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Noncovalent Association with Stress Protein Facilitates Cross-Priming of CD8\(^+\) T Cells to Tumor Cell Antigens by Dendritic Cells\(^1\)

Robert Kammerer, Detlef Stober, Petra Riedl, Claude Oehninger, Reinhold Schirmbeck, and Jörg Reimann\(^2\)

A viral onco gene carrying well-defined K\(^b\)/D\(^b\)-restricted epitopes was expressed in a heat shock protein (hsp)-associated or non-associated form in the murine tumor cell lines P815 and Meth-A. Wild-type SV40 large T-Ag (wtT-Ag) is expressed without stable hsp association; mutant (cytoplasmic cT-Ag) or chimeric (cT272-green fluorescent fusion protein) T-Ag is expressed in stable association with the constitutively expressed, cytosolic hsp73 (hsc70) protein. In vitro, remnants from apoptotic wtT-Ag- or cT-Ag-expressing tumor cells are taken up and processed by immature dendritic cells (DC), and the K\(^b\)/D\(^b\)-binding epitopes T1, T2/3, and T4 of the T-Ag are cross-presented to CTL in a TAP-independent way. DC pulsed with remnants of transfected, apoptotic tumor cells cross-presented the three T-Ag epitopes more efficiently when they processed ATP-sensitive hsp73/cT-Ag complexes than when they processed hsp-nonassociated (native) T-Ag. In vivo, more IFN-\(\gamma\)-producing CD8\(^+\) T cells were elicited by a DNA vaccine that encoded hsp73-binding mutant TAg than by a DNA vaccine that encoded native, non-hsp-binding T-Ag. Three- to 5-fold higher numbers of T-Ag (T1-, T2/3-, or T4-) specific, D\(^b\)/K\(^b\)-restricted IFN-\(\gamma\)-producing CD8\(^+\) T cells were primed during the growth of transfected H-2\(^d\) Meth-A/cT tumors than during the growth of transfected Meth-A/T tumors in F1 (b × d) hosts. Hence, the association of an oncogene with constitutively expressed, cytosolic hsp73 facilitates cross-priming in vitro and in vivo of CTL by DC that process material from apoptotic cells. The Journal of Immunology, 2002, 168: 108 –117.
class I molecules and/or their immunogenic presentation, although the mechanism that underlies these observations is not yet clear. We describe in vitro and in vivo experiments to test whether antigenic material from tumor cells (containing MHC class I-binding epitopes) taken up by DC can more efficiently cross-stimulate or cross-prime CTL when expressed in association with hsp73.

Materials and Methods

Mice

C57BL/6/Bom (B6) mice (H-2b), BALB/c/Bom mice (H-2d), and F1 (BALB/c × C57BL/6) mice were bred and kept under standard patent-free conditions in the animal colony of Ulm University (Ulm, Germany). Breeding pairs of these inbred strains were obtained from Bombholzard (Ry, Denmark). C57BL/6/Tap m1-lamp (B6 TAP1+/−) mice homozygous for the tap1 gene deletion were obtained from The Jackson Laboratory (stock no. 002944, Bar Harbor, ME). Male and female mice were used at 12–16 wk of age.

Cells and plasmids

The H-2d mastocytoma cell line P815 (TIB64) was obtained from the American Tissue Culture Collection (Manassas, VA). The BALB/c-derived fibrosarcoma Meth-A was provided by Dr. W. Deppert (Hamburg, Germany). The bovine papilloma virus-based vector BMGNeo, a gift from Drs. Y. Karasuyama and F. Melchers (Basel, Switzerland), was used to construct the BMG-large tumor Ag of SV40 (T-Ag) and BMGcT-Ag expression plasmids as previously described (29). The cT272 encompasses (mutant) green fluorescent protein (eGFP)-encoding vector BMGcT272-eGFP was constructed by inserting the cT272-encoding region into the commercially available pEGFP N1 vector (Clontech, Palo Alto, CA). The entire cT272-eGFP-encoding cassette was subcloned into the BMG vector. BMG/T, BMGcT272-eGFP, or BMGcT vector DNA or nonrecombinant BMGNeo vector DNA was transfected into P815 or Meth-A cells as previously described (36, 37). Lines, clones, and subclones of H-2b P815/T and P815xT, and H-2d Meth-A/T and Meth-A/xT with stable expression of the T-Ag protein were generated as previously described (29, 30, 36, 37). Hybridomas producing the anti-CD4 mAb YTS 191.1 or the anti-CD8 mAb RBL5 cells. The cytolytic assay with peptide-pulsed targets demonstrated that the CTL displayed T1, T2/3, and/or T4 epitope specificity and the respective (Kb or Db) restriction specificity.

