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Hyperthermia Enhances CTL Cross-Priming

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Dendritic cells (DCs) loaded with killed allogeneic melanoma cells can cross-prime naive CD8⁺ T cells to differentiate into melanoma-specific CTLs in 3-wk cultures. In this study we show that DCs loaded with killed melanoma cells that were heated to 42°C before killing are more efficient in cross-priming of naive CD8⁺ T cells than DCs loaded with unheated killed melanoma cells. The enhanced cross-priming was demonstrated by several parameters: 1) induction of naive CD8⁺ T cell differentiation in 2-wk cultures, 2) enhanced killing of melanoma peptide-pulsed T2 cells, 3) enhanced killing of HLA-A*0201 melanoma cells in a standard 4-h chromium release assay, and 4) enhanced capacity to prevent tumor growth in vitro in a tumor regression assay. Two mechanisms might explain the hyperthermia-induced enhanced cross-primming. First, heat-treated melanoma cells expressed increased levels of 70-kDa heat shock protein (HSP70), and enhanced cross-primming could be reproduced by overexpression of HSP70 in melanoma cells transduced with HSP70 encoding lentiviral vector. Second, hyperthermia resulted in the increased transcription of several tumor Ag-associated Ags, including MAGE-B3, -B4, -A8, and -A10. Thus, heat treatment of tumor cells permits enhanced cross-primming, possibly via up-regulation of both HSPs and tumor Ag expression.

Dendritic cells (DCs) loaded with killed allogeneic melanoma cells can cross-prime naive CD8⁺ T cells to differentiate into melanoma-specific CTLs in 3-wk cultures. In this study we show that DCs loaded with killed melanoma cells that were heated to 42°C before killing are more efficient in cross-priming of naive CD8⁺ T cells than DCs loaded with unheated killed melanoma cells. The enhanced cross-priming was demonstrated by several parameters: 1) induction of naive CD8⁺ T cell differentiation in 2-wk cultures, 2) enhanced killing of melanoma peptide-pulsed T2 cells, 3) enhanced killing of HLA-A*0201 melanoma cells in a standard 4-h chromium release assay, and 4) enhanced capacity to prevent tumor growth in vitro in a tumor regression assay. Two mechanisms might explain the hyperthermia-induced enhanced cross-primming. First, heat-treated melanoma cells expressed increased levels of 70-kDa heat shock protein (HSP70), and enhanced cross-primming could be reproduced by overexpression of HSP70 in melanoma cells transduced with HSP70 encoding lentiviral vector. Second, hyperthermia resulted in the increased transcription of several tumor Ag-associated Ags, including MAGE-B3, -B4, -A8, and -A10. Thus, heat treatment of tumor cells permits enhanced cross-primming, possibly via up-regulation of both HSPs and tumor Ag expression.


The use of hyperthermia in the therapy of cancer has been an object of clinical interest for many years (1). Hyperthermia seems to be particularly effective in combination with radiotherapy and/or radioimmunotherapy (reviewed in Refs. 2–4). The molecular mechanism by which hyperthermia leads to radiosensitization is not clear; however, activation of early response genes, heat shock factors, and subsequently heat shock proteins (HSPs) has been proposed to play a role (4, 5). HSPs constitute a superfamily of distinct proteins that are operationally named according to their molecular mass, e.g., 70-kDa HSP (HSP70). Most HSPs are expressed constitutively and are further induced under stress conditions, including temperature increase. HSPs chaperone newly synthesized polypeptides during protein folding and translocation to intracellular compartments and are important in the clearance of unfolded or improperly folded proteins (6, 7). In the context of the immune system, HSPs transfer antigenic peptides, thereby facilitating peptide presentation to CD8⁺ T cells (8–11). In this process, HSP70- or gp96-peptide complexes are internalized by APCs, including DCs (12, 13), through receptor-mediated endocytosis via CD91 (14, 15), scavenger receptor A (16), CD40 (17), lectin-like oxidized low-density lipoprotein receptor 1 (18), or TLR2/4 (19). Thus, we surmised that hyperthermia might contribute to tumor regression through enhanced cross-primming. In this study we show that loading dendritic cells (DCs) with killed melanoma cells that were heated before killing considerably enhances their immunogenicity and facilitates cross-primming of melanoma-specific CTLs.

