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Mast cell activation through co-aggregation of Fc-receptors and its impact on survival

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Abstract

The aggregation of high affinity IgE receptor FcεRI on a mast cell, activates the cell, leading to the release of inflammatory mediators contributing to acute and late phase allergic responses. Co-aggregating FcεRI with FcγRIIB, a low affinity receptor for IgG, have been shown to inhibit IgE induced release of inflammatory mediators. The hypothesis that the co-aggregation of receptors FcεRI and FcγRIIB initiates mast cell apoptosis was investigated. The activation and induction of Akt, FKHRL1, Bim and A1 protein, all associated with the regulation of mast cell survival were also studied. The hypothesis that co-aggregation of FcεRI and FcγRIIB would induce apoptosis in mast cells could not be proven but there were differences seen in the activation and induction of some of the signal proteins investigated.

Keywords
Mast cell activation, FcεRI, FcγRIIB, Mast cell apoptosis, Pro and anti-apoptotic proteins

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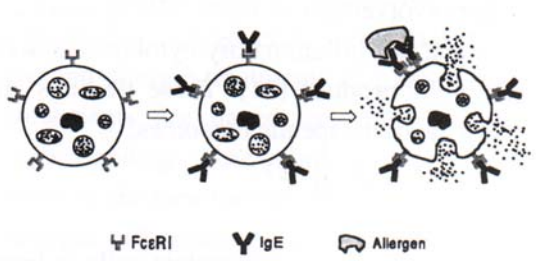
Mast cell activation through co-aggregation of Fc-receptors and its impact on survival

Maria Ekoff

Sammanfattning

Astma och allergi är två folksjukdomar som blir allt vanligare. Symptomen som uppstår vid en astma- eller allergiattack beror på aktiveringen utav en vit blodkropp, kallad mastcell. På mastcellens yta uttrycks receptorer som binder olika immunoglobuliner. Dessa immunoglobuliner binder till ett antigen som kan vara en allergiframkallande partikel. Om receptorer för immunoglobulin E (IgE) kallade FcεRI korsbinds enligt figuren, aktiveras mastcellen och frisätter inflammatoriska substanser som orsakar symptomen vi associerar med allergi. Aktiveringen som sker när FcεRI korsbinds kan inhiberas genom att FcεRI istället korsbinds till en receptor för immunoglobulin G (IgG) kallad FcγRIIB.



I detta examensarbete har jag undersökt om korsbindningen av FcεRI och FcγRIIB leder till ökad apoptos, så kallad programmerad självdöd. Dessutom har aktiveringen och uttrycket för signalproteinerna Akt, FKHRL1, Bim och A1, alla inblandade vid överlevnad och apoptos i mastcellen, studerats efter korsbindandet av FcεRI och FcγRIIB.

Resultaten visar att även om aktivering förhindras vid korsbindandet av FcεRI och FcγRIIB, så har inte ökad apoptos kunnat påvisas. Skillnader men även likheter i aktiveringen och uttrycket av proteinerna Akt, FKHRL1, Bim och A1 har påvisats och betydelsen återstår att undersöka. Korsbindandet av olika receptorer på mastcellens ytan och påverkan av signalvägar i mastcellen efter aktivering ger oss mer kunskap om de bakomliggande orsakerna till mastcellens överlevnad respektive apoptos. Detta kan vara betydelsefullt vid utvecklandet av nya och förbättrade behandlingsmetoder för allergi och astma.

Examensarbete 20p Molekylär bioteknikprogrammet

Uppsala Universitet Mars 2003

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Summary

Mast cells are multifunctional effector and regulatory cells of the immune system, derived from CD34+ progenitor cells of hematopoietic origin in the bone marrow. The mast cells are regarded as key effector cells in immediate hypersensitivity reactions and allergic disorders, when activated, amplifying both acute and long term local tissue responses to biological signals. During an acute or chronic inflammation the number of mast cells in the affected tissue will increase and there is a correlation between the number of mast cells and the severity of inflammation.

Receptors for the Fc part of immunoglobulin, FcRs, commonly trigger and regulate biological responses when aggregated on the cell surface. How the cell will respond depends mostly on what kind of Fc receptor that is aggregated and the cell type it is expressed on. The aggregation of high affinity IgE receptor, FcεRI, activates mast cells. This initiates a series of signaling events leading to the release of inflammatory mediators contributing to acute and late phase allergic responses. Co-aggregating FcεRI with FcγRIIB, a low affinity receptor for IgG, have been shown to inhibit the IgE induced mast cell degranulation and release of inflammatory mediators.

In this study, the hypothesis that the co-aggregation of FcεRI and FcγRIIB not only inhibits mast cell activation but in fact initiates mast cell apoptosis was tested. The phosphorylation pattern of Akt and FKHRL1 protein as well as the expression of the pro-apoptotic Bim protein were investigated, all associated with the signaling pathway downstream of phosphatidylinositol 3'-kinase (PI3-K). The PI3-K pathway can regulate various features of cellular function such as gene transcription, DNA synthesis and survival. Phosphorylated Akt was recently found to phosphorylate and thereby inactivate members of the transcription factor forkhead family such as FKHRL1. When phosphorylated by Akt, FKHRL1 moves into the cytoplasm and transcription is prevented. The protein Bim is under the transcriptional control of FKHRL1. In addition to these proteins, the mRNA induction of the anti-apoptotic A1 protein has been examined following co-aggregation of FcεRI and FcγRIIB.

Although degranulation and release is suppressed, the hypothesis that co-aggregation of FcεRI and FcγRIIB would induce apoptosis in mast cells could not be proven. Further studies will be needed in order to elucidate the effect on mast cell survival after co-aggregation of FcεRI and FcγRIIB. The phosphorylation of Akt is diminished upon co-aggregation of FcεRI and FcγRIIB compared to FcεRI aggregation. However, there is no difference in FKHRL1 phosphorylation between FcεRI aggregation and co-aggregating FcεRI and FcγRIIB. This indicates that the lower level of phosphorylated Akt is enough to establish the same level of phosphorylation of FKHRL1 as seen for FcεRI aggregation. The expression of Bim is upregulated to the same extent following FcεRI aggregation and co-aggregation of FcεRI and FcγRIIB.

A1 has previously been shown to be crucial in order for mast cells to survive FcεRI aggregation The results for A1 mRNA induction shows that A1 is clearly upregulated following FcεRI aggregation as well as co-aggregation of FcεRI and FcγRIIB. This raises the question of the role of upregulated A1 after co-aggregation of FcεRI and FcγRIIB.

Investigating the Fc receptor signaling pathways upon activation and examining the modes and actions of proteins such as Akt, FKHRL1, Bim and A1 will help to gain more knowledge about the mechanisms regulating mast cell survival and apoptosis. To be able to regulate the number of mast cells and their activity in the tissue, thereby effecting the severity of inflammation, will create new ways of treating mast cell disorders and possibly improve already existing methods.

Introduction

The origin and localization of mast cells

Mast cells are of hematopoietic origin, derived from CD34+ progenitor cells in the bone marrow. Mast cell progenitors are recruited from peripheral blood into the tissue where they mature, with the aid of stem cell factor (SCF) and locally produced cytokines, into multifunctional effector and regulatory cells of the immune system [1-3].

Situated in the tissue, mast cells are still capable of proliferating but the number of cells is kept relatively constant under normal conditions [4]. In order to optimize the interaction with pathogens and antigen, mast cells are often found in close proximity to the external environment. In tissues such as skin and mucosal surfaces mast cells are commonly associated with blood vessels and nerves [2].

The role of mast cells in inflammation

Mast cells are regarded as key effector cells in immediate hypersensitivity reactions where they upon activation amplify both acute and long-term local tissue responses to biological signals [5-8].

For a long time mast cells have been recognized as the critical effector cell mediating IgE-dependent allergic responses. Binding of the antigen (allergen) to IgE, already bound to its high affinity receptor, FcεRI, on the mast cell surface aggregates the receptors. This initiates a series of signaling events leading to the release of inflammatory mediators contributing to acute and late phase allergic responses (Fig.1) [2].

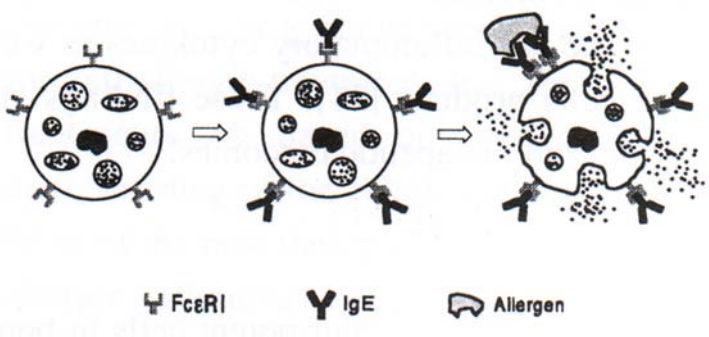


Figure 1. An IgE-dependent mast cell activation through FcεRI aggregation.

The activated mast cell releases preformed inflammatory mediators stored in the granules such as histamine, proteoglycans, proteases and cytokines. The degranulation process itself initiates synthesis of mediators, which consists of cytokines and lipid mediators [2]. The release of these mediators and cytokines lead to vasodilatation, bronchial and gastrointestinal smooth muscle contraction and recruitment of leukocytes, augmenting the inflammatory response [1].

