

Fc γ RI-Mediated Activation of Human Mast Cells Promotes Survival and Induction of the Pro-survival Gene Bfl-1

Mats Karlberg · Zou Xiang · Gunnar Nilsson

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Abstract Activation of mast cells through either Fc ϵ RI or Fc γ RI leads to release of mediators contributing to the inflammatory response. One of the biologic characteristics of mast cells in allergic pathology is that these cells have the capacity to recover and regranulate after aggregation of Fc ϵ RI. We have previously demonstrated that the pro-survival protein A1/Bfl-1 is required for mast cells to survive IgE-mediated activation. In the present study, we have investigated whether human mast cells show similar induction of *bfl-1* and activation-induced survival after aggregation of Fc γ RI. Human cord blood-derived mast cells were activated by aggregation of either Fc ϵ RI or Fc γ RI, and activation-induced survival and induction of *bfl-1* was measured. We found that aggregation of Fc γ RI-induced expression of *bfl-1* and caused a comparable activation-induced mast cell survival as Fc ϵ RI does. These data suggests that activation through Fc-receptors contrib-

ute to mast cell survival during antibody-dependent mast cell mediated inflammatory responses.

Keywords Bfl-1 · Fc ϵ RI · Fc γ RI · mast cell · survival

Introduction

Mast cells are highly specialized immune cells that are infamous for their pivotal role in allergic reactions [1]. Activation of mast cells by aggregation of the high-affinity receptor for IgE (Fc ϵ RI) leads to degranulation and release of preformed mediators, de novo synthesized pro-inflammatory lipid mediators, and both preformed and de novo synthesized cytokines and chemokines [2]. Antigen-mediated activation of mast cells is not only mediated via aggregation of Fc ϵ RI but can also be mediated via Fc γ RI. Treatment of human mast cells with interferon- γ induces expression of Fc γ RI (CD64), the high-affinity receptor for IgG [3]. Similarly to Fc ϵ RI-activation, aggregation of Fc γ RI leads to degranulation and secretion of lipid mediators and cytokines [4]. Thus, Fc-receptor-mediated activation of mast cells is not only restricted to IgE/T_{H2}-associated diseases but may also occur in T_{H1}-associated diseases where interferon- γ can promote Fc γ RI expression on mast cells that facilitate IgG-mediated mast cell activation.

Mast cells have a comparatively long lifespan in tissues [5], where they participate in inflammatory allergic reactions and in many other physiological and pathophysiological responses [6]. In an allergic reaction, activation of mast cells via aggregation of Fc ϵ RI induces activation-induced mast cell survival [7–9]. Degranulated mast cells are therefore able to recover and regranulate [10–13]. Thus, they can thereby be activated repeatedly and in that way perpetuate the allergic reaction. Activation-induced mast cell survival upon

M. Karlberg · Z. Xiang · G. Nilsson
Department of Genetics and Pathology, Uppsala University,
751 85 Uppsala, Sweden

M. Karlberg · G. Nilsson
Department of Medicine, Karolinska Institutet,
171 76 Stockholm, Sweden

G. Nilsson (✉)
Clinical Immunology and Allergy, Department of Medicine,
Karolinska Institutet,
171 76 Stockholm, Sweden
e-mail: gunnar.p.nilsson@ki.se

Present address:

Z. Xiang
The Cambridge Institute for Medical Research, University of
Cambridge School of Clinical Medicine, Addenbrooke's Hospital,
Cambridge CB2 2XY, UK

FcεRI aggregation is dependent on the pro-survival Bcl-2-family member A1 (mice)/Bfl-1 (human) [7, 14].

In this study, we describe that aggregation of FcγRI, expressed on interferon-γ treated human cord blood derived mast cells (CBMC), exhibit similar activation-induced survival and expression of *bfl-1* as FcεRI-activated mast cells do. Thus, the promotion of mast cell survival after activation is not restricted to FcεRI but takes place also after FcγRI activation.

