

Requirement of Inositol 1,4,5-Trisphosphate Receptors for Tumor-mediated Lymphocyte Apoptosis*

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Camia Steinmann[‡], Megan L. Landsverk[§], José M. Barral[‡],
and Darren Boehning^{‡,1}

From the [‡]Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555-0620 and the [§]Department of Pediatrics, University of Washington, Seattle, Washington 98195-6320

Tumor cells strategically down-regulate Fas receptor expression to evade immune attack and up-regulate expression of Fas ligand to promote apoptosis of infiltrating T lymphocytes. Many pathways leading to apoptotic cell death require calcium release from inositol 1,4,5-trisphosphate receptors (IP₃Rs). Here, we show that Fas-dependent killing of Jurkat T lymphoma cells by SW620 colon cancer cells requires calcium release from IP₃R. General suppression of IP₃R signaling significantly reduced SW620-mediated Jurkat cell apoptosis. Significantly, a specific inhibitor of apoptotic calcium release from IP₃R strongly blocked lymphocyte apoptosis. Thus, selective pharmacological targeting of apoptotic calcium release from IP₃R may enhance tumor cell immunogenicity.

Fas receptor is a member of the tumor necrosis factor- α superfamily of death receptors. Fas receptor binds to Fas ligand presented on another cell, initiating a series of events ultimately leading to the activation of proapoptotic proteases known as caspases. Fas ligand expression is restricted primarily to activated T cells and natural killer cells, where it is essential for activation-induced cell death and immune-mediated cytotoxicity. In addition, Fas ligand is constitutively expressed in select immunoprivileged tissues such as the eye and testis. Cancer cells can harness the Fas pathway to evade immune attack (1–3). Loss of Fas receptor function is frequently observed in human cancers, reducing the ability of infiltrating T cells and natural killer cells to kill cancerous cells (4–8). Modulation of Fas signaling for selective survival advantage has been documented in multiple tumor types, including colon, hepatocellular, ovarian and esophageal carcinomas, melanoma, and astrocytoma (1). Loss of Fas signaling by cancer cells is accomplished at the molecular level by transcriptional down-regulation of Fas receptor or the adaptor protein Fas-associated protein with

death domain and up-regulation of negative regulatory components of the Fas pathway such as FLICE-like inhibitory protein, Bcl-X_L, and Bcl-2 (2). Moreover, many cancer cells also up-regulate Fas ligand expression, which consequently results in increased T cell apoptosis and decreased infiltration (9–11). Thus, the identification of the molecular pathways modulating Fas signaling is an important goal in rational drug development that targets this adaptive strategy of cancer cells.

We have recently shown that intracellular calcium homeostasis is critical to the progression of apoptotic cell death. Inositol 1,4,5-trisphosphate receptor (IP₃R)² calcium channels regulate intracellular calcium concentration during apoptosis induced by death receptor ligation (12) and cellular damage (13). Apoptosis-specific signaling via IP₃R is mediated by cytochrome *c*, which binds to the channel after being released from mitochondria, resulting in augmented channel function (13). This ultimately leads to cytosolic and mitochondrial calcium overload and cell death. Fas receptor signaling utilizes cytochrome *c* release from mitochondria to amplify the apoptotic signal (14). Therefore, we hypothesized that inhibiting IP₃R-dependent apoptotic signaling in lymphocytes would protect them from apoptotic cell death induced by tumor cells expressing high levels of Fas ligand.

In this report, we show that inhibiting IP₃R function in Jurkat T-lymphoma cells is cytoprotective against apoptosis induced by co-culture with Fas ligand-expressing SW620 colon cancer cells. The IP₃R antagonist xestospongins C (XestC) and calcium buffering with BAPTA dose-dependently inhibited Jurkat cell apoptosis. Inhibiting exclusively IP₃R activation due to proapoptotic signaling in lymphocytes by blocking cytochrome *c* binding to IP₃R inhibited lymphocyte death with nanomolar affinity. Thus, specifically targeting proapoptotic signaling through the IP₃R in lymphocytes appears to be a promising therapeutic approach for enhancing tumor cell immunogenicity.

EXPERIMENTAL PROCEDURES

Materials—Peptide synthesis of the IP₃R fragment encoding the cytochrome *c* binding domain of the type I IP₃R isoform and conjugation to BODIPY-577/618 has been described elsewhere (15). This cell-permeant peptide is termed B-IP3RCYT. The aminomethoxy ester form of BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; BAPTA-AM), propidium iodide, and fura-2-AM were purchased from Molecular Probes (Eugene, OR). XestC and all other chemicals were purchased from Sigma-Aldrich.

