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ORIGINAL ARTICLE

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Mature dendritic cells are protected from Fas/CD95-mediated apoptosis by upregulation of Bcl-X_L

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Abstract The clinical use of dendritic cells (DC) as tumor vaccines is very much dependent on their survival potential. Members of the tumor necrosis factor (TNF) receptor superfamily and their ligands are involved in the regulation of cell death. Fas (CD95) is a representative protein that promotes apoptosis. The Bcl-2 family of proteins functions as an integrator of diverse pro- and anti-apoptotic signals. It has been found that DC maturation facilitates their survival, and thus has an anti-apoptotic function. However, little is known about the underlying mechanisms. We investigated the effects of TNF-α and lipopolysaccharide (LPS) on the expression of apoptotic molecules during differentiation and maturation of DC under serum-free conditions, and correlated this to the sensitivity to apoptosis by the Fas-mediated pathway. Indeed, DC activation effectively inhibited DC apoptosis, which was predominantly accompanied by the upregulation of Bcl-X_L and to a lesser extent Bcl-2, while Bax and FLICE inhibitory protein (FLIP) remained unchanged. In contrast, in the presence of serum FLIP was also upregulated. We conclude that under serum-free conditions, Bcl-X_L rather than FLIP plays the main role in protection against DC apoptosis.

Keywords Bcl-2 · Differentiation · FLIP · LPS · TNF-α

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Introduction

Hematopoetic dendritic cells (DC) are a unique population of antigen-presenting cells (APC) and also one of the most potent initiators and modulators of the immune response. Once peripheral immature DC are stimulated and capture an antigen, they begin to process it to form MHC–peptide complexes and migrate from the peripheral tissues to the T cell regions of the lymphoid organs [3, 42]. During this process, DC mature and upregulate various accessory molecules that enhance binding to and activation of T cells [9, 18]. This process is, however, defective in cancer patients and leads to an insufficient recognition by the host immune system of tumor antigens [19]. By in vitro conditioning this phenomenon can be reversed, leading to functional restoration. Several clinical studies have demonstrated that injection of small numbers of peripheral blood-generated DC pulsed with a relevant antigen induce a strong immune response in cancer patients, in some cases leading to tumor regression [12, 26, 28, 36]. The clinical use of DC as tumor vaccines is, nevertheless, dependent on their survival potential.

Apoptosis is critical for the control of homeostasis in a variety of tissues, including the immune system [1, 25, 35, 51]. A wide range of factors and molecules are involved in the regulation of apoptosis, including the Fas (CD95/Apo1)-mediated pathway that is responsible for the induction of cell death [27]. The death receptor Fas belongs to the tumor necrosis factor (TNF) receptor superfamily, and activates several downstream signal proteins such as caspases. Other members of the TNF receptor superfamily also include the TNF receptor itself and CD40 [14, 22, 24, 39, 40]. It is noteworthy that they have both cytotoxic as well as protective effects upon cells [15, 33, 37, 44, 45]. Other molecules involved in the control of apoptosis are members of the Bcl-2 family, with the molecules Bcl-2 and Bcl-X_L known to prevent apoptosis [10, 15], while Bax promotes it [30].

It has been found that several proteins of the TNF family that are expressed on activated and memory T cells can increase DC survival [2, 7, 50]. TNF-α, CD40 ligand (CD40L) and lipopolysaccharide (LPS) enhance the expression of MHC and accessory molecules such as CD80 and CD86. Furthermore, it has been shown that CD40L counteracts Fas-mediated apoptosis [5, 20]. However, little is known about the underlying mechanisms.

We investigated how TNF-α and LPS affect the sensitivity of DC to Fas-mediated apoptosis. We have shown that these molecules effectively inhibit Fas-mediated cell death and at the same time upregulate the expression of Bcl-2 and in particular Bcl-X_L, while FLICE inhibitory protein (FLIP) expression remains unchanged. These results suggest that Bcl-X_L plays a major role in the regulation of DC apoptosis.

