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Heat-Shock Induction of Tumor-Derived Danger Signals Mediate Rapid Monocyte Differentiation into Clinically Effective Dendritic Cells

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Abstract

Purpose: This study characterizes, biologically and clinically, a novel type of dendritic cells (DC) produced in the short term and called tumor antigen–presenting cells (TAPCells). In particular, we identified factors present in a lysate derived from heat-shocked allogeneic melanoma cells (TRIMEL) that are associated with TAPCells' enhanced capability to induce CD8^{+} T-cell responses *in vitro* and in vaccinated melanoma patients.

Experimental Design: First, extensive phenotypic and functional characterization of TAPCells was performed, followed by vaccination of 45 melanoma patients with four doses of TAPCells over a period of 2 months. Specific delayed-type hypersensitivity (DTH) reaction was analyzed posttreatment and correlated with overall survival rates. Furthermore, heat-shock (HS)-induced factors present in TRIMEL and their effects on DC activation were identified and studied.

Results: TRIMEL induced a committed, mature, DC-like phenotype in TAPCells and effectively activated melanoma-specific CD4^{+} and CD8^{+} T cells. Clinically, 64% of vaccinated patients showed positive DTH reaction against TRIMEL, and this was associated with improved overall survival. HS treatment of tumor cells increased calreticulin (CRT) plasma membrane translocation and induced the release of high-mobility group box 1 proteins (HMGB1). Both CRT and HMGB1 mobilization were associated with enhanced TAPCells' maturation and antigen (Ag) cross-presentation, respectively. DTH infiltration analysis revealed the presence of CD8^{+}/CD45RO^{+} T cells, thus confirming TAPCells' ability to cross-present Ags *in vivo*.

Conclusions: Our results indicate that lysates derived from heat-shocked tumor cells are an optimal source of tumor-associated Ags, which are crucial for the generation of DCs with improved Ag cross-presentation capacity and clinically effective immunogenicity. *Clin Cancer Res; 17(8); 2474–83. ©2011 AACR.*

Introduction

The activation of T lymphocytes against tumor cells requires antigen (Ag) presentation by dendritic cells (DC), which are strategically located within peripheral tissues in an immature state (1). After the interaction with pathogens or other inflammatory stimuli, DCs mature, upregulating several surface markers associated with Ag presentation, costimulation, and cell–cell adhesion (1). Maturation partly results from activation through pattern-recognition receptors (PRR; ref. 2) such as Toll-like receptors (TLR), which recognize well-conserved, pathogen-associated, molecular patterns (3). In a noninfectious context, certain endogenous factors, originating from necrotic or stressed cells induced by trauma, ischemia-related injuries, chemical insults, radiation, or excessive heat, can act as "danger signals," thus inducing an inflammatory response via PRRs on DCs (4). These signals include "eat-me signals," such as calreticulin (CRT) and damage-associated molecular patterns (DAMP) as heat-shock (HS) proteins, cellular nucleic acids, and the high-mobility group box 1 (HMGB1), recognized by TLRs, integrins, or scavenger receptors (5–7).

Given their low frequency in blood, DCs produced *in vitro* are differentiated from CD14^{+} monocytes cultured with cytokines (8, 9). These immature DCs require additional stimuli, which is provided by TNF-α or lipopolysaccharide (LPS), to achieve a mature DC (maDC)-like phenotype (9).

Until now, DC vaccines have shown encouraging immunologic results, although only a few have been accompanied

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Translational Relevance

Dendritic cell (DC)-based anticancer vaccines have shown an extraordinary ability to induce immunity, but this has low correlation with clinical effects. This constraint is probably due to tumor escape caused by dominant single antigens (Ag), absence of immunologic danger signals during immunization, or deficiencies in Ag-presentation by injected DCs. Optimal delivery of a wide-ranging pool of Ags coupled with the presence of factors promoting Ag cross-presentation to CTLs is critical for DC vaccine success. In this article, we describe the effect of an allogeneic melanoma cell lysate (TRIMEL) on the rapid differentiation of human monocytes into tumor antigen-presenting cells (TAPCells). In particular, heat-conditioned tumor lysate triggers the induction of calreticulin (CRT) and high-mobility group box 1 proteins (HMGB1), both of which act as danger signals, mediating an optimal Ag-presenting cell maturation and Ag cross-presentation. Importantly, TAPCells induce cellular responses in 64% of vaccinated patients, associated with a prolonged survival. These findings provide new insights into the design of potent and clinically effective DC-based tumor vaccines.

by durable clinical responses, which has caused disappointment in the medical and scientific communities (10–13). Therefore, over the past 5 years, a major emphasis has been placed on improving the design of DC vaccines to induce proper activation, better Ag presentation, and increased immunogenicity.

Optimal delivery of a wide-ranging pool of tumor-associated Ag (TAA) is critical for DC-based immunotherapy. Therefore, autologous tumor cell lysates, whole tumor cells, mRNA (10, 14–16), and allogeneic melanoma cell lysates (17–19) are valuable alternatives as TAA providers, with the latter representing a standardized applicable source of melanoma-associated Ag (MAA) that is useful in high-risk tumor-free patients. CTLs are crucial for tumor rejection and control of dissemination (20, 21). Because metastatic melanoma cells marginally express MHC class I (MHC-I), and not MHC-II, molecules (22, 23), their recognition requires an efficient Ag cross-presentation by DCs (24–26). DC cross-presentation is regulated by cytokines and TLR ligands during inflammation (24).

Recently, we demonstrated the effectiveness of standard DC immunization for improving long-term survival in patients with late-stage melanoma (17, 19). In the present article, we propose that a lysate of heat-conditioned allogeneic melanoma cells, named TRIMEL, provides a unique strategy to obtain, within 48 hours, efficient tumor Ag–presenting cells (TAPCells) with an maDC-like phenotype. In particular, HS treatment of tumor cells induces DAMPs, which provide activation signals that trigger a fast monocyte differentiation into maDCs.

Furthermore, tumor-associated DAMPs may be responsible for an efficient Ag cross-presentation by TAPCells,

thus mediating an optimal immune response in vaccinated patients. Overall, our results provide new insights into the design of more potent and clinically effective vaccines for treatment of melanoma.

