

**Rapid and large amount of autocrine IL-3 production
is responsible for mast cell survival by IgE
in the absence of antigen**

Short title: Autocrine IL-3 supports IgE-mediated BMMC survival

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Abstract

Crosslinking Fc ϵ RI on mast cells by IgE and antigen initiates cascades leading to anti-parasitic or allergic responses. It was recently reported that IgE without antigen (IgE(-Ag)) actively promotes mast cell survival. Although we have demonstrated that the ITAM within FcR γ is essential for IgE(-Ag)-induced mast cell survival, the underlying mechanism remains still unclear. Here, we investigated the mechanism of IgE(-Ag)-induced survival using mast cells lacking several downstream molecules. Lyn and Syk were essential, whereas Fyn, Gab2 and the PI3K-Akt pathway were not critical for survival. Failure of survival in FcR γ ^{-/-} BMMCs was rescued by co-culture with IgE-treated WT BMMCs, suggesting that survival is induced not directly through Fc ϵ RI signals. We found that the survival is predominantly mediated by high production of IL-3, evidenced by severe impairment of survival by anti-IL-3 and in IL-3^{-/-} BMMCs. The upregulation of Bcl-xL/Bcl-2 by IgE was abrogated in IL-3^{-/-} BMMCs, whereas the expression of histidine decarboxylase (HDC) was normally induced. These results indicate that IL-3 plays a crucial role for IgE(-Ag)-induced mast cell

survival, functioning in an autocrine manner by inducing the Bcl-xL/Bcl-2 *via* STAT5. We further suggest that IgE(-Ag)-mediated gene expression in mast cells is regulated at least two mechanisms: autocrine IL-3-dependent and –independent.

Introduction

The high affinity IgE receptor, Fc ϵ RI, exists as a tetramer composed of α and β monomers and a γ homodimer in rodent cells ¹. The β and γ chains contain an immunoreceptor tyrosine-based activation motif (ITAM) within their cytoplasmic domains. Fc ϵ RI can bind to IgE in the absence of Ag. This step has been thought as a passive “sensitization” prior to subsequent Ag crosslinking. However, several works revealed that IgE alone can actively promote upregulation of cell surface Fc ϵ RI ²⁻⁴ and mast cell survival ^{5,6}. Fc ϵ RI upregulation by IgE is caused by stabilization of Fc ϵ RI on the plasma membrane ^{7,8}, which requires the Fc ϵ RI α chain ^{9,10} but not Fc ϵ RI γ -ITAM ¹¹.

On the other hand, we have recently shown that IgE-induced mast cell survival is mediated through ITAM within the FcR γ chain, similar to degranulation by crosslinking by Ag plus IgE (IgE(+Ag)) ¹¹. Furthermore, we have also shown that a weak but sustained FcR γ signal is sufficient for mast cell survival by varying the strength of stimulation *via* a CD8/FcR γ chimera ¹². However, the precise signaling pathway elicited by IgE(-Ag) towards survival remains unclear,

compared with well-characterized IgE(+Ag)-induced signaling. Crosslinking of Fc ϵ RI by IgE(+Ag) initiates an activation signaling cascade *via* tyrosine phosphorylation of ITAMs by Src family protein tyrosine kinase, Lyn. Syk is then recruited to phospho-ITAMs within FcR γ , where it is activated to phosphorylate several key substrates such as LAT and SLP-76¹³. But the involvement of these signaling molecules in IgE(-Ag)-mediated survival has yet to be clarified.

IgE may induce survival signal directly through Fc ϵ RI. In addition to the classical pathway of Fc ϵ RI-mediated signaling described above, Fyn-mediated signal is also reported to be involved in Fc ϵ RI signal transduction¹⁴, through positively regulating the phosphorylation and function of Gab2. The absence of Gab2 severely impairs mast cell responses to IgE(+Ag)¹⁵. Gab2 also induces recruitment and activation of phosphoinositide 3-kinase (PI3K)¹⁶. In most cells, PI3K has a role in cell survival *via* activation of PI3K-dependent kinase 1 (PDK1) and Akt¹⁷, but the contribution of these pathways to IgE-induced survival remains undefined.

As for which effector molecule induces survival, Bcl-xL may play a role since

IgE alone prevents downregulation of Bcl-xL by IL-3 withdrawal ⁶. Whether signaling through FcεRI directly regulates Bcl-xL, however, is still unclear.

Alternatively, IgE-induced survival is recently reported to be mediated by soluble factors but it is still controversial ¹⁸. It is possible that cytokine(s) could contribute to this event, either individually or synergistically, in an autocrine manner ⁶. Although IL-3 and IL-4 are potent growth and survival factors for mast cells ¹⁸, whether these cytokine(s) are produced upon IgE(-Ag) at a level that is for supporting mast cell survival is unknown.

Here, we investigated the signaling pathway that mediates IgE-induced survival using BMMCs or fetal liver-derived mast cells (FLMCs) lacking several downstream molecules. Lyn and Syk were essential, whereas the Fyn, Gab2, PI3K p85α, and Akt pathway were dispensable. We further demonstrated that high level of autocrine production of IL-3 upon IgE(-Ag) stimulation and subsequent IL-3 signals are critical for the induction of Bcl-xL/Bcl-2 and survival in mast cells.

Materials and Methods

Mice

C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). $Fc\gamma R^{-/-}$ C57BL/6 mice were established as we have previously described ¹⁹. $Gab2^{-/-}$ mice were provided by Dr. T. Hirano (Osaka Univ. and RIKEN) ²⁰. $Fyn^{-/-}$ mice were provided by Dr. T. Yagi (Osaka Univ.) through the courtesy of Dr. S. Yuasa (Chiba Univ.) ²¹. $Lyn^{-/-}$ mice were provided by Dr. T. Yamamoto (Univ. of Tokyo) ²². These three mice were C57BL/6 x 129 mixed background. $PI3K\ p85\alpha^{-/-}$ C57BL/6 mice were provided by Dr. S. Koyasu (Keio Univ.) ²³. $Syk^{-/-}$ BALB/c mice ²⁴ were obtained through the courtesy of Dr. H. Yamamura (Kobe Univ.). IL-3-deficient mice were generated by gene targeting in embryonic stem cells which introduced a frameshift mutation into exon 1 of the IL-3 gene and replaced part of exon 3 with a PGK-neo cassette (V. Tybulewicz and P. Mee, unpublished data). Mice bearing the disrupted IL-3 gene were backcrossed to BALB/c for 10 generations, and intercrossed to generate $IL-3^{-/-}$ BALB/c mice.

