Direct Antigen Presentation and Gap Junction Mediated Cross-Presentation during Apoptosis[1]

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MHC class I molecules present peptides from endogenous proteins. Ags can also be presented when derived from extracellular sources in the form of apoptotic bodies. Cross-presentation of such Ags by dendritic cells is required for proper CTL responses. The fate of Ags in cells initiated for apoptosis is unclear as is the mechanism of apoptosis-derived Ag transfer into dendritic cells. Here we show that novel Ags can be generated by caspases and be presented by MHC class I molecules of apoptotic cells. Since gap junctions function until apoptotic cells remodel to form apoptotic bodies, transfer and cross-presentation of apoptotic peptides by neighboring and dendritic cells occurs. We thus define a novel phase in classical Ag presentation and cross-presentation by MHC class I molecules: presentation of Ags created by caspase activities in cells in apoptosis. ***The Journal of Immunology,*** **2009, 183: 1083–1090.**

The classical process of Ag presentation by MHC class I molecules is defined in detail. In brief, intracellular proteins (both resident proteins and defective ribosomal products (DRiPs))[5] (1, 2) are degraded in cells by the proteasome (3). Most proteasomal products are rapidly degraded by cytosolic peptidases (4) and only a fraction escapes degradation by binding to MHC class I molecules in the lumen of the endoplasmic reticulum after translocation by the peptide transporter TAP. This complex system thus guarantees that peptides are only presented by MHC class I molecules on cells expressing the Ag. Consequently, innocent bystander cells are not presenting such antigenic information and ignored by CTL.

This mechanism should be different when exogenous Ags are presented by MHC class I molecules of APCs. This so-called cross-presentation is essential for the induction of proper CTL responses (5). Dendritic cells (DCs) (but also other APCs) receive Ags from infected or tumor cells for presentation on their own MHC class I molecules to stimulate specific CTL populations. Ags then have to be transferred from one cell to an immune cell and access the MHC class I pathway of the latter. Various routes for cross-presentation have been proposed (reviewed in Ref. 6), and it is possible that they are all operational albeit with different efficiencies in different DC populations and other immune cells (7).

Still, Ags have to cross multiple membranes to enter the cross-presenting APCs. Ags have to be released from the (infected/tumor) donor cell and have to pass at least another membrane to enter the classical MHC class I Ag presentation pathway. No membranes have to be crossed when cytosolic antigenic peptides enter neighboring cells by diffusion through gap junctions (8). Gap junctions directly connect the cytosol of neighboring cells and allow the transfer of information in the forms of ions, metabolites, peptides, and other small molecules. Connexin43 (Cx43) is expressed by most cells including many immune cells (9). For example, human monocytes express Cx43 in response to "danger signals" such as LPS and IFN-γ. Human DCs, B cells, and T cells can also express Cx43. Gap junctions transfer cAMP between regulatory T cells and other T cells for immune-suppressive actions (10) and have different functions as well. When viral peptides from infected cells enter noninfected neighbors after gap junction transfer, the exchange of antigenic information can result in innocent bystander recognition. When such peptides are acquired by professional APCs after gap junction peptide transfer, cross-presentation will be the result (8). This has been shown for viral and tumor Ags (11, 12).

Apoptotic cells or apoptotic bodies can be an efficient source for cross-presented Ags (13, 14), and injection of apoptotic bodies in mice has been shown to efficiently activate CTL responses (15). Various cell types, such as macrophages and DCs, can recognize and remove apoptotic cells and apoptotic bodies by uptake through pinocytosis and phagocytosis processes (16). How Ags from apoptotic cells or bodies then enter the classical MHC class I pathway of DCs is unclear. Saponins may be required to disintegrate apoptotic vesicles inside phagosomes (15), but the Ags or Ag fragments still have to pass the phagocytic membrane to enter the classical MHC class I route.

It is unclear how long after induction of apoptosis that processes like Ag generation and degradation by proteasomes, peptide destruction by cytosolic peptidases, and TAP translocation ensue. If these continue, MHC class I Ag presentation of cytosolic Ags generated after onset of apoptosis should be possible. Apoptotic cells can couple through gap junctions to healthy

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[5] Abbreviations used in this paper: DRiP, defective ribosomal product; BMDC, bone marrow-derived dendritic cell; CLSM, confocal laser scanning microscopy; Cx43, connexin43; DC, dendritic cell; F-Casp9, FKBP-caspase-9-IRES-GFP; FKBP, FK506 binding protein; FRAP, fluorescence recovery after photobleaching; PARP, poly-(ADP-ribose) polymerase.

