

Conventional amphotericin B elicits markers of immunogenic cell death on leukemic blasts, mediates immunostimulatory effects on phagocytic cells, and synergizes with PD-L1 blockade

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ABSTRACT

Immunostimulatory regimens are a game changer in the fight against cancer, but still only a minority of patients achieve clinical benefit. Combination with immunomodulatory drugs and agents converting otherwise non-immunogenic forms of cell death into bona fide “immunogenic cell death” (ICD) could improve the efficacy of these novel therapies. The aim of our study was to investigate conventional Amphotericin B (AmB) as an enhancer of antitumor immune responses. In tumor cell line models, AmB induced ICD with its typical hallmarks of calreticulin (CALR) expression and release of high mobility group box 1 (HMGB1) as well as Adenosine 5'-triphosphate (ATP). Interestingly, in contrast to non-ICD inducing treatments, ICD induction led to up-regulation of PD-L1-expression by ICD experiencing cells, resulting in decreased maturation of dendritic cells (DCs). Blocking this PD-L1 expression on tumor cells could unleash full ICD effects on antigen presenting cells. Even at sub-toxic concentrations, AmB was able to enhance CALR on leukemic blasts, particularly on phagocytic monoblastic THP-1 cells, which also showed features of “M1-like” differentiation after AmB exposure. The ability of AmB to increase the immunogenicity of tumor cells was confirmed in vivo in a mouse vaccination experiment. In conclusion, we demonstrate that AmB can promote antitumor immune responses in a dose-dependent manner by ICD induction, surface translocation of CALR on leukemic blasts even at sub-toxic concentrations, and “M1-like” polarization of phagocytic cells, making it noteworthy as potential booster for cancer immunotherapy. We additionally report for the first time that PD-L1 expression may be a feature of ICD, possibly as a negative feedback mechanism regulating the maturation status of DCs and thus indirectly affecting T-cell priming.

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Introduction

To prevent adverse inflammatory effects with catastrophic consequences to the whole-body homeostasis, the physiological form of regulated cell death (RCD) is a process relatively “invisible” to the adaptive immune system, resulting in a silent or tolerogenic phagocytic clearance.¹ However, in some specific cases, cells that succumb to stress can display sufficient antigenicity and adjuvanticity to elicit an adaptive immune response, a process referred to as immunogenic cell death (ICD).^{2–4} This rare type of apoptosis with immunogenic abilities is usually preceded by premortal activation of the ER stress response and results in enhanced adjuvanticity by up-regulation and secretion of danger-associated molecular patterns (DAMPs). They act analogously to the pathogen-associated molecular patterns (PAMPs) by stimulation of pattern recognition receptors (PRRs) expressed by innate immune cells.^{2,5,6} Consequently, DAMPs released after ICD can

stimulate maturation of the antigen presenting cells (APCs) capable of eliciting adaptive immune responses, followed by the establishment of an immunological memory.^{5,7}

Endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) generation appear to be the common feature of most, if not all, ICD generating modalities.⁵ Unfolded protein response (UPR) occurs as response to ER stress to restore cell homeostasis, but can also shift cell fate toward death if ER stress cannot be controlled.⁵ The ICD is then associated with timely release of immunostimulatory DAMPs. The key DAMPs are (i) surface-exposed ER chaperones HSP70, HSP90, and especially calreticulin (CALR), which are able to activate innate immune cells and act as an “eat me” signal;^{2,8–10} (ii) extracellular ATP, a “find me” signal and an activator of the NLPR3 inflammasome,^{10,11} and (iii) extracellular HMGB1, which binds to Toll-like receptors (TLRs) 4 and 2 and the receptor for advanced glycosylation end products (RAGE).^{2,10,12} Together with other ICD-associated molecules

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as nucleic acids, cytokines like CXCL1 and CXCL10, or annexin A1 (ANAXA1), the annexin superfamily member and type I interferon signature, these are prerequisites that contribute to adaptive immunity.

Apart from the relative rarity of ICD inducing agents, the dose required to induce an immunological type of apoptosis is regularly much higher than the dose required to induce an “ordinary” apoptosis and the acceptable dose *in vivo*.¹³ Thus, treatments such as doxorubicin (Dox), which is a potent ICD inducer,^{14,15} likely mediate their therapeutic effects in part by stimulating the immune system, but are not capable of eliciting a strong enough immune response on their own to completely eliminate cancer cells in clinical practice. The lower than expected efficacy of ICD-inducing agents is also caused by the tumor microenvironment, which hinders the immunological effects of ICD.

AmB is thought to have some kind of antitumor activity that is not clearly understood.^{16–19} Considering its mechanism of action, characterized by generating ROS and ER stress,^{20,21} the increase of intracellular Ca²⁺ concentration,^{22,23} the production of nitric oxide (NO)²⁴ and IL-8 secretion²⁵ (all known as major factors leading to CALR surface expression^{8,11,14,26,27}), and ATP secretion,^{23,28,29} we found it tempting to study AmB in the context of ICD and stimulation of antitumor immune responses.

AmB used as an antifungal agent has almost disappeared in clinical practice following the introduction of less toxic liposomal formulations. However, unlike its liposomal form, conventional AmB is reported to possess additional significant immunomodulatory properties.²⁰ Interestingly, in virtually all studies investigating antifungal prophylaxis in leukemia patients, significantly better leukemia remission rates were obtained in the AmB-group than in the control arms.^{30,31}

In the present study, we show that AmB triggers ICD and shifts phagocytic cells toward an antitumor phenotype. AmB proved to be a potent stimulator of CALR surface translocation in leukemic blasts, particularly in THP-1 myeloid leukemia cells, even at sub-toxic concentrations that do not necessarily lead to cell death. Given the strong positive correlation between CALR expression and clinical outcome in leukemia patients,^{32,33} this is a remarkable finding. Interestingly, when we examined ICD induction by AmB and Dox, we observed that ICD is regularly accompanied by significant PD-L1 up-regulation, which prevents proper DC maturation, and that blocking of the enhanced PD-L1 expression can abrogate the inhibitory effects on DCs.

Materials and methods

Cell lines, reagents and antibodies

K562 (CML in blast crisis (DSMZ Cat# ACC-10, RRID: CVCL_0004)), MCF-7 (breast adenocarcinoma (DSMZ Cat# ACC-115, RRID:CVCL_0031)), Jurkat (T cell leukemia (DSMZ Cat# ACC-282, RRID:CVCL_0065)), THP-1 (acute monocytic leukemia (DSMZ Cat# ACC-16, RRID:CVCL_0006)), HT29 (colon adenocarcinoma (DSMZ Cat# ACC-299, RRID: CVCL_0320)) cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ).

The cell lines were cultured in RPMI 1640 medium (Gibco Cat# 61870–010). All media were supplemented with 10% heat-inactivated FBS (Gibco Cat# 10500–064) and 100 U/mL penicillin (Gibco Cat# 15140–122). MCF-7 was additionally supplemented with NEAA (Gibco Cat#11140-050) and HT29 cells were cultured only in McCoy5a (Gibco Cat# 26600–023).

For flow cytometric analyses, fluorescence labeled monoclonal antibodies: CALR – FITC (Enzo Life Sciences Cat# ADI-SPA-601-488, RRID:AB_10615501), CD11c–APC (BD Biosciences Cat# 559877, RRID:AB_398680), CD86–PE-Cy7 (BD Biosciences Cat# 561128, RRID:AB_10563077), CD83 – FITC (BD Biosciences Cat# 556910, RRID:AB_396534), CD80 – PE-Cy7 (BD Biosciences Cat# 561135, RRID: AB_10561688), HLA-DR -PE-Cy7 (BD Biosciences Cat# 560651, RRID:AB_1727528), TGM2 (Bio-Rad Cat# 0100–0582, RRID:AB_2240525, Goat Anti Rabbit IgG-FITC: Bio-Rad Cat# STAR121F, RRID:AB_567028), CD47-APC (Miltenyi Biotec Cat# 130–101-407, RRID:AB_2658401), PD-L1-PE-Cy7 (BioLegend Cat# 329718, RRID: AB_2561687), MDR1-APC (Miltenyi Biotec Cat# 130–107-473, RRID:AB_2656599), STAT1p-APC (BD Biosciences Cat# 612597, RRID:AB_399880), STAT3p-FITC (BD Biosciences Cat# 557814, RRID:AB_647098), CXCL10-APC (Miltenyi Biotec Cat# 130–104-963, RRID:AB_2651478) were used.

