Immune complex-mediated co-ligation of the BCR with FcγRIIB results in homeostatic apoptosis of B cells involving Fas signalling that is defective in the MRL/Lpr model of Systemic Lupus Erythematosus

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ABSTRACT

Negative regulation of B cell activation by cognate immune complexes plays an important homeostatic role in suppressing B cell hyperactivity and preventing consequent autoimmunity. Immune complexes co-ligate the BCR and FcγRIIB resulting in both growth arrest and apoptosis. We now show that such apoptotic signalling involves induction and activation of p53 and its target genes, the pro-apoptotic Bcl-2 family members, Bad and Bid, as well as nuclear export of p53. Collectively, these events result in destabilisation of the mitochondrial and lysosomal compartments with consequent activation and interplay of executioner caspases and endosomal-derived proteases. In addition, the upregulation of Fas and FasL with consequent activation of caspase 8-dependent death receptor signalling is required to facilitate efficient apoptosis of B cells. Consistent with this role for Fas death receptor signalling, apoptosis resulting from co-ligation of the BCR and FcγRIIB is defective in B cells from Fas-deficient MRL/MpJ-Fas^{lpr} mice. As these mice develop spontaneous, immune complex-driven lupus-like glomerulonephritis, targeting this FcγRIIB-mediated apoptotic pathway may therefore have novel therapeutic implications for systemic autoimmune disease.

Keywords: B lymphocytes, FcγRIIB, apoptosis, caspases, Fas

1. INTRODUCTION

B-lymphocytes are the principal mediators of the humoral (antibody) immune response: recognition of antigen (Ag) by the B cell Receptor (BCR) induces the clonal expansion and differentiation of B cells into antibody (Ab)-producing plasma cells [1]. Negative feedback inhibition of such responses by cognate IgG-containing immune complexes [2-6] is a result of co-ligation of the BCR with the low affinity Fcγ receptor, FcγRIIB, promoting the induction of growth arrest in the G_1 phase of the cell cycle and consequent commitment of the B cells to apoptosis [7, 8]. In addition to such homeostatic negative feedback inhibition of ongoing B cell responses, ligation of FcγRIIB by non-cognate immune complexes can induce B cell anergy and/or apoptosis and hence, FcγRIIB signalling acts to suppress B cell activation and potential autoimmunity [8, 9].

The precise mechanisms underlying apoptosis resulting from co-ligation of the BCR with FcγRIIB have yet to be delineated but we and others have previously reported recruitment of negative elements (eg SHIP, SHP, DOK, PTEN and PAC-1) that antagonise BCR signalling [10-13] as well as disruption of the mitochondrial membrane potential (MMP) [14], the loss of which is considered to be the so called ‘point of no return’, after which cells irreversibly engage the ‘intrinsic’ or mitochondrial apoptosis programme [15]. We now show that such FcγRIIB-driven apoptosis is mediated by both mitochondrial- and lysosomal-effector mechanisms and by upregulation of Fas and consequent coupling to caspase 8-dependent death receptor signalling.

2. MATERIALS AND METHODS

2.1. Animals

MRL/MpJ-*Fas*^{lpr} and the parental strain, MRL/MpJ were purchased from Harlan Laboratories (Oxon, UK) and then maintained with "in house" bred BALB/c mice at the Central Research Facility, University of Glasgow in accordance with UK Home Office Licence guidelines.

2.2. Antibodies, inhibitors and cell culture reagents

Cell culture reagents were purchased from Invitrogen Life Technologies (Paisley, UK) except for foetal bovine serum (FBS), which was obtained from Lonza (Lonza Biologicals, Cambridge UK). B cells were stimulated by F(ab')$_2$ fragments of goat-anti-mouse IgM antibodies at 50 μg/ml to ligate the BCR and intact rabbit anti-mouse anti-IgM antibodies at 75 μg/ml to co-ligate the BCR and FcγRIIB (Jackson Immunoresearch Laboratories, Stratech Scientific Ltd; Beds., UK) except where the following antibodies were indicated to ligate the BCR and FcγRIIB: monoclonal rat anti-mouse IgM (B7.6) at 50 μg/ml (BCR), monoclonal rat anti-mouse FcγRIIB (2.4G2) at 50 μg/ml and donkey anti-rat IgG (Jackson Immunoresearch Laboratories) at 75 μg/ml to cross-link the B7.6 and 2.4G2 antibodies [10]. Additional antibodies were: anti-BAD (C20), anti-Cathepsin-B (C-19), anti-GAPDH and anti-β-actin (Santa Cruz Biotechnology Inc. Heidelberg, Germany); anti-full length and anti-cleaved caspase 8, anti-phosphoSer15-p53, anti-p53 (1C12), anti-BID, (Cell Signaling Technology, Danvers, MA); anti-Fas (Clone 13, BD Biosciences, Oxford, UK or rabbit polyclonal Santa Cruz), anti-FASL (Clone 33, BD Biosciences; Armenian hamster anti-FasL (anti-

CD178) IgG antibody and isotype control, BioLegend, Cambridge Bioscience, Cambridge UK;) and anti-p53 (Calbiochem, Merck, Middlesex UK).

Inhibitors of caspase 8 (z-IETD-FMK), capase 9 (z-LEHD-FMK), caspase 2 (z-VDVAD-FMK), caspase 3 (z-DEVD-FMK), cathepsin B (EST) and calpain (inhibitor V) were purchased from Calbiochem (Merck) [16-18]. The pan-caspase inhibitor (Q-VD-OPhe [19]) was obtained from Imgenex Corp (Cambridge Bioscience) while E64d, Pepstatin A and Microcystin were from Enzo Life Sciences (Exeter UK).

2.3. DNA Synthesis ([^{3}H]-thymidine uptake) assay

Murine B cells (>95% B220$^+$) were purified from BALB/c (>9 weeks) spleens using the CD43-magnetic bead negative-selection method according to the manufacturer's instructions (Miltenyi Biotec, Surrey UK). B cells (2 x 10^5 cells/well) were stimulated as indicated (in triplicate) in RPMI-1640 medium containing 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 50 μM 2-mercapthoethanol,1% (v/v) non-essential amino acids and 1 mM sodium pyruvate for 48 h at 37^{o}C in 5% (v/v) CO_2 atmosphere and pulsed with 0.5 μCi/well [6-^{3}H]-thymidine (Amersham Pharmacia Biotech, GE Healthcare Life Sciences, Bucks., UK) for the last 4 h of culture before harvesting using a Betaplate 96-well liquid scintillation counter (Amersham)

2.4. Flow cytometry

All data were acquired with a FACSCalibur using CellQuest software (BD Biosciences) and analysed by FlowJo software (TreeStar Inc., Ashland, OR) as described previously [20, 21].

2.4.1. Phenotypic markers

Cells (0.5 x 10^6 cells/sample) were washed with ice-cold PBS containing 1% BSA and 2mM EDTA, 0.05% sodium azide and incubated with the indicated fluorochrome-conjugated Abs for 30 min at 4°C**.** Antibodies used for FACS analysis were anti-Fas, purified and FITC-conjugated (Jo2, BD Biosciences), anti-B220-APC (RA3-6B2) and anti-CD43-PE (1B11; BioLegend).

2.4.2. CFSE analysis

Cells (10^5 cells/sample) were resuspended in PBS containing 0.1% BSA and loaded with 2.5 μM CFSE for 10 min at 37°C. CFSE staining was detected by FACS and analysed using FlowJo software to identify the number of cell divisions undergone.

2.4.3. DNA Content Analysis

Cells were stained with propidium iodide (PI) solution (0.1% (w/v) sodium (tri) citrate, 0.1% (v/v) triton-X-100, 50 μg/ml PI, 50 μg/ml RNase A) for 30 min on ice.

2.4.4. MMP

Cells were stained with 2.5 μM $DiOC_6$ (Molecular Probes, Invitrogen Life Technologies) in PBS containing 0.1% BSA for 30 min at room temperature. The typical biphasic profiles enabled gating of cells into high (healthy) or low (apoptotic) MMP.

2.4.5. LMP

Lysosomal damage was assessed by acridine orange (1 μg/ml, AO, Sigma) or Lysotracker Red DND99 (100 nM, Invitrogen Life Technologies) assays [22, 23] following staining for 30 min or 1 h at 37°C in the dark, respectively.

2.4.6. Caspase activity

Cells (10^6 cells/well) were treated with the indicated stimuli at 37°C/5% CO_2 for up to 24 h before incubation with appropriate FITC-labelled caspase inhibitor (BioVision, Cambridge Bioscience) for 45 min.

2.5. Whole cell lysates and immune complexes

B cells (10^7 cells/sample) were lysed by the addition of ice-cold, modified RIPA buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 2% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 10 mM sodium orthovanadate plus 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml chymostatin, leupeptin, antipapain and pepstatin) and solubilised on ice for 30 min. Samples (1 mg/ml) were pre-cleared with protein-G-sepharose before being incubated with antibody (1-2 µg/sample) overnight at 4°C with constant rotation. Protein G-sepharose (10 µl) was then added for 2 h at 4°C and immune-complexes harvested by centrifugation (20,000 x g, 30 min, 4°C) followed by release from the sepharose beads by boiling in 50 mM Tris pH 6.8, 2% (v/v) bromophenol blue, 0.1% SDS and 10% v/v glycerol containing 2.5% 2-ME for 10 min at 100°C and pelleting of beads.

2.6. Cytosolic and membrane/organelle fractions

Membrane/organelle and cytosolic fractions (5-10 x 10^6 cells/sample) were prepared using the ProteoExtract Subcellular Proteome Extraction Kit from Calbiochem. Mitochondrial and cytosolic fractions ($5x10^7$ cells/sample) were prepared using the mitochondrial extraction kit from Active Motif (La Hulpe, Belgium). Nuclear and cytosolic fractions were prepared using the ProteoExtract Subcellular Proteome Extraction Kit from Calbiochem or in some experiments, using the Active Motif Nuclear Extract kit.

2.7. Western Blot Analysis

Protein (whole cell lysates) or cell equivalents (subcellular fractions) of samples were resolved on the XCell *SureLock* Mini-Cell kit with NuPAGE Novex high-performance pre-cast Bis-Tris gels and NuPAGE buffers and reagents (Invitrogen Life Technologies) at 200 V for 50 min. Proteins were transferred to nitrocellulose (Amersham) or PVDF (Millipore, Watford, UK) and membranes were blocked by incubating for 1 h in 5% non-fat milk or BSA in TBS/Tween (0.5 M NaCl and 20 mM Tris pH7.5 with 0.1% (v/v) Tween-20) at RT. Membranes were incubated with primary antibody diluted in 5% non-fat milk or BSA in TBS/Tween buffer overnight at 4°C, washed with TBS/Tween and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at RT. Membranes were then washed with TBS/Tween and protein bands were visualised using the ECL detection system. Quantification of the bands was performed using ImageJ software (National Institute of Health).