During an acute or chronic inflammation the number of mast cells in the affected tissue will increase and there is a correlation between the number of mast cells and the severity of inflammation [4]. In addition to allergic inflammation mast cells have also been suggested to play a role during certain protective immune responses to parasites and bacteria. This indicates that mast cells are important effector cells, shown to have a more versatile role in various immune responses apart from allergic inflammation [2].

The Fcε receptor I

Fc receptors (FcRs), the receptors for the Fc part of immunoglobulin, trigger and regulate biological responses by delivering signals when they are aggregated on the cell surface. How the cell will respond depends primarily on the cell type and what kind of Fc receptor being aggregated [9].

FcεRI, the high affinity receptor for immunoglobulin E (IgE) is one of the classic markers for mast cells, found on the cell surface at an early stage in mast cell development [10]. IgE binds via its Fc region to FcεRI with high affinity in a 1:1 ratio and the binding of IgE further upregulates the number of FcεRI present on the surface [11-13].

FcεRI is a member of the multisubunit immune response receptor (MIRR) family of cell surface receptors. It lacks intrinsic enzymatic activity but transduce intracellular signals through the association with cytoplasmic tyrosine kinases [14]. The receptor consists of a tetrameric proteincomplex; the IgE-binding α-chain, a β-chain and two disulfide-linked chains (Fig.2) [15].

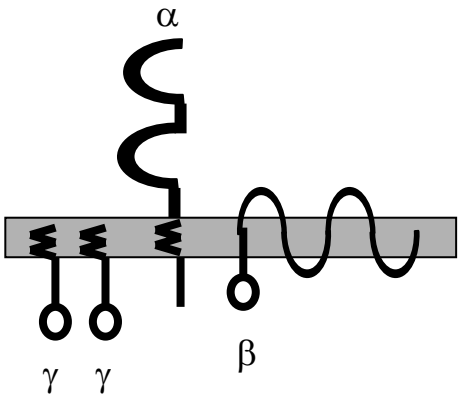


Figure 2. Schematic picture of the FcεRI, consisting of the IgE-binding α-chain, a β-chain and two γ-chains. Kindly provided by H. Wootz, adapted from Daëron, 1997 [9].

The β- and γ-subunits of the FcεRI each contain an immunoreceptor tyrosine-based activation motif (ITAM) which rapidly becomes phosphorylated on tyrosine after FcεRI aggregation [16]. The tyrosine phosphorylation of the ITAMs is mediated by the tyrosine kinase lyn and this leads to the recruitment and activation of tyrosine kinase syk. Once syk has been activated it can leave the receptor and phosphorylate downstream substrates. The signals generated by the FcεRI aggregation eventually lead to mast cell degranulation, secretion and changes in gene expression [9], [17].

Degranulation and survival

After an aggregation of the FcεRI, leading to mast cell degranulation and the release of inflammatory mediators and cytokines, the mast cells regranulates. Although the degranulation being a very harsh process for the cell it recovers within 3 to 48 hours after activation has taken place and can thereafter be activated again (Fig.3) [18-19].

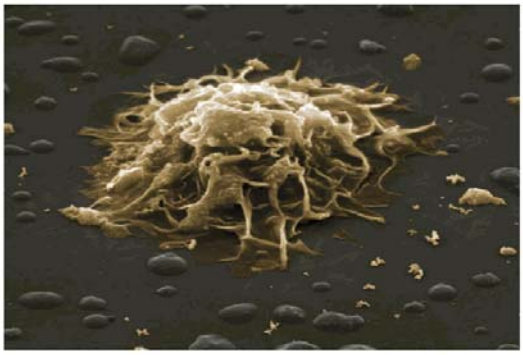


Figure 3. Degranulation, as a consequence of activation, is a harsh process for the mast cells. Photograph used with permission from Mats Block.

The ability of the mast cell to withstand the degranulation process give rise to a lot of intriguing questions: what is the mechanism by which the mast cell survives and what molecules are involved in this process? In order to answer these questions, we need to gain more knowledge about the signalling pathways controlling mast cell survival.

Signalling pathway: the transducing proteins Akt and FKHRL1

One intracellular signalling pathway that FcεRI aggregation activates is the phosphatidylinositol 3'-kinase (PI3-K) pathway [20]. This pathway can regulate various features of cell activation such as gene transcription, DNA synthesis and survival [21].

When PI3-K is activated by syk, it can via 3-phosphoinositide-dependent protein kinases phosphorylate the protein Akt also known as protein kinase B (PKB) [22]. Akt was recently found to phosphorylate and thereby inactivate members of the transcription factor forkhead family such as FKHRL1 [20] [23]. When phosphorylated by Akt, FKHRL1 remains in the cytoplasm and transcription is prevented. Unphosphorylated FKHRL1 is located in the nucleus, where it acts as a transcription factor for certain genes, some of them being pro-apoptotic members of the Bcl-2 family (Fig.4). Acting on this pathway might ensure the mast cell to survive the degranulation process [20].

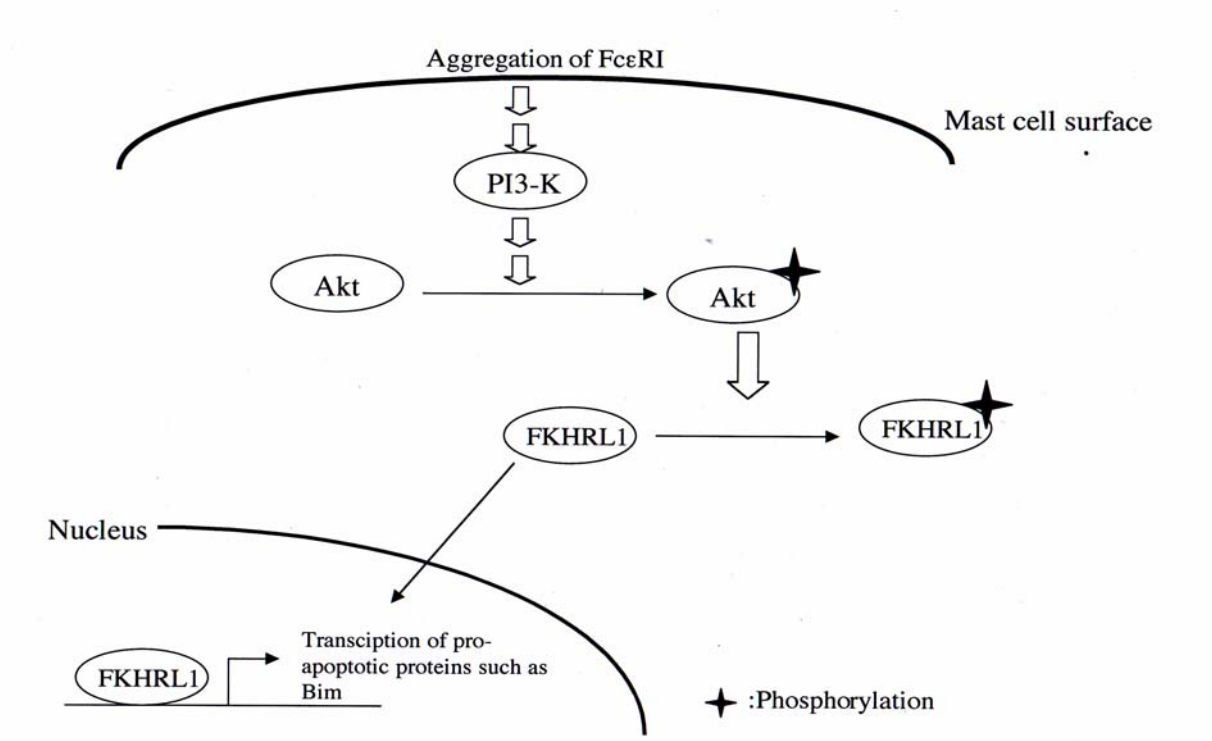


Figure 4. Schematic picture of one signalling pathway downstream of FcεRI aggregation. When PI3-K is activated it indirectly phosphorylates Akt protein. Phosphorylated Akt inactivates the transcription factor FKHRL1. Phosphorylated FKHRL1 remains in the cytoplasm, while unphosphorylated FKHRL1 is located in the nucleus. In the nucleus FKHRL1 acts a transcription factor for certain genes, some of them being pro-apoptotic members of the Bcl-2 family.

The Bcl-2 family members A1 and Bim

The Bcl-2 family of proteins consists of both pro-apoptic proteins and proteins having anti-apoptotic functions. The pro- and anti-apoptotic proteins of the Bcl-2 family can heterodimerize and titrate each other's functions. This suggests that the relative concentrations of the proteins might be an important factor in terms of cell survival versus apoptosis [24].

Anti-apoptotic members of the Bcl-2 family are needed for cell survival [25]. One of the murine pro-survival Bcl-2 family members is the anti-apoptotic protein A1. A1 is the only known Bcl-2 family member to be induced by inflammatory cytokines, suggesting a possible role of A1 in inflammatory processes [26]. A1 is strongly induced on mRNA and protein level when mast cells are activated through FcεRI aggregation. To survive an allergic activation A1 seems to be crucial since mast cells lacking A1 does not survive the process [27].

Another member of the Bcl-2 family is the pro-apoptotic Bcl-2 interacting mediator of cell death (Bim). Three isoforms of Bim have been characterised, Bim_{XL}, Bim_L and Bim_S [28]. Bim is known to be under the transcriptional control of the forkhead transcription factor FKHRL1 [29]. Containing a protein-interaction motif, Bim can heterodimerize to anti-apoptotic Bcl-2 members and thereby neutralise their pro-survival action [28].