Flow cytometric analyses

For surface staining cells were suspended in PBS/0.3% (w/v) BSA supplemented with 0.1% (w/v) sodium azide. Non-specific binding of Abs to Fr was blocked by preincubating cells with 1 μg/106 cells of the anti-CD16/CD32 mAb 2.4G2 (catalog no. 01240D, BD Pharmingen, San Diego, CA). Cells were incubated with 0.5 μg/106 cells of the relevant mAb for 30 min at 4°C, washed twice, and subsequently incubated with a second-step reagent for 15 min at 4°C. Cells were washed twice and analyzed on a FACScan (Becton Dickinson Biosciences, Mountain View, CA). Dead cells were excluded by propidium iodide staining. The following reagents and mAbs from BD Pharmingen were used: PE-conjugated anti-I-A* (catalog no. 06045A), biotinylated anti-H-2Db (clone no. 06232D), PE-conjugated anti-CD80 (B7-1; catalog no. 09605B), PE-conjugated anti-CD40 (catalog no. 09665B), FITC-conjugated anti-CD86 (B7-2; catalog no. 09215B), and FITC-conjugated and PE-conjugated anti-CD11c (catalog no. 553801 and 09705B). We furthermore used FITC-conjugated IgG1 mAb R3-34 (catalog no. 019534-028, BD Pharmingen), streptavidin-Red670 (catalog no. 019534-024, Life Technologies, Gaithersburg, MD).

Induction of apoptosis in tumor cells

Tumor cells were irradiated (1.5 MJ/ml/cm2) for either 2 or 5 min using the UV cross-linker 1800 (UV Stratalinker TM-1800, Stratagene). Cells were suspended in PBS to exclude the UVB-absorbing effect of phenol red in culture medium. Apoptosis was confirmed by annexin V staining. Cell lysates were produced by exposing tumor cells to four rapid freeze-thaw cycles until cell membrane integrity was lost. Cell debris was removed by centrifugation (30 min at 5000 × g). Aliquots of the lysates were used to pulse DCs.

Ag uptake by BMDC

Uptake of macromolecules was determined by incubating DC with 250 ng/ml FITC-dextran (catalog no. D-1845, Sigma) for 2 h at either 37 or 4°C. Cells were washed and labeled with PE-conjugated anti-CD11c or anti-MHC class II mAb. To study the uptake of apoptotic tumor cells by DCs, tumor cells suspended in PBS were stained for 10 min at 37°C with 5 μM green CFSE dye (catalog no. C-1157, Molecular Probes). Cells were washed three times in ice-cold PBS before induction of apoptosis. Apoptotic tumor cells incubated for 5 h postirradiation in medium were co-cultured with DCs at different DC/tumor cell ratios at either 4 or 37°C. DCs harvested from 18-h cocultures were stained by PE-labeled anti-CD11c or anti-MHC II mAb. In two-color flow cytometric analyses we determined the percentage of DC that had taken up green fluorescent material derived from apoptotic tumor cells. DC incubated at 4°C showed no uptake of material derived from tumor cells, indicating that we were dealing with active, temperature-dependent uptake and not passive adsorption of material to the cell surface directly.

Coculture of CTL with DC

DCs were harvested from cultures in which they were pulsed with titrated amounts of material from apoptotic tumor cells, extensively washed, and cocultured at (2.5–5 × 104 DC/well) with CTL (1–2.5 × 104 CTL/well) in 96-well U-bottom plates for 24 h. Culture supernatants were harvested and analyzed for IFN-γ by ELISA. In some experiments DCs were fixed with 1% paraformaldehyde in PBS for 10 min before the pulse with either remnants of apoptotic cells or peptides, washed, and cocultured with CTL.

Cytokines and cytokine detection by ELISA

For detection and capture of IFN-γ in supernatants by conventional double-sandwich ELISA, we used the mAb R4-6A2 (catalog no. 18181D, BD
Pharmingen) and biotinylated mAb XMGI.2 (catalog no. 18112D; BD Pharmingen). Murine IFN-γ was also obtained from BD Pharmingen (catalog no. 19301T). Extinction was analyzed at 405/490 nm on a TECAN, Crailshem, Germany) with the EasyWin software (TECAN). The detection limit of the ELISA for IFN-γ was 20 pg/ml.

Tumor cell transplantation

Tumor cells were washed three times in PBS, and 50 μl of the cell suspension was injected s.c. into the shaved right flank. Experimental groups consisted of four to six mice. Tumor development was followed by serial measurements of tumor size at two perpendicular diameters.

Determination of splenic CTL frequencies

Spleen cells (10^6/ml) were incubated for 60 min in RPMI medium with 5 μg/ml of the indicated peptide in round-bottom 96-well plates. Thereafter, 5 μg/ml brefeldin A (BFA; catalog no. 15870, Sigma) was added, and the cultures were incubated for an additional 4 h. Cells were harvested and surface-stained with PE-conjugated anti-CD8 mAbs (catalog no. 01045B; BD Pharmingen). Surface-stained cells were fixed with 2% paraformaldehyde in PBS before intracellular staining for IFN-γ. Fixed cells were resuspended in permeabilization buffer (HBSS, 0.5% BSA, 0.5% saponin, and 0.05% sodium azide) and incubated with FITC-conjugated anti-IFN-γ mAb (catalog no. 55441, BD Pharmingen) for 30 min at room temperature and washed twice in permeabilization buffer. Stained cells were resuspended in PBS×0.3% (w/v) BSA supplemented with 0.1% (w/v) sodium azide and analyzed by flow cytometry (FCM). The number of IFN-γ+ cells in 10^3 CD8+ cells was determined.

