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


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Running title: Conditioned tumor lysate induces effective DC-vaccines

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TRANSLATIONAL RELEVANCE

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

**Purpose:** Herein characterizes, biologically and clinically, a novel kind of short-term produced dendritic cells (DCs) named tumor antigen-presenting cells (TAPCells®). Particularly, 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 two months. Specific delayed type hypersensitivity (DTH) reaction was analyzed post treatment and correlated with overall survival rates. Furthermore, heat shock-induced factors present in TRIMEL and their effects on DC activation were identified and studied. **Results:** TRIMEL induced a committed mature DC-like phenotype on TAPCells and effectively activated melanoma-specific CD4⁺ and CD8⁺ T cells. Clinically, 64% of vaccinated patients showed positive DTH reaction against TRIMEL, associated with improved overall survival. Heat-shock 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, confirming TAPCells ability to crosspresent Ags in vivo. **Conclusions:** Our results indicate that lysates derived from heat-shocked tumor cells are an optimal source of tumor-associated Ags, crucial for the generation of DCs with improved Ag cross-presentation capacity and clinically effective immunogenicity.
INTRODUCTION

Activation of T lymphocytes against tumor cells requires antigen (Ag) presentation by dendritic cells (DCs), which are strategically located within peripheral tissues in an immature state (1). After the interaction with pathogens or other inflammatory stimuli, DCs mature, up-regulating several surface markers associated with Ag presentation, co-stimulation and cell-cell adhesion (1). Maturation partly results from the activation through pattern recognition receptors (PRRs) (2) like Toll like receptors (TLRs), which recognize well-conserved pathogen associated molecular patterns (3). In a non-infectious context, some endogenous factors arising from necrotic or stressed cells induced by trauma, ischemia-related injuries, chemical insults, radiation, or excessive heat, can act as “danger signals” inducing an inflammatory response via PRRs on DCs (4). These signals include “eat me signals” like calreticulin (CRT) and damage associated molecular patterns (DAMPs) as heat shock 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, in vitro-produced DCs are differentiated from CD14+ monocytes cultured with cytokines (8, 9). These immature DCs (iDC) require additional stimuli, provided by TNF-α or LPS, to achieve a mature DCs (maDC)-like phenotype (9).

Until now, DC vaccines have shown encouraging immunological results, although only few have been accompanied by durable clinical responses causing disappointment in the medical and scientific community (10-13). Therefore, over the past five years, a major emphasis has been placed in improving the design of DC-vaccines in order to induce proper activation, better Ag presentation and increased immunogenicity.

Optimal delivery of a wide-range pool of tumor-associated Ag (TAA) is critical for the DC-based immunotherapy. Therefore, autologous tumor cell lysates, whole tumor cells, mRNA
and allogeneic melanoma cell lysates (17-19) are valuable alternatives as TAA providers, the latter representing a standardized applicable source of melanoma-associated Ag (MAA), useful also in high-risk tumor-free patients. CTL are crucial for tumor rejection and control of dissemination (20, 21). Since 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). DCs cross-presentation is regulated by cytokines and TLR-ligands during inflammation (24).

Recently, we showed the effectiveness of standard DC-immunization for improving long-term survival in late-stage melanoma patients (17, 19). Herein, we propose that a lysate of heat-conditioned allogeneic melanoma cells, named TRIMEL, provides a unique strategy to obtain, in only 48 h, efficient tumor-Ag presenting cells (TAPCells) with maDC-like phenotype. Particularly, heat-shock (HS)-treatment of tumor cells induces DAMPs, which provides activation signals that trigger a fast monocyte differentiation into maDCs.