2.8. Cytochrome C, TransAM and FACE assays

Cytochrome C levels in cytosolic and mitochondrial fractions (cell equivalents) were measured by ELISA. p53 activity in nuclear extracts (2 µg) was measured using a TransAM kit. AKT activity and BAD phosphorylation status (5 x 10^5 cells/well) were measured by FACE assays. All kits were from Active Motif and performed according to manufacturer's instructions.

2.9. RT-PCR

Total mRNA was isolated using a RNeasy Mini Kit (Qiagen, Crawley, West Sussex) and DNAse I digested RNA transcribed into cDNA using the Superscript II Reverse Transcriptase system (Invitrogen Life Technologies) or High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems, Life Technologies). FASL Primers were from Applied Biosystems (Mm 00438864) and RT-PCR was performed using Eurogentec mastermix. The probes contained a reporter (FAM) and quencher (TAMRA) dye and PCR reactions were performed in the ABI-prism 7700 Sequence Detector or the 7900HT Fast Real-Time PCR System (Applied Biosystems) and amplification was performed using the following conditions: 2 min at 50°C, 10 min at 95°C, followed by a total of 45 two-temperature cycles (15 s at 95°C and 1 min at 60°C).

3. Results

3.1. Apoptosis resulting from co-ligation of the BCR and FcγRIIB can be blocked by the pan-caspase inhibitor Q-VD-OPhe

Co-ligation of the BCR and FcγRIIB (X) inhibits BCR-mediated proliferation (Fig. 1A): we, and others, have previously shown [14, 24] that this reflects G_0/G_1 growth arrest (Figs. 1Aii & 1B) and commitment to apoptosis consequent to disruption of the MMP and cytochrome C release from mitochondria to the cytosol (Figs. 1B-E). Although growth arrest was not reversed by treatment with the pan-caspase inhibitor, Q-VD-OPhe (Q; Figs. 1Aii & B) the dissipation of MMP, which could be detected within 6-18 h following BCR/FcγRIIB co-ligation (Fig. 1D), and apoptosis were suppressed to the levels observed in cells treated with the survival stimulus, IL-4 (Figs. 1B-D).

3.2. Co-ligation of BCR/FcγRIIB activates multiple caspases

To identify which caspases are involved in transducing apoptosis, we assayed for activation of initiator and executioner caspases and found that caspases 3, 8 and 9 were activated following BCR/FcγRIIB co-ligation as early as 6 h (Figs. 2A-C). Caspase 2 was also assayed at 18h and found to be active (results not shown). Pre-treatment with the pan-caspase inhibitor Q-VD-OPhe reduced the levels of BCR/FcγRIIB-stimulated caspase activation to those observed with the IL-4 alone, confirming that the caspase activation is specific and occurs as a result of BCR/FcγRIIB co-ligation.

Having ascertained that caspases 2, 3, 8 and 9 are activated upon BCR/FcγRIIB co-ligation, we investigated which, if any, of these play a crucial role in the resulting apoptotic programme. Although not as effective as the pan-caspase inhibitor, Q-VD-

OPhe (caspases 1-9 and 12), pre-treatment with the specific caspase 8 inhibitor (Z-IETD-FMK) suppressed BCR/FcγRIIB-mediated apoptosis to the levels observed in cells stimulated with the survival signal, IL-4. Similarly, but to a lesser extent, inhibitors of caspase 9 (Z-LEHD-FMK) and caspase 3 (Z-DEVD-FMK), but not that of caspase 2 (Z-VDVAD-FMK), also provided rescue from apoptosis (Fig. 3A), although the caspase 3 inhibitor has also been reported to inhibit human caspases 6-8 and 10, albeit at higher concentrations [18, 25]. By contrast, none of these selective inhibitors of caspases were able to significantly block MMP dissipation (Fig. 3B and results not shown), suggesting that caspases 3, 8 and 9 mediated their apoptotic effects independently and/or downstream of mitochondrial disruption. Moreover, as with Q-VD-OPhe, none of the selective caspase inhibitors tested were able to restore BCR-mediated cell proliferation as assessed by DNA synthesis (Fig. 3C).

3.3. BCR/FcγRIIB co-ligation induces lysosomal destabilisation

There is increasing evidence that in addition to effector caspases, proteases released from the endosomal/lysosomal system are also involved in executing apoptotic programmes [26]. Indeed, we have previously shown that Cathepsin B plays a key role in mediating BCR-driven apoptosis of the immature B cell lymphoma, WEHI-231 [21] and it has been more recently shown that germinal centre (GC) B cells undergo apoptosis which involves lysosomal destabilisation indicative of lysosomal membrane permeabilisation (LMP) and release of such proteases [27]. BCR/FcγRIIB co-ligation was found to cause LMP within 3-6 h (Fig. 4A) as indicated by both the AO and lysotracker assays and detection of cleaved Cathepsin B in the cytosolic compartment

(Fig. 4B). Consistent with LMP playing a role in transducing apoptosis resulting from BCR/FcγRIIB co-ligation, LMP was reduced to the levels observed in the presence of the survival signal IL-4 by the pan-caspase inhibitor Q-VD-OPhe (Fig. 4C). Perhaps rather surprisingly therefore, BCR/FcγRIIB-mediated apoptosis was not blocked by selective inhibitors of the endosomal-derived proteases Cathepsin B and Calpain (EST and Calpain Inhibitor, respectively) or the lysosomal protease inhibitors, E64d plus Pepstatin A (Fig. 4D). However, the partial rescue afforded by the caspase 9 inhibitor was substantially enhanced when the cells were treated with the caspase 9 inhibitor in combination with the inhibitors of Cathepsin B plus Calpain but not the inhibitors of lysosomal proteases, E64d plus Pepstatin A (Figs. 4D). Similar effects were observed when the cells were treated with the caspase 3 inhibitor, in concert with inhibitors of the endosomal or lysosomal proteases (data not shown). Although LMP appeared to be an earlier event than disruption of MMP, blocking of caspase or endosomal/lysosomal protease activity, either alone or in combination, did not prevent MMP dissipation (data not shown). Similarly, LMP was not prevented by treatment with any of the selective inhibitors of caspases found to suppress apoptosis, either alone or in combination with the inhibitors of endosomal/lysosomal proteases (data not shown).

3.4. Interplay of p53, Bid and Bad signalling is associated with mitochondrial and lysosomal disruption consequent to BCR/FcγRIIB co-ligation

The tumour suppressor, p53 mediates apoptosis via transcriptional regulation of genes such as pro-apoptotic Bcl-2 family members including Bad, Bid and Bax [28-32]. Consistent with this, BCR/FcγRIIB signalling upregulates p53 expression, phosphorylation and transactivation, relative to that seen in B cells stimulated via the

BCR or treated with IL-4 alone (Figs. 5A & B), and results in the upregulation of the p53 targets, Bid (Figs. 5C) and Bad (Fig. 5D). These Bcl-2 family members have been implicated in the oligomerisation of Bax resulting in dissipation of the MMP and LMP [15, 33].

In addition to being upregulated following BCR/FcγRIIB signalling, Bid is proteolytically cleaved to the truncated tBid product (Fig. 5C) that has been reported to translocate to the outer mitochondrial membrane to facilitate Bax/Bak pore formation [34, 35] and to the lysosomes to promote release of Cathepsin B [33, 36]. Consistent with its protective effects against MMP and LMP, the pan-caspase inhibitor Q-VD-OPhe inhibits BCR/FcγRIIB-mediated caspase 8 cleavage and tBid generation. By contrast, although Bid has been reported to be a caspase 8 target, the caspase 8 inhibitor (z-IETD-FMK) did not block generation of tBid (Fig. 5C), a finding that is consistent with its observed lack of protection against MMP and LMP (Fig. 3B & 4C).

Akt-mediated hyper-phosphorylation of Bad leads to its sequestration by 14-3-3 proteins and functional inactivation [29, 37]. Thus, the finding that B cells exhibiting higher levels of Bad expression in response to BCR/FcγRIIB crosslinking (Fig. 5D) also show reduced Bad phosphorylation and Akt activity (Fig. 6A), compared with those stimulated via the BCR, provides support for a role for Bad in destabilising mitochondrial and/or lysosomal membranes under such apoptotic conditions.

Moreover, p53 can act as an inducer of apoptosis independently of its transcriptional activity. Thus, it has been shown that p53 can translocate to the cytoplasm where it acts, either independently or in concert with Bid [38, 39] or Bad [40], on mitochondria and/or lysosomes to induce MMP disruption and/or LMP [33, 38, 40-

42]. Analysis of the cytosolic and nuclear fractions of B cells following co-ligation of the BCR/FcγRIIB revealed p53 to be found in both locations (Fig 6B) suggesting that under such apoptosis-inducing conditions, p53 is fulfilling both indirect gene induction and direct MMP/LMP-apoptotic functions. Further support for a direct p53-mediated LMP mode of action was provided by the finding that FcγRIIB- or BCR/FcγRIIB-, but not BCR, signalling resulted in the phosphorylation of p53 on Ser15 prior to onset of LMP (Figs. 5A & 6C) as this modification has been reported to act as a lysosomal targeting signal for p53 where it interacts with the lysosomal-associated apoptosis-inducing protein containing the pleckstrin homology and FYVE domains (LAPF) and triggers LMP associated with apoptosis [41]. Treatment of the cells with the pan-caspase or caspase 8 inhibitors did not suppress the cytosolic expression of p53.

3.5. BCR/FcγRIIB co-ligation modulates Fas/FasL expression and consequent death receptor signalling

Collectively, the above data suggest that caspase 8, Bad, Bid and p53 are major players in executing the apoptotic programme initiated by BCR/FcγRIIB co-ligation. As these are all targets of death receptor signalling [43] and activated B cells are known to express the death receptor, Fas on their surface [44-46], we next investigated whether co-ligation of BCR/FcγRIIB co-ligation modulates Fas and/or FasL expression rendering them susceptible to Fas/FasL mediated cell death.

BCR/FcγRIIB co-ligation was indeed found to substantially increase both the portion of B cells expressing Fas on their surface and also the levels of Fas expressed on the surface of such cells, compared to the levels detected on cells treated with IL-4, and this upregulation was caspase 8 independent (Fig. 7A). These findings were

corroborated by the analysis of total Fas protein expression, in the presence and absence of the caspase 8 and pan-caspase inhibitors, by Western blot analysis which confirmed that the increased expression following BCR/FcγRIIB co-ligation was caspase independent (Fig. 7B and results not shown).