Mast cell preparation

Bone marrow (BM) cells or fetal liver (FL) cells of each mouse were cultured in the presence of 30 ng/ml of IL-3¹². Four weeks after induction, >95% of cells were c-kit⁺ and FcεRI⁺ except for cells from FcRγ^{-/-} mice.

Construct

cDNA for wild-type and dominant negative forms of Akt (WT, T308/473A and K179D) were kindly provided by Dr. W. Ogawa (Kobe Univ.). Wild-type, constitutively active (CA; H299R, S711F)²⁵ STAT5a cDNA were provided by Dr. H. Wakao (Riken). Bcl-xL cDNA was provided by Dr. K. Eshima (Kitasato Univ.). Each fragment was subcloned into pMX-puro or pMX-IRES-GFP (Dr. T. Kitamura, Univ. of Tokyo). Retroviral-mediated gene transfer to BM cells and mature BMMCs were performed as previously described^{11,12}.

Antibodies

Anti-mouse IL-2R (PC61) and anti-mouse IL-4 (11B11) Abs were purchased

from BD Bioscience Pharmingen (San Diego, CA). Anti-mouse IL-3 polyclonal Ab was obtained from R&D Systems (Minneapolis, MN). Anti-Akt polyclonal Ab and anti-Erk Ab were from Cell Signaling Technology (Beverly, MA) and Promega (Madison, WI), respectively. Anti-dinitrophenyl (DNP) mouse IgE mAb (DNP H1- ϵ -26)²⁶ provided by Dr. F-T Liu was used in all assays unless otherwise described. Anti-TNP mouse IgE mAb, C38-2 and C48-2, were purchased from BD Bioscience (San Jose, CA). Each IgE was ultracentrifugated at 100,000 x g for 10 min to exclude aggregates just before use for assays. The IL-3 ELISA kit was from BD Bioscience Pharmingen.

Survival assays

Survival assays of BMMCs were performed as described previously¹². Briefly, 4×10^5 mast cells were cultured in 400 μ l of IL-3-free medium in the presence or absence of 1 μ g/ml of IgE (DNP H1- ϵ -26) on flat-bottom 48 well plates (FALCON, Lincoln Park, NJ). At indicated days, cells were stained with propidium iodide (PI) and live cells (PI-negative cells) counted by FACScalibur (BD Bioscience, San

Jose, CA).

RT-PCR

Total RNA was isolated from BMMCs and reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). Real time RT-PCR was performed as previously described ¹¹. Primers were as follows; Bcl-xL, (+)5'- AAT GAA CTC TTT CGG GAT GG-3'; (-)5'- CAG CCG CCG TTC TCC TGG AT-3'; Bcl-2, (+)5'- CCG GGA GAA CAG GGT ATG AT-3'; (-)5'- GCA CAG CGG GCA TTG GGT TG-3'; IL-3, (+)5'- ATA GGG AAG CTC CCA GAA CCT GAA CTC-3'; (-)5'-AGA CCC CTG GCA GCG CAG AGT CAT TC-3'; HDC, (+)5'-AGT CTG GCG AGA AGG GAA GG-3'; (-)5'- TCT GGG CAC TCA TAG GCA CA-3'; GAPDH, (+)5'-GCC GGT GCT GAG TAT GTC GT-3'; (-)5'- AGA TGA TGA CCC GTT TGG CT-3'.

Results

FcεRI signaling pathway required for the IgE(-Ag)-induced survival

To examine if FcεRI signaling pathways differ between IgE(+Ag) and IgE(-Ag) stimulation, we analyzed BMMCs lacking a series of signaling molecules which are known to be involved in IgE(+Ag)-induced degranulation. We have recently reported that the ITAM within FcRγ is essential for IgE(-Ag)-induced survival as well as IgE(+Ag)-dependent responses¹¹. ITAMs within the β and γ chains of the FcεRI receptor complex were known to be phosphorylated by Lyn upon IgE(+Ag) stimulation and provide binding sites for Syk²⁷. Lyn and Syk are recently shown as essential kinases for IgE (DNP-H1-ε-26)-induced mast cell survival in the absence of Ag²⁸. We also confirmed that IgE did not induce survival in mast cells lacking Lyn and Syk throughout six days after IL-3 depletion (Fig. 1A, 1B).

Recently, Parravicini et al. showed that Fyn positively controls the phosphorylation and function of Gab2¹⁴, which is required for FcεRI signaling by IgE(+Ag)¹⁵. Since Gab2 is associated with PI3K upon phosphorylation¹⁶, it is possible that the Fyn-Gab2 pathway may contribute to the IgE(-Ag) induced survival through PI3K-Akt activation. However, as shown in Fig. 1C and 1D, mast

cell survival was not impaired by the lack of Fyn or Gab2.

Next, we investigated the involvement of the PI3K-Akt pathway since this is involved in cell survival in various systems. p85 α is a major PI3K expressed in mast cells, and class I_A PI3K activity is almost abrogated in p85 α ^{-/-} mast cells²⁹. Although it is known that stem cell factor (SCF)-induced survival is impaired in p85 α ^{-/-} BMMCs^{29,30}, IgE-mediated mast cell survival was not altered (Fig. 1E). Since non-class I_A PI3Ks might be involved in this process, the contribution of Akt as their downstream molecule was further investigated by introducing dominant negative forms of Akt (AA and KD)³¹ into differentiated BMMCs by retroviral infection. Although mutant Akts were expressed at a several-fold excess compared to endogenous protein (Fig. 1F, left panel), all these transfected cells showed similar levels of survival in response to IgE (Fig. 1F). It should be noted both dominant negative forms of Akt suppressed IL-2 production induced by IgE(+Ag)(data not shown), consistent with previous report³². These results indicate that Fyn, Gab2, PI3K and Akt are not essential for IgE(-Ag)-induced mast cell survival.

IgE(-Ag)-mediated survival is mediated by soluble factor(s)

Next we examined whether IgE(-Ag)-induced survival operates through a direct intracellular signal through Fc ϵ RI or is mediated by secreted soluble factor(s). Fig. 2A shows that FcR $\gamma^{-/-}$ BMMC did not survive upon IgE(-Ag) stimulation as we have previously described ¹¹. However, when we added supernatant from IgE-treated FcR $\gamma^{+/+}$ BMMCs to FcR $\gamma^{-/-}$ BMMCs, significant survival was observed compared to cells given non-treated supernatant (Fig. 2B).