Apoptosis of tumor cells

Tumor cell death induced by AmB was monitored after incubation with indicated drug concentrations for indicated time periods. Dox at ICD inducing concentration (5 μmol) served as a positive control. Cisplatin (non-ICD inducer at apoptosis inducing low and high concentrations) was used as a negative control in some experiments.

Cell death was assessed by annexin V FITC (Life Technologies Cat# V132245) staining. Briefly, 10⁶ cells per sample were collected, washed in PBS (Gibco Cat# 14190–094), pelleted and resuspended in an incubation buffer containing annexin V-FITC antibodies. The samples were kept in the dark and incubated for 15 min prior to the addition of DAPI (Sigma Cat# D9542), and subsequent analysis was performed with a FACSCanto II (BD Bioscience).

Flow cytometric analysis

Tumor cells were plated on 12-well plates and treated with the indicated agents for indicated time periods. The cells were collected and washed twice with PBS, and then incubated for 30 minutes with the specified antibodies. Each sample was analyzed by a FACSCanto II flow cytometer (BD Bioscience) to identify cell surface expression of CALR, CD47, TGM2, MDR1 and PD-L1.

For the DC maturation experiments mAbs against the following molecules were used: CD80, CD83, CD86, CD11c and HLA-DR. For the intracellular status of STAT1p, STAT3p, and CXCL10 in the THP-1 cells, cells were stained with the indicated antibodies using the Fixation Buffer (BD Biosciences Cat#556455) and Perm Buffer III (BD Biosciences Cat# 558050, RRID:AB_2869118) and protocol.

Detection of HMGB1 release

Tumor cells were plated in 1 mL full medium appropriate for the cell type. Supernatants were collected at different time points, dying tumor cells were removed by centrifugation, and the supernatants were isolated and frozen immediately. Quantification of HMGB1 in the supernatants was assessed by ELISA (LSBio Cat# LS-F23444) according to the manufacturer's instructions.

Detection of ATP release

Following AmB- or Dox-treatments, extracellular ATP was measured in the conditioned media (serum-free) via an ATP assay Kit (Abcam Cat# AB83355) based on the phosphorylation of glycerol, following the manufacturer's instructions.

RT-PCR analysis

RNA Isolation, cDNA synthesis and realtime PCR analyses were performed as described previously.³⁴ Gene expression analyses were carried out using the realtime PCR system (LightCycler® 480, Roche) and 96-well microtiter plates. The expressions of the following genes were analyzed: CALR, MYC. Each cDNA sample was analyzed in duplicate and normalized against expression of the reference gene GAPDH. Relative quantification was performed applying the Δ CT or $\Delta\Delta$ CT method. The whole experiment was carried out on three different occasions.

Primers and probes (TIB Molbiol, Germany) used in this study:

GAPDH fwd 5' gAA ggT gAA ggT Cgg AgT C 3'

GAPDH rev 5' gAA gAT ggT gAT ggg ATT TC 3'

GAPDH-TM 5'-FAM CAA GCT TCC CGT TCT CAG CC
CalR-fwd 5' gAC CTC TGG CAG GTC AAG TC 3' CalR-rev 5'
TCA GCG TAT GCC TCA TCG T 3' CalR-TM 5'-FAM TGC
ACC ATC TTT GAC AAC TTC CTC ATC ACC

C-MYC rev 5' GTC GTT TCC GCA ACA AGT CCT CTT C

C-MYC fw 5'AAT GAA AAG GCC CCC AAG GTA GTT
ATC C For MYC – analysis Platinum SYBR Green qPCR Super
Mix -UDG was used.

IDC generation, co-culture of iDCs with tumor cells and FACS analysis of DC phenotype after interaction with killed tumor cells

The experiment was performed based on methods described by Fucikova et al.¹⁵ Peripheral blood mononuclear cells (PBMCs) were obtained from human blood of healthy donors by density gradient centrifugation using Ficoll-Paque™ PLUS (GEHealthcare Cat# 17-1440-02). The procedure was approved by the Local Ethical Committee (Ethikkommission, Ethikausschluss 1 am Campus Charité Mitte). Monocytes were isolated from the PBMCs using the Pan Monocyte Isolation Kit (Miltenyi Biotec Cat# 130-096-537). To generate immature DCs (iDCs), they were treated with IL-4 (Promocell Cat# C61420A) and granulocyte-macrophage colony stimulating factor (Miltenyi Biotec Cat# 130-095-372). The tumor cells were exposed as indicated to AmB or Dox. The extent of apoptosis was monitored by annexin V/DAPI staining. The cells were extensively washed prior to feeding to DCs. iDCs (Day 5) were fed tumor

cells at a DC/tumor cell ratio of 5:1. The phenotype of DCs cultured with tumor cells was monitored by flow cytometry. Tumor cells killed by AmB or Dox were co-cultured for 24 hours with iDCs. For some experiments, the killed tumor cells were pre-incubated for 1 hour with anti-PD-L1 mAb (BioLegend Cat# 329715, RRID:AB_11149486). The DCs were stained for 30 min at 4°C, washed twice in PBS and analyzed using a FACSCanto II (BD Biosciences). FMO (fluorescence minus one) and unstained, untreated controls were included.

ELISA to detect IL-12p70, IL-10, and IFN- γ

IDCs were co-cultured with killed tumor cells (as indicated above) or stimulated with lipopolysaccharide (LPS Cat# L2630) as a positive control. The culture supernatant was then harvested to detect the level of IL-12p70 and IL-10 secretion using ELISA kit (IL-12p70 ELISA kit (BioLegend Cat#431704); IL-10 ELISA kit (BioLegend Cat# 430604)) according to the manufacturer's instructions. After co-culture with cancer cells unpulsed or tumor-loaded DCs were added to autologous T cells at a ratio of 1:10. After 48 hours, the production of IFN- γ was measured by ELISA (BioLegend Cat# 430104) according to the manufacturer's instructions.

Anti-tumor vaccination

The animal study was conducted at the EPO GmbH Berlin-Buch, Germany in compliance with the United Kingdom Coordinated Committee on Cancer Research guidelines and has been approved and authorized by the Landesamt für Gesundheit und Soziales, Berlin, Germany (approval No. Reg 0010/19).

The experiment was performed based on the "gold standard" protocol for the in vivo assessment of ICD in immunocompetent mice as described previously.³⁵ Briefly, for cell vaccination a total of 3×10^5 CT26 cells (LGC Standards GmbH (Wesel, Germany)), treated for 24 h with AmB or AmB plus anti-PD-L1 mAb (clone 10 F.9G2, Hölzel GmbH, Köln, Germany, (for 1 h before injection)), were inoculated subcutaneously (s.c.) in 100 μ l PBS into the right flank of 6-8-week-old female BALB/c mice. Mice injected with PBS or CT26 cells treated with the established ICD inducer mitoxantrone served as a negative and as positive control respectively. One week after immunization, mice were challenged with 3×10^5 untreated vital CT26 cancer cells s.c. on the opposite (left) flank. Tumor growth was measured in two dimensions with a caliper. Tumor volumes (TV) were determined by the formula: $TV = (\text{width}^2 \times \text{length}) \times 0.5$. Mice in the "AmB + anti-PD-L1" group were additionally treated with 250 μ g anti-mouse PD-L1 antibody intraperitoneally on day 8, 12, and 16. Animals with tumors of $> 1.5 \text{ cm}^3$ or necrotic tumors, were sacrificed (humane endpoint). The study was terminated on day 26.

Statistical analysis

The data show the compiled results (mean and standard deviation) of at least three independent experiments. Statistical significance was determined using the unpaired t-test and Mann-Whitney-U-Test. Differences were considered significant when $*p < .05$.

