Biochemical and Biophysical Research Communications 421 (2012) 145–151

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A defect in cell death of macrophages is a conserved feature of nonobese diabetic mouse

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ARTICLE INFO

Article history:
Received 2 April 2012
Available online 9 April 2012

Keywords:
Autoimmune diabetes
Macrophage
Cell death
Apoptosis
NF-κB
Bcl-X_L

ABSTRACT

Impaired apoptosis in immune effector cells such as macrophages has been implicated in the development of autoimmune disease by promoting the breakdown of self-tolerance and the sustained production of cytotoxic molecules. Macrophages from nonobese diabetic (NOD) mouse, an animal model of human autoimmune diabetes, exhibit several defects that are causally linked to the onset and progression of the disease. In this context, we investigated whether NOD macrophages have a defect in a cell death pathway, and if that is the case, the mechanism underlying such dysregulation of cell death. We found that NOD macrophages were resistant to treatment with a broad spectrum of cell death stimuli, triggering both apoptotic and non-apoptotic death. Through analysis of intracellular signaling pathways along with the expression of apoptosis-related proteins, we found that atypical resistance to cell death was associated with an elevated expression of anti-apoptotic Bcl-X_L but not the NF-κB signaling pathway in NOD macrophages. Further, ABT-737, which can inhibit Bcl-X_L function, sensitized NOD macrophages to apoptosis induced by diverse apoptotic stimuli, thus restoring sensitivity to cell death. Taken together, our results suggest a macrophage-intrinsic defect in cell death as a potential mechanism that promotes an immune attack towards pancreatic β-cells and the development of autoimmune diabetes in NOD mice.

1. Introduction

Macrophages play pivotal roles in the initiation and progression of type 1 diabetes (T1D), an autoimmune disorder resulting from aberrant immune responses against insulin-producing pancreatic β-cells [1]. They were shown to be the primary source of inflammatory cytokines such as TNF-α in the early islet infiltrate [2] and required for the priming of diabetogenic $CD8^+$ T cells specific for a β-cell antigen [3]. Besides their role as antigen-presenting cell (APC), macrophages also have been recognized as the essential effector cells that directly induce β-cell death as producers of reactive oxygen species (ROS) and other soluble cytotoxic mediators [1,4]. Depletion of macrophages results in complete prevention of the disease in NOD mice [5], thus pointing to a key role for macrophages in the development of T1D.

Of note, several studies have demonstrated that macrophages from NOD mouse exhibit developmental and functional defects that would promote T1D development. These defects include an impaired ability to trigger regulatory T cell function [6] and a propensity to enhance Th17 cell generation and to produce elevated levels of IL-12 compared to reference strains of mice, including C57BL/6 (B6) [7,8]. Moreover, NOD macrophages entail an intrinsic defect in phagocytic clearance of apoptotic cells [9], which could promote inflammatory responses if dying cells are not removed in time but undergo secondary necrosis [10,11]. Thus, it has become evident that aberrant regulation of macrophages could play a pathogenic role in T1D and thereby precipitate the development of disease. Moreover, we recently demonstrated that NOD macrophages were resistant to apoptotic pathway by IFN-γ and lipopolysaccharide (LPS) [12] due to their hypo-responsiveness to IFN-γ [13]. Because death of activated macrophages could be a mechanism for controlling inflammation and potential tissue damage, apoptotic resistance of NOD macrophages may be an important risk factor to precipitate T1D development. Supporting this, the induction of macrophage apoptosis either by silica or clodronate liposomes abrogated the incidence of T1D in NOD mice [5,14]. However, at present little is known about dysregulation of apoptotic pathway in NOD macrophages.