Tumor-associated DAMPs may also be responsible for an efficient Ag cross-presentation by TAPCells, thus mediating an optimal immune response in vaccinated patients. Altogether, our results provide new insights into the design of more potent and clinically effective vaccines for melanoma treatment.
MATERIALS AND METHODS

Patients

Forty-five melanoma patients were vaccinated with TAPCells and followed-up from September 2006 until July 2010, according to 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 lysates preparation

TRIMEL is a cell lysate derived from three allogeneic melanoma cell lines Mel1, Mel2 and Mel3, prepared as described (19). Briefly, each cell line was HS-treated at 42°C for 1 h and then incubated for 2 h at 37°C. Obtained cells were mixed in equal amounts, and lysed through repeatedly freeze-thaw cycles in liquid nitrogen. The cell lysate was then sonicated and irradiated with a 60-Gy dose. The protein concentration was estimated by Bradford’s method using a Biophotometer (Eppendorf). Cell lysates from PBL, 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-leukaemia cells, and T2 cells (T cell leukemia/B cell hybrid) were all purchased from the American Type Culture Collection. CD40L-transfected mouse embryonic fibroblast NIH3T3, were kindly provided by Dr. Eduardo Villablanca (San Raffaele Institute, Milan, Italy).

TAPCells generation

Adherent monocytes isolated from melanoma patient’s PBMC were cultured in serum-free AIM-V medium (Invitrogen) with rhIL-4 (500 U/ml; US-Biological), and rhGM-CSF (800
U/ml; Shering Plough) for 22 h, and then stimulated for 24 h with TRIMEL (100 μg/ml) alone or plus 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 mAb, 1.75 μg (BD), anti-HMGB1 polyclonal Ab (pAb), 10 μg (Sigma-Aldrich), or an isotype control pAb (BD).

Generation of MAA-specific T cells

PBL or naïve CD4+ T cells were co-cultured with autologous TAPCells (20:1) in RPMI 1640 medium (Invitrogen) containing 10% FBS (Invitrogen) and fed every 2 days with rhIL-6 (5 ng/ml) (eBioscience) and rhIL-2 (100 U/ml) (ProSpec-Tany TechnoGene). T cells were re-stimulated with autologous Ag presenting cells (APCs) (30:1), and maintained with rhIL-2 (100 U/ml) for additional 10 days. CdL43-1 is an HLA-A2+-restricted T cell clone, Melan-A/MART-127–35-specific (26).

Flow Cytometry

The following Abs 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). The anti-MICA mAb (R&D Systems) were used together with a secondary FITC-conjugated goat anti-mouse IgG (R&D Systems). Samples were acquired on a FACSCalibur (BD), and analyzed using WinMDI 2.8 software.

ELISPOT and Proliferation assay

T cells (2 x 10⁶) were co-cultured with APCs at different effectors/target ratios overnight. IFN-γ secretion was tested by ELISPOT assay, as described (19). MHC I, and MHC II blocking were performed using mAb W6/32 (eBioscience) and mAb TÜ39 (BD Pharmingen),
respectively. Proliferation response was measured by $[^3]H$-thymidine uptake at 24 h 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, followed by incubation with purified mouse anti-human CRT mAb, aa270-390, (BD; 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 63x/1.4 oil objective.

**Western Blot**

Cell pellets from APCs or tumor cells were suspended at 4°C in RIPA lysis buffer plus protease and phosphatase inhibitors. Equal amounts of protein were separated by 12% SDS-PAGE, followed by western blotting and evaluated using anti-phospho-p65 (Cell Signaling); anti-IkBα (Santa Cruz Biotech); anti-HMGB1 (BD), and anti-MART-1 (Invitrogen) Abs. Anti-β-actin (Sigma-Aldrich) and anti-GAPDH (Cell Signaling) Abs were used as controls. Bands were visualized using 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 one month after the last immunization. Ten patients were also tested before vaccination. Skin tests were performed by i.d. 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 ≥ 5 mm at 48 h after injection.
In three DTH+ patients, an 8 mm excision at the DTH reaction was made, using a disposable biopsy punch (Delasco). Half 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) during three weeks and analyzed by flow cytometry.

**Statistical Analysis**

Student’s t, Dunn’s Multiple Comparison and Kruskal-Wallis tests were used for continuous variables. Survival curves were analyzed by Kaplan-Meier method and Logrank 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; MART1, 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 nine, and Mel2 eight out of ten MAA (data not shown). Addition of TRIMEL to AM, in the presence or absence of TNF-α, mediated up to three-fold induction of maturation markers associated with DC maturation such as; MHC-I, MHC-II, CD80, CD86, and CD83 (Fig. 1A and B). Additionally, 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 investigate whether TAPCells maintain their phenotypic properties after additional stimuli. TAPCells remained insensitive to stimulations with pro-inflammatory (LPS and Pam3Cys) or inhibitory (IL-10 and Dexamethasone) factors, thus demonstrating their committed mature phenotype (Suppl. Fig. 1A). Furthermore, reduced phagocytic capacity (Suppl. Fig. 1B), and increased release of IL-6, TNF-α and IL-10 (Suppl. Fig. 1C-E) confirmed maDC properties. Stimulation of TAPCells with CD40L-expressing fibroblasts induced augmented levels of the Th1-polarizing cytokine IL-12 (p70) (Suppl. Fig. 1F).

