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Protein kinase C signaling during T cell activation induces the endoplasmic reticulum stress response

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Abstract T cell receptor (TCR) ligation (signal one) in the presence of co-stimulation (signal two) results in downstream signals that increase protein production enabling naïve T cells to fully activate and gain effector function. Enhanced production of proteins by a cell requires an increase in endoplasmic reticulum (ER) chaperone expression, which is accomplished through activation of a cellular mechanism known as the ER stress response. The ER stress response is initiated during the cascade of events that occur for the activation of many cells; however, this process has not been comprehensively studied for T cell function. In this study, we used primary T cells and mice circulating TCR transgenic CD8$^+$ T cells to investigate ER chaperone expression in which TCR signaling was initiated in the presence or absence of co-stimulation. In the presence of both signals, *in vitro* and *in vivo* analyses demonstrated induction of the ER stress response, as evidenced by elevated expression of GRP78 and other ER chaperones. Unexpectedly, ER chaperones were also increased in T cells exposed only to signal one, a treatment known to cause T cells to enter the 'nonresponsive' states of anergy and tolerance. Treatment of T cells with an inhibitor to protein kinase C (PKC), a serine/threonine protein kinase found downstream of TCR signaling, indicated PKC is involved in the induction of the ER stress response during the T cell activation process, thus revealing a previously unknown role for this signaling protein in T cells. Collectively, these data suggest that induction of the ER stress response through PKC signaling is an important component for the preparation of a T cell response to antigen.

Keywords ER stress · Chaperones · T cell activation · Protein kinase C

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Introduction

The endoplasmic reticulum (ER) is a highly specialized network involved in regulating the synthesis, folding, and assembly of secretory and transmembrane proteins (Kaufman 2002; Kaneko and Nomura 2003; Groenendyk and Michalak 2005). Folding of newly synthesized proteins is mediated by a set of specific molecular chaperones that reside mainly in the ER lumen (Kuznetsov et al. 1996; Ma and Hendershot 2001). Increased cellular production of proteins requires a commensurate increase in a cell's protein folding capability to avoid the accumulation of unfolded proteins (Rao et al. 2002). To counteract this physiological stress and return the cell to homeostasis, an adaptive cellular mechanism known as the unfolded protein response or ER stress response is

initiated (Harding et al. 2002; Yoshida et al. 2006). The ER stress response both elevates ER chaperone protein expression to enhance the protein folding capacity of the ER and inhibits general protein translation to decrease the protein 'load' in the ER (Ellgaard and Helenius 2003). This is accomplished by three specific ER transmembrane proteins that act as ER stress transducers. Activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) control the expression of activated genes, while PKR-like ER kinase (PERK) maintains translational control through phosphorylation of eukaryotic translation initiation factor 2 on its α subunit (eIF2α) of serine 51 (Ron 2002). Glucose-regulated protein 78 (GRP78), the central regulator of the ER stress response, binds to these ER stress transducers and inhibits their signaling. During ER stress, however, GRP78 releases the transducers allowing activation of the ER stress response (Lee 2005).

Sustained ER stress that prevents restoration of cellular homeostasis may lead to apoptosis (Boyce and Yuan 2006). Indeed, ER stress response signaling may be initiated with pathological events, and has classically been associated with exposure to deleterious chemical stressors. More recently, ER stress response signaling has also been shown to occur during physiological cellular processes, such as muscle development (Nakanishi et al. 2005), osteoblast differentiation, and the functioning of hepatocytes (Wu and Kaufman 2006). The differentiation of B lymphocytes into antibody-producing plasma cells involves a physiological ER stress response to permit high levels of antibody production (Gass et al. 2004). Thus, different external signals may activate the physiological ER stress response as a fundamental component common to the differentiation and development of many tissues.

Recently, priming of CD4$^+$ T cells with peptides in the context of major histocompatibility complex (MHC) molecules has been reported to utilize components of physiological ER stress response signaling, including expression of stress response-induced genes (Scheu et al. 2006). Typically, two signals are required for a T cell to become fully activated. Signal one occurs when the T cell receptor (TCR) recognizes and binds to a peptide presented by a MHC molecule expressed on an antigen-presenting cell (APC; Davis et al. 1998; Lanzavecchia and Sallusto 2000). Signal two is generated by ligation of co-stimulatory molecules on the T cell and APC (Harding et al. 1992). If signal one occurs in the absence of signal two, T cells enter a state of antigen-specific tolerance or become 'nonresponsive', a state often termed anergy (Schwartz 1997; Powell et al. 1999).

Alternatively, T cells can be activated *in vitro* by stimulating with a combination of phorbol 12-myristate 13-acetate (PMA) along with the calcium (Ca^{2+}) ionophore, ionomycin (Io; Iwata et al. 1996; Mukherjee et al. 2006).

PMA specifically activates protein kinase C (PKC) upon entering the cell, while Io acts as a Ca^{2+} channel regulator by raising the intracellular level of Ca^{2+} (Iwata et al. 1996; Mukherjee et al. 2006). Activation of T cells by signal one and signal two via surface-mediated receptors or by direct stimulation with PMA and Io results in numerous downstream signals that activate pathways enabling T cells to proliferate and produce cytokines, such as IL-2 (Palmer et al. 1996; Slavik et al. 1999). This increase in protein production and protein folding activity enlarges the physiological demands on cellular protein folding machinery.

In the present studies, we investigated the contribution of ER stress response signaling to modulation of T cell function during various physiologically relevant immune states. In the body, T cells are exposed to a panoply of external signals that, in turn, transmit signaling cascades that are integrated into the appropriate cellular response. Here, we have identified PKC as an intracellular signal involved in the initiation of the ER stress response in T cells. Moreover, we demonstrate that signaling through the TCR alone is sufficient to generate the ER stress response, consistent with the proposal that 'nonresponsive' immune states, such as anergy or tolerance, require expression of select proteins to maintain the tolerant state (Harris et al. 2004; Safford et al. 2005). Thus, physiological ER stress appears to be an intrinsic cellular state that may be initiated by distinct activators involved in the precise regulation of cellular differentiation and function.

Materials and methods

Animals Six- to 10-week-old male BALB/c (*H2^d*; Charles River Laboratories, Wilmington, MA, USA) mice and C57BL/6 (*H2^b*) and CBA (*H2^k*) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used. Animals were housed in a viral-antibody-free facility and maintained in accordance with the guidelines of the University of Massachusetts Medical School Institutional Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences 1996).

T cell preparation Spleen and mesenteric lymph nodes were removed from BALB/c mice and processed aseptically. T cells were purified (93–97% TCRβ$^+$) as previously described (Rigby et al. 1996).

In vitro T cell stimulation Six-well culture plates (BD Falcon, Bedford, MA, USA) were incubated overnight with 10 μg of anti-CD3 monoclonal antibody (mAb; clone 17A2; BD Pharmingen, San Diego, CA, USA) per well in

phosphate-buffered saline (PBS) at 4°C. Purified T cells were cultured in plates rinsed with PBS at 6×10^6 cells per well in 3 ml of RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Hyclone, Logan, UT, USA), 1% Pen/Strep/Glut (Gibco, Carlsbad, CA, USA), and 0.1% β-mercaptoethanol (Gibco) at 37°C. Where indicated, soluble anti-CD28 mAb (1 μg/ml, clone 37.51; BD Pharmingen) was added. Control T cells were incubated with immobilized isotype-matched IgG mAb. For PMA (Calbiochem, San Diego, CA, USA) and Io (Calbiochem) stimulation, T cells were stimulated with 100 ng/ml PMA and 1 or 2 μg/ml Io for 20 h. For thapsigargin (Calbiochem) treatment, T cells were plated at 6×10^6 cells per well in 6 ml culture media with 2 μM thapsigargin. Calphostin C (Calbiochem) was added at 500 nM for the duration of activation for specified treatment groups. Control T cells were treated with the vehicle dimethyl sulfoxide (DMSO; Sigma-Aldrich).