Results

Cell death induced by AmB is associated with robust generation of ICD surrogate markers

AmB triggered tumor cell killing in a dose-dependent manner. Induction of early/late apoptosis analyzed by flow cytometry with annexin V-FITC and DAPI showed a substantial increase in apoptotic cells at a concentration of approximately 20 $\mu\text{g/ml}$ AmB in the solid and leukemic tumor cell lines (K562 (CML in blast crisis), MCF-7 (breast cancer), Jurkat (T cell leukemia), HT29 (colon cancer), THP-1 (acute monocytic leukemia)). Apoptosis results are shown for K562 cells (Figure 1a). K562 and THP-1 were of particular interest for most of our further experiments, as a strong correlation between calreticulin surface expression on leukemic blasts and clinical outcome has already been demonstrated.^{32,33}

Since the ability of apoptotic cells to undergo ICD is strongly dependent on the surface exposure of CALR associated with ER stress, we tested for CALR expression. Treatment of tumor cells with AmB

for 24 hours resulted in significant surface expression of CALR (2–4 fold increase compared with untreated cells), which was even higher than CALR membrane translocation induced by the well-known ICD inducer Dox (Figure 1(b,c)). CALR expression increased in a dose-dependent manner in the range of 20–100 $\mu\text{g/ml}$ AmB concentration.

This CALR expression was accompanied by a decrease in CD47 after AmB treatment, suggesting that AmB-treated cells are more likely to be phagocytosed and thus elicit an immune response (Figure 1(b,c)), because CD47 on dying tumor cells is a known “don’t eat me” signal. A similar expression pattern of CALR and CD47 after AmB treatment was observed in all cancer cell lines tested, suggesting that ICD mechanisms induced by AmB are wide-spread in many tumor entities (Figure 1c).

Notably, as in most studies, the expression of ICD markers was presented only for early apoptotic annexin V +/DAPI- cells. However, the fact that DAPI+ cells were usually excluded in ICD studies may underestimate their role in the subsequent immune response. Since we observed significantly higher CALR levels also in DAPI+ cells after treatment with ICD inducers, in contrast to treatment with the non-ICD inducer cisplatin, which hardly induced CALR expression in a population of DAPI+ late apoptotic/necrotic cells (Supplementary Fig. S1). In addition, we also measured HMGB1, a late-stage marker of ICD and a key DAMP, by ELISA in the supernatants of dying tumor cells and ATP, the autophagy-dependent release of which is another hallmark. The amounts of HMGB1 released from AmB-treated cells were significantly increased after 24 hours of treatment and continued to increase, with a maximum at 48 hours (Figure 1d). The effect was comparable in a leukemic (K562) and a solid (MCF-7) tumor cell line. Similarly, to CALR expression and HMGB1 release, AmB treatment also resulted in a significant increase of extracellular ATP after 24 hours of exposure (Figure 1e). Unlike HMGB1, the maximal effect was reached much earlier, after 4 hours of treatment

Effects of AmB at sub-toxic concentration

As shown above, AmB very effectively stimulated CALR translocation to the cell surface in early and late apoptotic cells after treatment with high doses of drug, resulting in a relatively rapid induction of apoptosis. In the therapeutic concentration range used for antifungal therapy^{18,36} around 2.5 $\mu\text{g/ml}$, an increase of CALR expression compared with untreated cells was observed only in two particular cases.

We tested effects of AmB at different drug concentrations and time points and observed that leukemic K562 blasts exposed to low and nontoxic AmB concentrations corresponding to those used for antifungal treatment or prophylaxis also began to express CALR on their surface. However, the CALR translocation occurred much later in that constellation (after approximately 7 days of incubation). The mRNA expression started to increase about 2 days earlier (Figure 2a) and the drug concentration required for detectable CALR surface expression started at the concentration of 2.5 $\mu\text{g/ml}$ (Figure 2a).

On the other hand, THP-1 acute monocytic leukemia blasts, a model cell line used to study phagocyte functions, showed increased CALR expression as early as 90 min of AmB exposure at 2.5 $\mu\text{g/ml}$. It could be enhanced by higher doses and was observed until approximately 48 hours of incubation. It was further confirmed at the mRNA level (10-fold increase at 4 hours). Not only did the AmB-treated monocytic blasts show a stronger CALR expression intensity, but a significantly higher number of AmB treated cells became CALR-positive in all cases (Figure 2b). Interestingly, we could show, that AmB activates phagocytic THP-1 cells. In parallel with CALR overexpression in THP-1 cells, AmB affected their STAT phosphorylation status. In contrast to STAT3, only STAT1 phosphorylation, which plays a role in type I and II IFN- γ signaling and is typical of the “M1-like” anti-tumor response of phagocytes, was enhanced after AmB treatment. The “M1-like” character of phagocytic cells activated by AmB was further confirmed by the increased production of another typical “M1-like” molecule CXCL10 (Figure 2c).

AmB induces a robust ICD reaction, but the concomitant upregulation of PD-L1 on tumor cells in response to AmB exposure impedes the activation effects on iDCs. However, the impaired ICD effects can be reversed by PD-L1 blockade

In order to test for the subsequent effects on iDCs, we examined the maturation status of dendritic cells and their ability to produce IL-12/IL-10 after co-culture with AmB-treated tumor cells (K562). Unexpectedly, we found that ICD induction by AmB resulted in only a very modest upregulation of the maturation and co-stimulatory molecules on DCs, most of which did not reach a significance level (Figure 3 and Supplementary Fig. S2) and was significantly lower than that observed after LPS treatment. The interaction between AmB-exposed cells and DCs also did not result in a significant release of IL-12p70 (Figure 4a, lined bars). Instead, we observed a significant increase of IL-10 secretion, which is known to have rather immunosuppressive properties (Figure 4a, lined bars). Curiously, exactly the same results were obtained with Dox-treated cells. Consequently, we