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http://dx.doi.org/10.1016/j.bbrc.2012.04.017

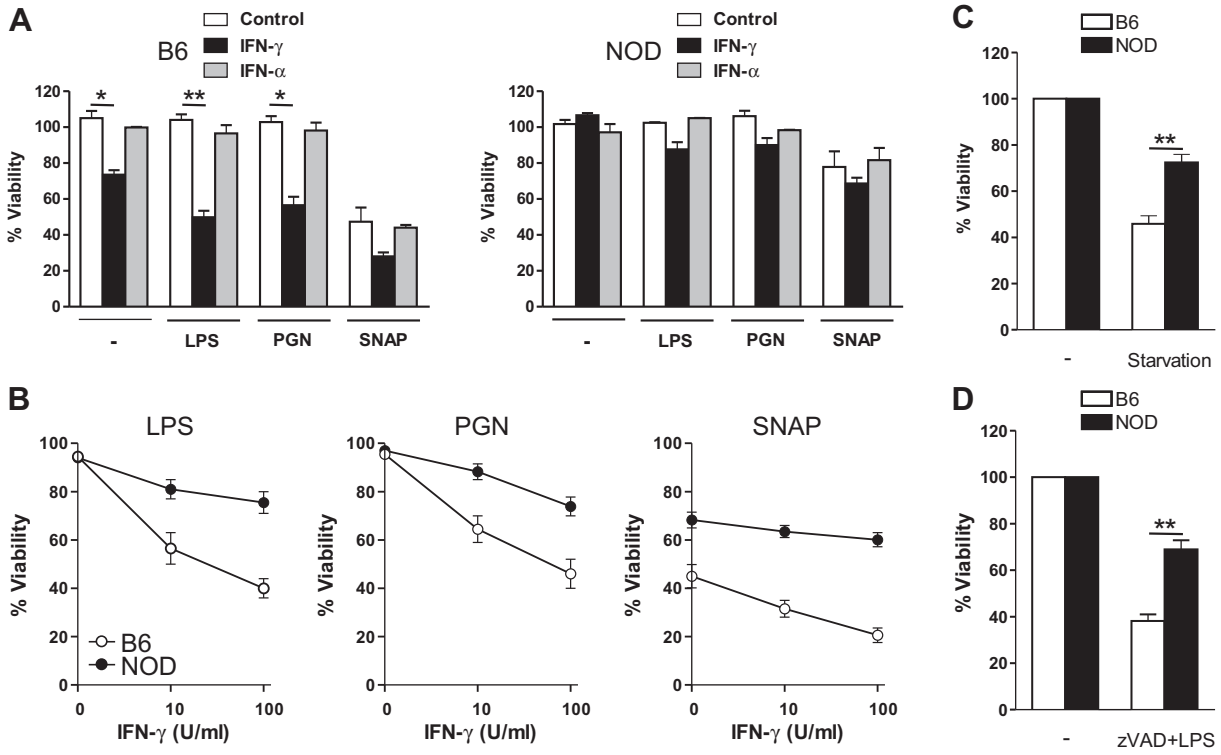

Fig. 1. NOD macrophages are resistant to a broad spectrum of cell death pathway. (A) Peritoneal macrophages of B6 (left panel) and NOD mice (right panel) were left untreated (−) or treated with LPS (100 ng/ml), PGN (1 g/ml), or SNAP (0.5 mM) in combination with IFN-γ (10 U/ml) or IFN-α (10 U/ml) for 48 h. Cell viability was assessed by crystal violet assay and shown as a percentage of untreated cells, (B) B6 and NOD macrophages were incubated with LPS (100 ng/ml), PGN (1 μg/ml), or SNAP (0.5 mM) in combination with increasing doses of IFN-γ for 48 h. Cell viability is shown as in panel A, (C) B6 and NOD macrophages were incubated for 36 h in a serum-containing medium (−) or a serum-free medium (Starvation). Cell viability is shown as in panel A k and (D) B6 and NOD macrophages were left untreated (−) or treated with LPS (100 ng/ml) for 12 h with zVAD (50 M) pretreatment for 1 h. Cell viability is shown as in panel A. Significant differences between groups are shown as $^{*}P < 0.05$ or $^{**}P < 0.01$.

Thus, the aim of the present study was to investigate whether NOD macrophages have an intrinsic defect in cell death pathway. Here, we provide evidence that NOD macrophages are resistant to treatment with a broad spectrum of death stimuli, triggering both caspase-dependent and caspase-independent cell death. Analysis of intracellular pathways revealed that such a defect was related to an aberrant expression of Bcl-X_L but was corrected by a small molecule inhibitor of anti-apoptotic Bcl-2 proteins, thus suggesting a potential therapeutic approach to treat T1D.