Materials and Methods

Cell lines

HLA-A*0201 Me275 and HLA-A*0201 Me290 melanomas were gifts from Drs. J.-C. Cerottini and D. Rimoldi (both from Ludwig Institute for Cancer Research, Lausanne, Switzerland). HLA-A*0201 SK-Mel24, HLA-A*0201 SK-Mel28, and T2 were obtained from American Type Culture Collection. Cell lines were maintained in RPMI 1640 (Invitrogen Life Technologies), 1% L-glutamine, 1% penicillin/streptomycin, and 10% heat-inactivated FCS. For T cell culture, FCS was replaced by 10% heat-inactivated human AB serum.

Reagents

GM-CSF (Berlex), soluble CD40L, IL-2, IL-7 and IL-4 (all from R&D Systems). Betulinic acid (BA) and DNA dye 7-aminoactinomycin D were purchased from Sigma-Aldrich. Peptides gp100209–217 (IMDQVPFSV), tyrosinase244–251 (YMDGTMSSQV), MART127–35 (AAGIGILTV), and NY-ESO123–33 (YMDGTMSQV) were synthesized by BioSynth. The gene vector Production Network.

Preparation of heat shock-killed melanoma cells

Melanoma cell lines were plated at 3 × 10⁵/ml and, after 24-h culture at 37°C, cultured at 42°C for 2 or 4 h, followed by exposure to 10 μg/ml BA for additional 24 h at 37°C. Without heating, cells were treated with 10 μg/ml BA for 48 h. Annexin V and PI staining was used to measure the death of tumor cells.

Determination of HSP expression

Cells were collected, washed twice with cold PBS, resuspended with lysis buffer supplemented with protease inhibitor mixture (0.1 mM PMSF, 1
μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin), and incubated on ice for 30 min with occasional mixing. The cell lysate was centrifuged at 12,000 rpm for 20 min at 4°C. HSP70 levels in the supernatant were detected by ELISA kit (Stressgen). The total cell protein in supernatant was examined with a Micro BSA protein assay reagent kit (Pierce). The HSP70 concentration in cell lysates is expressed as micrograms per milligram of protein in the supernatant.

**Confocal microscopy assay**

Cells were washed, harvested with PBS, mounted onto polylysine-pre-treated slides, fixed with 4% paraformaldehyde, and permeabilized with 0.5% saponin. Cells were stained with PE-conjugated CD8 mAb and analyzed using a FACSCalibur.

**Lentiviral vectors and transduction**

The HSP70-encoding lentiviral vector was derived from pRLRpkgEGFPp-sin18 (20) by replacing the enhanced GFP (EGFP) cDNA with a bicistronic unit encoding the HSP70 cDNA placed under control of the human phosphorilase kinase promoter and the EGFP cDNA using an internal ribosomal entry site strategy. The production of HIV-1-derived vectors was described previously (21). No replication-competent virus was detected in the concentrated lentiviral stocks. Target cells were exposed for 24 h to lentivector preparations with a multiplicity of infection of 10 in a volume of 500 μl in the presence of 4 μg/ml polybrene (Sigma-Aldrich). EGFP+ cells were then sorted by FACSVantage (BD Biosciences).

**Monocyte-derived DCs generation and Ag loading**

Monocytes were enriched by adherence from nonmobilized or G-CSF-mobilized PBMCs of HLA-A*0201+ healthy volunteers and cultured in RPMI 1640 supplemented with 10% FCS, GM-CSF (100 ng/ml), and IL-4 (25 ng/ml). Day 5 immature DCs were labeled with CD11c-allophycocyanin for 30 min at 4°C and coincubated with killed melanoma cells at a 1:1 ratio at 37°C. After 3 h, cells were washed with 0.05% trypsin/0.02% EDTA/PBS solution, CD11c+ DCs were sorted, matured with soluble CD40L (25 ng/ml). Day 5 immature DCs were labeled with CD11c-allophycocyanin and MHC class I tetramers (Milenyi Biotec). The enriched CD8+ T cells were sorted as CD8+CD45RA–CD27–CD45RO+ naive T cells (>95% purity) and cocultured with unloaded or loaded DCs at a 10:1 ratio supplemented with 10 IU/ml IL-7 in the first week and IL-2 in the second week. T cells were restimulated on day 7.