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

Then, we evaluated TAPCells capacity to elicit MAA-specific T lymphocytes from autologous PBL. Elicited T cells released IFN-γ after challenging with autologous TAPCells, or
with three allogeneic melanoma cell lines (Mel1, Mel2, and FM3D) 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 naïve CD4+ T cell proliferation (Fig. 2B).

Additionally, TAPCells induced IFN-γ release by a HLA-A2-restricted/MART-1 specific CD8+ T cell clone (CdL43-1) (Fig. 2C; Suppl. Fig. 2), showing their ability to cross-present MHC-I restricted MAA (Fig. 2C). In fact, MART-1-loaded, but not TRIMEL-loaded T2 cells activated 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 main issue to evaluate 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 (Suppl. Table 1). Sixty four percent of patients (29 out of 45) developed TRIMEL-specific DTH reaction (Fig. 3A), indicating CD4+ T cell activation. Importantly, CD8+ CD45RO+ memory T cells were detected by immunohistochemistry in DTH+ skin biopsies (Fig. 3B), which was further confirmed after expanding the T cells isolated from these biopsies (Fig. 3C). The majority of the patients showed a DTH+ reaction against control Ags (MULTITEST®) and only one patient, out of ten tested, displayed a weak spontaneous DTH response prior treatment (data not shown).
The median follow-up of the stage IV patients (n=32) was 33.6 months (range 13 to 47) with an overall median survival of 15.4 months (Fig. 3D). Stage IV/DTH+ patients (19 out of 32) had a prolonged median survival compared to non-responder ones (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 out of 11) remained metastasis free during the follow up (Suppl. Table 1).

**Tumor cell lysates mediate monocyte differentiation to maDCs.**

We investigated TRIMEL capacity to activate transduction signals associated to phenotypic changes in APCs. TRIMEL induced higher phosphorylation levels of NF-κB p65 than LPS (Fig. 4A). Additionally, 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 a conditioned allogeneic PBL cell lysates, significantly increased MHC-I, CD80 and CD83 expression on 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, and also 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). Additionally, 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, ruling out positive HMGB1 supernatant detection due to cell destruction or attrition (Suppl. Fig. 3A and B).

To evaluate the role of tumor-derived CRT and HMGB1 in APCs activation, we stimulated AM with TRIMEL pre-incubated with anti-CRT or anti-HMGB1 specific Abs. CRT blockage inhibited TRIMEL-mediated induction of MHC-II, CD80 and CD86 (Fig. 5D, upper panel). In contrast, MHC-I and CD83 expression was not affected on these cells (Fig. 5D, upper panel). TRIMEL pre-treatment with a HMGB1 neutralizing Ab reduced MHC-I expression on APCs, but the expression of other surface markers was not affected (Fig. 5D, lower panel). The involvement of CRT and HMGB1 in DC-mediated Ag cross presentation was also assessed. Stimulation of APCs from three melanoma patients with TRIMEL induced a five-fold increase of IFN-γ release by the CdL43-1 clone, compared to APCs stimulated with a non HS-treated melanoma cell lysate (Fig. 6A). No differences in endocytosis rates were detected in HS-treated (TRIMEL) vs. non HS-treated (melanoma lysate) (Suppl. Fig. 4). Interesting, pre-treatment 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) added to non HS-treated melanoma lysate, restored and improved Ag recognition by the clone CdL43-1 (Fig. 6B). This effect was blocked when the supernatant were pre-treated with an anti-HMGB1 Ab, suggesting a role for the released HMGB1 in the induction of Ag cross-presentation (Fig. 6B). Finally, HS-treated Mel2 cells 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 DCs ability to activate T cells.
DISCUSSION