CTL assays

Single-cell suspensions were prepared from spleens of mice in α-MEM tissue culture medium supplemented with 10 mM HEPES buffer, 0.1% (w/v) sodium azide and analyzed by gamma radiation counting. The percent specific release was calculated as ((experimental release – spontaneous release)/total release – spontaneous release) × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always <15% of the total counts. Data shown are the mean of triplicate cultures. The SEM of triplicate data was always <20% of the mean.

Results

Expression of wild-type, mutant, and chimeric SV40 T-Ag (T-Ag) in tumor cell lines

We cloned different constructs encoding Ags with the N-terminal, hsp-binding domain of the SV40 T-Ag into expression plasmids. These included 1) the wild-type, nuclear 90-kDa T-Ag (wt-T); 2) a mutant, cytoplasmic 85-kDa cT-Ag from which the NLS110–152 was deleted (cT); and 3) the fusion protein cT272-eGFP in which the N-terminus 1–272 aa of the cT-Ag fragment was fused in-frame to eGFP. The maps of these three Ags with their Dβ- or Kβ-restricted T-Ag epitopes are shown in Fig. 1A.

P815 and Meth-A cells were transfected with plasmid DNA encoding Ags with an N-terminal T-Ag domain. Stable transfectants were generated and cloned in vitro. From the lysate of the stable transfectants, proteins of the expected sizes were immunoprecipitated with an anti-T-Ag mAb that binds the extreme N-terminus of this viral nucleoprotein (Fig. 1, B and C). Anti-T-Ag mAb coprecipitated hsp73 from cells expressing mutant cT-Ag or chimeric cT272-eGFP, but not from cells expressing wt-T-Ag (Fig. 1, B and C). Western blot analyses with a panel of anti-hsp70 mAbs confirmed the association of mutant T-Ag with hsp73, confirming our previously published data (29–31). Tumor cells stably transfected with the cT272-eGFP-encoding plasmid DNA showed green cytoplasmic autofluorescence readily detectable by FCM (Fig. 1C). Hence, we generated a panel of stable transfectants from two different H-2d tumor cell lines that expressed the wild-type, a mutant, or a chimeric form of this viral oncoprotein.

FIGURE 1. Construction and expression of Ags with the N-terminal, hsp-binding domain of the SV40 T-Ag. A. Schematic representation of wt nuclear T-Ag (wt-T), cT-Ag, and chimeric cT272-eGFP Ag (encoding the N-terminal 272 aa of cT and the C-terminus of eGFP). The CTL-defined, Dβ- or Kβ-restricted T-Ag epitopes, hsp73 binding, and deletion of the nuclear localization sequence (NLS) are indicated. B, wt T-Ag (not associated with hsp73) and (hsp73-associated) cT-Ag are stably expressed by transfected P815 cells as detected after immunoprecipitation with mAb PAB108 in Coomassie-stained gels. Western analyses confirmed that the 70-kDa band is hsp73 as described in detail previously (29, 30). C, hsp-associated expression of cT272-eGFP in transfected, radiolabeled Meth-A cells was detected by immunoprecipitation with mAb PAB108 in SDS-PAGE gels (a); eGFP expression by Meth-A cells was confirmed by FCM analysis of transfectants (b).

110 STRESS-PROTEIN-FACILITATED CROSS-PRESENTATION
Uptake of material from apoptotic tumor cells by immature DC

Murine DC were generated from B6 bone marrow progenitors in GM-CSF-supplemented, serum-free cultures using an established protocol (38, 39). The large majority of the CD11c+ DC harvested from day 7 cultures expressed low levels of MHC class II and costimulator (CD40, CD80, CD86) molecules on the surface, indicating an immature phenotype. Only a minor fraction of 10–20% of the DC showed evidence of spontaneous maturation in culture expressing high levels of MHC class II and costimulator molecules on the surface. We measured FITC-dextran uptake by these marrow-derived DC. The majority of the immature (MHC-IIlow) DC, but not the mature (MHC-IIhigh) DC, efficiently endocytosed FITC-dextran at 37°C (Fig. 2A). Fluorescent material from CFSE-labeled, apoptotic P815 or Meth-A tumor cells was efficiently taken up by immature MHC-IIlow (but not mature MHC-IIhigh) DC at 37°C (Fig. 2B). Uptake of FITC-dextran (Fig. 2A) or CFSE-labeled, apoptotic tumor cells (Fig. 2B) was detected at 37°C, but not at 4°C. Hence, immature, metabolically active DC take up FITC-dextran or CFSE-labeled proteins from apoptotic tumor cells. The autofluorescent protein cT272-eGFP was expressed by transfected Meth-A tumor cells (Fig. 1C). This autofluorescent protein was detected in FCM analyses of immature CD11c+ MHC-IIlow DC cocultured with apoptotic, cT272-eGFP-expressing Meth-A cells (Fig. 2C). Uptake of the autofluorescent protein in tumor cell lysates was temperature and dose dependent (Fig. 2D). Hence, a substantial fraction of immature DC takes up hsps, antigenic oncoprotein from apoptotic tumor cells or tumor cell lysates.

