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Cardiovascular inflammation and lesion cell apoptosis: A novel connection via the interferon-inducible immunoproteasome

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Abstract

Objective—Increasing evidence suggests that chronic inflammation contributes to atherogenesis, and that acute inflammatory events cause plaque rupture, thrombosis, and myocardial infarction. The present studies examined how inflammatory factors, such as interferon-γ (IFNγ), cause increased sensitivity to apoptosis in vascular lesion cells.

Methods and Results—Cells from the fibrous cap of human atherosclerotic lesions were sensitized by interferon-γ (IFNγ) to Fas-induced apoptosis, in a Bcl-X_L reversible manner. Microarray profiling identified 72 INFγ-induced transcripts with potential relevance to apoptosis. Half could be excluded because they were induced by IRF-1 overexpression, which did not sensitize to apoptosis. IFNγ treatment strongly reduced Mcl-1, phospho-Bcl-2 (ser70), and phospho-Bcl-X_L (ser62) protein levels. Candidate transcripts were modulated by siRNA, overexpression, or inhibitors to assess the effect on IFNγ-induced Fas sensitivity. Surprisingly, siRNA knockdown of PSMB8 (LMP7), an 'immunoproteasome' component, reversed IFNγ-induced sensitivity to Fas ligation and prevented Fas/IFNγ-induced degradation of Mcl-1, but did not protect p-Bcl-2 or p-Bcl-X_L. Proteasome inhibition markedly increased Mcl-1, p-Bcl-2, and p-Bcl-X_L levels after IFNγ treatment.

Conclusions—While critical for antigen presentation, the immunoproteasome appears to be a key link between inflammatory factors and the control of vascular cell apoptosis, and thus may be an important factor in plaque rupture and myocardial infarction.

Keywords

myocardial infarction; apoptosis; stroke; restenosis; angioplasty; inflammation; Bcl-X_L; Bcl-2; Mcl-1; interferon-γ; STAT1; LMP7; PSMB8; Fas; immunoproteasome; microarray

Advancing age, the single strongest risk factor for cardiovascular disease, favors lesion formation in response to injuries resulting from hyperlipidemia, hypertension, and smoking. Particularly in early lesions, apoptosis of monocyte/macrophages contributes to the accumulation of extracellular lipid and proteins[1]. In later stages, smooth muscle cell-like (SMC-like)/myofibroblasts encapsulate the lipid-rich regions, and their persistence contributes

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to matrix accumulation and negative remodeling[2]. However, when apoptosis occurs in the cap of advanced lesions, the plaque is prone to rupture, thereby triggering thrombosis and myocardial infarction. Increased apoptosis is frequently associated with clinically unstable coronary artery lesions[3], and there is strong evidence that apoptosis is a major contributing factor to plaque instability and sudden coronary death[4, 5].

Inflammation is intimately linked to the Fas/Fas ligand system, which can either drive proliferation or induce apoptosis of lymphocytes based on the particular context[6]. Cells within human lesions express Fas (CD95), the receptor for Fas ligand, a potent pro-apoptotic factor[7]. Inflammatory regions, which contain lymphocyte-derived interferon-γ (IFNγ) and Fas ligand, exhibit the highest levels of SMC-like apoptosis[8]. Activated macrophages induce apoptosis in LDC[9] and SMC[10] by Fas/Fas ligand interactions. Fas-ligand deficient mice show excessive vascular repair[11], and Fas ligand-based gene therapy reduces intimal hyperplasia in animal models[12, 13]. Transcript profiling of human restenotic lesions observed activation of IFNγ signaling, and IFNγ receptor knockout mice have an attenuated neointimal response to injury[14], consistent with an evolving theory that vascular diseases have a chronic inflammatory component[15].

Cells grown from fibrous regions of human vascular lesions contain both apoptosis-sensitive and resistant cells, but apoptosis-resistant cells quickly dominate the culture[16]. Transcript profiling of lesion cells revealed that increased expression of Bcl-X_L is a major determinant of resistance to Fas-mediated apoptosis[16, 17]. Inflammatory cytokines, especially interferons, can increase the apoptotic response to Fas ligation in SMCs[7, 18, 19], endothelial cells[20], and other cell types[21]. The present studies employed a microarray-based approach to identify factors that mediate the pro-apoptotic effect of IFNγ on human LDC.

Methods

Cells

Human atherosclerotic lesions were acquired by carotid endarterectomy by IRB-approved protocols. Lesion-derived cells (LDC) were isolated by explant of the fibrous cap, and cultured in M199 with 10% fetal bovine serum (FBS) and gentamicin (50 μg/mL). Microarray analysis[16, 22] and α-actin positivity[23] indicate that LDC are SMC-like cells.

Apoptosis assay: Small molecule and siRNA inhibition

The sensitivity to apoptosis was examined by determining survival after challenge with a Fas-activating IgM (clone CH11, Upstate Biotechnology). LDC were plated at 50 K/24-well in M199 + 10% FBS for 24 hours prior to overnight serum reduction to 1% FBS. Small molecule inhibitors, such as compstatin (Tocris Bioscience), pefabloc (BioChemika), cathepsin S inhibitor (Z-FL-COCHO, Calbiochem), MG132 (Z-Leu-Leu-Leu-al, Sigma), or epoxomycin (Sigma) were added 4-6 hours prior to IFNγ. LDC were pretreated with siRNA pools (Dharmacon OnTargetPlus) containing 4 siRNAs in Dharmafect for 24-48 hours prior to IFNγ treatment. IFNγ (human recombinant, R&D Systems, 5 ng/50 IU/mL) was added for 20-24 hours before Fas-activating antibody (50-100 ng/mL). After 24 hours, MTT was added for 4 hours, the stained cells were dissolved in DMSO, and the level of reduced MTT was measured by O.D. at 570 nm in a plate reader[24].

