

Cognate MHC–TCR interaction leads to apoptosis of antigen-presenting cells

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Abstract: Antigen presentation to T lymphocytes has been characterized extensively in terms of T lymphocyte activation and eventual cell death. In contrast, little is known about the consequences of antigen presentation for the antigen-presenting cell (APC). We have determined the outcome of major histocompatibility complex class II-restricted peptide presentation to a specific T cell. We demonstrate that specific T lymphocyte interaction with peptide-presenting APCs led to apoptosis in the APC population. In contrast, T lymphocyte interaction with nonpeptide-loaded APCs or APCs loaded with monosubstituted peptide failed to induce T lymphocyte secretion of interleukin-2 and APC apoptosis. Phosphatidylserine externalization and mitochondrial depolarization were used to evaluate APC apoptosis. Fas/Fas ligand interactions were not required, but cytoskeletal integrity and caspase activation were essential for APC apoptosis. Antigen presentation leading to T lymphocyte activation is therefore coordinated with apoptosis in the APC population and could provide a mechanism of immune response regulation by eliminating APCs, which have fulfilled their role as specific ligands for T lymphocyte activation. This pathway may have particular importance for APCs, which are not sensitive to death receptor-induced apoptosis. *J. Leukoc. Biol.* 75: 1036–1044; 2004.

Key Words: MHC class II · T–B interaction · programmed cell death

INTRODUCTION

Major histocompatibility complex (MHC) class II-mediated presentation of peptide to CD4⁺ T lymphocytes leads to helper T lymphocyte activation and proliferation in response to recognition of a specific peptide. The MHC class II antigens are $\alpha\beta$ heterodimers, belonging to the immunoglobulin superfamily and constitutively expressed at the surface of professional antigen-presenting cells [APCs; including dendritic cells (DCs) and B lymphocytes]. MHC class II expression can be induced

by cytokines on a wide variety of nonprofessional APCs including epithelial cells and tumor cells [1–3]. Specific peptide presentation by MHC class II antigens to CD4-positive T lymphocytes plays a critical role in the generation of the cognate immune response. Much attention has been devoted to characterizing the events leading to T lymphocyte activation, which is typically followed by activation-induced cell death (AICD), as well as determining the role of molecules participating in this activation and death. AICD has a key role in the regulation of peripheral T lymphocyte homeostasis [4]. In contrast, little attention has been given to the consequences of antigen presentation for the survival of the APC.

Apoptosis of DCs has been reported as a consequence of cognate interaction with peptide-specific T lymphocytes, and such apoptosis was partially inhibited in the presence of a human leucocyte antigen (HLA)-DR monoclonal antibody (mAb) or a CD95/Fas mAb [5]. In vivo DC apoptosis was detected within the T cell-rich areas of lymph nodes [6]. Signal-transduction studies in APCs have used MHC class II mAb to mimic the peptide-specific T lymphocyte interaction, and diverse consequences have been reported in the APC, including proliferation [7], synthesis of costimulatory molecules [8], or cytokine release [9], depending on the origin of the APC and its state of activation and differentiation. Truncation of MHC class II molecules abrogates signal transduction and impairs antigen presentation to T cell clones [10, 11]. Programmed cell death of B lymphocytes can also be mediated by engagement of MHC class II molecules with specific mAb [12, 13]. Mature DCs are markedly more susceptible to MHC class II-mediated apoptosis than immature DCs [14, 15]. The impact of the maturity or the activation state of the APC has also been observed in B cells, as activated human B lymphocytes are more sensitive to MHC class II-mediated apoptosis than resting cells, and B lymphocytes isolated from fetal umbilical cord blood are resistant [16]. A recent study in a primate model examined HLA-DR-mediated apoptosis in vivo and confirmed that although HLA-DR molecules induce apoptosis of diverse B lymphoproliferations, no durable impact on haematopoiesis

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was observed [17]. These data support the notion of a selective sensitivity of mature/activated APCs to MHC class II-mediated apoptosis.

Although these data underline the therapeutic potential of MHC class II ligands in lymphoproliferative diseases, the physiological consequences for the APC of MHC class II molecule interaction with its principal physiological ligand, namely the peptide-specific T cell receptor (TCR), have not yet been clearly established. We therefore determined the impact of specific antigen presentation on the survival of the APC and characterized the mechanisms leading to cell death in the peptide-presenting APC population.

MATERIALS AND METHODS

Cells

T8.1 is a murine T cell hybridoma expressing human CD4 and a chimeric human–mouse TCR specific for a tetanus toxin peptide (tt830–843) restricted by HLA-DRB1*1102 or HLA-DRB1*1301, which has been characterized extensively [18–20]. T8.1 was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics, supplemented with 400 nM methotrexate, 1 mg/ml G418, and 50 μ M 2-mercaptoethanol. T8.1 recognition of a specific peptide has been widely used as a model to study biochemical events in the T lymphocyte triggered by antigen recognition [18, 20, 21].

The homozygous Epstein-Barr virus (EBV)-transformed B cell lines JVM and BM15, expressing HLA-DR11 (both are HLA-DRB1*1102), were maintained in RPMI with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics. The HLA-DR11-expressing murine L cells (L625.7) express endogenous B7-1 and intercellular adhesion molecule-2, were cultured in complete DMEM with 250 μ g/ml G418 [22], and have been shown to be efficient APCs of tt830–843 to T8.1 [21]. Raji is a MHC class II-positive (HLA-DR3/DR10) B-EBV cell line. Murine L1210 leukemia cells and murine Fas-expressing L1210 (kindly provided by Pierre Golstein, Centre d'Immunologie de Marseille Luminy, France) were cultured in RPMI with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics. All experiments were performed at least three times.

