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Differential susceptibility to CD95 (Apo-1/Fas) and MHC class II-induced apoptosis during murine dendritic cell development

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Abstract

Disappearance of antigen presenting cells (APC) from the lymph node occurs following antigen specific interactions with T cells. We have investigated the regulation of CD95 (Apo-1/Fas) induced apoptosis during murine dendritic cell (DC) development. Consistent with the moderate levels of CD95 surface expression and low, or absent, MHC class II expression, immature DC in bone marrow cultures were highly sensitive to CD95 induced apoptosis, but insensitive to class II mediated apoptosis. In contrast, mature splenic, epidermal and bone marrow derived DC were fully resistant to CD95 induced cell death, but sensitive to class II induced apoptosis. Although caspase 3 and 8 activation was detected in immature DC undergoing CD95L-induced apoptosis, the pan-caspase inhibitor zVAD-fmk did not inhibit the early events of CD95-induced mitochondrial depolarisation or phosphatidyl serine exposure and only partially inhibited the killing of immature DC. In contrast, zVAD-fmk was completely effective in preventing CD95L mediated death of murine thymocytes. Collectively, these data do not support a major role of CD95: CD95L ligation in apoptosis of mature DC, but rather emphasise the existence of distinct pathways for the elimination of DC at different stages of maturation. *Cell Death and Differentiation* (2000) **7,** 933–938.

Keywords: dendritic cell; apoptosis; CD95; MHC class II; caspases

Abbreviations: AMC, amino-methylcoumarin; BMDC, bone marrow derived dendritic cells; CCCP, carbamoyl cyanide m-chlorophenylhydrazone; CSN, culture supernatant; DC, dendritic cells; $DiOC_6$, 3,3′-dihexyloxacarbocyanide iodide; LC, Langerhans cell; $\Delta\Psi m$, mitochondrial membrane potential; PI, propidium iodide; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-(*O*-methyl)-fluoromethyl ketone

Introduction

Dendritic cells (DC) are potent activators of primary immune responses and are present as sentinel cells in most peripheral tissues.[1–3] In the context of inflammatory signals, DC migrate from sites of antigen exposure and efficiently process and present antigen to antigen-specific T cells encountered in regional lymph nodes. Mature DC do not appear to undergo cell division, have a limited lifespan *in vitro* and disappear from the lymph nodes soon after the completion of antigen presentation.[4] Since mature, interdigitating lymph node DC do not appear to re-enter the circulation of normal animals,[5–7] DC may undergo apoptotic cell death in the lymph node. As well as a mechanism for the removal of unwanted tissue, the deletion of DC from the lymph node may be important in the downregulation of immune responses following the successful priming of antigen-specific T cells. Recent evidence suggests that this process might occur following antigen-specific interactions with T cells,[4,8] therefore, molecules expressed by activated T cells are potential death inducing ligands for DC.

The CD95 molecule (Apo-1/Fas) is a member of the TNFR family expressed by many haematopoietic and stromal cells and is able to induce apoptosis upon crosslinking with its ligand (CD95L). Mice deficient in CD95 (*lpr*) or the CD95L (*gld*) show profound lymphoproliferative disorders, that appear to relate to inefficient removal of autoreactive lymphocytes (reviewed in[9]). The CD95 ligand is expressed on plasma cells[10] and on T cells activated via the TCR and has been demonstrated to play an important role in T-cell activation-induced cell death.[11–13] Upon trimerisation of surface expressed CD95 by its ligand, several intracellular signalling proteins are recruited to the death inducing signalling complex (DISC), including the adaptor protein FADD and caspase 8.[14] Caspase 8 is activated at the DISC, followed by processing of downstream caspases including caspase 3 and proteins, such as Bid, that activate the mitochondrial apoptotic signalling pathway.[15] These well characterised early events following CD95 receptor activation lead to the downstream apoptotic events of phosphatidylserine exposure, mitochondrial membrane potential dissipation, nuclear changes and ultimate cell death.

We have investigated the role of CD95 ligation for DC apoptosis. We show that immature DC are highly susceptible to CD95 induced cell death. In contrast, mature DC are insensitive to CD95 crosslinking, but susceptible to MHC class II induced apoptosis. The dependence of the susceptibility to death by CD95 ligation on the maturation state of DC might explain why only a sub-population of human DC have been previously reported as susceptible to CD95 ligation.[16–18] Together with our

other recent human data,[19] these results show that the state of DC differentiation can be an important predictor of DC sensitivity to various apoptotic signals.

