Imiquimod Exerts Antitumor Effects by Inducing Immunogenic Cell Death and Is Enhanced by the Glycolytic Inhibitor 2-Deoxyglucose

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The induction of immunogenic cell death (ICD) in cancer cells triggers specific immune responses against the same cancer cells. Imiquimod (IMQ) is a synthetic ligand of toll-like receptor 7 that exerts antitumor activity by stimulating cell-mediated immunity or by directly inducing apoptosis. Whether IMQ causes tumors to undergo ICD and elicits a specific antitumor immune response is unknown. We demonstrated that IMQ-induced ICD-associated features, including the surface exposure of calreticulin and the secretion of adenosine triphosphate and HMGB1, were mediated by ROS and endoplasmic reticulum stress. In a B16F10 melanoma mouse model, vaccinating mice with IMQ-induced ICD cell lysate or directly injecting IMQ in situ reduced tumor growth that was mediated by inducing tumor-specific T-cell proliferation, promoting tumor-specific cytotoxic killing by CD8+ T cells, and increasing the infiltration of various immune cells into tumor lesions. The ICD-associated features were crucial in the induction of specific antitumor immunity in vivo. The glycolytic inhibitor 2-deoxyglucose enhanced IMQ-induced ICD-associated features and strengthened the antitumor immunity mediated by IMQ-induced ICD cell lysate in p53-mutant cancer cells, which were IMQ-resistant in vitro. We conclude that IMQ is an authentic ICD inducer and provide a concept connecting IMQ-induced cancer cell death and antitumor immune responses.


INTRODUCTION

Immunogenic cell death (ICD) can be induced by various antitumor-tumor therapies that confer dying cells to vaccinate and reinforce the therapeutic effect by eliciting a specific immune response (Galluzzi et al., 2017). ICD is characterized by at least three hallmarks: (i) the pre-apoptotic, endoplasmic reticulum (ER) stress—dependent surface exposure of calreticulin (CRT); (ii) autophagy-dependent adenosine triphosphate (ATP) secretion during apoptosis; and (iii) the postapoptotic exodus of HMGB1 (Apetoh et al., 2007; Galluzzi et al., 2017). Surface-exposed CRT is engulfed by antigen-presenting cells via CD91; secreted ATP recruits antigen-presenting cells into the tumor bed by interacting with purinergic P2X7 receptors; and HMGB1 is recognized by toll-like receptor (TLR) 2, TLR4, and RAGE to stimulate maturation of antigen-presenting cells and promote tumor antigen presentation (Apetoh et al., 2007; Elliott et al., 2009; Ghiringhelli et al., 2009; Park et al., 2006). Although a limited number of clinically used anticancer drugs have been verified to induce ICD response, including cyclophosphamide, doxorubicin, mitoxantrone, and oxaliplatin (Pol et al., 2015), some anticancer drugs that stimulate antitumor immunity have not been proven as ICD inducers.

Imiquimod (IMQ), a synthetic TLR7 ligand, exerts both antitumor and antiviral activity. IMQ is a Food and Drug Administration—approved drug for the topical treatment of various skin malignances and viral warts (Chi et al., 2017). IMQ exerts its antitumor activity by indirectly stimulating TLR7 on dendritic cells (DCs) to activate cell-mediated immune responses and by directly inducing tumor apoptosis and autophagic cell death (Gorden et al., 2005; Huang et al., 2010; Schönherr et al., 2003). However, the relationship between IMQ-induced cell death and antitumor immune response is still unknown.

In this study, we not only revealed that IMQ can induce ICD in cancer cells in vitro and in vivo but also demonstrated targeting glycolysis with 2-deoxyglucose (2DG) could improve IMQ-induced ICD in p53-mutant cancer cells.
Figure 1. IMQ-induced cell apoptosis and ICD-associated features in various types of cancer cells. (a) Cell cycle distributions were analyzed by DNA content assay using flow cytometry analysis. (b, c) CRT surface exposure was analyzed by flow cytometry and immunocytochemistry. (d) The supernatants were collected at the indicated time points, and the extracellular ATP content was measured by ATP content assay. (e) After 48 hours of incubation with IMQ, the supernatants of these cells were collected to analyze the HMGB1 levels by ELISA experiments. (f) The expression of p-eIF2α, eIF2α, and β-actin was measured.
RESULTS

IMQ induces the expression of ICD-associated features in cancer cells

To assess the possibility that IMQ may induce ICD, we confirmed that the apoptotic sub-G1 populations in IMQ-treated BCC/KMC-1, AGS, HeLa, and B16F10 cells were increased in a time-dependent manner and remarkable at 24 hours after IMQ treatment (Figure 1a). IMQ markedly increased the levels of CRT on the cell surface (Figure 1b and c), the secretion of extracellular ATP (Figure 1d), HMGB1 release (Figure 1e), and the phosphorylation of eIF2α (Figure 1f). IMQ also stimulated ROS generation in these cells (Figure 1g), which has been reported to induce ER stress to trigger ICD (Krysko et al., 2012). We summarized that IMQ could induce the ICD characteristics and ROS production in various cancer cells.