Transcript profiling

Genome-wide microarray profiling was conducted of IFNγ-responsive transcripts in human LDC *in vitro*. Total RNA was prepared using RNAzol B and Qiagen RNeasy Mini columns. RNA quantity was assessed by spectrometry and RNA quality was assessed by Agilent Bioanalyzer. Total RNA (10 μg) was reverse transcribed, and biotin-labeled cRNA was

produced by T7 *in vitro* transcription (IVT). Labeled cRNA was fragmented and hybridized to U133A GeneChips (Affymetrix, 22,282 transcripts). The IFNγ response was evaluated in LDC from three different patients.

Data analysis

The raw data was summarized and normalized using GC-RMA in GeneSpring GX7. A paired *t*-test identified 1,500 transcripts, which were further filtered by a >1.5 fold-change and potential relevance to apoptosis. Gene lists were compared to published interferon-inducible gene lists using LOLA[25]. Pathway analysis was conducted using Ingenuity Pathway Analysis (Ingenuity Systems).

Retroviral overexpression

Full-length cDNA clones were obtained from Open Biosystems, or RT-PCR amplified from LDC mRNA. Inserts were subcloned into MSCV-IRES-eGFP retroviral vector, upstream of an encephalomyocarditis virus internal ribosome entry site (IRES) cassette linked to the enhanced green fluorescent protein (eGFP) reporter gene.

Quantitative RT-PCR (qRT-PCR)

qRT-PCR of candidate genes was conducted using SYBR Green fluorescence detected with an ABI 7300 real-time PCR. Standard dilutions of the cDNA were used to interpolate relative transcript abundance. HBOA and ZNF were used as control genes to compensate for minor variations in mRNA quality or quantity. Primer sequences are contained in Supplementary Table I.

Western blot analysis

Protein lysates from untreated or IFNγ treated cells were prepared with a protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). Protein was quantified by BCA (Pierce), and 50 μg of protein was run on a 12% SDS/PAGE gel and transferred to PVDF. Primary antibodies (Supplementary Table III) were detected by peroxidase-labeled secondary antibody and SuperSignal WestDura substrate (Pierce) on a Kodak 2000MM chemiluminescent detection system.

Results

Interferonγ sensitizes lesion-derived cells (LDC) to apoptosis in a Bcl-X_L reversible manner

Like SMC[17, 18, 26], human LDC are resistant to apoptosis induced by the Fas-activating CH11 IgM (50 ng/mL), causing <10% apoptosis (Figures 1, 3 and 4). However, 24 hour pretreatment with IFNγ (5 ng/mL) synergistically increases the apoptotic response to Fas ligation to 70% apoptosis. When the mitochondrial apoptosis pathway was suppressed with retroviral expression of Bcl-X_L[17], the small pro-apoptotic effect of IFNγ alone was reduced, and the synergistic effect of IFNγ and Fas ligation was eliminated (Figure 1).

Transcript profiling of IFNγ-regulated messages in LDC

A full genome microarray profiling was conducted of IFNγ-responsive transcripts in human LDC *in vitro*. Using CRL1999 (ATCC) and radial artery smooth muscle as reference, the array data indicates that LDC are transcript-positive for 5 consensus smooth muscle markers: smooth muscle α-actin (ACTA2), smooth muscle myosin heavy chain (MYH11), calponin 1 (CNN1), aortic preferentially expressed protein 1 (APEG1), and SM22α/transgelin (TAGLN). Upon IFNγ treatment, the overwhelming majority of affected transcripts were induced (Supplementary Figure I), with some increases as much as 100-fold (INDO). A paired *t*-test identified almost 1,500 possible genes, which were further filtered by a >1.5 fold-change (135

transcripts), and potential relevance to apoptosis (72 transcripts, Supplementary Table III). These 72 transcripts were prioritized by the strength of their association to apoptosis, and their occurrence in other IFNγ studies, using LOLA[25].

qRT-PCR confirmation of microarray results

Many of the key IFNγ-regulated transcripts identified by microarray profiling were confirmed by qRT-PCR of LDC treated with 5 ng/mL IFNγ for 24 hours prior to total RNA isolation (Supp. Table 3). Essentially all transcripts with a greater than 1.5-fold change by microarray were confirmed by qRT-PCR. Marked changes in caspases 1 and 7, IRF-1, STAT1, and proteasome component mRNAs were confirmed, with smaller changes (1.6 fold) in Fas (CD95), caspase 3 and 4 levels, and essentially unchanged levels of caspase 8.

Identification of IRF-1 inducible messages and IRF-1 effect on apoptosis by stable overexpression

Many of the transcriptional effects of IFNγ are mediated directly by STAT family members, and indirectly by induction of IRF-1, a potent transcription factor. IRF-1 was overexpressed in resistant LDC to partially mimic the effect of IFNγ. The majority of IFNγ-inducible messages were induced to a similar degree by IRF-1 overexpression (Supp. Table III). Consistent with prior reports, caspase 1 (interleukin converting enzyme, ICE)[27] was strongly induced by IRF-1 expression. However, several transcripts were not elevated by IRF-1, such as Fas (CD95), which is regulated by IRF-8[28]. Notably, PSMB8 (LMP7) was not induced in IRF-1 expressing cells, while other members of the proteasome group were slightly increased in IRF-1 expressers (2-fold), but markedly less than that induced by IFNγ (3.5 to 55-fold). Surprisingly, IRF-1 overexpression did not sensitize cells to apoptosis (not shown), and thus, the IRF-1-induced transcripts can be excluded as candidate factors.