IL-2 ELISA BALB/c T cells were cultured as described (above). Supernatants were harvested after 17 or 20 h and assayed for IL-2 production using a mouse IL-2 ELISA set (BD Pharmingen) according to manufacturer's instructions.

Western blotting T cells were lysed (Rigby et al. 1996) and protein concentrations determined by bicinchoninic acid (BCA) protein assay (Sigma-Aldrich). Protein (30 μg) was mixed with 4× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and Western blot analyses were performed as described (Rigby et al. 1996). Actin was used as a loading control. Band densities were measured by densitometry (ImageJ software, NIH, Bethesda, MD, USA). Density values are expressed as a ratio normalized to actin, and the 'fold change' is compared to control samples.

Flow cytometry Single-cell suspensions from spleen and mesenteric lymph node cells were washed and suspended in PBS containing 1% fetal clone serum (HyClone) and 0.1% sodium azide (Sigma-Aldrich). Samples were incubated in anti-CD16/32 for 10 min at 4°C, then incubated with specific antibodies. KB5 synchimera splenic cells were incubated with clonotypic DES mAb for 20 min. Samples were washed, incubated for 20 min with secondary mAb for DES, and labeled with fluorescent mAbs to cell-surface markers. BALB/c splenic and lymph node single cell suspensions were labeled with fluorescent mAbs to various cell-surface markers as described in the text. To detect GRP78, cells were permeabilized using Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer's directions. Cells were washed and incubated with Alexa Fluor 647-conjugated GRP78 mAb for 20 min. Labeled cells were washed, fixed with 1% paraformaldehyde (Polysciences, Warrington, PA, USA) in D-PBS and analyzed with a

FACSCalibur (BD Biosciences, San Jose, CA, USA) and FlowJo Software (PC version 5.5; Tree Star, Ashland, OR, USA). Lymphoid cells were gated according to their light-scattering properties.

Antibodies IgG2a developing reagent for Désiré (DES, clone R19–15), CD44 (clone IM7), CD8α (clone 53–6.7), CD69 (clone H1.2f3), 7-AAD, TCRβ (clone H57–597), GRP78 (clone 40), PDI (clone 34), and isotype control unconjugated or fluorochrome-conjugated anti-mouse mAbs were from BD Pharmingen. A Zenon Mouse IgG2a Labeling Kit, Alexa Fluor 647 (Invitrogen, Carlsbad, CA, USA), was used to label GRP78 mAb per the manufacturer's directions. A mouse hybridoma cell line secreting the KB5-specific clonotypic DES mAb (Hua et al. 1986) was a gift from Dr. J. Iacomini (Harvard Medical School, Boston, MA, USA). Anti-actin (clone C4), anti-ERp72, and anti-GRP94 (clone 9G10) antibodies were obtained from Chemicon International (Temecula, CA, USA), Calbiochem, and Stressgen (San Diego, CA, USA), respectively. Anti-rabbit, anti-rat, and anti-mouse IgG HRP conjugates were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MR1 hamster anti-mouse CD154 mAb was produced as tissue culture supernatant and purified by affinity chromatography (National Cell Culture Center, Minneapolis, MN, USA). Antibody concentration was determined by measurement of optical density and confirmed by ELISA (Iwakoshi et al. 2000). The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC, USA) and was uniformly <10 U/mg of mAb (Foy et al. 1993).

Synchimeras and tolerance induction To determine the *in vivo* ER stress response in antigen-activated alloreactive CD8^{+} T cells in a normal microenvironment, we used KB5 TCR transgenic hematopoietic "synchimeric" mice generated as described (Iwakoshi et al. 2001). The transgenic T cells that develop express an anti-H2-K[b]-specific TCR recognized by the mAb DES. These procedures have been documented to generate a stable population of DES^{+}CD8^{+} cells comprising 5–8% of PBMC within 8 weeks of bone marrow transplantation (Iwakoshi et al. 2001; Seung et al. 2003). Male KB5 synchimeric mice were treated with a single transfusion of C57BL/6 (H2-K[b]) mouse splenocytes, known as a donor splenocyte transfusion (DST), for full activation of transgenic DES^{+}CD8^{+} T cells or DST plus anti-CD154 mAb for induction of tolerance as described (Markees et al. 1998b; Iwakoshi et al. 2001).

Statistics Statistical analyses were performed with GraphPad Prism software (Graphpad Software, San Diego, CA, USA). Differences were compared by two-tailed unpaired *t* tests. Values of $p < 0.05$ were considered statistically significant.

Results

ER stress response is induced in T cells by thapsigargin We first confirmed the ability of T cells to generate an effective ER stress response in the presence of a known ER stress inducer, thapsigargin (TG). To do so, BALB/c mouse lymph node and splenic T cells (>93% purity) were treated with TG, and the expression of various ER chaperone proteins was quantified. Thapsigargin selectively inhibits the ER Ca^{2+}-ATPase, disrupting ER Ca^{2+} stores and leads to ER stress (McCormick et al. 1997). In T cells treated with TG, expression levels of the ER chaperones GRP78, GRP94, PDI, and ERp72 increased within 2 h (1.3- to 1.7-fold above the expression of vehicle (DMSO) treated control cells) and remained elevated (1.7- to 3.3-fold) through 24 h of treatment (Fig. 1). These data confirm that T cells can generate a robust ER stress response when treated with a known ER stress inducing chemical, thapsigargin.

ER stress response occurs after in vitro T cell activation Having established that freshly isolated T cells exhibit a functional ER stress response when treated with a chemical stressor, we next confirmed that this response would be induced by activation through the TCR with costimulation. To do so, we incubated purified T cells with both a mAb to CD3 (a TCR subunit that, when ligated, provides signal one) and the costimulatory anti-CD28 mAb for 17 h. As a negative control, a portion of the purified T cells were incubated with an immobilized isotype-matched IgG mAb. Lymphocytes were gated according to their forward scattering (FSC) and side scattering (SSC) light properties, a means of distinguishing viable cells from their nonviable coun-
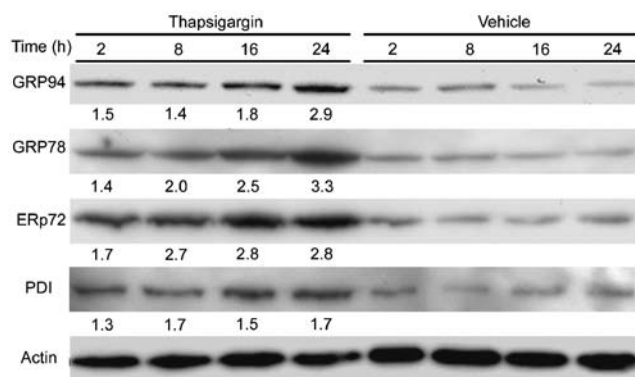
terparts. The percentage of viable lymphocytes following co-treatment with anti-CD3 plus anti-CD28 mAbs was 71.7%±0.6% (Fig. 2A). This treatment increased the percentage of T cells expressing the early activation marker CD69 from 3.8%±1.2% in cells treated with an immobilized isotype-matched IgG control mAb to 45.2%± 4.2% in co-treated cells (Fig. 2B, shaded region and black line, respectively).

