Microenvironment and Immunology

Proinflammatory Characteristics of SMAC/DIABLO-Induced Cell Death in Antitumor Therapy

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Abstract

Molecular mimetics of the caspase activator second mitochondria-derived activator of caspase (SMAC) are being investigated for use in cancer therapy, but an understanding of in vivo effects remains incomplete. In this study, we offer evidence that SMAC mimetics elicit a proinflammatory cell death in cancer cells that engages an adaptive antitumor immune response. Cancer cells of different histologic origin underwent apoptosis when transduced with lentiviral vectors encoding a cytosolic form of the SMAC mimetic LV-tSMAC. Strikingly, treatment of tumor-bearing mice with LV-tSMAC resulted in the induction of apoptosis, activation of antitumor immunity, and enhanced survival. Antitumor immunity was accompanied by an increase of tumor-infiltrating lymphocytes displaying low PD-1 expression, high lytic capacity, and high levels of IFN-γ when stimulated. We also noted in vivo a decrease in regulatory T cells along with in vitro activation of tumor-specific CD8+ T cells by dendritic cells (DC) isolated from tumor draining lymph nodes. Last, tumor-specific cytotoxic T cells were also found to be activated in vivo. Mechanistic analyses showed that transduction of cancer cells with LV-tSMAC resulted in exposure of calreticulin but not release of HMGB1. Nevertheless, DCs were activated upon engulfment of dying cancer cells. Further validation of these findings was obtained by their extension in a model of human melanoma using transcriptionally targeted LV-tSMAC. Together, our findings suggest that SMAC mimetics can elicit a proinflammatory cell death that is sufficient to activate adaptive antitumor immune responses in cancer. Cancer Res; 72(6); 1–11. ©2012 AACR.

Introduction

Deregulated apoptosis is a hallmark of many cancers (1). Several abnormalities have been described, including overexpression of inhibitor of apoptosis proteins (IAP; refs. 1–3) and failure of IAP antagonists to translocate from the mitochondria to the cytosol (4). Second mitochondria-derived activator of caspases (SMAC), also known as direct inhibitor of apoptosis-binding protein with low pl (DIABLO) is an IAP antagonist that is a potentially interesting therapeutic target. Cleavage of the mitochondrial targeting signal of SMAC is required for its translocation to the cytosol, in which SMAC binds to the baculovirus IAP repeat domain of IAPs (5). As such SMAC competes with caspase-3 and -9 for binding to IAPs, resulting in their release, cleavage of their substrates, and induction of apoptosis. This knowledge has led to the development of small molecules that mimic SMAC functions (6–9) and to the development of gene vectors encoding full-length SMAC (10), pro-SMAC (4, 11), or processed SMAC (tSMAC; ref. 12), illustrating that SMAC plays a pivotal role in the onset of cancer cell apoptosis.

Dendritic cells (DC) in the tumor environment are mainly immature (13), as such ideally equipped to engulf and process dying cells (14). However, tumor-derived factors keep DCs immature (13), trap them within the tumor (15) hence impair effective presentation of ingested antigens to T cells. Moreover, these immature DCs have immune suppressive rather than stimulating properties (16). It has been suggested that this blockade on antigen-presenting cells can be overcome by proinflammatory signals such as pathogen-derived factors (13). Because we showed that lentiviral vectors (LV) activate DCs through Toll-like receptors (TLR; refs. 17, 18), we evaluated whether lentiviral delivery of tSMAC (LV-tSMAC) to tumor cells induces apoptosis and boosts antitumor immunity. We report on the antitumor efficacy of this strategy, together with the immunologic mechanisms responsible for its outcome.
Materials and Methods

Mice, cell lines, and tumor-infiltrating lymphocytes

Female 6- to 12-week-old C57BL/6, DBA/2, and C3H3 mice were purchased from Harlan. OT-I mice that carry a transgenic CD8\(^+\) T-cell receptor specific for the MHC I-restricted ovalbumin (OVA) peptide SIINFEKL were a gift from B. Lambrecht (University of Ghent). C57BL/6 TLR4 KO mice were a gift from S. Akira (Hyogo College of Medicine). Animals were handled according to the Institutional Guidelines. Approval from the Ethical Committee for use of laboratory animals of the Vrije Universiteit Brussel was obtained.