Vaccination with IMQ-induced ICD tumor cell lysates (TCLs) provides antitumor immunity

We tested whether IMQ-treated TCLs could induce protective immunity against the same tumor challenge by vaccination (Kepp et al., 2014). Immunocompetent C57/B6 mice were vaccinated once (prime) or twice (boost) with IMQ-treated B16F10 TCLs. As shown in Figure 2, compared with the vehicle group, vaccination with IMQ-treated B16F10 TCLs significantly increased the median survival rate (Figure 2a) and percentage of tumor-free mice (five mice from the prime group) (Figure 2b). Tumor weights were assessed after 32 days of challenge. There were 14 mice in each group. The asterisk represents a significant difference between the vehicle and IMQ prime groups, and the double dagger represents a significant difference between the vehicle and IMQ boost groups. ICD, immunogenic cell death; IMQ, imiquimod; TCL, tumor cell lysate.

Figure 2. Vaccination with IMQ-induced ICD TCLs contributed to specific antitumor immunity. IMQ-treated B16F10 TCLs were harvested and injected into the right flanks of C57/B6 mice once (prime) or twice (boost) as described in the Materials and Methods section. The vehicle group received PBS injections. (a) The survival rate, (b) tumor-free outcomes, and (c) tumor volume were monitored. (d) Tumor weights were assessed after 32 days of challenge. There were 14 mice in each group. The asterisk represents a significant difference between the vehicle and IMQ prime groups, and the double dagger represents a significant difference between the vehicle and IMQ boost groups. ICD, immunogenic cell death; IMQ, imiquimod; TCL, tumor cell lysate.

by immunoblotting. (g) The intracellular ROS content was detected by DCFDA staining using flow cytometry. The data are expressed as the mean ± SEM of at least three independent experiments. ATP, adenosine triphosphate; CRT, calreticulin; DCFDA, 2’,7’-dichlorofluorescin diacetate; ICD, immunogenic cell death; IMQ, imiquimod; MFI, mean fluorescence intensity; N.D., not detected; p-eIF2α, phosphorylated eIF2α.
Figure 3. Vaccination with IMQ-induced ICD TCLs promoted T-cell responses and altered the populations of tumor-infiltrating leukocytes. (a) CD4⁺ and CD8⁺ lymphocytes were isolated from C57/BL6 mice with or without vaccination, and then the lymphocytes were cocultured with IMQ-treated B16F10 ICD TCL-treated imDCs. The cell proliferation of CD4⁺ and CD8⁺ T lymphocytes was analyzed by a BrdU incorporation assay. (b) The cytotoxic killing activity of isolated CD8⁺ T lymphocytes from C57/BL6 mice was determined by a cytotoxic T lymphocyte assay at an E:T ratio of 25:1, 50:1, or 100:1, and then the fluorescence of the supernatants was measured. (c–f) Splenocytes were harvested from C57/BL6 mice with or without twice vaccination and then the expression of IFNγ was detected in CD4⁺ or CD8⁺ cells (c) by intracellular cytokine staining or (d) by staining with fluorescence-conjugated CD4, CD8, and CD44 antibodies by flow cytometry analysis. Isolated CD8 T lymphocytes from these splenocytes were then infused into other B16F10 tumor-established C57/BL6 S-W Huang et al. Glycolysis Inhibition Enhanced Imiquimod-Induced Immunogenic Cell Death
Vaccination of IMQ-induced ICD TCLs increases T lymphocyte proliferation, cytotoxic killing, and the infiltration of immune cells into the tumor lesion

To investigate the immunomodulatory effect caused by IMQ-induced ICD TCLs, we analyzed whether IMQ-induced ICD TCLs could stimulate CD4⁺ and CD8⁺ T lymphocyte proliferation. As shown in Figure 3a, vaccination with IMQ-induced ICD TCLs (prime) significantly increased the proliferation of both CD4⁺ and CD8⁺ T lymphocytes. Mice that received two vaccinations exhibited a boosted effect. Next, we assessed whether IMQ-induced ICD TCLs could elicit specific cytotoxic T-cell killing. Compared with the nonvaccinated mice, the isolated CD8⁺ lymphocytes from mice vaccinated with IMQ-induced ICD TCLs (prime) dramatically increased the specific lysis ability of B16F10 cells (Figure 3b). Consistently, the boost group exhibited much higher specific lysis ability. We thereby evaluated the amounts of effector and memory T cells after vaccination with IMQ-induced ICD TCLs. We observed that not only CD4⁺/IFNγ⁺ and CD8⁺/IFNγ⁺ effector T cells but also CD4⁺/CD44⁺ and CD8⁺/CD44⁺ memory T cells were significantly increased in splenocytes from vaccinated mice (Figure 3c and d). Additionally, by comparing the B16F10 tumor-established mice that received adoptive transfer of CD8⁺ T cells from mice injected with freeze-thawed control cell lysate, we found that the infusion of CD8⁺ T cells from IMQ-induced ICD TCL-vaccinated mice significantly repressed tumor growth and caused some mice to exhibit tumor-free outcomes (60%, 3 of 5) (Figure 3e and f). This effect corresponded to the CD8⁺ T cells from vaccinated mice inducing B16F10 cell lysis after coculture. Finally, we observed that vaccination with IMQ-induced ICD TCLs significantly increased the number of CD8⁺, CD11c⁺, and IL-17A—producing cells in the B16F10 tumor sections but did not alter the CD4⁺ cell population (Figure 3g). These results suggest that IMQ-induced ICD might trigger the expansion of T lymphocytes, activation of specific cytotoxic T cells, and modification of tumor-infiltrating immune cells.