The Fcγ receptor IIB

In addition to FcεRI, other FcRs such as FcγRIIB have been shown to influence mast cell degranulation and secretory events. FcγRIIB, a low affinity receptor for the immunoglobulin G (IgG), is the first identified and most extensively studied member of the inhibitory receptor superfamily (IRS). The receptor exists as three alternatively spliced isoforms, all containing two extracellular Ig-like loops and an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Fig.5) [17].

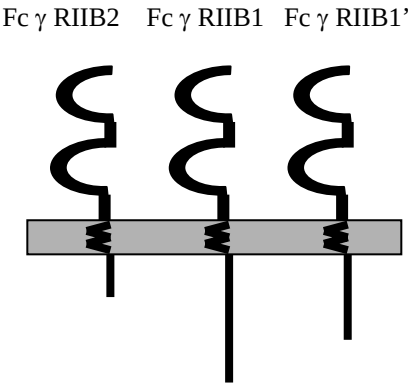


Figure 5. FcγRIIB exists as three alternatively spliced isoforms, all containing two extracellular Ig-like loops and an immunoreceptor tyrosine-based inhibitory motif (ITIM). Kindly provided by H. Wootz, adapted from Daëron, 1997 [9].

Activation through ITAM-containing receptors, such as FcεRI, can be inhibited by the co-aggregation with ITIM-containing receptors and hence the IgE induced release of mediators and cytokines is inhibited by the co-aggregation of FcεRI and FcγRIIB [30]. When FcεRI and FcγRIIB are co-aggregated, the FcεRI associated tyrosine kinase lyn phosphorylates the ITIM of FcγRIIB. The phosphorylated ITIM recruits SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP) that mediates the inhibitory signal (Fig.6) [31].

The mechanisms by which SHIP mediates the inhibitory function by blocking downstream signalling events required for FcεRI-mediated degranulation and gene expression in mast cells is not yet completely elucidated. One pathway that seem to be affected is the previously mentioned phosphatidylinositol 3'-kinase (PI3-K) pathway [32-34].

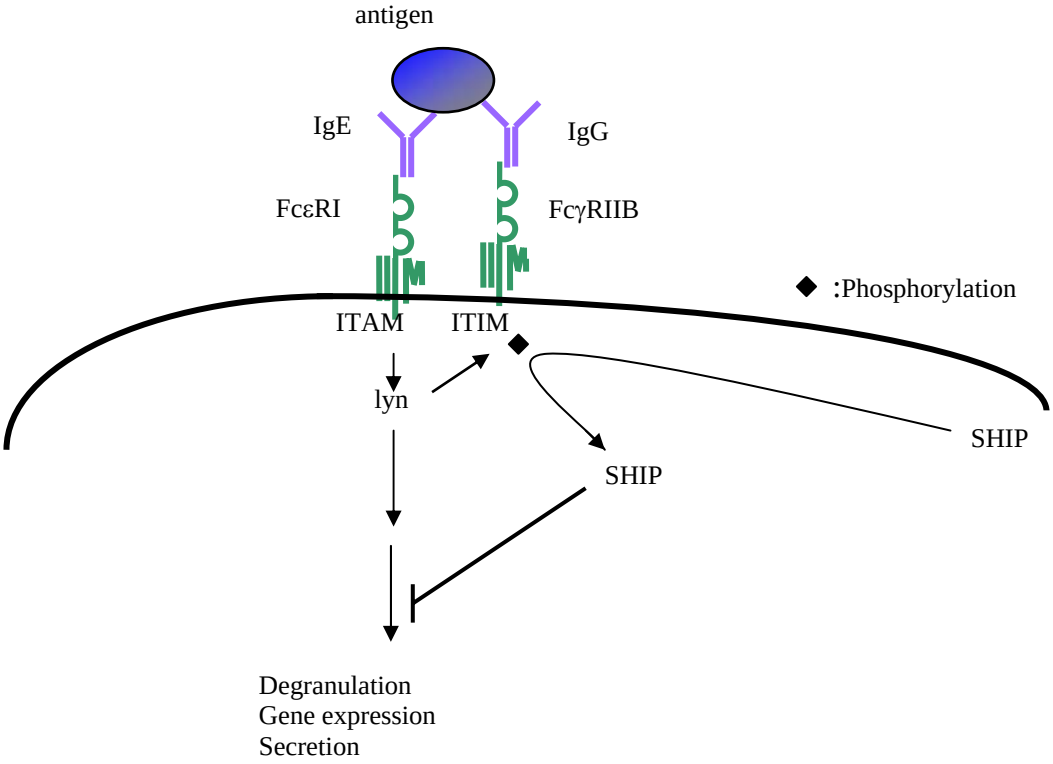


Figure 6. A schematic picture of the co-aggregation of FcεRI and FcγRIIB that inhibits the IgE induced release of mediators and cytokines mediated through FcεRI aggregation. Adapted from Ott V. L., 2000 [17].

Project background

Today most treatments for allergy and asthma involve drugs that relieve some of the symptoms associated with the inflammatory reaction seen after mast cell activation. Developing new ways of treating allergy is of great significance. A potential way of doing this is to regulate the number of mast cells and their activity in the tissue, thereby affecting the initiation and severity of inflammation.

One way of treating allergy is by Specific Immune Therapy (SIT). During SIT, a low concentration of the allergen is injected repeatedly for a long time period. As a consequence, the mast cells are desensitized and the allergic reaction towards the antigen ceases. Although SIT has been used since 1911 the specific mechanism behind it remains unknown [35-36]. To understand the mechanism behind SIT, the cause of its effects, could improve this method of treating allergy and also give some insight to the problem of some patients not responding to SIT.

A way in which immunotherapy might act is by effecting the number of mast cells. It has been shown that SIT leading to desensitization correlates to a decreasing number of mast cells [37]. Shifts in antibody production with a decline in IgE and high titers of IgG antibodies being raised during SIT have also been reported [38-40].
One hypothesis, possibly explaining the effects of SIT, is that antigen specific IgG and IgE antibodies generated during SIT bind to mast cells and induces apoptosis upon activation with allergen. When the allergen, complexed to the IgG antibodies, reaches the FcεRI-bound IgE antibodies it might co-aggregate the two receptors: FcεRI and FcγRIIB.

Project aim

The aim of my project is to test the hypothesis that the co-aggregation of FcεRI and FcγRIIB initiates mast cell apoptosis, this possibly explaining the effect of SIT. The co-aggregation of FcεRI and FcγRIIB is known to inhibit the mast cell activation, degranulation and release of mediators, seen during aggregation of FcεRI but the mast cell survival after co-aggregation has not been studied previously [30]. In addition to the impact on mast cell activation and survival, the effect on downstream signalling pathways such as the PI3-K pathway have been elucidated. This was done by investigating the transducing proteins Akt, FKHRL1 and Bim after co-aggregation of FcεRI and FcγRIIB as well as examining the induction of A1 upon activation.

Materials and Methods

Cell culture

The murine mast cell line C57 was cultured in RPMI-1640 medium supplemented with 10% filtered FBS (Fetal Bovine Serum), 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin and 50 µM 2-mercaptoethanol (all from Sigma Chemicals Co., St. Louis, MO, USA). The cell line was kept in a humidified incubator with 5% CO_2 at 37 °C and passaged twice a week. The C57 mast cell line has previously been characterized for FcγRII/FcγRIII expression [41]. All experiments were performed on this mast cell line.

Antibodies and Reagents

AffiniPure Rabbit Anti-Mouse IgG (H+L), Affinipure F(ab')$_2$ Fragment Rabbit Anti-Mouse IgG (H+L) and Affinipure F(ab')$_2$ Fragment Mouse Anti-Rat IgG (H+L) were all purchased from Jackson ImmunoResearch Laboratories, Inc. (Baltimore, USA). Purified Rat Anti-mouse CD16/CD32 (Fcγ III/II Receptor) monoclonal antibody (2.4G2) and Propidium Iodide (PI) Staining Solution were obtained from BD Biosciences (Heidelberg, Germany). Nonfat dry milk was purchased from Semper Foods AB (Stockholm, Sweden). Anti-Rabbit IgE, horseradish peroxidase (HRP) linked whole antibody (from donkey) was obtained from Amersham Biosciences (Uppsala, Sweden). LumiGLO reagent and peroxide, 10x Cell Lysis Buffer, antibodies directed against phospho-Akt (Serine(Ser) 473) and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies directed against phospho-FKHRL1 (Threonine(Thr) 32) and FKHRL1 were obtained from Upstate biotechnology (Lake Placid, NY, USA). Anti-Bim antibody was purchased from Affinity Bioreagents, Inc. (Golden, CO, USA). 4x NuPAGE Sample LDS Buffer and 10x NuPAGE Sample Reducing Agent were obtained from Invitrogen (Carlsbad, CA, USA), TriPure Isolation Reagent from Boehringer Mannheim (Mannheim, Germany) and Tween 20 from MERCK-Schuchardt (Hohenbrunn, Germany). Phosphate buffer saline (PBS) and Tris buffer saline (TBS) were both obtained from the medium preparation facility at the Rudbeck laboratory (Uppsala, Sweden). All other reagents were purchased from Sigma Chemical CO (St. Louis, MO, USA).

Antibody conjugation

Affinipure F(ab')$_2$ Fragment Mouse Anti-Rat IgG (H+L), F(ab')$_2$ MAR, was dialyzed twice against borate buffer saline (BBS) [0.17 M H_3BO_4, 0.12 M NaCl, pH 8.0] at 4 °C over night. After being dialyzed, 50 µl picrylsulfonic acid: 2,4,6-Trinitrobenesulfonic acid (TNBS) per mg of F(ab')$_2$ MAR, was added while stirring (on a vortex) at 10 mg/ml in BBS. The mixture was incubated for 2 h at room temperature (RT) on a Belly-Dancer (Stovall Life Science Inc., Greensboro, NC, USA), wrapped in aluminum foil (TNBS being light sensitive).