Antibodies, peptides, and reagents

The tt830–843 peptide (QYIKANSKFIGITE) and the alanine monosubstituted analog peptide (QYIKANSKAIGITE) were purchased from Neosystems (Strasbourg, France) and were more than 90% pure. Presentation of the specific peptide tt830–843 to the T lymphocyte T8.1 leads to interleukin-2 (IL-2) secretion and has been characterized extensively in previous studies [18, 19]. The caspase inhibitors Ile-Glu-Thr-Asp-fluoromethylketone (IETD-fmk) and Z-Val-Ala-Asp-fmk (zVAD-fmk) were purchased from Calbiochem (San Diego, CA). Disruption of the integrity of the actin cytoskeleton was performed in the presence of latrunculin, which inhibits actin polymerization, or in the presence of cytochalasin D, which disrupts actin polymers; both were obtained from Calbiochem. The agonist anti-human Fas antibody 7C11 (Immunotech, Marseille, France) was used to activate Fas/CD95-mediated cell death. The CellTracker orange 5-(and-6)-[[(4-chloromethyl) benzoyl] amino] tetramethylrhodamine (CMTMR) probe, the CellTracker green 5-chloromethylfluorescein diacetate (CMFDA) probe, and the marker of mitochondrial transmembrane potential ($\Delta\Psi_m$) 3,3-dihexyloxycarbocyanine iodide [DiOC₆(3)] were purchased from Molecular Probes (Eugene, OR). An annexin V–fluorescein isothiocyanate (FITC) apoptosis kit was used to detect apoptosis (Boehringer Mannheim, Meylan, France).

Characterization of conjugates

T8.1 cells were incubated with the CellTracker orange CMTMR probe at a final concentration of 5 μ M for 30 min at 37°C in serum-free medium, washed once, thereafter incubated for another 30 min in fresh medium to allow excess probe to diffuse, and finally washed twice in serum-free medium. The APCs (JVM,

BM15, Raji), preloaded or not for 2 h with the tt830–843 peptide (1 μ g/ml), were labeled following the same procedure with the CellTracker green CMFDA. APCs and T8.1 cells were then mixed at a 1:4 ratio in serum-free medium and allowed to form conjugates for 1 h. Conjugate formation was measured on a FACScan cytometer (Becton Dickinson, San Jose, CA) using the FL-1 gate for the CMFDA probe and the FL-2 gate for the CMTMR probe and was characterized by a doubly stained population.

Apoptosis assays

Annexin-V binding

APCs were loaded for 2 h at 37°C with 1 μ g/ml (or as indicated) of the specific tt830–843 peptide or with the monosubstituted peptide or were left untreated in complete medium before labeling with 5 μ M CMTMR for 30 min and washing three times. Labeling with CMTMR did not alter cell viability. T cells were mixed with APC at a 1:1 ratio in 200 μ l complete medium in flat-bottom microplates. After 24 h (or as indicated), cells were harvested and pelleted. Supernatants were collected and frozen at –80°C for measurement of IL-2 production. Cells were resuspended in annexin-V–FITC staining mix (following the manufacturer's instructions), incubated for 15 min, and then analyzed by flow cytometry. Annexin-V staining of APCs was assessed by gating on CMTMR-positive events.

Determination of $\Delta\Psi_m$

$\Delta\Psi_m$ was determined after overnight incubation of peptide-loaded, CMTMR-labeled APCs with T lymphocytes. Cells were labeled with DiOC₆(3) (40 nM) for 15 min at 37°C. The cells were washed and resuspended, and DiOC₆(3) fluorescence was analyzed by gating on the CMTMR-positive cells corresponding to the APCs.

IL-2 secretion assay

The IL-2 secretion assay was performed as described previously [18]. T8.1 lymphocytes (2×10^4) were incubated in duplicate wells with an equal number of peptide-loaded APCs in flat-bottom 96-well plates at 37°C. After 24 h, 50 μ l supernatants were harvested and analyzed for murine IL-2 production by enzyme-linked immunosorbent assay (R&D Minneapolis, MN). Results are expressed as optical density (O.D.) units.

Detection of active Fas ligand (FasL)

A tripartite apoptosis assay of “target” cells was designed to determine whether T lymphocytes expressed biologically active CD95 ligand (CD95L; FasL) as a consequence of peptide recognition and activation. In these experiments, APCs were loaded with peptide and cocultured with T lymphocytes in the presence of third-party CMTMR-labeled target cells. The target cells tested included a positive control for CD95L activity [CD95⁺ L1210 (L1210–Fas)], a negative control for CD95L activity [CD95[–] L1210 (L1210)], and the B lymphoid APCs used in peptide-presentation assays [CD95⁺ HLA-DR11⁺ B-EBV (BM15, JVM)].

APCs were peptide-loaded and mixed with T lymphocytes as above in the presence of CMTMR-labeled target cells. Apoptosis was detected by measuring annexin-V fluorescence of CMTMR-labeled target cells as above.

Treatment with inhibitors

Peptide-loaded APCs were pretreated for 30 min with latrunculin (1 μ M) or cytochalasin D (40 μ M) to disrupt the actin cytoskeleton of the APC before culture with T lymphocytes. In experiments using the cell-permeable, irreversible inhibitors of caspase activation IETD-fmk and zVAD-fmk, inhibitors (100 μ M) were present throughout the assay. None of the inhibitors used were toxic at the selected concentrations.