All IMQ-induced ICD-associated features are involved in the induction of specific antitumor immunity

Next, we determined which IMQ-induced ICD-associated molecules are required for activating specific antitumor immunity. As shown in Figure 4a and b, vaccination with IMQ-treated B16F10 ICD TCLs with CRT knockdown (treated with CRT small interfering RNA) or coinjection of the ATP receptor P2X7 antagonist suramin and the HMGB1 inhibitor glycyrrhizin significantly reduced the efficiency of tumor repression compared with vaccination...
with IMQ-induced ICD TCLs and decreased the survival rates and percentage of tumor-free mice after tumor challenge (Figure 4c and d). Mice vaccinated with IMQ-treated ICD TCLs with CRT knockdown and glycyrrhizin treatment and coinjected with suramin (CRT small interfering RNA + glycyrrhizin + suramin + IMQ) not only presented with higher tumor growth rates and tumor weights than mice injected with CRT small interfering RNA, glycyrrhizin, or suramin alone (Figure 4a and b) but also exhibited a much lower survival rate and percentage of tumor-free mice than the other groups (Figure 4c and d). Furthermore, we found that the direct injection of IMQ into established B16F10 tumors significantly repressed the tumor growth rate and tumor weight and that this effect could be partially abrogated by injection both suramin and glycyrrhizin (Figure 4e and f). Thus, IMQ-induced ICD characteristics, including CRT surface exposure, ATP secretion, and HMGB1 release, are critical in triggering specific antitumor immunity and contribute to prolonged protective outcomes in vivo.
IMQ-induced ICD-associated characteristics are mediated by ROS production and ER stress

ROS and ER stress are crucial for the ICD process to promote the expression of damage-associated molecular patterns (Krysko et al., 2012). We hypothesized that IMQ-induced ROS and ER stress may be involved in IMQ-induced ICD features. The ROS scavenger N-acetyl cysteine not only significantly decreased the IMQ-induced surface exposure of

**Figure 5.** IMQ induced ROS production and triggered ER stress to upregulate ICD-associated molecules. BCC and B16F10 cells were treated with 50 μg/ml IMQ with or without (a–c) NAC (2 mM) pretreatment for 30 minutes or (d–f) PB (1 mM) pretreatment for 1 hour. (a and d) The cells were harvested, and cell surface-exposed CRT was detected by flow cytometry. (b and e) The secretion of ATP was measured, and (c and f) the extracellular HMGB1 content was analyzed by ELISA. The data are expressed as the mean ± SEM of at least three independent experiments. ATP, adenosine triphosphate; BCC, basal cell carcinoma; CRT, calreticulin; ER, endoplasmic reticulum; ICD, immunogenic cell death; IMQ, imiquimod; NAC, N-acetyl cysteine; N.D., not detected; PB, sodium 4-phenylbutyrate.
CRT (Figure 5a) (Supplementary Figure S1a), secretion of ATP (Figure 5b), and release of HMGB1 (Figure 5c) in BCC/KMC1 and B16F10 cells but also attenuated ER stress-related proteins, including IRE1α, Grp78, CHOP, and phosphorylated-eIF2α, and decreased the expression of apoptosis-associated cleaved caspase-3 and cleaved PARP and the accumulation of autophagic LC3-II (Supplementary Figure S1b). We also found that treating IMQ-treated BCC/KMC1 and B16F10 cells with the molecular chaperone sodium 4-phenylbutyrate (Figure 5d–f) (Supplementary Figure S1c and S1d) or the PERK inhibitor GSK2606414 (Supplementary Figure S2) significantly downregulated all three ICD-associated features and reduced the ER stress-related and apoptotic proteins and LC3-II. These results indicate that IMQ-induced ICD-associated features are caused by IMQ-induced ROS production, which triggers ER stress and activates the PERK/eIF2α pathways in cancer cells.
2DG treatment enhances IMQ-induced ICD TCL-mediated antitumor immunity and ICD-associated features in p53-mutant cancer cells

Inhibiting glycolysis during chemotherapy has been reported to enhance the antitumor immune response (Bénétête et al., 2012). Moreover, targeting of aerobic glycolysis enhanced the antitumor activity of IMQ in both p53–wild-type and p53-mutant cancer cells (Huang et al., 2014). Thus, glycolysis inhibition might ameliorate IMQ-induced ICD in p53-deficient cancer cells. Treatment with the glycolysis inhibitor 2DG not only dramatically enhanced the IMQ-induced sub-G1 cell populations (Figure 6a) and promoted ROS production (Figure 6b) but also significantly increased ICD-associated features (Figure 6c–e) in p53-mutant SCC12 and 4T1 cells compared with single agent-treated cells. We found that BALB/c mice vaccinated with 2DG- and IMQ-cotreated ICD TCLs of 4T1 cells presented slower growth rates and weights of orthotopic transplanted 4T1 tumors than mice treated with either 2DG or IMQ alone (Figure 6g and h). Remarkably, this vaccination also maintained a higher survival rate and tumor-free outcome than the control or single agent-treated groups (Figure 6i and j). Importantly, vaccination with 2DG- and IMQ-cotreated ICD TCLs also reduced established tumor growth and weight compared with vaccination with single agent-treated cell lysate (Supplementary Figure S3). This cotreatment strategy did not protect against the same tumor cell challenge in immune-deficient nude mice (Supplementary Figure S4). These results suggest that the protective antitumor immunity induced by IMQ-treated TCLs is dependent on adaptive immunity, and the combined treatment of 2DG and IMQ strengthens the expression of ICD features and enhances vaccination-mediated specific antitumor immunity in p53-mutant cancer cells.