FIGURE 2. Uptake of tumor cell-derived material by immature, bone marrow-derived DC in vitro. A, Immature CD11c+ MHC-IIlow DC take up FITC-dextran. DC were incubated with 0.25 mg/ml FITC-dextran for 2 h at either 37 or 4°C. Cells were washed and labeled with either PE-conjugated anti-CD11c or PE-conjugated anti-MHC II mAb. B, CFSE-labeled, apoptotic P815 cells were cocultured with DC (at a ratio of 1:1) for 16 h at 37 or 4°C, harvested, washed twice, labeled with anti-MHC II mAb, and analyzed by FCM by gating on the appropriate population of cells using forward and side scatter. DC cocultured with unlabeled, apoptotic P815 cells showed similar fluorescence intensities than DC cocultured with CFSE-labeled apoptotic P815 cells at 4°C. C and D, Immature DC take up cT272-eGFP from apoptotic (C) or lysed (D) Meth-A cells that express cT272-GFP. C, Apoptotic Meth-A/cT272-eGFP cells were cocultured with DC (at a ratio of 1:1) overnight and analyzed by FCM. The percentage of cells in each quadrant of the dot plots is indicated. A representative experiment of four experiments with similar results is shown. D, Meth-A/cT272-eGFP cells (105) were lysed by four rapid freeze-thaw cycles, the lysates were cleared by centrifugation, and 100 μl of serial dilutions were added to DC for 3 h at 37 or 4°C. DC were harvested, washed twice, and analyzed by FCM. A representative experiment of three experiments with similar results is shown.

Ags from apoptotic tumor cells taken up by DC are cross-presented to MHC class I-restricted CTL

Polyclonal and multispecific (short term) CTLL were generated from vaccinated B6 mice as previously described (37). In an IFN-γ release assay these CTL lines showed specific reactivity against RBL5/T cells expressing T-Ag after transfection or against B6 DC pulsed with T-Ag-derived, antigenic peptides (Fig. 3A). We tested whether DC take up exogenous material from apoptotic tumor cells and cross-present peptides generated by processing these exogenous tumor-associated Ags in the context of MHC class I molecules. CTLL released IFN-γ in response to syngeneic DC that had taken up material from allogeneic, T-Ag-expressing, apoptotic P815 or Meth-A tumor cells (Fig. 3B). DC incubated with material from nontransfected, apoptotic tumor cells did not stimulate cytokine release by CTLL (Fig. 3B). Apoptotic material from nontransfected or transfected tumor cells did not stimulate CTLL in the absence of syngeneic DC (Fig. 3B). IFN-γ release was blocked by mAb to Dα and Kβ, demonstrating the MHC class I restriction of the response (data not shown). When DC were incubated with apoptotic material from transfected, allogeneic tumor cells at 4°C, they did not acquire the capacity to cross-present epitopes to CTL (data not shown). Similarly, paraformaldehyde-fixed DC incubated with apoptotic material from transfected, allogeneic tumor cells at 37°C were not recognized by Kβ/Dα-restricted, T-Ag-specific CTL, although peptide-pulsed, fixed DC stimulated CTL efficiently (Fig. 3C). The release of antigenic peptides by apoptotic tumor cells that bind to surface MHC I molecules of DC is thus not involved in this cross-presentation. Hence, uptake, processing, and
Antigens from apoptotic tumor cells taken up by DC are cross-presented to CTL across an allo-barrier