Methods

Cell Cultures

Human mast cells were cultured from umbilical cord blood CD34⁺ progenitor cells as described [15]. Briefly, these cells were obtained by culturing the cells for 8–10 weeks in RPMI 1640 medium with 10% fetal bovine serum (FBS), stem cell factor (SCF) 50 ng/ml (Biosource) and IL-6 10 ng/ml (PeproTech). The medium was changed weekly. Cultures were maintained at 37°C and 5% CO₂.

For high-affinity IgG receptor-dependent activation, mast cells were preincubated with interferon gamma (IFNγ) 15 ng/ml (PeproTech) for 24, 48, and 72 h. In one experiment, different concentrations of IFNγ were used (3, 15, 75, and 150 ng/ml). Cells were washed twice in PBS + 0.5%BSA, 0.1%NaN₃ before adding either FITC-conjugated anti-human CD64 (PharMingen) or FITC-conjugated mouse IgG1 negative control (Dako) as isotype control. The expression of FcγRI (CD64) was measured by flow cytometry analysis using a FACScan and Cell Quest software (Becton Dickinson).

Fc-Receptor Activation

Cells were incubated with F(ab')₂ mouse anti-human FcγRI mAb, 1 μg/ml (clone 32.2, Medarex East Annadale, NJ, USA) for 30 min at 37°C or human myeloma IgE (ND; kindly provided by Dr. Torbjörn Karlsson, PhaDia, Uppsala, Sweden), 1 μg/ml, for 16 h at 37°C. The cells were washed twice in PBS and resuspended in serum-free medium, containing 100 ng/ml SCF and 10 ng/ml IL-6. For cross-linking of FcγRI, mast cells were exposed to F(ab')₂ goat anti-mouse IgG, 1 or 10 μg/ml (Jackson ImmunoResearch). For cross-linking of FcεRI, a rabbit-anti-human IgE Ab, 2 μg/ml (provided by Dr. Torbjörn Karlsson) was used. Cells were incubated at 37°C for an additional 30 min for β-hexosaminidase release analysis or 4–5 h for isolation of RNA. For detection of β-hexosaminidase, an enzymatic colorimetric assay was used as described [16]. All non-treated cells were neither stimulated with IFNγ nor activated by cross-linking of FcγRI.

Cell Survival Assay

Cell survival was analyzed by fluorescence-activated cell sorting (FACS) analysis of propidium iodide (PI) uptake. The cells were kept under starvation of FBS and the cytokines IL-6 and SCF. Mast cells were either left untreated or activated by aggregation of FcεRI or FcγRI at the same time, as they were put under starvation. CBMC activated by FcγRI aggregation (IgGR CL) were pre-stimulated with IFNγ 15 ng/ml for 24 h. IgG1κ (PharMingen) 1 μg/ml was used as isotype control for sensitizing F(ab')₂ mouse anti-human FcγRI mAb. Both isotype CL and IgGR CL were cross-linked by F(ab')₂ goat anti-mouse IgG, 10 μg/ml. Cell viability was analyzed by PI exclusion after 38 h of starvation or after 12, 16, and 20 h of starvation for the kinetic study.

RNA Expression Analysis

RNA was isolated by TriPure isolation reagent (Roche Diagnostics GmbH) and reversibly transcribed into single-stranded complementary DNA (cDNA) using a First-Strand cDNA Synthesis Kit for RT-PCR (AMV) according to the manufacturer's protocol (Roche Diagnostics GmbH). The cDNA was amplified using PCR Core Kit (Roche Diagnostics GmbH) with specific primers for the Bfl-1 gene and glyceraldehyde-3-phosphate dehydrogenase [14]. The polymerase chain reaction (PCR) products were visualized by electrophoreses using 2% agarose gel containing 0.5 μg/ml ethidium bromide. RNase protection assay was performed according to RiboQuant System protocol, using the hAPO-2c probe template set (PharMingen), as previously described [14].