To date, several DC-vaccine studies have shown encouraging data on induced immunity, but only few correlate with clinical improvements (29). This constraint is probably due to tolerance induction by dominant tumor single peptides, absence of immunological danger signals during immunization, or deficiencies in Ag processing and presentation by injected DCs (30, 31). Here, APCs obtained by an original procedure were phenotypically and functionally characterized in vitro, and clinically tested. We showed that short-term cultured monocytes loaded with TRIMEL generated a committed maDC-like phenotype with high levels of co-stimulatory and Ag-presenting molecules, as well as release of pro-inflammatory cytokines. The rapid timing (48 h) for TRIMEL-induced in vitro monocyte differentiation into maDCs is closer to the physiological differentiation process (32, 33) than standard seven-days DC standard protocols. Additionally, TAPCells released the Th1-associated cytokine IL-12, a process dependent on CD40 stimulation. Synchronized IL-12 production by DCs in vivo is crucial, since IL-12 synthesis ends 24 h after exposure to maturation factors, thus becoming refractory to further activation (34). In this respect, we propose that TAPCells might receive CD40-mediated signals by T cells in vivo, favoring the Th1 polarization of the immune response. Moreover, 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 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.

TAPCells, tested in 45 stage I, III, and IV melanoma patients, proved to be well tolerated, and except for the presence of vitiligo in two cases, no major side effects were observed (Suppl. Table 1). The DTH+ reaction, detected in 64% of TAPCells-vaccinated patients after challenging
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 immune suppression, since most of 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 anti-tumor 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 since CTL-mediated immune responses are closely related to tumor clearance and patient survival (20, 21, 37). TRIMEL-specific DTH associated with in vivo CD4⁺/CD8⁺ memory T cell accumulation at the reaction site, along with reduced rates of progression and prolonged patient survival, linking ex vivo events with clinical anti-tumor 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). Our TAPCells activated MART-1-specific CD8⁺ T cells 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).
Herein, we demonstrated that TRIMEL not only provides a broad panel of shared Ag 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, particularly the transcription factor NF-κB associated to phenotypic changes. Furthermore, neither autologous nor allogeneic conditioned non-tumor 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 also lysates derived from prostate and colon cancer cells significantly increase MHC and costimulatory molecules expression on DCs. Although unexplained, tumor cells, but not primary cells, induce DCs maturation after necrosis, which may be associated to over-expression of DAMPs by human neoplasms (40, 41).

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

HS-treatment previous to the tumor cell lysis causes CRT translocation (most likely, 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 MHC-II and co-stimulatory molecules surface expression, but also reduces the capacity of CTLs to
recognize MAA on TAPCells associated to the induction of a deficient DCs 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 (Suppl. Fig. 4). Since murine bone marrow-derived DC precursors are different from human monocyte-derived DCs, perhaps they react differently to similar stimulus (47). Additionally, 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 (Suppl. Fig. 2B) indicate that preferentially HS-treated tumor cell lysates induce HMGB1 release related with APCs maturation, suggesting a role for released HMGB1 in DCs activation.

Simultaneously, blocking of HMGB1 inhibited MHC-I surface expression and Ag cross-presentation by TAPCells. Cross-presentation reconstitution by addition of supernatant from HS-treated melanomas to non-stressed lysate indicates that released HMGB1, but not the intracellular protein, is essential for activation of APCs. In this respect, HMGB1 suffers post-translational modifications that may allow its release, augmenting the interaction and APC activation (48) and may increase cross-presentation through interaction with TLR4 (5).

Taken together, 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 pre-conditioning of a tumor cell lysate triggers the induction of danger signals such as CRT and HMGB1 close 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 pro-inflammatory cytokines and the improvement of Ag cross-presentation, essential for the priming and activation of a CD8+ T cell-mediated immune response resulting in anti-tumor clinical effectiveness.
ACKNOWLEDGEMENTS

This work was supported by grants from the National Fund for Scientific and Technological Development (FONDECYT 1090238, 1090243, 3090044), the Fund for the Promotion of Scientific and Technological Development (FONDEF DO5I10366), and the Millennium Nucleus of Immunology and Immunotherapy (P04/030-F).
REFERENCES


FIGURE LEGENDS

Figure 1. TRIMEL induces a maDC phenotype on TAPCells. Surface markers expression 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 five 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 represents at least three independent experiments; bars indicate SD; *p < 0.05, **p < 0.01.