We then reconstituted FcR $\gamma^{-/-}$ BMMCs with a retrovirus vector encoding FcR γ -IRES-GFP. After infection, we stimulated the mixed bulk population cells with IgE in the absence of IL-3 and traced the viability of GFP⁻ (FcR $\gamma^{-/-}$) and GFP⁺ (FcR γ^{+}) cells. If IgE transmits the survival signal directly into Fc ϵ RI-bearing cells, GFP⁻ cells should not survive. However, both GFP⁻ and GFP⁺ cells exhibited comparable survival (Fig. 2C), indicating that survival was mediated indirectly by a factor derived from IgE-bound cells, as recently suggested ^{6,28}. To test whether these factor(s) require cell-cell contact to induce survival, a transwell assay was

performed. BMMCs were cultured in a transwell plate divided into upper and lower chambers by a permeable membrane. IgE was added in the lower chambers and the survival of cells in upper chambers was analyzed as indicated in Fig. 2D. IgE-treated $FcR\gamma^{+/+}$ BMMC clearly supported survival of $FcR\gamma^{-/-}$ BMMCs through permeable membrane (Fig. 2D, left panel), indicating that some kinds of soluble factor(s) cause BMMC survival in the absence of cell-cell contact.

Anti-IL-3 blocked IgE(-Ag)-induced mast cell survival

To investigate the soluble factor(s) responsible for mast cell survival induced by IgE, blocking antibodies against various cytokines were added during the survival assay. Although the addition of anti-IL-2R α and anti-IL-4 showed no inhibition, anti-IL-3 exhibited significant inhibition of IgE-induced survival in a dose dependent manner (Fig. 3A). This inhibition was not due to any direct cytotoxic effect since anti-IL-3 did not affect IL-4-induced survival (data not shown). The inhibitory effect of anti-IL-3 was observed throughout the culture periods (Fig. 3B).

IL-3 is preferentially produced upon IgE(-Ag) stimulation

Although there is a report that a low level of IL-3 mRNA is induced after IgE(+Ag) stimulation ³³, whether IgE(-Ag) also induces substantial levels of IL-3 has not been known. In our assay, IgE(+Ag) induced limited amounts of IL-3, whereas IgE(-Ag) induced substantial amounts of IL-3 (Fig. 3C) sufficient to support BMMC survival alone (Fig. 3D). Although large amounts of IL-6 and TNF were also induced by IgE, as previously reported ⁶, neither of these were sufficient to support mast cell survival (data not shown). The IL-3 production seemed mainly to be regulated at the transcriptional level since IL-3 mRNA was also rapidly induced and reached a peak level as early as 1h after IgE(-Ag) stimulation (Fig. 3E). These kinetics are consistent with the previous report showing that a 4h exposure to IgE is sufficient for enhancing survival ⁶.

IgE-induced survival is impaired in the absence of IL-3

To directly assess the role of endogenous IL-3 in mast cell survival, IL-3^{-/-}

BMMCs were examined. IgE-induced survival was severely impaired in IL-3^{-/-} BMMCs, although exogenous IL-3 supported survival (Fig. 4A). The expression levels of surface FcεRI and secretion of β-hexosaminidase by IgE(+Ag) was not reduced in IL-3^{-/-} BMMCs (data not shown), as previously reported³⁴. We next examined the roles of the IL-4 and IL-9, which can act as potent mast cell growth factors *in vitro*^{35,36} and *in vivo*^{37,38}. Because the receptors for these cytokines utilize a common γ chain (γ_c), we analyzed BMMCs lacking Jak3, an essential PTK associated with the γ_c chain. We have previously established Jak3^{-/-} mice and reported the failure of cytokines that utilize γ_c, including IL-4 and IL-9, to function in these mice^{19,36}. As shown in Fig. 4B, although exogenous IL-4 failed to induce mast cell survival in Jak3^{-/-} BMMCs, IgE induced normal survival comparable to littermate control. These results indicated that IL-3 but not “γ_c cytokines” such as IL-2, 4, 7, 9, 15 and 21, is responsible for the IgE-induced survival. We also examined another IgE clones, C38-2 and C48-2, which are reported as poor cytokine inducers²⁸. Survival was significantly induced, albeit weak, by these IgE clones, and was impaired in IL-3^{-/-} BMMCs (Fig. 4C).

IgE-induced upregulation of Bcl-xL and Bcl-2 was abrogated in IL-3^{-/-} BMMCs

IgE stimulation alone maintains the level of anti-apoptotic protein Bcl-xL after IL-3 withdrawal ⁶ and we also confirmed this in WT BMMCs (Fig. 5A, lane 1-3). However, IgE did not induce Bcl-xL expression in IL-3^{-/-} BMMCs (Fig. 5A, lane 5), although exogenous IL-3 did this normally (Fig. 5A, lane 6). We observed that IgE also upregulated mRNA for Bcl family including Bcl-xL and Bcl-2 in WT BMMCs but not in IL-3^{-/-} BMMCs (Fig. 5B). These results indicated that the upregulation of Bcl family by IgE is mediated through IL-3.

Introduction of Bcl-xL into IL-3^{-/-} BMMCs in a form of IRES-GFP expression vector conferred survival only in a bicistronic GFP⁺ cells, suggesting that Bcl-xL has the potential to induce mast cell survival (Fig. 5C). Consistent with this, STAT5, a known downstream component of the IL-3R signaling pathway, can activate Bcl-xL transcription ³⁹⁻⁴¹. We found that the expression of constitutively active (CA) but not wild-type (WT) STAT5a induced mast cell survival only in

GFP⁺ cells (Fig. 5D). We have previously shown that introduction of sustained Erk activation by active MEK is sufficient for the survival¹². However, survival induction by active MEK was observed not only in bicistronic GFP⁺ (active MEK⁺) cells but also in GFP⁻ (active MEK⁻) cells (Fig. 5E), indicating that Erk activation is required for the secretion of IL-3 but not for the signaling downstream of IL-3R. These results are highly suggestive of an upregulation of the Bcl-xL/Bcl-2 by IgE, mediated through autocrine IL-3 and IL-3R signaling rather than through a direct intracellular signal from FcεRI.

IgE(-Ag) also induces IL-3-independent pathway

Histidine decarboxylase (HDC), an enzyme for histamine synthesis, is also reported to be induced by IgE alone⁴². We asked whether the induction of HDC is also mediated through autocrine IL-3 similarly to Bcl-xL/Bcl-2 and survival. We found that the HDC mRNA was induced normally in IL-3^{-/-} BMMCs (Fig. 5F), indicating that HDC induction does not require IL-3, in clear contrast to Bcl-xL/Bcl-2 induction and survival.

Discussion

In the present study, the molecular mechanism of IgE-induced mast cell survival was investigated using gene-targeted mice.