Fas signalling is initiated by FasL-mediated receptor trimerisation which drives formation of the Death Inducing Signalling Complex (DISC) and activation of caspase 8 [47]. Consistent with reports that FasL has also been shown to be expressed by activated B cells [44], assessment of the levels of FasL expression by Western blot and q-RT-PCR analysis revealed that BCR/FcγRIIB stimulation (Figs. 7Bi-iii) also upregulates FasL mRNA and protein expression, relative to that seen following crosslinking of the BCR, in a caspase 8 independent manner, presumably to facilitate efficient induction of apoptosis via fratricide or suicide of immune complex-regulated B cells.

That Fas/FasL signalling plays a functional role in immune complex-driven apoptosis was shown by (a) the ability of anti-FasL blocking antibodies to suppress the extent of BCR/FcγRIIB-mediated apoptosis (Fig. 8A) and (b) the dysfunctional apoptotic responses of B cells from the MRL/MpJ-Fas^{lpr} mouse (7-20 weeks), which do not express, or upregulate Fas on their cell surface in response to co-ligation of the BCR/FcγRIIB (Fig. 8B). Such naïve, quiescent (CD43^{-}) B cells express the BCR and FcγRIIB at levels comparable to those of B cells from Fas$^{+/+}$ BALB/c mice and these receptors are downregulated to a similar degree following BCR/FcγRIIB co-ligation in both the Fas-deficient and wild type cells (Fig. 8C & D). However, MRL/Lpr B cells displayed reduced apoptosis and growth arrest (inhibition of DNA synthesis) resulting

from BCR/FcγRIIB co-ligation relative to cells from the parental MRL/MpJ and BALB/c mouse strains (Fig. 9A&B). Importantly, this reduction in apoptosis observed in MRL/MpJ-Fas^{lpr} B cells did not simply reflect modulation of the kinetics of induction of LMP, MMP or sub-diploid status of the cells in response to BCR/FcγRIIB co-ligation observed in BALB/c B cells (Fig. 9C), indicating involvement of additional Fas-specific signals.

4. Discussion

Negative regulation of B cell activation by cognate immune complexes plays an important homeostatic role in preventing B cell hyperactivity and consequent potential autoimmunity. Immune complex-mediated negative feedback inhibition results in both growth arrest and apoptosis and we now show that full activation of this apoptotic pathway requires the upregulation and activation of Fas death receptor signalling. Such apoptotic signalling is associated with caspase 8 activity, recruitment of both transcriptional-dependent and independent p53 apoptotic pathways and destabilisation of mitochondrial and endosomal/lysosomal compartments with consequent activation and interplay of executioner caspases and endosomal/lysosomal-derived proteases. The requirement for disruption of both mitochondrial- and lysosomal-integrity is evidenced by the finding that inhibitors of effector caspases 3 and 9 were only partially protective whilst those selective for the endosomal-derived proteases, cathepsin B and calpain, could only prevent apoptosis when used in combination with inhibitors of the various effector caspases.

The precise mechanisms involved in BCR/FcγRIIB-mediated MMP and LMP have not yet been fully delineated but involve gene induction of the p53 targets, Bad and Bid that presumably activate Bax to induce MMP and/or LMP, as well as direct destabilising effects of p53 on mitochondrial and/or lysosomal membranes [33, 38, 40-42]. Interestingly, p53 signalling has previously been shown to upregulate Fas expression both by transcriptionally-dependent [48] and -independent means, the latter reflecting increased trafficking of Fas from

intracellular stores to the cell surface [49], suggesting that it may be involved in BCR/FcγRIIB-coupled apoptotic signalling both upstream and downstream of Fas death receptor upregulation. Similarly, FasL upregulation and increased death receptor signalling has been reported to be a result of inhibition of Akt in Jurkat cells [50] and this may also be the case during FcγRIIB-mediated negative feedback inhibition, in which the B cells exhibit reduced Akt signalling [10, 51]. Thus, in addition to promoting the activities of the pro-apoptotic Bcl-2 family members, Bad and Bid, BCR/FcγRIIB-mediated suppression of Akt activity and induction of p53 signalling may act to expedite apoptosis of B cells undergoing negative feedback inhibition by immune complexes by upregulating Fas and FasL to facilitate fratricide/suicide of such B cells.

MMP dissipation and LMP are both reported to be promoted by the pore-forming Bcl-2 family member, Bax and its activator tBid whilst dephosphorylated Bad has, to date, been implicated only in transducing mitochondrial disruption [33, 52, 53]. Perhaps consistent with this, the pan-caspase inhibitor Q-VD-OPhe, which blocked Bid cleavage, could only partially protect against MMP whilst it could fully prevent LMP and apoptosis. It was at first sight perhaps surprising that caspase 8 did not mediate Bid cleavage as reported for Fas-mediated apoptosis of other cell types [34], but this finding was corroborated by the lack of protection against MMP and LMP afforded by the caspase 8 inhibitor, despite its ability to suppress apoptosis. Moreover, these data reflect earlier studies suggesting that death receptor-mediated caspase 8 signalling directly activates caspase 3 and other effector molecules in lymphoid cells [34, 35]. The identity of the caspase(s)

involved in Bid cleavage remains unclear, as none of the inhibitors of caspase 2, 3, 8 or 9 were alone able to block either MMP or LMP. However, this lack of effect of individual caspases may simply reflect the complex interplay of caspases, cathepsins and calpains resulting from MMP disruption and LMP required for BCR/FcγRIIB-mediated apoptosis as cathepsin-mediated tBid formation and consequent mitochondrial disruption has previously been shown to occur downstream of caspase-dependent LMP in other systems [54, 55]. Indeed, B cells appear to employ different effector mechanisms of apoptosis as tolerance checkpoints at distinct stages of development as we have previously shown that whilst BCR-mediated apoptosis of WEHI-231 immature B cells is dependent on cathepsin B, it can occur independently of caspase activation [21, 56]. By contrast, BCR-mediated apoptosis of PNA^+ GC B cells can be partially blocked by caspase inhibitors [56] and consistent with the later finding, apoptosis of human GC B cells requires the activation of both caspase and cathepsin activities, the cathepsin activity being downstream of caspase 3 and responsible for exonuclease activity and execution of apoptosis [57].

Nuclear export and targeting of p53 to the mitochondria and lysosomes for direct apoptotic signalling has been shown to occur by several mechanisms. Firstly, nuclear export of p53 (and its related family members, p63 and p73) and translocation to the mitochondria has been shown to reflect cleavage by a variety of caspases (caspases 3, 6, 7 and 8), generating truncated forms, some of which lack the nuclear localisation signal and exhibit enhanced apoptotic activity [58-60]. Nucleocytoplasmic shuttling of p53 also appears to be regulated

by several of the other apoptotic signals [42, 61, 62] induced by BCR/FcγRIIB co-ligation. Thus, whilst Bid and p53 have been reported to associate in order to facilitate nuclear export and induction of the mitochondrial pathway of apoptosis [38, 39], dephosphorylated Bad can also complex with, and target p53 to the mitochondria [40]. Similarly, following phosphorylation on Ser15, p53 is recruited to, and interacts with, LAPF at the lysosomes where it acts to destabilise lysosomal membranes [41, 63]. Collectively, the induction of caspase-, hypo-phosphorylated Bad, Bid and $p53^{pSer15}$ signals suggests that BcR/FcγRIIB–signalling may employ both Bid/Bad-dependent and independent mechanisms of trafficking p53 to the mitochondria and lysosomes and consequent induction of MMP and LMP.

FcγRIIB signalling is important, not only in regulating B cell activity, but also in maintaining B cell tolerance at late checkpoints of peripheral development, particularly those involving class-switched B cells and antibody-producing plasmablast/plasma cells. Thus, it has been proposed that the simultaneous activatory (Ag/BCR) and inhibitory (IgG/FcγRIIB) signals generated by immune complexes tethered to follicular dendritic cells in GC sets an activation threshold that ensures that only high affinity (non-autoreactive) B cells are capable of developing to antibody-producing B cells (reviewed in [64, 65]). Given the mitochondrial and lysosomal-components of FcγRIIB-mediated apoptosis demonstrated here, it is perhaps pertinent to the tolerance-maintaining function of FcγRIIB that it has previously been reported that apoptosis of GC B cells requires lysosomal destabilisation [27] and that Fas

signalling is involved in the deletion of low affinity BCR-expressing B cells in GC [66]. Consistent with this idea of FcγRIIB setting a tolerance-induction threshold, FcγRIIB-deficient mice on C57BL/6 backgrounds produce increased levels of antibodies of lower affinity and show increased susceptibility to induced and spontaneous autoimmune pathologies such as arthritis [67] and lupus-like glomerulonephritis [68]. Likewise, promoter and allelic polymorphisms resulting in low expression and/or functionality of FcγRIIB are associated with development of SLE in humans [64, 65]. Moreover, B cells from autoimmune prone-mice, including MRL mice, show reduced FcγRIIB expression and functionality on GC B cells and plasma cells [69-71] and reflecting this, retroviral transfer of FcγRIIB has been shown to restore tolerance in NZM2410 and BXSB mice [72]. Thus, although we showed comparable levels of the BCR and FcγRIIB in naïve, quiescent B cells from BALB/c and MRL/Lpr mice, we therefore additionally compared apoptosis resulting from co-ligation of the BCR and FcγRIIB in B cells from MRL/MpJ-Fas^{lpr} mice relative to that observed in the parental MRL/MpJ strain specifically to focus on the role of Fas rather than FcγRIIB levels: that immune complex-induced apoptosis of B cells is partially mediated via Fas death receptor signalling may therefore go some way to explaining development of the severe autoimmune glomerulonephritis in FcγRIIB-deficient mice as this is reminiscent of the lupus-like pathology exhibited by the MRL/MpJ-Fas^{lpr} mouse.