The mouse melanoma cell lines K1735-C4 (provided by I.J. Fidler, University of Texas), B16F0 and MO4 (provided by K. Rock, University of Massachusetts Medical Center), and HEK293T cells [American Type Culture Collection (ATCC)] were cultured in Dulbecco’s Modified Eagle Medium (Lonza), supplemented with 10% fetal bovine serum (Harlan), 2 mmol/L l-glutamine (L-Glu; Lonza), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin (PS; Lonza). The mouse mastocytoma cell line P815 (provided by C. Uytttenhove, Université Catholique de Louvain), the human colon carcinoma cell line Caco-2 (ATCC), the human breast carcinoma cell line MCF7 (ATCC), and the human melanoma cell lines 888-mel...
and 1087-mel (provided by S.L. Topalian, Surgery Branch, National Cancer Institute) were cultured in RPMI-1640 medium (Lonza) supplemented with FBS, l-Glu, and PS. No full authentication was carried out. Cell lines were tested for their known characteristics including expression of antigens and MHC molecules by reverse transcriptase PCR or flow cytometry. Their in vitro and in vivo growth characteristics were closely monitored.

Tumor-infiltrating lymphocytes (TIL L2D8), recognizing gp100 presented by HLA-A2 (provided by M. Dudley, Surgery Branch, NIH) were maintained in RPMI-1640 containing 1% human AB serum (PAA Laboratories), PS, L-Glu, and 6,000 IU/mL IL-2 (Chiron).

Production and characterization of LVs
The packaging plasmid pCMVΔR8.9 and the VSV.G encoding plasmid pMD.G were a gift from D. Trono (University of Geneva). The plasmids pHR'-trip-CMV-luc2-Ires-tNGFR-SIN, pHR'-trip-CMV-Ires-tNGFR-SIN, pHR'-trip-CMV-Ires-eGFP-SIN, and pSIN-Thy1.1 were described (18, 19). The survivin gene was obtained as a BamHI-BclI fragment from the plasmid pcDNA3.1-survivin (a gift from E. Conway, Katholieke Universiteit Leuven) and cloned into the BamHI linearized pHR'-trip-CMV-Ires-tNGFR-SIN. The human tyrosinase promoter (huTYR) was obtained from pGL3-huTYR2E/P (a gift from D. Nettelbeck, German Cancer Research Center; ref. 20) as a ClaI-BglII fragment.
and used to replace the cytomegalovirus (CMV) promoter in pHR’-trip-CMV-Ires-eGFP-SIN, after restriction digestion with ClaI-BamHI. The sequence encoding tSMAC was excised from pcDNA3.1-tSMAC (21) using BglII-XbaI and cloned into the BamHI-SpeI digested pHr’-trip-CMV-Ires-tNGFR-SIN or pHr’-trip-huTYR-Ires-eGFP-SIN. Enzymes were purchased from Fermentas. The production and characterization of LVs was described (18, 22).

Transduction of cells with LVs

In vitro transduction of 10^5 tumor cells was carried out at the indicated multiplicity of infection (MOI). Where indicated tumor cells were treated with 1 μg/mL dexamethasone (Organon) or 1 μg/mL mitoxantrone (Teva). Palpable tumors grown at the tailbase from 3 × 10^5 tumor cells were injected with 10^7 TU LVs. Where indicated mice were depleted of CD8⁺ T cells by i.p. injection of 50 μg of an anti-CD8 antibody 24 and 2 hours before treatment (5 mice per group). C, depletion of CD8⁺ T cells was verified by flow cytometry. The results shown are representative for 3 mice. D, the graph shows the growth curves of tumors injected with PBS (CTRL), LV-tNGFR, or LV-tSMAC in mice depleted or not of CD8⁺ T cells. E, to assess the presence of memory T cells, DBA/2 mice that were cured (Fig. 1C) were challenged with viable P815 cells. Naive mice served as a control. The graph shows the growth curves.
2 hours before treatment, or treated with a s.c. injection of 200 μg ZVAD-fmk (Bachem) or an i.p. injection of 5 U of apyrase (Sigma-Aldrich), 1 hour before and 24 hours after treatment.