By analogy with the response to IgE(+Ag)⁴³, IgE(-Ag)-induced survival also requires FcR γ -ITAM¹¹ and Syk²⁸. Lyn is also essential for the IgE-induced survival despite the observation that Lyn^{-/-} BMMCs show normal or increased responses to IgE(+Ag)^{31,44,45}. This has been interpreted to indicate that other Src family PTKs such as Fyn can compensate Lyn function by phosphorylating ITAM upon IgE(+Ag) stimulation⁴⁴. The reason for the differential requirement of Lyn in response to IgE(-Ag) and IgE(+Ag) is unclear. Recent reports suggested that IgE may cause crosslinking of Fc ϵ RI even in the absence of Ag^{12,28}. One possible explanation is that “weak” crosslinking of Fc ϵ RI by IgE may be insufficient to enable Fyn to phosphorylate FcR γ -ITAM instead of Lyn. This is consistent with the recent report that SPE-7, an IgE mAb that induces strong aggregation of Fc ϵ RI, could induce partial survival even in the absence of Lyn²⁸.

Fyn is reported to phosphorylate Gab2¹⁴, which is essential for the Fc ϵ RI

signaling by IgE(+Ag) through the recruitment of PI3K¹⁵. Activation of PI3K is known to be involved in cell survival *via* recruitment and activation of Akt in various cells¹⁷. However, our data demonstrated that Fyn, Gab2, PI3K p85 α and Akt were dispensable for IgE-induced survival.

We confirmed that survival is mediated mainly by a secreted soluble factor rather than direct intracellular signaling from Fc ϵ RI as recently suggested^{6,28}. Kalesnikoff et al. reported that a mixture of possible cytokines including IL-2, IL-3, IL-4, IL-6, IL-13 and TNF α could partially induce mast cell survival⁶. However, among candidate factors, γ_c cytokines including IL-4 or IL-9 were dispensable because Jak3^{-/-} BMMC exhibited normal survival in response to IgE (Fig. 4B). We found IL-3 to be secreted immediately after IgE treatment and that it supports survival of BMMCs.

Since the effect of IgE(-Ag) has recently been reported to be largely dependent on the individual clone of IgE, in addition to three IgE clones described here, we also examined the survival effect of SPE-7 which is reported to induce strong Fc ϵ RI aggregation and mast cell activation²⁸. Survival promotion in the

presence of SPE-7 was also impaired in IL-3^{-/-} mice (unpublished data), suggesting that the role of IL-3 in IgE(-Ag)-induced survival is not limited to particular IgE mAbs.

IgE(-Ag) is reported to induce sustained Erk activation⁶. We have recently reported, using a CD8/FcR γ chimera, that weak but sustained Erk activation favors survival¹². Subsequently, we have found that a large amount of IL-3 is secreted under the condition inducing survival in this system (unpublished data). In T cells, prolonged Erk activation is suggested to be critical for IL-2 production through c-Rel activation⁴⁶. Similarly, sustained Erk signaling may be transmitted to intensive IL-3 transcription in IgE(-Ag)-mediated survival of mast cells. Indeed, sustained Erk activation by active MEK induced enhancement of BMMC survival in a soluble factor-dependent manner. In addition, MEK inhibitor, PD098059, suppressed IgE-induced transcription of IL-3 and Bcl-xL but not IL-3-mediated Bcl-xL transcription (unpublished data).

IgE(-Ag) stimulation maintains Bcl-xL protein expression⁶. We found that IgE(-Ag) induces Bcl-xL expression through an indirect autocrine IL-3 pathway

rather than through direct intracellular downstream signaling of FcεRI.

In the downstream signaling pathway of the IL-3R, inactivation of Bad and transcription of Bcl-xL are regulated by Akt and STAT5, respectively^{39,47,48}. We found Akt activity is not required for IgE(-Ag)-induced survival. In addition, IL-3-induced mast cell survival as well as Bcl-xL/Bcl-2 expression was severely impaired in STAT5a/b-deficient mice⁴⁹. Consistent with this, Bcl-xL or constitutively active STAT5a is sufficient for the induction of mast cell survival. Thus, it is highly conceivable that IgE(-Ag) induces IL-3 production, which leads to upregulation of Bcl-xL through STAT5 activation.

Recently, it has been shown that IgE also triggers HDC induction, which is required for subsequent histamine release upon Ag crosslinking. In contrast to survival, we found that HDC upregulation does not require IL-3, although precise signaling pathway remains to be clarified. Taken from these results together with our previous report¹¹, IgE-induced mast cell responses appear to involve more than one signaling pathway. Upregulation of FcεRI expression following IgE stimulation is independent of FcRγ-ITAM, whereas other responses such as

survival and cytokine production are totally dependent on the ITAM¹¹. FcR γ -ITAM-dependent responses could be further dissected into IL-3-mediated responses such as survival, and IL-3-independent one including HDC induction. Therefore, we suggest that IgE-mediated responses are controlled by multiple mechanisms. Although the present data demonstrate the importance of IL-3 in IgE-induced survival, the contribution of other cytokine(s) cannot be excluded, since slight enhancement of survival was still observed in IL-3^{-/-} BMMCs (Fig. 4A).

IgE-induced mast cell survival has not been clearly verified *in vivo*¹⁸. The normal number of mucosal mast cells found in Fc ϵ RI α ^{-/-} mice indicates that this mechanism is not essential for normal mast cell homeostasis⁵⁰. However, Kitaura et al. recently reported that implantation of IgE-producing hybridoma cells in the mouse peritoneal cavity showed slightly increased survival of mucosal mast cells²⁸. Furthermore, recent works revealed that IgE(-Ag) plays a significant role in immune responses *in vivo*, possibly through chemokine/cytokine production from mast cells⁵¹. In addition, since infection of

helminths or nematodes is also known to induce mastocytosis⁵², the response to parasite infection may reflect more physiological function of IgE-mediated mast cell survival. On the other hand, parasite infection often promotes a rapid increase of non-specific IgE in addition to parasite-specific IgE⁵³. It is possible, therefore, that such IgE may induce mast cell survival in the absence of Ag in specific tissues. Although the role of IgE in parasite-induced mastocytosis is controversial⁵⁴⁻⁵⁶, splenic mastocytosis induced by primary infection of *Trichinella spiralis* is greatly impaired in IgE^{-/-} mice⁵⁷. Furthermore, Lantz et al. reported that mucosal mastocytosis is impaired in IL-3^{-/-} mice during *Strongyloides venezuelensis* infection³⁴. Together, these observations imply that IgE-induced autocrine production of IL-3 from mast cells may support mastocytosis by parasite infection, although its importance may differ depending on the type of parasite⁵⁶.

In humans, the presence of large amounts of total IgE contributes to the exacerbation of atopic diseases⁵⁸, possibly by promoting mast cell survival even in the absence of allergen. The *in vivo* significance of IgE(-Ag)-induced mast cell

survival and the contribution of autocrine growth factors to this situation in humans is now under investigation.

