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Imiquimod induces ER stress and Ca^{2+} influx independently of TLR7 and TLR8

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ABSTRACT

Endoplasmic reticulum (ER) stress is a physiological response to protein overload or misfolded proteins in the ER. Certain anti-cancer drugs, e.g. bortezomib and nelfinavir, induce ER stress implying that this could be a successful therapeutic strategy against several forms of cancer. To find novel ER-stress inducers we screened a panel of natural and synthetic Toll-like receptor (TLR) agonists against human keratinocytes and identified the anti-cancer drug imiquimod (IMQ) as a potent inducer of ER stress. Other TLR7 and TLR8 agonists, including resiquimod and gardiquimod, did not induce ER stress, demonstrating that IMQ induces ER stress independently of TLR7 and TLR8. We further confirmed this by showing that IMQ could still induce ER stress in mouse *Tlr7*$^{-/-}$ cells. IMQ also induced a rapid and transient influx of extracellular Ca^{2+} together with the release of Ca^{2+} from internal stores. Depletion of Ca^{2+} from the ER is a known cause of ER stress suggesting that IMQ induces ER stress via depletion of ER Ca^{2+}. The ER-stress inducing property of IMQ is possibly of importance for its efficacy in treating basal cell carcinoma, in situ melanoma, and squamous cell carcinoma. Our data could potentially be harnessed for rational design of even more potent ER-stress inducers and new anti-cancer drugs.

1. Introduction

Endoplasmic reticulum (ER) stress is a physiological response that occurs after protein overload, or accumulation of misfolded proteins, in the ER lumen. ER stress triggers the highly conserved unfolded-protein response (UPR) mediated by IRE1-dependent splicing of XBP1 mRNA, proteolytic processing of ATF6 and the phosphorylation of PERK [1,2]. This in turn is followed by the induction of ER-stress target genes including pro- or anti-apoptotic genes, chaperone genes and phospholipid-synthesis genes, e.g. *DDIT3* (CHOP), *ERDJ4*, *GRP78* (BiP), and *EPT1* [3]. Chronic and high levels of ER stress induce cell death whereas moderate or transient ER stress serves a protective role for stressed cells, e.g. during tumor growth [4]. Tipping the balance of ER stress towards cell death has recently emerged as a successful anti-cancer strategy. The anti-cancer drugs bortezomib, nelfinavir and tanespimycin all act via the induction of ER stress followed by apoptosis or reduced growth of cancer cells [5–8]. Since pharmacologically induced ER stress seems effective against certain forms of cancer, a search for additional potent ER-stress inducers is motivated.

Toll-like receptor (TLR) signaling can trigger the activation of XBP1, PERK and ATF6 in macrophages, but with little or no induction of classical ER-stress target genes [9]. In addition, TLR signaling also leads to splicing of XBP1 followed by induction of pro-inflammatory genes in synoviocytes and by apoptosis in intestinal stem cells [10,11]. Since skin responds with ER stress after exposure to several environmental factors, we hypothesized that keratinocytes, in contrast to macrophages, would be prone to a full ER-stress response after exposure to natural or synthetic TLR agonists [12–14]. Indeed, while screening TLR agonists against primary human keratinocytes we identified the synthetic TLR7-agonist imiquimod (IMQ) as a potent inducer of ER stress. IMQ is a purine analog with anti-cancer properties and is the prototypic compound of the imidazoquinolines, a group of small immunostimulatory molecules including resiquimod (RSQ) and gardiquimod (GDQ) [15]. IMQ triggers TLR7 signaling followed by the expression of pro-inflammatory factors such as IFNα, IL6 and TNF [16]. However, it is well established that IMQ also provokes TLR7-independent responses in skin. Topical IMQ cream (Aldara) induces strong responses in skin of *Tlr7*$^{-/-}$ and *Myd88*$^{-/-}$ mice independently of

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TLR7 and adaptive immune responses [17,18]. We discovered that IMQ induces ER stress in a TLR7-independent manner in keratinocytes and melanoma cells. Interestingly, other TLR7 and TLR8 agonists, including RSQ and GDQ, were unable to induce ER stress indicating that small structural changes in imidazoquinolines impacts their ER-stress inducing activity.

While trying to understand the mechanism whereby IMQ induces ER stress we discovered that IMQ induced a rapid and transient influx of extracellular Ca^{2+} and a release of intracellular Ca^{2+} stores. Depletion of Ca^{2+} from the ER lumen is a known cause of ER stress, mediated by the interference with Ca^{2+}-dependent chaperones. This suggests that IMQ induces ER stress via depletion of Ca^{2+} from the ER [19]. Identifying the receptor for IMQ that mediates the induction of ER stress could potentially lead to the discovery of endogenous ligands that control physiological ER-stress responses. Furthermore, the data provided here might be useful for rational design of strong ER-stress inducers and new anti-cancer drugs.

2. Material and methods

2.1. Cells

Neonatal human keratinocytes (Life Technologies) were cultured in keratinocyte-SFM medium (Gibco) supplemented with 0.2 ng/ml recombinant epidermal growth factor (EGF) and 30 μg/ml of bovine pituitary extract (BPE) (Gibco). The medium was additionally supplemented with 0.3 mM $CaCl_2$, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco). Human melanoma cell lines A375, SK-MEL-28 and MeWo (ATCC) were cultured in DMEM medium supplemented with 5% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (1 mM). Mouse splenocytes were isolated from WT and *Tlr7*$^{-/-}$ mice and were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (1 mM) and β-mercaptoethanol (50 μM).