Cell Culture—Jurkat T-lymphoma (clone E6-1) and SW620 colon adenocarcinoma cells were purchased and cultured according to the guidelines of American Type Culture Collection (ATCC, Manassas, VA). SW620 cells grow as adherent monolayers, whereas Jurkat cells grow in suspension. Jurkat cells were co-cultured with SW620 cells by exchanging the cul-

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¹ To whom correspondence should be addressed: 301 University Blvd., University of Texas Medical Branch, Galveston, TX, 77555-0620. Fax: 409-747-2200; E-mail: dfboehni@utmb.edu.

² The abbreviations used are: IP₃R, inositol 1,4,5-trisphosphate receptor; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; BAPTA-AM, aminomethoxy ester form of BAPTA; XestC, Xestospongins C; YFP, yellow fluorescent protein; RNAi, RNA interference.

ture medium of SW620 cells (80% confluent in a 10-cm dish) with 10 ml of Jurkat medium containing 1×10^6 Jurkat cells. Co-cultures were incubated for 24 h, after which cell death or caspase activity was determined in Jurkat cells. Preloading BAPTA or B-IP3RCYT into Jurkat cells was performed for 30 min at room temperature. Free BAPTA-AM or B-IP3RCYT were removed by medium exchange prior to addition to SW620 cells. XestC was similarly preincubated with Jurkat cells prior to co-culturing and remained in the culture medium for the duration of the co-culture experiment.

RNAi—Knockdown of the human IP₃R-1 in Jurkat cells and rescue with the rat IP₃R-1 isoform was done exactly as described elsewhere (12). The rat *itpr1* gene has several base substitutions within the targeted region of the human *ITPR1* gene allowing expression in the presence of this RNAi. Control experiments utilized an RNAi with several deletions and insertions (12). Co-transfection with yellow fluorescent protein (YFP) was utilized to identify cells containing the RNAi for calcium imaging and cell death assays (described below).

Calcium Imaging—SW620 cells grown on coverslips were loaded with fura-2-AM for 30 min at room temperature. Jurkat cells were loaded in suspension as described (12). Coverslips with fura-2-loaded SW620 cells were placed in an imaging chamber (Attofluor® cell chamber, Molecular Probes) and imaged at $\times 40$ magnification as described (12). Fura-2-loaded Jurkat cells ($\sim 1 \times 10^4$) were added by solution exchange. In $\sim 20\%$ of trials (6 of 28), a Jurkat cell came into direct contact with one or more SW620 cells in the field of view. To measure responses in Jurkat cells co-transfected with RNAi and YFP, images were acquired with a $\times 20$ objective, and $\sim 5 \times 10^4$ Jurkat cells were added to the imaging chamber containing SW620 cells. This methodology permitted imaging of the low abundance transfected Jurkat cells contacting SW620 cells with greater frequency at the expense of resolution. RNAi data are expressed as a histogram of the percentage of responding YFP-positive cells from a minimum of 15 cells, which were confirmed to directly contact an SW620 cell. A response was defined as an increase in 340/380 ratio greater than 0.5.

Caspase-3 Activity and Cell Death Determination—Caspase activity and cell death were determined specifically in Jurkat cells by removing the medium containing the Jurkat cells into a fresh tube. An additional rinse with culture medium was used to fully recover the Jurkat cells. Adherent SW620 cells remained attached to the culture plate after this rinse as determined by light microscopy. Jurkat cells were pelleted by centrifugation at $1000 \times g$, and cell death was determined by propidium iodide staining as described (12). Fluorometric determination of DEVDase (caspase-3 like) activity was performed on lysates prepared from Jurkat cells as described (12, 15).

Statistical Analysis—Statistical significance was determined by using unpaired two-tailed *t* tests on data points from at least three separate experiments. Data comparisons were considered significant if *p* was < 0.05 .