2. Materials and methods

2.1. Animals

C57BL/6 and NOD/Lt mice were purchased from The Jackson Laboratory. STAT1$^{-/-}$ mice were from the Taconic Farms and were backcrossed to C57BL/6 mice for 12 generations. Female 6–10-week-old NOD (pre-diabetic) and age-matched C57BL/6, STAT1$^{-/-}$ mice were used. Autoimmune diabetes develops in ~75% of NOD female mice by 1 year of age. All mice were housed and fed under a specific-pathogen-free condition. All animal experiments were done in accordance with the institutional guidelines of Samsung Medical Center.

2.2. Macrophages and reagents

Peritoneal macrophages and bone marrow-derived macrophages were prepared and cultured as described [15,16]. Adenovirus expressing human IκBα-superrepressor (SR) whose serines 32 and 36 were replaced with alanines (Ad5IκBα-SR) and control adenovirus (Ad5LacZ) were propagated and used as described [11]. Recombinant mouse TNF-α was from R&D Systems. A pancaspase inhibitior, zVAD-fmk, was from Enzyme Systems Products. All other chemicals were from Sigma unless indicated otherwise.

2.3. Assessment of cytotoxicity and apoptosis

Crystal violet assay for the measurement of macrophage death was performed as described [15]. Intracytoplasmic nucleosomes were quantitated as described [13], using the Cell Death Detection ELISA^{PLUS} Kit (Roche). Annexin V staining and TUNEL assays were performed using annexin V-FITC (BD Biosciences) and TUNEL assay kit (Roche), respectively, according to manufacturer's instructions.

2.4. Electrophoresis mobility shift assay (EMSA)

Macrophages in 6-well plates were left untreated or treated with 2×10^6 apoptotic, LA, PN cells (1:1 cell to macrophage ratio), LPS, or TNF-α. After 1 h, plates were washed and nuclear extracts were prepared using Nuclear Extract Kit (Active Motif). EMSA was then performed with Gel Shift Assay Systems (Promega) using the equal amounts (5 μg) of nuclear extracts and an NF-κB consensus oligonucleotide (Promega) as described [11].

2.5. Protein and RNA analysis

Lysates from macrophages were prepared and analyzed for Western Blot analysis as described [11]. Specific antibodies used were STAT1, Bcl-X_L, Bad (Cell Signaling Technology), Bax (Upstate), or actin (BD Biosciences). Total RNAs from macrophages, that had been unstimulated or stimulated with LPS for 4 h, were isolated using RNAwiz (Ambion). RNA analysis using real-time PCR was performed as described [17].

