Immunodominant AH1 Antigen-Deficient Necroptotic, but Not Apoptotic, Murine Cancer Cells Induce Antitumor Protection

Tania Løve Aaes, Hanne Verschuere, Agnieszka Kaczmarek, Liesbeth Heyndrickx, Bartosz Wiernicki, Iris Delrue, Bram De Craene, Joachim Taminau, Tinneke Delvaeye, Mathieu J. M. Bertrand, Wim Declercq, Geert Berx, Dmitri V. Krysko, Sandy Adjemian and Peter Vandenabeele

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Immunogenic cell death (ICD) occurs when a dying cell releases cytokines and damage-associated molecular patterns, acting as adjuvants, and expresses Ags that induce a specific antigenic immune response. ICD is studied mainly in the context of regulated cell death pathways, especially caspase-mediated apoptosis marked by endoplasmic reticulum stress and calreticulin exposure and, more recently, also in relation to receptor-interacting protein kinase–driven necroptosis, whereas unregulated cell death like accidental necrosis is nonimmunogenic. Importantly, the murine cancer cell lines used in ICD studies often express virally derived peptides that are recognized by the immune system as tumor-associated Ags. However, it is unknown how different cell death pathways may affect neoepitope cross-presentation and Ag recognition of cancer cells. We used a prolymphatic tumor vaccination model and observed that both apoptotic and necroptotic colon carcinoma CT26 cells efficiently immunized mice against challenge with a breast cancer cell line that expresses the same immunodominant tumor Ag. AH1, but only necroptotic CT26 cells would mount an immune response against CT26-specific neoepitopes. By CRISPR/Cas9 genome editing, we knocked out AH1 and saw that only necroptotic CT26 cells were still able to protect mice against tumor challenge. Hence, in this study, we show that endogenous AH1 tumor Ag expression can mask the strength of immunogenicity induced by different cell death pathways and that only necroptotic CT26 cells were still able to protect mice against tumor challenge. Hence, in this study, we show that endogenous AH1 tumor Ag expression can mask the strength of immunogenicity induced by different cell death pathways and that only necroptotic CT26 cells were still able to protect mice against tumor challenge. This work highlights necroptosis as a possible preferred ICD form over apoptosis in the treatment of cancer.
Besides adjuvanticity, the vaccination model requires TAA processing and recognition by the host (i.e., mouse) immune system and hence allows studying CTL cross-priming and activity (7, 8). Conveniently, many stable cancer cell lines express endogenous virally derived peptides that are recognized by the immune system as TAAs (9–11). CT26, a murine colon carcinoma cell line expressing the murine leukemia retrovirus (MuLV)–derived protein Gp70, is such an example. The Gp70 glycoprotein is one of two MuLV envelope-encoded gene products and harbors an immunodominant epitope corresponding to the sequence SPSYVYHQF (12–14). Gp70 is expressed and known to give rise to TAA-specific T cell responses in several murine cell lines, such as 4T1 mammary carcinoma, EL4 lymphoma, and B16 melanoma cells (15), but especially in CT26 cells, Gp70 is found to be the most abundant transcript (16).

In ICD studies, apoptosis is often induced in cancer cells by the use of chemotherapeutics (mitoxantrone, doxorubicin, oxaliplatin, etc.), photodynamic therapy, UV light, or gamma radiation (17, 18). However, especially in the case of chemotherapeutic triggers, it is difficult to distinguish between the immunogenic effects deriving from the cell death process and the direct effects of the drug on the immune system (19–24), although these can be of therapeutic relevance. To avoid such drug-mediated bystander effects, we used ligand-free Tet-ON–inducible constructs that allow studies of the immunogenic impact deriving only from the cell death process itself. To compare the impact of different cell death modalities on TAA expression and recognition, we used our previously reported immunogenic necroptosis system (25) and a similar Tet-ON–inducible apoptosis system. To date, inducible apoptosis has been shown with the active form of caspase-3 (26, 27), a dimerizing form of caspase-9 (28), caspase-8 (29), and a proteolytically activated form of Bid (tBid) (26) and Bim (30). However, common to all of these studies is that the TAA-specific immunogenicity was described only using cells overexpressing OVA, which is an exogenous Ag fulfilling the role of a model tumor Ag. Acknowledging the vast amount of literature on the immunodominant role of Gp70 and AH1 (31–36) in combination with our previous findings on immunogenic necroptosis using a ligand-free inducible system in the CT26 cell line (25), we aimed to study the immunogenic role of apoptosis in the same ligand-free inducible manner and compared it to necroptosis while taking into account that the AH1 peptide expression in these cells is likely to play an immunodominant role as an endogenously derived TAA.

In this study, we show that CT26 cells receiving an apoptosis stimulus give rise to a less prominent adaptive anti-AH1 immune response than in the case of necroptosis. Using AH1–deficient CT26 cells, we demonstrated that AH1 expression dictated the immunogenicity of cancer cells that had received an apoptosis stimulus. Although both apoptotic and necrotic CT26 cells would efficiently protect mice against challenge with another AH1-expressing cancer cell line, 4T1 mammary carcinoma, this immunogenicity was lost in apoptotic, but not necrotic, cells upon knockout (KO) of AH1, demonstrating tumor Ag immunodominance. Additionally, immunization of mice with necrotic CT26 cells revealed the involvement of CD4+ and CD8+ T lymphocyte–specific neopeptides likely to explain why necrotic cells would induce an Ag-specific immune response independent of the AH1 expression in the dead cell vaccine. Our findings highlight necroptosis as a potentially more immune response independent of the AH1 expression in the dead likely to explain why necroptotic cells would induce an Ag-specific and harbors an immunodominant epitope corresponding to the sequence SPSYVYHQF (12–14). Gp70 is expressed and known to give rise to TAA-specific T cell responses in several murine cell lines, such as 4T1 mammary carcinoma, EL4 lymphoma, and B16 melanoma cells (15), but especially in CT26 cells, Gp70 is found to be the most abundant transcript (16).