The mixture was fractionated by running a pre-packed disposable column PD-10 containing Sephadex G-25 medium (Amersham Biosciences), equilibrated with 1x phosphate buffer saline (PBS). Fractions were collected, equal to the excluded volume. The fraction which contains the conjugated TNP-F(ab')$_2$ MAR should be light yellow. The optical density (OD) was measured at 340 nm and 280 nm using a Philips UNICAM 8625 in order to determine the coupling ratio.

Activation

The mast cell line C57 was activated by FcεRI aggregation or FcεRI/FcγRIIB co-aggregation using two different systems, system I and II.
System I was used for activating cells in all experiments except for the Western experiments where System II was being used.

System I

Mast cells were resuspended in RPMI-1640 medium supplemented with 0.2% bovine serum albumin (BSA), 2 mM L-glutamine and 100 µg/ml penicillin/streptomycin at a concentration of 1x10^6 cells/ml. The cells were sensitized for 90 min at 37 °C by adding 0.1 µg/ml of monoclonal anti-dinitrophenyl (DNP) clone SPE-7 IgE mouse antibody. After washing the cells twice with pre-warmed 1xPBS, 750xg for 5 min at RT, the cells were suspended at a concentration of 1x10^6 cells/ml in the same medium as during the sensitization. The cells were activated by adding either 45 µg/ml of Rabbit Anti-Mouse IgG (co-aggregation of FcεRI and FcγRIIB) or 30 µg/ml of F(ab')$_2$ Fragment Rabbit Anti-Mouse IgG (aggregation of FcεRI) at 37 °C for time periods indicated.

Briefly, the addition of IgE sensitizes the mast cells by binding to its high-affinity receptor FcεRI. When adding F(ab')$_2$ Fragment Rabbit Anti-Mouse IgG (RAM-F(ab')$_2$) it binds to IgE bound to FcεRI and thereby aggregates FcεRI leading to the activation of the mast cell. However, Rabbit Anti-Mouse IgG (RAM-IgG) consisting of not only the F(ab')$_2$ Fragment of the antibody but also the Fc-part, allows the RAM-IgG to bind to both IgE (bound to FcεRI) and FcγRIIB. Binding to IgE with its F(ab')$_2$ Fragment and to FcγRIIB via its Fc-part causes a co-aggregation of FcεRI and FcγRIIB.

System II

Mast cells were resuspended in RPMI-1640 medium supplemented with 0.2% bovine serum albumin (BSA), 2 mM L-glutamine and 100 µg/ml penicillin/streptomycin at a concentration of 1x10^6 cells/ml. The cells were sensitized for 90 min at 37 °C by adding 0.1 µg/ml of monoclonal anti-dinitrophenyl (DNP) clone SPE-7 IgE mouse antibody (anti-DNP IgE) or by adding 0.1 µg/ml of anti-DNP IgE and 5 µg/ml of purified Rat Anti-Mouse CD16/CD32 (Fcγ III/II Receptor) monoclonal antibody (2.4G2 Ab).
After washing the cells twice with pre-warmed 1xPBS, 750xg for 5 min at RT, the cells were suspended at a concentration of 1x10^6 cells/ml in the same medium as during the sensitization. The cells were activated by adding 10 µg/ml of conjugated TNP-F(ab')$_2$ MAR (coupling ratio: 6) at 37 °C for time periods indicated.

Briefly, adding IgE alone or in combination with the 2.4G2 Ab sensitized the cells. When adding the 2.4G2 Ab (directed towards FcγRIII/FcγRII) as well as IgE, the 2.4G2 Ab ($F(ab')_2$ Fragment) binds to the FcγRIIB. Addition of the conjugated TNP-$F(ab')_2$ MAR to mast cells only sensitised with IgE will cause a FcεRI aggregation by TNP binding to IgE. Adding TNP-$F(ab')_2$ MAR to cells sensitised with IgE and 2.4G2 Ab will cause a co-aggregation of FcεRI and FcγRIIB since the conjugated TNP-$F(ab')_2$ MAR will bind to both IgE and 2.4G2 Ab. TNP will bind to IgE and the $F(ab')_2$ MAR fragment will bind to the 2.4G2 Ab.

In experiments where the phosphorylation pattern of Akt and FKHRL1 as well as total amount of these two proteins were measured, the mast cells were starved for approximately 24 hours at 37 °C in RPMI-1640 medium supplemented with 0.5% FBS before sensitization and activation. This in order to decrease the background levels of phosphorylation.

For Bim expression experiments, the mast cells were resuspended in RPMI-1640 medium supplemented with 10% filtered FBS (Fetal Bovine Serum), 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin and 50 µM 2-mercaptoethanol during both sensitisation and activation in order to decrease the background level of Bim. Mast cells analyzed by flow cytometry and counted using Trypan Blue Staining for survival analysis were also kept in this medium during sensitisation and activation.

N-acetyl-β-D-hexosaminidase release assay

The method measures enzymatic activity of the enzyme N-acetyl-β-D-hexosaminidase, which is present in mast cell granules. After mast cell activation it is released with similar kinetic to that of histamine. The enzyme converts the substrate, p-nitrophenyl-N-acetyl-β-$_D$-glucosaminide to p-nitrophenol, which can be measured spectrophotometrically.

The substrate, P-nitrophenyl-N-acetyl-β-$_D$-glucosaminide, was dissolved in citric acid buffer [0.08 M citric acid, pH 4.5] to a concentration of 2.738 mg/ml. The mixture was incubated, on a rocker platform for 30 min at 37 °C, in order to dissolve the substrate, and thereafter filtered (pore size: 0.45 µm). After activating the mast cells for 30 min, using either system I or II, 500 µl of the cell culture was transferred to a eppendorf tube and microcentrifuged at 500xg for 5 min at 4 °C. The supernatant, 60 µl/sample and in triplicates, was then transferred to a 96 well plate. Sixty µl of the substrate mixture was added to each well. The plate was incubated on a rocker platform for 2 h at 37°C. Glycine [0.2 M, pH 10.7], 120 µl/well, was added to stop the reaction and absorbance was measured at 405 – 490 nm using an Emax Precision Microplate Reader (Molecular device, Sunnyvale, CA, USA) and analysed by Softmax (version 2.02 for Macintosh, Molecular device).

Flow cytometry analysis

The Propidium Iodide Staining Solution (PI) is used to assess plasma membrane (PM) integrity. PI is a fluorescent dye that stains DNA. Cells that are viable or in the early stage of apoptosis maintain PM integrity whereas cells in the late stages of apoptosis or already dead cells do not, hence these cells are permeable to PI and can be detected. PI is detected in the orange range of the spectrum using a 562-588 nm band pass filter.

After activation of the mast cells, at time points 24, 48 and 72 h, 1 ml of cell culture containing approximately $0.5x10^6$ cells were transferred and incubated with 5 µl of Propidium Iodide (PI) Staining Solution [50 µg/ml] for 5 min on ice, in the dark, before analysed. Analysis of 10000 cells per sample was performed on a FACSort (Becton Dickinson, Mountain View, CA, USA) using CELLQuest (version 3.3, Becton Dickinson).

Trypan blue staining

Trypan Blue Staining uses the same principle as PI Staining and is commonly used for determining the viability of cells in a cell culture. At time points 24, 48 and 72 h after activation, mixed 50 µl of cell culture and 50 µl Trypan Blue Solution [0.4%] and incubated for 5 min at RT. Fifteen µl were transferred to a Bürker chamber and the percentage live cells (% live cells= number of live cells / total cell number) was determined from approximately a total of 100-200 cells.

Protein assay

Protein contents of cell lysates were measured using BCA Protein Assay Reagent Kit (PIERCE, Rockford, IL, USA) using the manufacturer's protocol (working range 20-2,000 µg/ml). The BCA Protein Assay is a colorimetric method for detection and quantification of total protein. The absorbance was measured at 540 nm in an Emax Precision Microplate Reader (Molecular device, Sunnyvale, CA, USA) and analysed by Softmax (version 2.02 for Macintosh, Molecular device).

Western blot

Lysates to be used for detection of the transducing proteins Akt and FKHRL1 and their phosphorylation patterns were prepared by ending the activation at time points 1, 5, 10 and 30 min. The activation was aborted by washing with ice cold 1xPBS at 490xg for 5 min at 4°C. A total amount of $2x10^6$ cells/time point were lysed by adding 100 µl of 2xSDS sample buffer [125 mM Tris-HCl (pH 6.8), 4%w/v sodium dodecyl sulfate (SDS), 20% glycerol, 0.02% w/v bromphenol blue, 50 mM dithiothreitol (DTT, added just before use)]. The lysates were kept on ice at all time, transferred to eppendorf tubes and sonicated for 2x7 seconds and then stored at -20°C. Before use, lysates were heated at 95°C for 5 min and centrifuged at 16000xg for 5 min. Ten µl/sample were loaded onto a NuPAGE Bis-Tris western gel(Invitrogen, Carlsbad, CA, USA).