Results

Mast Cells Upregulate FcγRI upon Treatment by IFNγ

It has previously been reported that the high-affinity receptor for IgG, FcγRI (CD64), is present in low amounts on the cell membrane of mast cells, but after IFNγ stimulation, the number of receptors increases [3]. To confirm these results, the expression of FcγRI on CBMCs after treatment with IFNγ was analyzed by flow cytometry. As shown in Fig. 1, we could demonstrate an upregulation of FcγRI on IFNγ-treated cells compared to non-treated cells. To analyze if the upregulation of FcγRI is time dependent, a kinetic study was performed where mast cells were treated with IFNγ for 24, 48, or 72 h. The upregulation of FcγRI was detected already after 24 h, and it did not differ much from the upregulation after 48 h (Fig. 1b). After 72 h of IFNγ-treatment, the upregulation of

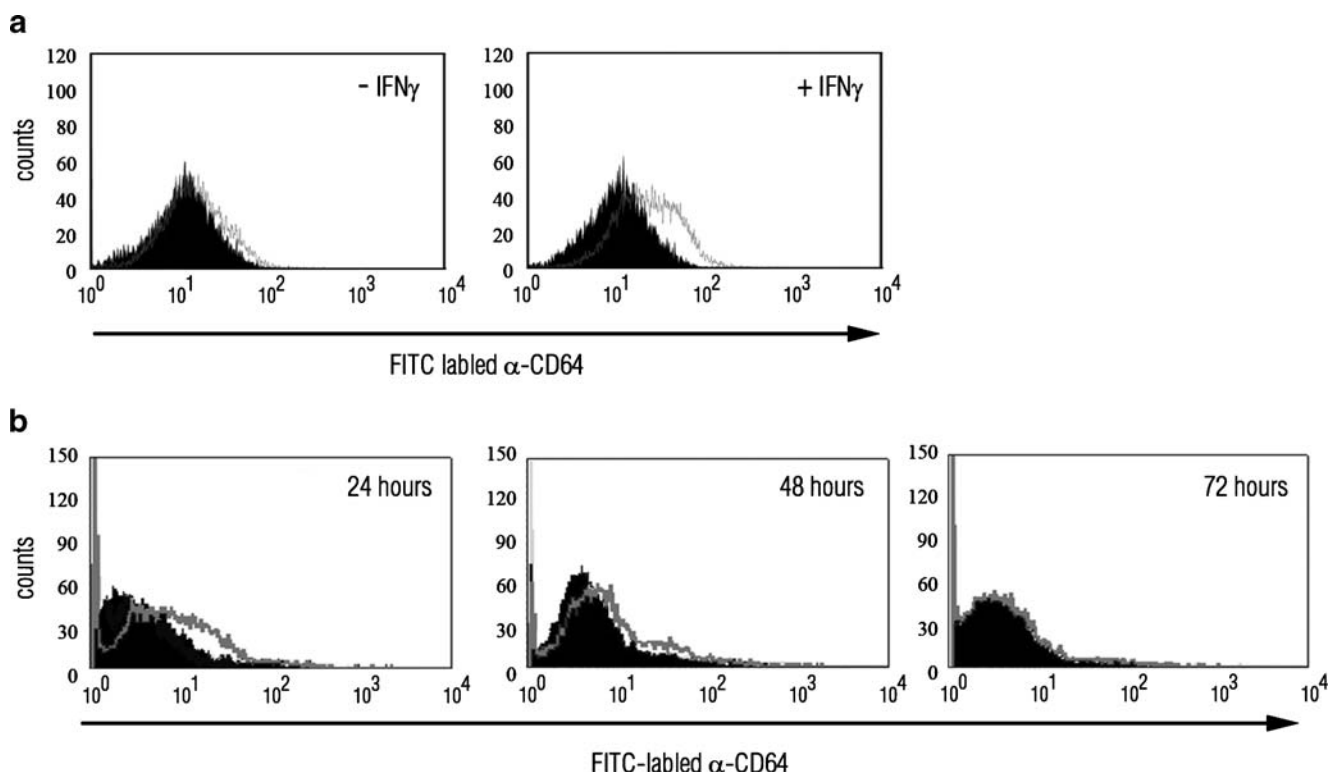


Fig. 1 IFN γ treatment induced an upregulation of Fc γ RI on CBMC. **a** CBMC treated with IFN γ , 15 ng/ml (*right*) for 24 h showed enhanced expression of Fc γ RI (CD64) compared to non-treated CBMC (*left*). *Open gray peaks* FITC-labeled α -CD64; *filled peaks* isotype control. **b** Kinetics of Fc γ RI expression. The induced

upregulation of Fc γ RI decreased again after 48 h, and at 72 h, the expression levels were equal to non-stimulated CBMC. *Filled peaks* Non-stimulated CBMC; *open gray peaks* IFN γ -stimulated CBMC. Data are from representative experiments from three donors.