The Fas-specific apoptotic signals that are defective in MRL/Lpr B cells are not clear but are unlikely to impact on LMP and MMP as Fas upregulation is

only apparent some 20-48 h post crosslinking of the BCR with FcγRIIB, whilst significant LMP and MMP can be detected within 3-6 h respectively. Indeed, our data clearly show that LMP and MMP is essentially intact in the absence of Fas signalling. Moreover, whilst inhibition of the Fas effector, caspase-8 suppresses BCR/FcγRIIB-mediated apoptosis, it does not block either LMP or MMP and consistent with this, we also find that the caspase 8 inhibitor does not suppress the generation of tBID required for Bak/Bax-mediated MMP. Rather, our preliminary data (unpublished results) suggest that BCR/FcγRIIB signalling stimulates AMPK activation to phosphorylate p53 on Ser15, resulting in p53 stabilisation and consequent induction of Fas expression and caspase 8 activation [73, 74]. Similar lag times between such p53 stabilisation (maximal 1-4 h) and induction of Fas and caspase 8 activation (maximal >20 h) have previously been reported [74] in models of DNA damage–induced apoptosis and are proposed to reflect cell cycle arrest resulting from transactivation of p21waf1. This lag period reflects the time course of BCR/FcγRIIB-mediated, p53-dependent upregulation of Fas expression that occurs subsequent to establishment of LMP and MMP. Collectively, therefore, these data suggest that Fas/FasL upregulation may be a marker of an absolute “point of no return” and that such late Fas-mediated apoptotic signals reflect direct caspase 8-dependent activation of caspase 3 and associated effector pathways that complement the early mitochondrial/lysosomal pathways to expedite rapid and full execution of apoptosis. Thus, dysfunction of such Fas-dependent FcγRIIB-mediated negative feedback and tolerance-inducing signals in the MRL/MpJ-

Fas^{lpr} mouse could provide an additional mechanism to explain the chronic activation of autoreactive B cells and consequent extremely high levels of autoantibodies characteristic of the lupus-like pathology of this model [75, 76].

Interestingly, FcγRIIB deficiency is also associated with an increased resistance in naïve mice to infections such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*, although challenge of immunised FcγRIIB-deficient mice with *S. pneumoniae* resulted in higher mortality due to septic shock [65]. FcγRIIB signalling therefore appears to be essential not only for maintaining B cell tolerance and homeostasis but also for promoting an optimal balance in fighting infection without incurring septic shock. It has been proposed that modulation of FcγRIIB levels/signalling might be a promising therapeutic strategy for combating systemic autoimmune disorders such as SLE [64, 65] but these interesting findings relating to the role of FcγRIIB in regulating responses to infection suggest that more selective targeting and consequent upregulation of Fas-dependent apoptotic pathways may provide the starting point for safer therapies.

5. Acknowledgements

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6. Figure Captions

Figure 1. Immune complex-mediated disruption of MMP and consequent apoptosis is caspase-dependent. In all panels, B cells were stimulated as indicated with either medium, IL-4 (10 ng/ml) and/or 50 µg/ml $F(ab')_2$ anti-mouse IgM (BCR) or 50 µg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 µg/ml anti-mouse IgG+IgM (BCR/FcγRIIB-X). Where indicated, cells were pre-treated with the pan-caspase inhibitor Q-VD-OPhe (X+Q, 10 µM) for 1 h. (Ai) CFSE-labelled B cells were stimulated for 72 h and cell proliferation tracked by the dilution of CFSE fluorescence. Percentages of cells within each generation were plotted for each condition. Data are representative of three independent experiments. (Aii) DNA synthesis after 48 h of stimulation was assessed by the [^{3}H] thymidine uptake assay and data are shown as counts per minute (cpm) ± standard deviation (SD) of triplicate values from a single experiment representative of at least five independent experiments where $p<0.001$ (BCR stimulated cells compared to the cells which received BCR/FcγRIIB-X co-ligation). (B) DNA content was determined by FACS analysis of PI staining. The extent of apoptosis is presented as the percentage of cells containing subdiploid DNA in the total cell population. (i) Representative PI profile obtained after FACS analysis. (ii) Data are presented as mean percentage of all cells in each cell cycle stage (± SD of triplicate values, $p<0.001$ for BCR/FcγRIIB-X in comparison with X+Q-VD-Ophe and IL-4) and are representative of at least five experiments. (C) MMP was assessed after 24 h of stimulation by FACS analysis of $DiOC_6$ staining (i) Representative $DIOC_6$ profile after FACS analysis. (ii) Data

are presented as mean percentage of cells with low MMP (low $DIOC_6$ staining) (± SD of triplicate values) and are representative of at least three experiments, $p<0.001$ in comparison with BCR/FcγRIIB-X. (D) Time course of MMP dissipation shown as mean percentage of cells with low MMP ± SD of triplicate values. Data are representative of three experiments, $p<0.001$ for BCR/FcγRIIB-X in comparison with X+Q-VD-OPhe (E) Mitochondrial and cytosolic extracts were prepared following stimulation for 48 h and assessed for the cytochrome C content by ELISA. Results are displayed as a ratio of cytochrome C levels in the cytosol as compared to the mitochondria.

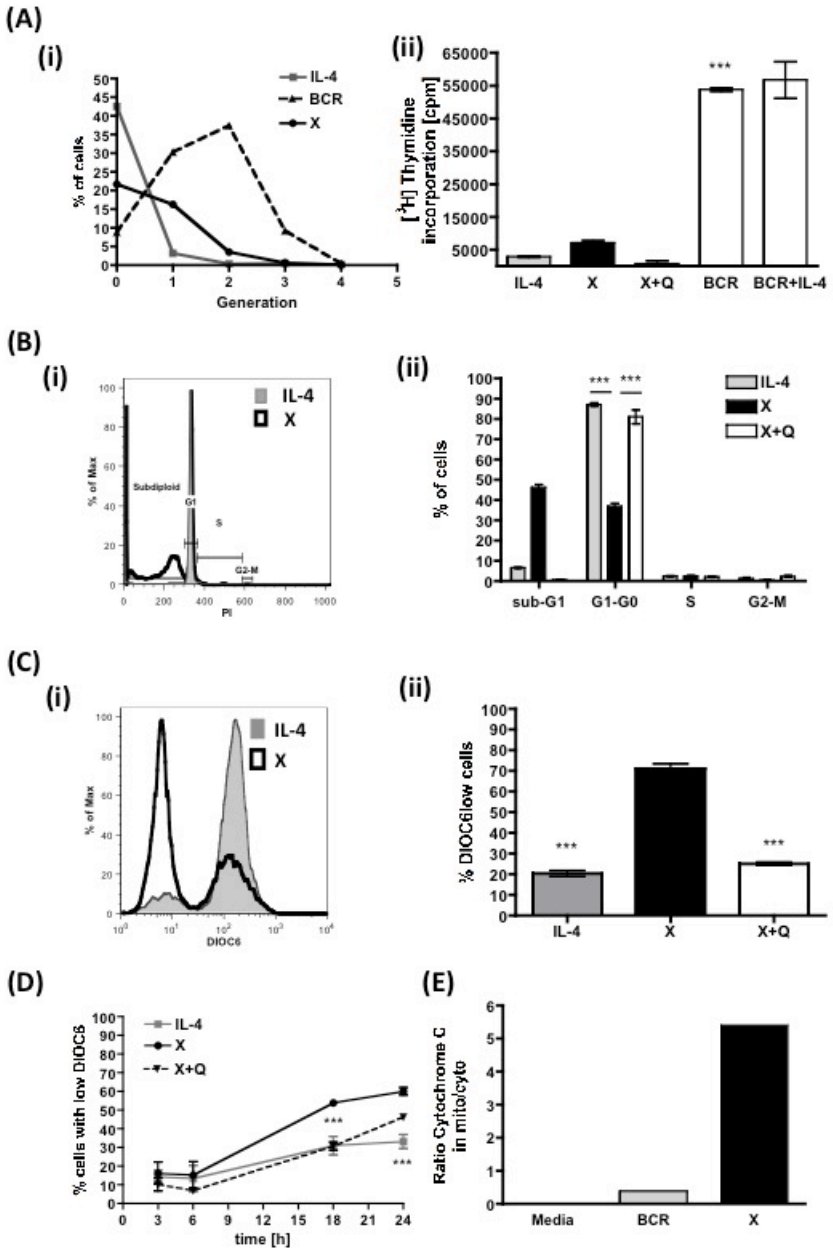
(A)
(i)
IL-4
BCR
X
% of cells
Generation
(ii)
[3H] Thymidine incorporation [cpm]
***
IL-4
X
X+Q
BCR
BCR+IL-4
(B)
(i)
IL-4
X
% of Max
Subdiploid
G1
S
G2-M
PI
(ii)
% of cells
***
***
IL-4
X
X+Q
sub-G1
G1-G0
S
G2-M
(C)
(i)
IL-4
X
% of Max
DIOC6
(ii)
% DIOC6low cells
***
***
IL-4
X
X+Q
(D)
% cells with low DIOC6
IL-4
X
X+Q
***
***
time [h]
(E)
Ratio Cytochrome C in mito/cyto
Media
BCR
X

Figure 1

Figure 2: Co-ligation of the BCR and FcγRIIB activates caspases. B cells were stimulated with either IL-4 (10 ng/ml) or 50 µg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 µg/ml anti-mouse IgG+IgM (BCR/FcγRIIB-X). Where indicated, cells were pre-treated with the pan-caspase inhibitor Q-VD-OPhe (X+Q, 10 µM) for 1 h. Caspase 3 (A), 8 (B) and 9 (C) activation was determined by FACS analysis of the appropriate FITC-conjugated caspase inhibitor. Representative histogram plots of the indicated FITC-inhibitor staining after 16 h of stimulation and the time courses of activation of the relevant caspases are shown where data are presented as percentage of cells that have caspase activity ± SD of triplicate values from a single experiment, representative of three independent experiments ($p<0.001$ BCR/FcγRIIB-X compared to either IL-4 or X+Q-VD-Ophe stimulated cells).