Results and Discussion

Expression of CD95 during murine bone marrow derived DC development

The expression of CD95 antigen during development of DC from bone marrow precursors was determined by labelling GM-CSF stimulated bone marrow cell cultures after 5, 8 and 13 days following culture initiation. As shown in Figure 1a, bone marrow cultures are heterogenous and contain both immature DC negative for surface expression of class II and class II$^+$ mature BMDC. However only immature DC expressed moderate levels of CD95 antigen, which was present on immature DC up until day 8. In this culture system, all immature BMDC had differentiated into class II$^+$/CD95$^-$ mature BMDC by day 13. We were unable to detect significant levels of CD95 on class II$^+$ fresh or cultured epidermal Langerhans cells (LC; Figure 1b). However, weak reactivity of CD95 mAbs was noted on LC with the RMF2 (Figure 2b) and Jo-2 (not shown) anti-CD95 mAb, but only after a prolonged culture period of 4 days. The CD95 epitopes did not appear to be lost during the obligate trypsin treatment for the isolation of fresh LC, since similar handling and trypsin treatment of 5-day BMDC did not result in detectable loss of CD95 mAb labelling (n=2; data not shown).

The identity of the large class II$^{-/+}$ cells present in the BMDC cultures[20] was further investigated by immunodepletion of mature CD86$^+$ DC from BM cultures and replating of the class II$^-$ population in fresh culture dishes. Differentiation of GM-CSF stimulated cells into a population of 10–20% class II^{++} mature DC occurred following only one overnight-culture step, confirming that DC precursors were contained within this class II$^{-/+}$ population (data not shown).

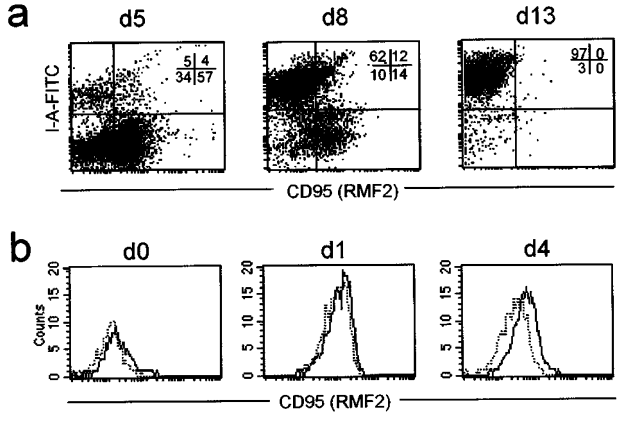

Figure 1 Expression of CD95 on (**a**) BMDC at days 5, 8 or 13 or (**b**) epidermal LC obtained immediately after isolation (day 0) or following 1 or 4 day culture. Cells were labelled as indicated with rat IgG control, anti-CD95 mAb RMF2 (revealed with PE goat anti-rat) *versus* FITC anti-I-Ad (clone AMS32.1). For (**a**) the quadrants were set to include 98% of isotype mAb stained cells and the per cent of positive stained cells in each quadrant is indicated. For the histograms in (**b**), LC were selectively gated as viable, class II$^+$ events. For (**b**) the isotype control staining is shown as a dotted line and CD95 staining as a solid line. Representative of at least two independent experiments for each cell type

Sensitivity of DC to CD95 and class II crosslinking

Different murine DC populations were next tested for their sensitivity to CD95 mediated apoptosis. Addition of anti-murine CD95 mAb or soluble huCD95L-CD8 (not shown) led to the induction of apoptosis in immature, but not mature, bone marrow derived DC (Figure 2a). In CD95-mAb treated BM cultures, a depletion of immature DC with concomitant upregulation of annexin on the remaining immature DC was noted. CD95L induced morphological changes in BMDC such as membrane blebbing and cytoplasmic disorganisation (Figure 3b). No differences in the morphology of apoptotic BMDC were noted using short (6 h) or longer incubations (16 h) with CD95L, possibly because late-stage apoptotic, or necrotic BMDC, were being removed by phagocytic cells, which were clearly visible within these preparations (Figure 3b; asterisk). This observed phagocytosis may account for the fact that we did not readily detect apoptotic DC in CD95L-treated cultures in the late apoptotic stage of nuclear condensation.