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**FIGURE 3.** Ags from apoptotic tumor cells taken up by DC are cross-presented to CTL across an allo-barrier. C57BL/6 (H-2^d)-derived DC were cocultured with allogenic, apoptotic (H-2^d)-Meth-A or P815 tumor cells expressing no viral Ag or expressing cT Ag after stable transfection. A. Polyclonal, short-term T-Ag-specific H-2^d T-CTL released IFN-γ when cocultured with either transfected, T-Ag-expressing H-2^d RBL5/T tumor cells or BMDC pulsed with the T-Ag-derived peptides T1, T2/3, and T4. Coculture of CTL with nonpulsed BMDC, BMDC pulsed with the irrelevant (K^b-binding) OVA peptide, or nontransfected RBL5 cells did not stimulate IFN-γ release by the CTL. B. The T-CTL used released IFN-γ when cocultured with H-2^d DC pulsed with H-2^d cT-Ag-expressing Meth-A/cT or P815/cT tumor cells. No IFN-γ was released when CTL were cocultured with allogeneic tumor cells without DC, or when CTL were cocultured with syngeneic DC pulsed with material from nontransfected Meth-A or P815 tumor cells. One experiment of five performed with similar results is shown. C. Paraformaldehyde-fixed BMDC pulsed with T-Ag-derived peptides stimulate IFN-γ release by CTL. No IFN-γ release was observed when CTL were cocultured with nonpulsed, fixed BMDC or fixed BMDC pulsed with either nontransfected or cT-Ag-transfected apoptotic Meth-A tumor cells.

**STRESS-PROTEIN-FACILITATED CROSS-PRESENTATION**

Cross-presentation of T-Ag from apoptotic tumor cells by DC is TAP independent

We tested whether processing of T-Ag-containing material from apoptotic tumor cells by DC for MHC class I-restricted peptide cross-presentation is TAP dependent. DC generated in vitro from marrow of either TAP-competent or TAP-deficient (TAPI^{−/−} knockout) C57BL/6 mice were pulsed with transfected, apoptotic tumor cells (that expressed either wt T-Ag or cT-Ag), washed, and cocultured with T-Ag-specific CTL. Comparable levels of IFN-γ were released by CTL that were cocultured with pulsed, TAP-competent (3150 ± 200 pg/ml) or TAP-deficient (2830 ± 140 pg/ml) DC (data not shown). Similar results were obtained in five independent experiments in which apoptotic (transfected) P815 or Meth-A cells expressing T-Ag were cocultured with TAP-competent or TAP-deficient DC, and the response was detected with different CTL. Hence, processing of apoptotic material from tumor cells for MHC class I-restricted epitope presentation by B6 DC is at least in part TAP independent in the T-Ab system. The data do not exclude the possibility that a minor component of the processing of apoptotic material for class I-restricted epitope presentation by DC is TAP dependent.

hsp-associated (mutant) cT-Ag from apoptotic tumor cells is more efficiently cross-presented by DC than non-hsp-associated (native) T-Ag

We tested whether the relative efficiency of cross-presentation of T-Ag epitopes differs when DC process apoptotic material and lysates from tumor cells that express either native (non-hsp-associated) wt T-Ag, or mutant, hsp-associated cT-Ag. A constant number of DC was pulsed either with titrated numbers of apoptotic wtT-Ag-transfected or cT-Ag-transfected tumor cells or with titrated amounts of lysate from wt T-Ag-transfected or cT-Ag-transfected tumor cells. In the P815 and Meth-A tumor cell systems, material from cT-Ag-expressing tumor cells was 2- to 8-fold more efficient than material from wt T-Ag-expressing tumor cells in supporting cross-presentation of CTL epitopes by DC. This was reproducibly seen in four independent experiments. The expressions of wt T-Ag and cT-Ag by the transfected tumor cell lines at the protein level were comparable (data not shown). Thus, different amounts of Ag expressed by the different tumor cell lines cannot explain the differences in the efficiency of cross-presentation in this system. It is evident that hsp-associated Ag has a superior ability to cross-present epitopes to CTL compared with non-hsp-associated Ag.

Further evidence for an involvement of cT/hsp complexes in facilitating transfer of antigenic information from tumor cells to DC was obtained when we treated lysates from tumor cells with 4 mM ATP before contact with DC (Fig. 5). As shown previously (29), incubation with 4 mM ATP disrupts hsp/cT complexes that

T1, T2/3, and T4 epitopes are presented by transfected P815 and Meth-A tumor cells expressing wt T-Ag or cT-Ag and by B6 DC pulsed with the respective peptides. We tested whether the three CTL-defined T1, T2/3, and T4 epitopes of T-Ag are presented by DC that take up and process material from T-Ag-bearing tumor cells. CTL lines specific for T1, T2/3, or T4 were established in vitro. The K^b or D^b restriction specificity of these lines was confirmed by mAb blocking studies (data not shown). B6-derived DC cocultured with apoptotic P815 or Meth-A cells that expressed T-Ag presented all three tested T-Ag epitopes to CTL (data not shown). Hence, we did not find limitations in the repertoire of epitopes that can be cross-presented by DC processing material from apoptotic T-Ag-bearing tumor cells.
can be immunoprecipitated from the lysate of transfected Meth-A/cT tumor cells (data not shown). Treatment of Meth-A/cT tumor cell lysates with ATP before pulsing DC reduced cross-presentation to the level seen with cross-presentation of the same CTL epitopes by DC pulsed with lysates from Meth-A/T tumor cells (Fig. 5). Hence, disruption of hsp/cT complexes in the lysate of tumor cells impairs the enhanced cross-presentation efficacy.