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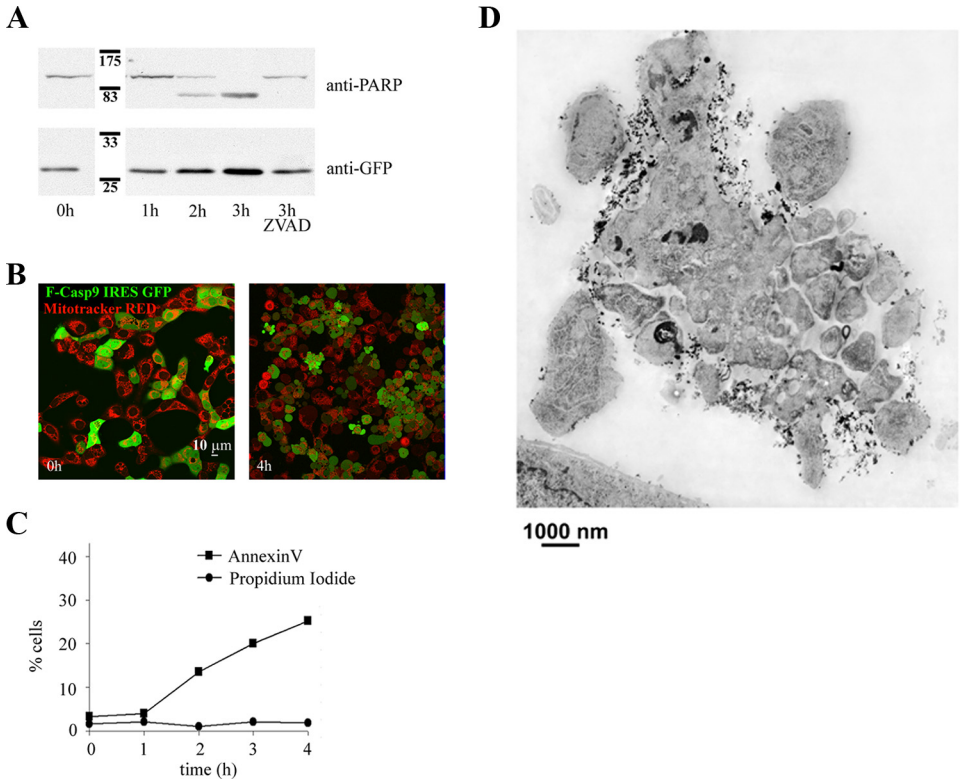

FIGURE 1. Selective induction of apoptosis. *A*, Lysates from A431/F-Casp9 cells were generated at different time points after AP20187 dimerizer exposure. Lysates were analyzed by SDS-PAGE and Western blotting with Abs against PARP and GFP as a loading control. Cleavage of PARP, a substrate of active caspase-3, can be observed 2 h after induction of apoptosis, which is inhibited by the pan-caspase inhibitor Z-VAD-FMK. *B*, Selective apoptosis induction by A431 cells expressing F-Casp9. These cells were cocultured with control A431 cells and stained with MitoTracker Red. The cells were analyzed by CLSM after 0 and 4 h exposure to the AP20187 dimerizer. Bar, 10 μm. *C*, Apoptosis and plasma membrane permeabilization were monitored by flow cytometry. A431/F-Casp9 cells were stained with annexin V-allophycocyanin and incubated with propidium iodide at different time points after induction of apoptosis by the dimerizer. *D*, F-Casp9-expressing A431 cells were exposed to the AP20187 dimerizer for 3 h while cultured in the presence of ruthenium red. Cells were fixed and sections were analyzed by electron microscopy. Bar, 1000 nm.

surrounding cells until very late stages of apoptosis (17–20), which suggests that gap junction-mediated cross-presentation of apoptotic peptides by healthy neighboring cells should be possible. Since the time span between apoptosis induction, caspase activation, and the actual morphological alterations that create apoptotic bodies may be more than 24 h (21), Ag presentation by MHC class I on apoptotic cells may be successfully performed. This may yield presentation of a unique peptide repertoire since active caspases cleave hundreds of substrates (22) in a way that is different from proteasomes. Caspases are not considered as proteases generating antigenic peptides. This may not be correct since tumors and other cells may have constitutive low caspase activities (23) and caspase activity is required for successful influenza infection (24). If direct presentation of caspase-generated Ags is possible, unique peptides will be presented by MHC class I molecules.

Here we have studied the processes in MHC class I Ag presentation after initiation of apoptosis. We used an apoptosis induction system (25) and showed that apoptotic cells maintain membrane integrity. We showed that most steps in the MHC class I Ag presentation route continue until late after apoptosis induction. Using a chimeric cytosolic construct containing an epitope released during apoptosis by activated caspase-3 or caspase-7, we showed that apoptotic cells can generate unique peptides for direct presentation and for cross-presentation by DCs and healthy neighboring cells after transfer through gap junctions.

Materials and Methods

Cells, Abs, peptides, and reagents

A431 cells were stably transfected with human Cx43 cDNA in pcDNA3 as described before (8). Mouse B16-F10 cells were stably transfected with human Cx43 cDNA. The mRFP-UbΔG76-DEVD-OVA$_{257–264}$ construct was made by PCR and sequence was verified. Construct was cloned in a normal or modified pEGFP-C1 vector (Clontech) or in an LZRS retrovirus construct. A431 and A431/Cx43 were transduced with the inducible apoptosis construct FK506 binding protein (FKBP)-caspase-9-IRES-GFP (F-Casp9) as described (25) and the additional mRFP-UbΔG76-DEVD-OVA$_{257–264}$. Transfectants were FACS sorted for equal GFP and mRFP expression levels. B3Z cells were used for OVA$_{257–264}$ presentation by H2-K^b. Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 mice (26). OVA$_{257–264}$-specific OT-I lymphocytes were obtained as described before (27). Most Abs are described (28) or commercially available. Mouse anti-OVA$_{257–264}$ peptide SIINFEKL was a gift from Dr. K. Rock.

Immunoblot analysis

A431 cells stably transfected with F-Casp9 and/or mRFP-UbΔG76-DEVD-OVA$_{257–264}$ were induced into apoptosis by 0.1 nM dimerizer AP20187, prevented by 50 μM caspase inhibitor Z-VAD-FMK (Sigma-Aldrich). Equal amounts of total proteins (20 μg) were analyzed by Western blotting.

Electron microscopy

The protocol was essentially as previously described (29). The sections were examined using a Philips CM10 microscope.