To determine whether T cell activation via signal one plus signal two also led to the induction of a physiological ER stress response, intracellular expression of the ER chaperone GRP78, an indicator of ER stress (Lee 2005), was quantified by flow cytometry. Because only ~50% of T cells are stimulated by the plate-bound mAbs in these *in vitro* incubations, GRP78 expression levels were separately measured in CD69^{high} and CD69^{low} subpopulations of T cells. After 17 h of co-treatment with anti-CD3 and anti-CD28 mAbs, intracellular GRP78 expression was significantly higher in T cells expressing high levels of CD69 (CD69^{high}) than in CD69^{low} cells (Fig. 2C). The relative amount of intracellular GRP78 protein in the CD69^{high} and CD69^{low} subpopulations is quantified by mean fluorescence intensity (MFI; Fig. 2D). As expected, T cells co-treated with anti-CD3 plus anti-CD28 mAbs secreted a robust amount of the IL-2 cytokine (Fig. 2E). These data indicate that full T cell activation resulting in cytokine secretion induces ER stress response signaling.

ER stress response occurs after partial T cell activation in vitro via signal one alone We next investigated the ER stress response in T cells exposed only to signal one (TCR ligation alone). Because such cells are generally thought to become 'nonresponsive', we hypothesized that this process would *not* invoke a classical ER stress response or, alternatively, would induce one qualitatively and/or quantitatively different from that associated with fully activated (signals one and two) T cells.

We first determined the expression of CD69 on T cells that received only signal one through treatment with anti-CD3 mAb alone. This treatment is known to lead to the expression of the CD69 activation marker (Shah et al. 2006), and we found a similar increase in CD69 expression on T cells incubated with anti-CD3 mAb alone (48.2%± 2.5%) compared to co-treated T cells (Fig. 2B). To ensure that these T cells had a functional phenotype of cells only receiving signal one, we measured their production of IL-2. As expected, T cells incubated with anti-CD3 mAb alone produced significantly less IL-2 compared to T cells exposed to both signal one and signal two (Fig. 2E).

We then tested directly our hypothesis that the ER stress response in these T cells would be different from that observed in the fully activated, cytokine-secreting T cells. Because apoptosis is a possible outcome of anti-CD3 mAb
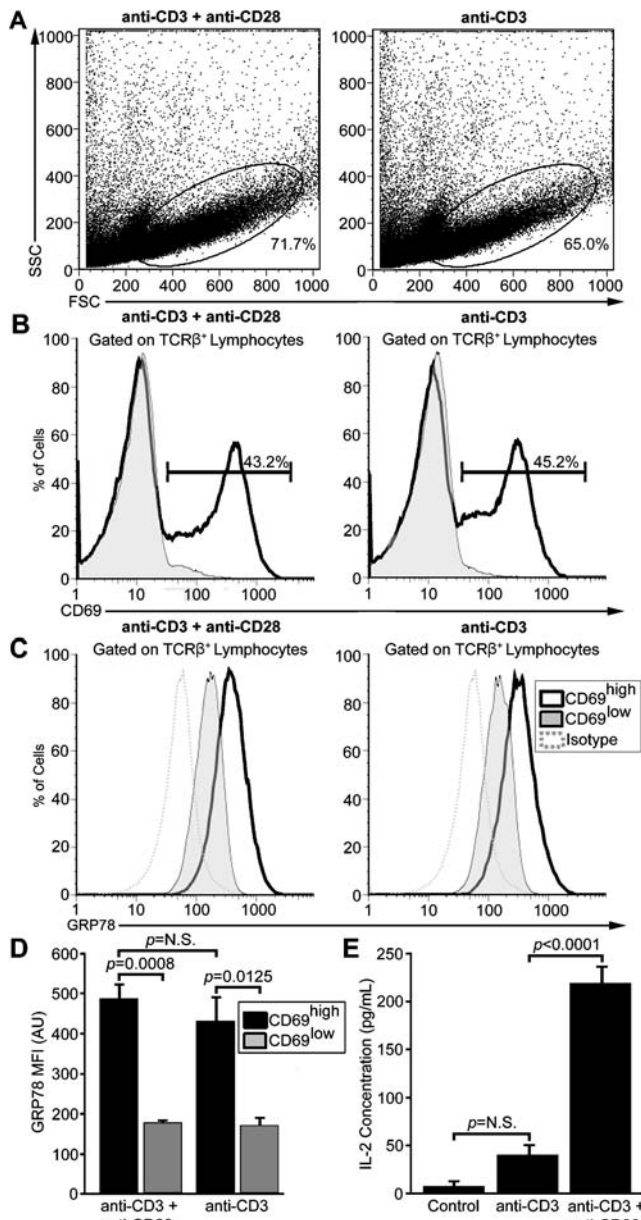

Fig. 1 Thapsigargin treatment of T cells induces ER stress. Purified T cells were treated with 2 μM thapsigargin (*TG*) or DMSO (*Vehicle*) for 2, 8, 16, and 24 h. TG- and DMSO-treated T cell protein lysates were analyzed by Western blot for ER chaperone expression. Band densities were measured by densitometry and values shown are 'fold changes' relative to control (=1.0) after actin normalization at the same time point. Data represent one of two independent time course experiments

Fig. 2 *In vitro* T cell activation induces an ER stress response. Purified T cells were co-treated with anti-CD3 plus anti-CD28 mAbs (*left*, *n*=3) or treated with anti-CD3 mAb alone (*right*, *n*=3) for 17 h as described in the Materials and methods section. **a** Representative flow dot plots depicting the forward scattering (*horizontal axis*) and side scattering (*vertical axis*) light properties of lymphocytes. Viable lymphocyte populations are *encircled*. **b** Flow cytometric analyses of the activation marker CD69 in treated T cells (*black line*). *Shaded regions* represent CD69 expression in T cells incubated with immobilized isotype matched IgG mAb (Control). **c** Intracellular GRP78 expression in CD69^{high} (*black line*) and CD69^{low} (*shaded reigion*) T cells. The GRP78 expression for isotype control staining (*dotted line*) is shown in each histogram. **d** *Bar graphs* displaying the mean of intracellular GRP78 protein expression (arbitrary units; AU) for CD69^{high} and CD69^{low} T cells. *Error bars* represent the SEM of triplicate samples. **e** IL-2 production was determined by ELISA on T cell supernatants obtained from three independent experiments for each treatment group. Samples were examined in triplicate and *error bars* represent SEM of experimental replicates. Data shown are from one of three independent experiments

alone treatment, 7-AAD was used to exclude the contribution of any ER stress response from nonviable T cells (data not shown). At 17 h, the intracellular GRP78 expression in T cells incubated with anti-CD3 mAb alone was significantly higher in CD69^{high} than in CD69^{low} T cells (Fig. 2C, D). Unexpectedly, however, intracellular GRP78 expression was not significantly different between CD69^{high} T cells in anti-CD3 plus anti-CD28 mAbs and anti-CD3 mAb alone treatment groups (Fig. 2D). Therefore, GRP78 expression correlates with signaling through the TCR but, at least initially, does not distinguish between incomplete activation (anti-CD3 mAb alone) and full T cell activation (anti-CD3 plus anti-CD28 mAbs) in which high levels of IL-2 are produced.