Materials and methods

Generation of monocyte-derived DC

Peripheral blood mononuclear cells (PBMC) were separated on Ficoll-Hypaque gradient (Amersham Pharmacia, Uppsala, Sweden) from peripheral blood buffy coats of healthy donors. Monocytes isolated by negative depletion using immunomagnetic selection ($CD14^+$ monocyte isolation kit MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer's instructions, were resuspended in X-vivo 15 (Biowhittaker, Rockland, Md.) supplemented with 50 ng/ml GM–CSF and 40 ng/ml IL-4 (both Schering-Plough, Stockholm, Sweden). To obtain DC with mature phenotype, 50 ng/ml of TNF-α (Cetus, Emeryville, Calif.) or 100 ng/ml of LPS was added for the last 24 h of culture. To induce apoptosis, anti-Fas antibody (CH-11; Medical & Biological Laboratories, Japan) or isotype control (mouse IgM; Dako, Dakopatts AB, Stockholm, Sweden) was added at 500 ng/ml and further incubated for 8 h.

Flow cytometric analysis

The generated DC were characterized by flow cytometry using a FACScan cytometer (Becton Dickinson, San Jose, Calif.). The following panel of monoclonal antibodies (mAbs) was used: CD83–PE (phycoerythrin), CD86–APC (allophycocyanin), CD80–FITC (fluorescein), CD1a–PE, HLA–DR/DQ/DM–FITC, HLA–ABC–PE, CD3–FITC, CD56–PE, CD19–PE (Pharmingen, San Diego, Calif.) and CD14–PC5 (Immunotech, Marseille, France). For determination of Fas (CD95) expression, monoclonal mouse anti-human CD95 antibody (clone DX2) was used. Annexin-V binding assay was performed according to the instructions supplied by the manufacturer.

Western blot analysis

Harvested cell populations ($2–5 \times 10^6$) were centrifuged and pelleted. The cell pellets were resuspended in ice-cold lysis buffer. Nuclei and unlysed cellular debris were removed by centrifugation at 14,000 *g* for 20 min. The supernatant was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and electrophoresed through 8% SDS–polyacrylamide gels. Proteins were electrotransferred onto polyvinylidene fluoride filter membrane (Immobilon-P; Millipore, Bedford, Mass.). Filters were incubated with primary monoclonal murine anti-human: Bcl-2, Bcl-X_L, Bax (Zymed, San Francisco, Calif.) and actin (Amersham, Bucks., UK) antibody, and polyclonal rabbit anti-human I-FLICE antibody (Pharmingen) for 1 h. Following incubation with secondary horseradish peroxidase (HPR)-conjugated anti-mouse immunoglobulin (Ig) or anti-rabbit Ig (Amersham) for 1 h, filters were developed with an enhanced chemiluminescence (ECL) system (Amersham).

Results

Activated DC resist Fas-induced apoptosis

To investigate how cell death is affected with regard to differentiation of monocytes into DC, immunomagnetically enriched monocytes were differentiated in the presence of GM–CSF and IL-4 for seven days. To obtain DC at the terminal stage of differentiation, LPS or TNF-α were added to the culture during the last 24 h. A major upregulation of the costimulatory molecules CD80 and CD86 as well as the activation molecule CD83 was detected. The CD1a marker was expressed independently