Cell biology of apoptotic cells

Confocal microscopy, in vitro peptidase assays were described before (4, 30). Annexin V and propidium iodide staining were according to the supplier (BD Biosciences). Protein synthesis during apoptosis was measured by pulse labeling cells for 30 min with ^{35}S-methionine at different time points after apoptosis induction. Adherent cells, nonadherent cells, and cell fragments were isolated and analyzed.

ATP in apoptotic cells was assayed by transient transfection of A431/Cx43 F-Casp9 with firefly luciferase and *Renilla* luciferase constructs. Forty-eight hours posttransfection, apoptosis was induced by dimerizer and cells were lysed and luciferase activity was measured (Promega).

Gap junctional coupling of apoptotic and healthy cells

A431/Cx43 F-Casp9 cells were cocultured with A431/Cx43 transiently transfected with H2B-mRFP on glass coverslips. Apoptosis was induced by 0.1 nM AP20187 (Ariad Pharmaceuticals). Cells were fixed and stained for Cx43. To show dye transfer between healthy and apoptotic cells, A431/Cx43 F-Casp9 mRFP-UbΔG76-DEVD-OVA$_{257–264}$-expressing cells were cocultured with A431/Cx43 cells and were loaded with 1 μg/ml calcein-AM (Molecular Probes) for 30 min at 37°C. Apoptosis was induced by 0.1 nM AP20187. Gap junctional coupling was assayed by photobleaching one cell in a small group of cells and recording fluorescence in the bleached cell (fluorescence recovery after photobleaching, FRAP) using confocal microscopy (Leica AOBS or Leica SPII).

Direct Ag presentation of apoptotic Ags

A431/Cx43 F-Casp9 cells stably expressing H2-K^b were transduced with mRFP-UbΔG76-DEVD-OVA$_{257–264}$. Apoptosis was initiated by 0.01 nM AP20187 under different conditions and cells were cocultured with B3Z cells. Presentation of OVA$_{257–264}$ epitope was measured by B3Z activation that was assayed by enzymatic conversion of the β-D-galactopyranoside analog chlorophenol red-β-D-galactopyranoside (CPRG) (Roche). Target cells (2×10^4) were seeded per 0.6 cm^2 well and 1×10^5 B3Z cells were added. After overnight coculture, CPRG was added and substrate conversion was measured at OD of 595 nm.

Intercellular peptide transfer and cross-presentation assays

mRFP-UbΔG76-DEVD-OVA$_{257–264}$-expressing A431/F-Casp9 cells either expressing Cx43 or not were cocultured with B16-F10/Cx43 cells and the cells were allowed to adhere and grow to 70% confluency. Apoptosis was induced by 0.01 nM AP20187 in the culture medium. B3Z cells were added overnight to assay OVA$_{257–264}$ presentation by B16-F10 cells. A431 or A431/Cx43 cells stably expressing F-Casp9 and mRFP-UbΔG76-DEVD-OVA$_{257–264}$ were labeled with calcein-AM at a concentration of 1 μg/ml for 1 h followed by extensive washing. Activated BMDCs were added and cells were cocultured for 18 h. Apoptosis was induced by 0.5 nM AP20187 for 4 h. Parallel cocultures without induction of apoptosis were used as control. Cells were collected by trypsinization and subsequently stained with allophycocyanin-conjugated anti-mouse CD11c Abs as a DC marker. BMDCs with high calcein fluorescence were FACS sorted and Ag cross-presentation was measured by incubating 1×10^4 BMDCs with 1×10^5 OT-I lymphocytes for 4 h followed by intracellular IFN-γ staining as described (27).

Results

Defining early apoptotic cells

If apoptotic cells are able to produce peptides for MHC class I Ag presentation, they have to maintain an active Ag degradation and MHC class I loading system and not release the antigenic proteins or peptides into the surrounding medium. Whether these processes continue between the initiation of apoptosis and the final (blebbing) phase that precedes apoptotic body formation is unclear.

We generated cells in which apoptosis could selectively be induced in a synchronized manner. An F-Casp9 construct was introduced in the human squamous carcinoma cell lines A431 (devoid of gap junctions) and A431/Cx43 where gap junctions were reintroduced by Cx43 expression (8). Apoptosis was initiated in both cell lines by the FKBP-binding chemical dimerizer AP20187 (25). Dimerization of the initiator caspase-9 induces autocleavage and activation. Activated caspase-9 then cleaves and activates effector caspase-3 and caspase-7 as required for apoptosis. The dimerizer compound AP20187 thus initiates apoptosis.