Fc γ RI had declined. As the difference in upregulation of Fc γ RI expression after 24 and 48 h was small, we chose to use IFN γ treatment for 24 h for the study. A dose-response experiment with different concentrations of IFN γ ranging from 3 to 150 ng/ml was also performed. The upregulation of Fc γ RI after stimulation with IFN γ did not differ between the concentrations tested (data not shown).

Fc γ RI Aggregation Induces Degranulation in IFN γ -Treated Mast Cells

As treatment of CBMC with IFN γ for 24 h showed an upregulation of Fc γ RI, we next investigated if aggregation of Fc γ RI induced mast cell degranulation. IFN γ -treated mast cells were first sensitized with F(ab')₂ mouse anti-human Fc γ RI mAb. Activation was then induced through cross-linking of two anti-Fc γ RI mAb by a F(ab')₂ goat anti-mouse mAb. Degranulation was confirmed by measuring the release of β -hexosaminidase (β -hex) in the supernatant. As shown in Fig. 2, the release of β -hex is highly increased from either Fc γ RI- or Fc ϵ RI-activated mast cells compared to mast cells not stimulated with IFN γ or activated by Fc receptor cross-linking. IgGR CL without IFN γ stimulation induced an insignificant release of β -hexosaminidase.

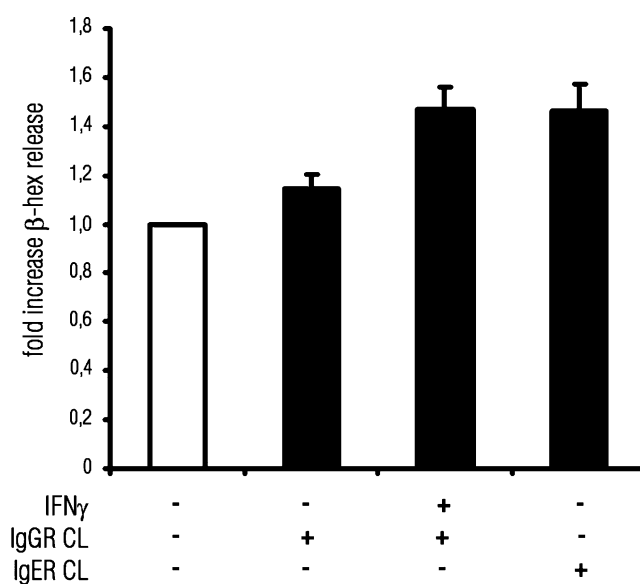


Fig. 2 Degranulation measured by release of β -hexosaminidase (β -hex) from Fc-receptor activated CBMCs. Activation either via IgG receptor cross-linking (*IgGR CL*) or IgE receptor cross-linking (*IgER CL*) induced an equally strong release of β -hex. CBMC activated via IgGR CL were treated with IFN γ , 15 ng/ml, 24 h before activation. Data are presented as mean fold increase ($\pm$ SEM) of β -hex release compared to spontaneous release from non-treated, non-activated CBMC (*white bar*) from three to five different donors.