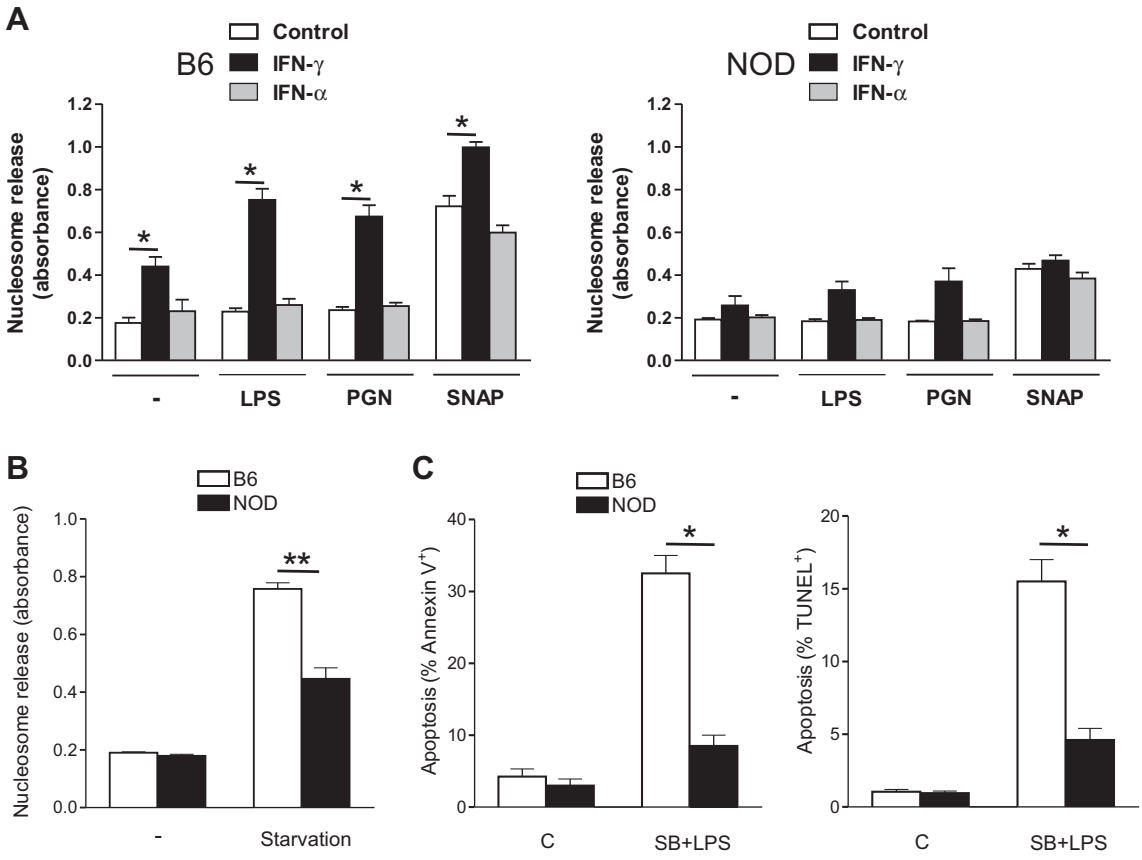

Fig. 2. NOD macrophages have a defect in apoptotic pathway. (A) Peritoneal macrophages of B6 (left panel) and NOD mice (right panel) were treated as in Fig. 1A for 24 h. The apoptotic nucleosomes in the cytoplasm were determined as described in Section 2. (B) B6 and NOD macrophages were incubated as in Fig. 1C for 18 h. Nucleosome release was determined as in panel A. (C) Bone marrow-derived macrophages of B6 and NOD mice were treated with LPS (100 ng/ml) for 8 h in the presence of SB202190 (SB; 10 M). Cells were analyzed by FACS after staining with annexin V-FITC (left panel) and under a fluorescent microscope after TUNEL staining (right panel). Significant differences are shown as $^{*}P < 0.05$ or $^{**}P < 0.01$.

2.6. Statistical analysis

All data were analyzed using GraphPad Prism v.4.00 (GraphPad Software Inc). Results are presented as means ± SEM. The comparisons between groups were performed using the independent sample *t*-tests. The statistical significance was defined as $P < 0.05$.

3. Results

3.1. NOD macrophages were resistant to a broad spectrum of cell death stimuli

To characterize apoptotic response of NOD macrophages, we first assessed the effect of IFN-γ on the death of activated macrophages. Compared with B6 macrophages, NOD macrophages were refractory to cell death induced by IFN-γ (10 U/ml) alone as well as IFN-γ/LPS combination (Fig. 1A), consistent with their hyporesponsiveness to IFN-γ [13]. Moreover, similar resistance of NOD macrophages to death was shown with IFN-γ/peptidoglycan (PGN) treatment (Fig. 1A). An increasing dose of IFN-γ (100 U/ml) in combination with the stimuli tested significantly reduced the viability of B6 macrophages but not NOD macrophages (Fig. 1B). IFN-α, LPS, PGN alone or in combination did not cause any noticeable effect on the viability of macrophages. To test whether IFN-γ hypo-responsiveness is the main mechanism underlying apoptotic resistance of NOD macrophages, we exposed NOD macrophages to nitric oxide (NO) donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP). NO has been implicated as a downstream effector molecule which is capable of triggering macrophage death by IFN-γ/LPS [18]. As shown in Fig. 1A, NOD macrophages were more resistant to NO-mediated cytotoxicity than B6 macrophages (~47% vs ~77%). IFN-γ also potentiated cytotoxic effect of SNAP on B6 macrophages, whereas it did not on NOD macrophages (Fig. 1A, B). Thus, these results suggest that NOD macrophages have a protective mechanism against NO-mediated cytotoxicity, which is independent of hyporesponsiveness to IFN-γ.

