). Our earlier finding that thapsigargin enhances BMMC survival (39), coupled with a very recent report that thapsigargin stimulates ROS production in mast cells (52), highlights the importance of increased Ca2+ and ROS levels to BMMC survival. Support for our model comes from both our finding that exogenous H2O2 enables IgE to enhance BMMC survival. Support for our model comes from both our finding that exogenous H2O2 enables IgE to enhance BMMC survival. Support for our model comes from both our finding that exogenous H2O2 enables IgE to enhance BMMC survival. Support for our model comes from both our finding that exogenous H2O2 enables IgE to enhance BMMC survival.

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