Present analyses suggest that IL-3 may become a possible target for the development of new treatments for allergic diseases, provided that further evidence for its significance in these diseases is clarified.

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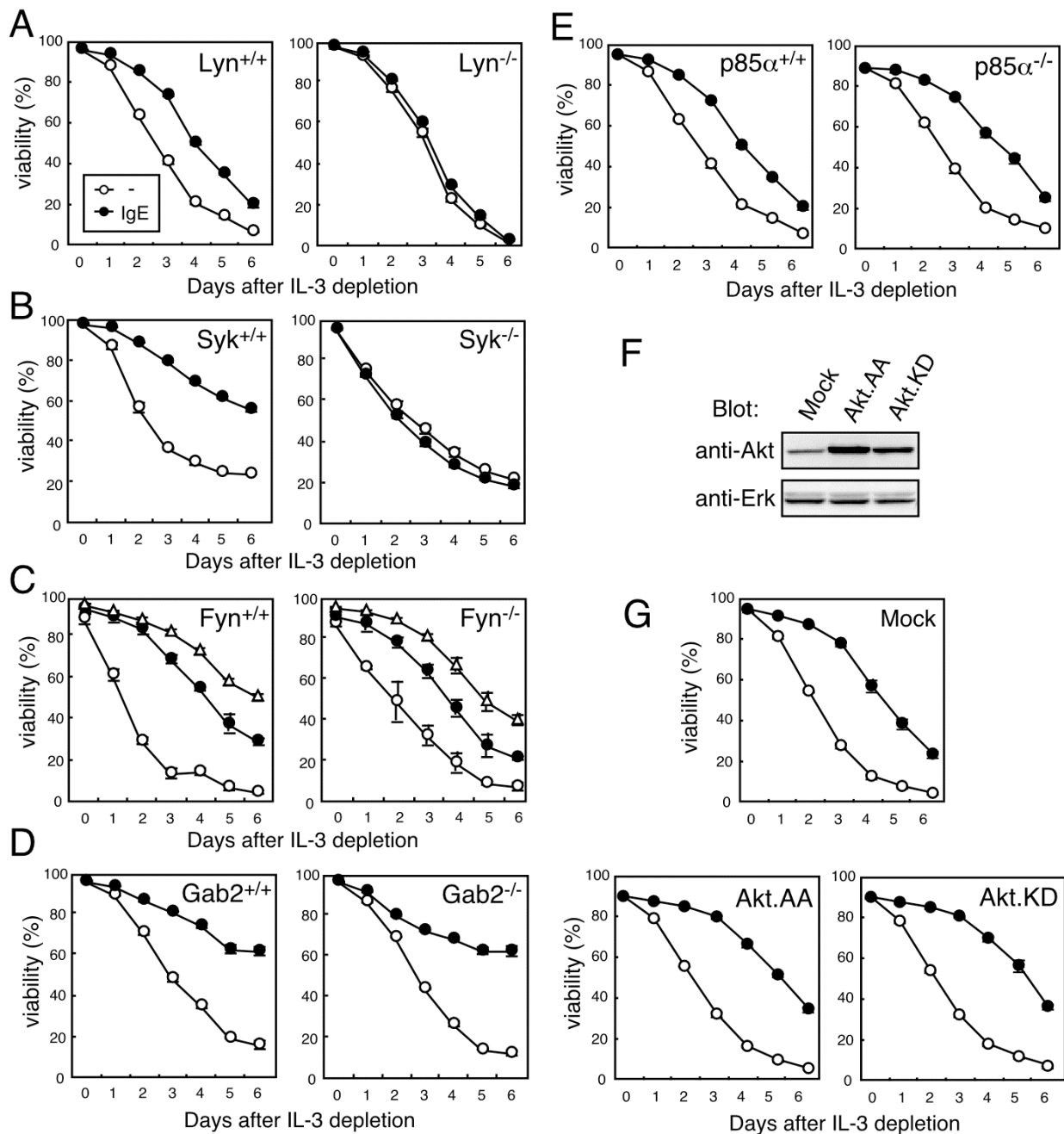


Figure 1. IgE-induced survival in BMMCs or FLMCs lacking Lyn, Syk, Fyn, Gab2 and PI3K p85α. Mast cell survival assay. Mast cells were prepared from bone marrow cells of mice deficient in A) Lyn, C) Fyn, D) Gab2 and E) PI3K p85α and fetal liver cells from B) Syk^{-/-} embryo (E13.5). Mast cell survival was compared with that of cells from littermate wild-type control mice that were prepared and cultured simultaneously as described in Materials and Methods. F) Effect of dominant negative Akt on IgE-induced BMMC survival. BMMCs retrovirally expressing dominant negative Akt (Akt.AA or KD) were blotted with anti-Akt and anti-Erk Abs as a

control (upper panel) and tested for survival (lower three panels). Relative intensity of AA and KD were 6.4 and 3.9 fold higher than that of pMX-puro vector (Mock), respectively.

Data were means $\pm$ SD for triplicated assays. Similar results were obtained from at least two independent mice pairs. IgE concentration was 0 μ g/ml (open circles), 1 μ g/ml (closed circles) and 10 μ g/ml (open triangles).