Lysates to be used for detection of the Bim protein were prepared by ending the activation after 24 h. The activation was aborted by washing with 1xPBS at 490xg for 5 min at 4°C. A total amount of $2x10^6$ cells/sample were lysed by adding 200 µl of 1xcell lysis buffer containing 20 µl 10xcell lysis, 2 µl phenylmethylsulfonyl fluoride (PMSF) and 178 µl H_2O. The lysates were kept on ice at all time, transferred to eppendorf tubes and sonicated for 4x5 seconds and then microcentrifuged for 10 min, 16000xg, at 4°C before storage at -20°C. The protein concentration of samples were determined using the BCA Protein Assay Reagent Kit (PIERCE) and were adjusted for in the following experiment, to ensure a equal loading. Briefly, 8.45 µl of sample lysate and 3.25 µl NuPAGE Sample LDS Buffer [4x] together with 1.3 µl NuPAGE Sample Reducing Agent [10x] were mixed and 10 µl/sample were then loaded onto a NuPAGE Bis-Tris western gel(Invitrogen).

The phosphorylation and/or the total amount of protein were studied by western blotting using a NuPAGE Bis-Tris western gel(Invitrogen). After electrophoresis the proteins were electrically transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences). The electrophoresis and transfer was performed according to the manufacturer's instructions for NuPAGE Electrophoresis System for Bis-Tris Gels. After transfer the membrane was washed in 25 ml of 1xTris Buffer Saline (TBS) for 5 min and thereafter blocked 1 h in 1xTBS containing 5% w/v nonfat dry milk and 0.1% Tween 20. The membrane was incubated over night at 4°C with the primary antibody. The primary antibody was dissolved in BSA 5% w/v –TBS, in order to detect phosphorylated FKHRL1 (Thr 32)(1:200 dilution), total FKHRL1 (1:500 dilution) or Bim (1:1000 dilution). For detection of phosphorylated Akt (Ser 473) or total Akt, the primary antibody was dissolved in the previously mentioned blocking buffer at a 1:1000 dilution. Following day the membrane was incubated with horseradish peroxidase-conjugated donkey-anti rabbit secondary antibody (1:2000 dilution) for 1 h at RT. The membrane was developed using enhanced chemiluminescence (ECL) system LumiGLO and exposure to a Hybond ECL film (Amersham Biosciences).

A membrane can be stored at 4°C and reused, if it is first stripped from antibodies. Briefly, membranes are incubated at 50°C for 30 min in strip buffer containing 3.34 ml H_2O, 6.25 ml 0.5M Tris (pH 6.8), 10.0 ml 10% w/v SDS and 340 µl 2-Mercaptoethanol. The membrane was washed in TBS supplemented with 0.1% Tween 20 for approximately 30 min and thereafter blocked for 1 h in blocking buffer. New primary antibody was then added.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Approximately $1x10^7$ cells were used for isolation of total cellular RNA using TriPure isolation reagent after the cells had been activated for 6h. RNA was reversibly transcribed into single stranded cDNA using a First Strand cDNA Synthesis Kit according to manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cDNA was synthesized from 1 µg RNA by using 2 µl of Oligo-p(dT)15 Primer in the presence of 1 µl RNAse Inhibitor, 0.8 µl AMV Reverse Transcriptase, 2 µl Deoxynucleotide Mix, 4 µl MgCl2, 2 µl 10x Reaction Buffer and 0.4 µl gelatin where the final volume was adjusted using sterile water to 20 µl. The reactions were incubated at 25°C for 10 min, 42°C for 60 min, 95°C for 5 min and finally 4°C for 5min.

The cDNA was amplified by using PCR Core Kit according to the manufacturer's protocol (Roche Diagnostics) with specific primers for the A1 gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [42-43]. GAPDH was used as a control of cDNA integrity. cDNA from MCP5/L cells (mouse mast cell line), activated through FcεRI aggregation, was used as a positive control for A1 expression. Briefly, 1.67 µl 10xPCR Buffer, 0.33 µl Deoxynucleotide Mix, 0.17 µl Taq DNA Polymerase, all provided in the PCR Core Kit, was added to 0.67 µl 10 µM A1 primer, 0.67 µl 0.33 µM GAPDH primer and 0.67 µl of cDNA. The total volume was adjusted to a final volume of 17.37 µl using sterile water. The cDNA was amplified by PCR for 30 cycles in a thermal cycler (Gene Amp PCR system 2400, Perkin Elmer), one cycle being composed of denaturation at 94°C for 30 seconds, annealing at 57°C for 45 seconds and elongation at 72°C for 1 min (7 min for the last elongation). The PCR products were visualized by electrophoresis using a 2% agarose gel containing 0.5 µg/ml ethylium bromide (EtBr) in a 1xTris-borate/EDTA (TEB) buffer at 100 V for approximately 45 min.

RNAse Protection Assay (RPA)

TriPure isolation reagent was used for isolation of total cellular RNA. RNAse protection assay was performed according to RiboQuant System protocol, using the mAPO-2 multi-probe set (PharMingen, San Diego, CA, USA). The gel was dried and exposed on Kodak film (Eastman Kodak Company, Rochester, NY, USA) with intensifying screens at –70°C but expression of RNA was also detected with a phosphoimager device and levels of expression were quantified using MacBas V2.2 Software (Fuji Photo Film, Co. Ltd., Japan).

Results

Activation

The mast cell line C57 was activated by FcεRI aggregation or co-aggregation of FcεRI and FcγRIIB using two different systems, system I and II. In order to measure the activation of the mast cells an N-acetyl-β-D-hexosaminidase release assay was used. The enzyme N-acetyl-β-D-hexosaminidase is present in mast cell granules and is released after mast cell activation with similar kinetic to that of histamine release. The absorbance measurement gave an indication of the level of mast cell activation after co-aggregation and aggregation, compared to unactivated control cells.

Using System I the mast cells were clearly activated when aggregating the FcεRI, yielding a 4-folded increase in absorbance compared to control cells incubated with IgE. However, co-aggregating the FcεRI and FcγRIIB resulted in ~60% decreased activation compared to that achieved with FcεRI (Fig 8). When the mast cells were activated with system II the aggregation of FcεRI yielded a 2-folded increase in absorbance compared to control cells incubated with IgE. However, co-aggregating the FcεRI and FcγRIIB resulted in a ~30% decreased activation compared to that achieved with FcεRI (Fig 9). This showed that both systems are functional and could be used in order to further study cell survival and signaling pathways after co-aggregation of FcεRI and FcγRIIB compared to FcεRI aggregation and unactivated cells.

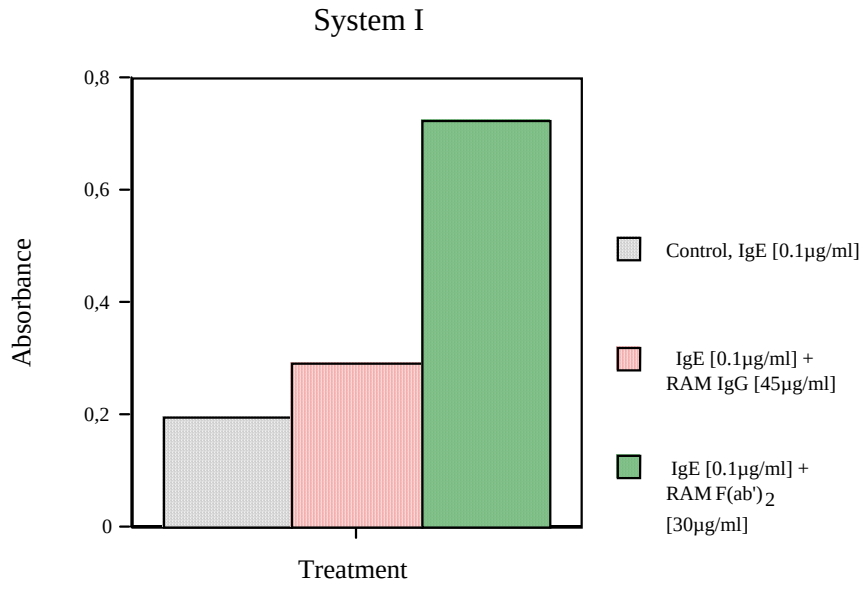


Figure 8. Murine C57 mast cells activated using system I. The release was measured after FcεRI and FcγRIIB co-aggregation, using IgE and Rabbit Anti-Mouse IgG (RAM-IgG) and compared to FcεRI aggregation using IgE and Rabbit Anti-Mouse $F(ab')_2$ Fragment IgG (RAM-$F(ab')_2$). Cells incubated with IgE, served as a background control. Two separate experiments were performed and representative results from one of the experiments are presented as mean ±SEM. The significance was tested using the Mann-Whitney test, $0.025>p>0.001$.