Activation-Induced Mast Cell Survival upon Aggregation of FcγRI

We and others have previously described that aggregation of FcεRI promotes mast cell survival [5, 7–9, 14]. Withdrawal of SCF induces apoptosis in mast cells in vitro as well as in vivo [17, 18], but upon FcεRI-aggregation, they survive better than non-stimulated cells. As shown in Fig. 3, activation of CBMC, pre-treated with IFNγ for 24 h, by aggregation of FcγRI promotes cell survival as detected by propidium iodide exclusion at 38 h in serum- and cytokine-deprived medium. The survival effect was not detected in either IFNγ-treated or non-stimulated mast cells being sensitized with a non-binding IgG1κ isotype control followed by cross-linking. Kinetic analysis show increased cell viability of IFNγ-treated mast cells activated by FcγRI aggregation compared to non-treated, non-activated mast cells in all donors at 12–20 h of serum and cytokine starvation (Fig. 3b–d). Similar survival data was detected after cross-linking of FcεRI (data not shown).

The Pro-survival Gene *bfl-1* is Upregulated upon FcγRI Activation

The pro-survival protein A1 has been shown to be upregulated in IgE-activated mouse mast cells [7]. Similarly is the human homologue *bfl-1* upregulated in human mast cells upon FcεRI aggregation [14]. Mast cells deficient in A1 do not exhibit activation-induced survival, proving the importance of this protein for mast cell survival during an allergic reaction [7]. RNA from IFNγ-stimulated human mast cells activated through aggregation of FcγRI was isolated, and the expression of *bfl-1* after 5 h activation was determined by reverse transcriptase (RT)-PCR. A clear induction of *bfl-1* could be observed in activated cells (Fig. 4a). The upregulation of *bfl-1* transcripts was confirmed by a ribonuclease protection assay (RPA; Fig. 4b). Quantification with phosphor-imager analysis revealed a two- to threefold increase of the expression of the *bfl-1* gene (Fig. 4c). Changes in gene expression of other Bcl-2 family members investigated (pro-survival, *bcl-*