Upregulation of multiple ER chaperones after in vitro TCR signaling in the absence of signal two To confirm the initiation of an ER stress response in activated T cells, protein lysates of purified T cells incubated for 17 h with anti-CD3 mAb alone or anti-CD3 plus anti-CD28 mAbs were analyzed by Western blot. Analogous to the results obtained using flow cytometry, Western blot analyses revealed that GRP78 protein expression was increased in T cells incubated with anti-CD3 mAb alone (2.1-fold) or with anti-CD3 plus anti-CD28 mAbs (2.1-fold) when compared to that observed in T cells incubated with the immobilized isotype-matched IgG mAb control (Fig. 3). The expression levels of the ER chaperone proteins GRP94, ERp72, and PDI were also increased to a similar amount after stimulation with either anti-CD3 mAb alone or in the
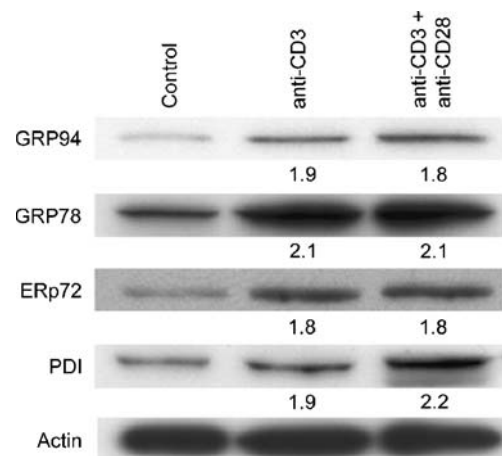

Fig. 3 ER chaperone expression increases during *in vitro* T cell activation. Purified T cells were treated with immobilized isotype matched IgG mAb (*Control*), anti-CD3 mAb alone, or with anti-CD3 plus anti-CD28 mAbs for 17 h as described in the Materials and methods section. Western blot analyses of ER chaperone proteins in lysates from T cells treated as indicated. Numbers represent densitometry of protein band densities after normalization to actin and comparison to control (=1.0). Data shown are from one of three independent experiments

presence of anti-CD3 plus anti-CD28 mAbs (Fig. 3). These data further reveal that an ER stress response leading to increased production of ER stress-associated chaperone proteins occurs *in vitro* in T cells activated with anti-CD3 mAb, irrespective of anti-CD28 mAb costimulation.

An ER stress response is induced by in vivo activation of T cells We next sought to determine if a functional ER stress response also occurs with T cell activation *in vivo*. To do so, we required a system in which we could readily identify a population of activated cells with well-defined kinetics. We elected to use KB5 synchimeric mice to investigate T cell activation *in vivo* (Iwakoshi et al. 2001; Thornley et al. 2006). These KB5 synchimeric mice were modified (as described in the Materials and methods section) to circulate a trace population (~5–8%) of TCR transgenic CD8^{+} T cells that specifically recognize the H2-K[b] antigen. To induce T cell activation *in vivo*, the KB5 synchimeric mice were given a donor splenocyte transfusion (DST) of H2-K[b]-expressing splenocytes from allogeneic C57BL/6 mice. This *in vivo* DST provides both signals one and two and uniformly activates only the transgenic population of CD8^{+} T cells. The transgenic CD8^{+} T cells can be distinguished from endogenous T cells by use of the DES mAb.

Spleen cells were recovered from KB5 synchimeric mice 2 days after injection of C57BL/6 splenocytes. Representative histograms illustrate that transgenic DES^{+}CD8^{+} T cells can be readily distinguished from endogenous (non-transgenic) DES^{−}CD8^{+} lymphocytes from the KB5 synchimeric mice (Fig. 4A). Transgenic DES^{+}CD8^{+} T cells from DST-treated mice exhibited an increased expression of the activation marker CD44 (91.2±2.4%) compared to the DES^{−}CD8^{+} lymphocyte population (29.3±6.7%; Fig. 4B, black line and shaded region, respectively). As expected, a few DES^{+}CD8^{+} T cells in untreated KB5 synchimeric mice expressed high levels of CD44 (14.4±5.7%; Fig. 4B).

To investigate the *in vivo* ER stress response in activated CD8^{+} T cells, expression of intracellular GRP78 protein was determined by flow cytometry. We observed that intracellular GRP78 protein expression was not significantly different between DES^{+}CD8^{+} T cells and non-transgenic DES^{−}CD8^{+} lymphocytes from untreated KB5 synchimeric mice (Fig. 4C,D). However, intracellular GRP78 protein expression was significantly greater in DES^{+}CD8^{+} T cells than in non-transgenic DES^{−}CD8^{+} lymphocytes from DST-treated mice (Fig. 4C,D). In addition, intracellular GRP78 protein expression of DES^{+}CD8^{+} T cells from KB5 synchimeric mice injected with the allogeneic cells was significantly greater than in DES^{+}CD8^{+} T cells from untreated mice (Fig. 4D). These data provide evidence of a physiological ER stress response after *in vivo* activation of antigen-specific CD8^{+} T cells.

An ER stress response characterizes in vivo tolerized T cells that receive signal one alone The KB5 synchimera mouse system has been used to study transplantation tolerance induction. In such studies, in addition to an injection of DST, the recipient mice also receives an anti-CD154 mAb to block CD40-CD154 interaction, thus preventing signal two by inhibiting expression of costimulatory molecules on the surface of the APC (Iwakoshi et al. 2001; Thornley et al. 2006). When anti-CD154 mAb is given, the majority of the alloresponsive KB5 transgenic T cells (approximately 66%) disappear (Iwakoshi et al. 2001; Thornley et al. 2006). Little is known, however, about the molecular phenotype of the T cells that are not deleted. We hypothesized that residual KB5 transgenic T cells in this tolerance induction system would display an 'activated' ER stress phenotype analogous to that which we had observed in T cells exposed *in vitro* to signal one alone.

Transgenic DES^{+}CD8^{+} T cells from these co-treated mice were stimulated as documented by an increased expression of the activation marker CD44 (80.6±1.6%; Fig. 4B, black line), while intracellular GRP78 protein expression in the DES^{+}CD8^{+} T cells was significantly increased compared to non-transgenic DES^{−}CD8^{+} lymphocytes (Fig. 4C,D). Furthermore, intracellular GRP78 protein expression in activated DES^{+}CD8^{+} T cells from KB5 synchimeric mice co-treated with DST plus anti-CD154 mAb was significantly greater than in DES^{+}CD8^{+} T cells from untreated KB5 synchimeric mice (Fig. 4D). Interestingly, the increase of GRP78 chaperone protein expression by flow cytometry in *in vivo* tolerized alloreactive CD8^{+} T cells (2.2-fold) was similar to that observed following *in vitro* activation using anti-CD3 mAb alone (2.5-fold), another protocol that activates T cells that fail to become fully functional (Fig. 2C). These data demonstrate the presence of a physiological ER stress response after *in vivo* tolerance induction ('nonresponsiveness') in CD8^{+} T cells. These observations suggest that allogeneic cells that are not actually deleted by the tolerance induction protocol have a molecular phenotype consistent with partial activation.

Ionomycin stimulation of T cells does not lead to ER stress response signaling T cell activation via TCR (or CD3) ligation is known to signal through pathways that regulate Ca^{2+} levels within the T cell (Long et al 2001). Because fluxes in Ca^{2+} levels are known to cause ER stress (McCormick et al. 1997), and we earlier showed TG treatment caused increases in ER chaperone expression (Fig. 1), we investigated whether stimulating T cells with Io, a Ca^{2+} channel regulator which raises intracellular Ca^{2+} levels and mimics certain components of TCR signaling, leads to activation of the ER stress response. To accomplish this, we stimulated purified T cells with DMSO (vehicle) or Io alone for 20 h.