**Western blot**

The preparation of cell lysates and protein quantification were described (23). Proteins (μg) were separated on a 15% SDS-PAGE and transferred to a nitrocellulose membrane.

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**Figure 4.** DCs play a role in the immunologic effect of LV-tSMAC. MO4-bearing mice were treated as described in the legend of Fig. 1D. Three days later, CD11c+ cells were sorted from tumor draining lymph nodes and used to stimulate sorted CD8+ OT-I cells. A, the following day, supernatants were tested for the presence of IFN-γ. B, proliferation of the stimulated CD8+ OT-I cells was evaluated by incorporation of 3H thymidine on day 4 of coculture. The stimulation index was calculated as the proliferation when stimulated divided by the steady state proliferation. The graphs summarize the results of 2 experiments.

**Figure 5.** Treatment of tumor cells with LV-tSMAC results in exposure of CRT but not HMGB1 or ATP release. A–C, MO4, K1735-C4, and P815 cells were transduced as described in the legend of Fig. 1A. Tumor cells treated with dexamethasone or mitoxantrone served as controls. A, the histogram overlays show the exposure of CRT 2 days posttransduction. The results shown are representative for 3 experiments. The graphs show the amount of HMGB1 (B) and ATP released by tumor cells treated as indicated (C). The results shown are representative for 3 experiments. D, mice-bearing MO4 tumors (5 mice per group) were treated as described in the legend of Fig. 1D. In addition, mice were treated with LV-tSMAC combined with apyrase. The graph shows the tumor growth. E, mice were immunized with Annexin V− LV-tSMAC−transduced MO4 cells or PBS (naïve). Five days later, mice were challenged with viable MO4 cells, after which tumor growth was monitored. The graph shows the growth curves.
The following primary antibodies were used: polyclonal rabbit anti-mouse/human antibodies against panIAP (R&D Systems), survivin, caspase-3 and -9 (Santa Cruz Biotechnology). An anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibody (Cell Signaling) was used for detection. An HRP-conjugated β-actin antibody (Cell Signaling) was used for normalization. Antibody binding was visualized with enhanced chemoluminescence (Pierce).

### In vivo bioluminescence imaging

In vivo bioluminescence imaging was carried out as described (24).

### Preparation of single-cell suspension

Single-cell suspensions were prepared after isolation of tumors with the GentleMACS single cell isolation protocol (Miltenyi Biotec).

### Flow cytometry

Staining of cellular markers was previously described (22). Mouse cells were stained with allophycocyanin (APC) conjugated antibodies against CD11c, CD8, and Foxp3; fluorescein isothiocyanate (FITC)-conjugated antibodies against CD86, CD40 511 and C6, and CD4; phycoerythrin (PE)-conjugated antibodies against CD11c, CD8, and Foxp3; and anti-rabbit IgG-APC (Abcam). Human cells were characterized with APC-conjugated antibodies against CD11c and PE-conjugated antibodies against CD80, CD83, CD86, and HLA-DR (BD Biosciences). Data were acquired on a FACSVerse or FACSJuno flow cytometer. A pool of Mouse DCs (22) were cultured at 5 × 10^5 DCs/mL with GFP^+ MO4 cells. The following day, their phenotype was evaluated and compared with immature DCs and DCs matured with 10 μg/mL poly(I:C) (Sigma).

### In vitro cytotoxicity assay

CD90^+ T cells were isolated from single-cell suspensions obtained from MO4 tumors by magnetic-activated cell sorting (MACS). These T cells, containing on average 81.2% ± 1.5% CD8^+ T cells (data not shown), were cultured in a 96-well round bottom plate at a 1:1 ratio with 2 × 10^5 B16F0 or MO4 cells labeled with 0.5 or 10 μmol/L carboxy-fluorescein diacetate succinimidyl ester (CFSE), respectively. The following day, the cells were collected, pooled per condition, and analyzed by flow cytometry. A pool of B16F0 and MO4 cells, which were not cocultured with T cells served as a control. The percentage specific lysis was calculated as described in the work of M. Dullaers and colleagues (25).