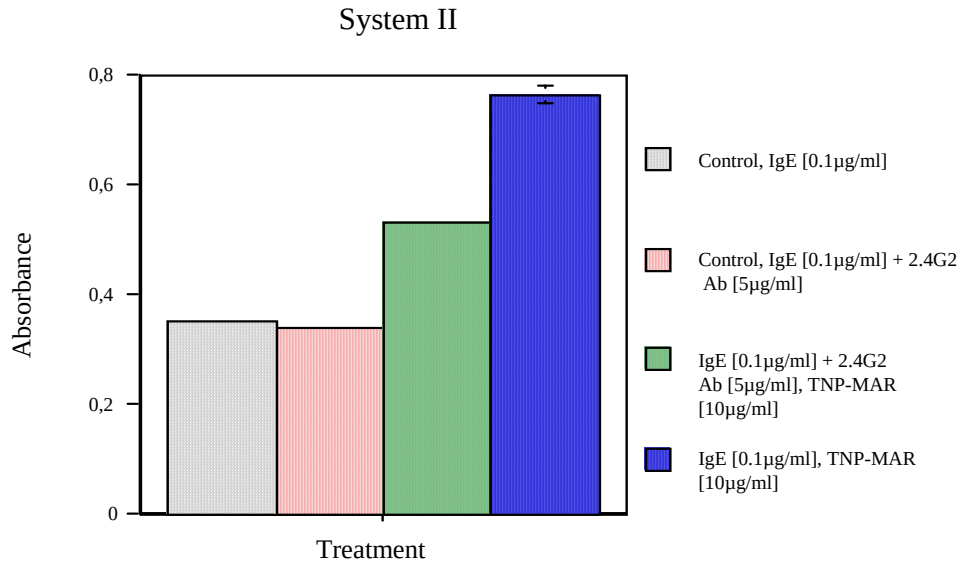


Figure 9. Murine C57 mast cells activated using system II. The release was measured after FcεRI and FcγRIIB co-aggregation, using IgE and 2.4G2 Ab with conjugated TNP-F(ab')$_2$ MAR and compared to FcεRI aggregation using IgE and TNP-F(ab')$_2$ MAR. Cells incubated with IgE served as a background control. Two separate experiments were performed and representative results from one of the experiments are presented as mean ±SEM. The significance was tested using the Mann-Whitney test, 0.025>p>0.001.

Survival

Flow cytometry analysis

Mast cell survival after co-aggregating FcεRI and FcγRIIB or FcεRI aggregation was measured using Propidium iodide (PI) staining solution, which is a fluorescent dye that stains DNA. Cells that are permeable to PI can be detected using flow cytometry.

The results showed that there is a ~5-10% difference in cell survival comparing the aggregation and co-aggregation to the survival seen for unactivated cells. Small, if any, difference was however seen in cell survival after co-aggregation of FcεRI and FcγRIIB compared to FcεRI aggregation (Fig.10).

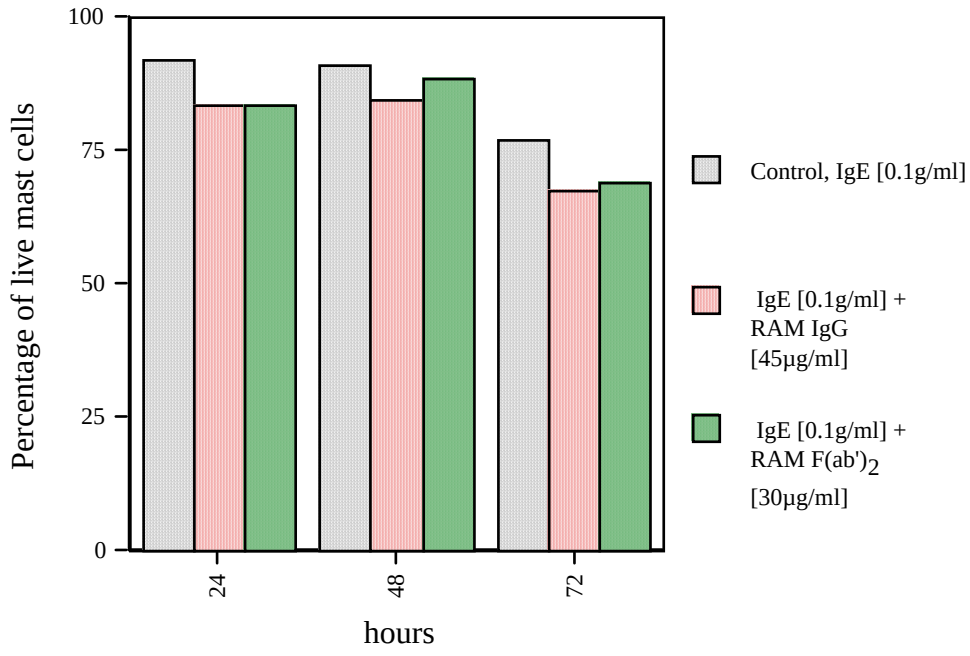


Figure 10. Mast cell survival seen after 24, 48 and 72 h using PI staining, analyzed by flow cytometry. Cell survival for FcεRI aggregation using IgE and Rabbit Anti-Mouse $F(ab')_2$ Fragment IgG (IgE + RAM -$F(ab')_2$) and co-aggregation of FcεRI and FcγRIIB using IgE and Rabbit Anti-Mouse IgG (IgE + RAM-IgG) was compared to unactivated cells (control, IgE).

Trypan Blue Staining

Trypan Blue Staining uses the same principle as PI Staining and is commonly used for determining cell viability.

The results showed that there was a small difference in cell survival between unactivated and FcεRI activated cells. However, there was a somewhat decreased survival seen when co-aggregating the FcεRI and FcγRIIB. This decrease ranged from ~5% at 24h up to ~20% at 72h (Fig.11).

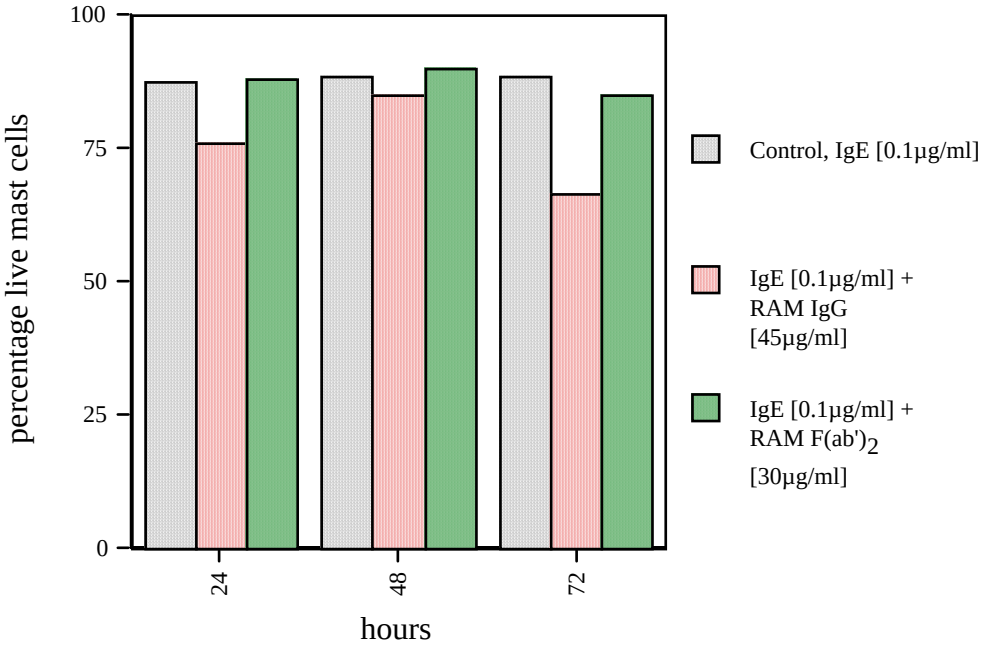


Figure 11. Mast cell survival seen after 24, 48 and 72 h using trypan blue staining. Survival after FcεRI aggregation using IgE and Rabbit Anti-Mouse F(ab')$_2$ Fragment IgG (IgE + RAM -F(ab')$_2$), co-aggregation of FcεRI and FcγRIIB using IgE and Rabbit Anti-Mouse IgG (IgE + RAM-IgG) and the survival of unactivated cells (control, IgE) was investigated.

Akt

Akt is a signaling transducing protein downstream of PI3-kinase. The PI3-kinase can via 3-phosphoinositide-dependent protein kinases phosphorylate Akt at three different sites, Serine 473 (Ser 473) being one of them. The phosphorylation of Akt at Ser 473 after FcεRI aggregation compared to co-aggregation of FcεRI and FcγRIIB was studied after 1, 5, 10 and 30 min of activation.

The results after using western blotting with an antibody directed toward the phosphorylation site, showed a clear and rapid phosphorylation of Akt within minutes after FcεRI aggregation. The phosphorylation site Ser 473 reached its maximum phosphorylation stage within 10 minutes. Phosphorylation is a reversible process and Akt seemed to be largely unphosphorylated after 30 minutes. The phosphorylation pattern after co-aggregation of FcεRI and FcγRIIB was different. Overall, less Akt was being phosphorylated and Ser 473 reached its maximum phosphorylation stage within 5 minutes. Akt was again unphosphorylated after 30 minutes (Fig.12a). The membrane was stripped and re-probed with a total Akt antibody to asses even sample loading (Fig.12b).

12.a)

A	A*	A	A*	A	A*	A	A*	A	A*
0		1		5		10		30 minutes	

12.b)

Figure 12. Western blot analysis showed the phosphorylation pattern of Akt protein, at Ser 473, and total Akt after 0, 1, 5, 10 and 30 min of activation with FcεRI aggregation, using IgE and conjugated TNP-F(ab')$_2$ MAR (A) or co-aggregation of FcεRI and FcγRIIB using IgE and 2.4G2 Ab with conjugated TNP-F(ab')$_2$ MAR (A*). a) Phosphorylation pattern of Akt at Ser 473. b) Total Akt. The result is representative of three independent experiments.

FKHRL1

Phosphorylated Akt can phosphorylate the forkhead protein FKHRL1. The phosphorylation of FKHRL1 at site Threonine 32 (Thr 32) was investigated, using the same samples that previously had been analyzed for Akt phosphorylation.