RESULTS AND DISCUSSION

SW620 colon carcinoma cells express high levels of Fas ligand and have been shown to be potent inducers of Jurkat

apoptosis in a Fas-dependent manner (16). Thus, we hypothesized that co-culture of Jurkat cells with SW620 cells would induce proapoptotic calcium release in Jurkat cells and that this calcium release would be required for lymphocyte apoptosis. To monitor intracellular calcium in co-culture experiments, SW620 cells were grown on coverslips and loaded with fura-2. Jurkat cells were also separately loaded with fura-2 to simultaneously image intracellular calcium in both cell types. After establishing baseline calcium levels in SW620 cells, Jurkat cells were added to the imaging chamber by solution exchange. In 6 of 28 trials, a Jurkat cell bound directly to one or more SW620 cells. As shown in Fig. 1, *A* and *B*, upon binding SW620 cells, a large transient rise in intracellular calcium is induced in Jurkat cells. Interestingly, after a short delay, calcium oscillations are induced in SW620 cells (Fig. 1, *A* and *C*). The functional significance of these oscillations in SW620 cells are unclear, but this finding is of significance since coupling of Fas ligand to calcium release in Fas ligand-presenting cells has not been demonstrated. Fas ligand microvesicles can induce calcium release in Jurkat cells via IP₃R calcium channels (12, 15). To determine whether the calcium response in Jurkat cells was due to IP₃R-mediated calcium release, we knocked down IP₃R levels with a previously characterized double-stranded RNAi oligonucleotide targeting the human IP₃R-1 isoform (12). Knockdown of IP₃R-1 completely abrogated Jurkat calcium release in response to SW620 cell binding (0/15 cells; Fig. 1*D*). Importantly, calcium release could be rescued by co-transfecting the rat *itpr1* cDNA (9/18 cells). As expected, Jurkat cells expressing control RNAi retained the ability to release calcium after contact with SW620 cells (10/16 cells). These results indicate that binding to SW620 cells induces IP₃R-dependent calcium release in Jurkat cells.

To examine the effects of modulators of IP₃R-dependent calcium release on SW620-dependent Jurkat apoptosis, we monitored caspase-3 activation. As expected, co-culture of Jurkat cells with SW620 cells induced significant activation of caspase-3 in Jurkat cells (Fig. 1*E*; #, *p* < 0.05 relative to Jurkat cells, which were not co-cultured with SW620 cells). To determine whether intracellular calcium contributed to this increase in caspase-3 activity, we loaded Jurkat cells with various concentrations of the calcium chelator BAPTA-AM to buffer cytosolic calcium levels. Significant attenuation of caspase-3 activation was observed by incubating the cells with 100 and 500 nM BAPTA-AM (*, *p* < 0.05 relative to Jurkat cells co-cultured with SW620 cells without pretreatment).

To determine whether the IP₃R is required for lymphocyte apoptosis, we incubated cells with various doses of the cell-permeant IP₃R inhibitor XestC, a selective blocker of IP₃R function when used at low micromolar concentrations (17, 18). XestC inhibited caspase-3 activation in Jurkat cells at concentrations ranging from 1 to 20 micromolar, indicating that IP₃R activity was required for caspase-3 activation (Fig. 1*E*). Importantly, XestC potentially blocked Jurkat cell death induced by co-culture with SW620 cells (Fig. 1*F*).

During apoptosis, calcium release from IP₃R is augmented by direct binding of cytochrome *c* to the channel (12, 13, 15). Cytochrome *c* binds to the cytosolic C-terminal “tail” of the IP₃R, decreasing the ability of calcium to inhibit the channel and pro-