RESULTS

Characterization of peptide-specific MHC class II-restricted T lymphocyte/APC conjugates

The interaction of tt830–843 peptide-loaded HLA-DR11-expressing APCs with specific T lymphocytes leads to the forma-

tion of APC/T lymphocyte conjugates. A doubly labeled cell population (indicated by a square inset in **Fig. 1**) characterizes the conjugate formation in the presence of peptide. In the absence of peptide, no conjugate formation is observed. APCs loaded with monosubstituted peptide also failed to form detectable conjugates with T lymphocytes (data not shown). Moreover, when a non-DR11 HLA class II-expressing APC is used (Raji HLA-DR3/DR10), no conjugate formation is observed.

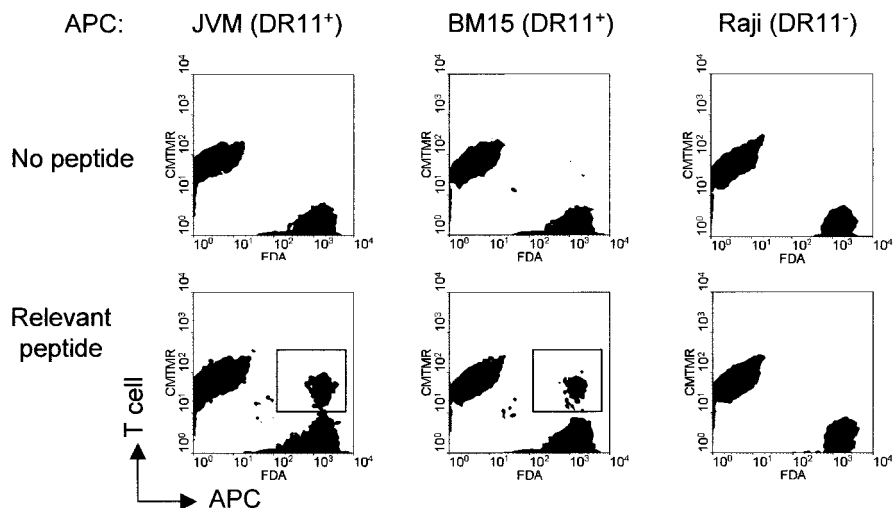
Detection of apoptosis in the APC population after peptide presentation

Efficient antigen presentation of tt830–843 peptide by HLA-DR11-expressing B lymphocyte BM15 was first confirmed by detecting IL-2 secretion by the T8.1 cells. **Figure 2A** shows that IL-2 secretion was dependent on the peptide-loading concentration and was observed at a peptide-loading concentration of 0.1 $\mu\text{g/ml}$. Whereas secretion of IL-2 was induced by the specific peptide at a concentration of 0.1 $\mu\text{g/ml}$, only a modest level of IL-2 secretion was detected in the presence of monosubstituted peptide, even at a 100-fold higher peptide concentration (10 $\mu\text{g/ml}$).

The APCs were labeled with the vital dye CMTMR after loading with specific or alanine-monosubstituted peptide and incubated with T8.1 for 24 h before apoptosis of the APCs was determined (CMTMR⁺, annexin-V⁺). **Figure 2B** demonstrates that apoptosis was detected in approximately half of the APCs that had been loaded with tt830–843 peptide (1 $\mu\text{g/ml}$), whereas APCs loaded with an equivalent concentration of alanine-monosubstituted peptide failed to undergo apoptosis. A low level of apoptosis in the presence of monosubstituted peptide was only observed when the APCs were incubated with a tenfold higher concentration peptide (10 $\mu\text{g/ml}$).

The mean level of apoptosis of APCs preloaded with 1 $\mu\text{g/ml}$ specific peptide compared with 1 $\mu\text{g/ml}$ alanine-monosubstituted peptide was determined as 51% (± 11.9) compared with 16.8% (± 4.9 ; n=5 separate experiments), demonstrating that apoptosis in the APC population is a consequence of specific peptide presentation.

Fig. 1. Detection of APC/T cell conjugates. T8.1 cells were incubated with the CellTracker orange CMTMR probe at a final concentration of 5 μM for 30 min at 37°C. The APCs (JVM, BM15, Raji), preloaded or not with the tt830–843 peptide (1 $\mu\text{g/ml}$), were labeled following the same procedure with the CellTracker green CMFDA. APCs and T8.1 cells were then mixed in serum-free medium and allowed to form conjugates for 1 h. Conjugate formation was measured on a FACScan cytometer (Becton Dickinson) using the FL-1 gate for the CMFDA probe (FDA) and the FL-2 gate for the CMTMR probe and was characterized by a doubly stained population (square inset). No APC/T conjugate formation was detected in the absence of specific peptide or when non-HLA-DR11-expressing APCs were used. One representative experiment of a series of 10 independent experiments is shown.



APC apoptosis occurs within 12 h of T–B interaction

The time course of apoptosis of peptide-loaded APCs cocultured with peptide-specific T lymphocytes was examined. Although apoptosis of the APCs (BM15) was negligible 6 h after interaction with T8.1, there was a marked increase in the proportion of annexin-V staining, reaching a maximum within 12 h (**Fig. 2C**). In contrast, apoptosis of APCs loaded with monosubstituted peptide was not increased at 12 h, and only a minor increase was detected at 24 h. In the following experiments, apoptosis in the APC population was determined after 24 h APC/T lymphocyte interaction. These data are consistent with the results of Grogg et al. [23], who examined CD4⁺ T cell-mediated killing of specific peptide-loaded APCs by a ⁵¹Cr-release assay and reported that apoptosis began after 6 h of cocubation and was maximal within 20–24 h.

Mitochondrial or plasma membrane apoptosis determination yields comparable levels of APC apoptosis

Mitochondria have been identified as playing a key role in programmed cell death, as permeabilization of the transmembrane mitochondrial membrane releases diverse proapoptotic proteins [24]. HLA-DR antibody stimulation of APCs leads to decreased $\Delta\Psi_m$ [14, 25], which can be detected readily by decreased fluorescence of the marker of transmembrane potential DiOC₆(3).