By comparison, thymocytes were found to be much more sensitive than BMDC to short term (6 h) CD95L treatment

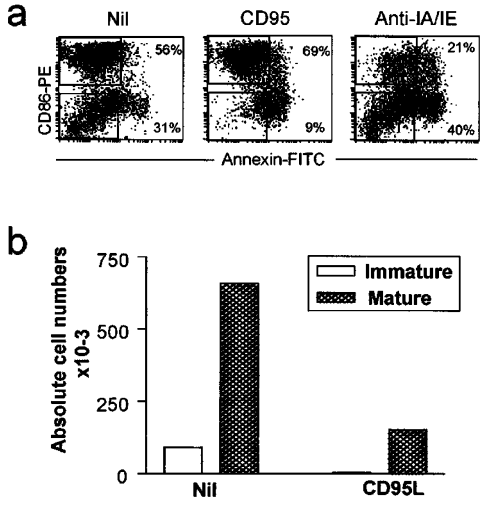

Figure 2 Differential regulation of CD95 and class II sensitivity during BMDC maturation. (**a**) BMDC at day 5 were incubated overnight in media alone (Nil), with CD95 mAb Jo-2, or with anti-class II mAb 2G9. Apoptotic cells were detected by staining with annexin-FITC and immature and mature DC distinguished by CD86-PE labelling. The per cent of cells contained within each quadrant is indicated. (**b**) Depletion of DC precursors by CD95L treatment. At day 0, 5×10^5 bone marrow cells were plated in 2 ml into each well of a 6-well plate. At day 5 the cultures were incubated with, or without, CD95L-CD8. After 3 additional days, the total number of viable cells present was determined in the haemocytometer by trypan blue exclusion and aliquots of the cultures were then stained with CD86-PE and PI and analyzed by FACS. The absolute numbers of immature or mature DC in each culture condition was estimated from the total haemocytometer counts and by the per cent immature (CD86$^-$) and mature (CD86$^+$) DC, as determined by FACS. In this experiment, CD95L treatment caused a 19 $\times$ and 4 $\times$ reduction in immature and mature DC cell numbers, respectively. One representative experiment of five is shown

(Figure 3d). Direct trypan blue counting of cell cultures used for cytospin experiments showed a 50–85% drop in absolute thymocyte numbers over the first 6 h of CD95 treatment, compared to a drop of only 0–30% in similarly treated DC cultures. Moreover, although all control thymocytes showed good viability and an even distribution of nuclear material, CD95L treatment induced severe to complete nuclear condensation (arrows) and the remnants of many necrotic thymocytes were also visible in cytospins (Figure 3d).

Through the CD95-mediated depletion of immature DC, there was apparent accumulation of DC in the mature, $CD86^+$ DC gate (Figure 2a). However, CD95L treated cultures showed decreased absolute numbers of mature DC when compared to untreated controls, showing that CD95 crosslinking had depleted the source of immature DC (see Figure 2b). Mature ($CD86^+$) BMDC were resistant to anti-CD95 treatment, but sensitive to MHC class II induced apoptosis (Figure 2a), a mechanism previously described for B cells[21] and more recently monocytes.[22] Investigation of splenic DC, or epidermal Langerhans cell (LC) as a source of tissue DC, also demonstrated that CD95 crosslinking did not trigger cell death in mature, 1–3-day cultured splenic DC or LC (data not shown), however, similar to mature BMDC, LC and splenic DC were highly sensitive to class II induced apoptosis[23]. Therefore, our data suggest that CD95-mediated apoptosis represents a mechanism mainly for the elimination of immature DC. It is possible that the downregulation of functional CD95 molecules on DC during development may be linked to a loss in clonal potential, since, in contrast to freshly isolated LC or mature bone marrow, early BMDC precursors are actively proliferating.[20,24] In agreement with the results presented here, CD95:CD95L interactions appear to play a role in the induction of apoptosis of proliferating human $CD34^+$ DC precursors.[18,25] Similarly, only activated T cells in the S-growth phase were reported as susceptible to CD95 crosslinking.[26,27]