Fig. 4 Increased GRP78 expression with *in vivo* T cell activation and *in vivo* tolerance induction. KB5 synchimeric mice were untreated (*left*, *n*=5), DST treated (*middle*, *n*=4) to induce *in vivo* activation, or DST plus anti-CD154 mAb co-treated (*right*, *n*=3) to achieve tolerance induction of transgenic DES$^+$CD8$^+$ T cells as described in the Materials and methods section. **a** Representative *flow dot plots* depicting CD8 expression (*horizontal axis*) and the anti-H2-K^b specific TCR recognized by the mAb DES (*vertical axis*) on lymphocytes obtained from the indicated treatment groups. DES$^+$CD8$^+$ T cells and DES$^-$CD8$^+$ lymphocyte populations are *encircled*. **b** Flow cytometric analyses of the activation marker CD44 on gated DES$^+$CD8$^+$ T cells (*black line*) and DES$^-$CD8$^+$ lymphocytes (*shaded region*). **c** Intracellular GRP78 expression in DES$^+$CD8$^+$ T cells (*black line*) and DES$^-$CD8$^+$ (*shaded region*) lymphocyte populations. The isotype control for GRP78 staining is indicated by a *dotted line*. **d** The mean of GRP78 protein expression displayed as *bar graphs* with *error bars* representing the SEM. Data are representative of four independent experiments
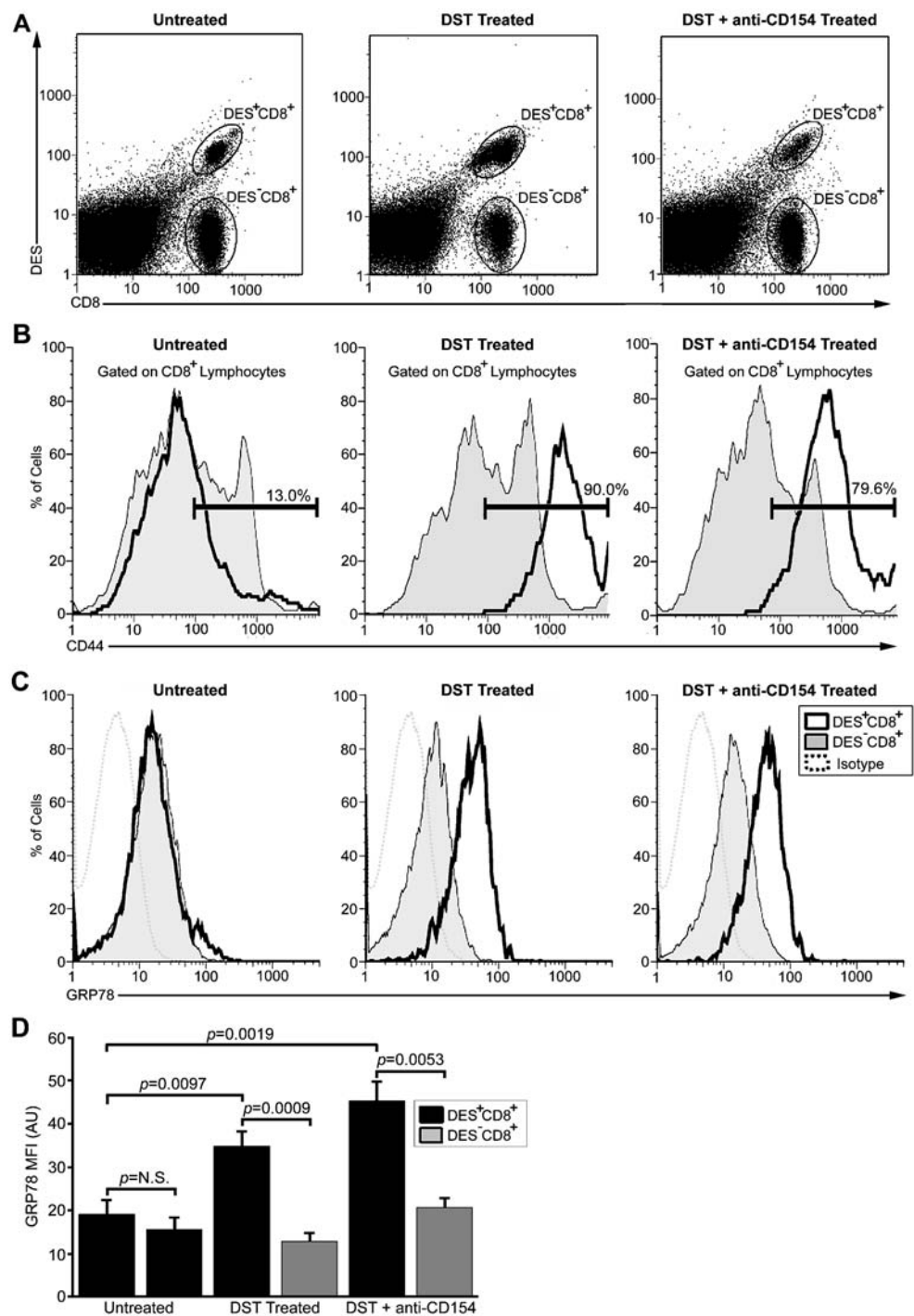
The percentage of viable lymphocytes after control (vehicle treated) or Io (1 μg/ml) treatment was 73.0%± 0.4% and 67.6%±3.7%, respectively (Fig. 5A). Only 5.2%± 4.3% of T cells had increased expression of CD69 after Io treatment, a number statistically similar to the percentage of cells (4.3%±0.3%) in the vehicle treatment (*p*=0.6135; Fig. 5B). This suggests that the small percentage of CD69^{high} T cells found in the vehicle and Io alone treatment groups may have been activated *in vivo* previous to their isolation. As expected, the small percentage of CD69^{high} T cells within the vehicle and Io alone treated populations had increased intracellular GRP78 protein expression compared to the CD69^{low} T cells (Fig. 5C,D). Interestingly, 47.5%± 0.2% of T cells incubated with a high dose of Io (2 μg/ml) showed increased expression of CD69 indicating this treatment is above the threshold necessary to initiate