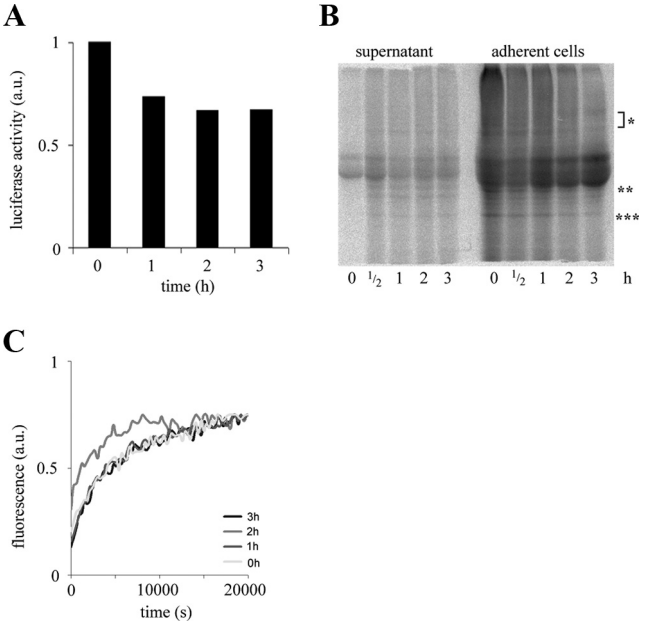

FIGURE 2. Requirements for MHC class I Ag presentation during apoptosis. *A*, ATP in apoptotic cells was monitored by expressing firefly luciferase and *Renilla* luciferase in A431 F-Casp9 cells. The cells were lysed at different time points after apoptosis induction by dimerizer, and luciferin conversion was measured by luminescence. *B*, Translation in A431/F-Casp9 cells was measured by labeling cells with ^{35}S-methionine for 30 min at different times after exposure to the dimerizer. Lysates from both detached as well as adherent cells were analyzed by SDS-PAGE and autoradiography. Asterisks indicate some protein bands appearing during apoptosis. *C*, Peptidase activity in apoptotic cells was detected in an in vitro peptidase assay. Cell lysates from A431/F-Casp9 cells exposed for various times to the dimerizer were made before addition of a 9-mer internal quenched fluorogenic peptide and fluorescence was recorded.

Apoptosis was visualized biochemically by generating lysates from F-Casp9-expressing A431 cells at different times after dimerizer exposure and detecting the caspase-3 substrate poly(ADP-ribose) polymerase (PARP). Cleavage of PARP was observed 2 h after induction of apoptosis when cell blebbing was not observed and could be efficiently inhibited by Z-VAD-FMK (Z-VAD), a pan-caspase inhibitor (Fig. 1*A*). Apoptotic cell blebbing was detected in almost all A431 cells expressing F-Casp9 exposed for 4 h to AP20187 (Fig. 1*B* and supplemental movie M1).[6] Apoptosis was confirmed by staining cells with annexin V at different time points after induction of apoptosis and analysis by flow cytometry. The number of annexin V-positive cells increased in time (Fig. 1*C*). This preceded cell permeabilization since propidium iodide was excluded from cells (Fig. 1*C*), even at time points when PARP was cleaved by caspases (Fig. 1*A*). To test whether apoptotic cells remained intact until the formation of apoptotic bodies, we used another technique. F-Casp9-expressing A431 cells were exposed to dimerizer AP20187 while cultured in the presence of ruthenium red. This dye produces electron-dense precipitates on negatively charged lipids and should reveal the integrity of the plasma membrane by exclusion of internal precipitates (supplemental Fig. 1). Analysis by electron microscopy did not reveal uptake of the dye by cells during apoptosis or in the resulting apoptotic bodies (Fig. 1*D*).

[6] The online version of this article contains supplemental material.