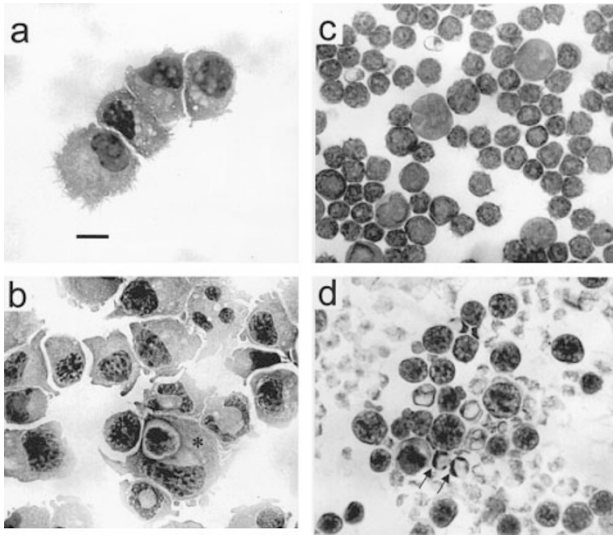

Figure 3 Morphology of CD95 induced apoptosis in BMDC. Cytospins were prepared from BMDC (**a**,**b**) or fresh thymocyte suspensions (**c**,**d**) after 6 h culture alone (**a**,**c**) or after 6 h treatment with 0.5 μg/ml CD95L (**b**,**d**). Cells were spun onto glass slides and photographed under oil at 100$\times$ power and the scale bar in (**a**) represents 10 μm. DC uptake by a phagocyte in CD95L treated suspensions is labelled in (**b**; asterisk). Ultracondensed nuclei in CD95L treated thymocytes are arrowed in (**d**)

Mitochondrial involvement of CD95-mediated apoptosis in immature BMDC

In order to elucidate the role of mitochondria in CD95-mediated apoptosis of immature BMDC apoptosis and to further check the resistance of mature DC to CD95 crosslinking, we examined dissipation of mitochondrial membrane potential ($\Delta\Psi$m) in immature and mature DC populations upon CD95 crosslinking. As shown in Figure 4, although both immature and mature DC populations showed reduced $\Delta\Psi$m upon treatment with the mitochondrial electron transport chain uncoupler CCCP, only immature DC suffered a loss in $\Delta\Psi$m following CD95 crosslinking. These results show that mitochondrial signals are also involved in the CD95-mediated signal transduction pathways in immature BMDC, as previously shown for various transformed cell lines by Scaffidi *et al*.[28]

Caspase requirement for CD95-mediated apoptosis in immature DC

The critical involvement of caspases in death receptor-mediated apoptosis has been demonstrated.[9] Having established that a population of immature DC was susceptible to CD95-induced apoptosis, we next determined whether caspase activation was a requirement for CD95-mediated death in DC. Using fluorometric determination of caspase activity in cell lysates, caspase 3 and 8 were found to be activated in both DC and thymocytes treated with CD95L (Figure 5). However, even at high concentrations (250 μM), the pan-caspase inhibitor zVAD-fmk only partially ($\leqslant$50%) inhibited CD95-mediated death in immature DC, while in contrast, 25 μM of zVAD-fmk was sufficient to fully inhibit CD95 killing of murine thymocytes (Figure 6). Interestingly, our timecourse analyses showed that although partially effective in inhibiting CD95-mediated DC death (as deter-

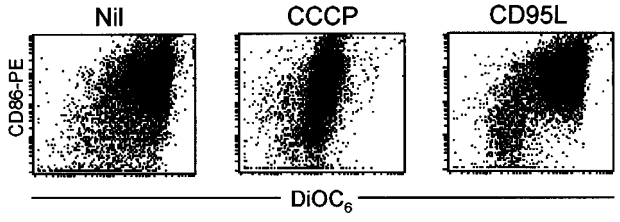

Figure 4 CD95L treatment induces loss in mitochondrial membrane potential ($\Delta\Psi$m) only in immature DC. Day 8 BMDC were incubated overnight in the absence or presence of CD95L-CD8 and $\Delta\Psi$m was determined by $DiOC_6$ staining. Following $DiOC_6$ staining, cells were briefly counter stained with CD86-PE (5 min; RT) to reveal immature ($CD86^-$) and mature DC ($CD86^+$) and the cells analyzed by FACS. Histograms show levels of $DiOC_6$ staining on control untreated DC (Nil), CD95L-CD8 treated (CD95L) or control DC pre-treated with 87 μM CCCP (CCCP) to artificially abolish $\Delta\Psi$m. One of three experiments