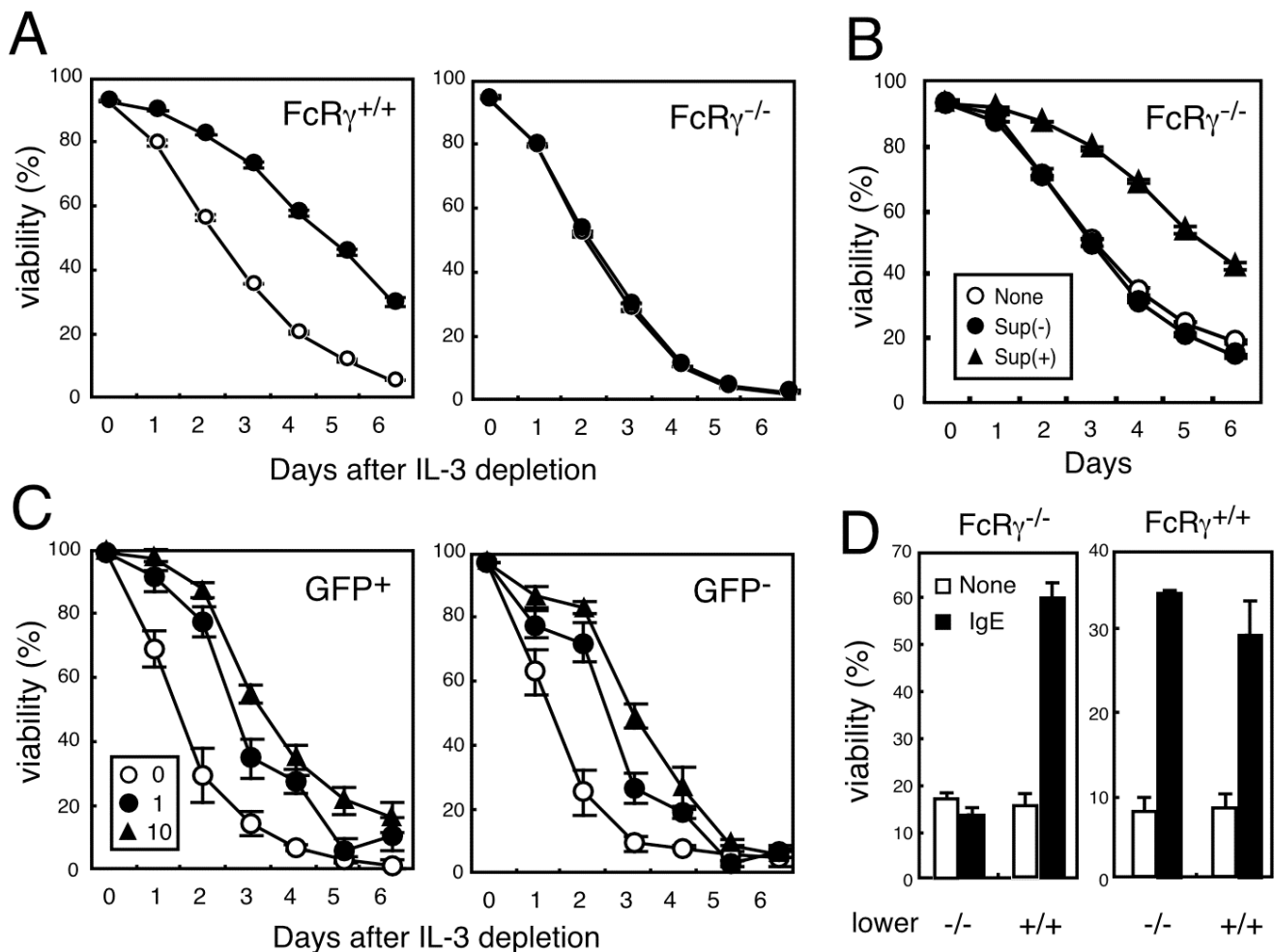


Figure 2. IgE-induced mast cell survival is mediated by soluble factor(s).

A) and B) Survival of $Fc\epsilon R\gamma^{-/-}$ BMMC supplemented with supernatant of IgE(-Ag)-treated $Fc\epsilon R\gamma^{+/+}$ BMMCs. BMMCs from $Fc\epsilon R\gamma^{+/+}$ and $Fc\epsilon R\gamma^{-/-}$ mice were cultured in the absence (A) and presence (B) of 100% supernatant from IgE-treated (1 μ g/ml, Sup(+)) or untreated (Sup(-)) WT BMMC for 12 h, and then tested in the survival assay.

C) IgE-induced survival of $Fc\epsilon RI^{-/-}$ BMMCs when co-cultured with $Fc\epsilon RI^{+/+}$ BMMCs. BMMCs were

infected with pMX-FcR γ -IRES-GFP and the viability of the bulk population were analyzed for both Fc ϵ RI $^{+}$ (GFP $^{+}$) and Fc ϵ RI $^{-}$ (GFP $^{-}$) populations.

D) Co-culture of FcR $\gamma^{-/-}$ and FcR $\gamma^{+/+}$ BMMCs in transwell chambers. Either FcR $\gamma^{-/-}$ or WT (FcR $\gamma^{+/+}$) BMMCs were cultured in upper and lower chambers with indicated combinations in the presence (closed column) or absence (open column) of 1 μ g/ml of IgE. The viability of cells in the upper chamber was analyzed 4 days after IL-3 depletion.

Data are expressed as the mean $\pm$ SD for triplicate assays. Similar results were obtained from three independent experiments.