Western blot confirmation of selected IFNγ-inducible proteins

Western blot analysis of the key proteins confirmed that antigen levels were elevated after IFNγ treatment (Figure 2). STAT1 protein was strongly elevated by IFNγ. Although Bcl-X_L and Bcl-2 protein, potent anti-apoptotic molecules, were only slightly decreased by IFNγ, their phosphorylated forms, p-Bcl-X_L (ser62) and p-Bcl-2 (ser70), were more markedly decreased. P-Bcl-2 (ser70) is a more active anti-apoptotic molecule[29], while p-Bcl-X_L (ser62) is less active[30].

Consistent with the strong elevation of its mRNA, caspase 1 (ICE) protein was strongly and consistently elevated by IFNγ. Both pro- and cleaved caspase 7 forms were elevated by IFNγ treatment. Caspases 8, but not caspases 3 and 9, was slightly elevated by IFNγ. Fas levels were increased by IFNγ although the levels in some untreated lines (E126) was greater than IFNγ-treated levels in other lines, suggesting that Fas levels alone do not determine sensitivity. Confirming prior work[19], the cell surface expression of Fas, determined by flow cytometry, was increased two-fold in IFNγ-treated cells, although among different cell lines Fas surface expression was poorly correlated with apoptotic sensitivity (not shown).

Despite a two-fold increase in Mcl-1 mRNA levels, there was a marked decrease in Mcl-1 protein levels, which could be explained either by translational repression or increased ubiquitin-mediated proteosomal degradation[31]. Consistent with its mRNA levels, there was a marked increase in PSMB8/LMP7 protein antigen in IFNγ-treated cells.

Retroviral overexpression of candidate apoptosis modulators

A smaller set of candidate genes were examined by stable retroviral overexpression to evaluate their impact on apoptotic sensitivity. Surprisingly, caspase 1 (ICE), which has been reported

to mediate apoptotic effects of interferon and IRFs[27] in other cell types[32], did not alter sensitivity to Fas ligation when overexpressed in LDC[17]. Other candidate transcripts: butrophylin (BTN3A3), caspase 7, ceramide kinase (CERK), G1P2, GBP1, IFITM1, phospholipid scramblase (PLSCR1), TRIM22, and VAMP4, were likewise ineffective.

Small-molecule inhibition

Certain IFNγ-induced targets were amenable to small-molecule inhibition. The complement pathway was strongly modulated by IFNγ, with induction of C1QR1, C1R, C1S, C3, C4A, and H Factor 1 (HF1). However, compstatin (10-50 μM), a complement inhibitor, was ineffective at blocking the IFNγ effect. Cathepsin S, which has known pro-apoptotic effects, was blocked with Z-FL-COCHO (0.02-2 μM, Calbiochem), but no effect on the IFNγ response was observed. NK4/IL32 is a ligand and activator for PR3, a proapoptotic serine protease. PR3 was blocked by the serine protease inhibitor Pefabloc (1-100 μM), but apoptotic sensitivity was unaltered.

siRNA-mediated knockdown of candidate apoptotic modulators

Using 4 siRNAs per target transcript, 14 candidate transcripts were targeted, including ADAR, ApoL6, caspase 7, caspase 9, CD47, CRADD/RAIDD, IL32/NK4, PKR, RIPK2, UBE1L, without abrogating the IFNγ-induced sensitization to apoptosis. However, as a positive control, siRNA-mediated knockdown of Bid completely blocked the IFNγ effect (not shown).

siRNA knockdown of PSMB8 blocks IFNγ-induced sensitivity

Unlike the prior 14 candidates, which serve as excellent controls for non-specific effects of siRNA, PSMB8/LMP7 knockdown clearly reversed IFNγ-induced sensitivity. As shown in Figure 3A, LDC treated with 5 ng/mL of IFNγ prior to Fas ligation (CH11, 50 ng/mL) show an increased sensitivity to apoptosis (83% survival reduced to 39% survival by INFγ) which is almost fully reversed (restored to 72% survival) by knockdown of PSMB8/LMP7 (50 nM siRNA), but not by knockdown of PSME1 (PA28). A 1:1 mix of siRNA to PSMB8/LMP7 and PSME1 (25 nM each) was equally effective at reversing the IFNγ effect. Non-targeted siRNA did not alter the response to IFNγ. Further, knockdown of PSMB9 (LMP2) or PSMB10 (MECL1), or combined knockdown, was ineffective in blocking IFNγ-induced sensitivity to Fas ligation (not shown). These experiments were repeated five times with similar results.

The specificity of the siRNA effect was evaluated by qRT-PCR for the target transcripts and closely related members of the immunoproteasome. As shown in Figure 3B, the siRNA was very specific, reducing the IFNγ-induced target transcripts to below their basal levels with only minimal effects on other members of the immunoproteasome. For further confirmation, siRNA to PSMB8/LMP7 reduced IFNγ-induced PSMB8/LMP7 antigen to unstimulated levels, as shown in Figure 3C. However, neither PSME1, nor non-targeted siRNA blocked IFNγ-induced PSMB8/LMP7 antigen levels. While PSMB8/LMP7 was essentially completely blocked, the induction of Fas antigen was not blocked by any of the siRNAs, and despite the elevated Fas levels, the PSMB8/LMP7 knockdown blocked sensitivity to apoptosis.