### In vitro T-cell stimulation

CD11c^+ cells were isolated from the tumor draining lymph node as described (18). OVA-specific CD8^+ T cells were obtained from the spleen of OT-I mice by positive selection (Miltenyi Biotec). These cells were cocultured at a stimulator to responder ratio of 1:10. Supernatants were collected the following day, to determine IFN-γ secretion by ELISA (eBioscience). Proliferation of T cells was determined 4 days later by incorporation of 3H thymidine (22).

Mouse DCs (22) were cultured at 5 × 10^5 DCs/mL in the presence of 50% supernatants of dying tumor cells or Annexin V^+ dying MO4 cells (MACS Dead Cell Removal Kit; Miltenyi Biotec). The following day, their phenotype was evaluated and compared with immature DCs and DCs matured with 10 μg/mL poly(I:C) (Sigma).

Human DCs from healthy HLA-A2^+ donors (26) were cocultured at 5 × 10^5 DCs/mL with GFP^+ HLA-A2^− Annexin V^+ 888-mel cells. The following day, their phenotype was evaluated and compared with immature DCs and DCs matured with inflammatory cytokines (26). DCs that had engulfed dying cells (eGFP^+, CD45^−, and CD11c^+) were FACS sorted (FACSaria; BD Biosciences) and used to stimulate TIL L2D8 in a 96-well round bottom plate at a stimulator to responder ratio of 4:1. IFN-γ secretion was measured a day later by ELISA (eBioscience).

### In vivo cytotoxicity assay

Tumor-bearing mice received an i.v. injection of 10^6 CD8^+ OT-1 cells 1 day before treatment. The in vivo

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**Table 1.** DCs are activated upon incubation with Annexin V^+ cells

<table>
<thead>
<tr>
<th></th>
<th>Wild-type DCs</th>
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<tbody>
<tr>
<td></td>
<td>No</td>
<td>Poly(I:C)</td>
<td>Supernatants</td>
<td>Annexin V^+</td>
</tr>
<tr>
<td></td>
<td>tSMAC</td>
<td>Mitoxantrone</td>
<td>tSMAC</td>
<td>Mitoxantrone</td>
</tr>
<tr>
<td>CD40</td>
<td>511 ± 36</td>
<td>692 ± 94</td>
<td>546 ± 57</td>
<td>1135 ± 354</td>
</tr>
<tr>
<td>CD80</td>
<td>4398 ± 794</td>
<td>8401 ± 3033</td>
<td>3279 ± 3</td>
<td>1181 ± 5391</td>
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<tr>
<td>CD86</td>
<td>1628 ± 298</td>
<td>4754 ± 2181</td>
<td>1446 ± 93</td>
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<tr>
<td>MHC II</td>
<td>12857 ± 1452</td>
<td>24158 ± 5689</td>
<td>10779 ± 1657</td>
<td>27091 ± 6757</td>
</tr>
</tbody>
</table>

NOTE: Supernatants or Annexin V^+ cells obtained from LV-tSMAC-transduced or mitoxantrone-treated MO4 cells were added to immature wild-type or TLR4 KO DCs. Flow cytometry was carried out 48 hours later to evaluate the expression of CD40, CD80, CD86, and MHC II. Immature DCs and poly I:C–activated DCs served as controls. The table shows the fluorescence intensity of the markers as mean ± SEM of 2 experiments.
cytotoxicity assay was conducted 5 days posttreatment as described (25).

High mobility group box 1 ELISA and ATP assay

Cellular release of HMG1 and ATP was measured in supernatants collected 24 to 72 hours after transduction, using the ELISA kit from Shino Test Corporation and the luciferin-based ENLITEN ATP Assay from Promega, respectively.