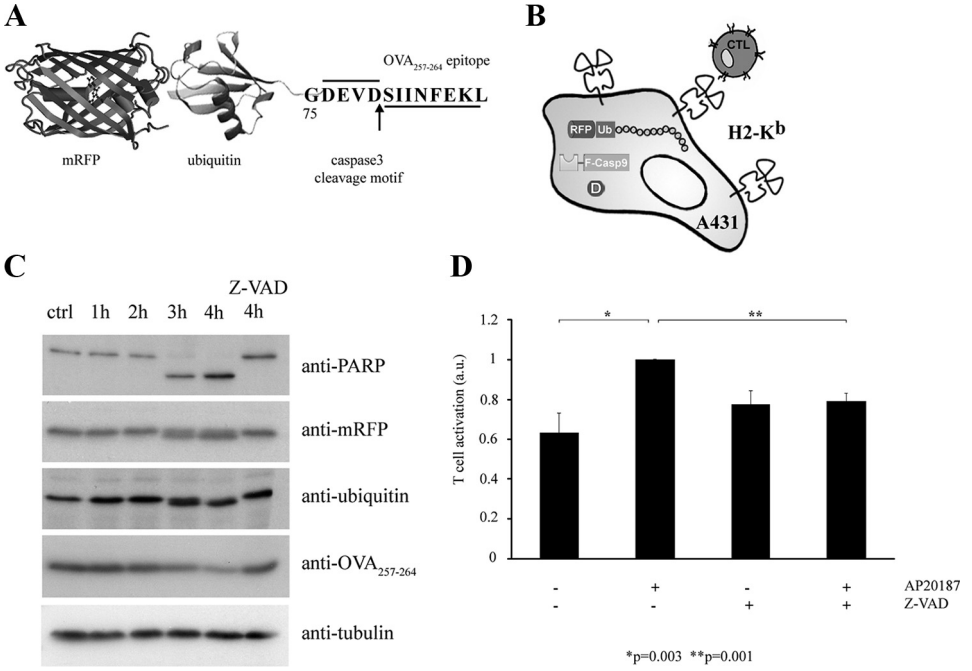

FIGURE 3. Direct Ag presentation of apoptotic Ags. *A*, Domain structure of the mRFP-UbΔG76-DEVD-$OVA_{257–264}$ construct for peptide release by caspase activity. The last glycine of ubiquitin was replaced by DEVD, the recognition and cleavage motif for activated caspase-3 and caspase-7. *B*, Experimental strategy. By expressing the mRFP-UbΔG76-DEVD-$OVA_{257–264}$ construct and F-Casp9 in A431 cells that express H2-K^b, direct presentation of Ags after apoptosis induction can be assayed. Ag presentation should then be dependent on the dimerizer AP20187 to induce apoptosis and should be inhibited by the pan-caspase inhibitor Z-VAD. *C*, Release of the $OVA_{257–264}$ peptide (SIINFEKL) after induction of apoptosis was tested biochemically. A431 cells stably transfected with F-Casp9 and mRFP-UbΔG76-DEVD-$OVA_{257–264}$ were sentenced to apoptosis by dimerizer exposure, and cell lysates were collected at different time points after induction of apoptosis for analyses by SDS-PAGE and Western blotting. To validate that cleavage was the result of caspase activities, cells were incubated with the dimerizer in the presence of the caspase inhibitor Z-VAD for 4 h. Membranes were probed with Abs indicated, and anti-tubulin Abs were used as loading control. *D*, Direct presentation of $OVA_{257–264}$ peptide by cells undergoing apoptosis. mRFP-UbΔG76-DEVD-$OVA_{257–264}$-expressing A431/H2-K^b F-Casp9 cells were incubated with dimerizer AP20183 in the absence or presence of Z-VAD, as indicated. The cells were cocultured for 18 h with OVA-specific T cells harboring the TCR response *LacZ* gene to determine T cell activation. The result is an average of three independent experiments. The response was corrected for spontaneous activation (T cells only). The maximal response was arbitrarily set at 1.0. Shown are means ± SD; ∗, $p = 0.003$ and ∗∗, $p = 0.001$).

Processes required for Ag processing and presentation during apoptosis

Successful class I Ag presentation requires a series of metabolic processes. Whether these continue after apoptosis initiation is unclear. One essential factor is ATP, which requires mitochondrial integrity. To detect ATP concentrations in cells during apoptosis, A431/F-Casp9 cells were transiently transfected with constructs for firefly luciferase, which requires ATP for light formation, and *Renilla* luciferase, which does not require ATP for light formation. Apoptosis was induced by dimerizer addition, and cell lysates were generated at different time points before luciferase activities were measured. Firefly luciferase signals were observed in all samples, with some 30% drop over a 3-h period of apoptosis, indicating that intracellular ATP continued to be present for more than 3 h after induction of apoptosis (Fig. 2*A*).

Protein synthesis is essential for DRiPs and MHC class I formation. Protein synthesis during apoptosis in F-Casp9-expressing A431 cells was determined by metabolically labeling cells with ^{35}S-methionine for 30 min at different time points after exposure to the dimerizer. Incorporation of radioactive amino acids into proteins was monitored by SDS-PAGE and autoradiography. Radioactive amino acids were incorporated in proteins for at least 3 h after induction of apoptosis, indicating that protein synthesis continued (Fig. 2*B*). Novel protein bands appeared during apoptosis, possibly by protein modifications by the unleashed caspases.

Intracellular peptidases remove most peptides before contacting MHC class I molecules (30). Caspase activities may add to total peptidase activities but may also decrease this when peptidases are targeted for destruction. The peptidase activities in apoptotic cells were measured by in vitro peptidase assays (30). Internally quenched fluorescent peptides were added to cytosol from cells exposed for different times to the dimerizer and appearance of fluorescence was recorded. Degradation of the peptides will spatially separate quencher and fluorophore, resulting in fluorescent signals (30). No differences in peptidase activity were observed for 9-mer peptides (Fig. 2*C*). These data suggest that many processes involved in MHC class I Ag processing and presentation persist after apoptosis induction.

Direct Ag presentation of apoptotic Ags

Cells may succeed in presenting Ags by MHC class I molecules between apoptosis initiation and actual cell destruction as the final step in apoptosis since many processes continue. We constructed an antigenic fragment that could be released only by caspase activities by using a variant of the GFP-Ub-peptide construct (8). The ultimate glycine (G76) of ubiquitin was replaced by a DEVD sequence that is recognized by caspases. We constructed a vector expressing the chimeric protein mRFP-UbΔG76-DEVD-$OVA_{257–264}$ (Fig. 3*A*). The resulting fusion protein is stable under normal conditions, and the peptide following the modified ubiquitin is not removed by deubiquitinating proteases. The chimeric protein is 37 kDa in molecular mass, allowing free diffusion through the nuclear pore. Hence, it is present in both the nucleus and cytosol. The proteasomal caspase-like activity represents only a minor activity,