Mcl-1 is anti-apoptotic for LDC and regulated by PSMB8/LMP7

To identify the specific apoptotic regulators that were modulated by PSMB8/LMP7, candidate factors with a known modulation by the proteasome were analyzed by Western blot. SiRNA knockdown of PSMB8 consistently elevated Mcl-1 protein levels, which was most pronounced with combined IFNγ/Fas challenge, compared to PSME1 or non-targeted siRNA (Fig. 4A). The inhibitor of apoptosis proteins (IAP1, IAP2, XIAP), which have been previously described to be proteasome-regulated[33], were not elevated by PSMB8 knockdown (IAP1 shown, Fig

4A). Likewise, Bcl-2, pBcl-2, and pBcl-X_L were not elevated by PSMB8/LMP7 knockdown (Fig. 4A).

To determine whether regulation of Mcl-1 levels was a viable mode of apoptotic regulation in LDC, Mcl-1 was knocked down by siRNA and the effect on Fas-induced apoptosis was determined. Mcl-1 knockdown reduced survival by approximately 50% and increased the apoptotic response to Fas ligation from 32% to 55%, yielding a combined increase in killing from 32% to 77% (Fig. 4B, Left). Western blot confirmed essentially complete knockdown of Mcl-1 antigen without an effect of a non-targeted control siRNA (siCon). Conversely, retroviral overexpression of Mcl-1 markedly reduced the sensitivity to apoptosis (72% to 46% death) in the presence of IFNγFig. 4B, Right). However, the IFNγ-induced degradation of Mcl-1 severely limited the degree to which Mcl-1 could be overexpressed, as measured by Western blot (Fig. 4B). Thus, Mcl-1 is a potent survival factor, and Mcl-1 levels are increased by PSMB8 knockdown.

Mcl-1 levels are protected by proteasome inhibition

To confirm that Mcl-1 is proteasomally degraded, LDC were IFNγ-treated in the presence or absence of proteasome inhibitors for 24 hours prior to protein harvest. Co-treatment with IFNγ and MG132 or epoxomycin strikingly increased levels of Mcl-1 above untreated levels, and modestly increased the levels of p-Bcl-2 and p-Bcl-X_L (Fig. 5). After proteasome inhibition, p-Bcl-2 antigen accumulates in higher mass forms consistent with ubiquitinated protein.

Discussion

The control of apoptosis during vascular repair may be a key determinant of both lesion progression and plaque rupture. Thus, understanding the immune and inflammatory influences on apoptosis in vascular cells may facilitate preventative and therapeutic strategies. A systematic examination of potential IFNγ targets revealed the unexpected role of the immunoproteasome component PSMB8/LMP7 as a major component of IFNγ-induced sensitivity. The immunoproteasome has been almost exclusively studied in the context of its role in antigen presentation, although recent studies in double MECL1 and LMP7 deficient T cells suggests that T cell proliferation is also affected by immunoproteasome activity[34].

PSMB8/LMP7 is a well-known stress[35] and IFNγ-inducible component of the immunoproteasome. The PSMB8 protein product, LMP7, replaces the constitutive β5 subunit, hence its alternate designation as β5i. PSMB8 has been reported to be induced by IFNγ through IRF-1[36], although our promoter analysis indicates there is only a single IRF-1 site (-2580 to TSS), but five STAT sites (-4574, -4563, -3406, -2160, -1148)[37], which is consistent with the present data that IRF-1 overexpression did not induce PSMB8, while IFNγ strongly induced it (>10×). As shown in Figure 6, the three catalytic units of the constitutive proteasome are replaced after IFNγ treatment, as well as key changes to the regulatory subunits (PA28 α and β). The result is an ‘immuno’proteasome which cleaves proteins into short peptide antigens for presentation by MHC I[38]. The immunoproteasome, however, can activate NFκB and degrade IκB[39].

The present results suggest a straightforward mechanism for the apoptotic sensitivity associated with immunoproteasome induction. Mcl-1, a potent prosurvival factor, is ubiquitinated by the MULE ubiquitin ligase and degraded by the proteasome[31, 40]. While other substrates could be affected, it is clear that induction of immunoproteasome activity accelerates Mcl-1 degradation, thereby reducing the survival ability of the cell. Evidence from siRNA knockdown of PSMB8, Mcl-1, and overexpression of Mcl-1 strongly suggests a significant role for this mechanism in mediating the pro-apoptotic effects of IFNγ via the immunoproteasome. Additional effects of

IFNγ on p-Bcl-2 and Fas receptor levels may also contribute importantly to the overall sensitivity to apoptosis.