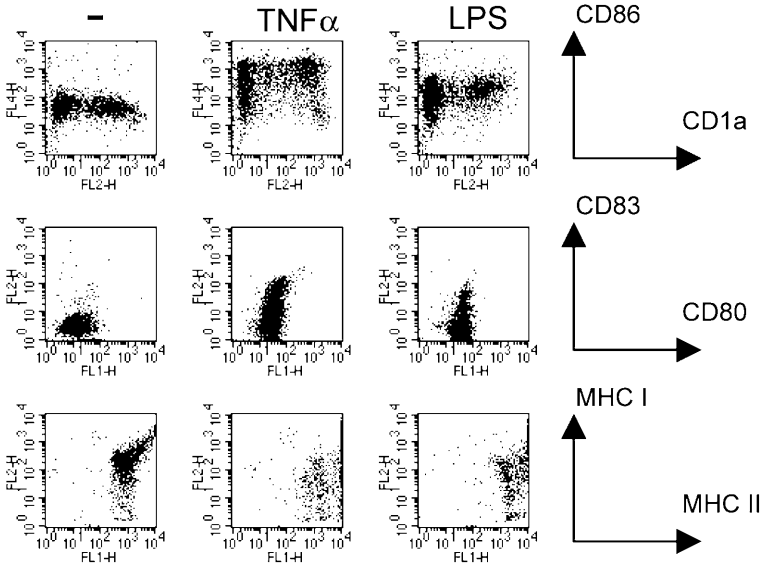

Fig. 1 Mature DC show higher levels of costimulatory molecules but not CD1a. Monocytes were isolated from normal donors and enriched by immunomagnetic selection. Monocytes were further differentiated into immature DC in X-vivo 15 medium supplemented with IL-4 (40 ng/ml) and GM–CSF (50 ng/ml). To induce maturation, TNF-α (50 ng/ml) or LPS (100 ng/ml) were added to the cultures during the last 24 h. Cells were incubated with fluorochrome-conjugated antibodies in PBS at 4°C for 30 min prior to flow cytometric analysis. A typical result is shown from at least four independent experiments

of activation (Fig. 1). To analyze their susceptibility to Fas-induced apoptosis, DC were subsequently incubated with anti-Fas mAb CH11 or isotype-matched control antibody for 8 h. Staining for Annexin-V and 7-AAD showed that immature DC exhibited higher levels of spontaneous apoptosis compared to TNF-α- or LPS-treated DC. Viability of immature DC was 79% compared to 86% and 90% for TNF-α- and LPS-treated DC respectively (Fig. 2A). Upon Fas triggering, this difference was even more pronounced, as 65% of the immature DC were still viable compared to 76% and 88% for TNF-α- and LPS-treated DC (Fig. 2B). These results extend the findings of others [32, 48], that TNF-α or LPS treatment rescues DC from Fas-induced cell death and apoptosis. To elucidate whether these differences could be explained by diverse Fas (CD95) expression, DC were stained with anti-Fas antibody and analyzed by flow cytometry. Others have shown that Fas expression on $CD34^+$-derived DC is enhanced following LPS or TNF-α treatment [34]. However, no differences were observed in Fas expression between immature and TNF-α-treated monocyte-derived DC (Fig. 2C).

Activated DC express higher levels of anti-apoptotic molecules

Conflicting information exists about the significance of different apoptosis-regulating proteins responsible for the pronounced protection from cell death of terminally differentiated DC. We analyzed by western blot the expression of apoptotic molecules throughout the culture period. No significant differences were detected in the levels of these molecules between day 0 and 8 (data not shown). However, upon stimulation with TNF-α or LPS a strong upregulation in protein level of Bcl-X_L was detected. Bcl-2 was also upregulated to a lesser extent, while no change in the level of FLIP was detected (Fig. 3A). This finding was contradictory to the published results of others when DC was cultured in the presence of bovine serum [23, 29, 48]. Therefore, we also examined FLIP expression under these culture conditions. Indeed, DC stimulated with TNF-α or LPS cultured in the presence of serum displayed a significantly upregulated level of FLIP (Fig. 3B). These results suggest that rather than FLIP, Bcl-X_L is the main regulatory protein responsible for the marked protection of Fas-induced apoptosis under serum-free conditions.

Discussion

In earlier clinical trials of DC cancer vaccines, mainly immature DC were used [21, 26, 43]. In some instances, even DC cultured in the presence of bovine serum were employed [28]. Lately, it has become apparent that mature DC are more suitable for clinical application. This concept is based on evidence that due to a higher expression of accessory molecules, mature DC are better APC and also that this phenotype does not revert in vivo. Based on the results reported here, these cells are also more resistant to cell death.