DISCUSSION

In this study, we demonstrated that IMQ is an ICD inducer, with evidence that IMQ induces ROS production to trigger ER stress and subsequently promotes the expression of ICD-associated features, including surface-exposed CRT, ATP secretion, and HMGB1 release, all of which are essential for activating specific antitumor immune responses against tumor cells, in apoptotic cancer cells. Additionally, we also suggest that the use of glycolytic inhibitors may strengthen the antitumor efficiency of IMQ by enhancing the ICD response in p53-mutant cancer cells.

ER-Golgi transport is required for CRT surface translocation after immunogenic insults (Zitvogel et al., 2010). Autophagy is essential for ATP secretion during ICD (Martins et al., 2014). ROS induction and ER stress also mediate the expression of ICD-associated features (Krysko et al., 2012). Consistently, we demonstrated that IMQ-induced ICD-associated features were mediated by ROS production and ER stress through the PERK/eIF2α pathway. We confirmed that IMQ-induced CRT exposure occurs through the ER-Golgi transport process using brefeldin A (Supplementary Figure S5a). We also showed that the induction of autophagy contributes to ATP secretion and IMQ-induced CRT surface exposure during IMQ-induced ICD by knockdown of the autophagy-essential gene BECN1 and adding the autophagy inhibitor 3MA or bafilomycin A1 (Supplementary Figure S5b and c). Early-stage autophagy inhibition suppressed ICD-associated CRT exposure (Li et al., 2016). Thus, IMQ-induced CRT exposure during ICD may be mediated by the ER-Golgi transport process and autophagic machinery in cancer cells. However, whether autophagy induces the surface exposure of CRT during IMQ-induced ICD needs to be clarified in the future.

The immunogenicity of ICD-associated features is dependent on the stimulus. Mitoxantrone- and oxaliplatin-induced ATP secretion, but not CRT exposure or HMGB1 release, is required to activate the ICD-associated antitumor immune response (Michaud et al., 2011). We found that CRT exposure, ATP secretion, and HMGB1 release by IMQ each partially contributed to antitumor immunity. However, the combined inhibition of these ICD-associated molecules did not completely prevent the subsequent activation of the antitumor immune response. A recent study demonstrated that the activation of RIG-1 by its ligands induced ICD and triggered specific antitumor immunity through the production of type I IFN but not CRT or HMGB1 (Duewell et al., 2014). In fact, the IMQ receptor TLR7 also mediates type I IFN production via its signaling cascade (Petes et al., 2017). Despite the absence of reports indicating IMQ as the ligand for RIG-1, some studies have shown that IMQ penetrates cells and concentrates in lysosomes (Russo et al., 2011). Thus, IMQ might interact with RIG-1 in the cytosol or with TLR7 in the endosome and lysosome to induce type I IFN production in cancer cells and to elicit part of ICD-mediated antitumor immunity.