Consistent with a possible *in vivo* relevance for the present results, important changes in the ubiquitin-proteasome system are observed in age-related atherosclerosis[41]. Stroke-prone, unstable carotid artery lesions exhibit elevated inflammatory markers and increased proteasome activity[42]. There are well-known changes in proteasome and immunoproteasome activities during the aging process[43, 44], which might result from inflammatory stimuli, interferon activity, and result in altered apoptotic sensitivity. Likewise, changes in the immunoproteasome response to interferon is a feature of senescent cells[45]. While a general connection between inflammation, atherosclerosis, and myocardial infarction is well established, the precise molecular connections are only beginning to be elucidated. For instance, epidemiological evidence suggests that influenza infection is a strong risk for myocardial infarction[46]. Likewise, influenza[47] and other viral infections[48] are potent activators of the immunoproteasome. Combined, the present results identify a novel, and potentially important connection between immune activation and the control of vascular apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

DISC
death-inducing signaling complex

HEPES
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IFNγ
interferon–gamma

LDC
lesion-derived cells

MTT
3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide

eGFP
enhanced Green Fluorescent Promoter

FACS
fluorescence-activated cell sorting

GAPDH
glyceraldehyde 3-phosphate dehydrogenase

PAGE
polyacrylamide gel electrophoresis

qRT-PCR
: quantitative reverse transcriptase-polymerase chain reaction

SDS
: sodium dodecyl sulfate

TBS
: Tris-buffered saline

TBST
: TBS-Tween-20

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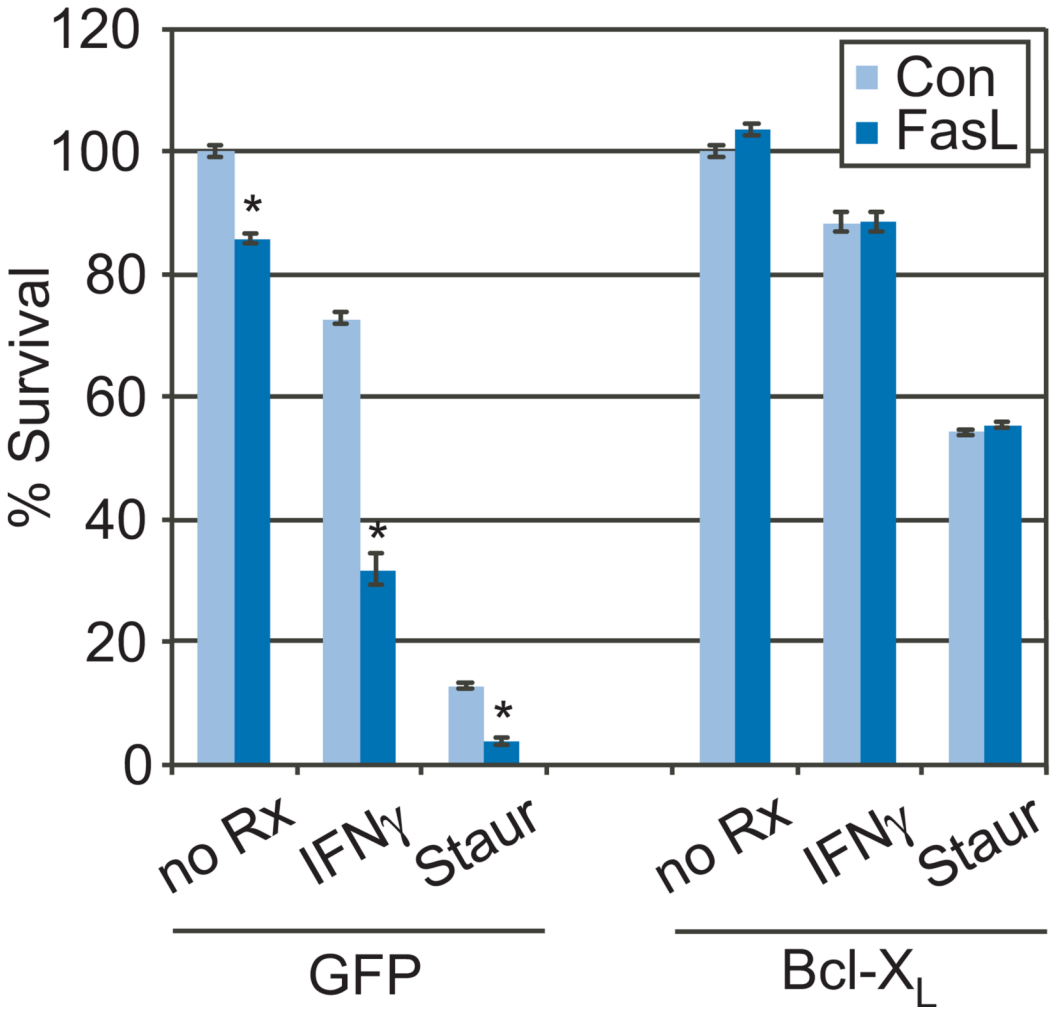

Figure 1. Bcl-X_L overexpression overrides IFNγ sensitization

Lesion cells were stably infected with eGFP or Bcl-X_L with eGFP and sensitivity to Fas ligation was examined. IFNγ (5 ng/mL) was preincubated for 24 hours prior to activating anti-Fas CH11 monoclonal IgM (FAS) for 24 hours. Viable cells were quantitated by MTT (O.D. 570 nm). Staurosporine (STAUR, 20 μM) was added in place of IFNγ as a positive control. Data is expressed as percent of control survival (n=4 per group±s.e.m.). No Rx is no treatment. Asterisks are $p<0.001$ by *t*-test.