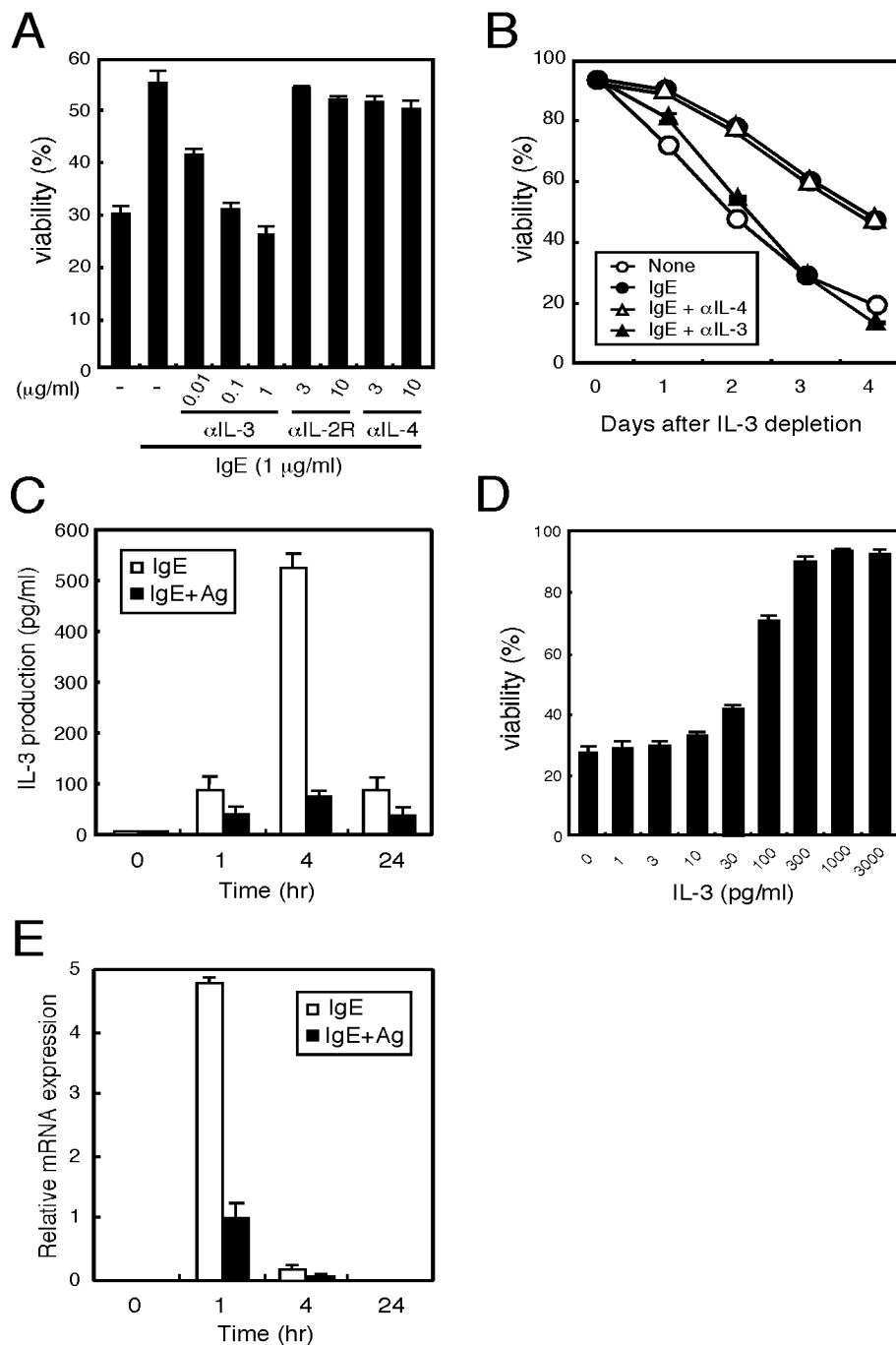


Figure 3. IL-3 is crucial for IgE-induced mast cell survival.

A) Inhibition of IgE(-Ag)-induced survival by anti-IL-3. The survival assay was performed with 1 µg/ml of IgE using WT BMMCs at day 3 of culture in the presence of graded amounts of anti-IL-3, anti-IL-2R or anti-IL-4.

B) Inhibition of IgE(-Ag)-induced survival by anti-IL-3 throughout the culture periods. Survival

assays were performed with 1 μ g/ml of IgE for 4 days in the presence or absence of 1 μ g/ml of anti-IL-3 and 10 μ g/ml of anti-IL-4 Abs.

C) The levels of IL-3 upon stimulation by IgE(-Ag) and IgE(+Ag). WT BMMCs were cultured with 1 μ g/ml of IgE (IgE) or 10 ng/ml of DNP-HSA after sensitization with 10 μ g/ml of IgE at 4°C for 1h (IgE+Ag). At the times indicated, the concentration of IL-3 in the culture supernatants was determined by high sensitivity ELISA.

D) BMMC survival by exogenous IL-3. WT BMMCs were cultured in the presence of graded amount of recombinant IL-3 for 3 days.

E) Induction of IL-3 mRNA upon IgE(-Ag) stimulation. mRNA levels of IL-3 were determined by realtime RT-PCR and expressed as fold induction over the value of IgE(+Ag) at 1h after normalization with the values for GAPDH.

Data are expressed as means $\pm$ SD for triplicate assays. Similar results were obtained from at least three independent experiments.

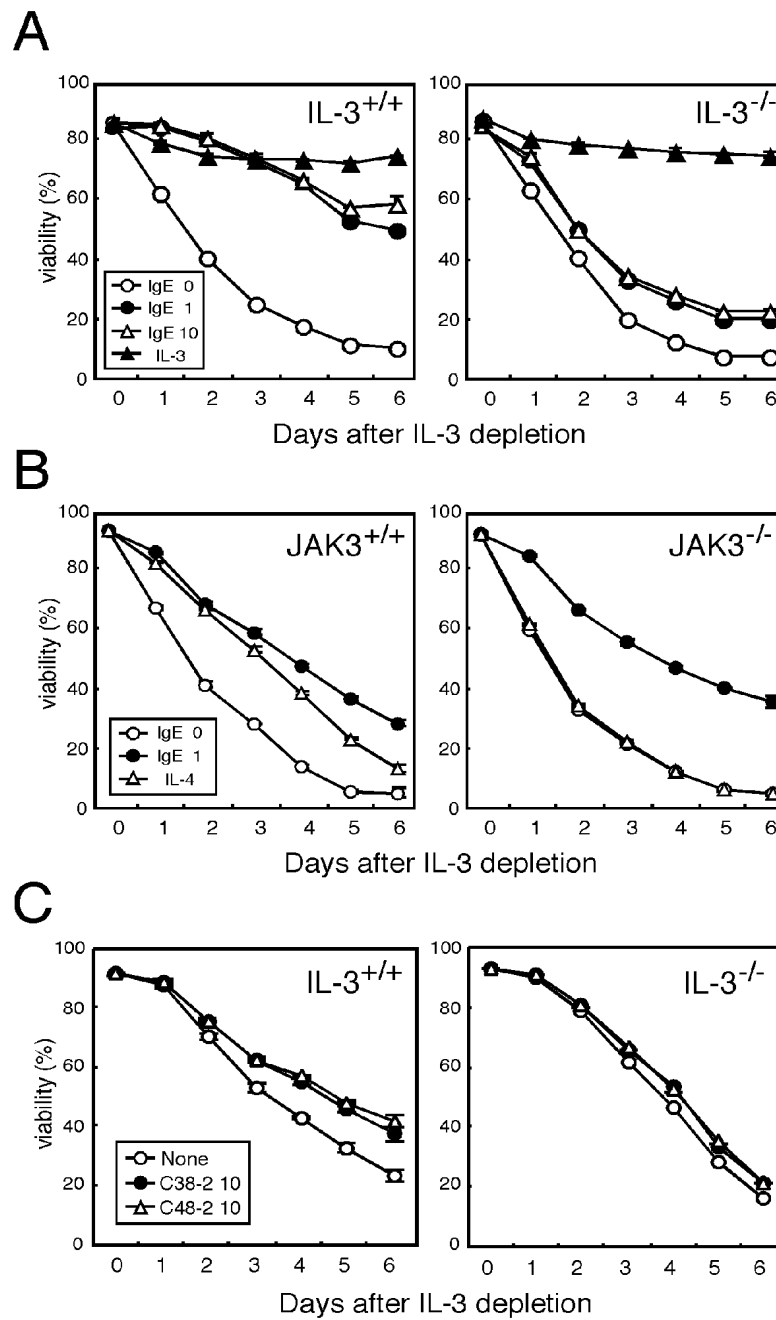


Figure 4. IL-3^{-/-} BMMCs show impaired survival upon IgE stimulation

A) IgE-induced survival of IL-3^{-/-} BMMCs. Survival of BMMCs from WT (IL-3^{+/+}) and IL-3^{-/-} mice was assayed in the presence of 10 ng/ml of IL-3 or IgE (DNP-H1-ε-26)(μg/ml).