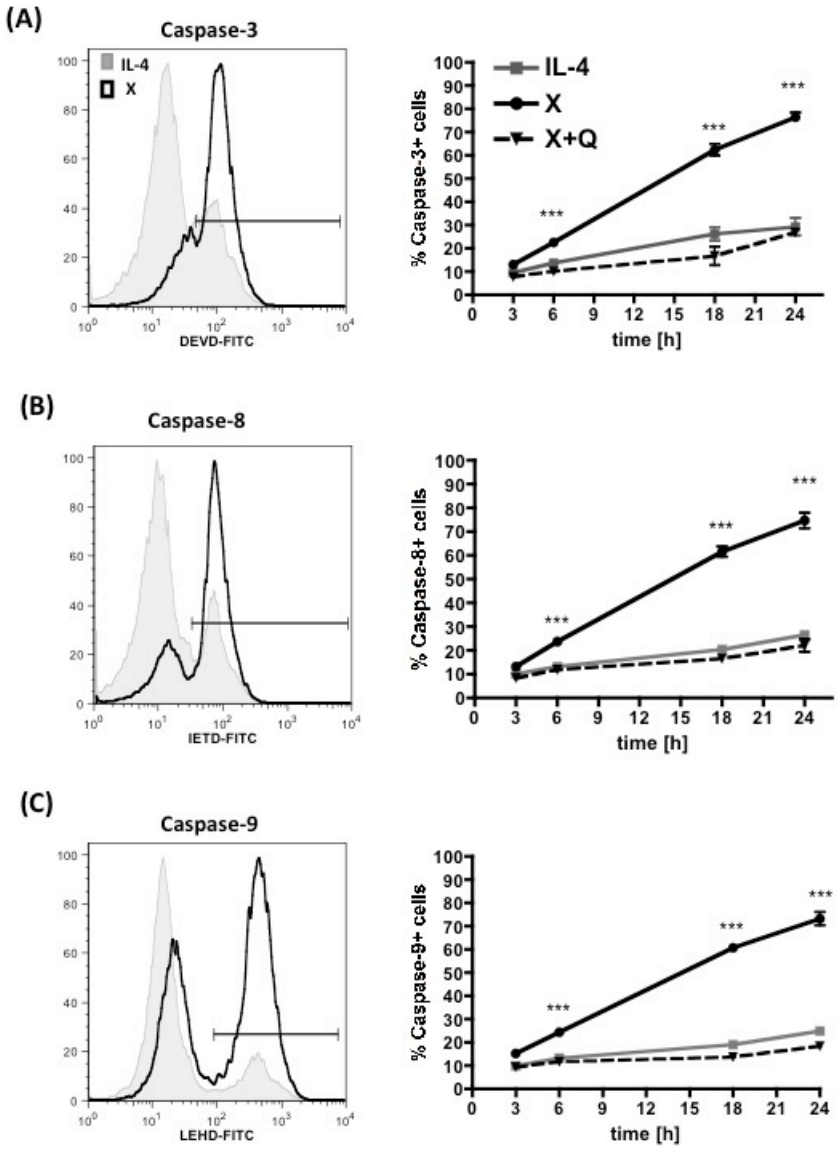
(A)
Caspase-3
IL-4
X
DEVD-FITC
IL-4
X
X+Q
% Caspase-3+ cells
time [h]
(B)
Caspase-8
IETD-FITC
% Caspase-8+ cells
time [h]
(C)
Caspase-9
LEHD-FITC
% Caspase-9+ cells
time [h]

Figure 2

Figure 3: Caspase 8 inhibition blocks apoptosis induced by co-ligation of BCR with FcγRIIB. B cells were stimulated with either medium, IL-4 (10 ng/ml) or 50 µg/ml $F(ab')_2$ anti-mouse IgM (BCR) or 50 µg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 µg/ml anti-mouse IgG+IgM (BCR/FcγRIIB-X). In addition, cells were pre-treated with the pan-caspase inhibitor Q-VD-OPhe (10 µM) or selective Caspase 2, 3, 8, or 9 inhibitors (10 µM) for 1 h prior to stimulation, as indicated. (A) Apoptosis at 24 h was assessed by PI staining and data presented as percentage of cells containing subdiploid DNA in total cell population. Data are presented as the mean of the mean values from three independent experiments ± SEM ($p<0.001$, compared to BCR/FcγRIIB-X). (B) MMP at 24 h was determined by FACS analysis of $DIOC_6$ staining and data are presented as the mean percentage of cells with low MMP ± SD of triplicate values from a single experiment representative of at least three independent experiments. ($p<0.001$, for X+Q or IL-4 stimulated cells compared to the cells stimulated through BCR/FcγRIIB-X). (C) DNA synthesis was assessed after 48 h of stimulation data are shown as mean cpm ± SD of triplicate values from a single experiment representative of two independent experiments ($p<0.001$, compared to the cells stimulated through BCR).

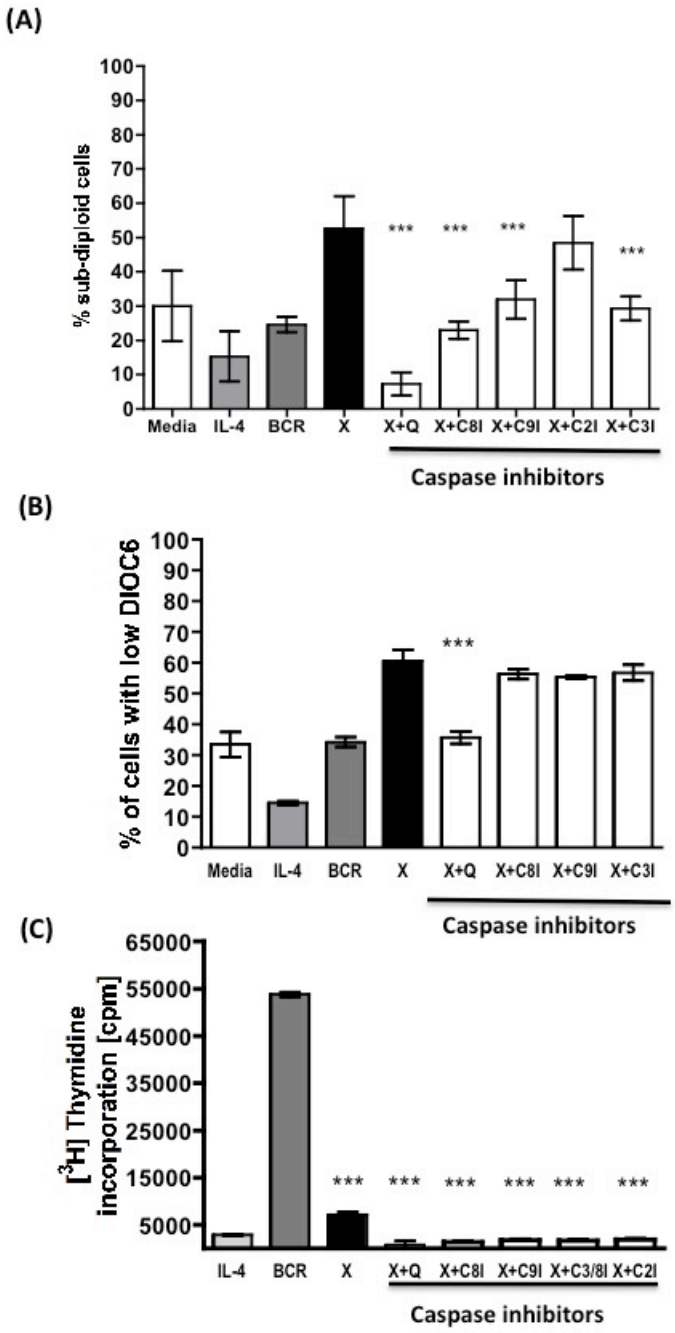
(A)
% sub-diploid cells
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
Media
IL-4
BCR
X
X+Q
X+C8I
X+C9I
X+C2I
X+C3I
Caspase inhibitors
(B)
% of cells with low DIOC6
100
90
80
70
60
50
40
30
20
10
0
***
Media
IL-4
BCR
X
X+Q
X+C8I
X+C9I
X+C3I
Caspase inhibitors
(C)
[3H] Thymidine incorporation [cpm]
65000
55000
45000
35000
25000
15000
5000
***
***
***
***
***
***
IL-4
BCR
X
X+Q
X+C8I
X+C9I
X+C3/8I
X+C2I
Caspase inhibitors

Figure 3

Figure 4. BCR/FcγRIIB co-ligation induces LMP. B cells were stimulated in the presence of either IL-4 (10ng/ml) or 50 µg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 µg/ml anti-mouse IgG+IgM (BCR/FcγRIIB-X) for 16 h or the indicated time course (panels A, C & D). B cells were stimulated with IL-4 (10 ng/ml) or 50 µg/ml B7.6 anti-IgM plus 50 µg/ml 2.4G2 anti-FcγRIIB followed by 75 µg/ml of donkey anti-rat IgG to crosslink B7.6 and 2.4G2 leading to coligation of BCR and FcγRIIB (panel B). Where indicated, cells were also pre-treated with pan-caspase inhibitor Q-VD-OPhe (10 µM) or the selective caspase (caspase 8 z-IETD-FMK 10 µM and caspase 9 z-LEHD-FMK 10 µM) or endosomal (EST 10 µM /Calpain inhibitor 10 µM) or lysosomal (E64d 10 µM + pepstatin A 10 µg/ml) inhibitors either alone or in combination for 1 h prior to stimulation. (A) Representative plots showing gating strategy for enumerating the percentage of 'pale' cells indicative of LMP as assessed by FACS analysis following staining with either acridine orange (AO) or Lysotracker Red DND99. In addition the time course of LMP is shown where data are presented as mean percentage ± SD of 'pale' cells (stained with Lysotracker Red DND99) at each time point from a single experiment representative of at least three independent experiments ($p<0.001$ BCR/FcγRIIB-X compared to either IL-4 or X+Q-VD-Ophe stimulated cells). (B) Cytosolic and membrane/ organelle fractions were prepared following stimulation for 16 h and analysed by Western blotting for Cathepsin B (C-19) expression. Fractions were probed for LAMP-1 (lysosomal maker) and GAPDH (cytosol marker) as quality control for fraction preparation. Data are representative of three independent experiments. (C) LMP was assessed by the

% pale cells (stained with Lysotracker Red DND99) after 16 h. Data are mean values ± SD of triplicate values from a single experiment representative of at least three independent experiments ($p<0.001$, compared to BCR/FcγRIIB-X). (D) Apoptosis (sub-diploid staining) was assessed by FACS analysis of PI staining of DNA content where data are mean ± SD of triplicate values from a single experiment representative of 3 independent experiments ($p<0.001$, for X+C9I compared to X+C9I+EST+Calp.Inh).