ICD-induced alterations in immune cell infiltration have been correlated with tumor outcomes (Garg et al., 2016; Gebremeskel and Johnston, 2015). We found that vaccination with IMQ-treated TCLs increased the number of CD8+, CD11c+, and IL-17A—producing cells in tumor lesions. The boost vaccination not only further increased the infiltration of these immune cells but also significantly increased the proliferation of tumor-specific T lymphocytes and CD8+ lymphocyte—mediated cytotoxic killing compared with the prime vaccination. Moreover, the infusion of CD8+ T cells from vaccinated mice into other tumor-established mice could cause obvious tumor suppression. Vaccination did not affect CD4+ T lymphocyte infiltration into tumor sections, but proliferation was increased. A recent study indicated that CD4+ T lymphocytes are required for generating functional CD8+ T memory cells that might facilitate efficient immune surveillance (Laidlaw et al., 2016). Moreover, the activation of CD4+ T helper type 1 cells promoted CD8+ cytotoxic T lymphocytes (CTLs) localized into tumors and eradicated tumor cells (Huang et al., 2007). Therefore, we speculate that IMQ-induced ICD TCLs might stimulate CD4+ T cells to activate CD8+ CTLs to overcome the subsequent challenge with tumor cells. Studies have shown that T helper type 17–polarized cells effectively mediate the eradication of B16 melanoma (Muranski et al., 2008). The rapid
Figure 6. 2DG enhanced IMQ-induced ICD-associated features and antitumor immunity in p53-mutant cancer cells. 4T1 and SCC12 cells were treated with 50 μg/ml IMQ with or without 2DG (10 mM) for 48 hours. (a) The apoptotic population, (b) ROS production, (c) cell surface-exposed CRT, (d) extracellular ATP content, (e) released HMGB1, and (f) status of p-eIF2α were analyzed, and the data are expressed as the mean ± SEM of at least three independent experiments. IMQ-treated 4T1 cells with or without 2DG treatment and control cells were harvested for vaccination with the boosting method. The vehicle group received PBS injections. (g) Tumor volume, (h) tumor weight, (i) the survival rate, and (j) the number of tumor-free mice were evaluated. There were 10 mice in each
infiltration of IL-17A—expressing γδ T cells after chemotherapy precedes the accumulation of CTLs within the tumor bed and is essential for the subsequent activation of antitumor immunity (Ma et al., 2011). Additionally, the increased amounts of intratumoral CTLs and T helper type 17 cells are associated with a good prognosis in patients with glioblastoma (Garg et al., 2016), and tumor-infiltrating CD4+ T cells are increased when mice are treated with the WT1 combination vaccine compared with the WT1 CTL peptide alone (Nakata et al., 2018; Shklovskaya et al., 2016). The in situ differentiation of CD11c+ DCs promoted by dying tumor cells could locally prime T cells and induce antitumor immunity (Ma et al., 2013). Therefore, we hypothesized that the induction of an efficient CTL response and the eradication of tumors may be mediated by the increased infiltration of CD8+, CD11c+, and IL-17A—producing cells after vaccination with IMQ-induced ICD TCLs, although the amounts of CD4+ cells were not altered. However, the involvement of IL-17A in the antitumor immune response of IMQ-induced ICD should be investigated in future.

We demonstrated that IMQ activated p53-dependent apoptosis (Huang et al., 2016) and that targeting aerobic glycolysis enhanced IMQ-induced cell death in p53−/− wild-type and p53-mutant cancer cells (Huang et al., 2014). Here, the inhibition of glycolysis by 2DG strengthened IMQ-induced apoptosis and ICD-associated features in p53-mutant SCC12 and 4T1 cells. Additionally, compared with vaccination with single agent-treated TCLs, vaccination with 2DG- and IMQ-cotreated 4T1 ICD TCLs greatly protected mice against the same challenge and established tumors. Consistently, a study showed that combining glycolysis inhibition with chemotherapy elicits an antitumor immune response by inducing ER stress and CRT surface exposure. However, the immunogenicity of ATP and HMGB1 was not evaluated (Bénéteau et al., 2012). Cotreatment with 2DG and IMQ significantly increased CRT exposure and promoted ATP secretion and HMGB1 release in SCC12 and 4T1 cells. We also discovered that extracellular ATP and HMGB1 uniquely affected IMQ-induced ICD TCL vaccination. Therefore, it is possible that 2DG may also enhance the release of ATP and HMGB1 in IMQ-treated cancer cells to increase immunogenicity and strengthen the antitumor immune response.

Monobenzone can induce melanoma and melanocyte immunogenicity by inducing tyrosinase- and MART-1-containing CD63+ exosomes following the induction of melanosome oxidative stress (van den Boorn et al., 2011). The combination of monobenzone and IMQ induces systemic melanocyte/melanoma antigen-specific immunity (Teulings et al., 2018). In contrast, the antitumor activity of 2DG is through the inhibition of glucose metabolism and the induction of metabolic stress (Simons et al., 2009), and it has been indicated that 2DG induces oxidative stress and cell killing in tumor cells (Coleman et al., 2008; Shutt et al., 2010; Vibhuti et al., 2013). We demonstrated that vaccination with 2DG- and IMQ-cotreated ICD TCLs exerted much more efficient specific antitumor immunity against breast cancer than single agent-treated TCLs. We found that this effect was associated with increased ICD features and ROS production. Although 2DG and monobenzone can trigger oxidative stress in cancer cells, in fact, there are no available reports indicating that monobenzone is an ICD inducer and affects glucose metabolism; distinct from 2DG, targeting hexokinase could induce metabolic stress and upregulate some signs of ICD to enhance antitumor immunity. However, we cannot rule out the possibility that monobenzone might also be an ICD inducer that can also trigger ROS production, which is important for enabling damage-associated molecular patterns. In this study, we provide a distinct strategy that can be used to enhance IMQ-induced ICD by combining 2DG to overcome IMQ-resistant tumors. We also looked forward to apply this strategy in the treatment of cancers in the future.

To the best of our knowledge, this is the first study to demonstrate that IMQ induces ICD in vitro and in vivo. Our findings provide not only a concept connecting IMQ-induced cancer cell death and antitumor immune responses that helps us explain how IMQ works in clinical treatments but also potential strategies for treating malignancies with IMQ using ICD-based immune therapy.