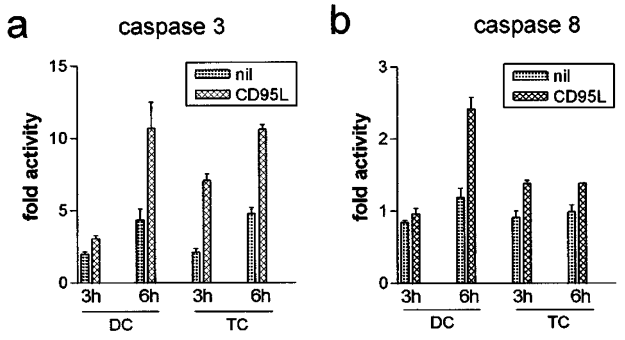

Figure 5 Caspase activation in immature DC and thymocytes following CD95 crosslinking. Caspase 3 (**a**) and caspase 8 (**b**) activation was determined in triplicate dendritic cell (DC) or thymocyte (TC) lysates either untreated (stippled) or treated with CD95L (hatches) for the indicated times. The means (±S.D.) of plotted fold activity are the ratios of cell lysate caspase activities divided by that for substrate and buffer alone. Representative of 2–4 experiments

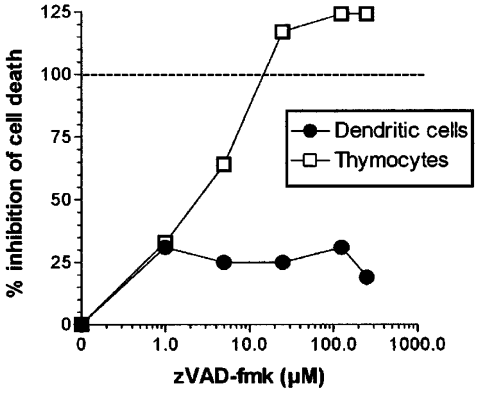

Figure 6 Inhibition of CD95L killing of DC by pan-caspase inhibitor zVAD-fmk. BMDC (circles) or thymocytes (squares) were treated overnight with CD95L-CD8 in the presence of varying concentrations of zVAD-fmk. DC were counterstained to identify $CD86^-$ immature DC and the per cent of live cells determined by PI exclusion and FACS analysis. The per cent inhibition of cell death for gated, immature DC and total thymocytes was plotted as: 100 × (inhibitor and CD95L-treated − CD95L-treated)/(control − CD95L-treated). One of three similar titration experiments

mined by PI uptake), zVAD-fmk failed to inhibit the early events of decreased $\Delta\Psi$m and phosphatidyl serine exposure in PI^- DC (Figure 7, *n*=4).

Recently, Scaffidi *et al*,[28,29] have characterised cell types on the basis of their response to CD95 crosslinking. Type I cells form a death inducing signalling complex (DISC) that generates a large amount of active caspase 8 at the DISC level – sufficient to initiate caspase 3 cleavage and further downstream events leading to cell death. In type II cells, caspase 8 activation at the DISC level is delayed and full activation of both caspase-8 and caspase-3 occurs mainly after the loss of mitochondrial membrane potential. Only in type II cells are inhibitors of mitochondrial depolarisation (Bcl-2 or Bcl-x_L) effective in inhibiting apoptosis.[28] However, in both cell types, caspase inhibitors prevent cell death. Interestingly, in BMDC, neither the CD95-mediated mitochondrial depolarisation, nor the cell death was totally blocked by caspase inhibition (Figures 6 and 7). Together, these results emphasise the existence of distinct signalling pathways for CD95-mediated apoptosis in different cell types.

Species, as well as maturation and cell type differences, may also alter the nature of CD95-mediated killing: In contrast to the data obtained here on immature murine BMDC, treatment of human monocyte-derived DC with TNF-related apoptosis inducing ligand (TRAIL) or CD95L leads to induction of apoptosis of a subpopulation of immature, but not mature DC, which is fully inhibited by 40 μM zVAD-fmk (our unpublished data). Similarly, a genetic caspase 10 deficiency in humans has been reported to limit the removal of DC from the lymph node,[30] suggesting that at certain developmental stages, human DC critically require caspase function for the induction of death receptor-mediated apoptosis.