Statistical analysis

Results are expressed as mean ± SEM. A one-way ANOVA followed by a Bonferroni multiple comparison test was carried out. Sample sizes and number of times experiments were repeated are indicated in the figure legends. The number of asterisks in the figures indicates the statistical significance as follows: * P < 0.05; ** P < 0.01; *** P < 0.001.

Results

Induction of apoptosis by LV-tSMAC results in enhanced survival of tumor-bearing mice

To evaluate whether LV-tSMAC induces tumor cell death, we transduced MO4, K1735-C4, and P815 cells in vitro with LV-tSMAC or LV-tNGFR. Mock transduced cells served as controls. Cell viability was assessed 3 days later, showing induction of cell death upon transduction with LV-tNGFR. The latter was enhanced when tSMAC was introduced (Fig. 1A–C).

Because LV-tSMAC induced cell death in vitro, we next evaluated its therapeutic efficacy. When compared with PBS- or LV-tNGFR–treated mice, we observed a delayed tumor growth in mice treated with a single intratumoral injection of LV-tSMAC in the M04 and K1735-C4 model (Fig. 1D and E). Importantly, treatment of P815-bearing mice resulted in complete tumor regression (Fig. 1F).

To evaluate the mechanism behind this prolonged survival, we assessed the induction of apoptosis in vivo upon treatment of M04 tumors. Three days posttreatment, tumors were isolated and cell lysates prepared for Western blot analysis. We showed downregulation of survivin, upregulation of caspase-3 and -9 but no differences in IAP expression in mice treated with LV-tSMAC (Fig. 1G). To further assess the role of survivin and caspases, we treated MO4-bearing mice with LV-tSMAC alone or combined with injection of LV-survivin or ZVAD-fmk. Mice treated with PBS or LV-tNGFR served as controls. Only mice treated with LV-tSMAC showed a delayed tumor outgrowth (Fig. 1H).

The therapeutic effect of LV-tSMAC involves effector T cells

In vivo bioluminescence imaging carried out upon intratumoral injection of LV-luc2, showed modest transduction of tumor cells in situ (Fig. 2A). The low in situ transduction efficiency was confirmed in flow cytometry carried out on cells obtained from LV-Thyl.1–injected tumors (Supplementary Fig. S1A). These data suggest that it is unlikely that induction of cell death is the sole mechanism explaining the therapeutic potency of LV-tSMAC, leading us to investigate whether LV-tSMAC induces antitumor immune responses.

We evaluated the presence of DCs, T cells, regulatory T cells (Treg), and myeloid-derived suppressor cells (MDSC) in the M04 tumor environment by flow cytometry. We observed no differences in DC or MDSC numbers between the differently treated mice neither in the tumor nor in the spleen (data not shown). Importantly, we observed that LV-tSMAC–treated tumors contained a higher number of TILs, which expressed low PD-1 levels compared with TILs from PBS or LV-tNGFR–treated tumors. Moreover, a reduction in Treg numbers was observed in LV-tSMAC–treated tumors (Fig. 2B and C, Supplementary Fig. S1B).

To evaluate the function of CD8+ TILs, we transferred OT-I cells to M04-bearing mice 1 day before treatment. Three days later, CD8+ T cells were isolated from the tumor and cocultured with SIINFEKL-pulsed DCs. We observed that TILs isolated from LV-tSMAC–treated mice secreted high levels of IFN-γ (Fig. 2D). Moreover, these TILs showed high specific lysis of target cells in the in vitro cytotoxicity assay (Fig. 2E, Supplementary Fig. S1C).

Subsequently, we evaluated the induction of OVA-specific CTLs upon LV-tSMAC treatment. Therefore, CD8+ OT-I cells were transferred to M04-bearing mice 1 day before treatment. Five days later, an in vivo cytotoxicity assay was conducted, showing potent lysis of target cells in mice treated with LV-tSMAC (Fig. 3A and B). Depletion of CD8+ T cells (Fig. 3C) before treatment was carried out to confirm the role of

Table 1. DCs are activated upon incubation with Annexin V⁺ cells (Cont’d.)