**MATERIALS AND METHODS**

**Detection of ICD-associated features**

Surface-exposed CRT on cells was detected with an anti-CRT antibody followed by a FITC-conjugated secondary antibody using flow cytometry analysis. The content of ATP and HMGB1 in supernatants was detected by using an ATPlite Luminescence Assay System (PerkinElmer, Waltham, MA) and an HMGB1 ELISA kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

**Vaccination procedure**

For vaccination, C57BL/6 mice or BALB/c nude mice were subcutaneously injected with 100 μl of IMQ-induced ICD TCLs from B16F10 or 4T1 cells into the left flank only once during 2 weeks as the prime group and once a week for 2 weeks as the boost group, respectively. One week after the final vaccination, the right flank or mammary gland was subcutaneously injected with 1 x 10^6 of the same live cells. For the therapeutic assay, BALB/c mice were subcutaneously injected with 1 x 10^5 4T1 cells into the mammary gland. After one week, mice were injected with 100 μl of 2DG, IMQ, or 2DG- and IMQ-cotreated 4T1 TCLs once a week for 2 weeks. C57BL6 mice were subcutaneously injected with 1 x 10^5 live B16F10 cells into the right flank, and mice were infused with CD8+ T cells from IMQ-induced ICD TCLs vaccinated mice through the tail vein after 4 days or injected with IMQ with or without sunitinib or glycyrrhizin after 4, 6, and 8 days. Tumor formation and tumor volume were monitored, and tumor weight was measured after sacrifice.

**Mixed lymphocyte reactions**

Bone marrow cells from the tibias and femurs of C57BL/6 mice were flushed and seeded with 20 ng/ml recombinant murine...
granulocyte macrophage colony-stimulating factor (ProSpec, Rehovot, Israel). The culture medium was changed every 2 days. Loosely adherent, clustered cells were used on day 6 as immature DCs, and then the immature DCs were subjected to mixed lymphocyte reaction experiments. Allogeneic responder T cells were isolated with a MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany) and cocultured with IMQ-treated B16F10 TCL-treated immature DCs for 72 hours. The proliferation of T cells was determined using a BrdU-based cell proliferation kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions.

**CTL assay**

CD8+ splenocytes from the test mice were used as effector cells. The B16F10 target cells were labeled with bis(acetoxymethy)2,2’6,2’-terpyridine-6,6’dicarboxylic reagent (DELFIa, Perkin Elmer) for 30 minutes at 37 °C. Target cells were cocultured with effector cells at the indicated ratios of effector:target cells, from 25:1 to 100:1, for 2 hours at 37 °C. The percent of specific lysis was calculated according to the following formula: \[
\text{spontaneous release (counts)} / \text{maximum release (counts)} \times 100.
\]

**Detection of effector and memory T lymphocytes**

Splenocytes were harvested from the tested mice. Effector T cells were detected by using fluoresce-conjugated CD4- or CD8- and IFNY-specific antibodies. For memory T cells, fluoresce-conjugated CD4 or CD8 and CD44 antibodies were used. The populations of CD4+/IFN\(\gamma\)+ and CD8+/IFN\(\gamma\)+ effector or CD4+/CD44+ and CD8+/CD44+ memory cells were analyzed by flow cytometry.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.12.039.

**REFERENCES**


SUPPLEMENTARY MATERIALS AND METHODS

Reagents and antibodies
Imiquimod (IMQ) was obtained from InvivoGen (San Diego, CA). Propidium iodide, suramin, glycyr rhizin, 4-phenylbutyrate, GSK2606414, N-acetyl cysteine, 2',7'-dichlorofluorescein diacetate, and 2-deoxyglucose were obtained from Sigma (St. Louis, MO). Antibodies specific for cleaved caspase-3, cleaved PARP, phosphorylated PERK, PERK, calreticulin (CRT), pan-cadherin, Grp78, phosphorylated eIF2α, eIF2α, CHOP, IRE1α, and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX). The LC3 antibody was purchased from Novus (Littleton, CO).

Cell lines
Human basal cell carcinoma BCC/KMC-1 cells, human gastric cancer AGS cells, and mouse breast cancer 4T1 cells were cultured in RPMI medium. Human cervical carcinoma HeLa cells and mouse melanoma B16F10 cells were cultured in DMEM. Human squamous cell carcinoma SCC12 cells were cultured in DMEM/F12. All media contained 10% fetal bovine serum.

Cell viability and DNA content assays
Cell viability and DNA content assays were performed as previously described (Huang et al., 2016).

Immunoblot analysis
Protein extraction and immunoblotting were performed as previously described (Huang et al., 2016).

Immunocytochemistry and immunohistochemistry
Immunocytochemistry and immunohistochemistry were performed as previously described (Huang et al., 2016).