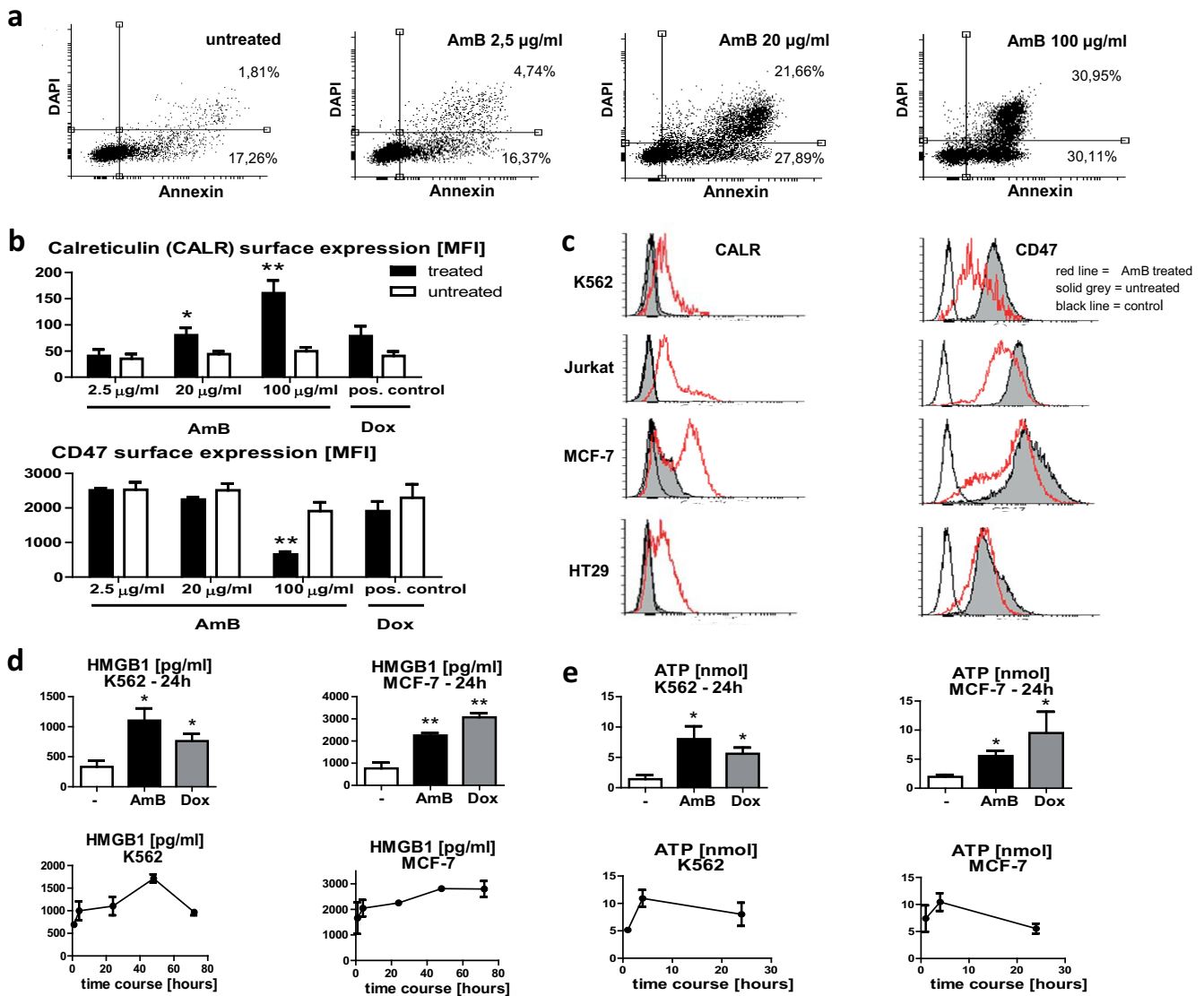


Figure 1. Amphotericin B (AmB) induces surrogate markers of immunogenic cell death at toxic concentrations. (a) Cytotoxicity of AmB. Tumor cell death of a human erythroleukemic cell line (K562) was measured after exposure for 24 h to different AmB concentrations. The percentage of early (annexin V+/DAPI-) and late (annexin V+/DAPI+) apoptotic cells was determined using flow cytometry. Each experiment was done at least in triplicate. (b) AmB induced apoptosis is associated with enhanced surface translocation of calreticulin and decreased CD47 expression. CALR expression was analyzed on K562 cells after exposure to indicated AmB concentrations by flow cytometry and compared to untreated cells. Doxorubicin treated cells were used as a positive control. DAPI+ cells were excluded. The same cells were analyzed for CD47 surface expression („don't eat me"-signal). Results are means of three replicates \pm standard deviation. * $p < .05$, ** $p < .01$ compared to untreated control. (c) Effect of AmB on different tumor cell lines. Induction of ICD features by AmB is a general effect and not cell line dependent. CALR/CD47 expression after AmB treatment tested by flow cytometry showed similar behavior in different hematological and solid tumor cell lines. One representative experiment of at least three replicates is shown. (d) HMGB1 secretion after AmB treatment. HMGB1 was measured in the culture supernatant after 24 h AmB treatment of K562 cells and MCF-7 cells and compared to untreated cells. Doxorubicin treated cells were used as a positive control. Results are means of three replicates \pm standard deviation. * $p < .05$, ** $p < .01$ compared to untreated control. Time dependence of HMGB1 Secretion in K562- and MCF-7 cells is shown below. (e) ATP secretion after AmB treatment. ATP was measured in the culture supernatant after 24 h AmB treatment of K562- and MCF-7 cells, and compared to untreated cells. Doxorubicin treated cells were used as a positive control. Results are means of three replicates \pm standard deviation. * $p < .05$, ** $p < .01$ compared to untreated control. Time dependence of ATP secretion in K562- and MCF-7 cells is shown below.

could not detect any increase of IFN- γ secretion after stimulation of autologous T cells with DCs pulsed with AmB- or Dox-treated cells, as an indicator of T cell activation triggered by ICD (Figure 4b, lined bars).

Because AmB-treated cells led to lower than expected activation and maturation of dendritic cells despite the strong induction of ICD markers, and because this phenomenon was not limited to AmB alone but also occurred with another ICD inducer, Dox, we searched for possible reasons for the unexpected negative regulatory ability of

cells experiencing ICD. We assessed the expression of several immune inhibitory- or tumor resistance-markers available in our laboratory. Three molecules: MDR1, TGM2 (not shown) and PD-L1 were significantly elevated in response to both AmB- and Dox-treatment (Figure 5a).

Encouraged by the fact that in a recent study PD-L1 expression by NK-cells negatively affected the maturation process of dendritic cells,³⁷ we repeated our maturation experiments after blocking of PD-L1 on AmB- and Dox-treated cells with a monoclonal antibody. In this way, DC

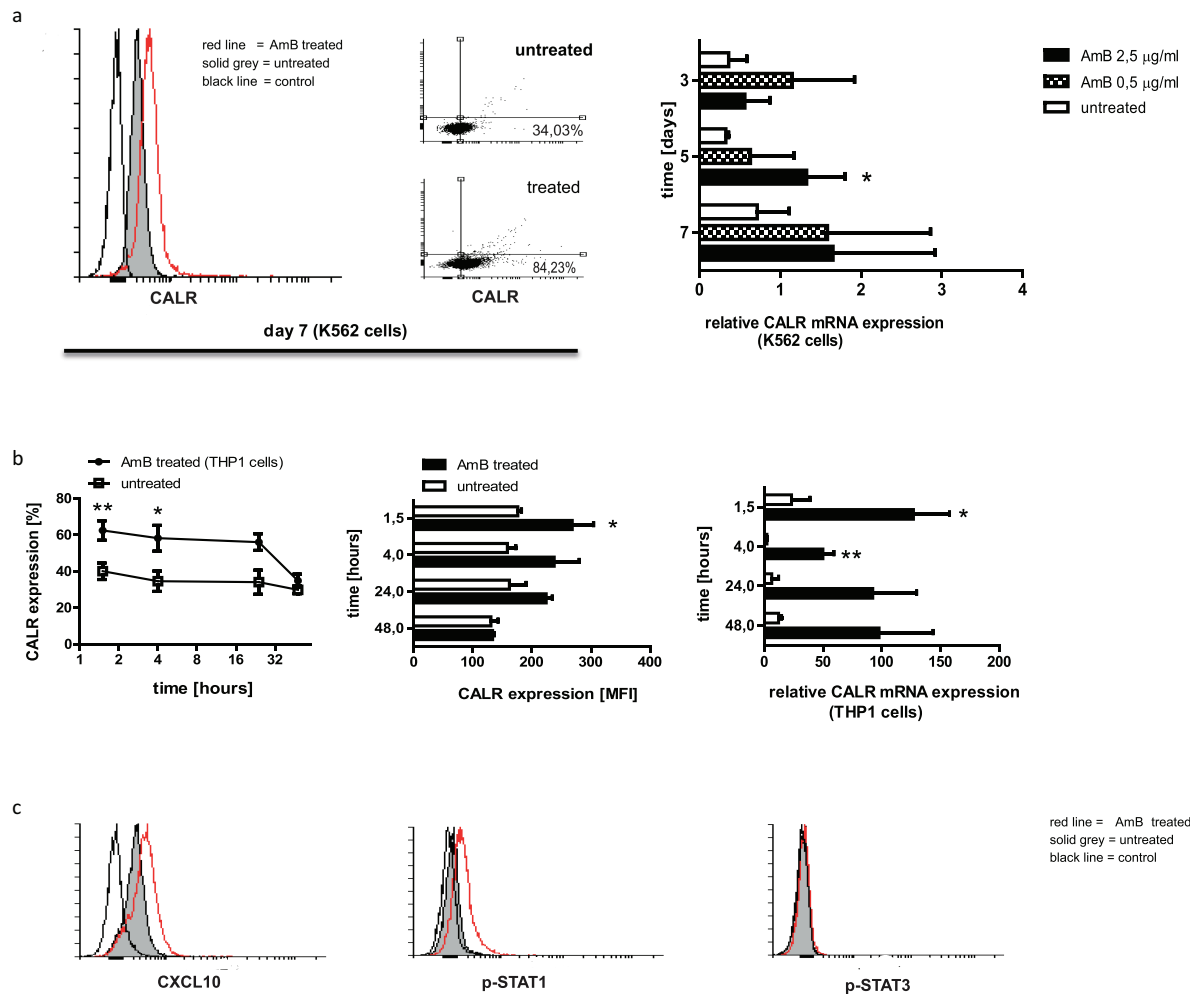


Figure 2. Immunomodulatory effects of Amphotericin B at sub-toxic concentrations. (a) Calreticulin expression (MFI) and positivity (percent cells) after 7 days of AmB treatment at not toxic concentration (2.5 $\mu\text{g}/\text{ml}$). CALR expression was elevated on K562 cells treated with AmB at low/not toxic concentration (used in the clinical practice for antifungal prophylaxis) after 7 days of exposure to AmB (measured by flow cytometry). CALR mRNA expression was additionally analyzed at the indicated time points. Gene expressions were normalized against GAPDH. Results are means of three replicates \pm standard deviation. * $p < .05$ compared to untreated control. (b) Effect of AmB on THP-1 cells at not toxic concentration (2.5 $\mu\text{g}/\text{ml}$). THP-1 cells were cultivated in the presence or absence of 2.5 $\mu\text{g}/\text{ml}$ AmB, respectively. The percentage of CALR positive cells, MFI of CALR surface expression and CALR gene expression (qPCR, relative expression, $2^{-\Delta\text{Ct}}$ method) were measured after 90 min, 4 h, 24 h, and 48 h of drug exposure. Gene expressions were normalized against GAPDH. Results are means of three replicates \pm standard deviation. * $p < .05$, ** $p < .01$ compared to untreated control. (c) AmB activates phagocytic cells toward “M1-like” phenotype. CXCL-10 production of THP-1 cells was induced by AmB after 24 h of incubation. Determination by flow cytometry after membrane permeabilization. p-STAT1 was enhanced in the same cells. p-STAT3 was not affected in the same cells. One representative experiment of three replicates is shown for each marker.

significantly upregulated co-stimulatory molecules such as CD80 and CD86 as well as HLA-DR (about 2-fold) (Figure 3, red lines and black filled bars). The DC maturation was not due to anti-PD-L1 antibody, because treatment of iDCs with the PD-L1 mAb alone did not result in DC maturation (Supplementary Fig. S3).