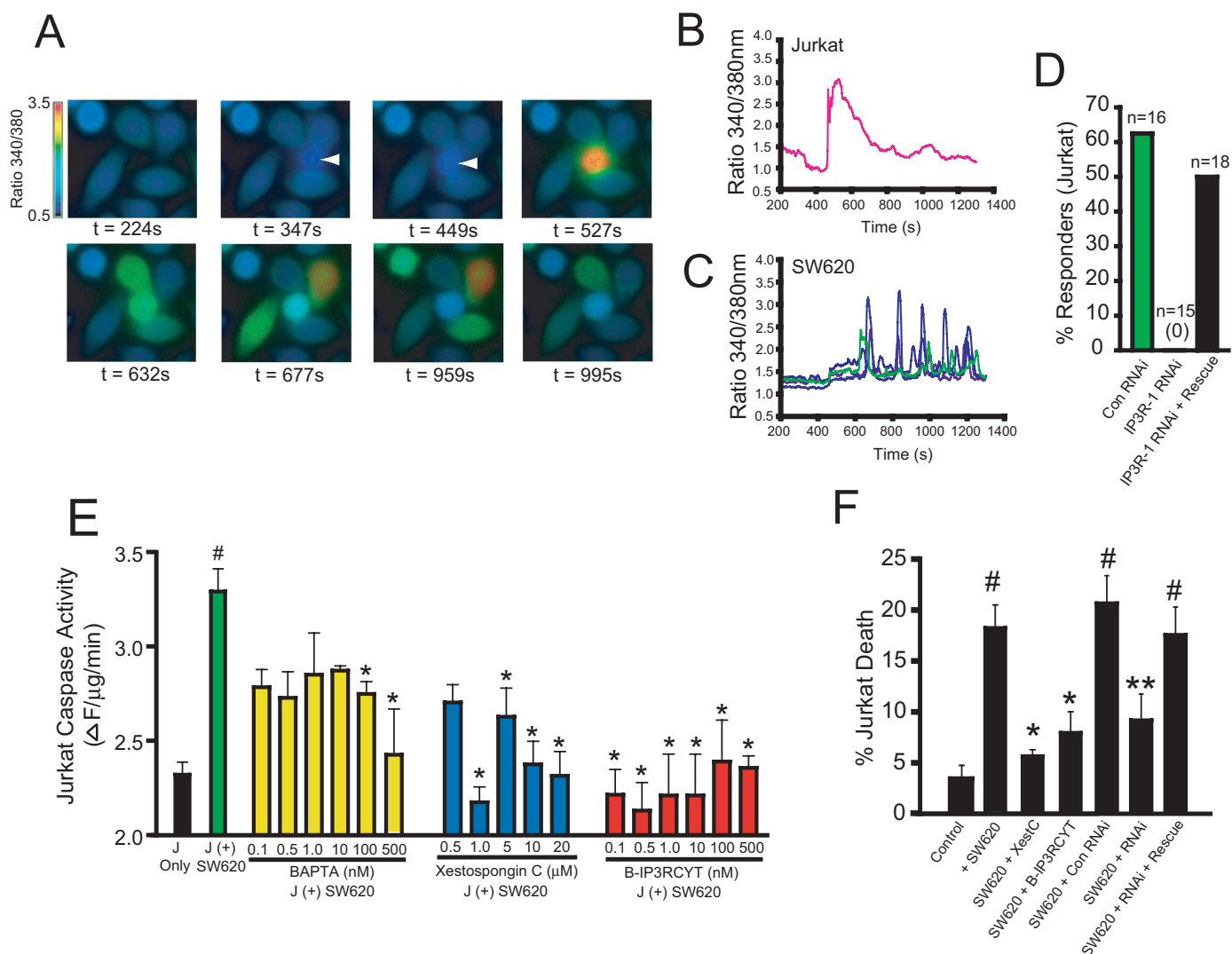


FIGURE 1. Calcium release from IP₃R is required for SW620-mediated killing of Jurkat cells. *A*, Fura-2 calcium ratio imaging of SW620/Jurkat co-cultures. The first panel shows only adherent SW620 cells. At a time of 347 s, a single Jurkat cell (second panel; indicated by an arrow), comes into contact with four SW620 cells, which leads to a rise in intracellular calcium. *B*, calcium response in the Jurkat cell shown in *A*. *C*, calcium responses of the four SW620 cells shown in *A*. Qualitatively similar results were observed in all trials in which a Jurkat cell contacted at least one SW620 cell (data not shown). *D*, histogram of the percentage of Jurkat cells, which released calcium in response to binding SW620 cells after transfection with control RNAi (*Con RNAi*), IP₃R-1 RNAi, or co-transfection with IP₃R-1 RNAi and the cDNA encoding the rat IP₃R-1. The total number of transfected cells that contacted an SW620 cell is indicated. No cells expressing IP₃R-1 RNAi responded (0/15). *E*, caspase-3-like activity (DEVDase activity) in Jurkat cells co-cultured with SW620 cells or cultured alone (black bar). Green, no pretreatment before co-culture for 24 h with SW620 cells. Yellow, Jurkat cells were preincubated with the indicated concentrations of BAPTA-AM before co-culture. Blue, Jurkat cells were preincubated with the indicated concentrations of xestospongine C. Red, Jurkat cells were preincubated with the indicated concentrations of B-IP3RCYT. See "Experimental Procedures" for details. *J* = Jurkat. *F*, cell death in Jurkat cells prior to (Control) or 24 h after co-culture with SW620 cells (+SW620). The effects of 1 μM XestC and 10 nM B-IP3RCYT were tested by preincubation prior to co-culture. The indicated RNAis were transfected 24 h before co-culture as described elsewhere (12). All data represent the mean \pm S.E. # indicates $p < 0.05$ relative to control (no incubation with SW620 cells). * indicates $p < 0.05$ relative to co-culture with SW620 cells. ** indicates $p < 0.05$ relative to co-culture with SW620 + control RNAi.