**51Cr release assay**

Targets were labeled with Na51CrO4 for 1 h at 37°C. T2 cells were pulsed with four melanoma peptides (gp100, Tyr, MART1, and MAGE3) for 3 h before labeling. A 4-h standard killing assay was performed as described previously (22). For blocking, 51Cr-labeled targets were coincubated with 10 μg/ml purified mouse anti-human HLA-ABC mAb (clone W6/32; DakoCytomation), HLA-DR mAb (clone G46-6; BD Biosciences), or matched mouse IgG isotypes (clones G155-157 or G46-6; BD Biosciences) in a 96-well plate for 30 min. The mean of triplicate wells for each sample was calculated, and the percentage of specific 51Cr release was determined according to the following formula: % specific 51Cr release = 100 × (experimental 51Cr release – spontaneous release)/(maximum 51Cr release – spontaneous release).

**Tumor inhibition assay**

Tumor cell lines were transduced with lentiviral vector encoding EGFP as described previously (23). Cell lines were suspended at a concentration of 5 × 105/ml with RPMI 1640 medium containing 10% AB serum. Primed T cell lines were suspended at 106/ml. Targets and T cells were coincubated in a 96-well, U-bottom plate for 0, 4, 24, 48, and 72 h in a 200-μl total volume. At each time point, cell mixture was harvested and washed with 0.05% trypsin/0.02% EDTA/PBS solution for 5 min. Cell pellets were stained with PE-conjugated CD8 mAb and analyzed using a FACSCalibur. Trypan blue exclusion was used to count live cells using light microscopy.

**Tetramer staining**

The iTag MHC HLA-A0201 tetramers gp100(IMDQVPFSV), MAGE3 (FLWGPRALV), tyrosinase (YMDGTMSQV), MART1 (ELAGIGILTV), and MAGEA10 (GLYDGMEHL) were purchased from Beckman Coulter. Primed T cell lines were stained with PE-conjugated tetramer for 30 min and with anti-CD8 or anti-CD3 mAb for another 30 min at room temperature.

**Recall assay**

After two stimulations with melanoma body-loaded DCs, CD8+ T cells were plated with peptide-pulsed autologous DCs at a 10:1 ratio. T cells were analyzed after 7 days of culture.

**Microarray analysis**

Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions, and RNA integrity was assessed using an Agilent 2100 bioanalyzer. From 5 μg of total RNA, double-stranded cDNA containing the T7-dT (24) promoter sequence (Operon) was generated as a template for in vitro transcription, single-round amplification with biotin template for in vitro transcription, single-round amplification with biotin

![FIGURE 1.](http://www.jimmunol.org/) Priming of CTLs able to kill melanoma cell lines. a, 51Cr release from HLA-A*0201 Me275 melanoma cells and control K562 cells after 4-h coculture with primed HLA-A*0201+ CD8+ T cells. CTLa, T cells cultured for 2 wk with cold Me275 body-loaded DCs; CTLb, T cells cultured for 2 wk with hot Me275 body-loaded DCs. Shown are the mean and SD of three experiments. b, 51Cr release from HLA-A*0201 Me290 melanoma cells. The T cells are the same as in a. Shown are the mean and SD of three experiments. c, T cells primed by DCs loaded with cold Me275 cells (CTLcold) or hot Me275 cells (CTLhot) were cocultured with Me290-EGFP target cells at a 20:1 ratio for 4, 24, 48, or 72 h. Viable tumor cells were counted by trypan blue exclusion using light microscopy. Shown are the mean and SD of three experiments.
the percentage of double-positive population (CD8+ Tetramer+) in the total CD8 population (CD8+).
b. Primed T cells were restimulated once by DCs pulsed with PSA-A (CTLm-2R/PSA+DCs) and analyzed 7 days after restimulation. c. Primed T cells were restimulated once by DCs pulsed with each of the four melanoma peptides (CTLm-2R/Me1+DCs) and analyzed 7 days after restimulation. The data shown are representative of two experiments.

**FIGURE 2.** Priming of melanoma-specific CTLs: tetramer binding assay. Priming was performed against HLA-A*0201+ Me290 cells as described in Fig. 1. a. Tetramer staining on day 7 after the second stimulation; 50,000 cells were acquired for each sample. Shown is the percentage of double-positive population (CD8+ Tetramer+) in the total CD8 population (CTLm-2R).