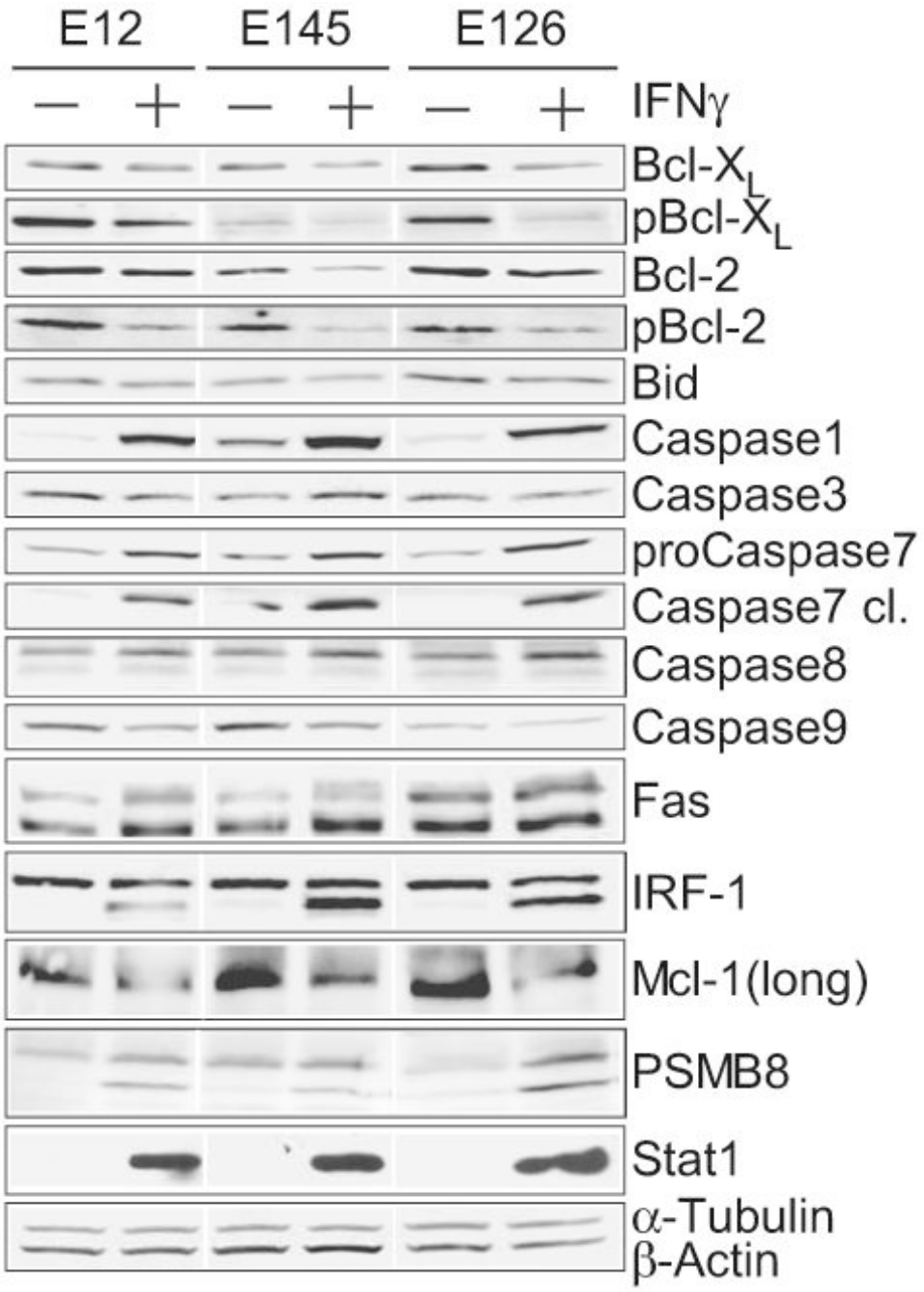

Figure 2. IFNγ effects on candidate apoptosis-related proteins

Transcripts identified from microarray analysis, and additional apoptosis-related proteins were confirmed by Western blot in three separate LDC lines (E12, E145, E126) in the absence (-) or presence (+) of IFNγ (5 ng/mL, 24 hours). The specific antibodies used are listed in Supplementary Table II. Mcl-1 antibody is polyclonal rabbit (Cell Signaling), which identifies the 37 kD anti-apoptotic long form.