longing calcium release (15). Peptides derived from the cytochrome *c* binding domain of IP₃R, when introduced into cells, inhibit calcium-dependent apoptosis without altering agonist-induced calcium release (12, 13, 15). Coupling of one of these peptides to the hydrophobic dye BODIPY 577/618 renders the peptide cell permeant, retaining antiapoptotic activity (12, 15). Thus, we tested whether this peptide, termed B-IP3RCYT, could block SW620-dependent cell death of Jurkat cells. As shown in Fig. 1*E*, B-IP3RCYT inhibited caspase-3 activation in Jurkat cells at nanomolar concentrations. This result is consistent with the *in vitro* observation that IP3RCYT has a higher affinity for cytochrome *c* than the cytosolic tail of IP₃R and can inhibit Fas-dependent cell death induced by Fas ligand

microvesicles or the cytotoxic drug staurosporine at nanomolar concentrations (15). Importantly, the B-IP3RCYT peptide also inhibited Jurkat cell death induced by SW620 co-culture (Fig. 1*F*). Thus, specifically blocking apoptotic calcium release via the IP₃R inhibits lymphocyte apoptosis induced by Fas ligand-expressing cancer cells. Finally, RNAi-mediated knockdown of IP₃R-1 also inhibited Jurkat cell death, and these effects were reversed by rescue with the rat *itpr1* cDNA (Fig. 1*F*).

The ability of tumor cells to attain immunoprivileged status *in vivo* by up-regulating Fas ligand has remained relatively controversial (9, 11, 19). In this report, we demonstrate *in vitro* that Fas-dependent killing of a lymphocyte cell line by cancer cells requires calcium release from IP₃R. Identification of the IP₃R as

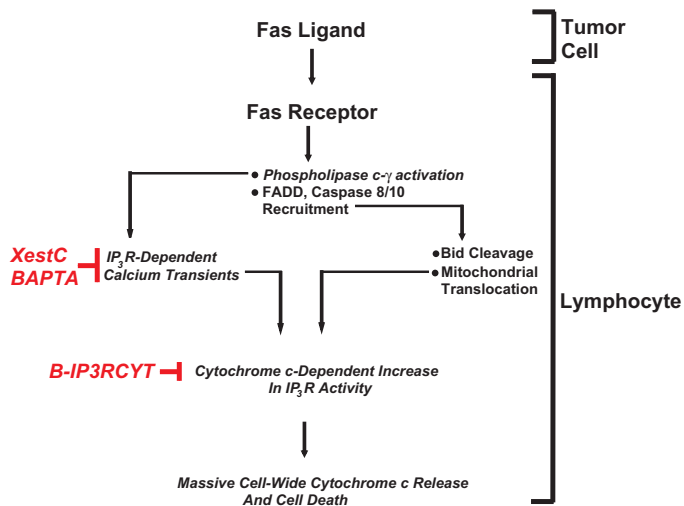


FIGURE 2. Calcium and tumor-induced lymphocyte apoptosis. Fas ligand expressed on tumor cells activates Fas receptor on infiltrating lymphocytes. This causes activation of IP₃R by coupling of Fas receptor to phospholipase C- γ and subsequent production of IP₃ (12). The canonical components of the death-induced signaling complex are also recruited to Fas receptors such as Fas-associated death domain (*FADD*) and caspase 8/10. Caspase 8/10 activation induces Bid activation and translocation to mitochondria, sensitizing them to calcium-induced cytochrome *c* release. Cytochrome *c* subsequently binds to IP₃R, causing further calcium release and mitochondrial calcium overload. Blocking IP₃-dependent elevations in cytosolic calcium with Xestospingon C or BAPTA inhibits lymphocyte apoptosis. Specifically blocking cytochrome *c* binding to IP₃R also inhibits lymphocyte apoptosis without modifying agonist-induced calcium release, suggesting a viable target for therapeutic intervention.

a mediator of lymphocyte apoptosis induced by a tumor cells highlights a novel Fas-dependent pathway that warrants further investigation *in vivo* (Fig. 2). Significantly, we found that lymphocyte apoptosis could be inhibited by specifically blocking apoptotic calcium release from IP₃R. Lymphocyte apoptosis was inhibited by B-IP3RCYT when placed in the culture medium at low nanomolar concentrations. Since B-IP3RCYT exhibits no significant cellular toxicity and does not modify agonist-induced calcium release (12, 15), targeting IP₃R/cytochrome *c* interactions appears to be an attractive target for enhancing the immunologic response of tumors that have

acquired adaptive changes in death receptor signaling to promote survival.

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