To further investigate this differential response of cell death, we next assessed the death of macrophages induced by serum starvation. Macrophages were incubated for 36 h in a serum-free medium to undergo apoptotic death [19]. Compared with B6 macrophages, NOD macrophages exhibited an increased resistance to apoptosis following serum withdrawal (Fig. 1C). Because NO can induce both apoptotic and necrotic death in macrophages via several mechanisms [20], we determined whether NOD macrophages are refractory to different mode of death other than apoptosis. We previously demonstrated that treatment of macrophages with pancaspase inhibitor zVAD rapidly induces death of LPS-stimulated macrophages in a caspase-independent manner [15]. This type of death process critically relied on the intracellular generation of ROS, and the majority of dying cells showed both necrosis-like changes and autophagic changes [15,21]. After treatment with zVAD/LPS, NOD macrophages were more resistant to death than B6 macrophages (~38% vs ~68%) (Fig. 1D). Collectively, these results suggest that NOD macrophages have an intrinsic defect in death pathway, which confers a survival advantage on NOD macrophages against a broad spectrum of death stimuli.

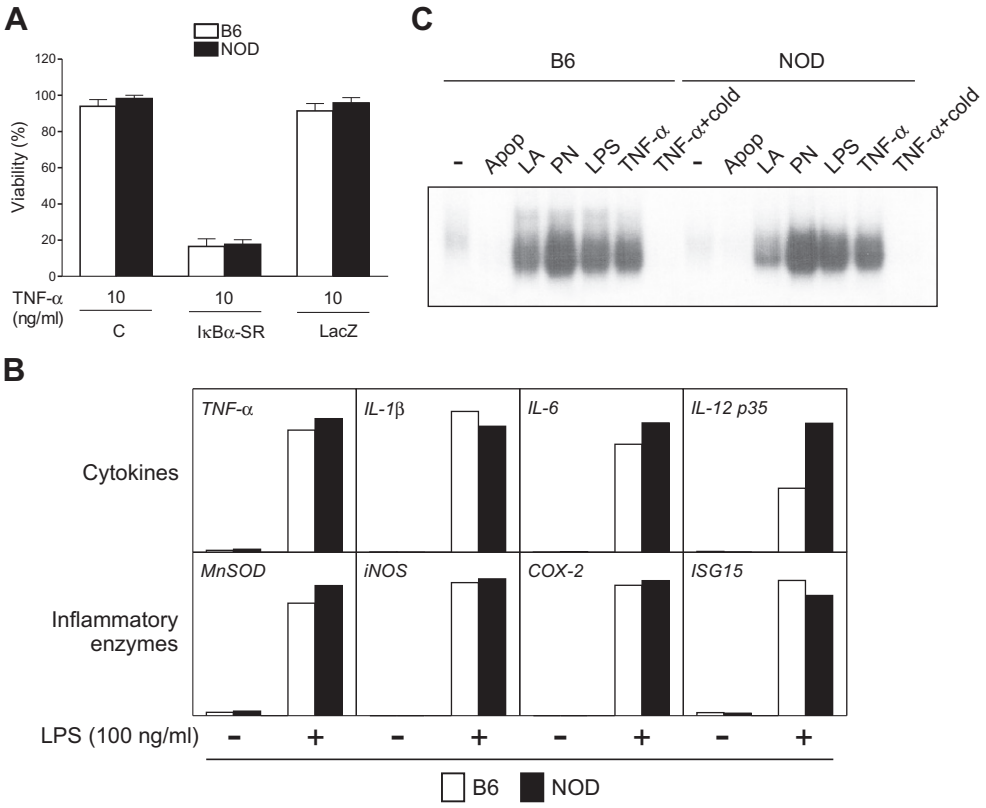

Fig. 3. NF-κB is not critical for apoptotic resistance of NOD macrophages. (A) Peritoneal macrophages of B6 and NOD mice were left untreated (C) or infected with adenovirus (multiplicity of infection of 50) harboring IκBα-SR or LacZ for 24 h and then treated with TNF-α (10 ng/ml) for 48 h. Cell viability is shown as in Fig. 1A. (B) B6 and NOD macrophages were left untreated (−) or treated with MIN6N8 insulinoma cells [apoptotic (Apop), late apoptotic (LA), and primary necrotic (PN)], LPS (100 ng/ml), or TNF-α (10 ng/ml) for 1 h. NF-κB DNA binding activity was determined by EMSA. The specificity of binding was evaluated by competition with added wild-type κB oligonucleotide (cold). (C) Bone marrow-derived macrophages of B6 and NOD mice were treated with LPS (100 ng/ml) for 4 h. Relative expression of 8 NF-κB-inducible genes was determined by real-time PCR.