Figure 2. TAPCells induces an anti-melanoma T cell response in vitro. A, PBL-derived T cells, pre-stimulated with autologous TAPCells, were co-cultured with autologous TAPCells, allogeneic melanoma (Mel1, Mel2 and FM3D), or with control cells (K-562 and NIH3T3). Specific mAb against MHC-I and MHC-II were used for blocking CD8+ and CD4+ T cells respectively. IFN-γ release was measured by ELISPOT. B, naïve CD4+ T cells, pre-stimulated with autologous TAPCells, were co-cultured with autologous AM or TAPCells at different ratios. The proliferative response was determined by [3H]-thymidine incorporation in two different donor samples (MT76 and MT65). Data represent counts per minute (c.p.m.); n=3 experiments. C, the HLA-A2+–restricted MART-1–specific clone (CdL43-1) was co-cultured 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 co-cultured with the CdL43-1 clone. IFN-γ release was measured by ELISPOT. A, C, and D, data
represent mean spots/1×10^4 effectors cells of at least three independent experiments; bars indicate SD; *p < 0.05, **p < 0.01.

**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 i.d. challenged with 150 μl of TRIMEL, MULTITEST (positive control), or with saline solution (negative control). The DTH reaction was evaluated after 48 h. A, representative pictures 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.

**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 expression were analyzed by Western blot. The graph shows IκBα levels normalized vs. GAPDH. C, CD86 and CD83 expression were analyzed by flow cytometry in AM, AM stimulated with TNF-α, TRIMEL, TRIMEL plus TNF-α (TAPCells), or with a lysate from conditioned autologous or allogeneic PBLs (with or without TNF-α). Data show the average percentages of MFI in relation to TAPCells. D, CD80 and CD83 expression were 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 three independent experiments; bars indicate SD; *p < 0.05, **p < 0.01.

Figure 5. HS pre-conditioning of cells composing TRIMEL is crucial for DAMPs 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 previous to the cell lysis) or cell lysate 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 blot for HMGB1. A-C, data are representative of at least three independent experiments with similar results; β-actin and albumin expression were used as controls. D, surface expression of maDC markers were analyzed by flow cytometry on AM incubated with TRIMEL, or with TRIMEL pre-incubated with an anti-CRT mAb (anti-CRT) (upper panel), or with an anti-HMGB1 pAb (lower panel). Isotype pAb was used as IgG control. Data represent the average increase of MFI in relation to AM, n=3 independent experiments; bars indicate SD; *p < 0.05.

Figure 6. CRT and HMGB1 from HS-treated melanoma cells enhance MAA cross-presentation. A, HLA-A2+-AM pre-incubated 24 h with TRIMEL or with a non-HS-treated melanoma cell lysate (Melanoma Lysate) were co-cultured with the CdL43-1 clone for 12 h. 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 24 h with TRIMEL alone, or with TRIMEL pre-incubated with an anti-HMGB1 pAb (anti-HMGB1) or an IgG control. Additionally, same
cells were incubated 24 h with a non-HS-treated melanoma cell lysate (melanoma lysate), in the presence or not of a supernatant derived from HS-treated melanoma cells (conditioned supernatant), or pre-incubated with an anti-HMGB1 or IgG control Abs. Treated APCs were co-incubated for 12 h with the CdL43-1 clone and IFN-γ release measured by ELISPOT. C, HLA-A2⁺-AM were co-cultured for 24 h with Mel2 cells, HS-treated or not, pre-incubated with an anti-CRT pAb (anti-CRT) or a pre-immune serum (P.I.S.). Cells were co-cultured for 12 h with the CdL43-1 clone and IFN-γ release measured by ELISPOT. Data represent mean spots/10,000 effectors cells of at least three independent experiments; bars indicate SD; *p < 0.05, **p < 0.01.
Figure 3
Clinical Cancer Research

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

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Clin Cancer Res  Published OnlineFirst February 3, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-2384

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/03/21/1078-0432.CCR-10-2384.DC1

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