Materials and Methods

Patients

Forty-five melanoma patients were vaccinated with TAPCells and followed up from September 2006 until July 2010, in accordance with the described protocol (19). The study was performed in agreement with the Helsinki Declaration and approved by the Bioethical Committee for Human Research of the Faculty of Medicine, University of Chile. All patients signed an informed consent.

Cell lines and cell lysate preparations

TRIMEL is a cell lysate derived from 3 allogeneic melanoma cell lines—Mel1, Mel2, and Mel3—prepared as described (19). Briefly, each cell line was HS-treated at 42°C for 1 hour and then incubated for 2 hours at 37°C. Cells obtained were mixed in equal amounts and lysed through repeated freeze–thaw cycles in liquid nitrogen. Thereafter, the cell lysate was sonicated and irradiated with a 60-Gy dose. The protein concentration was estimated by Bradford's method using a biophotometer (Eppendorf). Cell lysates from peripheral blood lymphocytes (PBL) and prostate and colon cancer cells were prepared following the same protocol, using commercial available cell lines. Two allogeneic primary renal cancer cell lines, established at the University of Chile, were used for renal cancer cell lysate preparation. The melanoma cell line FM3D was kindly provided by Dr. J. Zeuthen (Cancer Society, Copenhagen, Denmark). THP-1 monocytic/macrophagic cells, K-562 myelogenous leukemia cells, and T2 cells (T-cell leukemia/B-cell hybrid) were all purchased from the American Type Culture Collection. The CD40L-transfected mouse embryonic fibroblast NIH3T3 was kindly provided by Dr. Eduardo Villablanca (San Raffaele Institute, Milan, Italy).

TAPCells generation

Adherent monocytes isolated from peripheral blood mononuclear cells (PBMC) of melanoma patients were cultured in serum-free AIM-V medium (Invitrogen), with recombinant human interleukin 4 (rhIL-4: 500 U/mL; US-Biological), and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF: 800 U/mL; Shering Plough) for 22 hours and then stimulated for 24 hours with TRIMEL (100 μg/mL) alone or with rhTNF-α (20 U/mL; US-Biological; TAPCells), or with medium only [activated monocytes (AM)]. In some experiments, TRIMEL was additionally incubated with anti-CRT monoclonal antibody (mAb), 1.75 μg (BD Biosciences), anti-HMGB1 polyclonal Ab (pAb), 10 μg (Sigma-Aldrich), or an isotype control pAb (BD Biosciences).

Generation of MAA-specific T cells

PBL or naive CD4$^+$ T cells were cocultured with autologous TAPCells (20:1) in RPMI 1640 medium (Invitrogen) containing 10% FBS (Invitrogen) and nourished every 2 days with rhIL-6 (5 ng/mL; eBioscience) and rhIL-2 (100 U/mL; ProSpec-Tany TechnoGene). T cells were restimulated with autologous Ag-presenting cells (APC; 30:1), and maintained with rhIL-2 (100 U/mL) for an additional 10 days. CdL43-1 is a human leukocyte antigen (HLA) A2$^+$-restricted T-cell clone, which is Melan-A/MART-1$_{27-35}$ specific (26).

Flow cytometry

The following antibodies (Ab) were used for cell staining: anti-HLA-ABC, HLA-DR, CD80, CD83, DC-SIGN, DEC-205, CD11c, CD86, CCR7, CD4, CD8, and CD45RO (eBioscience); and CD14 (BD Biosciences). The anti-MICA mAb (R&D Systems) were used together with a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG; R&D Systems). Samples were acquired on a FACSCalibur (BD Biosciences) and analyzed using WinMDI 2.8 software.

ELISpot and proliferation assay

T cells (2×10^4) were cocultured overnight with APCs at different effectors/target ratios. IFN-γ secretion was tested by the enzyme-linked immunosorbent spot (ELISpot) assay, as described (19). MHC-I and MHC-II blocking was performed using mAb W6/32 (eBioscience) and mAb TÜ39 (BD Pharmingen), respectively. Proliferation response was measured by [^{3}H]-thymidine uptake at 24 hours according to standard methods (Topcount NXT; PerkinElmer).

Immunofluorescence staining

For intracellular and surface staining, melanoma cells were fixed with 70% cold methanol or 4% paraformaldehyde, respectively, and this was followed by incubation with purified mouse anti-human CRT mAb, amino acids 270–390 (BD Biosciences; Transduction Laboratories) and a secondary FITC-conjugated goat anti-mouse Ab (Invitrogen). Confocal image stacks were captured with a Zeiss LSM-5, Pascal 5 Axiovert 200 microscope, using LSM 5 3.2 software and a Plan-Apochromat 63×/1.4 oil objective.

Western blot

Cell pellets from APCs or tumor cells were suspended at 4°C in radioimmunoprecipitation assay (RIPA) lysis buffer with added protease and phosphatase inhibitors. Equal amounts of protein were separated by 12% SDS-PAGE, followed by Western blotting and then evaluated using anti-phospho-p65 (Cell Signaling), anti-IκBα (Santa Cruz Biotech), anti-HMGB1 (BD Biosciences), and anti-MART-1 (Invitrogen) Abs. The anti-β-actin (Sigma-Aldrich) and anti-GAPDH (Cell Signaling) Abs were used as controls. Bands were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences), and the ratio protein of interest/internal control was determined by densitometry (ImageJ software).

Skin test and T-cell isolation

All patients were assessed for *in vivo* delayed-type hypersensitivity (DTH) reactions to TRIMEL 1 month after the last immunization. Furthermore, 10 patients were tested prior to vaccination. Skin tests were performed by intradermal injection of 150 μL TRIMEL (2 μg/μL), 150 μL of MULTITEST cell-mediated immunity (Pasteur-Mérieux), and 150 μL of saline solution at different sites. Positive reaction was defined as skin induration of 5 mm or greater at 48 hours after injection.

In 3 DTH$^+$ patients, an 8-mm excision at the DTH reaction was made, using a disposable biopsy punch (Delasco). Half of the tissue sample was fixed in paraformaldehyde 1%, and paraffin-embedded specimens were immunostained with primary Abs against CD4, CD8, and CD45RO (Dako). T cells were isolated from the other half by mechanical disruption and incubated with IL-2 (250 U/mL) for 3 weeks and then analyzed by flow cytometry.