It is likely that this feature also has relevance in vivo. Mature DC in contrast to immature DC might have an

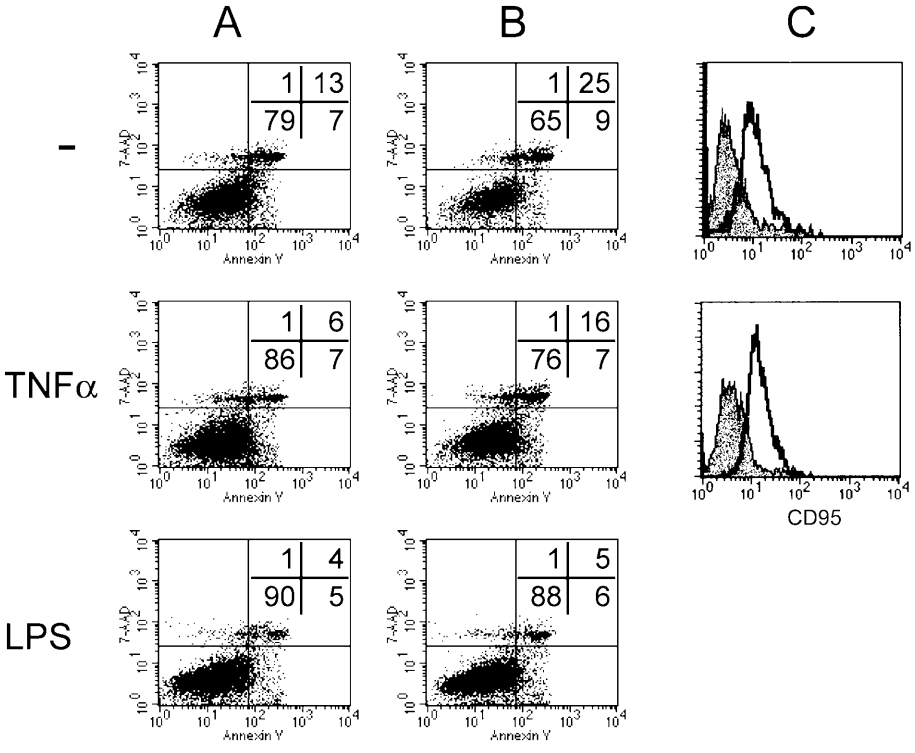

Fig. 2 Activated DC resist Fas-induced apoptosis. Monocytes were differentiated under serum-free conditions into immature DC in the presence of IL-4 and GM–CSF. **A** Spontaneous apoptosis of immature and mature DC. AnnexinV and 7-AAD were measured after 8 h of IgM control (500 ng/ml) antibody binding. **B** Fas-induced apoptosis of immature and mature DC, AnnexinV and 7-AAD were measured 8 h after anti-Fas (clone CH11; 500 ng/ml) binding. **C** Flow cytometric analysis of CD95 expression on immature and TNF-α-treated DC. *Filled* and *open histograms* represent isotype control and cells stained with anti-human CD95 antibody (clone DX2), respectively. One out of three independent experiments is shown

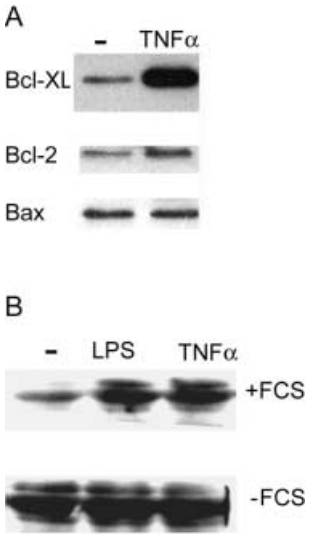

Fig. 3 Western blot analysis of immature or mature DC. Monocytes were immunomagnetically enriched and further differentiated into immature DC. During the last 24 h, TNF-α (50 ng/ml) was added to induce maturation. **A** Cells were differentiated under serum-free conditions. Cell lysates were prepared from day 8 DC and analyzed for expression of Bcl-X_L, Bcl-2 and Bax by western blot. **B** Analysis of FLIP of immature and mature DC in the presence or absence of fetal calf serum (FCS). One out of three independent experiments is shown

intrinsic escape mechanism, protecting them from the immunologically fatal contact with T cells and other cytotoxic cells such as natural killer (NK) cells and activated macrophages in the lymphoid tissue. Otherwise, DC could become degraded soon after reaching the lymphoid organs and their antigen-presenting capacity might be suboptimal. It has been reported that mature DC are far less targeted by autologous NK cells than immature DC [49]. Nevertheless. it is very difficult to speculate how these results can be transferred to the in vivo situation, since tissue cultures with or without bovine serum do not constitute physiological conditions.