Fig. 5 Stimulation of T cells with ionomycin does not activate ER stress response signaling. Purified T cells were treated with Vehicle (*left*, *n*=4), Io (1 μg/ml, *middle*, *n*=2), and Io (2 μg/ml, *right*, *n*=2) for 20 h as described in the Materials and methods section. **a** Representative *flow dot plots* depicting the forward scattering (*horizontal axis*) and side scattering (*vertical axis*) light properties of lymphocytes. Viable lymphocytes are *encircled*. **b** Surface expression of CD69 activation marker in treated T cells (*black line*) or CD69 isotype matched IgG control expression (*shaded region*). **c** The expression of intracellular GRP78 protein in CD69^{high} (*black line*) and CD69^{low} (*shaded region*) T cells. The isotype IgG mAb control for GRP78 is depicted as a dotted line in each histogram. **d** The mean of intracellular GRP78 protein expression in CD69^{high} and CD69^{low} T cells with *error bars* representing the SEM of duplicate samples. **e** Secretion of IL-2 was measured by ELISA on T cell supernatants harvested from two independent experiments. Samples were examined in triplicate and *error bars* represent SEM of experimental replicates. Data shown are representative of two experiments
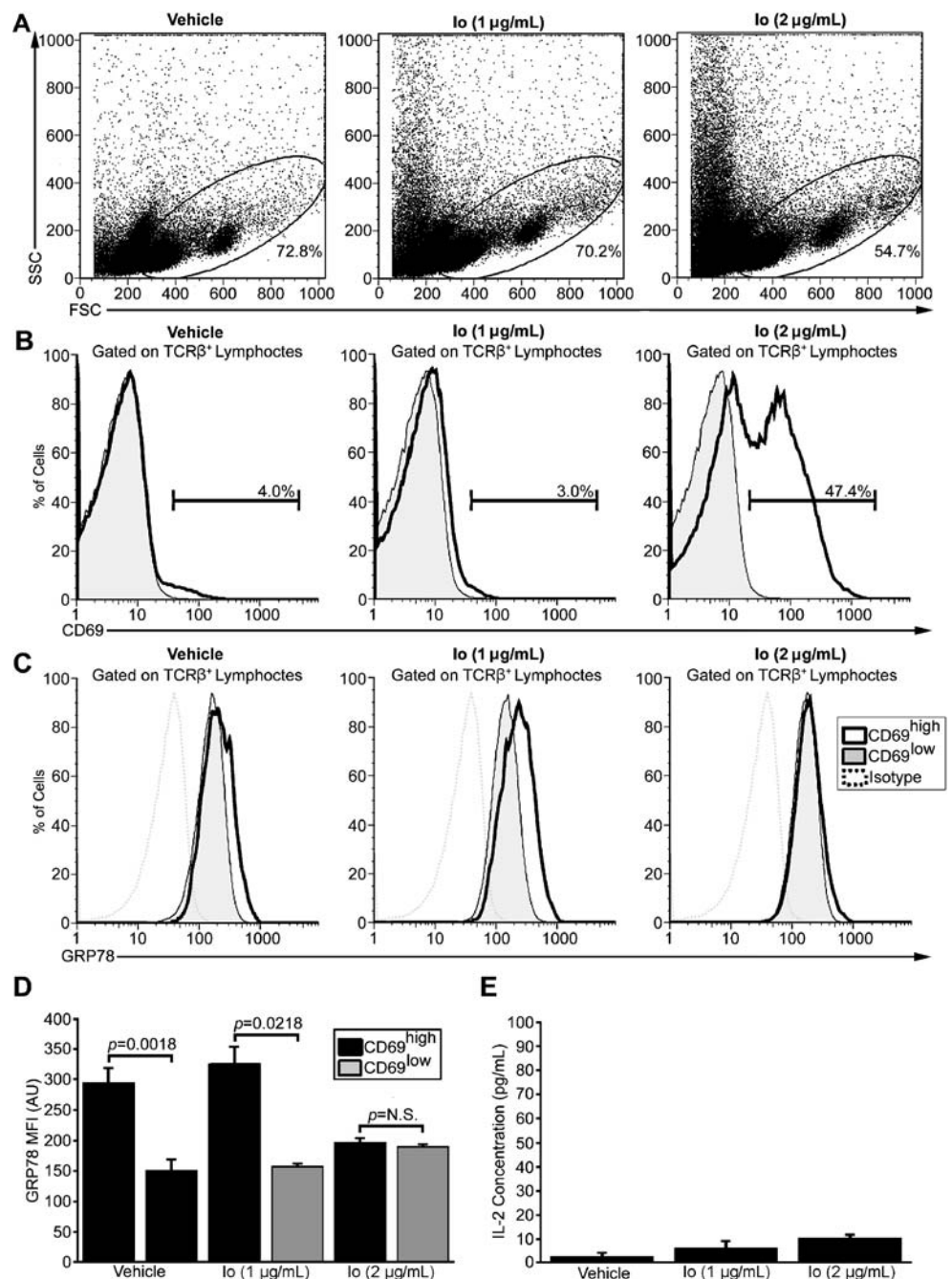
activation (Fig. 5B). However, the intracellular GRP78 protein expression within these CD69^{high} T cells was not statistically different from that of CD69^{low} T cells (Fig. 5C, D), demonstrating that upregulation of this activation marker by Io treatment can occur in the absence of ER stress response signaling. In addition, as anticipated, vehicle or Io-treated T cells did not produce high levels of IL-2 (Fig. 5E). These data indicate that Ca^{2+} signaling downstream of TCR or CD3 ligation is not responsible for ER stress response signaling during T cell activation.

PKC signaling in T cells initiates the ER stress response In addition to Ca^{2+} fluxes occurring downstream of TCR or CD3 ligation, signaling pathways that specifically activate PKC are initiated (Long et al. 2001). We next investigated whether PKC activation could initiate ER stress response signaling. For these studies, we stimulated T cells for 20 h with PMA, a specific activator of PKC, in the presence or absence of the PKC inhibitor, calphostin C.

In T cells treated with PMA alone (100 ng/ml), the majority of cells (95.7%±0.4%) showed increased expres-

sion of the activation marker CD69 (Fig. 6B). In addition, the CD69^{high} T cells had an increased expression of intracellular GRP78 protein that was statistically different from their CD69^{low} T cell counterparts (Fig. 6C,D). These data indicate that PKC activation alone is sufficient to induce the ER stress response. To validate the specificity of this response, T cells were next stimulated with PMA in the presence of the PKC-specific inhibitor, calphostin C (500 nM). The percentage of cells that were CD69^{high} remained elevated (91.6%±0.07%; Fig. 6B). In contrast, however, expression of intracellular GRP78 protein in these CD69^{high} T cells was not elevated and did not differ significantly from that of the CD69^{low} T cells (Fig. 6C,D). In addition, the intracellular GRP78 protein expression in CD69^{high} T cells from the PMA treatment group was significantly higher than in the presence of calphostin C (Fig. 6D), indicating that inhibition of PKC blocks the ER stress response. As expected, PMA treatment in the presence or absence of calphostin C did not result in high levels of IL-2 secretion from the T cells (Fig. 6E). These data demonstrate that activating PKC in T cells results in ER stress response signaling, even in the absence of IL-2 cytokine production.

PKC inhibition during T cell activation with PMA and Io diminishes ER stress response signaling and IL-2 production Treatment of T cells with PMA combined with Io has been shown to cause cellular proliferation and enhanced IL-2 secretion (Truneh et al. 1985). Within the lymphocyte gate (77.5%±2.5%; Fig. 6A) for PMA and Io treated cells, 95.8%±0.4% of the T cells increased expression of CD69 (Fig. 6B), indicating the majority of the T cells were activated. These T cells secreted very high levels of IL-2 (Fig. 6E) and the CD69^{high} T cells displayed an intracellular GRP78 protein level that was significantly higher than the few CD69^{low} T cells within the population (Fig. 6C,D).

Similar to T cells treated with PMA in the presence of calphostin C, treatment of T cells with PMA and Io in the presence of the PKC inhibitor had little effect on the expression of the activation marker CD69 (92.3%±0.8%; Fig. 6B), thus indicating these cells were receiving the initial T cell activation stimuli. However, blocking PKC activation with the inhibitor caused the cells to secrete significantly less IL-2 into the supernatant compared to PMA- and Io-treated cells in the absence of the inhibitor (Fig. 6E). This was not a secretory defect caused by the inhibitor because an intracellular protein enzyme-linked immunosorbent assay (ELISA) also revealed a dramatic decrease in intracellular IL-2 within T cells that were treated with PMA and Io in the presence of the inhibitor (data not shown). Furthermore, intracellular GRP78 expression within the CD69^{high} T cells did not differ significantly from that in the CD69^{low} T cells (Fig. 6C,D). These data

indicate PKC activation in the initiation of ER stress response signaling in T cells and suggest that signaling downstream of the TCR (signal one) leading to PKC activation is sufficient to cause increased ER chaperone expression, independent of IL-2 production and secretion.

The expression of multiple ER chaperones increases after PKC activation To further demonstrate ER stress response signaling in T cells after PKC activation, we examined the expression of multiple ER chaperones by Western blot analyses. The expression of multiple ER chaperones was not substantially increased during Io treatment in comparison to the expression in vehicle-treated T cells (Fig. 7). However, quantification of ER chaperone expression revealed that PKC activation with PMA or PMA combined with Io increases GRP94, GRP78, ERp72, and PDI [1.5- to 2.3-fold above the expression of vehicle (DMSO) treated T cells]. Interestingly, simultaneous treatment of T cells with PMA or PMA combined with Io and the PKC inhibitor, calphostin C, effectively decreases the expression of multiple ER chaperones to levels comparable with vehicle-treated T cells (Fig. 7). These data further reveal that PKC activation leads to increased production of multiple ER stress chaperone proteins.