Conclusion

Our findings demonstrate that both the expression of CD95 antigen and sensitivity to CD95-induced apoptosis are downregulated during DC maturation. This raises the possibility that *in vivo*, only DC precursors are susceptible to CD95-mediated apoptosis. The significance for such a death

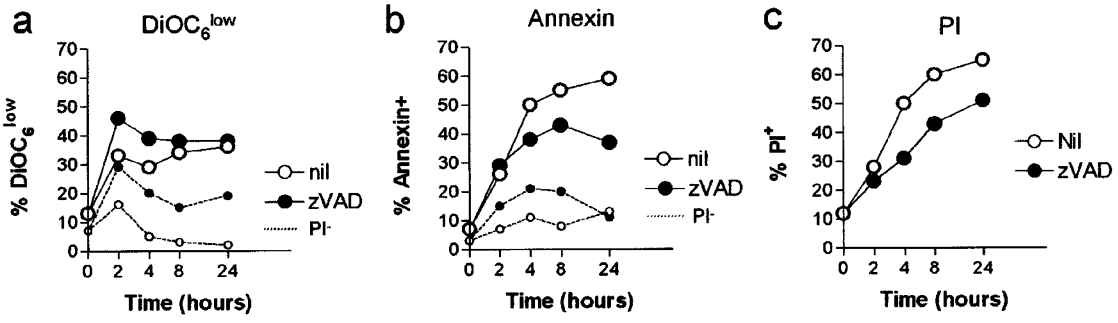

Figure 7 Time course analysis of the effect of zVAD-fmk on CD95-mediated loss in (**a**) mitochondrial membrane potential ($\Delta\Psi$m),(**b**) phosphatidyl serine exposure and (**c**) cell death. Day 5 BMDC were incubated for the indicated times with 0.5 μg/ml of CD95L-CD8, without (nil; open circles) or with 40 μM zVAD-fmk (zVAD; filled circles). $\Delta\Psi$m, annexin staining and PI uptake was measured for immature DC as described in Materials and Methods. Solid lines represent electronic gating of total, immature DC and the dotted lines show gating for PI^-, immature DC. One of four representative experiments is shown

pathway can only be speculated on at this stage, but may reflect the importance of deletion mechanisms for rapidly dividing cells, as a protection against malignant transformation. Together with other recent work from our group which shows that DC (but not CD40-ligated DC) are highly sensitive to a caspase-independent apoptosis via class II cross-linking[23], we conclude that *in vivo*, class II ligation might represent a more important pathway of mature DC death than CD95 ligation. We are currently investigating the nature of the CD95L and class II-induced pathways of DC apoptosis that are maintained in the presence of the pan-caspase inhibitor zVAD-fmk.

Materials and Methods

Media and monoclonal antibodies

Media used throughout the study was RPMI-1640 (R10) for spleen and bone marrow DC or IMDM (I10; for Langerhans cells supplemented with 10% foetal calf serum (Pan), 50 μM β-mercaptoethanol, 200 μM glutamine, 100 μg/ml penicillin, 50 μg/ml streptomycin and 200 U/ml of GM-CSF (Strathmann Biotech, Hannover, Germany), or 5% of culture supernatant from murine GM-CSF secreting Ag8653 myeloma line.[31] FITC-AMS32.1 (mouse anti-I-Ad) and Jo-2 (hamster anti-CD95) were both from Pharmingen (Hamburg, Germany) and RMF2 (rat anti-CD95) from Coulter Immunotech (Marseille, France). Fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated goat anti-mouse, rat and hamster Ig were all from Pharmingen. The 2G9 mAb[32] (rat IgG2a anti-I-A/I-E) was a kind gift from Professor Knop, (Department of Dermatology, University of Mainz, Germany).

Mice and DC isolation

BALB/c or C57BL/6 mice used throughout this study were bred in our own animal facilities at the University of Würzburg and normally used between the ages of 3 and 8 weeks. Bone marrow derived DC were generated exactly as previously described.[20] Briefly, $2-4\times10^6$ bone marrow cells (without red cell lysis or removal of contaminating mature cell lineages by mAb and complement lysis) were plated in 10 ml of R10 plus GM-CSF, with addition of 10 ml fresh media at day 3 and exchange of 10 ml media at day 6 and day 8. Langerhans cells (LC) were isolated from the mice ears exactly as described.[33] Briefly, split ears were trypsinised and epidermal cells (EC) released from the epidermal sheets by gentle knocking on a metal sieve. EC were then cultured at 2×10^7 per Falcon 3003 dishes in 10 ml of I10 plus GM-CSF for 2–3 days prior to enrichment of LC by 13% Nycodenz gradient (ρ=1.068). For analysis of cultured induced changes in CD95 expression, 3×10^6 fresh EC were cultured in 3 ml of I10 plus GM-CSF per well in 6-well plates. At various times, non-adherent cells were harvested (without gradient enrichment) and LC identified in these suspensions by FACS analysis of class II or CD86 expression.