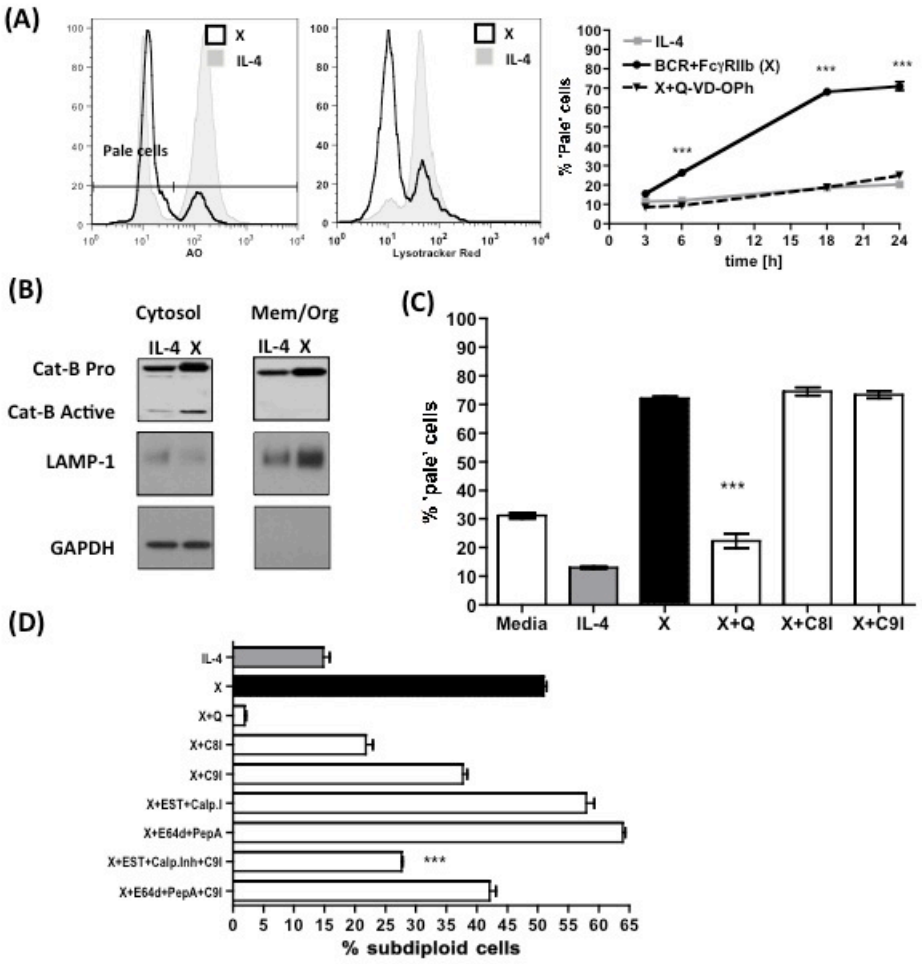
(A)
X
IL-4
Pale cells
AO
Lysotracker Red
% 'Pale' cells
IL-4
BCR+FcγRIIb (X)
X+Q-VD-OPh
time [h]
(B)
Cytosol
Mem/Org
IL-4 X
IL-4 X
Cat-B Pro
Cat-B Active
LAMP-1
GAPDH
(C)
% 'pale' cells
Media
IL-4
X
X+Q
X+C8I
X+C9I
(D)
IL-4
X
X+Q
X+C8I
X+C9I
X+EST+Calp.I
X+E64d+PepA
X+EST+Calp.Inh+C9I
X+E64d+PepA+C9I
% subdiploid cells

Figure 4

Figure 5. Co-ligation of BCR/FcγRIIB induces a complex interplay of p53, Bid and Bad signals. B cells were stimulated with medium or IL-4 (10 ng/ml) or 50 µg/ml B7.6 anti-IgM (BCR) or 50 µg/ml B7.6 anti-IgM plus 50 µg/ml 2.4G2 anti-FcγRIIB followed by 75 µg/ml of donkey anti-rat IgG to crosslink B7.6 and 2.4G2 leading to coligation of BCR and FcγRIIB (panels A & C). B cells were stimulated with either medium, IL-4 (10 ng/ml) or 50 µg/ml $F(ab')_2$ anti-mouse IgM (BCR) or 50 µg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 µg/ml anti-mouse IgG+IgM (BCR/FcγRIIB-X) in panels B and D. Where indicated, cells were pre-treated with either the caspase 8 (C8I, z-IETD-FMK 10µm) or pan-caspase (Q-VD-OPhe, 10µM) inhibitor for 1 h. (A) WCL were prepared after 24 h stimulation and analysed by Western blotting for p53 expression. Probing with β-actin was used as loading control. Data are representative of at least three independent experiments. (B) Nuclear extracts were prepared at 48 h post stimulation for assay of p53 transactivation activity by a modified ELISA (TransAM) method. Data are displayed as the mean level of p53 activity ± SD where n=3 from a single experiment representative of 3 independent experiments. WCL were prepared 24 h post stimulation and caspase-8 and Bid cleavage (C) were assessed by Western blotting. Levels of housekeeping genes β-actin and GAPDH were used as loading controls (C). Data are representative of three independent experiments. (D) Levels of Bad expression were determined by Western Blotting (24 h; i) or the FACE ELISA method (48 h; ii) where data are displayed as mean Bad signal ± SD of triplicate values of a single experiment representative of three independent experiments.

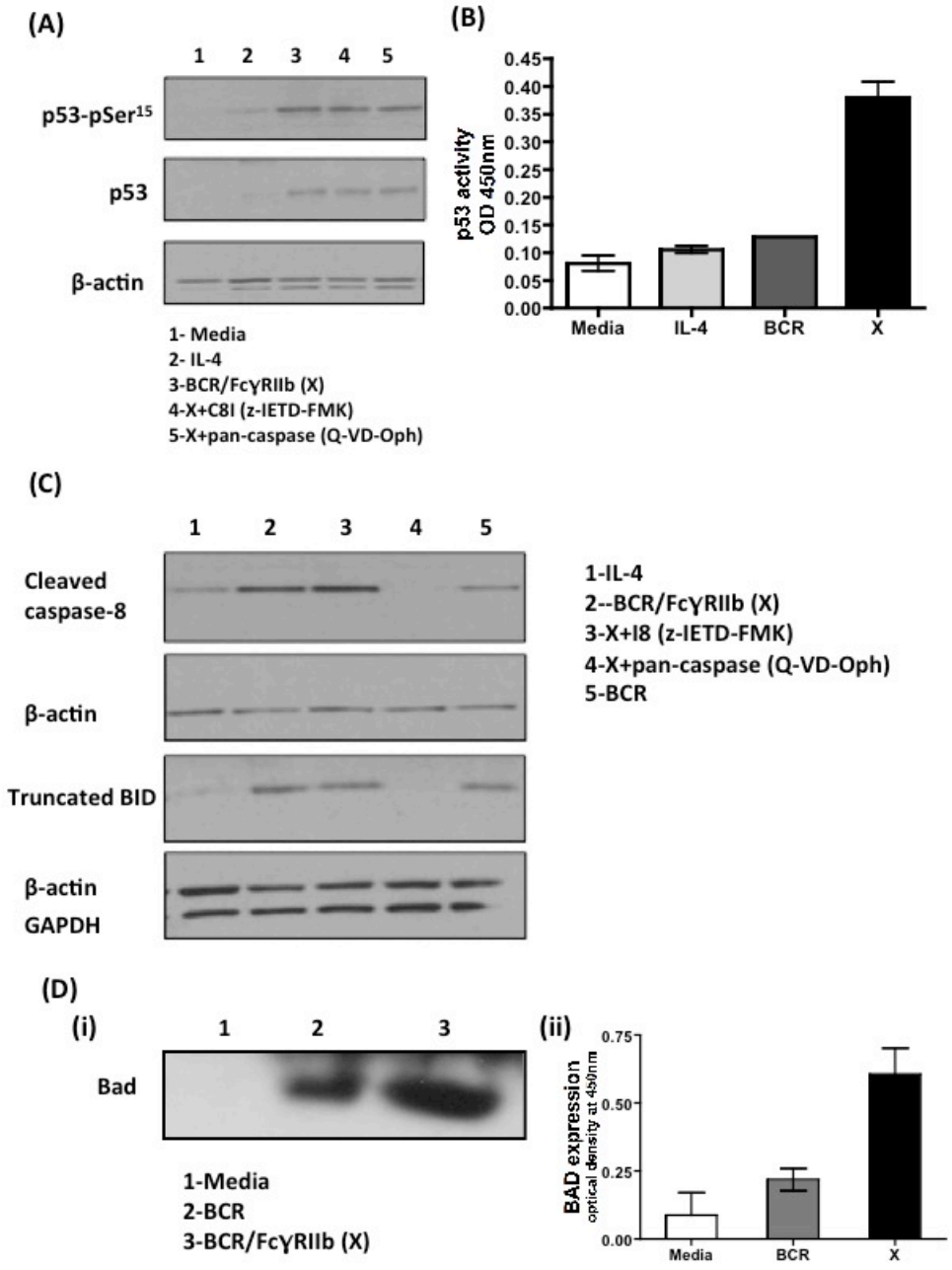
(A)
1 2 3 4 5
p53-pSer15
p53
β-actin
1- Media
2- IL-4
3-BCR/FcγRIIb (X)
4-X+C8I (z-IETD-FMK)
5-X+pan-caspase (Q-VD-Oph)
(B)
p53 activity OD 450nm
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Media
IL-4
BCR
X
(C)
1 2 3 4 5
Cleaved caspase-8
β-actin
Truncated BID
β-actin
GAPDH
1-IL-4
2--BCR/FcγRIIb (X)
3-X+I8 (z-IETD-FMK)
4-X+pan-caspase (Q-VD-Oph)
5-BCR
(D)
(i)
1 2 3
Bad
1-Media
2-BCR
3-BCR/FcγRIIb (X)
(ii)
BAD expression
optical density at 450nm
0.75
0.50
0.25
0.00
Media
BCR
X

Figure 5

Fig. 6. Coligation of BCR/FcγRIIB induces nuclear export of p53 and Ser15 phosphorylation. B cells were stimulated with either medium, IL-4 (10 ng/ml) or 50 μg/ml $F(ab')_2$ anti-mouse IgM (BCR) or 50 μg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 μg/ml anti-mouse IgG+IgM (BCR/FcγRIIB-X) in panels A and C. B cells were stimulated with IL-4 (10 ng/ml) or 50 μg/ml B7.6 anti-IgM plus 50 μg/ml 2.4G2 anti-FcγRIIB followed by 75 μg/ml of donkey anti-rat IgG to crosslink B7.6 and 2.4G2 leading to coligation of BCR and FcγRIIB in panel B. Where indicated, cells were pre-treated with the phosphatase (microcystin 1 μM) inhibitor for 1 h. (Ai) pBad/Bad levels were determined using the FACE ELISA method. The data are displayed as a ratio of the mean phospho-Bad to mean Bad signal and are from a single experiment representative of two independent experiments. (Aii) Akt activation was assessed by the ratio of pAkt/Akt expression determined using the FACE ELISA method. Cells treated with microcystin assessed the cumulative AKT phosphorylation over 48 h whereas cells without microcystin were used to assess the phospho-AKT levels at 48 h. The data are displayed as a ratio of the mean pAKT to mean AKT signal and are from a single experiment representative of four independent experiments. (B) Cytosolic and nuclear fractions were prepared 16 h post-stimulation and analysed by Western Blot for p53 expression. Fractions were probed for HDAC-1 (nuclear maker) and GAPDH (cytosolic marker) expression for a quality control for fraction preparation. Data are representative of at least three independent experiments. (C) p53-containing immune complexes were subjected to Western

Blot analysis with an anti-phospho-p53 (Ser15) antibody and then stripped and re-probed with an anti-p53 antibody.