The activating effect of PD-L1 blockade on tumor cells undergoing ICD was supported by an increase of the IL-12p70 secretion by matured DCs (Figure 4a, black filled bars). Moreover, DCs pulsed with AmB- or Dox-treated and PD-L1-blocked cells provoked activation of T cells as evidenced by significantly increased supernatant IFN- γ levels measured by ELISA (Figure 4b, black filled bars).

These results led us to the assumption that the observed PD-L1 expression is a specific immunosuppressive mechanism in ICD to prevent an overwhelming immune response after stress-induced cell death. Of note, both Dox and AmB

induced a remarkable PD-L1 overexpression on dying cells (Figure 5a). In contrast, cisplatin, which was unable to stimulate ICD, demonstrated by its inability to induce CALR surface translocation at low and high doses (Figure 5a), did not alter the PD-L1 expression (Figure 5a) supporting the hypothesis that PD-L1 is up-regulated in response to ICD, but not in case of regular apoptosis.

Considering that the MYC oncogene regulates the expression of the immune checkpoint proteins³⁸ and that there is a link between ER stress and MYC³⁹ as well as between ER stress and ICD,⁵ we also quantified MYC and CALR mRNA. We found a concordant time- and dose-dependent increase of both mRNAs in response to AmB (Figure 5b), supporting the notion that ER stress and ICD are associated with activation of MYC and up-regulation of PD-L1.

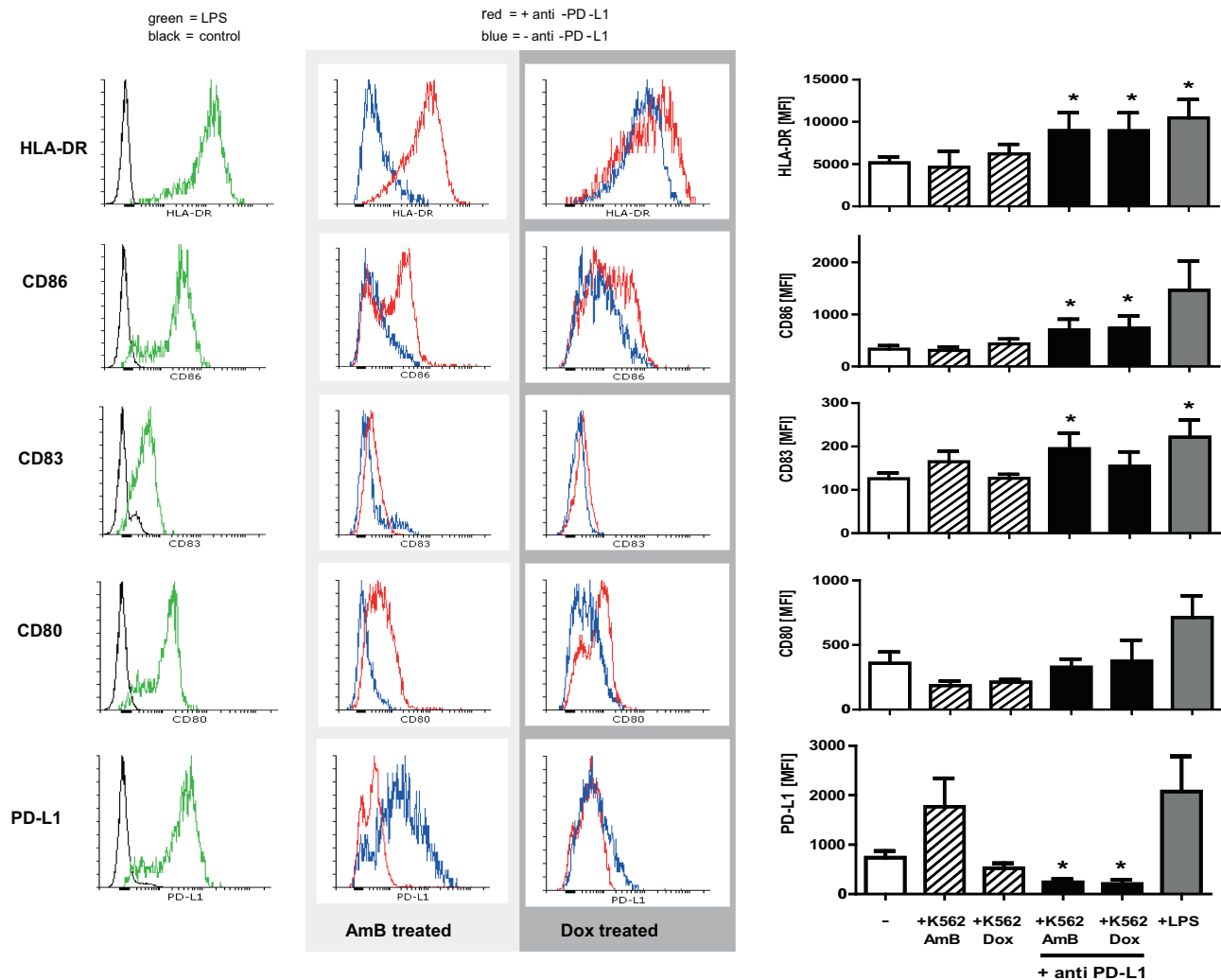


Figure 3. PD-L1 blocking on cancer cells succumbing to ICD reverses their incapability to fully promote DC maturation. Immature DCs (Day 5) were cultured for 24 h with K562 cells killed by AmB or Dox without blocking or after blocking of PD-L1 on dying cells. After 24 h, the expression of HLA-DR, CD86, CD83, CD80 and PD-L1 by DCs was analyzed by flow cytometry. LPS matured DCs served as a positive control. Representative histograms of at least three experiments are shown. MFI results are means of three replicates \pm standard deviation. * $p < .05$, ** $p < .01$ compared to immature DCs

Increase of tumor cell immunogenicity induced by AmB treatment leads to protection against tumor challenge *in vivo*

To explore whether AmB-treated tumor cells have the ability to activate an effective antitumor immune response *in vivo*, we performed the “gold standard” protocol for the *in vivo* assessment of ICD in immunocompetent mice,³⁵ using the established CT26 – Balb/c syngeneic mice model as described previously.⁴⁰ One week after immunization with AmB-treated CT26 cells, mice were re-challenged with living tumor cells on the opposite site and the tumor growth was monitored (Figure 6a). Tumor growth was significantly delayed in recipients, which have been immunized by AmB-treated CT26 cells (Figure 6b), substantiating our *in vitro* results and hypothesis that AmB can induce ICD. Notably, tumor growth was considerably more controlled than in our positive control (vaccination with mitoxantrone-treated cells, an established ICD inducer) (Figure 6b). Following our previous *in vitro* results, which also suggested that a more robust immune answer could be achieved by blocking of ICD induced PD-L1 expression on

dying tumor cells, anti-PD-L1 mAb was combined with immunization of AmB-treated CT26. Recipients were additionally treated three times (day 8, 12, and 16) with the anti-PD-L1 mAb intraperitoneally in the course of the study. A tendency to further delay in tumor growth could be supposed by this combination treatment (mean tumor volume 318mm³ versus 392.25 mm³ in the “AmB alone” group, respectively) (Figure 6b), however did not reach significance in the observation time period.