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determined using a NanoDrop 1000 spectrophotometer according to the manufacturer’s recommendations. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed with anti-FKBPI2 (PA-026A; Thermo Fisher Scientific), anti-RIPK3 (R2477; Sigma-Aldrich), anti-caspase-8 (MAB3429; Abnova), anti-cleaved caspase-8 (9429; Cell Signaling Technology), anti-caspase-3 (9662; Cell Signaling Technology), anti-cleaved poly(ADP-ribose) polymerase (9544; Cell Signaling Technology), anti-actin (69100; MP Biomedicals), anti-iFKBP12 (ab34667; Santa Cruz Biotechnology), anti-iBcl-2 (sc-377; Santa Cruz Biotechnology), anti-RIPK3 (2283; ProSci); anti-tubulin HRP (ab21058; Abcam), anti-FADD (sc-6036; Santa Cruz Biotechnology), anti-RIPK1 (610459; BD Biosciences), anti-MCL1 (MABC604; MilliporeSigma), and anti–phospho-MCL1 S345 (ab196436; Abcam). Secondary Abs included Amersham ECL HRP-linked sheep anti-mouse IgG (NA931; GE Healthcare), donkey anti-rabbit IgG (NA934; GE Healthcare), donkey anti-goat IgG (SC-2020), or goat anti-rat IgG (NA935; GE Healthcare). After incubation with the appropriate secondary Abs conjugated to HRP, blots were revealed using ECL Western blotting substrate (32106; Pierce Biotechnology) or Western Lightning Plus-ECL (NEL105001EA; PerkinElmer).

**Annexin V staining and cell death analysis by flow cytometry**

Cells were washed in annexin V binding buffer (556454; BD Biosciences), followed by staining with SYTOX Blue Nuclear Acid Stain (S11348; Molecular Probes) and aliphophycocyanin annexin V (550474; BD Biosciences) or annexin V–Alexa Fluor 488 conjugate (A13201; Molecular Probes). Samples were run on a BD FACSVerse or BD LSR II flow cytometer. The data were analyzed using FlowJo 10.6.1 (Becton Dickinson).

**Cletreculin exposure**

CT26 cells were collected after the indicated stimulation (with mitoxantrone or doxyBB) and washed once in ice-cold FACS buffer (PBS [Gibco Dulbecco PBS (DPBS), 14190-094; Life Technologies] plus 0.5% FCS [Life Technologies]). Cletreculin staining was performed in two steps: first with a rabbit anti-cletreculin Ab (92156; Abcam) for 30 min at 4˚C and next with a donkey anti-rabbit Ab coupled with DyLight 488 for another 30 min at 4˚C. Before acquisition on a BD FACSVerse flow cytometer (BD Biosciences), the cells were resuspended in FACS buffer with SYTOX Blue Dead Cell Stain (S11348; Molecular Probes). The data were analyzed with FlowJo 10.6.1 (Becton Dickinson).

**ATP and HMGB1 release**

Supernatant was collected and cleared from cells by centrifugation (1500 rpm for 5 min in the case of HMGB1). ATP was quantified using an ATP Bioluminescent Assay Kit (Sigma-Aldrich) and measured with the GloMax96 Microplate Luminometer (Promega). HMGB1 was measured with an ELISA kit (IBL International, Hamburg, Germany) and was quantified using the IMARK Microplate Absorbance Reader (Bio-Rad Laboratories), followed by analysis with a Four Parameter Logistic Curve Fit (MyAssays).

**Cytokine release**

Cells were seeded in six-well plates, left to adhere for 18 h, and then treated with doxy or doxyBB for another 18 h. Supernatant was collected and cleared from dying tumor cells by centrifugation (400 × g for 5 min). Cytokine quantification was performed using a bead-based multiplex immunosassay (ProcartaPlex; eBioscience) in accordance with the manufacturer’s instructions. Measurements were performed with Bio-Plex 200 Systems (Bio-Rad Laboratories) and analyzed with the Bio-Plex Manager software.

**CRISPR/Cas9 gp70 gene editing**

*Streptococcus pyogenes* WT Cas9 (pSpCas9(BB)-2A-GFP) was obtained from Addgene (plasmid no. 48138). The single guide RNAs (sgRNAs) targeting the AH1 region of gp70 were designed using the CRISPR Design Tool (http://crispr.mit.edu) and were ordered with Thermo Fischer Scientific. The sgRNA sequences are listed in Table I. The Cas9 vector was digested with BamH I and the sgRNA oligo was annealed and cloned into the BpiI-digested Cas9 vector. The cloning protocol by F. Ann Ran was largely followed (37). The sgRNA Cas9 plasmid was transfected into CT26 cells via jetPRIME transfection reagent (catalog no. 11-145, Polypuls-transfection). For each transfection, 2 μg of plasmid was added per 250,000 cells. The medium was replaced after 4 h, and 4 h after transfection, the cells were harvested. Single-cell sorting was performed on a FACSArria III from BD Biosciences. Effective genomic deletion was confirmed with genomic PCR and with a pair of primers flanking the deletion, followed by Sanger sequencing, and the allele editing was analyzed using TIDE (http://tide-calculator.nki.nl). The PCR and sequencing primers used are listed in Table II.

**Real-time quantitative PCR analysis**

RNA was isolated using the RNAeasy Plus Mini Kit according to the manufacturer’s instructions (74134; QIAGEN). RNA concentration was measured on the NanoDrop 1000 spectrophotometer. For the inflammatory cytokine profile analysis, the RNA quality was assessed using Agilent RNA 6000 Nano Reagents and Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA synthesis was performed with SensiFAST cDNA Synthesis Kit (BIO-65054; Bioline) or with iScript cDNA Synthesis Kit (170-8891; Bio-Rad Laboratories). AH1 gene expression was assessed with four Ah1 gene-specific primers (Table II) using SensiFAST SYBR No-ROX kit (BIO-98020; Bioline) on a LightCycler 480 instrument (Roche). Pigs1 and Pigs2 gene expression was assessed with TaqMan probes (Mm00477214_m1 and Mm00478374_m1; Applied Biosystems) using TaqMan Fast Advanced Master Mix (444558; Applied Biosystems) on a LightCycler 480 instrument (Roche). Data were analyzed with qbase+ 3.1 (Biogazelle) generating normalized relative quantities. The results shown represent the fold increase compared with the values obtained in the untreated or nontransfected control samples.