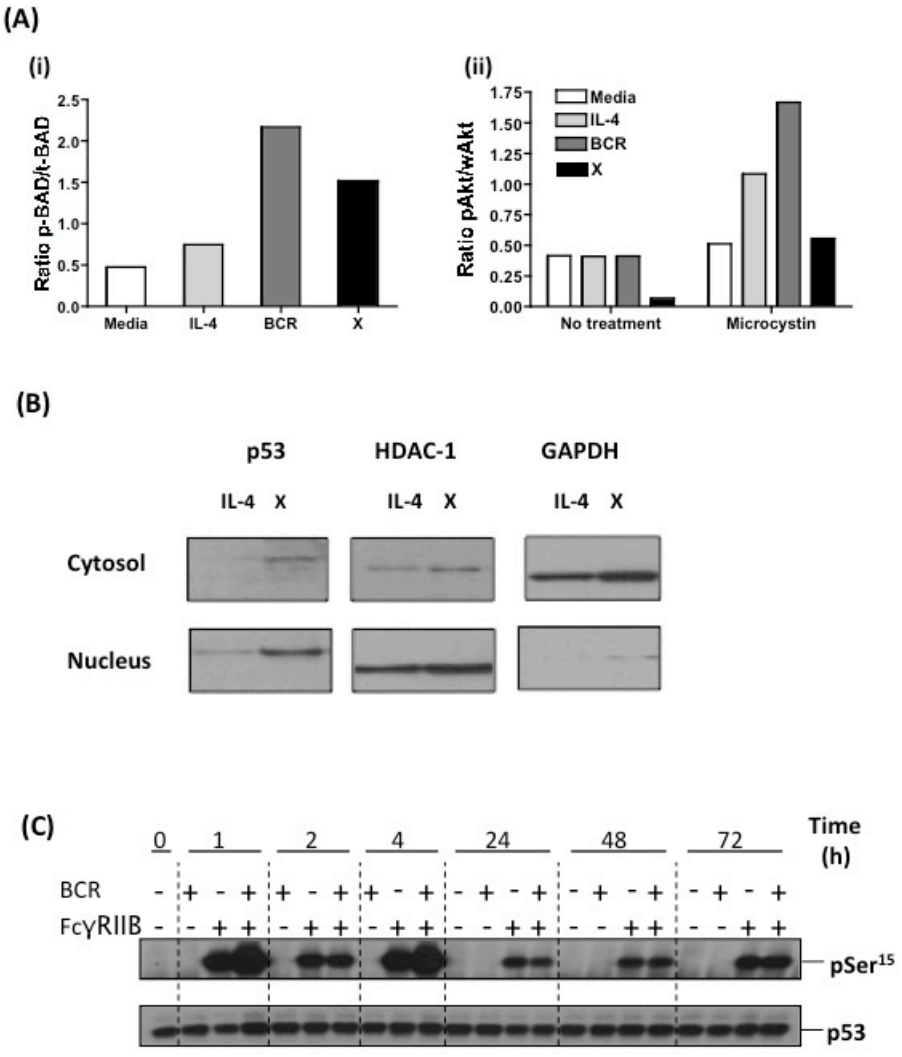

Figure 6

Figure 7. Co-ligation of the BCR with FcγRIIB upregulates Fas expression.

B cells were stimulated with either medium, IL-4 (10 ng/ml) or 50 µg/ml $F(ab')_2$ anti-mouse IgM (BCR) or 50 µg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 µg/ml anti-mouse IgG+IgM (BCR/FcγRIIB-X) in panels A & Biii. B cells were stimulated with IL-4 (10 ng/ml) or 50 µg/ml B7.6 anti-IgM (BCR) or 50 µg/ml B7.6 anti-IgM plus 50 µg/ml 2.4G2 anti-FcγRIIB followed by 75 µg/ml of donkey anti-rat IgG to crosslink the B7.6 and 2.4G2 leading to coligation of BCR and FcγRIIB (Bi & ii). Where indicated, cells were pretreated with a caspase 8 inhibitor (C8I, z-IETD-FMK 10 µM). (A) Representative plots of Fas surface expression detected on cells 24h after stimulation with (i) IL-4 or BCR/FcγRIIB co-ligation, (ii) BCR/FcγRIIB alone or in combination with caspase 8 inhibitor. Data shown are representative of at least three independent experiments. (B) WCL were prepared 24 h post-stimulation and levels of Fas and FasL assessed by Western blot analysis (Bi). Levels of the housekeeping gene β-actin were used as a loading control. (Bii) The intensities of bands representing FasL expression between different stimulation conditions were compared using ImageJ software. Data are presented as a ratio of the densitometric values of bands containing FasL and β-actin for each stimulation condition. Data are representative of at least three experiments. (Biii) mRNA levels of FasL expression were detected by TaqMan® quantitative RT-PCR using GAPDH as endogenous control. Data is shown as mean % expression relative to GAPDH ± SEM of triplicate values.

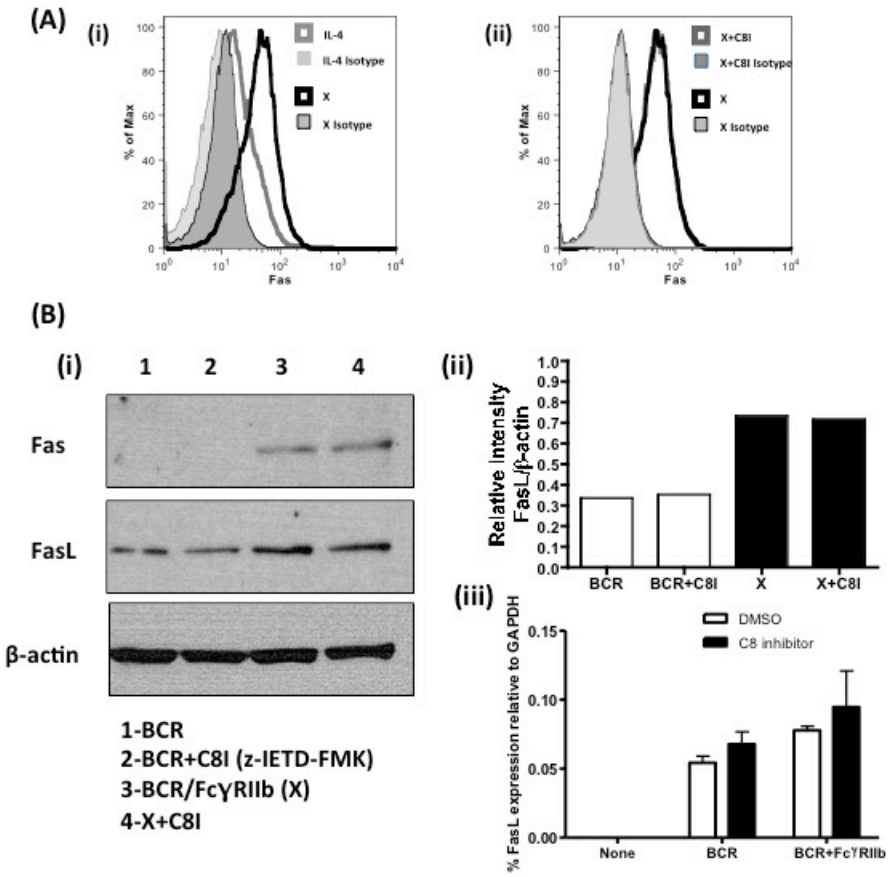
(A)
(i)
IL-4
IL-4 Isotype
X
X Isotype
% of Max
Fas
(ii)
X+C8I
X+C8I Isotype
X
X Isotype
% of Max
Fas
(B)
(i)
1
2
3
4
Fas
FasL
β-actin
1-BCR
2-BCR+C8I (z-IETD-FMK)
3-BCR/FcγRIIb (X)
4-X+C8I
(ii)
Relative Intensity FasL/β-actin
BCR
BCR+C8I
X
X+C8I
(iii)
DMSO
C8 inhibitor
% FasL expression relative to GAPDH
None
BCR
BCR+FcγRIIb

Figure 7

Figure 8. Fas signalling contributes to apoptosis induced by co-ligation of BCR/FcγRIIB. B cells were cultured in the presence of 50 µg/ml $F(ab')_2$ anti-mouse IgM or 50 µg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 µg/ml anti-mouse IgG+IgM. Where indicated cells were treated with 5 or 10 µg/ml of anti-FasL blocking antibody or as a control, the equivalent concentration of Armenian hamster IgG. (A) DNA content was assessed by FACS analysis of PI staining, and apoptosis was determined as percentage of cells containing subdiploid DNA content within the total cell population for each treatment condition. Data are representative of two independent experiments. (B & C) Resting thymocytes (T; B) and B cells, either resting (B0; B & C) or following co-ligation of the BCR/FcγRIIB for 44 h (B44; B) from BALB/c or MRL/MpJ-*Faslpr (*MRL/Lpr*)* mice were analysed for Fas (black line; B) and BCR (IgM, black line, isotype control, grey shading; C) surface expression by FACS analysis. Analysis of Fas staining relative to an appropriate isotype control (results not show) or following "Fas blocking" with unlabelled anti-Fas antibody (grey shading), revealed that the low level of Fas staining observed in naive B cells, but not thymocytes, from BALB/c and MRL/Lpr mice was non-specific. Data are representative of at least three independent experiments. (D) Resting thymocytes (T) and B cells, either resting (B0) or following co-ligation of the BCR/FcγRIIB for 24 (B24) or 40 h (B40) from BALB/c or MRL/MpJ-*Faslpr (*MRL/Lpr*)* mice were analysed for Fas, FcγRIIB (CD32) and BCR (IgM heavy chain) by Western Blot analysis, with GAPDH expression as a loading control. Data are representative of at least three independent experiments.