Statistical analysis

Student's *t*-, Dunn's multiple comparison, and Kruskal–Wallis tests were used for comparison of continuous variables. Survival curves were analyzed by the Kaplan–Meier method and log-rank test using Stata 7.0 software (Stata Corp). Differences were considered significant when $P \leq$ 0.05.

Results

TRIMEL induces differentiation of activated monocytes into DCs with a mature phenotype

The expression of the most common MAA, such as MART-1, gp100, tyrosinase, NY-ESO1, MAGE1, MAGE3, MC1R, MCSP, survivin, and Her2/neu (27, 28), was detected in the melanoma cell lines constituting TRIMEL. Mel1 and Mel3 expressed 9, and Mel2 8, out of 10 MAA (data not shown). The addition of TRIMEL to AM, in the presence or absence of TNF-α, mediated up to 3-fold induction of maturation markers associated with DC maturation such as MHC-I, MHC-II, CD80, CD86, and CD83 (Fig. 1A and B). In addition, TAPCells showed increased expression of DC-associated endocytic receptors DEC-205 and DC-SIGN and enhanced surface expression of MICA and CCR7 (Fig. 1C). In contrast, CD14 expression was marginal on TAPCells when compared with PBMC and THP-1 cells (Fig. 1C).

Mature DCs are phenotypically stable (1); therefore, we investigated whether TAPCells maintain their phenotypic properties after additional stimuli. TAPCells remained insensitive to stimulations with proinflammatory (LPS and Pam3Cys) or inhibitory (IL-10 and dexamethasone) factors, thus demonstrating their committed mature phenotype (Supplementary Fig. S1A). Furthermore, reduced phagocytic capacity (Supplementary Fig. S1B) and increased release of IL-6, TNF-α, and IL-10 (Supplementary Fig. S1C–E) confirmed maDC properties. Stimulation of TAPCells with CD40L-expressing fibroblasts induced

Figure 1. TRIMEL induces an maDC phenotype on TAPCells. Expression of surface markers on APCs treated under different conditions was assessed by flow cytometry (CD11c$^+$ cells gated). A, representative dot plots of AM and TAPCells of at least 5 independent experiments; numbers refer to the mean fluorescence intensity (MFI). B and C, MFI average increase in relation to AM or monocytes of AM treated with different stimuli, fresh monocytes, PBMC, THP-1 cells, and TAPCells. Data represent at least 3 independent experiments; bars indicate SD; *, $P < 0.05$; **, $P < 0.01$.
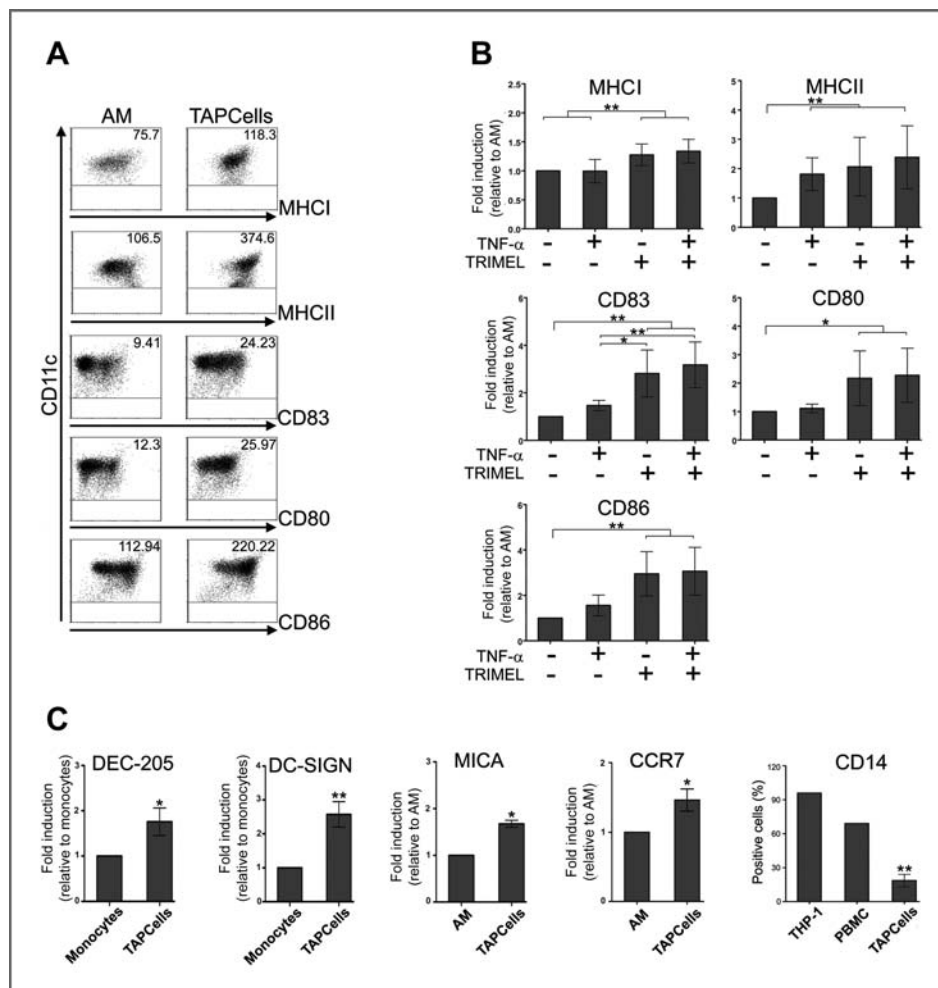
augmented levels of the Th1-polarizing cytokine IL-12 (p70; Supplementary Fig. S1F).