Figure 3 demonstrates that APC apoptosis was detected at the mitochondrial level in addition to the plasma membrane level. A mean level of 33.6% (± 8.9) of apoptotic APCs was detected when tt830–843 peptide-loaded APCs were cocultured overnight with specific T lymphocytes compared with 17.1% (± 2.8 ; n=4) in the absence of peptide. Moreover, comparable levels of APC apoptosis were detected whether phosphatidylserine externalization or decreased $\Delta\Psi_m$ was taken as a readout. The mean apoptosis detected by annexin V binding was 30.7% (± 5.1) for peptide-loaded APCs compared with 12.3% (± 3.6) for nonpeptide-loaded APCs in this series of experiments. The decreased mitochondrial membrane po-

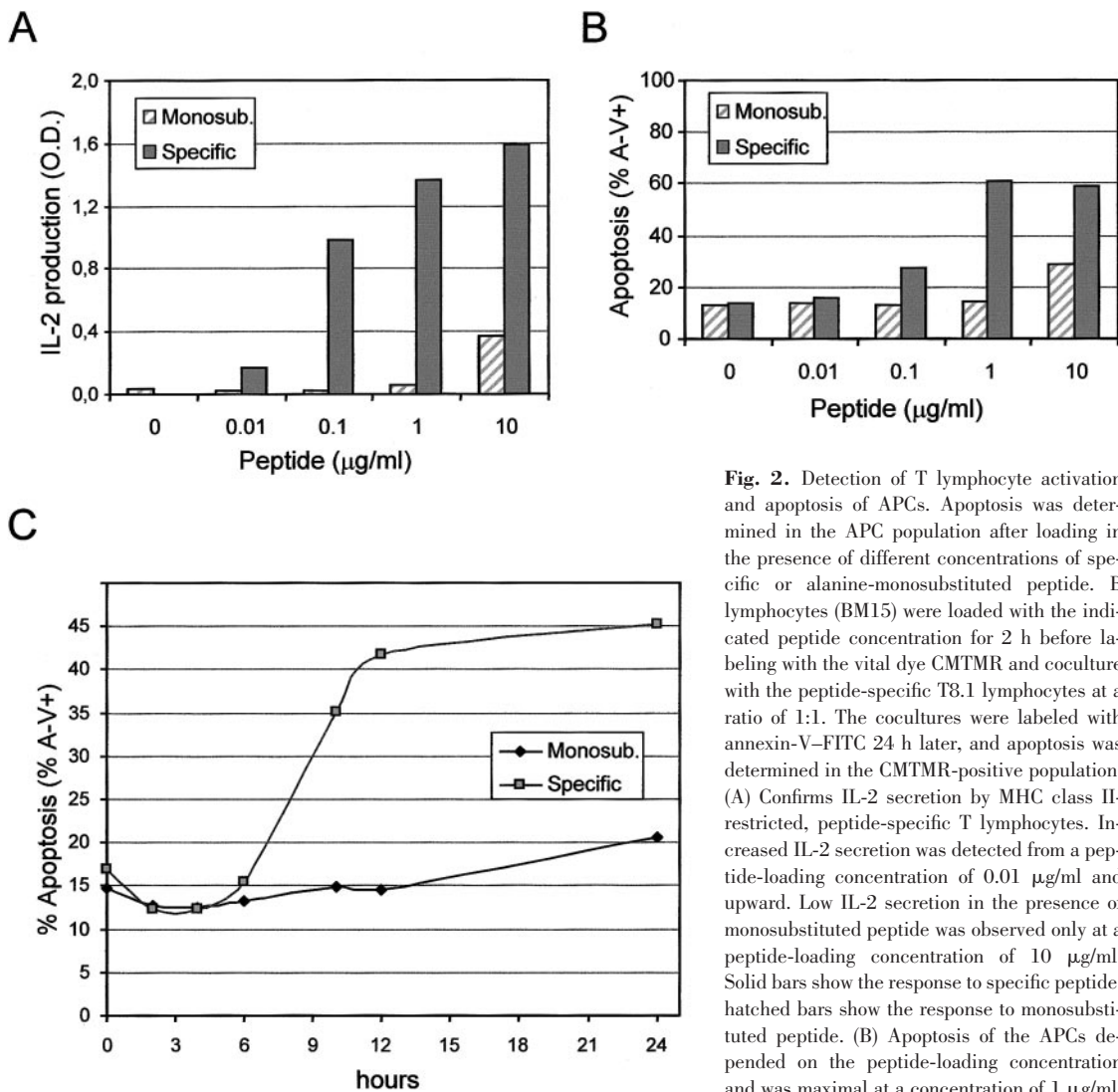


Fig. 2. Detection of T lymphocyte activation and apoptosis of APCs. Apoptosis was determined in the APC population after loading in the presence of different concentrations of specific or alanine-monosubstituted peptide. B lymphocytes (BM15) were loaded with the indicated peptide concentration for 2 h before labeling with the vital dye CMTMR and coculture with the peptide-specific T8.1 lymphocytes at a ratio of 1:1. The cocultures were labeled with annexin-V-FITC 24 h later, and apoptosis was determined in the CMTMR-positive population. (A) Confirms IL-2 secretion by MHC class II-restricted, peptide-specific T lymphocytes. Increased IL-2 secretion was detected from a peptide-loading concentration of 0.01 µg/ml and upward. Low IL-2 secretion in the presence of monosubstituted peptide was observed only at a peptide-loading concentration of 10 µg/ml. Solid bars show the response to specific peptide; hatched bars show the response to monosubstituted peptide. (B) Apoptosis of the APCs depended on the peptide-loading concentration and was maximal at a concentration of 1 µg/ml. Solid bars show the response to specific peptide; hatched bars show the response to alanine-monosubstituted peptide. These experiments are representative of five independent experiments. (C) APC apoptosis is maximal within 12 h of coculture with peptide-specific T lymphocytes. The time course of apoptosis was determined in APCs cocultured with T lymphocytes, and the percentage of apoptosis in the presence of specific peptide compared with monosubstituted peptide was determined. Increased apoptosis of APCs loaded with specific peptide was apparent from 12 h and remained stable at 24 h. (■) Apoptosis of APCs in response to specific peptide presentation; (◆) apoptosis of APCs in response to presentation of monosubstituted peptide. A-V+, Annexin-V+.