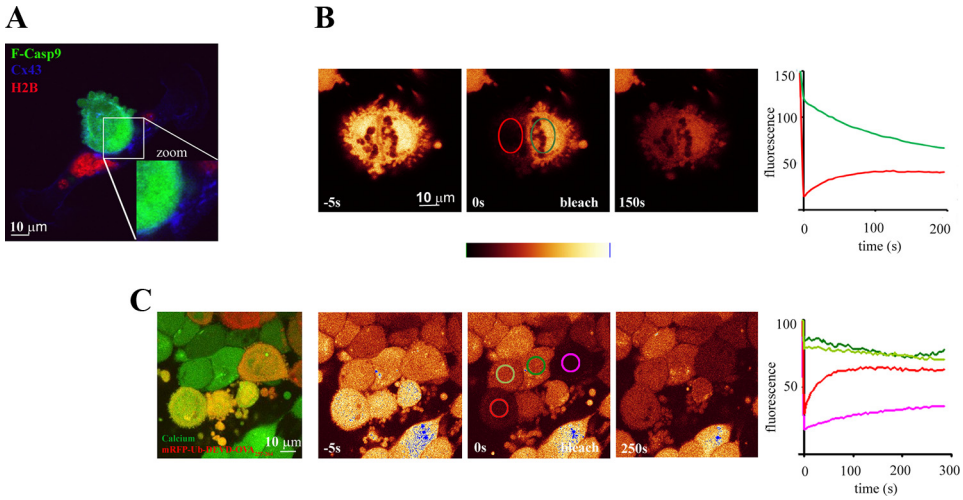

FIGURE 4. Gap junctional coupling of apoptotic and healthy cells. *A*, A431/Cx43 F-Casp9 cells expressing GFP were cocultured with A431/Cx43-expressing H2B-mRFP cells, and apoptosis was induced by 3 h of exposure to dimerizer. Fixed cells were stained with Abs against Cx43 and imaged by CLSM. The zoom-in shows membrane contact between two cells. The GFP of the F-Casp-9 cells (in green), Cx43 (in blue), and mRFP (in red) are indicated. Bar, 10 μm. *B*, Gap junctional coupling of apoptotic cells as shown by FRAP experiments. A431/Cx43 cells were loaded with calcein-AM, and apoptotic cells were identified on the basis of morphology (blebbing). Fluorescence was bleached in one cell followed by time-lapse microscopy. Fluorescence intensities are shown in the glow over-under mode. Calcein fluorescence recovery in the bleached cell (red trace) at the cost of the coupled nonbleached cells (green trace) was monitored and quantified. Bar, 10 μm. Details are found in supplemental movie M2. *C*, Gap junctional coupling of apoptotic and healthy cells was determined in FRAP experiments. A431/Cx43 F-Casp9 mRFP-UbΔG76-DEVD-OVA$_{257–264}$ cells were cocultured with A431/Cx43 cells and all cells were loaded with calcein-AM. The mRFP label thus marked the cell sensitive to the apoptosis inducer AP20187 dimerizer. Apoptosis was induced for 2 h by exposure to dimerizer. The calcein fluorescence in the indicated cells (in the red and pink circle) was bleached, and recovery of fluorescence in the same cells was determined by time-lapse microscopy using CLSM. The left image shows a merge of the cells to reveal the F-Casp9 mRFP-UbΔG76-DEVD-OVA$_{257–264}$-expressing cells (in yellow), which were undergoing apoptosis. Time-lapse images are shown in glow over-under mode. *Right panel*, Quantification of fluorescence recovery (red and pink) and loss (dark and light green) in the indicated cells where the area of quantification is indicated in corresponding colors. Bar, 10 μm. Details are shown in supplemental movie M3.

suggesting that the proteasome will not easily cleave directly before the OVA$_{257–264}$ sequence. In the absence of cytosolic aminopeptidase activities (30), the OVA$_{257–264}$ sequence will not be generated and presented in healthy cells but can be released by activated caspase-3 and caspase-7. This construct was introduced in A431 cells expressing H2-K^b and the F-Casp9 construct to induce apoptosis by the dimerizer (Fig. 3*B*). Western blot analysis of lysates from cells at different time points after apoptosis induction showed cleavage of PARP as well as a faster migrating band for mRFP or ubiquitin. Further staining with an anti-OVA$_{257–264}$ peptide Ab showed a reduced signal following induction of apoptosis, indicating that the OVA$_{257–264}$ epitope was released by caspase activities since it could be inhibited by the pan-caspase inhibitor Z-VAD (Fig. 3*C*).

We then tested whether the OVA$_{257–264}$ peptide, as released by caspases, could be presented by H2-K^b in cells primed for apoptosis, which can be detected by the B3Z CTL (31). Since DRiPs are a major source of MHC class I peptides (1, 2), such DRiPs will also be generated from the mRFP-UbΔG76-DEVD-OVA$_{257–264}$ construct. Consequently, some T cell activation occurred when this construct was expressed in nonapoptotic cells. OVA$_{257–264}$ peptide presentation by H2-K^b on A431 cells was significantly increased only when apoptosis was induced by the dimerizer and presentation was inhibited by Z-VAD (Fig. 3*D*), while necrotic cells that expressed mRFP-UbΔG76-DEVD-OVA$_{257–264}$ as well as apoptotic cells without mRFP-UbΔG76-DEVD-OVA$_{257–264}$ did not give additional T cell responses (data not shown). This suggests that caspases can release new peptides for presentation by MHC class I molecules on cells sentenced to apoptosis.

Gap junctional coupling of apoptotic and healthy cells

Apoptotic bodies are efficient in initiating cross-presentation and priming (16), but apoptotic peptides may also be transferred by gap junctions. To test whether cells primed for apoptosis are still coupled via gap junctions, A431/Cx43 F-Casp9-containing cells were cocultured with A431/Cx43 cells transiently transfected with histone H2B-mRFP for detection. Apoptosis was induced by dimerizer before cells were fixed and stained with Abs against Cx43. Confocal laser scanning microscopy (CLSM) analyses detected Cx43 clusters between healthy and dying cells (Fig. 4*A*) until apoptotic cells were fragmented into apoptotic bodies, when these contacts were lost (not shown).

To show that these gap junctions are still open to allow diffusion of small substrates between cells, FRAP experiments were performed to visualize coupling of cells through gap junctions. Cocultured cells were loaded with calcein-AM. Once converted by intracellular hydrolases, calcein is captured in the cell and can move to other cells only via functional gap junctions. Apoptosis was induced by edelfosine (alkyl-lysophospholipid), resulting in characteristic morphological changes. The calcein fluorescence in one cell was photobleached, and fluorescence recovery as a result of calcein diffusion from the neighboring cell was monitored (Fig. 4*B*; see supplemental movie M2). Apoptotic cells can still be coupled by gap junctions until gap junction contacts are destroyed as the result of apoptotic body formation (not shown).

Transfer of calcein between healthy and apoptotic cells was monitored in an analogous experiment. A431/Cx43 cells expressing the F-Casp9 cassette and mRFP-UbΔG76-DEVD-OVA$_{257–264}$ were cocultured with A431/Cx43 cells. Both were loaded with calcein-AM in this coculture experiment. Apoptosis was induced by dimerizer addition for 2 h before the FRAP experiments were performed. Green fluorescent calcein from healthy cells exchanged with the bleached dye in apoptotic cells (expressing the mRFP-ubiquitin chimera), indicating that communication via gap junctions still occurred after initiation of apoptosis (Fig. 4*C*; see supplemental movie M3).