**Real-time PCR**

RT-PCR was conducted with Applied Biosystems predesigned assays for MAGE-B3, MAGE-B4, MAGE-A8, and MAGE-A10 using the ABI PRISM 7700 sequence detection system (Applied Biosystems). A GAPDH predesigned control reagents kit was used for the endogenous control. Reactions were set up in duplicate using the One-Step RT-PCR Kit (Applied Biosystems) according to the manufacturer’s recommended protocol with 100 ng of total RNA. Cycling conditions were as follows: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, then 60°C for 1 min. Relative expression of target genes was calculated using the comparative threshold cycle method according to ABI’s protocol.

**Two-dimensional fluorescence difference gel electrophoresis (2-D DIGE)** (24)

Heated and unheated bodies were solubilized in whole-cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, and 5 mM magnesium acetate), followed by a 10,000 × g spin for 10 min at 4°C. The protein concentration was determined by bicinchoninic acid protein assay. Six hundred micrograms of each sample was precipitated and desalted using the ReadyPrep 2D clean up kit (Bio-Rad). Uniform protein labeling was achieved by means of CyDye DIGE Fluros using the minimal dye labeling protocol (Amersham Biosciences). Briefly, 200-μg samples from heated and unheated bodies were resuspended in whole-cell lysis buffer and individually labeled with Cy3 (200 pmol) or Cy5 (200 pmol) dyes, respectively. Similarly, 100 μg of each sample was pooled and labeled with Cy2 (200 pmol). Labeling occurred on ice in the dark for 30 min; reactions were quenched with the addition of 10 mM lysine. Sixty-six micrograms of each labeled sample was then combined and allowed to rehydrate an 11-cm Ready Strip IPG strip (Bio-Rad; pH 3–10) overnight at room temperature in the dark. Hydrated strips were loaded onto a PROTEAN isoelectric focusing cell (Bio-Rad) and separated in the first dimension overnight. Second-dimension separation was achieved by electrophorating the samples from the strips onto 10–15% gradient second-dimension SDS-polyacrylamide gels. All samples were run in triplicate. Gels were scanned using a Molecular Imager FX Pro Plus Phospho/Fluorimager (Bio-Rad) equipped with three lasers (488, 532, and 635 nm). Individual fluorescent images were overlaid and assessed using PDQuest 2D software (Bio-Rad). Spots were excised labels, using the Enzo RNA Transcript Labeling Kit (Affymetrix). Biotinylated cRNA targets were purified using the sample cleanup module and subsequently hybridized to human Affymetrix U133A GeneChips according to the manufacturer’s standard protocols. GeneChips containing 22,283 probe sets, represented by 10–20 unique probe pairs (perfect match and its corresponding mismatch), allowed detection of 14,500 different genes and established sequence tags. Arrays were scanned using a laser confocal scanner (Agilent). For each chip, raw intensity data were normalized to the mean intensity of all measurements on that array and scaled to a target intensity value of 500 (TGT) in Affymetrix Microarray Suite 5.0. With the aid of GeneSpring software version 6.1, the measurement for each gene per sample array was divided by the median of that gene’s measurement from all samples in the GeneSpring experiment. Then a raw data cutoff was applied at 100 to be above the background threshold.

**FIGURE 3.** Cross-priming of melanoma-specific CTLs. a. Priming against HLA-A*0201+ Me290 cells was performed as described in Fig. 1. 51Cr release was measured from T2 cells pulsed with a mixture of the four melanoma peptides (MART-1/Melan A, gp100, tyrosinase, and MAGE-3 (T2 + 4P)) or a control PSA peptide (T2 + PSA) or were used unpulsed (T2). Shown are the mean and SD of three experiments. b, TIA with T cells as described in a; the growth rates of peptide-pulsed T2-EGFP cells were calculated using the following formula: % growth rate = (% EGFP+/population at time points)/% EGFP+ population at 0 h) × 100%. The tumor growth rate at 0 h was defined as 100%. Results represent the mean value of three experiments. c, Priming against HLA-A*0201+ mimicked cells was performed as described in Fig. 1. 51Cr release data are the mean and SD of three experiments.
from a SYPRO Ruby (Bio-Rad)-stained companion gel run in parallel with 200 μg of either heated or unheated unlabeled lysates and subjected to a limited tryptic digest and identified by MALDI-TOF (Applied Biosystems 4700 MALDI-ToF; University of Georgia Proteomics Facility).