Discussion

Intrigued by several previous reports attributing antitumor properties to AmB^{16–19} and by its mechanisms of action known (as described in the introduction) to promote cell surface expression of CALR,^{8,11,14,26,27} which is one of the key molecules of ICD and, when expressed on tumor cells, positively correlates with favorable prognosis,^{32,33,41,42} we wanted to address the questions whether AmB can contribute to an effective antitumor immune response and, if so, by what mechanisms.

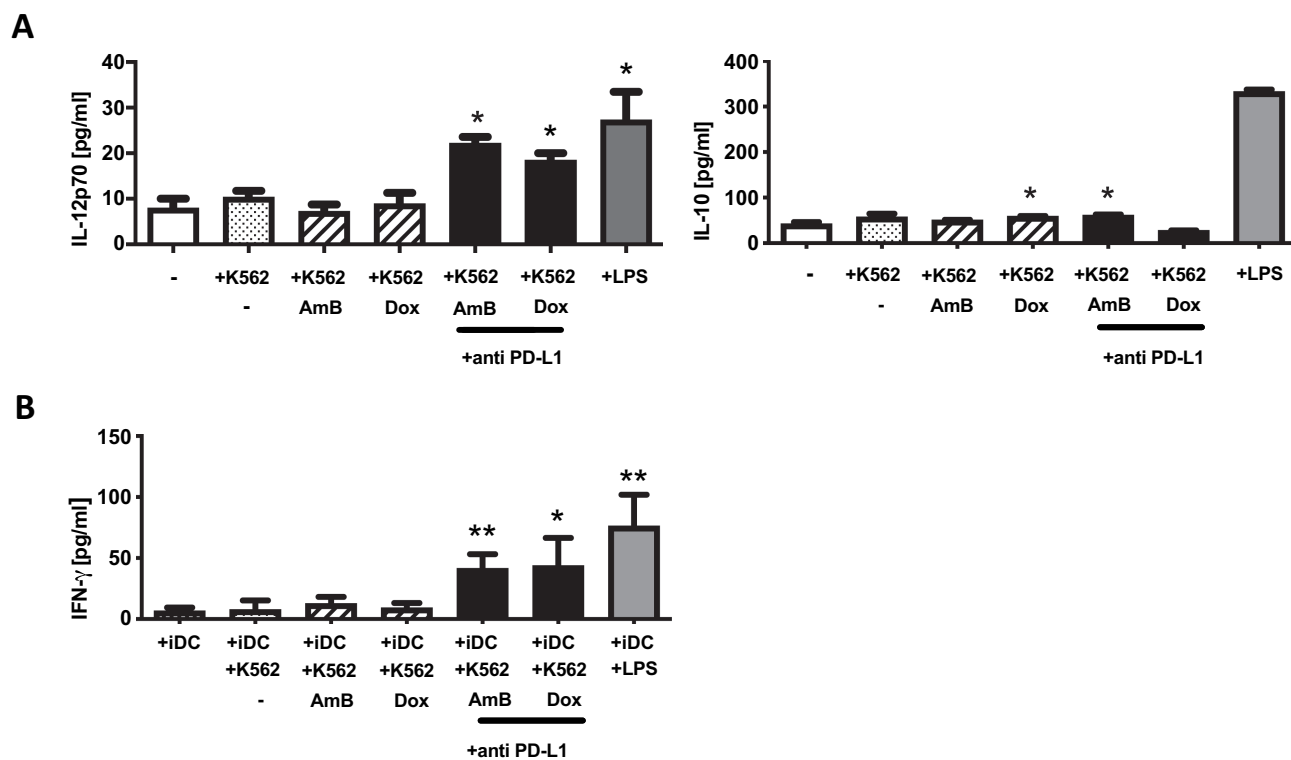


Figure 4. DCs pulsed with AmB- or Dox-treated tumor cells demonstrate enhanced immune stimulating activity after blocking of PD-L1 on cells succumbing to ICD. (a) Secretion of IL-12p70 and IL-10 after co-culture of iDCs (day 5) for 24 h with AmB- or Dox-treated cells after or without PD-L1 blocking (ELISA). (b) Secretion of IFN- γ by autologous T cells after co-culture for 48 h with DCs pulsed with AmB- or Dox-treated cells after or without PD-L1 blocking (ELISA). The values are means of three replicates \pm standard deviation compared to iDCs (A) or T+ iDC (B). * $p < .05$, ** $p < .01$, *** $p < .001$

AmB is not only a TLR2 and TLR4 agonist,⁴³ that directly enhances NK cell cytotoxicity,⁴⁴ It can increase autophagic activity,²⁸ and has immunomodulatory effects. It affects both ATP-secretion and IFN-1 β -production,^{23,29} and influences the so-called type I interferon signature,⁴⁵ processes that are also associated with ICD.

In the present study, AmB induced a robust CALR expression in all cell lines examined. This CALR expression was inversely correlated with a “don’t eat me” -signal CD47, the down-regulation of which promotes tumor cell uptake by phagocytes.⁴⁶ A closer look at CALR expression induced by AmB revealed some interesting aspects. Not different to other ICD inducers such as Dox, a significant CALR enhancement on dying cells was first detected at high and toxic concentrations. However, when investigating the leukemic blast line K562, we noticed an increase of CALR already after treatment at low, i.e. clinically applied, AmB-concentrations. In this case, however, it occurred only after longer cultivation time with AmB. Moreover, in another leukemia cell line, the acute monocytic leukemia cell line THP-1, early CALR induction was observed after only 90 min at therapeutic AmB concentration. These observations are intriguing because elevated levels of CALR on malignant blasts correlate with improved prognosis in patients with AML^{32,33,47} and CALR exposure can predict a cellular immune response against tumor cells as shown by Fucikova et al.⁴¹ Furthermore, AML patients with blasts spontaneously expressing CALR are characterized by higher activation of NK cells and patients with high levels of CALR on malignant

blasts and the NK cell activator receptor on NK cells have superior overall survival compared with all other patient subgroups.^{41,47}

On the other hand, THP-1 is a model cell line commonly used to study phagocytosis. As observed by Feng et al., TLR-activated phagocytic cells increase their surface CALR expression and can “eat” cancer cells using their own calreticulin as a guide.⁴⁸ In this situation, CALR expression is more an activation marker of phagocytes that mediates recognition and phagocytosis of neighboring tumor cells.⁴⁸ THP-1 cells reacted very sensitive in response even to low AmB doses. The observed CALR overexpression is likely associated with the differentiation of these blasts and is at least in part an expression of the AmB action as a TLR agonist that can activate phagocytic cells. This activation was further confirmed by enhanced phosphorylation of STAT1 and increased production of CXCL10, typical features of the “M1-like” antitumor response. We therefore hypothesize that the tumor cell uptake after AmB therapy may be enhanced by the induction of CALR expression in parallel with CD47 down-regulation on ICD experiencing tumor cells and, on the other hand, by TLR associated activation of phagocytes leading to CALR expression on their surface.

We found a case report describing an unexpected complete response of chemotherapy-resistant acute myelomonocytic leukemia after addition of AmB antifungal therapy.¹⁷ Based on our observations, it could be suggested that the (myelo-) monocytic leukemia forms should be particularly sensitive to AmB co-therapy.