tential confirms apoptosis detected by annexin-V binding. This also demonstrates that a mitochondrial pathway to cell death is operational in APC following cognate interaction.

CD95/CD95L interactions were not required for APC apoptosis

We then examined the mechanisms mediating apoptosis in the APC population. Previous studies have revealed a partial contribution of CD95/Fas to APC apoptosis [5] and that EBV+ B lymphocytes are sensitive to CD95-mediated apoptosis [26]. As BM15 expresses CD95 and is sensitive to apoptosis induced by the CD95 mAb 7C11 (data not shown), we determined whether biologically active CD95L was expressed by T8.1 lymphocytes after recognition of specific peptide and whether APC apoptosis was mediated by CD95/CD95L interaction with CD95 expressed by the APC.

We performed tripartite antigen-presentation experiments composed of APCs (BM15) loaded with specific or monosubstituted t830-843 peptide, T8.1 T lymphocytes, and an additional target population, which was labeled with CMTMR to identify the target population and then to detect apoptosis of the target population by annexin-V binding (see Fig. 4). Positive and negative control targets for the role of CD95L-mediated apoptosis were provided by the murine leukemia L1210, stably transfected to express CD95 (L1210-Fas, positive control, or the wild-type L1210, which does not express CD95, negative control) [27]. Antigen presentation was performed as above but in the presence of the third party target population at a ratio of 1:1:1 for the peptide-loaded APCs, T8.1, and the target population (L1210, L1210-Fas, nonpeptide-loaded BM15, or nonpeptide-loaded JVM). Murine FasL fully cross-reacts with human Fas and vice versa [28].

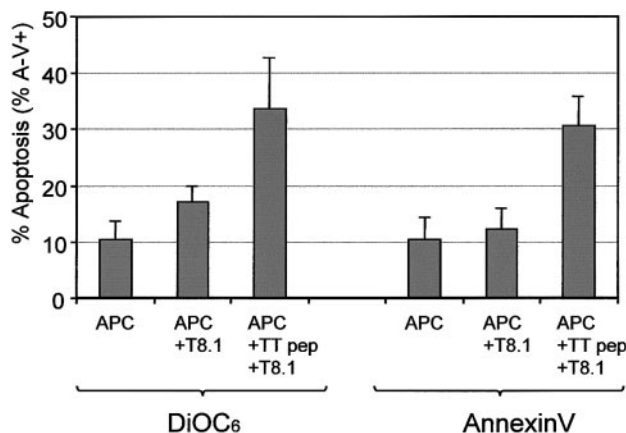


Fig. 3. APC apoptosis detection at the mitochondrial and the plasma membrane levels. The mean level of apoptosis was determined by measuring decreased DiOC₆(3) fluorescence or annexin-V-FITC fluorescence in the CMTMR-positive APC population. Specific apoptosis of peptide-loaded APCs was comparable whether mitochondrial or plasma membrane events were examined. A mean level of 33.6% of apoptotic APCs was detected with DiOC₆(3) when tt830–843 peptide-loaded APCs were cocultured overnight with specific T lymphocytes compared with 30.7% when apoptosis was detected in parallel by annexin V binding (n=4).

Presentation of specific peptide by BM15 to T8.1 led to efficient apoptosis of the L1210–Fas target cells (93.6% apoptosis; Fig. 4), whereas the L1210 target cells were not affected. When APCs were loaded with monosubstituted tt830–843 peptide, no apoptosis was induced in L1210–Fas, indicating that specific peptide recognition is required for expression of active CD95L. This result is consistent with the IL-2 production by the T8.1, confirming that T lymphocyte activation had taken place. However, when the same experiment was performed using nonpeptide-loaded HLA-DR11-expressing B lymphocytes (JVM or BM15) as nonpeptide-loaded targets, neither underwent apoptosis (Fig. 4). Thus, although the CD95L produced by the T8.1 lymphocytes upon peptide recognition is functional, apoptosis of the APC population via Fas/FasL interactions was not implicated by these experiments.

CD95-negative APCs undergo caspase-dependent apoptosis after peptide presentation

To further examine the role of CD95 in APC apoptosis, we replaced the antigen-presenting, Fas-positive B lymphocytes by the HLA-DR11⁺, Fas⁻ fibroblasts L625.7. First, we verified that L625.7 do not undergo Fas-mediated apoptosis. **Figure 5A** shows that the CD95 mAb (7C11) induced apoptosis of the L1210–Fas cells, whereas 7C11 failed to induce apoptosis in L625.7 or in CD95-negative L1210 cells.

The efficiency of peptide-loaded L625.7 in antigen presentation was confirmed by measuring IL-2 secretion by T8.1 lymphocytes (Fig. 5B and ref. [29]). Apoptosis of specific peptide-loaded L625.7 fibroblasts was determined after coculture with T8.1, and annexin-V binding was increased in 75–80% in L625.7 cells (Fig. 5C). Therefore, APCs, which do not express Fas, still undergo apoptosis as a consequence of peptide presentation.