Induction and inhibition of apoptosis in DC

BMDC cultures were harvested at day 5–8 and replated at 5×10^5 cells/ml with R10 plus GM-CSF into 24- or 96-flat-well plates (Falcon). The hamster anti-mouse CD95 Jo-2 mAb was included in cultures at 1–2 μg/ml. Human CD95L-CD8[34] also binds murine CD95 and was used at 0.5–10 μg/ml. Class II mediated apoptosis of mature DC[23] was induced by incubating DC cultures overnight with 10% 2G9 mAb culture supernatant. The pan-caspase inhibitor zVAD-fmk (Bachem, Heidelberg, Germany) was added to DC cultures at the indicated concentrations.

Cytocentrifuge cell preparations

Care was taken to preserve apoptotic morphology throughout cytospin preparation by gentle handling on ice and using a low speed for the cytocentrifuge step. Control or CD95L treated cells were washed once in cold PBS and 100 μl of cells at $5-7\times10^5$/ml in PBS were spun for 6 min at 250 r.p.m. onto glass slides using a Shandon cytocentrifuge. Cell spots were air dried, stained within 48 h using the Papanicolaou stain, mounted with Permount and photographed under oil at 100$\times$ magnification using a Zeiss Axiophot microscope camera system.

Determination of apoptosis in DC

Externalisation of phosphatidylserine on the DC membrane was detected by FITC-annexin staining (30 min, 4°C; Pharmingen). In order to identify LC in epidermal suspensions or mature DC in bone marrow cultures, DC were labelled with 5 μg/ml of CD86-phycoerythrin (CD86-PE; Pharmingen). Propidium iodide (PI; 5 μg/ml) was added immediately prior to analysis by FACS. Staining of DC by FITC-annexin, CD86-PE and PI was detected in the FL-1, FL-2 and FL-3 channels, respectively, using a FACS-SCAN (Becton Dickinson) equipped with a single argon laser emitting at 488 nm and calibrated and compensated by eye. Data was analyzed using Cell Quest^{TM} software (Becton Dickinson). Since many necrotic DC (PI^+) were lost (possibly by phagocytosis in the bone marrow cultures; see Figure 3, or by lysis during cell washing steps), analysis of cell death for some experiments was calculated from the absolute numbers (Figure 2b), or per cent (Figure 6) of remaining live DC (PI or trypan blue excluding cells) following CD95L or CD95 mAb treatment. Mitochondrial membrane potential was determined as previously described.[35] Briefly, DC were cultured with or without CD95 mAb or CD95L, washed and then incubated at 37°C with 3,3′-dihexyloxacarbocyanide iodide (87 nM; $DiOC_6$) in media for 15 min. Following $DiOC_6$ labelling, BMDC were counterstained (5 min; RT) with 5 μg/ml CD86-PE to distinguish immature ($CD86^-$) from mature ($CD86^+$) BMDC. Stained cells were then washed in cold PBS and resuspended in 200 μl PBS with 5 μg/ml PI. $DiOC_6$ labelling was detected in FL-1. In some experiments, the mitochondrial membrane potential of DC was artificially reduced by incubating BMDC in carbamoyl cyanide m-chlorophenylhydrazone (50 μM; CCCP; 10 min; 37°C) prior to $DiOC_6$ labelling.

Detection of caspase 3 and 8 activation

Caspase 3 and 8 activity was determined by a fluorometric substrate cleavage method.[36] BMDC or thymocytes were treated for 3 h or 6 h with 0.5 μg/ml of CD95L-CD8 construct, harvested and washed once in PBS. Cell pellets were lysed in 20 mM TRIS, 137 mM NaCl, 1% NP-40 detergent, 10% glycerol, 1 mM PMSF, 0.15 U aproptinin at pH 8.0. Protein content was determined by Bradford assay. Fluorometric substrate (25 μg/ml; DEVD or IETD-amino-methylcoumarin; AMC) was added to each well containing 200 μg of protein in a total volume of 200 μl and incubated at 37°C for 2 h. Release of free AMC was monitored fluorometrically at an emission wavelength of 460 nm.

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