Results

DCs loaded with hot melanoma bodies rapidly yield CTLs able to kill melanoma cells

We have previously shown that naive CD8+ T cells require three rounds of stimulation with DCs loaded with killed melanoma cells to generate detectable melanoma-specific CTLs, as determined by the killing of melanoma cells in a chromium release assay (25). However, it took only two rounds of stimulation to generate melanoma-specific CTLs when HLA-A*0201 CD8+ T cells were stimulated with autologous HLA-A*0201 DCs loaded with killed melanoma cells that were heated for 4 h at 42°C (hot bodies; n = 3; Fig. 1a). Primed CD8+ T cells also killed HLA-A*0201+ Me290 melanoma cells (n = 3; Fig. 1b), suggesting priming against Ags shared between these melanoma cell lines. The killing was CTL mediated, because no lysis of NK-sensitive K562 cells was found. Killing of melanoma cells was restricted by their expression of MHC class I, because pretreatment of target cells with MHC class I-blocking mAb W6/32 resulted in >60% inhibition of Me275 and Me290 killing at different E:T cell ratios (not shown).

The enhanced priming was confirmed in the tumor inhibition assay (TIA) (23). CD8+ T cells primed with hot Me275 melanoma body-loaded DCs were considerably more efficient than those primed with unheated cold Me275 body-loaded DCs in controlling the growth/survival of Me290 melanoma cells, as determined by trypan blue exclusion assay and viable cell count using light microscopy (Fig. 1c). When CD8+ T cells primed with hot melanoma body-loaded DCs were cocultured with EGFP-expressing Me290 melanoma cells, the fraction of EGFP+ melanoma cells was >80% decreased after 4 h of culture compared with an ~20% decrease in cocultures with CD8+ T cells primed with cold melanoma body-loaded DCs (not shown). The observed decrease in the fraction of viable tumor cells was specific to melanoma,

FIGURE 4.

Overexpression of HSP70. Heated and unheated melanoma cells were lysed in 8 M urea under reducing conditions. Proteins from heated and unheated bodies were labeled with the fluorescent Cy-3 and Cy-5 dyes, respectively. Labeled lysates were combined and run on a broad-range (pI 3–10) isoelectric focusing IPG strip, followed by 10–15% gradient second-dimension SDS-polyacrylamide gels in tricine. a, Gels were scanned using a Molecular FX Pro-Plus multiamaging system to resolve the Cy3-heated (red) and Cy5-unheated (green) samples. Proteins whose expression remained unchanged by heating appear yellow after overlay, proteins whose expression was down-regulated by heating appear green, and those proteins up-regulated by heating appear red. b, Predicted peptides from mass spectra fragmentation pattern identified using Applied Biosystems 4700 MALDI-TOF mass spectrometer of most differentially expressed protein in heated vs unheated Colo cell bodies. c, Fluorescence staining of HSP70 (red) and confocal microscopic analysis of Me290 cells either unheated (37°C) or heated at 42°C for 2 and 4 h. d, ELISA analysis of HSP70 protein expression in cell lysates relative to the total protein amount (ordinate) in heated melanoma cells.
because the survival/growth of NK-sensitive K562 cells was not altered (not shown). Thus, loading DCs with melanoma cells exposed to hyperthermia enhances their immunogenicity, because only two stimulations were necessary to induce naive CD8\(^+\) T cell differentiation into melanoma-specific CTLs.