**Surveyor assay**

Cells were harvested, and DNA was extracted with QuickExtract DNA Extraction Solution 1.0 (QE0950; Epicentre) according to the manufacturer’s instructions. One microliter of the lysate was used per 10 μl PCR reaction, in which a 629-bp region spanning the AH1-encoding sequence was amplified using Kapa HiFi HotStart ReadyMixPCR Kit (KK2602; Kapa Biosystems). The PCR product was purified using CleanPCR beads (CPCR-0050; CleanNa). Four hundred nanograms of purified RNA was used for the Surveyor Mutation Detection Kit for Standard Gel Electrophoresis according to the manufacturer’s instructions (706020; Integrated DNA Technologies). The samples were analyzed by separation on a 2% Tris-acetate-EDTA–agarose gel and imaged by staining with ethidium bromide. To sequence the mutant clones, the cleaned PCR products were analyzed by sequencing using a nested primer. Primer sequences are given in Table II.

**Generation of mouse bone marrow–derived dendritic cells**

Bone marrow–derived dendritic cells (BMDCs) were differentiated from the femurs and tibias of 7-wk-old BALB/c WT mice for 8 d using RPMI 1640 culture medium (Life Technologies) supplemented with 5% heat-inactivated FCS, 1-glutamine (0.03%), sodium pyruvate (0.4 mM), 2-ME (50 μM), and murine GM-CSF (20 ng/ml). Fresh culture medium was added on day 3, and on day 6, the medium was refreshed. On day 8, only the suspension cells were collected to be used in the phagocytosis and coculture assays.

**Phagocytosis and BMDC surface marker expression**

The phagocytosis assay was performed entirely according to the protocol as previously described (25, 38). Cell death was induced in the CT26 FADD cells for 18 h. The dead cells were collected, washed in RPMI 1640 culture medium, and cocultured with 50,000 BMDCs in three different ratios, 1:1, 1:10, and 1:20 (BMDC/CT26 cells). Coculture was performed for 18 h at 37˚C in six-well plates with 2.5 ml RPMI 1640 culture medium. Control cells were left untreated or stimulated with 100 ng/ml LPS (L2630; Sigma-Aldrich). After coculture, all cells were collected, spun down (400 × g for 6 min at 4˚C), and washed once in PBS (Gibco DPBS, 14190-094; Life Technologies) plus 0.5% FCS. Dead cells were excluded from the flow cytometry analysis by staining with SYTOX Blue (S11348; Molecular Probes). Maturation of BMDCs were analyzed by immunostaining using aliphophycocyanin–anti–CD11c (550261; BD Pharmingen), FITC–anti–MHC class II (MHCIId; 11-5321; eBioscience), PE-Cy7–anti–CD86 (550261; BD Pharmingen), and mouse Fc block (553142; BD Pharmingen). Gating strategy is as follows: single cells were obtained by gating on forward scatter area versus forward scatter height, followed by side scatter area versus side scatter height. Dead cells were excluded by SYTOX Blue. Mature BMDC cells were identified as a percentage of CD11c+MHC II+CD80+. All samples were acquired on the BD FACSVerse flow cytometer and analyzed with FlowJo 10.6.1 (Becton Dickinson).

**PGE2 ELISA**

PGE2 was measured directly in conditioned medium from CT26 FADD AH1 KO or CT26 DDR3 AH1 KO cells that were left untreated, treated with 50 μM ibuprofen (710280; Cayman Chemical), and/or induced to cell death with doxy/doxyBB for 18 h in total. PGE2 quantification was performed using a PGE2 ELISA Kit (514010; Cayman Chemical) in accordance with the manufacturer’s instructions. Measurements were performed with the iMark Microplate Absorbance Reader (Bio-Rad Laboratories).
Mice
Female BALB/c WT mice (6–7 wk-old) were purchased from Harlan Laboratories or from Charles River Laboratories and female BALB/c Foxn1nu mice (6-wk-old) were bought from Janvier Labs. All mice were housed in specific pathogen-free conditions, and all experiments were performed according to the guidelines of the local Ethics Committee of Ghent University.

In vivo prophylactic tumor vaccination
CT26 cells were seeded on 15-cm petri dishes or in tissue culture-treated flasks, and cell death was induced via the inducible systems for 18–24 h. Where indicated, the cells were pretreated for 30 min prior to cell death induction with 5 μM TPCA-1 (2559; Trocis Bioscience) or for 1 h with 50 μM butyron (710280; Cayman Chemical). After cell death induction, the cells were collected, washed once in PBS (Gibco DBPS, 14190-094, Life Technologies), and resuspended in the desired concentration in PBS. Mice were inoculated s.c. with the indicated amount of CT26 cells or with PBS on the left flank side. Accidental necrosis was induced by subjecting CT26 cells, already resuspended in the desired PBS volume, to 5 × freeze-thaw (F/T) cycles (dry ice/37°C). On day 8 after vaccination, the mice were challenged s.c. on the opposite flank with 5 × 10⁴ untreated CT26 WT or F/T CT26 cells. Tumor growth on the challenge side was evaluated using an electronic caliper for up to 4 wk after the challenge. All mice, including the Foxn1nu mice, vaccinated with F/T necrotic cells were tumor free on the vaccination site. In the Foxn1nu mice, all other vaccinations would give rise to tumor formation on the vaccination site. Despite the high cell death percentage, vaccination with necrotic cells would not, in most cases (up to 80–100%), develop a tumor on the vaccination site. Apoptotic FADD AH1KO cell immunizations would rarely (0–25%) give rise to a vaccination tumor. Apoptotic CT26 FADD immunizations would form a tumor on the vaccination site in 50–75% of the mice, except for Fig. 1C, in which all the mice were tumor free on the vaccination site. Because of the variations of tumor growth on the vaccination site and no obvious correlation between vaccination tumor growth and challenge tumor protection, all mice were included in the Kaplan–Meier curves throughout the paper despite the tumor growth on the vaccination site. Mice were sacrificed when a tumor on the challenge site (or, in some cases, on the vaccination site) became necrotic or reached a volume (V) of 2000 mm³; if tumor free on the challenge site on the day of sacrifice, this is shown as a symbol on the horizontal part of the Kaplan–Meier curve. The volume was calculated using the following formula: V = the numerical value of π × ⅓ × tumor length × tumor height × tumor width (39).