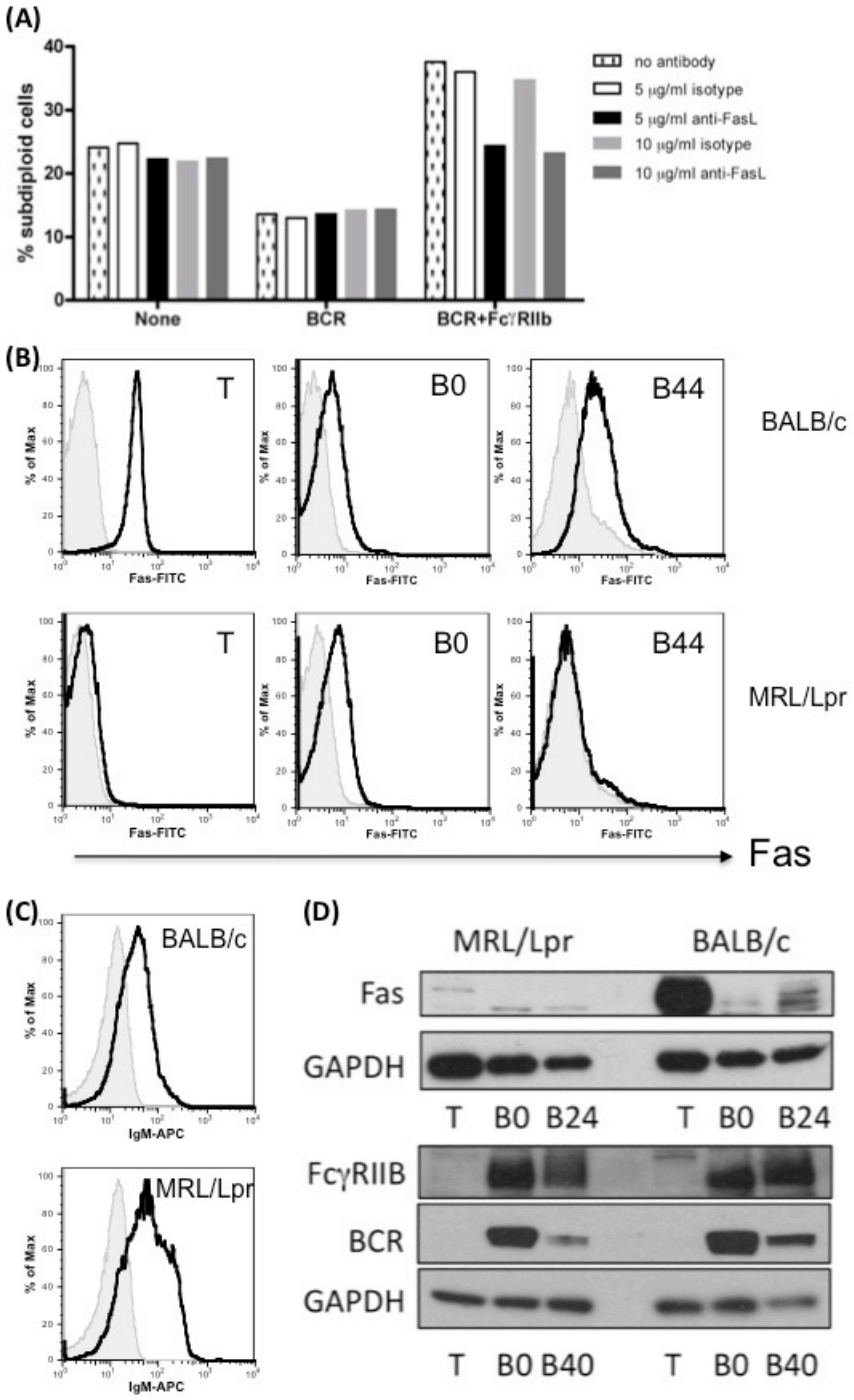
(A)
% subdiploid cells
40
30
20
10
0
None
BCR
BCR+FcγRIIb
no antibody
5 μg/ml isotype
5 μg/ml anti-FasL
10 μg/ml isotype
10 μg/ml anti-FasL
(B)
T
B0
B44
BALB/c
MRL/Lpr
% of Max
Fas-FITC
Fas
(C)
BALB/c
MRL/Lpr
IgM-APC
(D)
MRL/Lpr
BALB/c
Fas
GAPDH
T B0 B24 T B0 B24
FcγRIIB
BCR
GAPDH
T B0 B40 T B0 B40

Figure 8

Figure 9. **Apoptosis induced by co-ligation of BCR/FcγRIIB is reduced in MRL/Lpr B cells.** B cells were stimulated in the presence of IL-4 (10 ng/ml) or 50 μg/ml $F(ab')_2$ anti-mouse IgM or 50 μg/ml $F(ab')_2$ anti-mouse IgM in combination with 75 μg/ml anti-mouse IgG+IgM. (A) DNA content was assessed by FACS analysis of PI staining and the extent of apoptosis was determined as percentage of cells containing subdiploid DNA content within the total cell population for each treatment condition for B cells from (i) MRL/MpJ and (ii) MRL/MpJ-*Faslpr (*MRL/Lpr*)* mice. (iii) Data represent the relative levels of subdiploid cells induced by BCR/FcγRIIB coligation (normalised against values from BCR-stimulated cells) in MRL/MpJ-*Faslpr (*MRL/Lpr) versus MRL/MpJ B cells and are expressed as the mean values ± SEM relative to the normalised levels found in the MRL/MpJ cells and are pooled from 4 experiments with B cells from MRL/MpJ-*Faslpr (*MRL/Lpr*)* mice. (B) DNA synthesis at 48 h was assessed by the [^{3}H]thymidine assay of B cells from (i) MRL/MpJ and (ii) MRL/MpJ-*Faslpr (*MRL/Lpr) mice. (iii) Data (normalised against values from BCR-stimulated cells) represent the mean ± SEM percentage of BCR responses obtained by coligation of FcγRIIB pooled from 4 experiments using B cells from BALB/c, MRL/MpJ and MRL/MpJ-*Faslpr (*MRL/Lpr*)* mice (** $p<0.01$, * $p<0.05$). (C) LMP (i), MMP dissipation (ii) and DNA content (iii) were measured at the indicated time and data presented as means ± SD of triplicate values from a single experiment representative of two independent experiments. ($p<0.001$ for BCR/FcγRIIB-X stimulated BALB/c cells in comparison to MRL/MpJ-*Faslpr (*MRL/Lpr*)* mice).

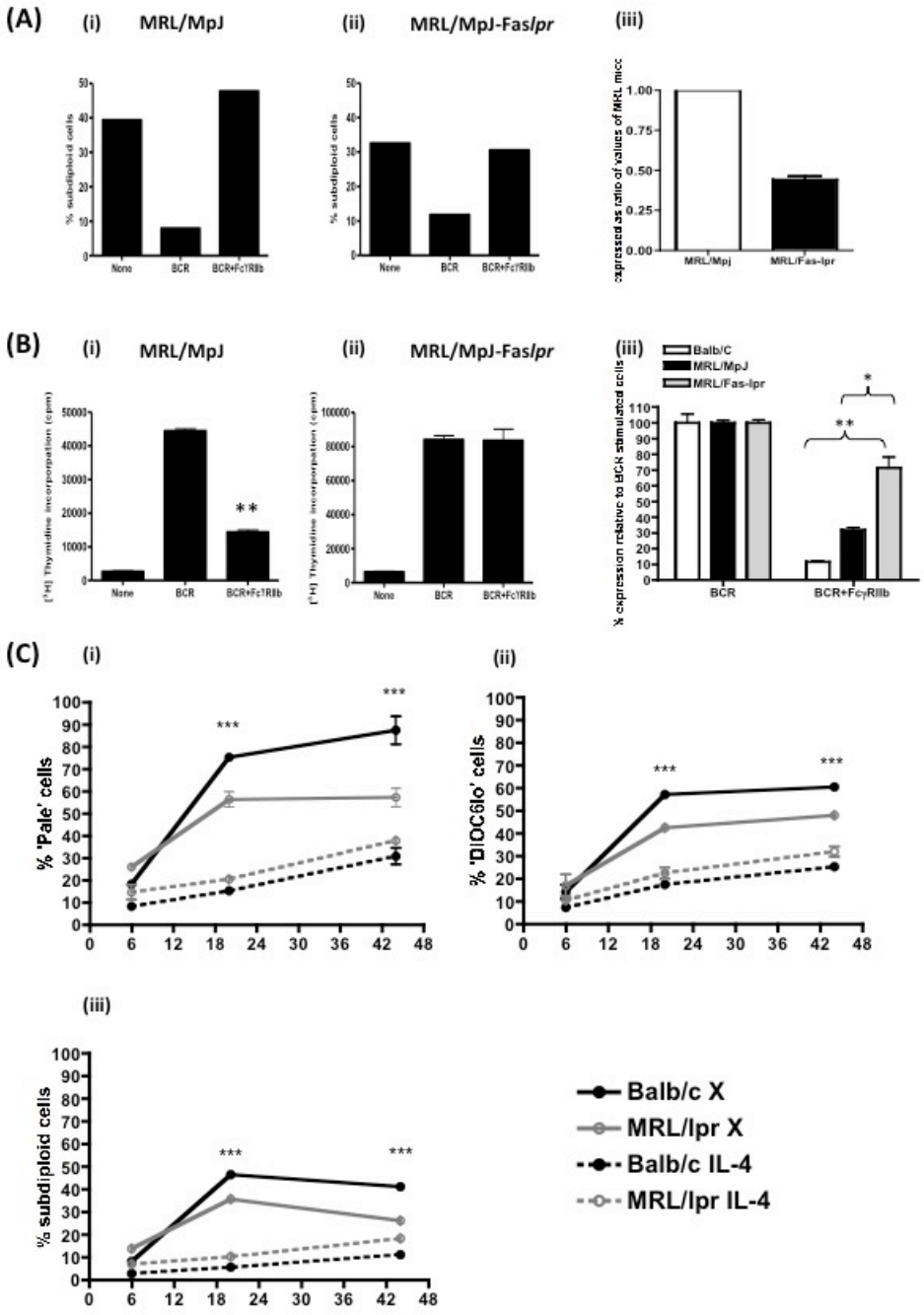
(A)
(i) MRL/MpJ
(ii) MRL/MpJ-Fas*lpr*
(iii)
% subdiploid cells
None
BCR
BCR+FcγRIIb
MRL/Mpj
MRL/Fas-lpr
(B)
(i) MRL/MpJ
(ii) MRL/MpJ-Fas*lpr*
(iii)
[³H] Thymidine incorporation (cpm)
Balb/C
MRL/MpJ
MRL/Fas-lpr
BCR
BCR+FcγRIIb
(C)
(i)
(ii)
(iii)
% 'Pale' cells
% 'DIOC6lo' cells
% subdiploid cells
Balb/c X
MRL/lpr X
Balb/c IL-4
MRL/lpr IL-4

Figure 9

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