The maximum phosphorylation of FKHRL1 occurred within 5 minutes and thereafter declined, reaching background levels again after 30 minutes (Fig.13a). This holds true for both ways of activating the cells, co-aggregation of FcεRI and FcγRIIB as well as FcεRI aggregation, and the level of phosphorylation was the same. The membrane was stripped and re-probed with a total FKHRL1 antibody to asses even sample loading (Fig.13b).

13.a)

A	A*	A	A*	A	A*	A	A*	A	A*
0		1		5		10		30 minutes	

13.b)

Figure 13. Western blot analysis showed the phosphorylation pattern of FKHRL1 protein, at Thr 32, and total FKHRL1 after 0, 1, 5, 10 and 30 min of activation with FcεRI aggregation, using IgE and conjugated TNP-F(ab')$_2$ MAR (A) or co-aggregation of FcεRI and FcγRIIB using IgE and 2.4G2 Ab with conjugated TNP-F(ab')$_2$ MAR (A*). a) Phosphorylation pattern of FKHRL1. b) Total FKHRL1. The result is representative of three independent experiments.

Bim

The pro-apoptotic Bcl-2 interacting mediator of cell death, Bim, is known to be under the transcriptional control of the forkhead transcription factor FKHRL1. Bim contains a protein-interaction motif and is therefor able to heterodimerize to anti-apoptotic Bcl-2 members and thereby neutralises their pro-survival action. When FKHRL1 is phosphorylated it is not able to act as a transcription factor for Bim. Since Bim is transcriptonally regulated by FKHRL1, the cells were activated for approximately 24 hours using either FcεRI aggregation or co-aggregation of FcεRI and FcγRIIB.

By western blotting it was found that two isoforms of Bim: Bim_{XL} and Bim_{L} were upregulated, compared to unactivated control cells incubated with either IgE alone or IgE together with 2.4G2 antibody (FcγRII/FcγRIII). The level of Bim expression was more or less the same after using either FcεRI aggregation or co-aggregation of FcεRI and FcγRIIB (Fig.14). It appeared as if Bim_{XL} consisted of two bands, the upper one possibly due to phosphorylation. The upper band of Bim_{XL} was slightly stronger for one of the controls, unactivated cells incubated with IgE and 2.4G2 antibody.

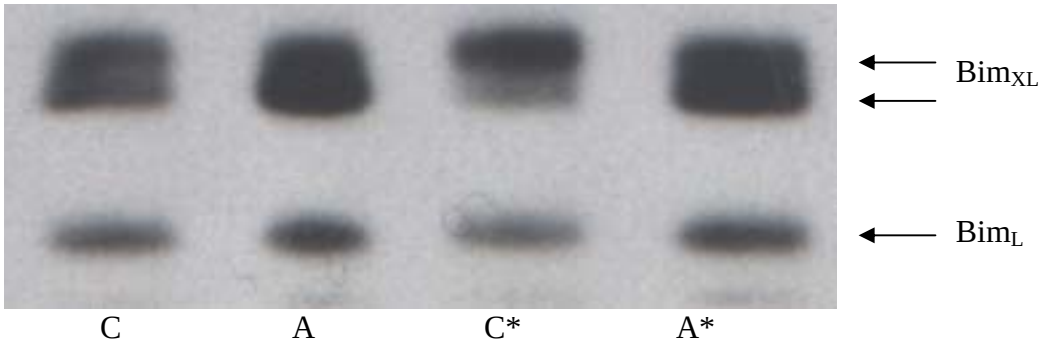


Figure.14 Western blot analysis showed the expression of Bim protein after 24 h of activation.
C: Unactivated cells incubated with IgE
A: FcεRI aggregation using IgE and conjugated TNP-F(ab')$_2$ MAR
C*: Unactivated cells incubated with IgE and 2.4G2 antibody
A*: Co-aggregation of FcεRI and FcγRIIB using IgE and 2.4G2 antibody with conjugated TNP-F(ab')$_2$ MAR.
The result is representative of three independent experiments.

A1

A1 is one of the murine pro-survival Bcl-2 family members. It is known that A1 is strongly induced on mRNA and protein level following mast cell activation through FcεRI aggregation. To investigate if A1 is upregulated on mRNA level after co-aggregation of FcεRI and FcγRIIB A1 expression was analysed by RT-PCR. The expression of the housekeeping gene GAPDH was used as a control of cDNA integrity (result not shown). The mast cells were activated for 6 hours using FcεRI aggregation or co-aggregation of FcεRI and FcγRIIB.

The result showed that A1 was upregulated upon FcεRI aggregation (Fig.15). In contrast, no upregulation could be observed upon co-aggregating FcεRI and FcγRIIB.

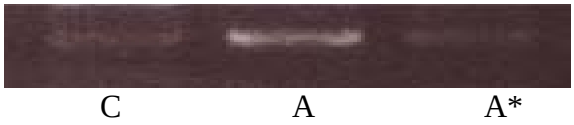


Figure 15. RT-PCR showed the A1 mRNA level after 6 h of activation with FcεRI aggregation using IgE and Rabbit Anti-Mouse F(ab')$_2$ Fragment IgG (A) or co-aggregation of FcεRI and FcγRIIB using IgE and Rabbit Anti-Mouse IgG (A*) compared to control cells incubated with IgE (C). The result is representative of two independent experiments.

To investigate this further, a RNAse protection assay (RPA) was performed. RPA is a highly sensitive and specific method for the detection and quantification of mRNA. mRNA from cells activated for 6 hours using FcεRI aggregation or co-aggregation of FcεRI and FcγRIIB was used. The multi-probe template set used for RNAse protection assay also contains other anti-apoptotic as well as pro-apoptotic proteins of the bcl-2 family besides A1.

A1 was upregulated both when activating the mast cells through FcεRI aggregation and co-aggregation of FcεRI and FcγRIIB (Fig.16a). Quantification of the RPA, using a phosphoimager device showed that A1 is upregulated ~1:9 for the co-aggregation of FcεRI and FcγRIIB and ~1:12 for FcεRI aggregation compared to unactivated control cells incubated with IgE (Fig.16b).

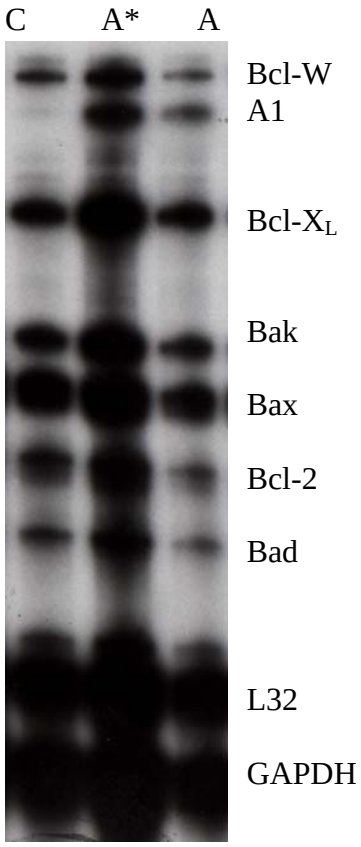
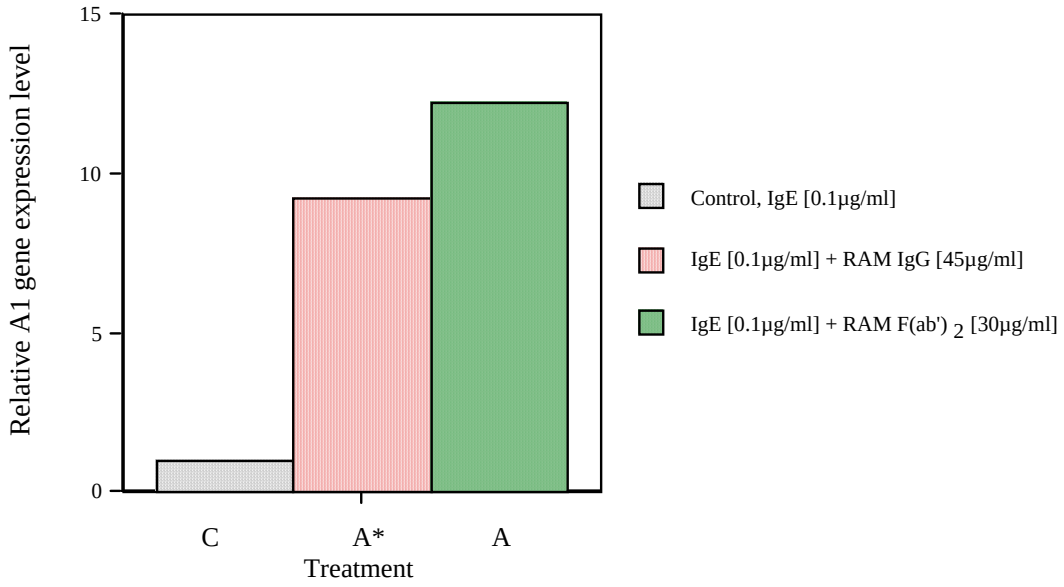


Figure 16. a) The RNAse protection assay showed the expression of bcl-2 family members upon mast cell activation.
C: Unactivated cells incubated with IgE
A*: Co-aggregation of FcεRI and FcγRIIB using IgE and Rabbit Anti-Mouse IgG (IgE + RAM-IgG).
A: FcεRI aggregation using IgE and Rabbit Anti-Mouse $F(ab')_2$ Fragment IgG (IgE + RAM -$F(ab')_2$).