IFN-γ ELISPot assay
Mice were injected s.c. with 3 × 10⁶ apoptotic CT26 FADD cells, necrotic CT26 DDR3 cells, or PBS at the tail base. Twenty-one days after immunization, the mice were sacrificed, and the spleens and inguinal draining lymph nodes were isolated. The spleens were treated twice with ACK Lysing Buffer (10-548E; Life Technologies), and the cells were spun down three times. Lymphocytes from the draining lymph nodes were resuspended in PBS containing 100U/ml penicillin and 100 μg/ml streptomycin and 100 μg/ml gentamycin. Twenty thousand lymph node cells were plated per well on a capture anti-IFN-γ Ab-coated 96-well PVDF bottomed plate (MSIPS4510; MilliporeSigma), and the cells were restimulated for 24 h with either 5 μg/ml of AH-1 peptide (PSPSYVYQFG, AS-64798; tebu-bio), 5 μg/ml of neoepitopes (a mixture consisting of CT26-M20 [PLLPFYPP-DEALEIGLELNSALPPT], CT26-M26 [VILLQAPGSPSYATLQ-PAAQMLTPP], CT26-M03 [DKPLRRNNSYTSYIMAICGM-PLDSFRA], CT26-M37 [EVIQTSSKYYMDVIAESAWLLELAPH], and CT26-M27 [EHHRRAGLVDFAIQGKFGRGKHF], or 4 μg/ml Con A type IV (C2010; Sigma-Aldrich), which served as a positive control. The murine IFN-γ ELISPot assay was performed according to the manufacturer’s instructions (862,031,010; Dianoche). Spots were imaged using an AEL VIS 4 Plate ELISPOT Reader V2.1 and quantified manually. The presented values (each dot) are the mean values of technical triplicates. For each mouse, spots counted in wells with AH-1 peptide (or neoepitope)–stimulated cells had only the background RPMI 1640 culture medium stimulation subtracted. The overall mean (black horizontal bar) and 95% confidence interval (error bars) for each immunization is calculated for all of the mice from two independent experiments.

Statistical analysis
Statistical analysis was performed in GraphPad Prism (v8.2.1). All prophylactic tumor vaccination experiments and the corresponding Kaplan–Meier survival curves showed the timeline for tumor occurrence on the challenge site. Calculations to compare two groups were done using the log-rank Mantel–Cox test. The phagocytosis assay was analyzed by a Mann–Whitney nonparametric t test. The BMDC activation and maturation assay was analyzed using a two-way ANOVA test. Gp70 real-time quantitative PCR (RT-qPCR) results were analyzed by log transformation of the normalized values and two-way ANOVA Tukey multiple comparisons tests. The IFN-γ ELISPot assays were all analyzed with an ordinary one-way ANOVA with Tukey multiple comparisons test. The DAMP measurements (ATP, HMGB1, and cytokines) were analyzed using a two-tailed Mann–Whitney nonparametric t test. PGE₂, ELISA and Pgd1/2 RT-qPCR results were analyzed with one-way ANOVA test with Holm-Sidak multiple comparisons test. Significant p values are indicated as follows: *p < 0.0332, **p < 0.0021, ***p < 0.0002, and ****p < 0.0001. No exclusion criteria were used.

Results
FADD-induced apoptosis is immunogenic in vitro and in vivo
It has been reported that overexpression and oligomerization of FADD can result in necrotic cell death in conditions in which caspase-8 is absent or blocked (40). However, depending on the cell line used, FADD oligomerization also serves as a platform for apoptosis induction (41). The BALB/c mouse–derived colon carcinoma cell line CT26 is commonly used in ICD studies (17, 25, 42, 43), so we chose to stably transfect this cell line with a FADD-dimerizing construct (CT26 FADD). For this purpose, we used a Tet-ON doxy-inducible construct of Flag-tagged FADD coupled to two copies of FKBP (FK506-binding protein), which allows oligomerization of FADD upon the addition of a dimerizing compound (B/B) (Supplemental Fig. 1A). Upon costimulation with doxyBB, the cells undergo apoptotic cell death, which is associated with the activation of the apoptotic executioner caspases as measured by DEVD-AMC proteolysis and which could be inhibited by pretreatment with a pan-caspase inhibitor, zVAD-fmk (Supplemental Fig. 1B). We also confirmed, as previously partially shown (25), that molecular characteristics of apoptosis (44–47) were present in the cell lysates following doxyBB stimulation; these included cleaved caspase-8, cleaved caspase-3, and cleaved poly(ADP-ribose) polymerase (Supplemental Fig. 1C). By time-lapse microscopy, we observed clear morphological features of apoptosis in the form of apoptotic bodies (48) prior to plasma membrane permeabilization (Supplemental Fig. 1D, Supplemental Video 1). And finally, we detected complex IIB formation, which has been shown to consist of FADD DD interaction with RIPK1 and FADD death effector domain interaction with caspase-8 (45, 49–51) but only upon FADD oligomerization by B/B (Supplemental Fig. 1E, 1F). Therefore, we conclude that oligomerization of FADD induces apoptosis in CT26 cells.