Discussion

Activation of T cells results in the upregulated expression of numerous proteins essential for differentiation and for effector functions (Rothstein and Sayegh 2003). Many of these proteins, such as cytokines, chemokines, and cell surface molecules, participate in the activation process, and thus distinguish activated T cells from their naïve precursors (Schwartz 1990). Considering the exigency on protein folding and production, it is conceivable that fully activated T cells require more efficient protein handling in the ER. Therefore, initiation of the ER stress response and upregulation of ER chaperones as a physiological consequence of TCR signaling is a logical step to augment the protein-folding ability of the ER. Consistent with this, our data demonstrate upregulation of ER chaperones during *in vitro* and *in vivo* activation of T cells through signal one and signal two. Since 'partial' activation of T cells (signal one only) resulted in reduced production of the important cytokine, IL-2, we hypothesized that the ER stress response in this case would be qualitatively or quantitatively different. Unexpectedly, however, we found that ER chaperones were also increased in cells that we purposefully treated to omit (in our *in vitro* studies) or block (in our *in vivo* tolerance model) signal two. Lastly, we demonstrate that activation of PKC, a serine/threonine protein kinase
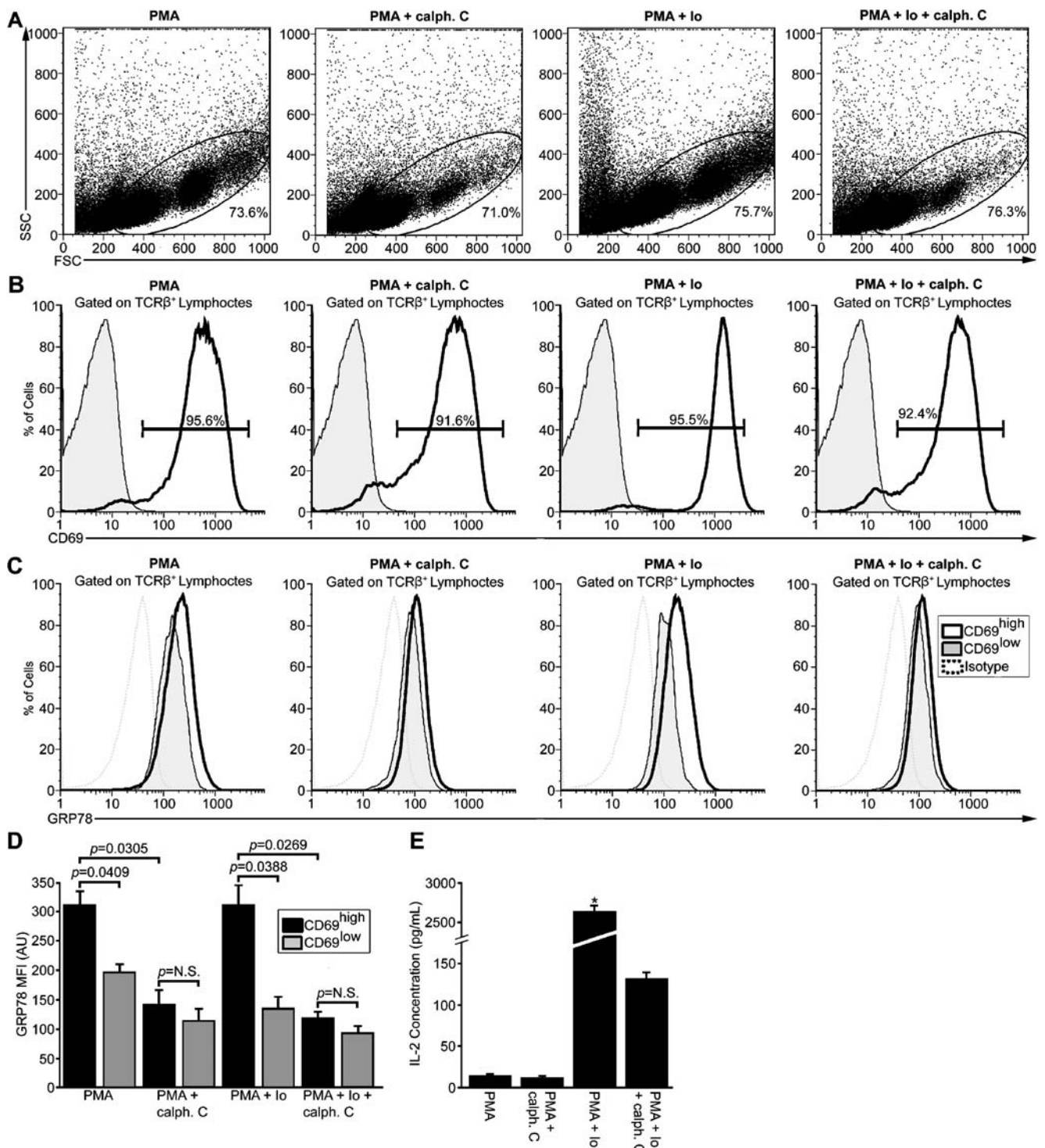
Fig. 6 Activating PKC with PMA treatment initiates ER stress signaling in T cells. Purified T cells were treated with PMA (*n*=2), PMA + calphostin C (*n*=2), PMA + Io (*n*=2), or PMA + Io + calphostin C (*n*=2) for 20 h as described in the Materials and methods section. **a** Representative *flow dot plots* depicting the forward scattering (*horizontal axis*) and side scattering (*vertical axis*) light properties of lymphocytes. Viable lymphocyte populations are *encircled*. **b** Representative histograms indicating expression of the activation marker CD69 (*black line*) and isotype IgG control for CD69 expression (*shaded region*). **c** Intracellular GRP78 expression for CD69high (*black line*) and CD69low (*shaded region*) T cells. Depicted in each histogram is the isotype IgG mAb control for GRP78 (*dotted line*). **d** Bar graphs displaying the mean of GRP78 protein expression in CD69high and CD69low T cell populations shown with *error bars* representing the SEM of duplicate samples. **e** IL-2 secretion by T cells measured by ELISA on supernatants from two independent experiments for each treatment group. *, *p*<0.0001 for PMA + Io vs. all other treatment groups. Shown are mean and SEM of triplicate samples. Data shown are representative of two experiments
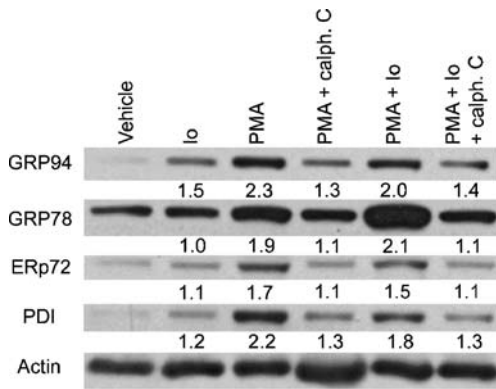
Fig. 7 PKC inhibition during T cell activation prevents ER stress response signaling. Western blot analyses of ER chaperone proteins in lysates obtained from T cells stimulated with DMSO (vehicle), Ionomycin (Io), PMA, PMA with calphostin C (PMA + calph. C), PMA + Ionomycin (PMA + Io), and PMA + Ionomycin with calphostin C (PMA + Io + calph. C). Numbers represent densitometry of protein band densities after normalization to actin and comparison to control (=1.0). Data shown are from one of two independent experiments

found downstream of TCR signaling, is involved in the initiation of ER stress response signaling during the T cell activation process (Fig. 8).