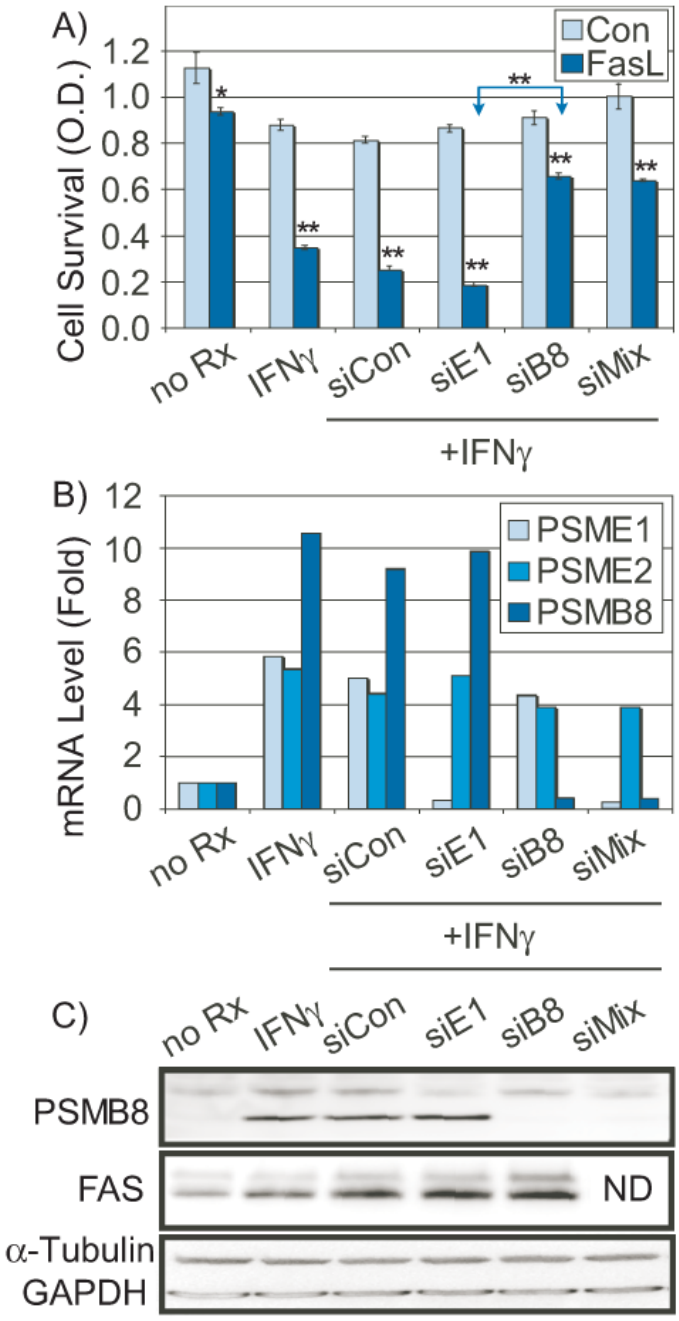

Figure 3. The effect of siRNA-mediated knockdown of PSMB8 on IFNγ-induced apoptotic sensitivity

LDC were pretreated either with non-targeted siRNA (siCon), or with siRNA to PSME1 (siE1, 50 nM), PSMB8 (siB8, 50 nM), or a mixture of both (siMIX, 25 nM each). **Panel A)** IFNγ-induced apoptotic sensitivity to Fas ligation was then determined by MTT survival. Bars are mean ± s.e.m. (n=4, *=p<0.05, **=p<0.001). **Panel B)** mRNAs were analyzed for effective knockdown of the target transcripts, as well as related transcripts as controls for non-specific effects. Transcript levels of PSME1, PSME2, and PSMB8 were quantitated by qRT-PCR. **Panel C)** Western blot of PSMB8/LMP7, FAS, and control proteins: α-tubulin (α-Tubu) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). ND=not determined.

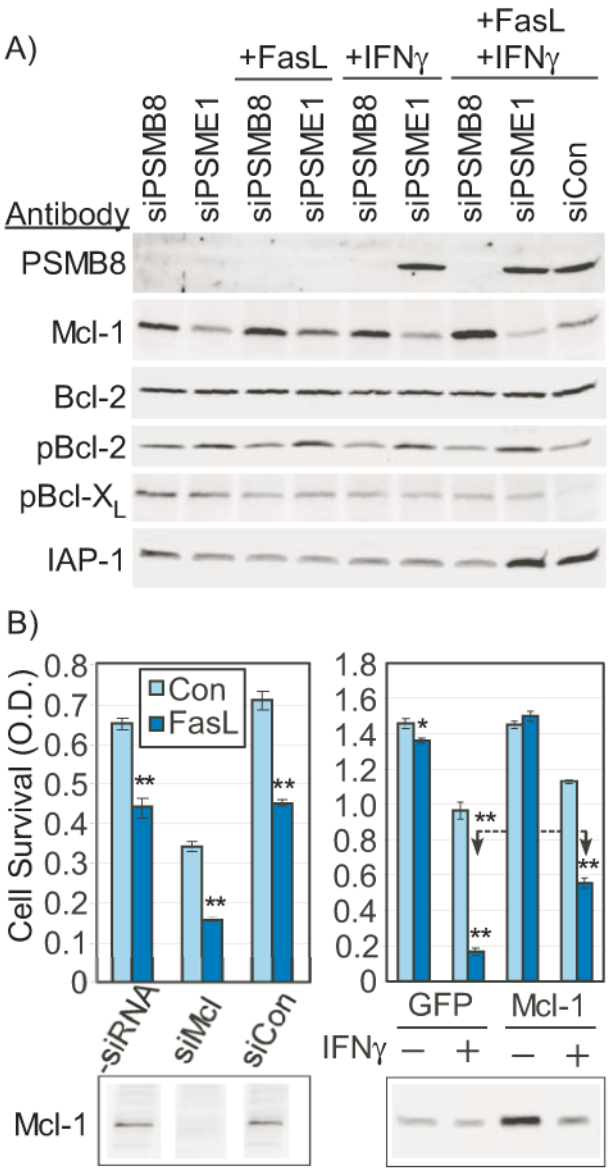

Figure 4. Mcl-1 is regulated by PSMB8 and modulates apoptosis in LDC

Panel A) LDC were pretreated with siRNA to PSMB8, PSME1, or non-targeted siRNA (siCon) for 48 hours prior to protein harvest and Western blot for candidate apoptosis regulators. Mcl-1 antibody is mouse monoclonal (Santa Cruz). Other antibodies are specified in Supplementary Table II. **Panel B)** Left panel: Mcl-1 was knocked down by siRNA (siMcl, 50 nM) 48 hours prior to examining sensitivity to apoptosis induced by Fas ligation. Bars are mean optical density (O.D.) of MTT compared to untreated control (Con) or cells pretreated with non-targeted siRNA (siCon, 50 nM). Right panel: Mcl-1 was over-expressed and compared to GFP-only expressing cells for sensitivity to apoptosis triggered by Fas ligation in the presence or absence of 24 hour pretreatment with IFNγ (5 ng/mL). Lower panels demonstrate parallel Western blots for Mcl-1 (mouse monoclonal). Bars are mean O.D. (n=4, *=p<0.05, **=p<0.001).