Upon efficient ICD induction, cross-priming and activation of CTLs will prevent tumor growth on the challenge site of mice that received an ICD vaccination (6). We induced apoptosis in CT26 FADD cells and collected them after 18 h when the majority of cells showed an apoptotic or secondary necrotic phenotype (Fig. 1A). We injected the cells as a vaccine into syngeneic naive BALB/c mice, which 8 d later, were challenged with untreated CT26 WT cells on the opposite flank (Fig. 1B). Vaccination with 3 × 10⁶ FADD-apoptotic CT26 cells proved to be highly immunogenic. All the vaccinated mice, which were also tumor free on their vaccination site, were completely protected against tumor challenge on the opposite flank side (Fig. 1C). The immunogenicity of the FADD-apoptotic CT26 cells was confirmed in vitro in coculture experiments with BMDCs (52). BMDCs engulfed the apoptotic CT26 cells to a significantly higher degree than untreated CT26 FADD cells (Fig. 1D, 1E), and overnight coculture of apoptotic CT26 FADD cells with BMDCs resulted in an upregulation of DC activation markers CD80 and MHCII (53), whereas the untreated CT26 FADD cells had no significant influence on the BMDC activation status (Fig. 1F, 1G). The release and exposure of DAMPs such as HMGB1, ATP, and calreticulin have been
proposed as crucial key factors for ICD (17, 42, 54, 55). We confirmed the release of the former two but did not find evidence for calreticulin exposure in our apoptotic model (Supplemental Fig. 1G–I).

Altogether, these results show that oligomerization of FADD overexpressed in the murine CT26 colon carcinoma cell line induces apoptosis as characterized by caspase-8 and -3 cleavage (25) and apoptotic blebbing. After cell death induction, coculture with BMDCs induces an efficient uptake of the apoptotic cells and up-regulation of BMDC maturation and activation markers. Finally, the apoptotic cells are also immunogenic in an in vivo setting, in which they protect mice with a high efficiency against tumor challenge in a prophylactic tumor vaccination model using syngeneic BALB/c WT mice.

**Antitumor immunity in response to apoptosis or necroptosis is independent of NF-κB activity in the dying cancer cells**

RIPK1 and NF-κB signaling have been reported to mediate cross-priming of CD8+ T cells in response to immunization with apoptotic mouse embryonic fibroblasts (29). Apoptosis induced by FADD oligomerization in CT26 cells caused an increase in the expression of several NF-κB–regulated inflammatory genes (Fig. 2A), many of which were similar to those induced by necroptosis in CT26...
DDR3 cells (25). Previously, we have shown that DDR3 induced to undergo necroptosis, were equally immunogenic, both under conditions with pronounced (+B/B) and minor (−B/B) NF-κB activity (25). Among its many gene targets, NF-κB drives the transcription of two chemokines CXCL1 and CXCL2, of which we measured a substantial increase in the supernatant of mainly apoptotic but also necroptotic CT26 cells (Fig. 2B, 2C). To test the concept of NF-κB activity contributing to the immunogenicity of dying CT26 cells, we used a selective inhibitor of IκB kinase 2 (56) prior to cell death induction in CT26 cells and used these cells in the prophylactic antitumor vaccination model. TPCA-1 pretreatment efficiently inhibited NF-κB activity in both apoptotic (Fig. 2D) and necroptotic (Fig. 2E) conditions. However, in the cell death vaccination model, blocking NF-κB activity showed no effect on the immunogenic properties of neither apoptotic nor necroptotic cancer cells (Fig. 2F, 2G).

Altogether, these results show a significant NF-κB activation and NF-κB–induced gene transcription profile in both apoptotic CT26 FADD and necroptotic (doxyBB-induced) CT26 DDR3 cells. However, upon blocking NF-κB activity with TPCA-1 in the dying cells and using these cells in the prophylactic tumor vaccination model in vivo, we did not observe any change in antitumor immunogenicity, hence demonstrating that the mere apoptotic or necroptotic cells used for prophylactic vaccination are sufficient to mount an antitumor response.

Necroptotic, but not apoptotic, CT26 cells elicit an immunogenic response directed toward both AH1 and neoantigenic epitopes

The MuLV-derived glycoprotein, Gp70, and the Gp70-derived antigenic peptide, AH1, have been used in antitumor studies, in which they showed strong vaccination boost potential (33, 34, 57). Interestingly, Gp70 is also one of the most expressed genes in the entire CT26 transcriptome (16). Thus, we speculated that knocking out AH1 from the CT26 genome by means of CRISPR/Cas9 technology (Supplemental Fig. 2A) would affect the immunogenicity of CT26 cells. Following transfection of CT26 FADD and CT26 DDR3 cells with an AH1-targeting Cas9–sgRNA plasmid (Table I),
we generated AH1-deficient clones and verified the KO efficiency by measuring AH1 transcript levels (Supplemental Fig. 2B) and indels in the AH1KO clones (Supplemental Fig. 2C) (Table II). Although our original results showed that FADD-dimerized induction of apoptosis in CT26 cells gave rise to a strong immunogenic antitumor response (Fig. 1C), vaccination with 1.5 × 10⁶ apoptotic cells was no longer immunogenic in the absence of AH1 expression, whereas mice vaccinated with necroptotic AH1KO cells were protected for a longer time period against a CT26 tumor challenge (Fig. 3A). Increasing the cell number used for vaccination to 3 × 10⁶ cells did not increase the immunogenicity of AH1-deficient apoptotic cells, which remained different from nonimmunized PBS-injected mice (Fig. 3B). Although the immunogenicity was lower in the necroptotic cells that did not express AH1 than in the parental necroptotic cell line, the tumor-free survival of mice that received a vaccination with AH1KO necroptotic cells was significantly longer than those that received an apoptotic AH1KO immunization (Fig. 3B). Gp70 and AH1 is expressed by a number of different mouse strains and murine cell lines (15, 58). To check the cross-reactivity of the CT26 prophylactic vaccinations, we immunized BALB/c WT mice with apoptotic (Fig. 3C) or necroptotic (Fig. 3D, 3E) CT26 cells expressing or not (AH1 KO) the immunodominant AH1 tumor Ag, but this time, we challenged the mice with the BALB/c-syngeneic breast cancer and AH1-expressing cell line 4T1. Apoptotic CT26 cells would efficiently protect mice against a 4T1 tumor challenge, but this tumor protection was solely dependent on AH1 expression in the dead cell immunization (Fig. 3C). Immunization with necroptotic CT26 cells induced by doxyBB (Fig. 3D) or doxy (Fig. 3E) also efficiently protected mice against 4T1 tumor challenge. Despite a slight difference in the level of tumor protection, AH1 expression did not seem to fully determine AH1immunogenicity in the absence of AH1 expression, necroptotic doxy-induced CT26 DDR3 cells still protected significantly more mice against 4T1 tumor challenge than in the case of apoptotic CT26 FADD immunization (Fig. 3F).