Specific priming of IFN-γ-producing CD8+ T cells by DNA vaccination is facilitated by hsp-associated T-Ag expression

B6 mice were vaccinated with pCI expression vector DNA encoding either wt T-Ag or (hsp-binding) cT-Ag. Mice were injected once either i.m. with 100 µg nonpackaged DNA or intradermally with 1 µg particle-coated DNA using the gene gun. The frequencies of class I-restricted, T-Ag-specific, IFN-γ-producing CD8+ T cells were measured in the spleen 2 wk postvaccination; spleen cells were restimulated in vitro for 5 h in the presence of BFA with T-Ag-derived peptides, washed, surface stained for CD8, fixed, and intracellularly stained for IFN-γ. The number of IFN-γ-producing CD8+ T cells per 10^5 splenic CD8+ T cells was measured by FCM. The data in Fig. 6 show that 2- to 3-fold more T-Ag-specific CTL were detectable in the spleen of mice that were immunized by cT-expressing plasmid DNA than in those expressing wt T-Ag. This was most striking in vaccinations by the intradermal route, in which Th2-biased immunity usually prevails. The stable association of a viral Ag with hsp73 during its in situ expression thus seems to facilitate priming of CTL.

A growing tumor cross-primes CTL

The nontransfected or transfected mastocytoma cell line P815 (derived from DBA/2 H-2^d mice) was highly tumorigenic in syngeneic hosts; a single s.c. injection of 10^7 cells resulted in rapidly progressing tumors in all transplanted mice (37, 43). Progressive, lethal tumors developed when 10^7 nontransfected or transfected Meth-A cells were injected s.c. into syngeneic BALB/c or F_1 (BALB/c x B6) hosts. Transfer of 10^7 or 10^8 (nontransfected or transfected) Meth-A cells into (semi)syngeneic hosts led to transient tumor growth, followed by subsequent rejection in most adoptive hosts. Spontaneous regression of tumors derived from (transfected or nontransfected) Meth-A tumor cells was dependent on CD8+ and CD4+ T cells, as progressively growing tumors developed in transplanted BALB/c or F_1 hosts depleted of T cells by in vivo Ab treatment (data not shown). Tumors developing in F_1 mice from transfected Meth-A/cT transplants were reproducibly smaller and showed earlier regression (data not shown).

Tumors transiently outgrowing from Meth-A/T and Meth-A/cT transplants cross-primed CTL (Fig. 7, A and B). Transfected H-2^d tumor cells growing in F_1(d x b) hosts primed H-2^b-restricted, T-Ag-specific CTL that specifically lysed T-Ag-expressing H-2^b targets (Fig. 7A). This specific cytolytic reactivity of CTL was lower when it was primed by a growing, T-Ag-expressing tumor.
than when it was primed by DNA-based vaccination. In addition, cross-primed CTL from lymph node or spleen released IFN-γ when specifically restimulated in vitro with T-Ag-expressing H-2b stimulator cells (Fig. 7B). Similar data were obtained when 10⁷ P815/T or P815/cT tumor cells were injected into allogeneic B6 mice (data not shown). These data indicate that Ags expressed endogenously by a tumor growing in vivo can cross-prime a functional CTL response.

A growing tumor expressing hsp-associated cT-Ag more efficiently cross-primes CTL than a growing tumor expressing wild-type T-Ag

The data in Fig. 4 indicate that Meth-A/cT tumor cells cross-stimulate T-Ag-specific CTL responses in vitro more efficiently than Meth-A/T tumor cells. Tumor regression was dependent on CD8⁺ T cells. F₁ mice transplanted with Meth-A/cT tumor cells showed less aggressive tumor growth and early regression than F₁ mice transplanted with Meth-A/T tumor cells. This indicates that tumor growth-controlling T-Ag-specific CTL are primed in the system. The frequencies of K⁺- or D⁺-restricted CD8⁺ T cells specific for the T1, T2/3, and T4 epitopes of the T-Ag cross-primed by a growing tumor were determined directly ex vivo. Spleen cells from F₁ mice that rejected a nontransfected Meth-A, transfected Meth-A/T, or transfected Meth-A/cT tumor or from mice that were vaccinated with a pCI/T DNA vaccine were restimulated in vitro for 5 h with the T1, T2/3, or T4 peptide, washed, surface stained for CD8, fixed, and intracellularly stained for IFN-γ. The number of IFN-γ-producing CD8⁺ T cells per 10⁵ splenic CD8⁺ T cells was determined by FCM. In Meth-A/cT tumor-bearing F₁ hosts we detected a larger number of T-Ag-specific CTL than in Meth-A/T tumor-bearing animals (Fig. 8). The data shown in Fig. 8 demonstrate that 2- to 3-fold more CTL specific for all three T-Ag epitopes tested were detected in the spleen of mice that rejected Meth-A/cT tumors compared with mice that rejected Meth-A/T tumors. These data confirm in a direct ex vivo readout that hsp-associated cT-Ag more efficiently cross-primes CTL in vivo than native T-Ag not associated with hsp. Taken together, these data indicate that the association of Ag with hsp facilitates in vitro and in vivo cross-priming of CTL to Ag endogenously expressed by tumor cells.