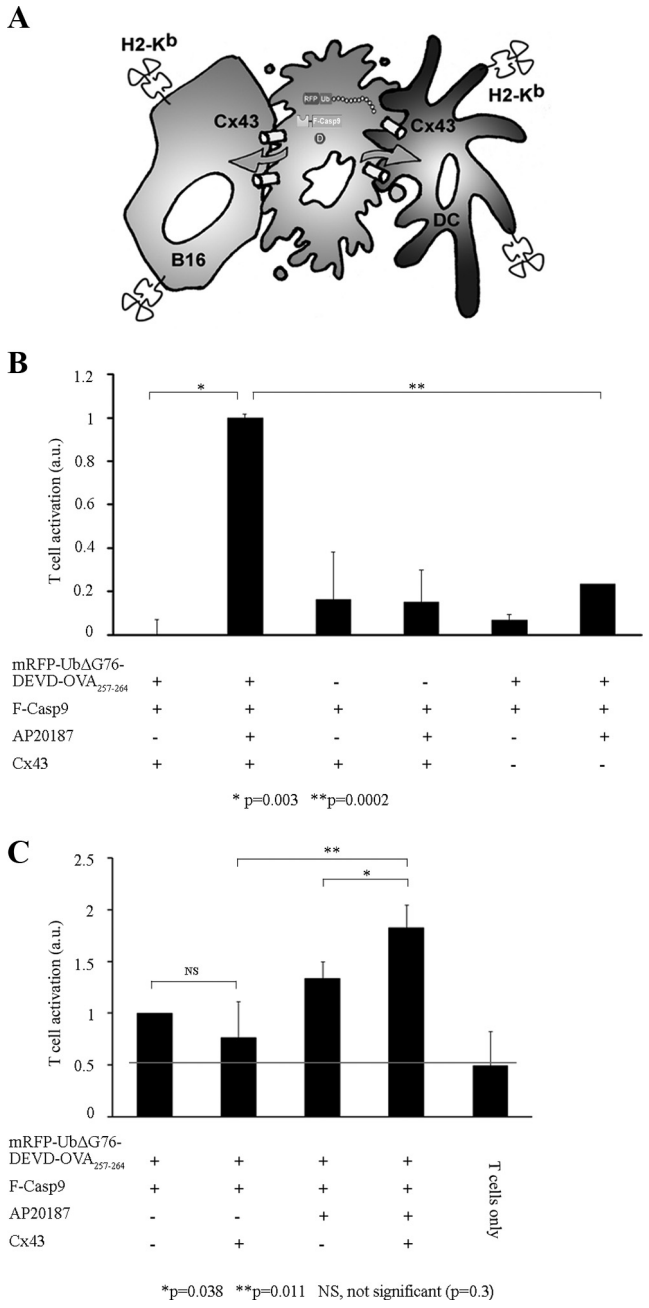

FIGURE 5. Intercellular transfer of apoptosis specific peptides for cross-presentation. *A*, Experimental set-up. A431 cells containing Cx43 were transfected with the apoptosis inducing F-Casp9 construct as well as mRFP-UbΔG76-DEVD-OVA$_{257–264}$ (SIINFEKL) to release peptide in a caspase-dependent manner. These cells were cocultured with H2-K^b and Cx43-expressing B16-F10 melanoma cells or BMDCs from C57BL/6 mice, and cross-presentation was tested by T cell assays using H2-K^b restricted SIINFEKL recognizing T cells. *B*, Gap junction-dependent cross-presentation of apoptotic Ags. The A431 cells either containing Cx43 or not and F-Casp9 as well as mRFP-UbΔG76-DEVD-OVA$_{257–264}$ were cocultured with Cx43-expressing B16-F10 melanoma cells before apoptosis was induced by the dimerizer AP20187. The different conditions are indicated below the diagram. Cross-presentation of SIINFEKL was detected by B3Z T cells, and the T cell response was corrected for spontaneous activation (from B16-F10 cells cocultured with A431/Cx43 F-Casp9 cells). The result is an average of two independent experiments. The maximal response was arbitrarily set at 1.0. Shown are means ± SD; ∗, $p = 0.003$ and ∗∗, $p = 0.0002$). *C*, Cross-presentation of apoptosis-generated peptides by BMDCs. BMDCs were isolated and activated for 24 h with TNF-α and IFN-γ to up-regulate expression of Cx43. A431 and A431/Cx43 cells stably transduced with F-Casp9 as well as mRFP-UbΔG76-DEVD-OVA$_{257–264}$ were loaded with calcein-AM and cocultured with activated BMDCs for 18 h before apoptosis was induced by 4 h of exposure with dimerizer AP20187. A control condition without induction of apoptosis was included, as indicated below the bars. Equal numbers of BMDCs that received the calcein signal from the A431 cells were isolated by FACS sorting, and T cell activation was measured with OT-I lymphocytes. The result is an average of three independent experiments, and the T cell response from A431/F-Casp9 mRFP-UbΔG76-DEVD-OVA$_{257–264}$ cocultured without apoptosis was set arbitrarily at 1.0. Shown are means ± SD; ∗, $p = 0.038$ and ∗∗, $p = 0.011$.