Recently, it was demonstrated that the therapeutic immunogenic response to necroptosis can be driven by neoepitopes (59, 60). To look further into the efficacy of the inducible necroptotic versus apoptotic cancer cell vaccines, we tested the AH1-deficient CT26 cells for their capacity to elicit an immune response to a mix of CT26-specific neoepitopes, consisting of four mutated MHCII-binding peptides and one MHC class I (MHC I)–binding peptide (60). Vaccination with necroptotic doxyBB AH1-expressing CT26 cells induced an AH1-specific adaptive immune response as measured by the production of IFN-γ from the draining lymph nodes (Fig. 3G) or splenocytes (Fig. 3H) from immunized mice. Apoptotic CT26 cells did not induce significantly increased IFN-γ levels whether in the presence or absence of AH1 expression (Fig. 3G-I). When we stimulated lymphocytes with neoepitopes, only the lymph nodes isolated from mice that had received the doxy-induced necroptotic CT26 vaccination responded in the IFN-γ ELISpot assay (Fig. 3I). AH1 and the tested neoepitopes are presented on MHC I or MHCII molecules (14, 60); thus, to examine the contribution of specific immune cells to the immunogenicity of apoptotic and necroptotic CT26 cells, we vaccinated athymic BALB/c mice that lack the T lymphocyte compartment. Immunization with neither apoptotic nor necroptotic CT26 cells could protect mice that lacked T lymphocytes against a challenge with CT26 WT cells (Supplemental Fig. 2D–G). Athymic mice immunized with doxy-induced necroptotic CT26 DDR3 cells showed a slight delay in tumor growth on the challenge site compared with nonvaccinated mice and mice vaccinated with apoptotic CT26 cells (Supplemental Fig. 2F, 2G); however, eventually all the mice, irrespective of the vaccination, showed the first tumor growth signs within 8 d after tumor challenge.

Altogether, apoptotic CT26 FADD cells efficiently immunize BALB/c WT mice against challenge with two different AH1-expressing cancer cell lines, CT26 and 4T1, but this immunogenicity appears to rely solely on AH1 expression in the apoptotic cells used for

<table>
<thead>
<tr>
<th>Table I. CRISPR/Cas9 guide sequences</th>
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<tr>
<td><strong>Target Gene</strong></td>
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<tr>
<td>AH1</td>
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The guide and reverse complement sequences (without the protospacer adjacent motif) were used for CRISPR/Cas9 gene editing of the CT26 cell line to create AH1KO cells.

### Table II. Gp70/AH1 primer sequences

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Sanger sequencing</td>
<td>5'-CCGTCCTCAGTACCTACTCCA-3'</td>
</tr>
<tr>
<td>Nested 1</td>
<td></td>
</tr>
<tr>
<td>PCR amplification</td>
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</tr>
<tr>
<td>471-bp fragment, forward</td>
<td>5'-CTCTCTAGATACCCCAAGAGTCTGATGTTATG-3'</td>
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<tr>
<td>471-bp fragment, reverse</td>
<td>5'-CCATAGGTGCTCTCCATATAGTAGGGCCAC-3'</td>
</tr>
<tr>
<td>629-bp fragment, forward</td>
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<tr>
<td>629-bp fragment, reverse</td>
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</tr>
<tr>
<td>RT-qPCR</td>
<td></td>
</tr>
<tr>
<td>No. 4, forward</td>
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</tr>
<tr>
<td>No. 4, reverse</td>
<td>5'-GAACCAACAATCTCAGTCTGATGTT-3'</td>
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<td>No. 5, forward</td>
<td>5'-AGGCTCATTGGGCAAAGAA-3'</td>
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<tr>
<td>No. 5, reverse</td>
<td>5'-TGTTGGAAACGTGCTAAGTGGC-3'</td>
</tr>
<tr>
<td>No. 6, forward</td>
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</tr>
<tr>
<td>No. 6, reverse</td>
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<tr>
<td>No. 7, forward</td>
<td>5'-AAAGGAGGAGGTTTGCTGCT-3'</td>
</tr>
<tr>
<td>No. 7, reverse</td>
<td>5'-GTTGGCCAGCTATCCCGG-3'</td>
</tr>
</tbody>
</table>

Primer sequences used for Sanger sequencing, PCR amplification, and RT-qPCR.
FIGURE 3. Necroptotic, but not apoptotic, CT26 cells give rise to an immunogenic response directed toward both AH1 and neoantigens. (A) Prophylactic vaccination of BALB/c WT mice with 1.5 × 10^6 or (B) 3 × 10^6 dead CT26 cells expressing or not (AH1 KO) the endogenous tumor Ag AH1 and challenge with 5 × 10^5 untreated CT26 WT cells. Mice tumor free after 2 mo were rechallenged on day 60 (indicated with a gray dotted vertical line) with 5 × 10^5 CT26 WT cells. Pool of three to four independent experiments; group sizes are indicated on the figures. (C–F) Prophylactic vaccination of BALB/c WT with 3 × 10^6 dead CT26 cells expressing or not (AH1 KO) the endogenous tumor Ag AH1 and challenge with 5 × 10^5 untreated 4T1 WT cells. Pool of three independent experiments with n = 11–13 per group. (G–I) IFN-γ ELISpot assays using the draining inguinal lymph nodes (G and I) or spleens (H) from nonimmunized (PBS) mice or mice vaccinated with necroptotic (DDR3) or apoptotic cells (FADD) cells. Each dot represents one mouse, and the mean and error bars represent the overall mean + 95% confidence interval (CI) of two independent experiments. (G and H) The lymphocytes and splenocytes were restimulated with AH1 peptide ex vivo, and IFN-γ spots were counted and background subtracted. *p < 0.0332, **p < 0.0021, ****p < 0.0001. ns, not significant (p < 0.1234).
vaccination. Vaccination with necroptotic CT26 DDR3 cells provides a stronger immunogenic phenotype as seen by the IFN-γ production from AH1- or neoepitope-stimulated immune cells. In the absence of AH1 expression, the necroptotic CT26 cells also show a more efficient vaccination potential than apoptotic cells against 4T1 tumor challenge.