<table>
<thead>
<tr>
<th>TLR4 KO DCs</th>
<th>Supernatants</th>
<th>Annexin V⁺ cells</th>
</tr>
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<tbody>
<tr>
<td>No</td>
<td>Poly IC</td>
<td>tSMAC</td>
</tr>
<tr>
<td>578 ± 3</td>
<td>709 ± 42</td>
<td>451 ± 78</td>
</tr>
<tr>
<td>3005 ± 110</td>
<td>7611 ± 2979</td>
<td>3012 ± 81</td>
</tr>
<tr>
<td>1430 ± 84</td>
<td>5820 ± 2834</td>
<td>1285 ± 31</td>
</tr>
<tr>
<td>8407 ± 1125</td>
<td>21146 ± 5921</td>
<td>7775 ± 1147</td>
</tr>
</tbody>
</table>

Cytotoxicity assay was conducted 5 days posttreatment as described (25).

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Figure 6. Uptake of LV-tSMAC transduced Annexin V⁺ human melanoma cells enables DCs to activate TILs. A–E, 888-mel cells were transduced at MOI 5 with transcriptionally targeted LV-eGFP or LV-tSMAC. Mock-transduced cells served as a control (CTRL). Where indicated cells were treated with dexamethasone or mitoxantrone. A, 3 days later, cells were stained for Annexin V/7-AAD. One experiment out of 3 is shown. The histogram overlays in (B) show the exposure of CRT 2 days posttransduction. The results shown are representative for 3 experiments. C and D, the graphs show the amount of HMGB1 (C) and ATP (D) released by tumor cells treated as indicated. The results shown are representative for 2 experiments. E and F, Annexin V⁺ and GFP⁺ cells were sorted and added to immature DCs. Uptake of dying cells was monitored by flow cytometry. The graph depicts the percentage of CD11c⁺ and GFP⁺ cells and summarizes the results of 4 independent experiments. E, flow cytometry was applied to evaluate the expression of maturation markers on DCs that engulfed dying cells. Immature DCs and DCs activated with proinflammatory cytokines served as controls. The results shown are a summary of 3 experiments. F, DCs (HLA-A2⁺) that engulfed Annexin V⁺ cells (HLA-A2⁺/C0) were sorted and cocultured with TIL L2D8. TILs cocultured with nonmanipulated DCs and 1087-mel (HLA-A2⁺) served as a negative and positive control, respectively. The graph depicts the production of IFN-γ by TILs. The results shown are representative for 3 experiments.
CTLS in the therapeutic effect of LV-tSMAC. Mice depleted of CD8\(^+\) T cells and treated with LV-tSMAC showed a comparable tumor growth as control mice, whereas mice that were not depleted of CD8\(^+\) T cells and treated with LV-tSMAC showed a reduced tumor growth (Fig. 3D).

To assess the generation of a memory immune response, DBA/2 mice that were cured of their tumor (Fig. 1F) were rechallenged with viable P815 cells. In contrast to naive mice, these mice did not develop a tumor (Fig. 3E).

**DCs play a role in the immunologic effect of LV-tSMAC**

We next addressed whether DCs isolated from tumor draining lymph nodes can activate OVA-specific T cells. Three days after treatment, sorted CD11c\(^+\) cells were cocultured with CD8\(^+\) OT-1 cells. When compared with CD11c\(^-\) cells from control mice, CD11c\(^+\) cells from LV-tSMAC–treated mice stimulated CD8\(^+\) OT-1 cells to produce high levels of IFN-\(\gamma\) (Fig. 4A) and to strongly proliferate (Fig. 4B).

**DCs are activated upon engulfment of LV-tSMAC transduced dying cells despite the absence of immunogenic cell death**

To identify the mechanisms underlying the observed immune activation, we evaluated the exposure of CRT and release of HMGB1 and ATP by tumor cells 24 to 72 hours after *in vitro* transduction. Exposure of CRT on tumor cells transduced with LV-tSMAC but not on tumor cells treated with PBS or LV-tNGFR was observed 48 hours after transduction (Fig. 5A). However, we did not observe any release of HMGB1 or ATP by the *in vitro* transduced tumor cells, although both were released when tumor cells were treated with mitoxantrone (Fig. 5B and C). To further address the involvement of ATP in the therapeutic effect of LV-tSMAC, we treated M04-bearing mice with LV-tSMAC alone or in combination with the enzyme apyrase to degrade ATP. Mice treated with PBS or LV-tNGFR served as controls. Mice treated with LV-tSMAC in the presence of apyrase showed a similar delay in tumor growth as mice treated with LV-tSMAC (Fig. 5D).