Small interfering RNA transfection
A human CRT small interfering RNA (Santa Cruz Biotechnology) was transfected into B16F10 cells by using Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA). After the indicated time, the cells were treated with or without pretransfection with the CRT small interfering RNA (80 nM) for 48 hours or pretreatment with glycyr rhizin (3 mM) for 24 hours. 4T1 cells were treated with 50 μg/ml IMQ for 48 hours with or without 10 mM 2-deoxyglucose. After scraping, centrifuging, and washing with PBS twice, 1 x 10^7 B16F10 or 4T1 cells per milliliter were suspended in PBS and then repeatedly freeze-thawed four times. After centrifugation, the supernatants were collected and stored for further vaccination. For the IMQ boost groups, C57BL/6 mice or BALB/c nude mice were subcutaneously injected with 100 μl of IMQ-induced immunogenic cell death TCLs from B16F10 or 4T1 cells into the left flanks once a week for 2 weeks. For the IMQ prime groups, the mice were injected only once during the second week. One week after the final vaccination, the subcutaneous region of the right flank or mammary gland was injected with 1 x 10^7 of the same live cells and monitored for tumor formation. For the therapeutic assay, BALB/c mice were subcutaneously injected with 1 x 10^4 4T1 cells into the mammary gland. After one week, mice were injected with 100 μl of 2-deoxyglucose, IMQ, or 2-deoxyglucose— and IMQ-cotreated 4T1 immunogenic cell death TCLs once a week for two weeks. For the CD8 T-cell transplantation test, C57BL/6 mice were subcutaneously injected with 1 x 10^7 B16F10 cells into the right flank, and mice were infused with CD8 T cells from IMQ-induced immunogenic cell death TCLs vaccinated mice through the tail vein after 4 days or injected with IMQ (12.5 mg/kg) with or without suramin (2.5 mg/kg) or glycyr rhizin (2 mg/kg) after 4, 6, and 8 days. Tumor formation was monitored. Tumor volume was calculated according to the following formula: volume = (length x width^2) x π/6. The survival rate and number of tumor-free mice were recorded. Tumor weights and images were immediately recorded on the indicated day after sacrifice.

Detection of effector and memory T lymphocytes
Splenocytes were harvested from the tested mice. To detect effector T cells, cells were stimulated with phorbol 12-myristate 13-acetate (25 ng/ml) and the Golgi inhibitor brefeldin A (1 μg/ml) for 4 hours and then stained with fluorescence-conjugated CD4 or CD8 antibodies for 1 hour at 4 °C. Then, intracellular cytokine staining was performed using a fluorescence-conjugated IFNγ-specific antibody. To detect memory T cells, the isolated splenocytes were stained with fluorescence-conjugated CD4- or CD8- and CD44-specific antibodies for 1 hour at 4 °C. The effector T lymphocytes presented as CD4^+IFNγ^+ and CD8^+IFNγ^+ cells and the memory T cells presented as CD4^+CD44^+ and CD8^+CD44^+ cells by flow cytometry analysis.

Preparation of tumor cell lysates (TCLs) and vaccination
B16F10 cells were treated with 50 μg/ml IMQ for 24 hours with or without pretransfection with the CRT small interfering RNA (80 nM) for 48 hours or pretreatment with glycyr rhizin (3 mM) for 24 hours. 4T1 cells were treated with 50 μg/ml IMQ for 48 hours with or without 10 mM 2-deoxyglucose. After scraping, centrifuging, and washing with PBS twice, 1 x 10^7 B16F10 or 4T1 cells per milliliter were suspended in PBS and then repeatedly freeze-thawed four times. After centrifugation, the supernatants were collected and stored for further vaccination. For the IMQ boost groups, C57BL/6 mice or BALB/c nude mice were subcutaneously injected with 100 μl of IMQ-induced immunogenic cell death TCLs from B16F10 or 4T1 cells into the left flanks once a week for 2 weeks. For the IMQ prime groups, the mice were injected only once during the second week. One week after the final vaccination, the subcutaneous region of the right flank or mammary gland was injected with 1 x 10^7 of the same live cells and monitored for tumor formation. For the therapeutic assay, BALB/c mice were subcutaneously injected with 1 x 10^4 4T1 cells into the mammary gland. After one week, mice were injected with 100 μl of 2-deoxyglucose, IMQ, or 2-deoxyglucose— and IMQ-cotreated 4T1 immunogenic cell death TCLs once a week for two weeks. For the CD8 T-cell transplantation test, C57BL/6 mice were subcutaneously injected with 1 x 10^7 B16F10 cells into the right flank, and mice were infused with CD8 T cells from IMQ-induced immunogenic cell death TCLs vaccinated mice through the tail vein after 4 days or injected with IMQ (12.5 mg/kg) with or without suramin (2.5 mg/kg) or glycyr rhizin (2 mg/kg) after 4, 6, and 8 days. Tumor formation was monitored. Tumor volume was calculated according to the following formula: volume = (length x width^2) x π/6. The survival rate and number of tumor-free mice were recorded. Tumor weights and images were immediately recorded on the indicated day after sacrifice.

Detection of effector and memory T lymphocytes
Splenocytes were harvested from the tested mice. To detect effector T cells, cells were stimulated with phorbol 12-myristate 13-acetate (25 ng/ml) and the Golgi inhibitor brefeldin A (1 μg/ml) for 4 hours and then stained with fluorescence-conjugated CD4 or CD8 antibodies for 1 hour at 4 °C. Then, intracellular cytokine staining was performed using a fluorescence-conjugated IFNγ-specific antibody. To detect memory T cells, the isolated splenocytes were stained with fluorescence-conjugated CD4- or CD8- and CD44-specific antibodies for 1 hour at 4 °C. The effector T lymphocytes presented as CD4^+IFNγ^+ and CD8^+IFNγ^+ cells and the memory T cells presented as CD4^+CD44^+ and CD8^+CD44^+ cells by flow cytometry analysis.