3.2. NOD macrophages exhibited a defect in apoptotic pathway

To gain an insight into the underlying mechanism, we next determined whether the resistance of NOD macrophages to cell death is indeed associated with apoptotic changes. To this end, we measured the accumulation of histone-complexed DNA fragments, a hallmark of apoptosis, in the cytoplasm of macrophages. As shown in Fig. 2A, the level of nucleosome release was inversely correlated to the viability of B6 and NOD macrophages, thus confirming apoptotic resistance of NOD macrophages. Likewise, NOD macrophages exhibited increased resistance to apoptosis upon serum starvation (Fig. 2B). Previous work showed that inhibition of p38 MAPK by a chemical inhibitor (SB202190) induces apoptosis of LPS-stimulated macrophages, which recapitulates the pathogenic effect of anthrax lethal factor [16]. Compared with B6 macrophages, NOD macrophages were refractory to apoptosis induced by SB202190/LPS combination, as jugged by a significant decrease in the percentage of cells that were positive for annexin V and TUNEL (Fig. 2C). Thus, these results suggest that NOD macrophages have a defect in apoptotic pathway, thus protecting them from diverse apoptotic stimuli.

3.3. NF-κB did not contribute significantly to apoptotic resistance of NOD macrophages

To study mechanism of such apoptotic resistance, we next assessed the role of NF-κB activation. It has been shown that stimuli such as LPS and TNF-α rapidly activate NF-κB, which plays a key role in the anti-apoptotic response as well as in the production of cytokines [22]. NF-κB is also one of the main antioxidant regulators via an expression of Mn-superoxide dismutase (SOD). First, we infected macrophages with Ad5IκBα-SR that inhibits NF-κB activation via resistance to proteasome degradation [11]. B6 and NOD macrophages were equally susceptible to apoptosis induced by TNF-α combined with an Ad5IκBα-SR infection (Fig. 3A). The viability of macrophages was not affected by TNF-α combined with a control Ad5LacZ infection. EMSA assay indicated that nuclear translocation of NF-κB was not significantly different between B6 and NOD macrophages exposed to various stimuli except for late apoptotic (LA) cells (Fig. 3B), probably due to a defect of NOD macrophages in phagocytic clearance of apoptotic cells [9]. By using quantitative real-time PCR (Q-PCR), we next determined the expression of several cytokines and inflammatory enzymes that are known to be induced by NF-κB activation. As shown in Fig. 3C, this analysis resulted in no strain differences in the expression of genes tested except for IL-12 as expected [7]. Thus, these results suggest that differential response of cell death is unlikely related to NF-κB activation but attributed to other signaling molecule(s).

3.4. Association of apoptotic resistance with dysregulated Bcl-X_L expression in NOD macrophages

We next investigated whether such a defect in cell death could be due to an aberrant expression of anti-apoptotic proteins including the inhibitor of apoptosis (IAP) and Bcl-2 families. Proteins of the IAP and Bcl-2 families play a critical role in the modulation of macrophage sensitivity to death signals [23,24]. Of the anti-apoptotic genes tested by Q-PCR, the expression of Bcl-X_L but not