TAPCells activate melanoma-specific CD8$^+$ T cells by cross-presenting MAA

Thereafter, we evaluated TAPCells' capacity to elicit MAA-specific T lymphocytes from autologous PBL. Elicited T cells released IFN-γ after being challenged with autologous TAPCells, or with 3 allogeneic melanoma cell lines (Mel1, Mel2, and FM3D), thus supporting their ability to induce the activation of T cells (Fig. 2A). Neither the NK-sensitive cell line K-562 nor the murine cell line NIH3T3 induced IFN-γ release (Fig. 2A). Elicited T-cell populations were 80% CD4$^+$ and 20% CD8$^+$, and their activity was blocked with anti MHC-I or anti MHC-II mAbs (Fig 2A). In addition, TAPCells, but not AM, induced autologous naive CD4$^+$ T-cell proliferation (Fig. 2B).

Furthermore, TAPCells induced IFN-γ release by an HLA-A2–restricted/MART-1–specific CD8$^+$ T-cell clone (CdL43-1; Fig. 2C; Supplementary Fig. S2), demonstrating their ability to cross-present MHC-I–restricted MAA (Fig. 2C). In fact, MART-1-, but not TRIMEL-, loaded, T2 cells activated

the CdL43-1 clone (Fig. 2D), ruling out the possibility that TRIMEL contains soluble peptides that exogenously bind to MHC-I.

TAPCells induce MAA-specific immune response in patients

Based on our previous findings using TRIMEL-loaded standard DCs (19), a major issue to be evaluated was whether TAPCells induce MAA-specific cellular immune responses in patients. To this end, 45 melanoma patients were immunized with TAPCells, producing no significant adverse reactions (Supplementary Table 1). In this evaluation, 64% of patients (29/45) developed TRIMEL-specific DTH reaction (Fig. 3A), indicating CD4$^+$ T-cell activation. Significantly, CD8$^+$CD45RO$^+$ memory T cells were detected by immunohistochemistry in DTH$^+$ skin biopsies (Fig. 3B), which was further confirmed after expansion of T cells isolated from these biopsies (Fig. 3C). The majority of the patients showed a DTH$^+$ reaction against control Ags (MULTITEST), and only 1 patient, out of 10 tested, displayed a weak, spontaneous DTH response prior to treatment (data not shown).
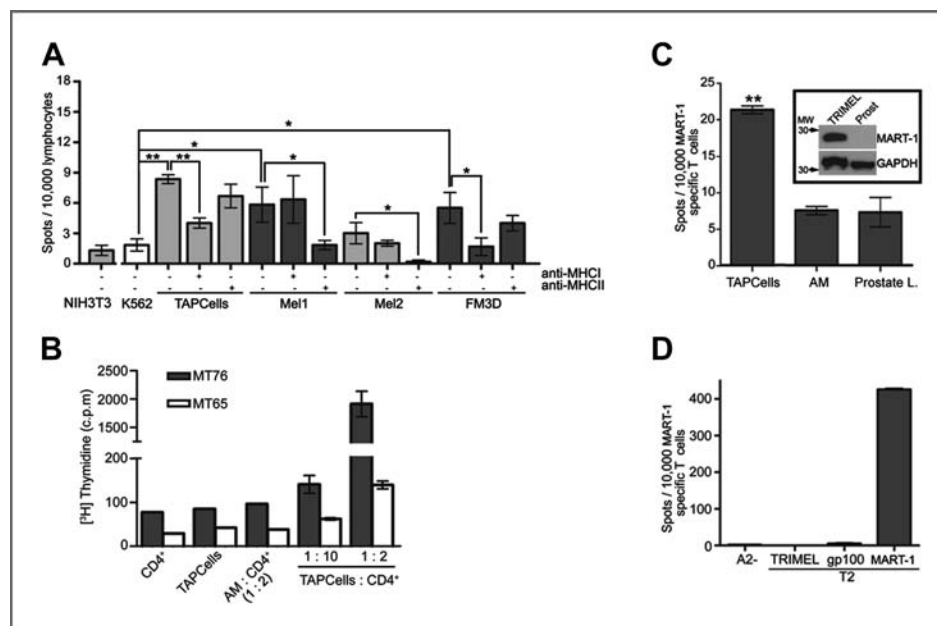
Figure 2. TAPCells induces an antimelanoma T-cell response *in vitro*. A, PBL-derived T cells, prestimulated with autologous TAPCells, were cocultured with autologous TAPCells, allogeneic melanoma (Mel1, Mel2, and FM3D), or with control cells (K-562 and NIH3T3). Specific mAbs against MHC-I and MHC-II were used for blocking CD8+ and CD4+ T cells, respectively. IFN-γ release was measured by ELISpot. B, naive CD4+ T cells, prestimulated with autologous TAPCells, were cocultured with autologous AM or TAPCells at different ratios. The proliferative response was determined by [³H]-thymidine incorporation in 2 different donor samples (MT76 and MT65). Data represent counts per minute (cpm); $n = 3$ experiments. C, the HLA-A2+–restricted MART-1–specific clone (CdL43-1) was cocultured with HLA-A2+ TAPCells, AM, or AM loaded with a lysate of HS-conditioned prostate cancer cells plus TNF-α (Prostate-L). IFN-γ release was measured by ELISpot. The Western blot shows MART-1 expression in the respective lysates (TRIMEL and prostate). D, HLA-A2− TAPCells (A2−) or T2 cells incubated with TRIMEL, MART-1, or with gp100 peptides were cocultured with the CdL43-1 clone. IFN-γ release was measured by ELISpot. A, C, and D, data represent mean spots/1 × 10⁴ effectors cells of at least 3 independent experiments; bars indicate SD; *, $P < 0.05$; **, $P < 0.01$.

The median follow-up of stage IV patients ($n = 32$) was 33.6 months (range: 13–47), with an overall median survival of 15.4 months (Fig. 3D). Stage IV/DTH+ patients (19/32) had a prolonged median survival compared with nonresponders (stage IV/DTH−). Indeed, while stage IV/DTH− patients had a median survival of 9.5 months, 57.9% of stage IV/DTH+ patients survived during the follow-up period (Fig. 3D). Importantly, 81.1% of stage III patients (9/11) remained metastasis free during the follow-up (Supplementary Table S1).