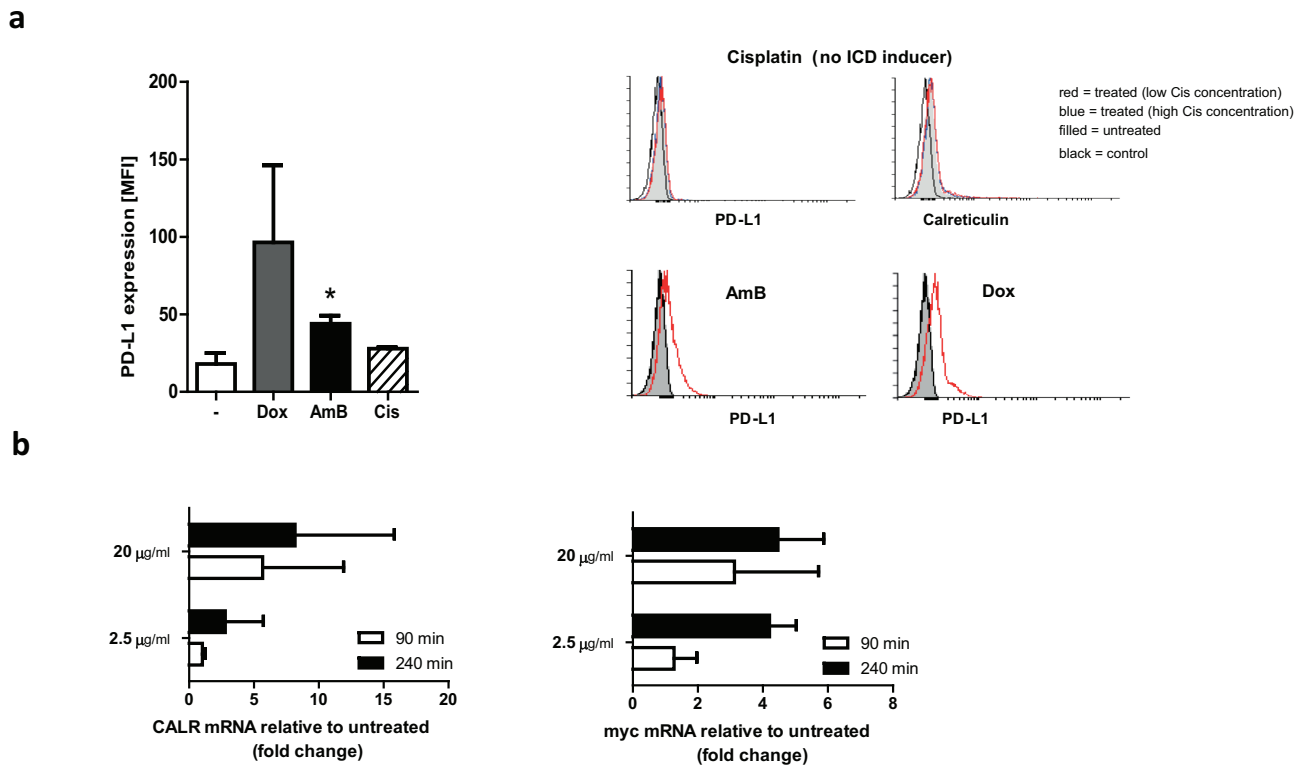


Figure 5. ICD induction is regularly accompanied by enhanced PD-L1 expression. (a) PD-L1 induction by ICD inducer Dox and AmB was analyzed at concentrations leading to CALR translocation. Additionally, K562 cells were treated with low (5 µg/ml) and high (25 µg/ml) apoptosis inducing cisplatin concentrations and analyzed for PD-L1 and CALR expression by flow cytometry. One representative histogram of three experiments is shown. PD-L1 MFI values were compared to untreated. * $p < .05$. The values are means of three replicates \pm standard deviation. (b) Calreticulin- and Myc -mRNA expression (analyzed by qPCR, $2^{-\Delta\Delta Ct}$ method relative to untreated) after AmB treatment at different concentrations and time points. The values are means of three replicates \pm standard deviation.

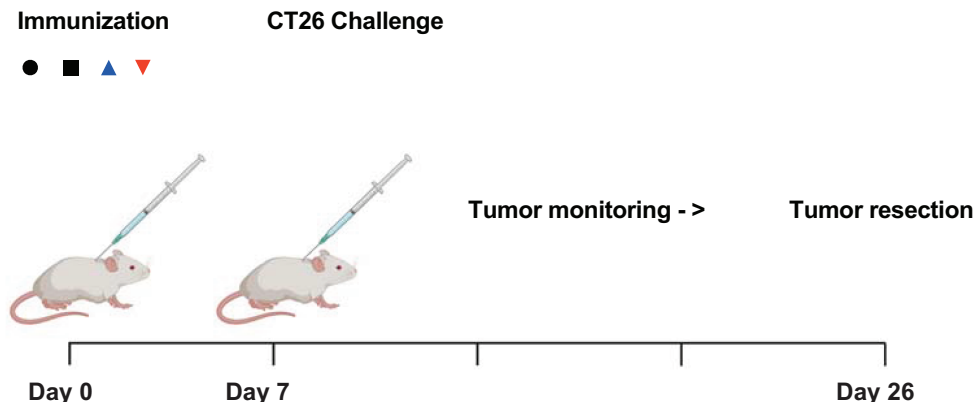
Fortunately, there are some clinical data from previous clinical studies that investigated antifungal prophylaxis with AmB in leukemia patients. We conducted a literature search for studies investigating AmB for antifungal prophylaxis and treatment in leukemia patients. Since the effect of AmB on tumor therapy was not the subject of these studies, data on leukemia remission rates were mostly only available as supplementary information. To illustrate the observed correlations, we generated graphs based on the data collected in the studies outlined in the following (Figure 7). Remarkably, in an early study conducted in 1978, the rate of achieving a complete remission was significantly higher in the group receiving AmB than in the control group, 77 versus 40% respectively ($p = .016$).³⁰ In a later study, adults with acute leukemia were randomly assigned to antifungal prophylaxis with AmB or fluconazole. Although fewer fungal infections and fewer side effects occurred in the fluconazole group, the AmB group achieved considerably better leukemia remission rates. Eighty-nine per cent of the AmB patients achieved a complete remission of their leukemia compared with 80% in the fluconazole group. The remission rates during initial induction therapy were 92% compared to 81%.³¹ In subsequent years, conventional AmB has been replaced by formulations with fewer side effects, and these observations have been largely neglected. In a recent study, continuous administration of AmB over 24 hours was investigated for antifungal treatment of AML patients receiving intensive chemotherapy, and it was claimed that prolonged administration was better tolerated. One group

had exposure to AmB (at any treatment time point), while the control patients were never exposed to AmB. The estimated overall survival probability at 5 years was 0.493 in the AmB and 0.291 in the control patients.⁴⁹ These results are consistent with an early experimental analysis that demonstrated a synergistic effect of AmB and chemotherapy against transplantable AKR leukemia in a syngeneic mice model. The addition of AmB to chemotherapy resulted in long-term survival of mice inoculated with leukemic cells in a substantial number of cases. Furthermore, the survivors were resistant to re-challenge with leukemic cells, at a dose that caused fatal leukemia in all untreated controls.¹⁹ This last observation strongly suggests an immunological process and resembles effects of cells succumbing to ICD.

It has been previously described by others that AmB can affect autophagy and ATP secretion.^{23,28,29} We confirmed an increase of ATP secretion and also observed an increase of HMGB1 secretion at concentrations that result in CALR trafficking to the cell surface.

To our surprise, despite the solid generation of danger signals known to enhance the adjuvanticity of dying cells by AmB treated cells, the co-cultured DCs showed only a modest maturation tendency, while exhibiting elevated PD-L1 expression and a moderate increase of IL-10 secretion. Interestingly, treatment with doxorubicin as an ICD control^{14,15} also did not result in more robust DC activation. Therefore, we suspected a general effect unrelated to AmB itself to be responsible for this phenomenon. A closer look at other previous studies

a



b

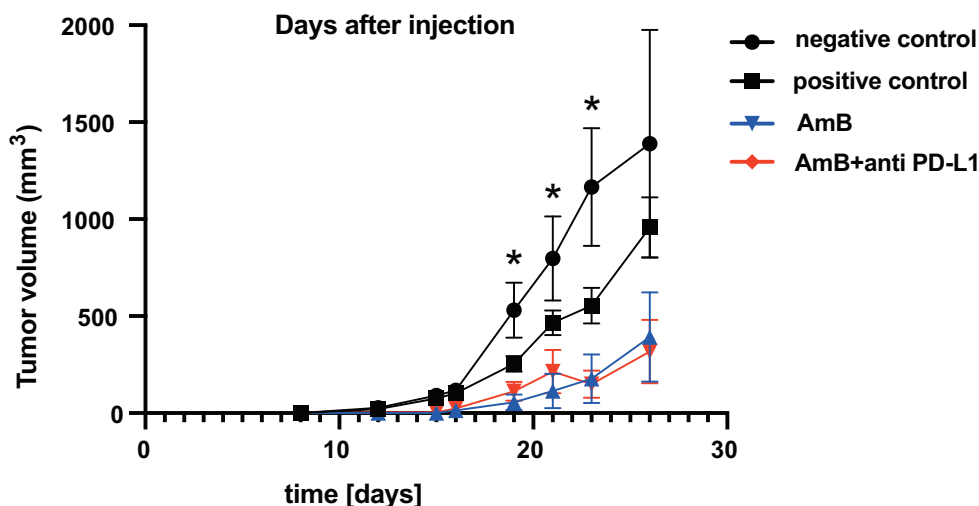


Figure 6. Re-challenge with CT26 resulted in significantly delayed tumor growth after vaccination with AmB-treated CT26. (a) Scheme of in vivo immunization. CT26 cells treated with AmB (at ICD-inducing concentration), AmB + anti-PD-L1, mitoxantrone (positive control), or PBS alone (negative control) were inoculated subcutaneously in Balb/c mice. After 7 days, mice were re-challenged with live 3×10^5 CT26 cells and tumor growth was monitored. Anti PD-L1 treatment was repeated three times on day 8, 12, and 16 in the “AmB + anti-PD-L1” group. (b) Growth dynamics of re-challenging tumors were monitored until day 26. Mean tumor volumes of each group ($n = 6$) were quantified every 2–3 days. Data are presented as mean value \pm SEM. * $p < .05$ (Mann-Whitney-U-Test).