Maturation of DC can be influenced by a variety of factors. TNF-α, LPS and CD40L stimulate the final maturation of DC [7, 8, 11], while IL-10 blocks it [6]. In our study, either TNF-α or LPS upregulated the expression of MHC II and CD86, which was correlated with the enhancement of the expression of CD83. Expression of these molecules is associated with a mature phenotype and an increase in the antigen-presenting capacity [4]. TNF-α, TNF-related activation-induced cytokine (TRANCE), FasL and CD40L are the TNF ligand family members that provide activation signals in APC such as B cells, macrophages, and DC [41, 46, 50]. In general, members of the TNF receptor family are recognized as killer molecules. They activate caspases, enzymes that are critically involved in the death process, and this activation is further amplified by intracellular mitochondria-associated mechanisms. Conflicting evidence on the susceptibility of DC to Fas-induced cell death exists. It has been reported that immature monocyte-derived DC are sensitive [20] as well as resistant [48] to Fas ligation. On the other hand, mature DC after treatment with TNF-α or CD40L have been generally considered to be resistant to apoptosis [6, 8]. The culture conditions, mainly the presence of bovine serum, might explain some of the conflicting findings. Willems et al. reported that DC do not undergo apoptosis upon CD95 ligation unless sensitized with cyclohexamide. Other studies suggest that DC or monocytes are not at all susceptible to Fas-induced cell death [29, 31, 34]. However, the majority of experiments have been performed on DC cultured in the presence of serum. In our study, for the future clinical application of DC, we deliberately employed serum-free conditions and found that even though DC expressed similar levels of Fas, immature DC were more susceptible to Fas-induced apoptosis.

To further investigate the underlying mechanism, we examined the expression of several proteins from the Bcl-2 family and FLIP in the DC preparations. Our results demonstrated that while Bcl-2 was moderately upregulated, a more pronounced upregulation of Bcl-X_L was observed during maturation of DC. Meanwhile, Bax and FLIP expression remained unchanged. Conflicting observations exist in the literature on the latter point. It has been reported that CD40L and TRANCE upregulate Bcl-X_L but not Bcl-2 [46, 50], and that FLIP is responsible for the increase in DC survival [23, 29, 34, 48]. Leverkus et al. reported that immature DC harvested on days 4 and 7 exhibited similar levels of FLIP, while DC harvested on day 7 were less susceptible to Fas-induced apoptosis [23]. They furthermore suggested that in addition to FLIP, other regulatory mechanisms might also account for the resistance of DC to death ligands [23]. Most studies have suggested that Bcl-2 is a poor inhibitor of CD95-mediated apoptosis, whereas Bcl-X_L has been shown to rescue B cells from anti-Ig-induced apoptosis [47]. We have shown that FLIP is strongly upregulated in DC in the presence of serum. Yet, when DC are cultured in the absence of serum, Bcl-X_L rather than FLIP seems to be the major regulatory protein of DC apoptosis. It may be speculated that due to its localization, Bcl-X_L could play a more significant role than Bcl-2 in protection from apoptosis. Bcl-2 is predominantly membrane-associated [16], whereas Bax is mostly located in the cytosol [17] and Bcl-X_L is present in both of these locations [13, 17]. Both Bcl-2 and Bcl-X_L form heterodimers with Bax, which renders Bax inactive [38, 52]. Therefore Bcl-X_L seems more flexible and capable of binding Bax more easily than Bcl-2, and thus blocks apoptosis more efficiently.

Taken together, our results demonstrate that under serum-free conditions the maturation of monocyte-derived DC protects them from CD95-mediated apoptosis. This effect is mediated not only via Bcl-2 but especially by the upregulation of Bcl-X_L.

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