Apoptosis of the APC is regulated by the actin cytoskeleton

Optimal peptide presentation requires the integrity of the actin cytoskeleton of the APC [30]. Studies of APC apoptosis induced by stimulation with MHC class II mAb demonstrated that disruption of the actin cytoskeleton by cytochalasin B and/or cytochalasin D pretreatment inhibited B lymphocyte and DC apoptosis [25, 31]. We therefore examined the impact of disruption of the actin cytoskeleton on apoptosis of the peptide-loaded APC by pretreating with the cell-permeable marine toxin latrunculin, which binds monomeric G actin and

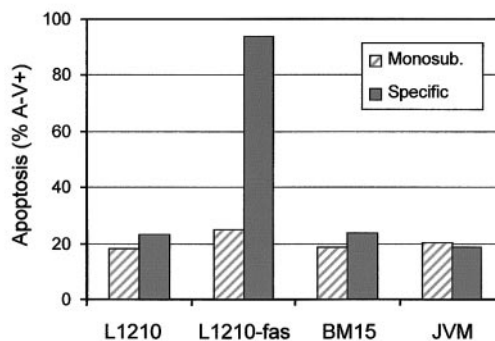
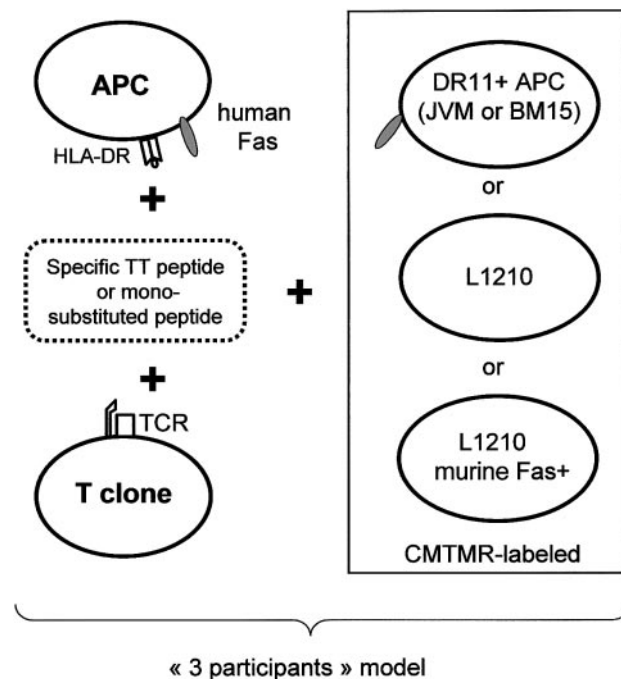


Fig. 4. APC apoptosis does not require CD95/Fas–FasL interaction. The role of biologically active CD95L was directly determined in a tripartite assay. CMTMR-labeled, nonpeptide-loaded APCs were used as targets in cocultures of peptide-loaded APCs with T lymphocytes. L1210 and L1210–Fas were used in parallel as CD95L-resistant and -sensitive targets, respectively. The L1210–Fas cell line underwent apoptosis, demonstrating production of CD95L after specific peptide presentation. Under the same conditions, the CD95-expressing, nonpeptide-loaded APCs (BM15 and JVM) did not undergo apoptosis. Solid bars show apoptosis of the target cells in the presence of APC presenting specific peptide; hatched bars show the response in the presence of APCs presenting monosubstituted peptide. These data are representative of three independent experiments.

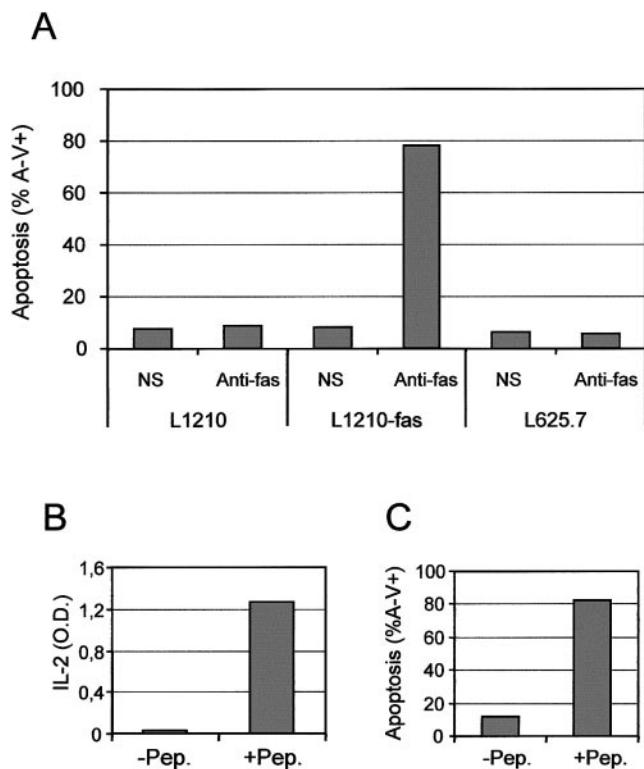


Fig. 5. CD95-negative APCs undergo apoptosis after peptide presentation to T lymphocytes. (A) L625.7 fibroblasts are resistant to apoptosis in the presence of anti-CD95 mAb (7C11). The L1210 and L1210-Fas cell lines were used as negative and positive controls for Fas-mediated apoptosis. Apoptosis was determined by annexin-V binding. NS, Not stimulated. (B) HLA-DR11-expressing L625.7 fibroblasts present specific peptide to T8.1, evaluated by IL-2 secretion by T8.1. (C) Apoptosis of peptide-loaded L625.7 fibroblasts 24 h after the beginning of coculture with T8.1.

thereby inhibits microfilament formation. A second approach to disrupting the actin cytoskeleton was taken by pretreating APCs with the cell-permeable fungal toxin cytochalasin D, which disrupts F-actin polymerization. Apoptosis was detected by reduced DiOC₆(3) fluorescence or annexin V binding. **Figure 6** reveals that actin cytoskeleton disruption of the APCs inhibits JVM and BM15 apoptosis induced by a cognate interaction. These data reveal that the APC is not a passive target for killing by the activated T lymphocyte but rather that APC signals, in which the actin cytoskeleton is implicated, are essential for programmed death of the APC.