shows that, analogous to our observations, ICD agents usually achieve only moderate phenotypic maturation of DCs, and in the vaccination models, LPS or poly(I:C) are typically added as additional maturation stimuli after co-culture of DCs with tumor cells.^{15,50} The question of what factors limit DC activation despite the strong DAMP stimuli, led us to the observation that PD-L1 is overexpressed on dying cells after both AmB- and Dox-treatment. PD-L1 appears to be involved in the cross-talk between dying and phagocytic cells.⁵¹ Blockade of PD-L1 on DCs has been shown to enhance T cell activation,⁵² but relatively little is known about the role of PD-1 on immature DCs. PD-1 is expressed on immature DCs, and maturation leads to its downregulation and increase of PD-L1.^{51,52}

Remarkably, blocking of PD-L1 after ICD induction caused significantly greater expression of maturation-associated molecules and release of the pro-inflammatory cytokine IL-12, resulting in the activation of IFN- γ -producing T cells. In a recent study,³⁷ NK cells overexpressing PD-L1 regulated DC maturation in a very similar manner, confirming that PD-L1/PD-1 interactions on iDCs can be of relevance for DC activation.

In contrast to AmB- or Dox-treatment, PD-L1 expression was not increased on cells killed by non-ICD inducer cisplatin,^{13,53} suggesting a link between increased PD-L1 expression and ICD. This observation contradicts previous study, describing that cisplatin was able to induce

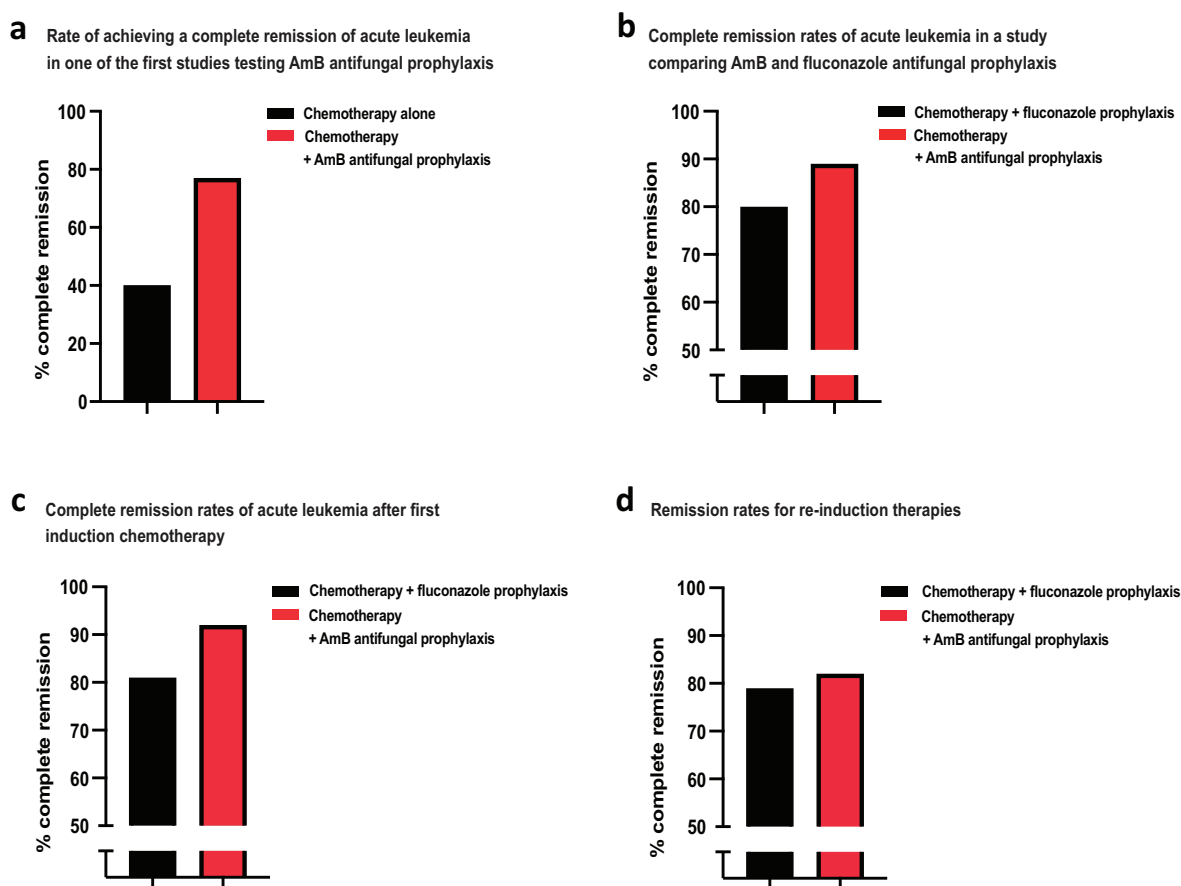


Figure 7. Clinical evidence of a probable additional effect of AmB on treatment of acute leukemia based on a literature search of major databases for studies investigating conventional AmB treatment in leukemia patients. (a) Rate of achieving a complete remission of acute leukemia in one of the first studies testing AmB antifungal prophylaxis,³⁰ $p = .0016$ (b) Complete remission rates in a study comparing AmB and fluconazole antifungal prophylaxis.³¹ (c) Complete remission rates in acute leukemia after first induction therapy.³¹ (d) Remission rates of re-induction therapies.³¹ All graphs illustrate results stated as additional information in the cited publications investigating AmB for antifungal prophylaxis (A, B, C, and D).

a significant PD-L1 up-regulation in head and neck squamous-cell carcinoma cells.⁵⁴ However, in that study, PD-L1 overexpression was also accompanied by a strong CALR induction, suggesting that cisplatin unexpectedly induced ICD in this experiment. There is an overwhelming evidence that ICD can sensitize to PD-1/PD-L1 blockade,⁵⁵ supporting our observations. To complete the puzzle, we hypothesized that MYC is a link between ER stress and PD-L1 expression. MYC can be directly and indirectly regulated by UPR,³⁹ and it increases the expression of PD-L1.³⁸ MYC-expression at the mRNA level exactly paralleled activation of CALR, but further sufficient experiments are needed to test this hypothesis.

The ability of AmB to enhance the immunogenicity of tumor cells *in vivo* was confirmed by a “gold standard” experiment for the assessment of ICD in immunocompetent mice. We did not observe any further improvement by combing the “AmB vaccine” with anti-PD-L1 mAb, however the duration time of this study might have been too short to detect significant differences between these two groups.

Overall, we conclude that conventional AmB treatment leads to an enhanced expression and secretion of several factors crucial for ICD, such as CALR, and shifts phagocytic cells toward an antitumor phenotype. Accordingly,

conventional AmB is a promising (and already approved) drug for developing of effective combinatorial anti-cancer therapies benefiting from an enhancement of the immunogenicity. In this context, we also demonstrate that cells exposed to stress/ICD upregulate immunosuppressive molecules like PD-L1, likely to raise the threshold for the subsequent adaptive immune response and thereby prevent tissue damage. There is strong evidence that ICD can sensitize to immune checkpoint therapy.⁵⁵ AmB acts as an effective ICD inducer and we demonstrate a mechanistic rationale for combining it with a checkpoint inhibitor to disrupt reactive PD-L1-mediated immunosuppression.

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Disclosure statement

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Data availability statement

The data that support the findings of this study are available from the corresponding author, [GK], upon reasonable request.

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