**DCs loaded with hot melanoma bodies promptly prime CD8\(^+\) T cell-binding peptide-MHC class I tetramers**

To determine the specificity of primed CD8\(^+\) T cells, we analyzed their ability to bind melanoma peptide-MHC class I tetramers. As shown in Fig. 2, after two stimulations with hot HLA-A\(^*\)0201\(^+\) Me290 melanoma body-loaded DCs, 0.42% of CD8\(^+\) T cells were specific for MART-1/Melan A (Fig. 2a). However, other specificities could be barely detected upon acquisition of 5 \(\times\) 10\(^4\) CD8\(^+\) T cells for analysis. At least two explanations could be considered: 1) the T cells were primed only against MART-1; or 2) the elicited repertoire is broad, but at the low frequency for a given peptide, and therefore it may be difficult to detect a T cell with a particular specificity. To address this, we have analyzed the presence of recall memory CD8\(^+\) T cells. To this end, naive CD8\(^+\) T cells were primed in 2-wk cultures with DCs loaded with hot Me290 melanoma bodies. On day 7 after the second stimulation, the T cells were washed, restimulated with autologous DCs pulsed either with each of the four melanoma peptides or with a control PSA peptide and analyzed after an additional 7 days of culture. As shown in Fig. 2b, the frequency of melanoma tetramer-binding CD8\(^+\) T cells did not change after restimulation with PSA peptide-pulsed DCs. However, a boost with melanoma peptide-pulsed DCs resulted in the expansion of melanoma-specific CD8\(^+\) T cells (Fig. 2c). Thus, the frequency of MART-1/Melan A tetramer-binding CD8\(^+\) T cells increased from 0.42 to 1.49%, and T cells with other specificities were clearly detectable: 0.35% MAGE-3-specific CD8\(^+\) T cells, 0.25% gp100-specific CD8\(^+\) T cells, and 0.16% tyrosinase-specific CD8\(^+\) T cells. Similar results were obtained in two experiments with CD8\(^+\) T cells primed against hot HLA-A\(^*\)0201\(^+\) Me290 melanoma bodies and when HLA-A\(^*\)0201\(^+\) T cells were cross-primed against HLA-A\(^*\)0201\(^+\) SK-Mel28 melanoma cells (data not shown). Thus, DCs loaded with hot bodies were more efficient in priming melanoma-specific CD8\(^+\) T cells than DCs loaded with cold bodies.

**DCs loaded with hot melanoma bodies cross-prime melanoma-specific CTLs**

We next determined whether the CD8\(^+\) T cells primed with hot body-loaded DCs can kill T2 cells pulsed with peptides derived from melanoma differentiation Ags: MART-1/Melan A, gp100, tyrosinase, and MAGE-3. CD8\(^+\) T cells primed with hot HLA-A\(^*\)0201\(^+\) Me290 melanoma bodies killed melanoma peptide-pulsed T2 cells with 40% specific lysis in a \(^{51}\)Cr release assay at an E:T cell ratio of 40:1 (Fig. 3a). The killing was specific, because T2 cells pulsed with a control PSA peptide were not killed. As expected, CD8\(^+\) T cells primed with cold Me290 melanoma body-loaded DCs were unable to kill peptide-pulsed T2 cells (Fig. 3a). The capacity of primed CD8\(^+\) T cells to recognize melanoma Ags was also confirmed in the TIA. There, CD8\(^+\) T cells from 2-wk cultures with loaded DCs were cocultured with EGFP-expressing T2 cells that were unpulsed, pulsed with control PSA peptide, or pulsed with a mixture of the four melanoma peptides. Primed CD8\(^+\) T cells induced an \(\sim\)50% decrease in the fraction of EGFP\(^+\) melanoma peptide-pulsed T2 cells after 4-h coculture, and the fraction of EGFP\(^+\) T2 cells remained low over 48 h of coculture (Fig. 3b). This effect was specific, because the survival of control T2 cells (either unpulsed or PSA pulsed) was not altered (Fig. 3b and not shown). Again, CD8\(^+\) T cells primed with hot melanoma body-loaded DCs were more efficient than those primed with cold melanoma body-loaded DCs (Fig. 3b). Indeed, after 2-wk culture, the latter were unable to control the survival/growth of melanoma peptide-pulsed T2 cells in three independent experiments (Fig. 3b).

Induction of melanoma-specific CTLs was confirmed in a cross-priming experiment. There, HLA-A\(^*\)0201\(^+\)CD8\(^+\) T cells were stimulated twice with DCs loaded with hot melanoma bodies derived from HLA-A\(^*\)0201\(^+\) SK-Mel28 cells. As shown in Fig. 3c, primed CD8\(^+\) T cells killed melanoma peptide-pulsed T2 cells with 48 \pm\% specific lysis (E:T cell ratio, 40:1; \(n = 3\)), but not PSA peptide-pulsed T2 cells, indicating cross-priming. Thus, loading DCs with melanoma cells exposed to hyperthermia enhances the cross-priming of melanoma-specific CTLs.