Tumor-cell lysates mediate monocyte differentiation to maDCs

We investigated the capacity of TRIMEL to activate transduction signals associated with phenotypic changes in APCs. TRIMEL induced higher phosphorylation levels of NF-κB p65 than LPS (Fig. 4A). In addition, TRIMEL and TNF-α, but not a conditioned allogeneic PBL lysate, induced degradation of IκBα (Fig. 4B). Moreover, TRIMEL, but not autologous or allogeneic PBL cell lysates, prepared under the same conditions, induced CD86 and CD83 surface expression on CD11c+ DC populations, even in the presence of TNF-α (Fig. 4C). Tumor lysates derived from conditioned prostate and colon cancer cells, but not conditioned allogeneic PBL cell lysates, significantly increased MHC-I, CD80, and CD83 expression in CD11c+ cells (Fig. 4D).

CRT and HMGB1 are involved in TAPCells' phenotypic maturation and cross-presentation, respectively

Recently, the mobilization of CRT to the cell membrane, induced by cell stress, has been associated with increased endocytosis of stressed cells by APCs as well as with improved tumor immunogenicity in a murine model (5). HS treatments induced robust CRT translocation from intracellular compartments to the cell membrane in all TRIMEL melanoma cell lines (Fig. 5A). In addition, HS induced the release of HMGB1 by cell lines composing TRIMEL, but not by PBL (Fig. 5B and C). HS-treated melanoma cell lines were negative for Annexin V staining, thus ruling out positive HMGB1 supernatant detection due to cell destruction or attrition (Supplementary Fig. S3A and B).

To evaluate the role of tumor-derived CRT and HMGB1 in APC activation, we stimulated AM with TRIMEL preincubated with anti-CRT- or anti-HMGB1–specific Abs. CRT blockage inhibited TRIMEL-mediated induction of MHC-II, CD80, and CD86 (Fig. 5D, top panel). In contrast, MHC-I and CD83 expressions were not affected in these cells (Fig. 5D, top panel). TRIMEL pretreatment with a HMGB1-neutralizing Ab reduced MHC-I expression on APCs, but the expression of other surface markers was not affected (Fig. 5D, bottom panel). Furthermore, the involvement of CRT and HMGB1 in DC-mediated Ag cross-presentation was assessed. Stimulation of APCs from 3 melanoma patients with TRIMEL induced a 5-fold
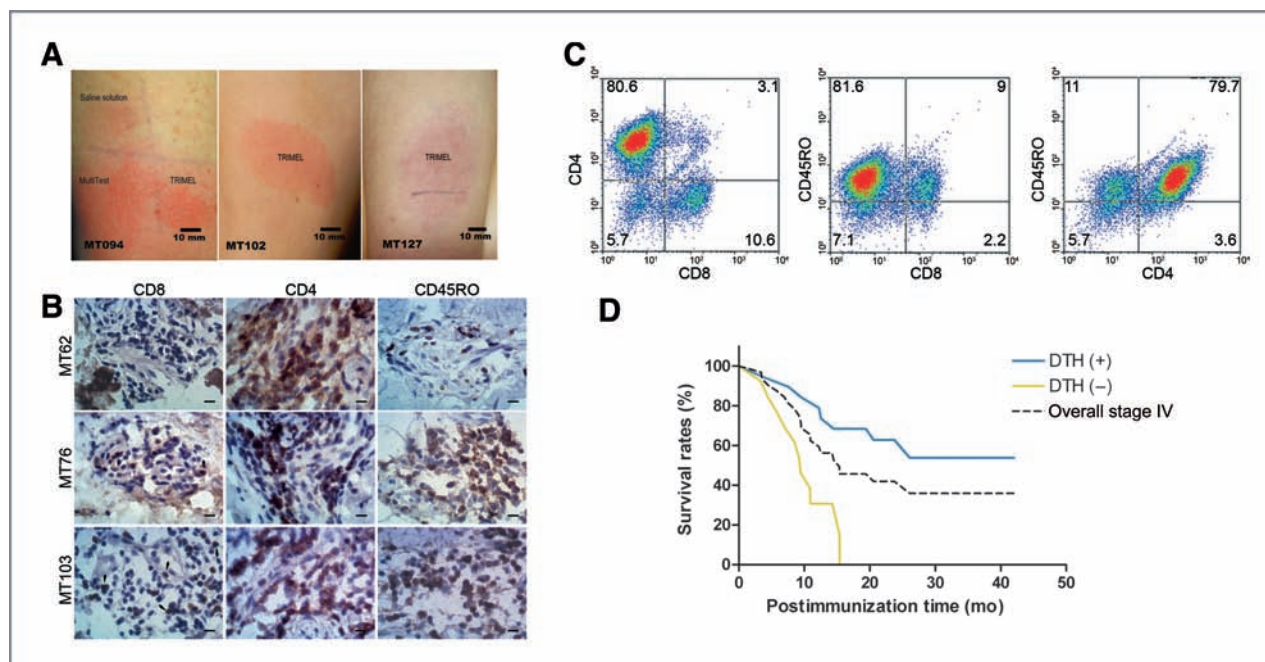
Figure 3. TAPCells vaccine triggers TRIMEL-specific DTH response, which is associated with improved survival among melanoma patients. One month after the last dose of TAPCells, vaccinated patients were intradermally challenged with 150 μL of TRIMEL, MULTITEST (positive control), or with saline solution (negative control). The DTH reaction was evaluated after 48 hours. A, representative photographs of DTH$^+$ reaction (patients MT94, MT102, and MT127) are shown. CD4$^+$CD45RO$^+$ and CD8$^+$CD45RO$^+$ memory T cells were detected in TRIMEL-DTH$^+$ biopsies obtained from patients MT62, MT76, and MT103 by immunohistochemistry (B) and flow cytometry (C). D, Kaplan–Meier survival curves of stage IV melanoma patients after TAPCells vaccination protocols, grouped according to their DTH response. Statistical difference between survival curves, $P < 0.001$.

increase of IFN-γ release by the CdL43-1 clone, compared with APCs stimulated with a non–HS-treated melanoma cell lysate (Fig. 6A). No differences in endocytosis rates were detected in HS-treated (TRIMEL) versus non–HS-treated cell lysates (melanoma lysate; Supplementary Fig. S4). Interesting, pretreatment with an anti-HMGB1–specific Ab attenuated TRIMEL-mediated IFN-γ release by the CdL43-1 clone (Fig. 6B). Moreover, supernatant from HS-treated melanoma cells (TRIMEL), when added to non–HS-treated melanoma lysate, restored and improved Ag recognition by the clone CdL43-1 (Fig. 6B). This effect was blocked when supernatants were pretreated with an anti-HMGB1 Ab, and this suggests a role for the released HMGB1 in the induction of Ag cross-presentation (Fig. 6B). Finally, HS-treated Mel2 cells that were treated with an anti-CRT mAb significantly reduced IFN-γ release by the CdL43-1 clone (Fig. 6C; $P < 0.01$), indicating that HS induction of CRT translocation improved the ability of DCs to activate T cells.