2.2. Quantitative RT-PCR

Total RNA was isolated using TRIzol (Life Technologies) using chloroform and isopropanol precipitation. After one wash in 70% ethanol the concentration of total RNA was determined using NanoDrop 1000 (Thermo Scientific). Total RNA was reverse transcribed in cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or iScript cDNA synthesis kit (Bio-Rad). Gene expression levels were determined by real-time PCR using SYBR reagent on an Mx3005P (Agilent Technologies) or TaqMan probes (Thermo Scientific) on a LightCycler 96 (Roche). Primer sequences and more information on SYBR and TaqMan assays are available on request.

2.3. Calcium measurements

A375 were loaded with Fluo-3 AM (1 μM) (Thermo Scientific) in PBS (0.9% Ca^{2+}) buffer containing pluronic acid (0.02%) and

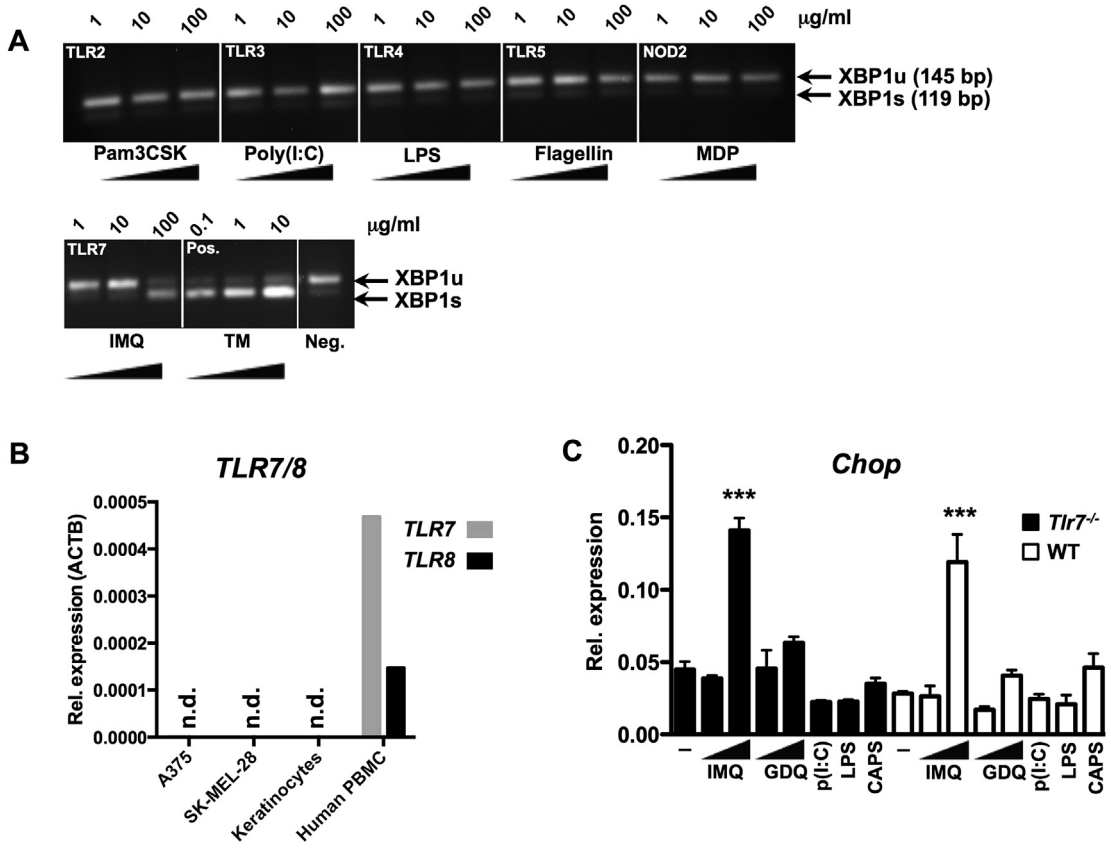

Fig. 1. IMQ induces a TLR7-independent ER-stress response. Using primary human keratinocytes we screened a panel of TLR agonists to identify novel inducers of ER stress. We used XBP1 splicing as a marker for ER stress and identified IMQ as a potent inducer of ER stress in human keratinocytes (A). Primary human keratinocytes and human melanoma cells do not express TLR7 or TLR8, clearly indicating that the IMQ-induced ER-stress response is independent of TLR7 and TLR8 (B). To further verify that the IMQ-induced ER-stress response occurs independently of TLR7 we stimulated mouse *Tlr7*$^{-/-}$ and WT splenocytes with IMQ (C). Indeed, IMQ induced an ER-stress response both in *Tlr7*$^{-/-}$ and WT cells as measured by induction of the ER-stress gene CHOP (Ddit3). The potent TLR7-agonist GDQ could not induce ER stress in neither *Tlr7*$^{-/-}$ nor WT cells. (One-way ANOVA followed by Dunnett's post-test, *** = $p < 0.01$.)