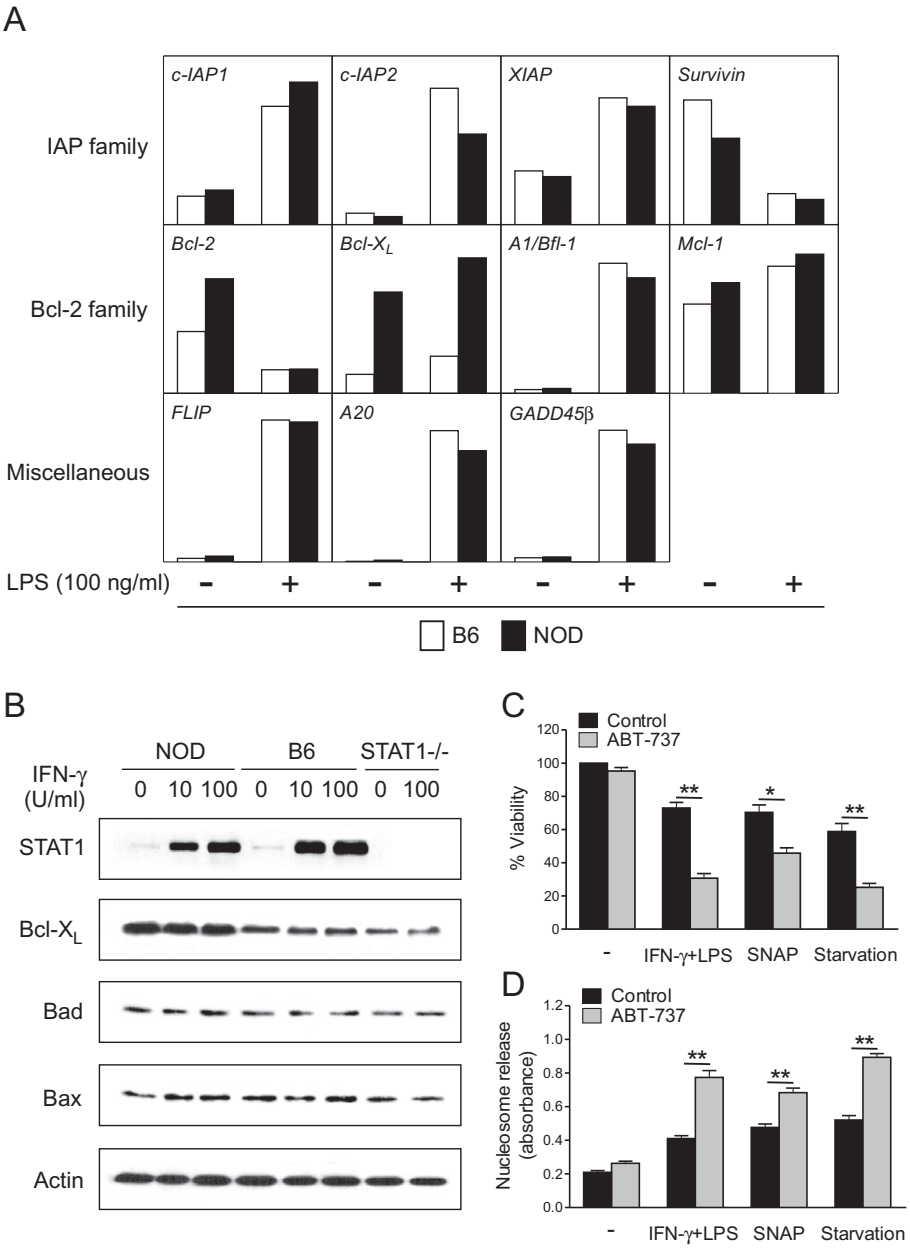

Fig. 4. Dysregulated expression of Bcl-X_L is associated with apoptotic resistance of NOD macrophages. (A) Bone marrow-derived macrophages of B6 and NOD mice were treated as in Fig. 3C. Relative expression of the indicated genes was analyzed by real-time PCR. (B) Peritoneal macrophages of B6, NOD, and STAT1$^{-/-}$ mice were left untreated (0) or with increasing doses of IFN-γ for 24 h. Total protein level of STAT1, Bcl-X_L, Bad, Bax, and actin was analyzed by Western blot analysis. (C and D) NOD macrophages were left untreated (−) or treated as in Fig. 1(C) and Fig. 2(D) with or without ABT-737 (10 M) pretreatment for 1 h. Cell viability (C) and nucleosome release (D) are shown. Significant differences are shown as $^{*}P < 0.05$ or $^{**}P < 0.01$.

other genes was substantially upregulated in both resting and LPS-stimulated NOD macrophages compared to B6 macrophages (Fig. 4A). Other miscellaneous genes such as FLIP, A20, and GADD45β were similarly expressed in B6 and NOD macrophages (Fig. 4A). The increased basal expression of Bcl-X_L in NOD macrophages was also shown at the protein level, which was not affected by various doses of IFN-γ treatment (Fig. 4B). Analysis of macrophages from B6 and STAT1$^{-/-}$ mice revealed that the expression of Bcl-X_L was independent of IFN-γ receptor pathway (Fig. 4B). In addition, no strain difference was observed in total levels of pro-apoptotic proteins such as Bad and Bax that are important players in macrophage apoptosis [25,26]. Although we cannot rule out the involvement of other apoptosis-related proteins, these results collectively suggest that the dysregulated expression of Bcl-X_L likely contribute to the apoptotic resistance of NOD macrophages.