Both apoptotic and necroptotic cells secrete a mixture of immunosuppressive and immunostimulatory adjuvants

Classically, apoptosis and caspase activation are linked with an immunosuppressive phenotype (61, 62). We therefore sought to investigate whether the AH1-deficient CT26 cells presented an immunosuppressive cytokine profile, which could explain their reduced immunogenicity and differences between immunogenic AH1KO apoptotic and necroptotic cells. We were able to detect a mixture of immunosuppressive, proinflammatory, and chemotaxtractant cytokines in the supernatant of both apoptotic and necroptotic cells (Fig. 4A, Supplemental Table I). Albeit low levels, apoptotic cells secreted more IL-6 but also more CXCL1, CXCL2, and CXCL10 than their necroptotic counterparts, regardless of the AH1 expression status (Fig. 4B–E). Whereas IL-10 was produced in higher concentrations in necroptotic CT26 DDR3 cells, IFN-β secretion was higher in apoptotic CT26 FADD cells (Supplemental Fig. 3A, 3B), suggesting that there is no clear immunosuppressive cytokine profile linked to apoptotic cells in our models. Production of PGE₂ is driven by cyclooxygenases (COX) and is known to suppress immunity and support tumor growth (63); hence, we tested whether blocking COX activity would increase the immunogenicity of apoptotic and necroptotic CT26 tumor vaccines. For this purpose, we used ibuprofen, which is reported to inhibit the constitutive active COX-1 and the inducible COX-2 (64, 65). PGE₂ secretion was not detectable in response to an apoptosis stimulus, but PGE₂ was secreted in high amounts by doxyBB-induced necroptotic DDR3 cells, and this secretion was efficiently blocked by pretreatment with ibuprofen (Fig. 4F). The PGE₂ secretion was likely to be due to COX2 activity rather than COX1 because we measured a similar increase in Pgs2, but not Pgs1, gene expression in the same doxyBB-induced necroptotic lysates (Supplemental Fig. 3C, 3D). Pretreatment with ibuprofen prior to cell death induction did not improve the vaccination potential of apoptotic cells (Fig. 4G). In the case of necroptosis, we also did not observe any difference in tumor protection following immunization with COX-inhibited necroptotic cells (Fig. 4H, 4I); hence, COX inhibition did not result in any significant changes in immunogenicity between apoptotic and necroptotic AH1-deficient CT26 cells (Fig. 4J).

Altogether, these results show that apoptotic CT26 cells, also in the absence of dominant AH1 tumor Ag expression, secrete a mixture of proinflammatory, immunosuppressive, and chemotaxtractant cytokines and, at times, in higher amounts than their necroptotic counterparts. Additionally, the decreased immunogenicity of AH1-deficient apoptotic CT26 cells cannot be attributed to COX activity in the dying cells.

Discussion

In this study, we show that ligand-free inducible apoptosis in a murine colon carcinoma cell line can elicit an efficient antitumor immune response. Using a similar cell death induction strategy, we also show that necroptosis, unlike apoptosis, gives rise to an antitumor immunogenic response directed toward not only an endogenous TAA, namely AH1, but also to a mixture of CT26-specific neoepitopes. Our experimental approach includes a model of drug-independent apoptosis in CT26 cells initiated by overexpression and oligomerization of FADD, which induces classical caspase-dependent apoptosis and is associated with the release of DAMPs (ATP and HMGB1), NF-κB activation, and secretion of chemokines (CXCL1, -2, and -10). The apoptotic CT26 FADD cells are phagocyted by BMDCs and lead to their phenotypic activation as measured by CD80 and MHCII cell surface expression. Immunizing mice with these apoptotic CT26 FADD cells, we observed a complete protection against a challenge with live tumor cells. This adaptive immunity response was independent of NF-κB activity in the dying cells but relied on the expression of an endogenous MuLV-derived tumor Ag, AH1. These results demonstrate that apoptosis as a cell death modality can be immunogenic when induced in a ligand-free manner but that in the absence of an immunodominant epitope, the immunogenicity of these dead cells is lost.

As a comparative cell death model to FADD-induced apoptosis, we used another inducible system, CT26 DDR3, in which necroptosis induction triggers an adaptive anti-AH1 tumor Ag immune response (25). Using these necroptotic CT26 DDR3 cells induced by either doxy (DD and RIPK3 overexpression) or doxyBB (additional oligomerization of DD) (25) in a prophylactic tumor vaccination model, we now show that the antitumor protective immune response was independent of NF-κB activity in the necroptotic cells. As opposed to the FADD-induced apoptosis, vaccination with necroptotic CT26 cells induced not only a strong AH1-specific adaptive immune response but also neoepitope-specific immunogenicity. The neoepitope-induced immune response was also present, albeit to a lower extent, upon vaccination with AH1-deficient necroptotic CT26 cells.

It has been reported that inducible necroptosis in OVA-expressing 3T3 fibroblast cells is more potent than apoptosis in activating an Ag-specific immune response and that cross-priming of T cells relies on RIPK1-driven NF-κB activity in necroptotic cells (29). Contrary to the FADD-induced apoptosis, vaccination with necroptotic CT26 cells induced not only a strong AH1-specific adaptive immune response but also neoepitope-specific immunogenicity. The neoepitope-induced immune response was also present, albeit to a lower extent, upon vaccination with AH1-deficient necroptotic CT26 cells.