To further evaluate a role for HMGB1, we evaluated the phenotype of wild-type and TLR4 KO DCs incubated with tumor cell–conditioned media or Annexin V\(^+\) cells from LV-tSMAC– or mitoxantrone-treated tumor cells. The phenotype of both DC types was not altered when compared with immature DCs incubated with media obtained from LV-tSMAC–transduced tumor cells. As expected, incubation of wild-type DCs but not TLR4 KO DCs with medium obtained from mitoxantrone-treated cells induced upregulation of maturation markers. When both DC types were cultured in the presence of Annexin V\(^+\) cells, we observed an upregulation of maturation markers. Of note, this was most pronounced upon coculture with Annexin V\(^+\) cells from LV-tSMAC–transduced tumor cells (Table 1).

As we could not evidence a role for ATP or HMGB1 but showed that uptake of LV-tSMAC–transduced Annexin V\(^+\) cells by DCs results in their activation, we addressed whether mice could be protected from a subsequent challenge with viable MO4 cells by immunization with Annexin V\(^+\) LV-tSMAC–transduced MO4 cells. Naive mice served as a control. In contrast to naive mice, we observed that immunized mice showed a delay in tumor growth (Fig. 5E).

**Uptake of LV-tSMAC–transduced dying human melanoma cells enables DCs to activate T cells**

To translate the mouse data to a human melanoma model, we first evaluated whether the expression of tSMAC can be restricted to melanoma cells with the huTYR promoter. Using GFP as a reporter, we showed that the huTYR promoter restricted the expression to tyrosinase\(^+\) cells (Supplementary Fig. S2A). However, the expression of GFP was low when compared with the expression upon transduction with CMV-containing LVs. Therefore, we increased the MOI used for transduction, showing high GFP expression when an MOI of 5 was used (Supplementary Fig. S2B). Importantly, transduction of melanoma cells with targeted LVs encoding both tSMAC and GFP at MOI 5 resulted in induction of apoptosis (Fig. 6A). We next evaluated the exposure of CRT and release of HMGB1 and ATP by 888-mel cells transduced with LV-tNGFR or LV-tSMAC. Mock-transduced cells served as a control. Similar to our observations with mouse tumor cells, we observed expression of CRT (Fig. 6B) but no release of HMGB1 (Fig. 6C) or ATP (Fig. 6D) after transduction with LV-tSMAC.

Subsequently, we showed the uptake of dying 888-mel cells by a significant fraction of immature HLA-A2\(^+\) DCs (Supplementary Fig. S2C, Fig. 6E). Phenotypic analysis of these DCs showed an enhanced expression of CD80 and CD83 (Fig. 6F). Finally, we showed that these DCs stimulate production of high levels of IFN-\(\gamma\) by HLA-A2/gp100-specific T cells (Fig. 6G).

**Discussion**

In this study, we report on the potency of LV-tSMAC to induce apoptosis of tumor cells of different histological origin, *i.e.* melanoma and mastocytoma cells. We demonstrated the anti-tumor efficacy of this strategy together with the underlying immunologic mechanisms. To our knowledge, we are the first to demonstrate that LV-tSMAC induced cell death results in anti-tumor immunity, which is mediated by a crosstalk between DCs and T-cells.