Apoptosis of the APC is caspase-dependent

The caspase family of proteases is a key actor of apoptosis induced by death receptor-mediated and death receptor-independent apoptosis [32]. We therefore determined whether caspase activation was implicated in apoptosis of the APC population. Peptide-loaded APCs (BM15) were pretreated with the irreversible, cell-permeable tetrapeptide caspase inhibitors zVAD-fmk (a broad spectrum caspase inhibitor, ref. [33]) and IETD-fmk (selective for the effector caspase-8, ref. [34]). Tetrapeptide caspase inhibitors were used at 100 μ M, a concentration that we have previously validated to inhibit CD95-mediated apoptosis without inducing toxicity in B-EBV cell lines [26]. The results in Figure 6 demonstrate that apoptosis of

APCs, following cognate interaction with a peptide-specific T lymphocyte, was strongly inhibited in the presence of zVAD-fmk or IETD-fmk. These data reveal that caspase activation is required for APC apoptosis and that caspase-8, the membrane proximal effector caspase, is implicated.

DISCUSSION

This study examines the impact of antigen presentation on the survival of the APCs and reveals coordinate T lymphocyte activation and apoptosis of a significant proportion of the APC population (at the levels of the plasma membrane and the mitochondria) following cognate APC/T interaction. The MHC class II molecules have a well-defined role in the immune response, directly, by activating CD4⁺ T lymphocytes, and indirectly, by providing help for CD8⁺ T lymphocytes. Whereas AICD of T lymphocytes and the role of CD95/Fas in such apoptosis have been characterized [35], the outcome of antigen presentation for the APC has not been studied extensively. CD4⁺ cytolytic T lymphocyte cytotoxicity has been described via Fas/FasL interactions in murine models [36–38]. However, a Fas-independent pathway of CD4⁺ T cell cytotoxicity has been demonstrated in human B lymphocytes [39], which is supported by our data, demonstrating apoptosis of the APC in the absence of Fas/FasL interactions. We show that a dynamic interaction between the peptide-specific T lymphocyte and the APC is necessary, as cytoskeletal integrity of the APC was required. Signal transduction via immunoreceptors and remodeling of the cytoskeleton are closely linked (reviewed in ref. [40]), and the role of the APC actin cytoskeleton in the formation of the immune synapse has been demonstrated [41]. As our data show that the APC apoptosis depends on a peptide-specific interaction with the T lymphocyte, the inhibition of APC apoptosis by cytoskeletal disruption could be a result of the lack of formation of a correctly ordered immune synapse.

The data in this paper reveal the coordination of T lymphocyte activation with apoptosis of a sizable portion of APCs. The death of the APC could therefore play a parallel role with AICD in the T lymphocytes for the termination of the cognate immune response. Although the APCs all expressed the appropriate HLA-DR for peptide presentation, and regardless of the peptide-loading concentration or the duration of coculture, an upper limit of APC apoptosis was always reached. The mechanism of regulating this limit is unknown, and inefficient interaction of part of the APC population with T lymphocytes or protection of part of the population could be invoked. In a physiological response to an exogenous antigen, this is likely to represent a minor proportion of APCs undergoing apoptosis, but if this proportion represents those APCs presenting appropriate peptide, such cell death would provide a valuable mechanism for limiting ongoing T lymphocyte activation.

The specificity of the apoptosis in the peptide-presenting population is undeniable, given the lack of apoptosis in the absence of peptide or in the presence of monosubstituted peptide. These data are consistent with a previous study of antigen presentation using the HLA-DR11⁺ L625.7 fibroblasts