The importance of PKC during T cell activation has been comprehensively investigated, and PKC has been found to play key roles in activating numerous downstream signaling pathways of the TCR (Berg-Brown et al. 2004; Redig and Platanias 2007). After TCR or CD3 ligation, downstream signaling events, leading to the production of diacylglycerols (DAG) and inositol triphosphates, occurs. It has been shown that these molecules play an active

regulatory role in PKC activation (Long et al. 2001). By using calphostin C, a highly specific inhibitor of PKC that functions by competing for the DAG binding site on PKC's regulatory domain (Cohen et al. 2006), we now show a previously uncharacterized link between PKC activation and ER stress response signaling in T cells. Although full activation of T cells is required for production of IL-2, our data establish that the initial stimuli through the TCR leading to PKC activation, or direct activation of PKC with PMA treatment, is sufficient to initiate activation of the ER stress response. As TCR signaling is well characterized, further insights into T cell functioning may be discovered by dissection of the ER stress response in these cells.

In a recent study, APC-stimulated CD4$^+$ T cells utilized elements of the ER stress response, including increased expression of ER stress response genes, during their differentiation into effector cells (Scheu et al. 2006). The authors speculated that ER stress response signaling, as part of a general integrated stress response, may be induced by imbalances in nutrients after CD4$^+$ T cell clonal expansion and differentiation or, alternatively, that TCR stimulation activates ER stress response signaling in preparation for effector function. Here, we extend those findings and demonstrate that stimulation of T cells through the TCR alone is sufficient to induce the expression of ER stress response proteins, even in the absence of differentiation. Although consistent with a potential preparatory function of the ER stress response before T cell differentiation and proliferation, ER stress response signaling may alternatively be activated due to the misfolding of proteins upregulated by TCR stimulation or secondary factors that disrupt
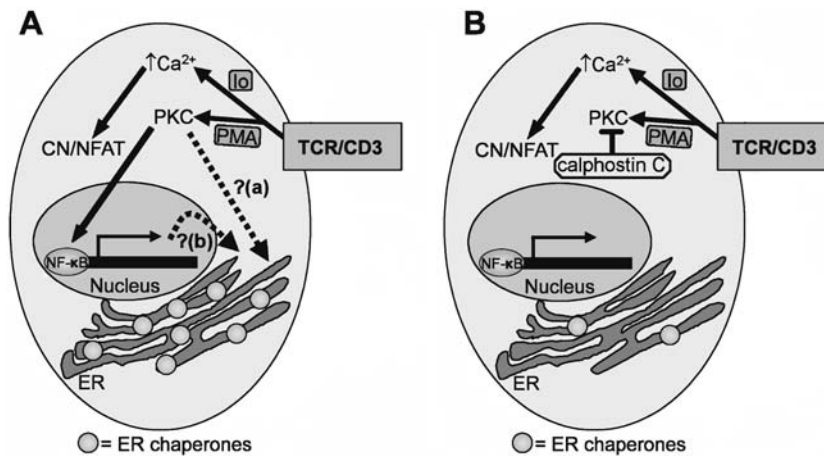

Fig. 8 A model for enhanced ER chaperone expression after PKC signaling downstream of TCR/CD3 ligation. **a** Activating T cells through the TCR/CD3 complex initiates downstream signaling events that activate PKC and increases the intracellular Ca^{2+} concentration. PKC activation and increased intracellular Ca^{2+} in T cells can be mimicked through *in vitro* treatment with PMA and Io treatment, respectively. Increasing intracellular Ca^{2+} activates calcineurin (CN) and the transcription factor, NFAT, but did not initiate ER stress response signaling in T cells. However, the ER stress response did

occur with PKC activation, as evidenced by enhanced ER chaperone expression. It remains to be determined if (**a**) activated PKC directly signals or interacts with ER molecules to initiate ER stress response signaling or (**b**) PKC through activation of the transcription factor NF-κB (Lucas et al. 2004) indirectly enhances ER chaperone expression. **b** The highly specific PKC inhibitor, calphostin C, caused a significant reduction in ER chaperone expression, thus indicating PKC signaling is necessary for initiation of the ER stress response during T cell activation

ER homeostasis. Further studies are necessary to determine the exact role for ER stress response induction after TCR engagement.

During the differentiation of B cells into high-rate immunoglobulin secreting plasma cells, the expression of ER chaperones is increased in preparation for antibody secreting activity (Gass et al. 2002; Iwakoshi et al. 2003). Signaling through the B cell receptor (BCR) alone, while insufficient to allow B cell differentiation into plasma cells, is also a physiological inducer of the ER stress response and causes an increase in ER chaperone expression (Skalet et al. 2005). In an analogous manner, we found that ER chaperone expression in T cells was elevated by TCR signaling. Although PKC has been shown to be required for BCR signal transduction (Guo et al. 2007), the relative contribution of PKC to the increase of ER chaperones in B cells has not been linked. We show that inhibiting PKC function during T cell activation results in a decreased amount of ER chaperones. Therefore, initiation of the ER stress response by signaling through the TCR or BCR appears to play an essential preparatory role in the adaptive immune response, and PKC signaling downstream of these receptors may be intimately involved in the preparation.

Besides activating PKC and subsequent induction of the ER stress response, signaling through the TCR complex without co-stimulation in naïve T cells can lead to anergy in CD4$^+$ T cells (Powell et al. 1999) or tolerance in CD8$^+$ T cells (Iwakoshi et al. 2000). Anergy in CD4$^+$ T cells is a form of tolerance in which antigen-exposed CD4$^+$ T cells become unresponsive to subsequent antigenic stimulation, even in the presence of co-stimulation (Macian et al. 2004). Maintenance of this anergic state has been shown to depend on the *de novo* synthesis of distinct proteins, including early growth response (Egr)-2 and Egr-3 proteins (Harris et al. 2004; Safford et al. 2005). Our data indicate that expression of ER chaperones in T cells only receiving signal one does not differ from the expression in fully activated T cells. Therefore, we suggest that tolerant T cells are in an immunological 'active' state that requires increased levels of ER chaperones for the folding and assembly of proteins necessary for initiating and maintaining a 'nonresponsive' phenotype. These studies further indicate that increased expression of ER chaperones may be a general mechanism employed by numerous cell types in response to extracellular and intracellular signaling for modulation of cellular functioning.

To investigate the induction of ER stress response signaling in T cells in the context of physiologically relevant immune events, we evaluated *in vivo* ER stress response signaling during activation and tolerance induction. We used an established mouse model in which administration of DST has been shown to fully activate TCR transgenic CD8$^+$ T cells (Iwakoshi et al. 2001;

Thornley et al. 2006). However, DST administration in the presence of an anti-CD154 mAb has been shown *in vivo* to induce tolerance and enhance long-term allograft survival in mice, rats, and nonhuman primates (Kirk et al. 1997; Markees et al. 1998a; Kenyon et al. 1999; Elster et al. 2001). Our results show for the first time that *in vivo* antigen-exposed CD8$^+$ T cells that are destined to become either activated or tolerant undergo a physiological ER stress response. The importance of PKC during the *in vivo* induction of a physiological ER stress response are currently under investigation to determine if qualitative or quantitative differences are observed in antigen-exposed T cells that differ in their ultimate fate.

TCR ligation leads to signals that are necessary, but not sufficient, for fully functional T cell activation. Our data reveal an induction of the ER stress response in T cells after TCR ligation (signal one), even in the absence of co-stimulation (signal two). Induction of the ER stress response in activated and tolerant T cells may play an integral, underlying role in the initiation of an immune response. In addition, we now link PKC, an important mediator of downstream TCR signaling, as a key molecule in the initiation of the ER stress response, as inhibiting PKC function failed to induce ER stress response signaling. Further studies characterizing ER stress response induction and PKC activation downstream of TCR ligation may uncover novel therapeutic targets in this signaling pathway. These targets could be used to suppress or promote activation of T cells for the treatment of immune-mediated diseases and disorders.

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