By definition, an adaptive immune response depends on the recognition of tumor Ags, and depending on the type of cancer, different tumor Ags or neoantigens have been identified (67). Previous studies reported the endogenously expressed retrovirus-derived tumor Ag in CT26 cells, Gp70, and its immunodominant epitope (AH1) to be recognized by CTLs when presented on MHC I molecules (14). The immunogenic role of AH1 has been further substantiated in different vaccination studies using recombinant AH1 or AH1-mimicking peptides (8, 33, 34, 57, 68), and Gp70 expression is not confined to CT26 cells only but is also found in other murine cell lines, such as 4T1, B16, and EL4 (9, 10, 15). Consolidating these findings with our immunogenic observations using dying CT26 cells as a prophylactic tumor vaccination led us to demonstrate that the high expression of the
immunodominant AH1 was indispensable for the immunogenicity of apoptotic CT26 cells, whereas necroptotic CT26 cells could engage also neoepitopes. Vaccination with both AH1-proficient apoptotic and necroptotic CT26 cells protected against 4T1 tumor challenge in syngeneic BALB/c mice, but under AH1-deficient vaccination conditions, only necroptosis still showed the remaining protection against challenge with CT26 or 4T1 WT cancer cells. This demonstrates the immunodominant phenotype of AH1 expression in FIGURE 4.

**FIGURE 4.** Immunostimulatory and immunosuppressive adjuvants are secreted by apoptotic and necroptotic CT26 cells. (A) Heatmap representation based on the mean of values of up to three independent cytokine assays. The cytokine levels were measured in supernatants of nonstimulated (untreated [UT]) or dead cell supernatants from apoptotic or necroptotic CT26 cells in the presence (+) or absence (−) of AH1 expression. (B) IL-6, (C) CXCL1, (D) CXCL2, and (E) CXCL10 cytokine expression measured in the supernatants of nonstimulated (UT) or dead cell supernatants from apoptotic or necroptotic CT26 cells in the presence (WT) or absence (AH1KO) of AH1 expression. (B–E) Data points represent each of the four biological replicates measured in one ProcartaPlex assay. (F) PGE2 secretion measured in CT26 FADD/DDR3 AH1 KO supernatants by ELISA. Mean values + SD of three biological replicates measured in one assay. (G–J) Prophylactic vaccination of BALB/c WT mice with 3 × 10^6 dead AH1 KO CT26 cells with or without ibuprofen pretreatment and challenge with 5 × 10^5 untreated CT26 WT cells. Data represents one vaccination experiment with n = 6 per group. *p < 0.0332, **p < 0.0002, ***p < 0.0001. ns, not significant (p < 0.1234).

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these murine cancer cell lines, especially in apoptotic conditions. In necrotic conditions, cross-presentation of neoepitopes is still able to elicit a persisting antitumor response against CT26 cells. It is quite remarkable that vaccination with AH1-deficient necrotic CT26 colon carcinoma cells are still able to prevent tumor growth of an unrelated 4T1 breast carcinoma cell line. It can be speculated that either other unknown Ags shared by CT26 and 4T1 are at stake and favored by necrotic cell death or necrotic in contrast to apoptotic tumor cells may be associated with elucidation of an Ag-independent antitumor response involving NK cells or innate lymphoid cells (69). This hypothesis is subject to further research.

Our work highlights the importance of immunodominant TAA s in the CT26 cancer cell line and demonstrates the antigenic contribution to ICD, which in addition to DAMPs, induce efficient antitumor immunity. The balance between adjuvantivity and antigenicity in ICD models is an issue that has been previously discussed in a therapeutic tumor treatment setting, in which reported chemotherapeutic ICD inducers showed no involvement of adaptive immune cells in the eradication of spontaneous tumor growth (70). However, our study now illustrates the impact of different intrinsically induced cell death modalities on the antigenicity of cancer cells in a prophylactic antitumor vaccination setting. Our results are in line with a recent report in which it was demonstrated that necrotic CT26 cells, induced by tumor electroporation with an MLKL-encoding mRNA, gave rise to an efficient antitumor immune response directed against CT26-specific neoepitopes (59, 60). However, in that study, the response of apoptotic cells against the dominant AH1 Ag was not evaluated. Related to ICD adjuvantivity, caspase-3 activation and apoptosis induction by radiation therapy has been shown to stimulate the secretion of PGE2 (71). Apoptotic caspases were also demonstrated to suppress type I IFN production (72, 73), and recently, it was reported that caspase-3 has an inhibitory effect on the immunogenicity of cancer cells that are dying in response to radiation therapy (74, 75). In our Tet-ON–inducible system, despite the NF-κB and caspase-3 activity measured in the FADD–apoptotic colon carcinoma cell model, we observed neither an increase in COX-2 gene expression nor secretion of PGE2. We did, however, measure a slight increase in IFN-β production, but not IFN-α secretion, upon caspase-3 activation, but because treatment with a pan-caspase inhibitor (i.e., Z-VAD-fmk) would completely block apoptosis in these cells, we did not further evaluate whether the type I IFN response would become more profound upon caspase-3 inhibition or knockdown as recently published (74). Additionally, as opposed to the radiation-induced models that take days for apoptosis to occur (71, 74), our Tet-ON–inducible apoptosis model is an extremely fast system; it kills the cells within just a few hours following FADD induction and oligomerization by B/B administration. Hence, the added role of type I IFNs to immunogenicity in our system is probably neglectable, yet it is worth further investigation.

It can be speculated that, in the absence of AH1 expression, other (e.g., virally derived) endogenous tumor Ags expressed in CT26 cells account for the Ag-dependent immune responses. Indeed, in the CT26 cell line, other mutations have been identified that can lead to the formation of antigenic peptides, which are predicted to bind to MHC molecules and to be recognized by CD4+ and CD8+ T cells (16, 60). Identifying a pattern of antigenic epitopes that are induced under various cell death stimuli can become a highly valuable tool for future immunotherapeutic cancer treatments in the clinic. However, already at the discovery phase, our findings emphasize the importance of endogenous immunodominant Ags in cancer cell lines used for ICD and cancer immunology studies but also how the cell death modality determines the strength of the immune response to alternative neoepitopes.

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Disclosures
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References