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**Mixed lymphocyte reactions**

Bone marrow cells from the tibias and femurs of C57BL/6 mice were flushed and seeded at 1 × 10⁶ cells/well in RPMI 1640 medium supplemented with 20 ng/ml recombinant murine granulocyte macrophage colony-stimulating factor (ProSpec, Rehovot, Israel). The culture medium was changed every 2 days. Loosely adherent, clustered cells were used on day 6 as immature dendritic cells (DCs). All cells were resuspended, centrifuged, and seeded at 2 × 10⁶ cells/well for 24 hours. Then, the immature DCs were subjected to mixed lymphocyte reaction experiments. The purity of the immature DCs was routinely >85%, as confirmed by CD11c staining. Allogeneic responder T cells were isolated with a MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). Immature DCs were treated for 24 hours with or without IMQ-treated B16F10 TCLs. After harvesting, 5 × 10⁵ DCs were added to 1 × 10⁵ allogeneic T cells in a flat-bottomed 96-well culture plate. During the final 16 hours of the 72-hour culture period, the proliferation of T cells was determined using a BrdU-based cell proliferation kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions.

**Statistical analyses**

Three independent experiments were conducted in each study, and assay conditions were performed in triplicate. Data were analyzed using Student’s t-test, and statistically significant differences were indicated as a P-value of 0.05 and are expressed as *P < 0.05; **P < 0.01; ***P < 0.001; #P < 0.05; ##P < 0.01; ###P < 0.001; ×P < 0.05; ××P < 0.01; ×××P < 0.001; §P < 0.05; §§P < 0.01; and §§§P < 0.001 in different compared groups described in figure legends of each experiment.

**SUPPLEMENTARY REFERENCE**

Supplementary Figure S2. Pharmacological inhibition of PERK attenuated IMQ-induced ICD-associated features. (a) BCC and B16F10 cells were treated with IMQ (50 μg/ml) with or without GSK2606414 (80 nM) for 1 hour; then, surface-exposed CRT was detected by flow cytometry. (b) The cellular distribution of CRT and pan-Cad was observed by immunocytochemistry, (c) the extracellular ATP content was determined by an ATP content assay, (d) the release of HMGB1 was determined by ELISA, and (e) the expression of IRE1α, Grp78, p-eIF2α, eIF2α, CHOP, LC3, cleaved PARP, cleaved caspase-3, and β-actin was examined by immunoblotting. The data are expressed as the mean ± S.E.M. of at least three independent experiments. BCC, basal cell carcinoma; CRT, calreticulin; ICD, immunogenic cell death; IMQ, imiquimod; pan-Cad, pan-cadherin; p-eIF2α, phosphorylated eIF2α.
Supplementary Figure S3. 2DG enhanced IMQ-induced ICD TCL-induced antitumor immunity against established tumors. 4T1 cells were treated with IMQ (50 μg/ml) with or without 2DG (10 mM). After 48 hours, TCLs were collected for vaccination. One week after 4T1 tumor cell implantation, mice were vaccinated using the boosting method. The vehicle group received PBS injections. (a) Tumor volume was monitored. (b) Tumor weights were measured 32 days after tumor cell implantation. There were eight mice in each group. The asterisk represents a significant difference between the vehicle and 2DG groups; the double dagger represents a significant difference between the vehicle and IMQ groups; and the pound symbol represents a significant difference between the vehicle and 2DG + IMQ groups. 2DG, 2-deoxyglucose; ICD, immunogenic cell death; IMQ, imiquimod; TCL, tumor cell lysate.
Supplementary Figure S4. Vaccination with the combination of 2DG and IMQ-induced ICD TCLs did not protect against tumor challenge in an immune-deficient mouse model. 4T1 cells were treated with IMQ (50 µg/ml) with or without 2DG (10 mM). After 48 hours, TCLs were collected for the vaccination assay with the boosting method in nude mice. Two weeks after the first vaccination, the mice were challenged with live 4T1 cells. The vehicle group received PBS injections. In these mice, (a) tumor volume and (b) tumor-free outcomes were monitored. (c) Tumor weights were measured 32 days after challenge. There were 10 mice in each group. 2DG, 2-deoxyglucose; ICD, immunogenic cell death; IMQ, imiquimod; TCL, tumor cell lysate.
Supplementary Figure S5. Autophagy is involved in IMQ-induced CRT surface exposure and ATP secretion. BCC cells were treated with IMQ (50 μg/ml) (a) in the presence or absence of BFA (5 μg/ml) for 24 hours and (b) with or without pretreatment with 1 mM 3MA or 10 μM BAF for 1 hour; then, CRT surface exposure was detected with flow cytometry. (c) BCC cells were transfected with BECN1 or a control siRNA (Scr), followed by IMQ (50 μg/ml) treatment for 4 hours; then, the extracellular ATP content was determined. The data are expressed as the mean ± SEM of at least three independent experiments. 3MA, 3-methyladenine; ATP, adenosine triphosphate; BAF, bafilomycin A1; BCC, basal cell carcinoma; BFA, brefeldin A; CRT, calreticulin; IMQ, imiquimod; Scr, control small interfering RNA; siRNA, small interfering RNA.