Previously McNeish and colleagues (12) showed that transduction of ovarian cancer cells with adenoviral vectors encoding tSMAC resulted in induction of tumor cell death. Our data extend these findings to melanoma and mastocytoma cells, which is of importance as it has been suggested that the role of SMAC can vary depending on the cell type (27). Of note, we achieved substantial induction of cell death using an MOI as low as 1. We hypothesize that the high efficiency we observed is attributed to the use of LVs as a delivery system. We previously showed that LVs activate TLR3 (17, 18), which is important as it has been described in melanoma that TLR3 engagement leads to a TRIF-dependent activation of caspase-8 hence results in induction of apoptosis (16). We indeed observed that transduction of melanoma cells with LVs encoding a reporter had an effect on melanoma cell death. Importantly, it was described for...
melanoma (16) and other cancer types (28) that SMAC mimicry and TLR3 triggering synergize to induce apoptosis. This could explain the potency of LV-tSMAC in our tumor models. Moreover, a single injection of $10^7$ TU of LV-tSMAC was sufficient to prolong the survival of tumor-bearing mice. Hereewith, we confirm the feasibility of delivering LVs to tumor cells in situ to induce tumor cell death, a strategy that was previously explored by others, delivering among others suicide genes (29, 30).

On the basis of the low in situ transduction efficiency, we hypothesized that the prolonged survival might be due to an immunologic component resulting from the LV-tSMAC–induced cell death. A first observation that corroborates this hypothesis is the infiltration of effector T cells within tumors treated with LV-tSMAC. Moreover, these T cells showed a low expression of PD-1, a receptor expressed on so called Treg in mice. Our observation that these T cells in contrast to the highly PD-1$^+$ T cells from control animals, could be stimulated in vitro to produce high amounts of IFN-$\gamma$ and kill target cells confirms that PD-1 is critical in the suppression of TILs (32, 33). In addition, we observed only a low percentage of CD4$^+$ CD25$^+$ Foxp3$^+$ T cells. These Treg actively contribute to inhibition of effector T cells and ACPs through various mechanisms (34–36). Importantly, both the infiltration of CD8$^+$ T cells (37) and the “loss” of Treg (38) in the tumor environment have been shown to be predictive for therapeutic outcome. We next showed that CD11c$^+$ cells from tumor draining lymph nodes stimulate T cells that recognize the model antigen OVA, which is expressed by MO4 cells. This observation suggests that DCs take up dying tumor cells, migrate to draining lymph nodes, process tumor antigens, and acquire a mature phenotype that enable them to activate antigen-specific T cells. Although Bonnotte and colleagues (39) succeeded in showing the presence of DCs that had engulfed FITC$^+$ dying tumor cells in draining lymph nodes, attempts to visualize trafficking of DCs that had taken up GFP$^+$ dying MO4 cells using a similar experimental set up failed in our hands. Consequently, we were unable to analyze the phenotype of DCs that engulfed dying cells in vivo. However, in vitro analysis of wild-type and TLR4 KO DCs that were exposed to dying cell–conditioned media or Annexin V$^+$ cells showed that DCs are activated only when dying cells are engulfed. This suggests that LV-tSMAC does not induce immunogenic cell death as defined by the exposure of CRT (40), release of HMGB1, and ATP (41), as the latter would result in DC activation through interaction with TLR4 and/or the receptor P2X7. Indeed, we only observed exposure of CRT, although we did not observe release of HMGB1 or ATP (42).

Using a human melanoma model, we showed that human DCs engulf dying cells, resulting in their maturation and presentation of the tumor cell–derived antigen gp100 to establish TILs. This observation strengthens our view that the induction of antitumor immune responses is dependent on the capacity of DCs to engulf dying cells, mature, and migrate toward T cells to present tumor antigen–derived peptides. A final observation that strengthens this view is the induction of CTLs in vivo.

Our data show the potential of LV-tSMAC as a strategy to target the tumor from within. Despite its promise, the clinical application of this type of therapy must be approached with caution (43). Undoubtedly a prerequisite for the translation of LV-based strategies to the clinic is restricting the expression of transgene to target cells. As shown, the latter can be accomplished by the use of target cell–specific promoters. Moreover, strategies to direct the transduction pattern of LVs using cell-specific nanobodies will further improve the safety and potential efficacy of LVs as an off-the-shelf therapeutic (44, 45).

In conclusion, LV-tSMAC–induced tumor cell death elicits antitumor immune responses mediated by a cross-talk between DCs and T cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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