Discussion

To date, several DC vaccine studies have shown encouraging data on induced immunity, but only a few have correlated with clinical improvements (29). This constraint is probably due to the induction of tolerance by dominant single tumor peptides, the absence of immunologic danger signals during immunization, or deficien-cies in Ag processing and presentation by injected DCs (30, 31). In the present study, APCs obtained by an original procedure were phenotypically and functionally characterized *in vitro* and clinically tested. We showed that short-term culture of monocytes loaded with TRIMEL generated a committed maDC-like phenotype with high levels of costimulatory and Ag-presenting molecules, as well as release of proinflammatory cytokines. The rapid timing (48 hours) for TRIMEL-induced *in vitro* monocyte differentiation into maDCs is closer to the physiologic differentiation process (32, 33) than standard 7-day DC standard protocols. In addition, TAPCells released the Th1-associated cytokine IL-12, a process dependent on CD40 stimulation. Synchronized IL-12 production by DCs *in vivo* is crucial because IL-12 synthesis ends 24 hours after exposure to maturation factors, thus becoming refractory to further activation (34). In this regard, we hypothesize that TAPCells might receive CD40-mediated signals by T cells *in vivo*, thus favoring the Th1 polarization of the immune response. Moreover, the CCR7 surface expression indicates that TAPCells can migrate to the lymph nodes, an essential requirement for the activation of adaptive immunity (35).

Functionally, TAPCells elicit MAA-specific T cells *in vitro* by inducing CD4$^+$ T-cell proliferation and activation of MART-1–specific CD8$^+$ T cells, indicating that they cross-present Ags, an important asset in DC-based immunotherapy.
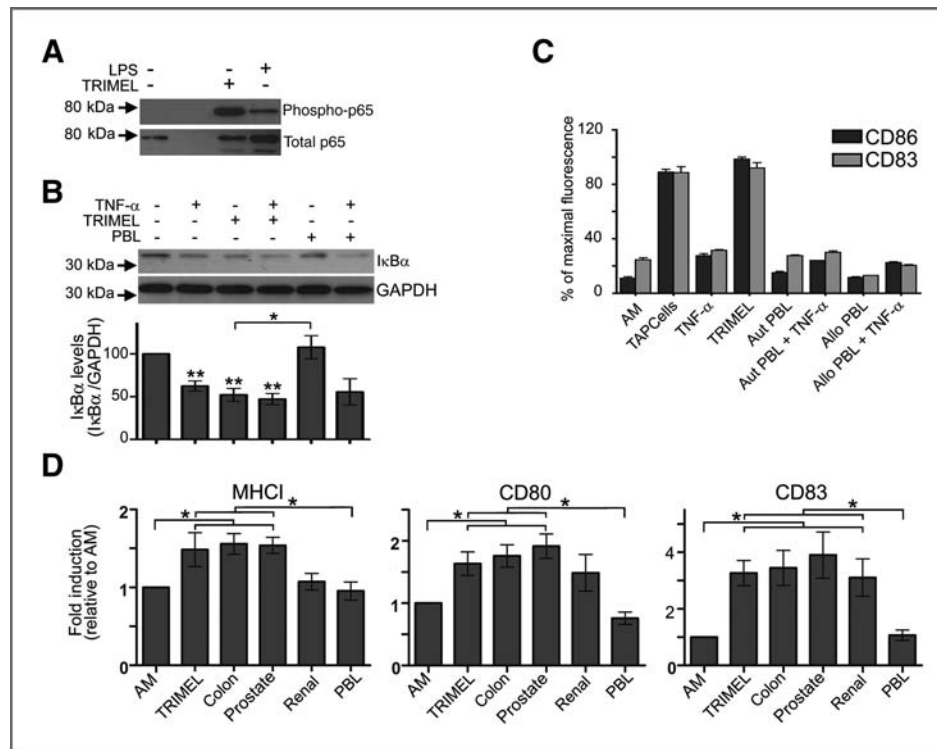
Figure 4. Lysates from conditioned tumor cells, but not from normal cells, induce a rapid differentiation of monocytes into maDCs. A, total and phosphorylated p65 expression and (B) IκBα and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) expression were analyzed by Western blot. The graph shows IκBα levels normalized versus GAPDH. C, CD86 and CD83 expression was analyzed by flow cytometry in AM, AM stimulated with TNF-α, TRIMEL, TRIMEL plus TNF-α (TAPCells), or with a lysate from conditioned autologous (Aut) or allogeneic (Allo) PBLs (with or without TNF-α). Data show the average percentages of mean fluorescence intensity (MFI) in relation to TAPCells. D, CD80 and CD83 expression was evaluated by flow cytometry in AM, AM incubated with TRIMEL, or with HS-conditioned cell lysates (from PBL, colon cancer, prostate cancer, and renal cancer cells). Data represent the average increase of MFI in relation to AM. C and D, CD11c$^+$ gated. A–D, data from at least 3 independent experiments; bars indicate SD; *, $P < 0.05$; **, $P < 0.01$.

TAPCells, tested in 45 patients with stage I, III, and IV melanoma, proved to be well tolerated, and, except for the presence of vitiligo in 2 cases, no major side effects were observed (Supplementary Table S1). The DTH$^+$ reaction, detected in 64% of TAPCells-vaccinated patients after challenge with TRIMEL, reflects the induction of a powerful cellular response against the lysate and constitutes an excellent prognostic marker for clinical outcome, reflecting a break of tolerance that correlates with prolonged survival of responder patients. The lack of response observed in a 36% cohort seems to be more associated with tolerance against TRIMEL Ags than with immunosuppression because most of the patients developed DTH reactions against a positive control (MULTITEST). In fact, as we previously demonstrated, DTH$^-$ patients accumulate regulatory TGF-β producing CD4$^+$ T-cell populations (19). Alternatively or concomitantly, these patients may have a genetic predisposition that limits their antitumor immune response.