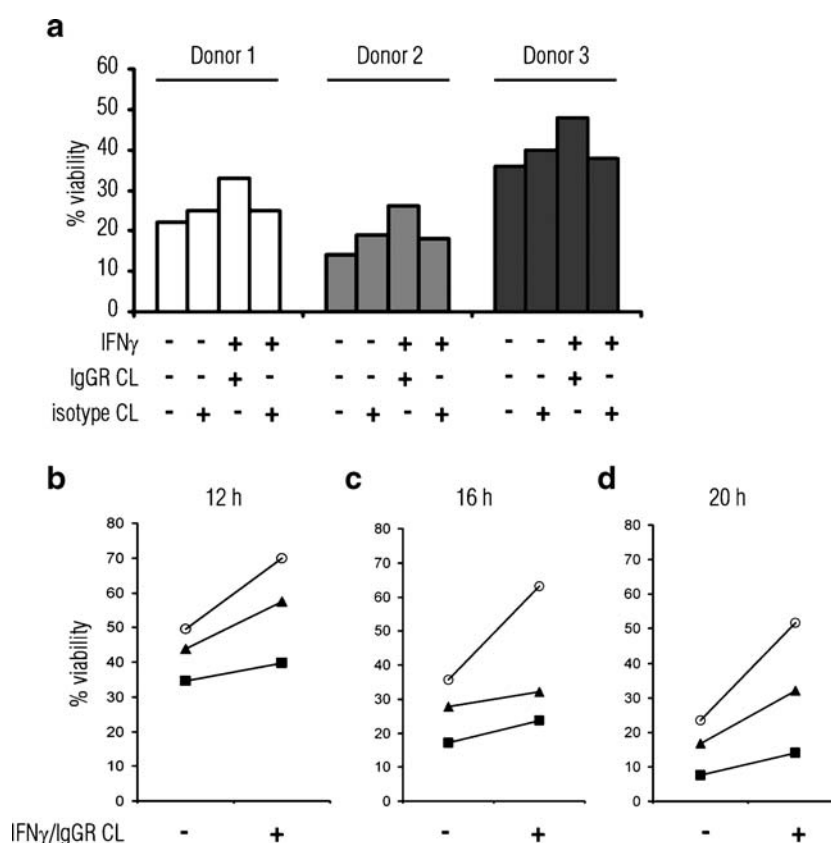


Fig. 3 Activation-induced mast cell survival after aggregation of FcγRI. Apoptosis was induced by withdrawal of SCF, IL-6 and serum. **a** IFNγ stimulated CBMC, sensitized by F(Ab')₂ mouse α-human FcγRI and cross-linked by F(Ab')₂ goat α-mouse IgG (*IgGR CL*) had augmented cell viability compared to non-stimulated/non-activated CBMC. An isotype for the sensitizing F(Ab')₂ was included as control for unspecific cross-linking of F(Ab')₂ goat α-mouse IgG (isotype CL). Viability was measured by PI exclusion at 38 h of

starvation and activation. **b–d** Kinetics of cell viability. IFNγ stimulation 24 h followed by IgGR CL promoted an increased cell survival compared to non-stimulated, non-activated control CBMC analysed after **b** 12 h, **c** 16 h, and **d** 20 h of serum and cytokine starvation. IgGR CL mast cells were pre-treated with IFNγ for 24 h. *Open circles*, *closed triangles*, and *closed squares* represent three different donors dissimilar to the three donors in **a**.

Fig. 4 Gene expression of bcl-2 family members after Fc-receptor activation. **a** Activation of CBMC via IgGR CL for 5 h promoted an upregulation of Bfl-1 mRNA analyzed by RT-PCR. **b** Gene expression of pro-survival and pro-apoptotic members of the bcl-2 family 4 h after activation via IgGR CL or IgE RCL. **c** Quantification of differences in gene expression compared to non-activated CBMC revealed a twofold increase in *bfl-1* in both IgGR CL and IgE RCL cells. Expression of the other genes analyzed did not show any significant changes. In **a**, comparable results were obtained in three separate experiments. In **b** and **c**, the RPA was performed three times. Quantification data is presented as mean ($\pm$ SEM) relative gene expression compared to non-activated cells.

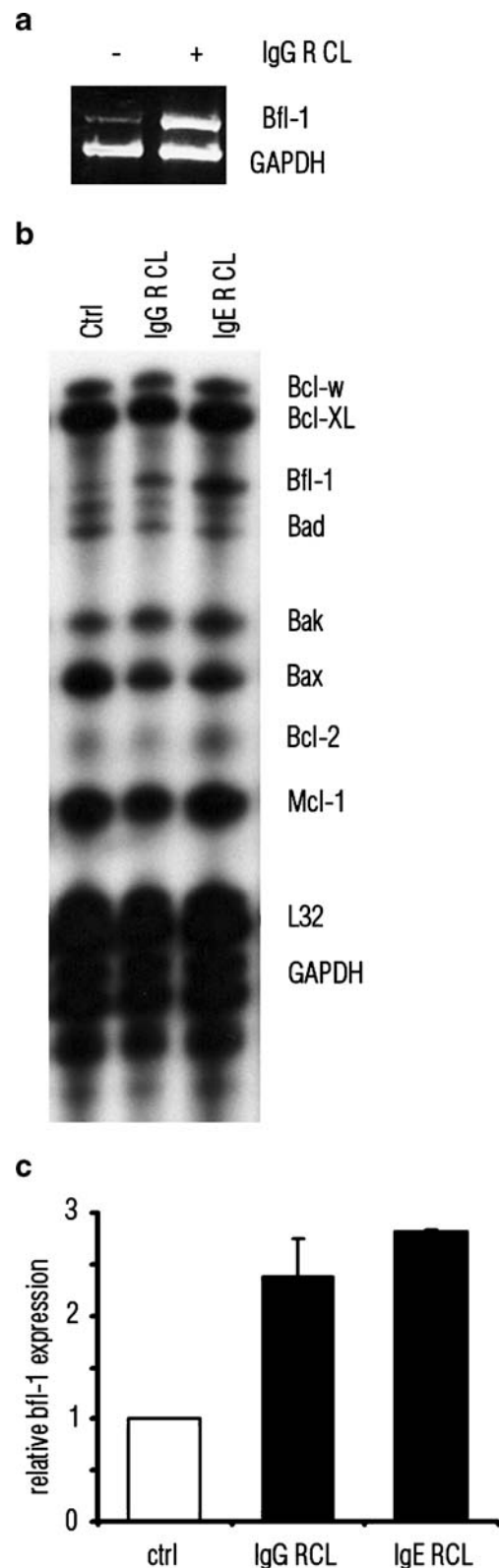
w, *bcl-X_L*, *bcl-2*, and *mcl-1*, and pro-apoptotic, *bad*, *bak*, and *bax*) were too small to be taken as significant evidence for upregulation or down-regulation of respectively gene.