16.b) The quantification of A1 expression showed that A1 is upregulated ~1:9 for the co-aggregation of FcεRI and FcγRIIB (IgE + RAM-IgG) (A*) and ~1:12 for FcεRI aggregation (IgE+RAM-F(ab')$_2$) (A) compared to unactivated control cells incubated with IgE (C). The phosphoimaging signals presented in 16.a) are shown as A1 gene expression relative to the average expression of the house keeping genes GAPDH and L32. Data are normalised such that the level of A1 from the control cells is given a value of 1.

Discussion

Mast cells are recognized as the critical effector cell mediating IgE-dependent allergic responses. During an acute or chronic inflammation the number of mast cells in the affected tissue will increase and there is a correlation between the number of mast cells and the severity of inflammation [3].

Aggregation of the receptors for the Fc part of immunoglobulin, FcRs, commonly trigger and regulate biological responses of cells on which they are expressed. How the cell will respond depends mostly on what kind of Fc receptor being aggregated and cell type [9]. Aggregation of the high affinity IgE receptor FcεRI on mast cells initiates a series of signaling events leading to the release of inflammatory mediators contributing to acute and late phase allergic responses [2]. FcγRIIB, a low affinity receptor for IgG, has been shown to negatively influence mast cell degranulation and secretory events [17]. When FcεRI and FcγRIIB are co-aggregated, the mast cell activation mediated through FcεRI is inhibited; hence there is no IgE induced release of mediators and cytokines [31].

In this study, the hypothesis that the co-aggregation of FcεRI and FcγRIIB not only inhibits mast cell activation but in fact initiates mast cell apoptosis was tested. Previously, FcγRIIB aggregation and co-aggregation with the B cell receptor (BCR) on B cells has been shown to induce apoptosis [44]. The effect on downstream signalling pathways, such as the PI3-K pathway, was also elucidated by investigating the proteins Akt, FKHRL and Bim after co-aggregation of FcεRI and FcγRIIB. The PI3-K pathway can regulate various features of cellular processes such as gene transcription, DNA synthesis and survival [21]. Earlier, co-aggregation of FcγRIIB and the B cell receptor (BCR) on B cells as well as the co-aggregation with the receptor for stem cell factor, Kit, present on mast cells, have been shown to affect the PI3-K pathway, inhibiting the activation of Akt [45-46]. The mRNA induction of the anti-apoptotic A1 protein has also been examined following co-aggregation of FcεRI and FcγRIIB.

Activation and Survival

The co-aggregation of FcεRI and FcγRIIB is known to inhibit the mast cell degranulation and release of mediators seen during aggregation of FcεRI [30]. The mast cell survival after co-aggregation has not been studied previously but a report has shown that FcεRI aggregation promotes survival after activation, rescuing the mast cells from apoptosis [27].

In this study, using release of mediators as a measurement of mast cell activation, the co-aggregation of FcεRI and FcγRIIB clearly suppressed the level of release compared to the levels of release seen during FcεRI aggregation. This is consistent with previous findings and both activation systems (System I and II) show this pattern of release inhibition when co-aggregating FcεRI and FcγRIIB.

The results from the survival experiments analyzed by flow cytometry gave no clear evidence for a change in mast cell survival after co-aggregating FcεRI and FcγRIIB compared to the survival after aggregating FcεRI. The activated cells have a slightly lower survival percentage compared to unactivated cells. The results from trypan blue staining present a somewhat different picture. Co-aggregation of FcεRI and FcγRIIB decreases the survival with 5-20% compared to FcεRI aggregation and unactivated cells, which are quite similar in survival percentage throughout the whole experiment.

The two methods for assessing cell survival, propidium iodide staining and trypan blue staining use the same principle of detecting apoptosis. By staining DNA they make apoptosis detectable. However, the two methods might not have the same sensitivity in detecting the apoptotic cells, making the interpretations of these survival experiments difficult. Comparing a set of experiments have also presented some problems since the results have varied to some extent even within the methods, the differences in survival apparently being small, if at all detectable.

These inconclusive experiments have not proven the hypothesis that co-aggregation of FcεRI and FcγRIIB would induce apoptosis in mast cells nor could they confirm the survival promoting effect previously seen when aggregating FcεRI on mast cells [27]. Further studies will be needed in order to elucidate the effect on mast cell survival after co-aggregation of FcεRI and FcγRIIB.

Akt, FKHRL1 and Bim

One signaling pathway affected by FcεRI aggregation is the PI3-K pathway. Activated PI3-K can via 3-phosphoinositide-dependent protein kinases phosphorylate the protein Akt [22]. Akt was recently found to phosphorylate and thereby inactivate members of the transcription factor forkhead family such as FKHRL1 [20], [23]. Unphosphorylated FKHRL1 is located in the nucleus, where it acts a transcription factor for certain genes [20]. The pro-apoptotic Bcl-2 interacting mediator of cell death (Bim) is a member of the Bcl-2 family and is known to be under the transcriptional control of the forkhead transcription factor FKHRL1 [28-29].

In order to investigate how some of the PI3-K pathway transducing proteins are affected by the co-aggregation of FcεRI and FcγRIIB compared to FcεRI aggregation, the phosphorylation kinetics of Akt and FKHRL1 as well as the regulation of Bim expression were examined.

Using the FcεRI aggregation to activate the mast cells, Akt becomes rapidly phosphorylated at the Ser 473 site investigated with its maximum phosphorylation occurring at 10 minutes. However, the phosphorylation pattern after co-aggregation of FcεRI and FcγRIIB showed that Akt was being phosphorylated to a lower extent and Ser 473 reached its maximum phosphorylation stage within 5 minutes. These results demonstrate that co-aggregation of FcεRI and FcγRIIB reduces the phosphorylation of Akt compared to FcεRI aggregation.

The forkhead protein FKHRL1 is, as well as Akt, subject to rapid phosphorylation on Thr 32 reaching its maximum phosphorylation stage within 5 minutes and there is no difference in phosphorylation levels comparing FcεRI aggregation and co-aggregation of FcεRI and FcγRIIB. This demonstrate that co-aggregation of FcεRI and FcγRIIB and FcεRI aggregation result in the same phosphorylation pattern of FKHRL1. There is a detectable background level of phosphorylation observable on the western blot, indicating a balance between phosphorylated and unphosphorylated FKHRL1 present in unactivated cells. The two bands seen after using the total FKHRL1 antibody are probably due to a distinct separation of phosphorylated and unphosphorylated FKHRL1, the total FKHRL1 antibody detecting both.

Given that co-aggregation of FcεRI and FcγRIIB reduces the phosphorylation of Akt compared to FcεRI aggregation one might expect the phosphorylation of FKHRL1 to be decreased in phosphorylated FKHRL1 due to less phosphorylated Akt. However, since the phosphorylation of Akt is not totally abrogated there might still be enough phosphorylated Akt able to phosphorylate FKHRL1 to the same extent.

After FcεRI aggregation and co-aggregation of FcεRI and FcγRIIB the two isoforms Bim_{XL} and Bim_{L} of Bim were upregulated. It appeared as if Bim_{XL} consisted of two bands, the upper one possibly due to phosphorylation. The level of Bim expression was more or less the same after using either FcεRI aggregation or co-aggregation of FcεRI and FcγRIIB. These results demonstrate that Bim is upregulated to a similar level using either FcεRI aggregation or co-aggregation of FcεRI and FcγRIIB compared to unactivated control cells. The upper band of Bim_{XL} was slightly stronger for unactivated cells, incubated with IgE and 2.4G2 antibody, compared to that of unactivated cells incubated with only IgE. This is a result that needs to be further investigated.

The finding that Bim_{XL} consists of two bands indicates that Bim has been phosphorylated. Previous studies have shown that Bim_{XL} and Bim_{L} are phosphorylated upon IL-3 treatment of murine hematopoietic cells [47]. Phosphorylation of Bim has not been reported in mast cells before and the effect on the pro-apoptotic mode of Bim is not known. However, it has been reported that nerve growth factor (NGF) down-regulate Bim in murine neuronal cells and also suppresses its pro-apoptotic activity by phosphorylation [48].

A1

RT-PCR confirmed that A1 mRNA was induced after FcεRI aggregation, compared to co-aggregation of FcεRI and FcγRIIB and unactivated cells where only low levels of A1 mRNA could be detected. This confirms previous studies having reported that A1 is strongly induced on mRNA level upon activation of the mast cell through FcεRI aggregation [27]. In order to investigate this further the RNAse protection assay method was used. RPA is a highly sensitive and specific method for the detection and quantification of mRNA. The RPA showed that A1 mRNA was upregulated both when activating the mast cells through FcεRI aggregation and co-aggregation of FcεRI and FcγRIIB.

The quantification of A1 mRNA showed that A1 is upregulated ~1:9 for the co-aggregation of FcεRI and FcγRIIB and ~1:12 for FcεRI aggregation. These results show that A1 is clearly upregulated following FcεRI aggregation and co-aggregation of FcεRI and FcγRIIB. Since A1 previously has been shown to be crucial in order for mast cells to survive FcεRI aggregation it raises the question of the role of upregulated A1 after co-aggregation of FcεRI and FcγRIIB [27].

Investigating the Fc receptor signaling upon activation and examining the modes and actions of transducing proteins will help to gain more knowledge about the effect FcγRIIB have on the FcεRI induced signalling, regulating mast cell survival and apoptosis. Although degranulation and release being affected when co-aggregating FcεRI and FcγRIIB, it has been shown that the downstream signalling proteins FKHRL1, Bim and A1 and the action of Akt protein seem to be largely unaffected compared with FcεRI aggregation. This shows that only certain signalling pathways are affected by the co-aggregation of FcγRIIB and FcεRI whereas others remain largely unaffected.

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