Although the DTH reaction has been associated mainly with memory CD4$^+$ T-cell–mediated response (36), our *in vivo* data also showed CD8$^+$ memory T-cell infiltration in DTH$^+$ biopsies. This finding may be very important because CTL-mediated immune responses are closely

related to tumor clearance and patient survival (20, 21, 37). TRIMEL-specific DTH is associated with *in vivo* CD4$^+$/CD8$^+$ memory T-cell accumulation at the reaction site, together with reduced rates of progression and prolonged patient survival, thus linking *ex vivo* events with clinical antitumor responses.

The capability of APCs to activate CD8$^+$ T cells depends on Ag cross-presentation mechanisms. In this process, exogenous Ags captured by endocytosis are released to the cytoplasmic compartment of DCs and routed to the MHC-I Ag-presentation pathway (24, 25, 38). In the present study, TAPCells activated MART-1–specific CD8$^+$ T cells, thus demonstrating their cross-presentation capacity. The cross-presentation process is regulated by external factors, including danger signals provided by tumor cells, which may act through TLR ligands and other receptors on DCs (24, 39–41).

In this experiment, we demonstrated that TRIMEL not only provides a broad panel of shared Ags to DCs but also is essential for the acquisition of TAPCells' functional phenotype. Thus, TRIMEL but not a conditioned allogeneic PBL lysate, activates transduction signal pathways on APCs, in particular, the transcription factor NF-κB associated with phenotypic changes. Furthermore, neither autologous nor
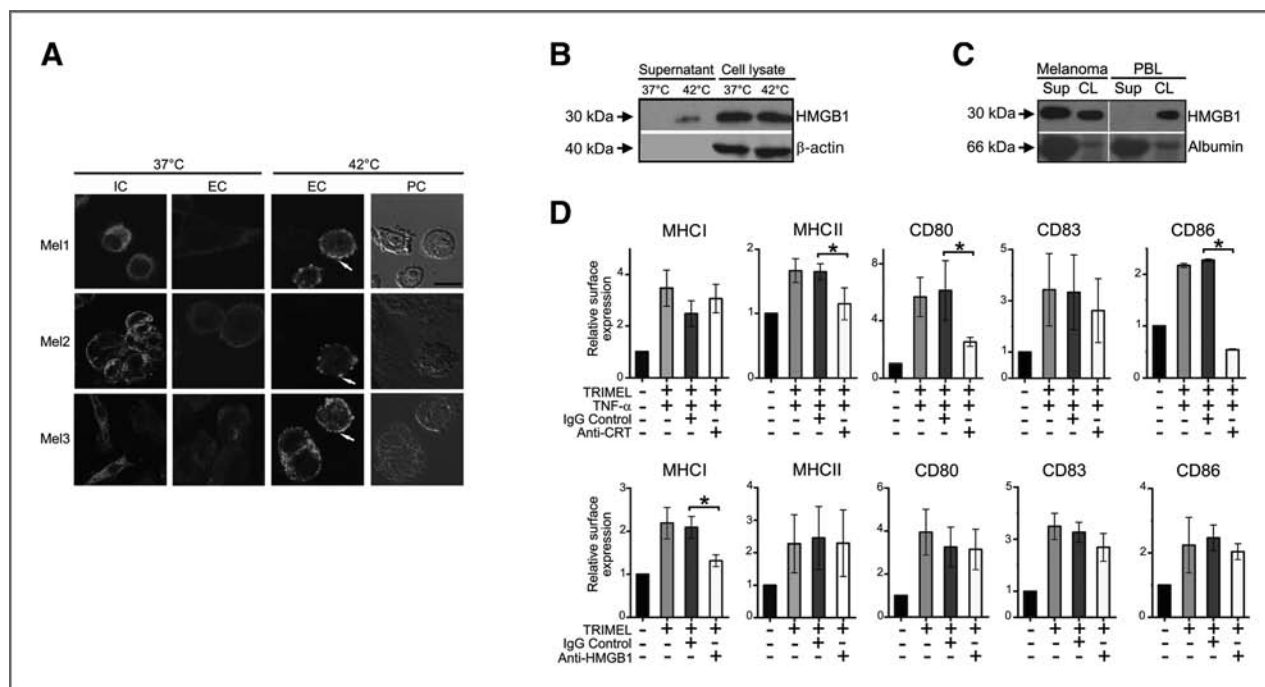
Figure 5. HS preconditioning of cells composing TRIMEL is crucial for DAMP induction. A, HS-treated (42°C) or untreated (37°C) melanoma cells were analyzed for CRT expression by intracellular (IC) or extracellular (EC) immunofluorescence staining. Arrows indicate CRT accumulation at the plasma membrane. PC, phase contrast; scale bar, 70 μm. B, supernatants (collected prior to the cell lysis) or cell lysates obtained from melanoma cells (Mel1, Mel2, and Mel3), HS-treated or not, were analyzed by Western blot for HMGB1. C, supernatants (Sup) or cell lysates (CL) from HS-treated melanoma cells were analyzed by Western blotting for HMGB1. A–C, data are representative of at least 3 independent experiments with similar results; β-actin and albumin were used as controls. D, surface expression of an maDC markers was analyzed by flow cytometry on AM incubated with TRIMEL or with TRIMEL preincubated with an anti-CRT mAb (anti-CRT; top) or with an anti-HMGB1 pAb (bottom). Isotype pAb was used as IgG control. Data represent the average increase of mean fluorescence intensity in relation to AM, $n = 3$ independent experiments; bars indicate SD; *, $P < 0.05$.

allogeneic conditioned, nontumor cell lysates induced surface expression of maturation markers, suggesting that factors present in the tumor cells induce the differentiation process. This may be a general property of some tumors because lysates derived from prostate and colon cancer cells significantly increase expression of MHC and costimulatory molecules in DCs. Although unexplained, tumor cells, but not primary cells, induce DC maturation after necrosis, which may be associated with overexpression of DAMPs by human neoplasms (40, 41).