FIGURE 7. Cross-presentation of T-Ag epitopes by tumor cells growing in vivo. Transfected or nontransfected Meth-A cells s.c. transferred into F₁ hosts (10⁶ cells/mouse) induced transient tumor growth. Spleen cells or lymph node (LN) cells from rejecting mice were restimulated in vitro with RBL5/T cells. A, Splenic CTL derived from F₁ mice transplanted with 10⁶ transfected (but not nontransfected) Meth-A cells lysed RBL5/T targets; nontransfected RBL5 targets were not lysed (data not shown). Splenic CTLs from F₁ mice immunized with pCI/T DNA vaccine were used as a positive control. Data from a representative experiment from three independent experiments are shown. B, IFN-γ release of spleen and LN cells from mice transplanted with 10⁶ Meth-A/cT cells and cocultured in vitro with RBL5/T cells or RBL5 cells.

FIGURE 8. H-2b tumors expressing hsp-associated T-Ag cross-prime T-Ag-specific, H-2b-restricted, IFN-γ-producing CD8⁺ T cells efficiently. T-Ag-specific CD8⁺ T cell frequencies were determined in spleens of F₁ mice transplanted with wtT-Ag- or cT-Ag-expressing Meth-A cell transfectants. Mice transplanted with nontransfected Meth-A cells were the negative controls; mice immunized with pCI/T were the positive controls. Spleen cells (4 × 10⁶) were incubated with 5 μg/ml T1, T2/3, or T4 peptide (in the presence of BFA) for 5 h before the surface staining for CD8 and intracellular staining for IFN-γ. We analyzed 10⁵ CD8⁺ T cells for IFN-γ expression. Double-positive CD8⁺ IFN-γ⁺ T cells were considered peptide-specific T cells. The mean number of double-positive IFN-γ⁺ CD8⁺ T cells per 10⁵ splenic CD8⁺ T cells ± SD of three individual mice are shown.
Discussion

The amino-phospholipid translocase-dependent appearance of phosphatidylserine in the outer leaflet of the membrane of cells is an early event signaling apoptosis (44). Apoptotic cells are rapidly cleared by macrophages and DC that recognize them through platelet-activating factor- and TGF-β-dependent phosphatidylserine recognition (45), CD36 (46), αvβ3 (15) or αvβ5 integrins (46), C-type lectin receptors, or Fc receptors (47). Uptake of apoptotic cells or their remnants can operate through phagocytosis (15, 48), endocytosis (17, 33), or macropinocytosis. Immature DC are most efficient in engulfing remnants from apoptotic cells (15, 33), which is confirmed by our data described in Fig. 2. Although Ag-containing material can be transferred between viable DC (18, 49), and viable cell may transfer antigenic material to DC (18, 50–52), we detected no uptake of fluorescent protein by DC cocultured with viable, transfected Meth-A/ct2722-eGFP or P815/ct2722-eGFP tumor cells (data not shown).

Uptake of exogenous antigenic material by APC can lead to processing and class I-restricted cross-presentation of epitopes to CTL. Although macrophages efficiently take up apoptotic cells or their remnants, they are deficient in cross-presenting Ags from apoptotic cells to CTL (3, 15, 53). Exceptions to this rule have been reported (48). Lympohid (CD8α−), but not myeloid (CD8α+), DC have been shown to cross-prime CTL (54). We used myeloid DC generated in vitro from bone marrow for our cross-presentation experiments. Processing of exogenous material from apoptotic cells for class I-restricted peptide presentation is in some Ag systems apparently TAP dependent (17, 48, 55). Our data indicate that cross-presentation of Kb- and Dβ-binding T-Ag peptides by DC is at least partially TAP-independent, confirming reports of TAP-independent cross-priming of CTL in a number of well-defined Ag systems (reviewed in Ref. 32). The nature of the Ag and/or the functional state of the APC may play a role in regulating TAP-dependent vs -independent epitope presentation. Uptake of exogenous material from cells by DC stimulates CD4+ T cell responses (50, 56), and cross-primming CTL responses by apoptotic tumor cells in vivo has been shown to be CD4+ Th cell dependent (1, 5). Our data described here and published previously (37, 43) demonstrate that the rejection of Meth-A and P815 tumors is CD4+ and CD8+ T cell dependent, confirming the helper dependence of cross-primed CTL responses. We found that all three epitopes of the T-Ag (T1, T2/3, T4) that bind to D6 or K6 molecules are cross-presented to CTL in vitro and in vivo in the investigated tumor system. This is in contrast to the TAP-independent presentation of endogenously expressed, hsp-associated T-Ag. We have described that the two D6-restricted, but not the K6-restricted, T-Ag epitopes were efficiently expressed by TAP-deficient, transfectected tumor cells (30). The described data indicate that all CTL-defined antigenic information of a viral onconege expressed endogenously by tumor cells can be transferred by apoptotic tumor cells to immature DC, processed, and cross-presented in the context of MHC class I molecules to CTL.