Discussion

In this study, we describe for the first time that Fc γ RI-activated mast cells, similarly to Fc ϵ RI-activated mast cell, exhibit activation-induced survival and expression of the pro-survival gene *bfl-1*. This functionality of mast cells has previously not been analyzed, mainly because rodent mast cells do not express Fc γ RI [19]. However, as described in this study and previously by Okayama et al. [3], IFN γ -treatment of human mast cells induce Fc γ RI expression, thereby making IgG-receptor mediated activation possible. Expression of Fc γ RI on mast cells in situ has been demonstrated in psoriasis, a disease associated with IFN γ [20, 21]. Thus, antigen-mediated activation of mast cells is not only possible in T_{H2}-environments with cytokine secretion promoting IgE-synthesis and upregulation of Fc ϵ RI but also in diseases associated with a T_{H1}-environment with induction of Fc γ RI and IgG production.

Both IgE- and IgG-mediated activation of mast cells cause degranulation, a process that can be followed by recovery and regranulation [10–13]. A1/Bfl-1 appears to be crucial for Fc-mediated activation-induced mast cell survival, as mast cells deficient in A1 does not exhibit activation-induced survival [7], and A1^{-/-} mice have less mast cells and clinical symptoms after allergic provocation (unpublished). A1 is rather unique among the Bcl-2 family members, as it can be transcriptionally regulated via Fc-receptor activation, B- or T-cell-receptor activation [7, 22, 23] and by cytokines [24, 25]. The specific upregulation of A1 in mast cells lacking other Bcl-2 family members also suggests a unique regulation of A1 transcription and possible function ([26] and Ullerås et al., submitted for publication).

Activation-induced mast cell survival is not a general mechanism, promoting survival in mast cells despite triggering mechanisms. As described in this study and previously [7, 14], Fc-receptor activation promote survival



and induction of A1/Bfl-1. Similarly, treatment with ionomycin causing increased intracellular calcium levels has the same effect [7]. In contrast, treatment with the mast cell secretagogue Compound 48/80 does not induce A1 upregulation or promote survival in bone marrow derived murine mast cells [7], neither does adenosine, despite its effect on degranulation [27]. Thus, there appears to be a correlation between Fc-receptor activation and the induction of A1/Bfl-1 and activation-induced survival.

To conclude, antigen-mediated activation of mast cells through aggregation of either FcεRI or FcγRI leads to degranulation, induction of the pro-survival gene *bfl-1*, and promotion of cell survival. These results suggest that activation-induced mast cell survival not only occur in IgE-associated diseases like allergy but also in diseases where IgG is the predominant immunoglobulin.

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