**Enhanced cross-priming is associated with overexpression of HSP70**

Heat treatment has long been shown to induce the expression of HSP, which have been shown to transfer tumor-associated peptides over 104 CD8\(^+\) T cells were transduced with HSP70 encoding vector-transduced SK-Mel28 melanoma cells (data not shown). Thus, DCs loaded with hot bodies were more efficient in priming melanoma-specific CD8\(^+\) T cells than DCs loaded with cold bodies.

**FIGURE 5.** Overexpression of HSP70 in melanoma cells results in enhanced cross-priming. a, EGFP expression in nontransduced, control vector-transduced, and HSP70 encoding vector-transduced SK-Mel28 melanoma cells. Confocal microscopy and flow cytometric analysis were performed. b, ELISA measurement of HSP70 in lysates of transduced cells. c, Priming against HLA-A\(^*\)0201\(^+\) SK-Mel28 cells was performed as described in Fig. 3d. Killing of melanoma peptide-pulsed T2 cells was determined. T cells were cultured with unloaded DCs (CTL1), DCs loaded with killed melanoma cells transduced with control vector (CTL2), or DCs loaded with killed melanoma cells transduced with HSP70-expressing vector (CTL3).
To assess the difference in the proteomic profile of heat-treated and unheated tumor bodies, we used 2-D DIGE. As shown in Fig. 4a, a single protein was highly overexpressed in the lysates of heated cells relative to unheated ones. This spot was excised and subjected to a limited tryptic digest, and fragments were sequenced by tandem MALDI. This protein was identified as HSP70 (Fig. 4b). An analysis of the predicted molecular mass and pI of this protein confirmed that it correlated well with the position of the unknown spot by 2-D DIGE.

DIGE data were confirmed by analysis of HSP70 protein expression by immunofluorescence and confocal microscopy (Fig. 4c). Heating tumor cells for 2 or 4 h was associated with considerably increased protein expression. These results were confirmed in several melanoma cell lines by measuring HSP70 protein expression by ELISA, where at least a 2-fold increase in protein expression was found (Fig. 4d).

To determine whether controlled overexpression of HSP70 in melanoma cells would enhance the cross-priming of melanoma-specific CTLs, HLA-A*0201neg SK-Mel28 cells were transduced with lentiviral vector encoding EGFP and/or EGFP-HSP70. As shown in Fig. 5a, >95% melanoma cells were transduced, as measured by EGFP fluorescence. Furthermore, ELISA indicated that transduction with HSP70-encoding vector led to increased expression of HSP70 protein (Fig. 5b). Thus, naive HLA-A*0201 CD8+ T cells were primed in 2-wk cultures with DCs loaded with killed SK-Mel28 cells overexpressing, or not, HSP70. Their CTL function was assessed by measuring their capacity to kill melanoma peptide-pulsed T2 cells. As shown in Fig. 5c, CTLs primed with HSP70-overexpressing SK-Mel28 bodies showed increased killing of melanoma peptide-pulsed T2 cells compared with control cultures. These results suggest that the enhanced cross-priming with heated melanoma cells is at least partially explained by the increased expression of HSP70.

Enhanced cross-priming is associated with overexpression of tumor-associated Ags

Our original hypothesis was that the enhanced cross-priming would be due to enhanced expression of HSPs. This would be
consistent with studies demonstrating that purified HSP70, HSP60, and gp96 act as immune adjuvants for cross-priming with antigenic proteins or peptides (10, 11). However, microarray analysis of control and HSP70-transduced SK-Mel28 cells showed increased transcription of several tumor Ags, including MAGE-A10 (Fig. 6a). Therefore, we measured by real-time PCR the expression of 12 genes encoding different members of the MAGE tumor Ag family. These included MAGE-B3, MAGE-A8, MAGE-B4, and MAGE-A10 (Fig. 6, b–h). The expression of several genes from the MAGE family was highly increased (up to $10^3$-fold for MAGE-B3) and was sensitive to actinomycin D, confirming active transcription (Fig. 6, b–h).

We identified HLA-A*0201-restricted peptides derived from MAGE-A10 (28) and analyzed whether DCs loaded with heat-treated bodies would prime CTLs specific for this epitope. We found that HLA-A*0201 T cells primed against hot HLA-A*0201 Me290 or HLA-A*0201neg SK-Mel28 cells displayed a reproducibly higher frequency of MAGE-A10 tetramer-binding CD8 T cells than CTLs primed against unheated melanoma cells (Fig. 7). Thus, hyperthermia-increased transcription of tumor Ags might contribute to enhanced cross-priming.