Cross-presentation of CTL-defined epitopes does not usually lead to cross-priming of T cells, but often induces tolerance (reviewed in Refs. 1 and 14). Maturation of immunostimulatory DC could be induced by pulsing with material derived from necrotic tumor cells, but not primary tissue cells or apoptotic cells, suggesting that necrosis (but not apoptosis) provides a control critical for the initiation of immunity (6). Different anti-inflammatory effects of apoptotic cells have been described. CD95-mediated apoptosis of lymphoid cells leads to the rapid production of IL-10 (57, 58). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production by the release of TGF-β, PGs, and platelet-activating factor (53). C-reactive protein and the classical complement components can promote noninflammatory clearance of apoptotic cells (59). In contrast, cytokines and CD40-dependent signals can stimulate maturation of immunostimulatory DC that process engulfed material from apoptotic cells (5, 59). We have demonstrated in vivo that CTL are cross-primed against T-Ag epitopes in mice by growing Meth-A or P815 tumors. It is unknown which signals drive the maturation of DC into an immunostimulatory phenotype in this system. Stress proteins are known to induce DC maturation and to be effective adjuvants for vaccines (60–65). We propose that the associating of an onconege with hsp facilitates the generation of immunostimulatory DC required for cross-priming CTL and preventing induction of tolerance.

Evidence has been reported that the stress protein gp96 participates in the cross-presentation of Ags of cellular origin (64). An hsp70-like chaperone is involved in cross-priming T cell immunity by DNA vaccination (63). hsp70 released from dying tumor cells and taken up directly by DCs may be involved in direct chaperoning Ags into DCs (60). Exosomes, i.e., vesicles secreted by DC upon fusion of late multivesicular endosomes with the plasma membrane, induce potent anti-tumor immune responses in mice, resulting in the regression of established tumors (34). A major exosome component is hsp73 (hsc70) (33). hsp73 stably associated with mutant or truncated T-Ag seems to play a role in Ag chaperoning. We have shown that stable, noncovalent binding of mut- tant, truncated, or chimeric T-Ag to hsp73 enhances expression, facilitates access of the complexes to an alternative (TAP-independent) processing pathway for MHC class I-restricted peptide presentation, and supports priming of Ab responses against endog- enous Ags when used as a DNA vaccine (reviewed in Ref. 32). Here we report a novel feature of the system: DC-dependent cross-priming of CTL was facilitated in vitro and in vivo by associating a viral onconege with hsp73. Although suggestive evidence for a role of hsp molecules of the 70- and 90-kDa family is available from different experimental systems, the molecular mechanism underlying these phenomena remains to be elucidated.

Is it the mutation, but not the hsp association, of T-Ag that facilitates cross-presentation of its CTL epitopes in the tumor model we studied? Mutant or truncated Ag (fragments) may give rise to defective ribosomal products that may represent a major source of antigenic peptides for MHC class I molecules (66). We have shown that expression of C-terminal fragments of the T-Ag (that contain some of the CTL epitopes studied here) is difficult to achieve and does not lead to presentation of CTL epitopes (29, 30). Although not formally excluded, a mutation or truncation per se thus does not seem to convey to an Ag a greater efficacy to cross-present its epitopes.

Our tumor transplantation experiments indicate that mice with a transiently growing tumor expressing hsp-associated mutant viral Ag contain 3-fold more onconege-specific CTL than mice carrying a tumor expressing the native (non-hsp-associated) variant of the onconege. Class I-restricted CTL against all three epitopes tested were more frequent in mice carrying tumors expressing the hsp-bound CT-Ag variant. This was seen in transplantation experiments using transfected Meth-A or P815 tumor cells. The Meth-A cell inoculum of 105 cells/mouse allowed transient tumor growth followed by rejection of the tumor in >90% of the transplanted mice. All mice injected with 105 (transfected or nontransfected) Meth-A cells developed progressively growing, lethal tumors. Mice that had rejected a (transfected or nontransfected) Meth-A tumor transplant also rejected an inoculum of 107 Meth-A cells, suggesting priming of tumor-associated Ag-specific immunity (data not
shown). Tumor-draining DC have been shown to efficiently cross-prime CTL in vivo to tumor-associated Ags, although the tumor grows progressively and eventually kills its host. This suggests failure to prevent tumor growth in the effector phase, but not in the induction phase (67). In addition to facilitating Ag transfer between tumor cells and DCs and making DC immunostimulatory, additional immunomodulatory protocols will be required to make CTL-mediated tumor rejection more efficient.

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