B) IgE(-Ag)-induced survival in Jak3^{-/-} BMMC. WT (Jak3^{+/+}) and Jak3^{-/-} BMMC were tested for survival in the presence of IgE or 10 ng/ml of IL-4.

C) Survival of IL-3^{-/-} BMMCs induced by other IgE clones. Survival induced by 10 µg/ml of anti-TNP IgE (C38-2) and anti-TNP IgE (C48-2) was assayed as described in (A).

Data are expressed as means ± SD for triplicate assays. Similar results were obtained from three independent pairs.

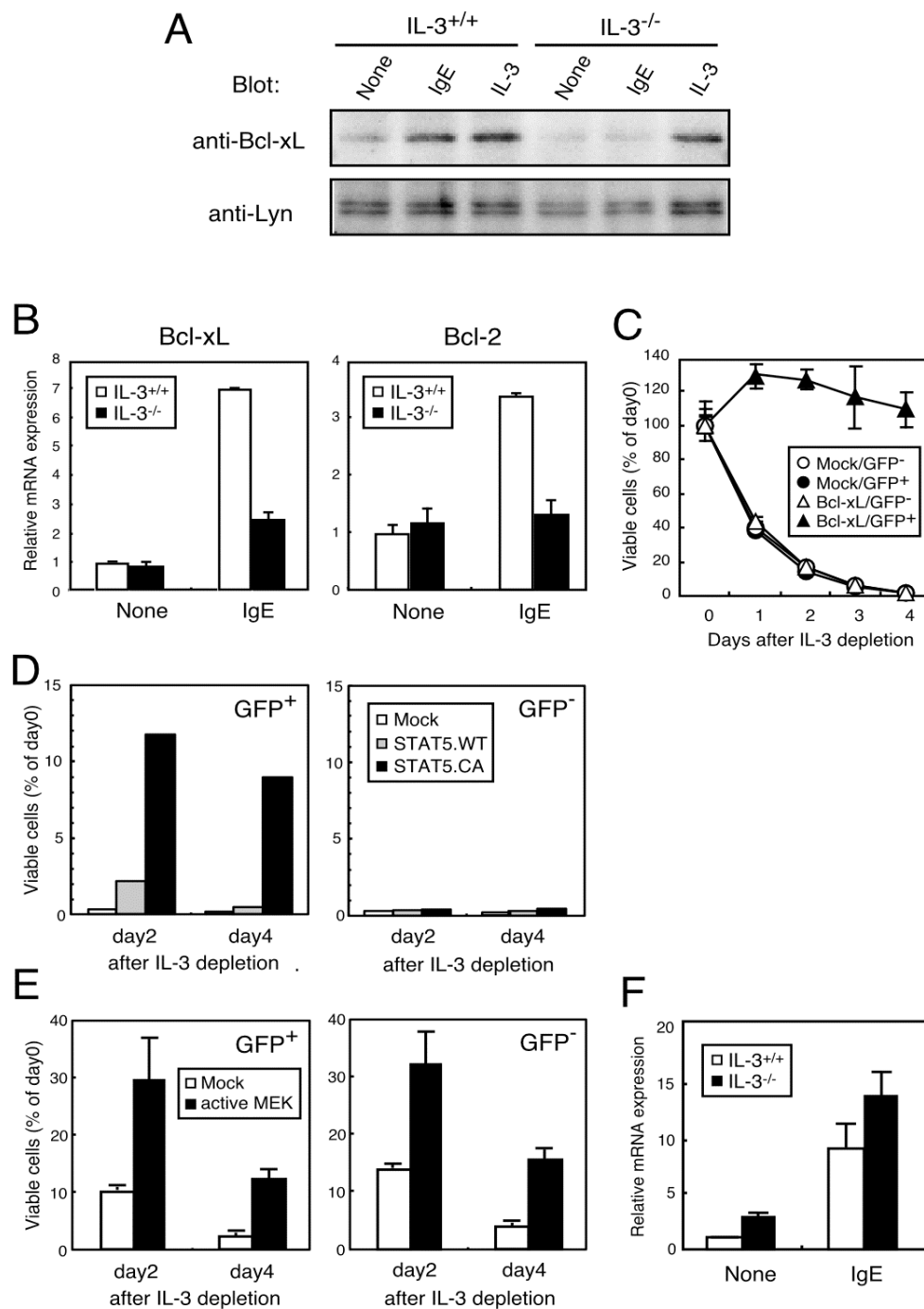


Figure 5. IgE(-Ag)-induced expression of Bcl family are impaired in IL-3^{-/-} BMMC

A) Expression of Bcl-xL protein upon IgE(-Ag) stimulation in IL-3^{+/+} or IL-3^{-/-} BMBCs. BMBCs were cultured with IL-3-depleted medium alone (None), 1 μ g/ml of IgE (IgE) or 10 ng/ml of IL-3 (IL-3) for 24h. Cell lysates were analyzed by Western blotting with anti-Bcl-xL (upper panel) and

anti-Lyn (lower panel) Abs as a control.

B) Expression of Bcl-xL and Bcl-2 upon IgE(-Ag) stimulation in IL-3^{+/+} or IL-3^{-/-} BMMCs. WT BMMCs (open column) and IL-3^{-/-} BMMCs (closed column) were cultured in the absence (None) or presence of 1 µg/ml of IgE (IgE) for 2h. Relative mRNA levels of Bcl-xL and Bcl-2 were determined by realtime RT-PCR and expressed as fold induction over untreated cells after normalization with the values for GAPDH.

C) Bcl-xL expression is sufficient for inducing survival in IL-3^{-/-} BMMCs. Mature IL-3^{-/-} BMMCs were infected using pMX-IRES-GFP retroviral vector either alone (Mock) or together with Bcl-xL (Bcl-xL) as described in Materials and Methods. 1 day after infection, cells were cultured in the absence of IL-3 and cell viability analyzed for GFP positive (left panel) or GFP negative (right panel) population at the indicated periods.

D) STAT5 activation is sufficient for inducing mast cell survival. Mature wild-type BMMCs were infected with vector alone (Mock), wild-type STAT5a (WT), or constitutively active STAT5a (STAT5a.CA). Cell viability was analyzed for GFP positive (left panel) or GFP negative (right panel) population at indicated periods.

E) Indirect effect of active MEK on BMMC survival. Wild-type BMMCs were infected with vector alone (Mock) or MEK.dSESE (active MEK). Cell viability was analyzed as described in (D).

F) Expression of HDC mRNA upon IgE(-Ag) stimulation in IL-3^{+/+} or IL-3^{-/-} BMMCs. Each BMMCs were cultured as described in B). Relative mRNA expression was determined as indicated in B).