Interestingly, APCs loaded with a lysate composed of nonstressed melanoma cells showed a reduced capacity to activate MAA-specific CTLs as compared with HS conditioning, prior to the cell lysis. In this regard, DCs primed with a HS-treated tumor cell lysate, followed by an *in situ* boost with radiofrequency thermal ablation, prevent local tumor recurrence in a murine model (42). The use of cell lysates as Ag source produces clinical responses in several tumor models (43–46), suggesting that our tumor cell conditioning may have an impact on the design of more optimal protocols.

HS treatment prior to the tumor cell lysis causes CRT translocation (most probably, from the endoplasmic reticulum) to the plasma membrane and HMGB1 release to the extracellular media. We showed that HS-induced CRT translocation to the plasma membrane directly contributes to maturation of TAPCells. Specific blockage of CRT not only inhibited surface expression of MHC-II and costimulatory molecules but also reduced the capacity of CTLs to recognize MAA on TAPCells associated with the induction of a deficient DC phenotype. Although CRT translocation caused by cytostatic drugs, or other stresses, increases both the phagocytic capacity and immunogenicity of DCs in a murine model (5), we could not detect differences in the endocytic capacity mediated by CRT-membrane mobilization (Supplementary Fig. S4). Because murine bone marrow–derived DC precursors are different from human monocyte–derived DCs, perhaps, they react differently to similar stimulus (47). In addition, our results show that tumor cells, but not normal PBL, release HMGB1 after HS treatment. Interestingly, the lack of released HMGB1 in HS-treated PBL supernatants and their low expression in renal tumor lysate (Supplementary Fig. S2B) indicate that HS-treated tumor cell lysates preferentially induce HMGB1 release related with APC maturation, thus suggesting a role for released HMGB1 in activation of DCs.

Simultaneously, blocking of HMGB1 inhibited MHC-I surface expression and Ag cross-presentation by TAPCells. Cross-presentation reconstitution by the addition of supernatant from HS-treated melanomas to nonstressed lysate
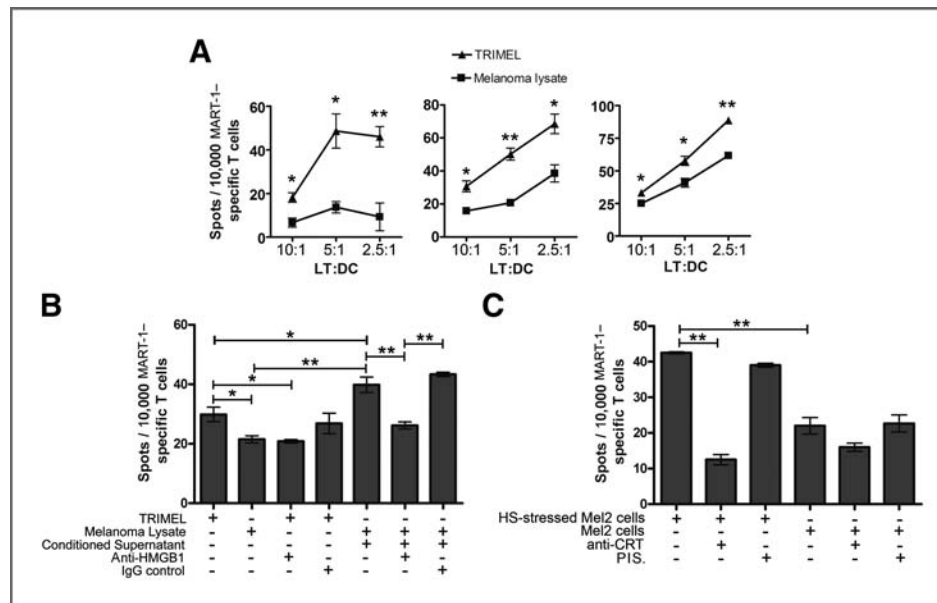
Figure 6. CRT and HMGB1 from HS-treated melanoma cells enhance MAA cross-presentation. A, HLA-A2$^+$ AM preincubated for 24 hours with TRIMEL or with a non–HS-treated melanoma cell lysate (melanoma lysate) were cocultured with the CdL43-1 clone for 12 hours. IFN-γ release was measured by ELISpot. Each graph corresponds to different donors; *, $P < 0.05$; **, $P < 0.01$. B, HLA-A2$^+$ AM were incubated for 24 hours with TRIMEL alone, TRIMEL preincubated with an anti-HMGB1 pAb (anti-HMGB1), or an IgG control. Furthermore, the same cells were incubated for 24 hours with a non–HS-treated melanoma cell lysate (melanoma lysate) in the presence or absence of a supernatant derived from HS-treated melanoma cells (conditioned supernatant) or preincubated with an anti-HMGB1 or IgG control Abs. Treated APCs were coincubated for 12 hours with the CdL43-1 clone, and IFN-γ release was measured by ELISpot. C, HLA-A2$^+$ AM were cocultured for 24 hours with Mel2 cells, whether HS-treated or not, and preincubated with an anti-CRT pAb (anti-CRT) or a preimmune serum (PIS). Cells were cocultured for 12 hours with the CdL43-1 clone, and IFN-γ release was measured by ELISpot. Data represent mean spots/10,000 effectors cells of at least 3 independent experiments; bars indicate SD; *, $P < 0.05$; **, $P < 0.01$.

indicates that released HMGB1, but not the intracellular protein, is essential for activation of APCs. In this regard, HMGB1 suffers posttranslational modifications that may allow its release, augmenting the interaction and APC activation (48), and this may increase cross-presentation through interaction with TLR4 (5).

Altogether, the findings presented here provide new insights into the design of more potent and clinically effective DC-based tumor vaccines. Importantly, we have shown, for the first time in a human model, that HS preconditioning of a tumor cell lysate triggers the induction of danger signals such as CRT and HMGB1 that are closely related with an optimal APC activation. In fact, TRIMEL works as a potent Th1 response mediator, favoring the induction of DC maturation, the release of proinflammatory cytokines, and the improvement of Ag cross-presentation, which are essential for the priming and activation of a

CD8$^+$ T-cell–mediated immune response resulting in anti-tumor clinical effectiveness.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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