Discussion

In this study, we show that heat treatment of melanoma cells before their killing enhances their immunogenicity, resulting in increased cross-priming of melanoma-specific CTLs. Thus, DCs loaded with heat-treated and killed melanoma cells are more efficient than DCs loaded with unheated and killed melanoma cells in cross-priming naive CD8 T cells. This could be demonstrated in several assays, including 1) enhanced killing of HLA-A*0201 melanoma cells in a standard 4-h chromium release assay, 2) enhanced capacity to prevent melanoma cell growth in vitro in a tumor inhibition assay, 3) enhanced killing of melanoma peptide-pulsed T2 cells, and 4) increased frequency of CD8 T cells binding melanoma peptide-MHC tetramers.

![Image](http://www.jimmunol.org/)

**FIGURE 7.** DCs are loaded with unheated (cold; a and c) or heated (hot; b and d) killed melanoma cells, either HLA-A*0201 Me290 (a and b) or HLA-A*0201-negative SKmel 28 (c and d). After two stimulations, HLA-A*0201 CD8 T cells are boosted with peptide-pulsed DCs. Flow cytometry staining with MAGE 10 tetramer. Percentage of tetramer binding CD3 T cells. Representative of two experiments with T cells from two different donors.

The most straightforward explanation for the enhanced cross-priming is that increased expression of HSPs allows the transfer of more tumor peptide onto DCs MHC class I (10). Indeed, HSPs are well recognized as peptide chaperones, and purified HSP70 permits cross-presentation of melanoma peptide(s) to melanoma-specific T cell clones (12, 13). Accordingly, we found that heat-treated melanoma cells display increased expression of several HSPs, including HSP70. Additional confirmation of the direct role of HSPs came from the demonstration that overexpression of HSP70 in melanoma cells by means of lentiviral vector could at least partially reproduce increased cross-priming.

Yet, recent studies by Yewdell and colleagues (29) suggest that cross-priming might be based on the transfer of proteasome substrates rather than peptides. This concept offers an additional mechanism by which hyperthermia could enhance cross-priming, i.e., enhanced tumor-associated Ag expression. Indeed, heat treatment of melanoma cells results in the enhanced transcription of several tumor-associated Ags including those from MAGE family (MAGE B3, B4, and A10) as well as tyrosinase. Such enhanced expression of tumor Ags, if followed by increased translation and protein expression, could contribute to enhanced cross-priming in our system. We were able to confirm this by analyzing priming against MAGE-A10 for which an HLA-A*0201-restricted peptide has been identified by cloning CTLs from a patient with metastatic melanoma (28). Thus, hyperthermia increases both the magnitude and the breadth of elicited melanoma-specific CD8 T cell immunity. The molecular mechanisms responsible for increased tumor-associated Ags transcription will need to be identified.

Finally, modulation of DC Ag-presenting function and/or maturation could also contribute to enhanced cross-priming (30). Hyperthermia-treated or HSP70-overexpressing killed cells could differ from untreated killed tumor cells with respect to their surface components. This could lead to differential capture by DCs and delivery into distinct intracellular compartments. Indeed, scavenger receptors, such as lectin-like oxidized low-density lipoprotein receptor 1, are involved in HSP70 binding to DCs and subsequent cross-presentation of delivered Ags (18). Furthermore, heat-treated killed tumor cells might enhance DC maturation and costimulatory molecule expression. The enhanced DC maturation upon exposure to tumor material rich in HSPs would be consistent with previous findings (31).

Our results will have direct clinical applications in DC- or T cell-based tumor immunotherapy. Indeed, the increased immunogenicity of DC vaccines proposed in this study might allow 1) shortening the time necessary for T cell elicitation/expansion for adoptive T cell therapy protocols, and 2) limiting the number of DCs per injection and/or the times of DC injections in DC-based immunotherapy protocols. Finally, these results might contribute to our understanding of mechanisms explaining the benefits of hyperthermia in cancer therapy.

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Disclosures

J.B. has been a consultant for Argos Therapeutics and is a scientific founder of ODC Therapy, two private companies, and has stock options for both. A.K.P. is a scientific founder of ODC Therapy, a private company, and has stock options. These private companies are related to DC vaccines,
and neither one has in any way supported the study, the results of which are described in the current manuscript.

References


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