Next, we studied whether inhibition of Bcl-X_L function modulates the apoptotic resistance of NOD macrophages. To this end, we assessed the apoptosis of NOD macrophages after incubation with IFN-γ/LPS, SNAP, or serum starvation in the absence and presence of ABT-737, a potent inhibitor of Bcl-2, Bcl-X_L, and Bcl-w protein function [27]. Interestingly, ABT-737 treatment sensitized NOD macrophages to apoptosis induced by death stimuli tested (Fig. 4C, D), thus suggesting that a defect in apoptotic pathway associated with Bcl-X_L overexpression can be reversed by ABT-737 treatment.

4. Discussion

Here, we report that NOD macrophages are resistant to treatment with a broad spectrum of death stimuli, inducing both apop-

totic and non-apoptotic death pathway. Mechanistic study indicated that such a defect was unlikely related to IFN-γ hypo-responsiveness and NF-κB activation but could be attributed to an aberrant expression of anti-apoptotic Bcl-X_L protein. The relevance of this finding was further supported by the sensitization of NOD macrophages to apoptosis by ABT-737 treatment.

Bcl-X_L plays a crucial role in protective mechanism in activated macrophages [23], thus rendering macrophages viable in the microenvironment of inflammatory lesions that are rich in cytotoxic inflammatory mediators. Bcl-X_L is known to be a potent repressor of both apoptosis and non-apoptotic death such as necrosis and autophagy [19,23,28,29], thus suggesting a causal link between the dysregulated expression of Bcl-X_L and the resistance of NOD macrophages against diverse death stimuli. According to our results, marked increase in basal expression of Bcl-X_L as well as its induced expression might be important for such resistance. The expression of Bcl-X_L has been shown to be under regulation according to the state of cellular differentiation or activation [30,31]. In this respect, it is interesting to note that NOD macrophages manifest a defect in cellular differentiation [6] although exact mechanism underlying the dysregulation of Bcl-X_L expression remains to be determined. Of note, activated T cells from NOD mice also exhibit an increased expression of Bcl–X_L and resistance to apoptosis following withdrawal of IL-2 despite different regulation of Bcl-X_L expression [32]. Thus, these suggest a possibility of Bcl-X_L as a common molecule that confers a survival advantage on various immune cells in NOD mouse.

Of special interest is the finding that blockade of Bcl-X_L function by ABT-737 could sensitize NOD macrophages to apoptosis induced by diverse apoptotic stimuli. There are evidences that Bcl-2 pathway-associated dysregulation of apoptosis in lymphocytes and APCs contributes to the development of autoimmune diseases [33,34]. Thus, restoration of apoptotic sensitivity to death signals in the context of autoimmunity may constitute a novel therapeutic approach. In fact, administration of ABT-737 was shown to significantly reduce disease severity in animal models of autoimmune arthritis and lupus [27]. Given the dysregulated expression of Bcl-X_L in T cells [32] and macrophages from NOD mouse, Bcl-2 family protein may represent a promising target to treat T1D.

In conclusion, we report that NOD macrophages have an intrinsic defect in death pathway that is causally related to an increased expression of Bcl-X_L. Macrophages appear to play a pivotal role in T1D by enhancing the function of diabetogenic T cells [35] or producing cytotoxic molecules to β-cells [4]. Although actual contribution of dysregulated expression of Bcl-X_L to T1D remains to be determined, the resistance of activated NOD macrophages to various forms of death may contribute to the pathogenesis of T1D. Thus, our results provide a new insight into the pathogenesis of T1D with respect to the resistance of macrophages to cell death.

Acknowledgments

This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (NRF-2011-0014138) and Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Korea (A110893). M.-S. Lee is the recipient of a grant of Bio R&D program of Korean Ministry of Science & Technology (2008-04090) and Global Research Laboratory Grant (K21004000003-10A0500-00310).

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