Intercellular transfer of apoptosis-specific peptides for cross-presentation

If gap junctions remain functional and Ags are generated in cells in apoptosis, gap junction-mediated cross-presentation of apoptotic Ags should be an option. Gap junctions could then be used to transfer antigenic peptides from cells undergoing apoptosis to healthy neighbors including specialized APCs such as dendritic cells. To test whether peptides generated in apoptotic cells could diffuse through gap junctions for presentation by neighboring cells, a coculture experiment was performed. A431/Cx43 F-Casp9 cells were transiently transfected with mRFP-Ub-DEVD-OVA$_{257–264}$, cocultured with B16-F10/Cx43 mouse melanoma cells expressing H2-K^b molecules, and Ag presentation was quantified by B3Z T cell activation (Fig. 5*A*). Since the A431 cells do not express H2-K^b, presentation of OVA$_{257–264}$ could only occur after transfer to B16-F10/Cx43 cells. As controls, B16-F10/Cx43 cells were cocultured with A431/F-Casp9 cells (lacking gap junctions) in the absence or presence of dimerizer. T cells were only activated when mRFP-UbΔG76-DEVD-OVA$_{257–264}$ was expressed in cells primed for apoptosis and required Cx43 gap junctions (Fig. 5*B*). This suggests that epitopes generated in apoptotic cells can be transferred via gap junctions for cross-presentation by healthy neighboring cells.

To test whether antigenic fragments generated in apoptotic cells could be transferred to DCs for cross-presentation, C57BL/6 mouse BMDCs were isolated and activated with TNF-α and IFN-γ to up-regulate the expression of Cx43 (32). Then A431/F-Casp9 cells either expressing Cx43 or not and stably transduced with mRFP-UbΔG76-DEVD-OVA$_{257–264}$ were loaded with the dye calcein-AM. The activated BMDCs were cocultured with calcein loaded donor cells for 18 h. Apoptosis was then initiated by dimerizer and cells were further cocultured for another 4 h. BMDCs from both apoptotic coculture and healthy control coculture experiments that acquired the dye calcium were isolated by FACS sorting. Presentation of OVA$_{257–264}$ by H2-K^b of calcein-positive C57BL/6 BMDCs was essayed by OVA$_{257–264}$-specific H2-K^b-restricted OT-I T cells. The OVA$_{257–264}$ fragment was cross-presented by BMDCs after apoptosis induction of the donor cells. Cross-presentation of OVA$_{257–264}$ by BMDCs was consistently better when the A431 cells expressing Cx43 created functional gap junctions with BMDCs (Fig. 5*C*). However, cross-presentation also occurred, albeit consistently less efficiently, when cells failed to form gap junctions, as with the A431 control cells. This suggests that BMDCs can acquire antigenic information from apoptotic cells for cross-presentation by at least two different routes. One employs gap junctions, and the other one could be the result of apoptotic cell uptake for MHC class I Ag presentation. Of note, cross-presentation of OVA$_{257–264}$ by the melanoma cell line B16-F10 was entirely dependent on gap junctions, probably because this cell line lacks cell biological alternatives more specific to DCs, such as phagocytosis.

Discussion

Here we have shown that most processes involved in MHC class I Ag presentation are still functional during the early phases of apoptosis. The activated caspases can generate unique antigenic peptides for presentation by apoptotic cells but also by neighboring cells for cross-presentation. Since gap junctions are still operational between apoptotic cells and neighboring healthy cells, until gap junction contacts are destroyed during apoptotic body formation, peptides generated in apoptotic cells can use this route for cross-presentation to the neighboring healthy cells or APCs.

It has been shown that autoantigens can be generated by the collaborative efforts of activated caspases and proteasome, which may result in lymphodepletion in HIV-infected patients (33). Of note, various tumor cells express basal levels of caspase activities (23) or they transiently activate effector caspases without cell death (34). Such tumor cells then contain cytosolic proteases other than the proteasome for the generation of novel antigenic fragments, especially since the caspase-specific activity accounts for 2–22% of proteasome degradation, and the trypsin and chymotrypsin activities account for the remainder (35). This process may further expand the presented peptide repertoire by including tumor-specific peptides, not because these Ags are tumor specific but because they are generated by selective proteases. This may also be applicable to influenza-infected cells that require activation of caspase-3 for virus propagation (24). The number of experimentally identified caspase substrates is increasing (36, 37), and the products may be further trimmed by cytosolic peptidases and the proteasome to yield unique peptides (38).

Apoptosis is usually a warning (danger) signal that can be induced in many circumstances such as during infections, and apoptotic cells or bodies are major sources for Ag cross-presentation. Apoptotic bodies are hardly detected by pathology staining, as apoptotic bodies are quickly cleared by resident macrophages and DCs. Our study defines a new pathway in which antigenic peptides can be transferred via gap junctions and hence cross-presented by bystander DCs during the early phase of apoptosis, before the formation and clearance of apoptotic bodies. We showed that gap junctions can transfer such peptides into healthy neighboring cells. However, this is not the only mechanism for transfer of apoptotic Ags, since the BMDCs, unlike the B16-F10 melanoma cells, also received information from apoptotic A431 cells, which are unable to form gap junctions. The exact mechanism for this process is unclear but may involve the ability of DCs to phagocytose apoptotic bodies or cells. How the phagocytosized apoptotic bodies and their Ags and antigenic fragments are handled in DC phagosomes is unclear, although various mechanisms like phagosome-endoplasmic reticulum transfer and recycling MHC class I molecules have been proposed (39–41). Apoptotic information is transferred to DCs for cross-presentation by at least two mechanisms, one involving gap junctional transfer of peptides generated by novel cytosolic proteases in the MHC class I Ag generation pathway, and the other one involving a potentially more DC-specific pathway.

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Disclosures

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