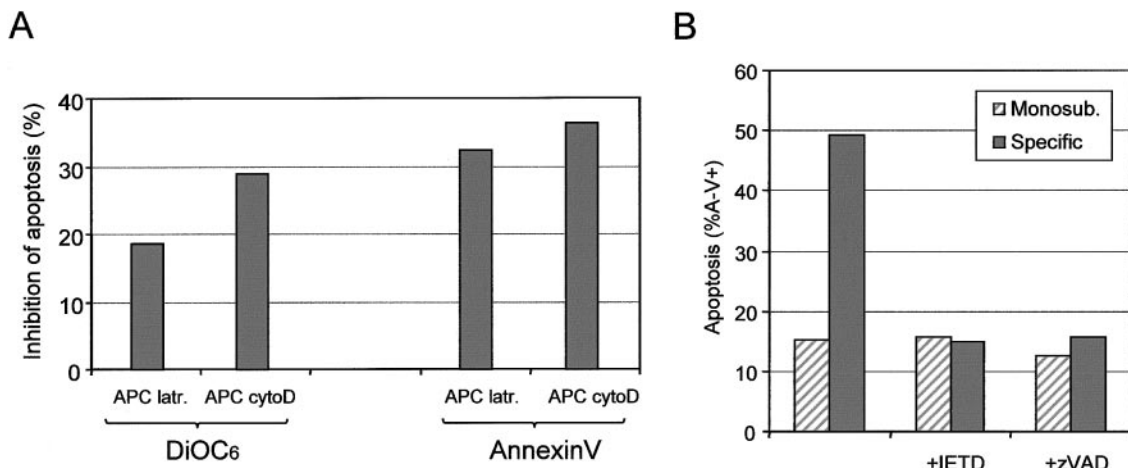


Fig. 6. Role of the actin cytoskeleton in APC apoptosis. Peptide-loaded APCs were pretreated with latrunculin (latr.; 1 μ M) or cytochalasin D (cytoD; 40 μ M) for 30 min before coculture for 24 h with T8.1. APC apoptosis detection at the mitochondrial [DiOC₆(3)] and the plasma membrane levels (annexin-V) indicated a decrease of APC apoptosis (20–35%) after inhibition of the actin cytoskeleton. Caspase activation is required for APC apoptosis. The nonreversible, cell-permeable tetrapeptide caspase inhibitors zVAD-fmk and IETD-fmk were examined for their ability to inhibit apoptosis of peptide-loaded APCs cocultured with T lymphocytes. IETD-fmk (100 μ M) and zVAD-fmk (100 μ M) reduced apoptosis in the APCs to the level observed in the presence of the alanine-monomonosubstituted peptide. These data are representative of two independent experiments.

in which specific peptide presentation to T cells led to anoikis (loss of anchorage) [29].

We have taken two approaches to determine whether Fas/FasL interactions were responsible for APC death after peptide presentation. The first used CD95/Fas-expressing APCs used as third-party targets for FasL expressed by the T cell and did not provide evidence of a requirement for Fas/FasL in mediating death of APCs. A further line of argument comes from the studies using HLA-DR11⁺-expressing fibroblasts, which do not express CD95 but nonetheless undergo apoptosis after presentation of a specific peptide. The physiological role for a CD95-independent pathway of APC apoptosis could be particularly relevant for CD95-insensitive APCs, the mature DC representing a key example [15]. Our previous data revealed a CD95-independent cell-death pathway mediated via HLA-DR in B lymphoproliferations, which failed to express CD95 [26]. The current data allow us to confirm that a CD95-independent death pathway can be initiated in the APC following interaction with a peptide-specific T lymphocyte.

Caspase activation has previously been dissociated from CD95 signaling, and a recent example is provided by the apoptosis of Jurkat T lymphoma cells after interaction with human monocyte-derived DCs. The apoptosis is mediated by caspase-8 activation independently of the death receptors, which depend on Fas-associated death domain (FADD) recruitment [42]. Fas-independent activation of caspase-8 plays a crucial role in apoptosis of B-chronic lymphocytic leukemia cells killed by cytotoxic drugs or γ irradiation [43], and CD95-independent caspase activation has been documented in APCs [32, 44].

The requirement for the localization of CD95 in specific lipid-enriched membrane microdomains for the induction of apoptosis via recruitment of FADD and downstream activation of the caspase cascade was described recently [45]. The mechanism for the caspase-mediated apoptosis of the APCs could depend on the localization of HLA class II proteins in lipid-

rich microdomains, which has been revealed as necessary for signal transduction via MHC class II [46, 47]. Engagement of HLA-DR enhances its recruitment to lipid microdomains [48] and could thus contribute to caspase recruitment and activation.

This study demonstrates mitochondrial depolarization in the APC following interaction with a specific T lymphocyte. Although many death pathways initiated by mitochondrial depolarization were initially described as caspase-independent, it is increasingly clear that release of mitochondrial proteins can lead to activation of caspase-dependent and -independent pathways [49]. Mitochondrial proteins released by an apoptotic stimulus, which can regulate caspase activation, include Omi/Htra2, cytochrome C, apoptosis-inducing factor, and endonuclease G [24]. Mitochondrial depolarization in the APC induced by a cognate interaction could therefore lead to the release of mitochondrial proteins regulating caspase activation. The mechanism by which APC/T interaction regulates mitochondrial depolarization has not yet been identified. Engagement of MHC class II molecules with mAb mediated translocation of the proapoptotic protein kinase C- δ isoenzyme from cytosolic to mitochondrial and nuclear fractions in mature DCs [50]. Engagement of MHC class II molecules in a cognate APC/T cell interaction could also induce such activity.

Similarities between MHC class II mAb-driven apoptosis and apoptosis mediated by a MHC class II-restricted cognate interaction with a T lymphocyte include the pathway of cell death (mitochondrial depolarization, phosphatidylserine externalization, need for the integrity of the actin cytoskeleton, lack of requirement for Fas/FasL interaction) and the upper limit of APC death, even in the presence of excess ligand. The major difference lies in the caspase activation implicated in APC apoptosis following a cognate interaction. This indicates the limitations of using mAb as ligands to mimic cell–cell interactions and may be a result of the contribution of accessory molecule signaling in the APC/T interaction.

Although the apoptosis of APCs induced by MHC class II mAb binding is of clear, therapeutic potential [17], the data in the current study are informative in terms of the survival of the APC after stimulation with a physiological ligand and provide a physiological basis for the apoptotic pathway initiated by MHC class II mAb binding. Finally, the point at which APC apoptosis would occur in an in vivo immune response remains to be considered. We and others have previously reported the sensitivity of mature/activated APCs (B lymphocytes and DCs) in comparison with resting/immature cells and that some protection is offered by CD40 signaling [25]. The initial, productive MHC class II-restricted peptide presentation to a specific T lymphocyte could represent the phase in which the APC would be most resistant to apoptosis. In contrast, peptide-presenting APC/T interactions post-T cell activation could represent the phase in which APC apoptosis would take place. Studies addressing the relationship among antigen internalization, processing, presentation, and APC apoptosis will improve our understanding of the regulation of APC survival.

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