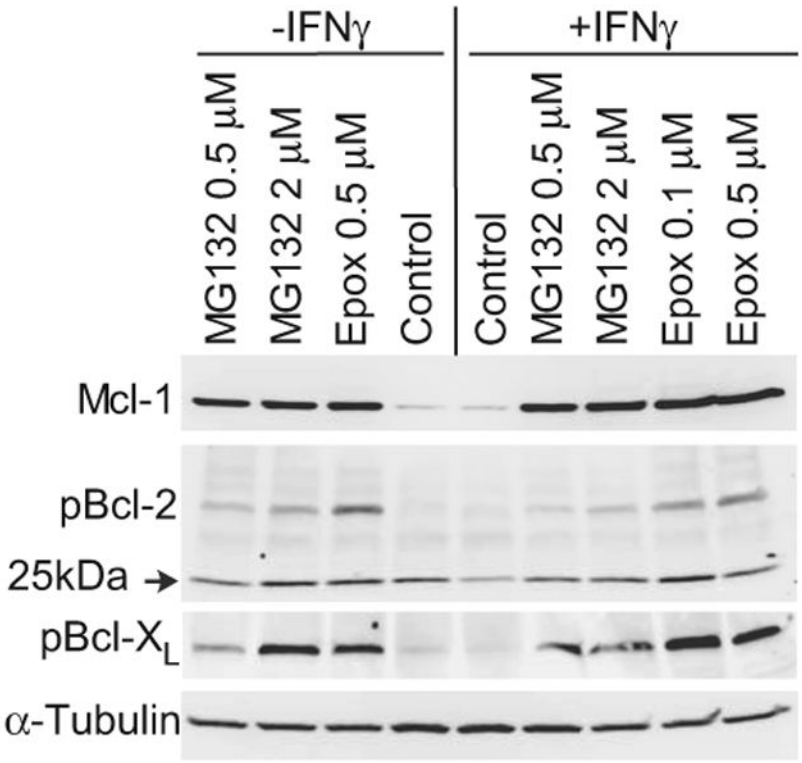

Figure 5. Effect of proteasome inhibition on Mcl-1, p-Bcl-2, and p-Bcl-X_L levels

LDC were treated with proteasome inhibitors MG132 or epoxomycin alone (left lanes), or in combination with IFNγ (5 ng/ml) for 24 prior to protein collection and Western blot for Mcl-1 (mouse monoclonal), p-Bcl-2(ser70), or p-Bcl-X_L(ser62). Equal protein loading of the lanes is confirmed by antibody to α-tubulin.

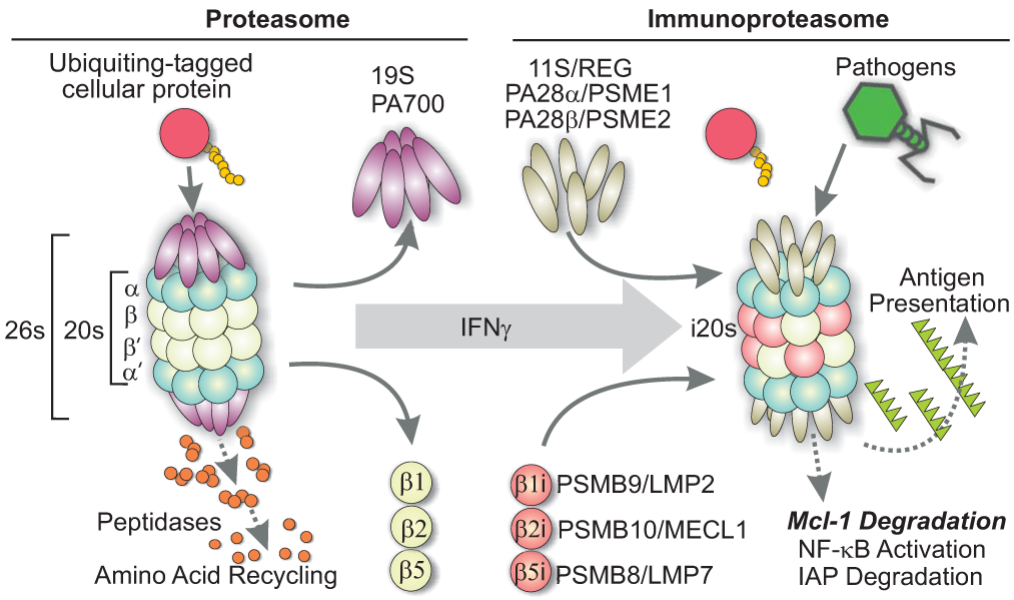

Figure 6. Schematic representation of the interferon-induced immunoproteasome conversion

The constitutive 26S proteasome is composed of the 19S regulatory and the 20S catalytic subunits, each having several protein units. In immune activated cells, interferons and other activators trigger transcriptional increases in at least 5 subunits which cooperatively form an immunoproteasome with entirely new catalytic subunits (β1i, β2i, β5i), and new regulatory subunits PA28 α/β/γ. The immunoproteasome has a different substrate specificity and catalytically produces larger peptide fragments, which are better, suited to presentation by MHC class I. Its role in apoptosis could be via degradation of apoptotic inhibitors such as Mcl-1.