The Serine-threonine Kinase Inositol-requiring Enzyme 1α (IRE1α) Promotes IL-4 Production in T Helper Cells*

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Background: IRE1α is a kinase important for the misfolded protein response.
Results: IRE1α promotes IL-4 production by stabilizing IL-4 mRNA, and IRE1α-specific inhibitor 4μ8C suppresses IL-4 production.
Conclusion: IRE1α is a positive regulator of IL-4 production by T helper cells.
Significance: Cytokine IL-4 has been implicated in allergic response. Our discovery that IRE1α promotes IL-4 production implies that IRE1α inhibition has a therapeutic potential in allergic disease treatment.

The inositol-requiring enzyme 1α (IRE1α) is a serine-threonine kinase that plays crucial roles in activating the unfolded protein response. Studies suggest that IRE1α is activated during thymic T cell development and in effector CD8+ T cells. However, its role in regulating T helper cell differentiation remains unknown. We find that IRE1α is up-regulated and activated upon CD4+ T cell activation and plays an important role in promoting cytokine IL-4 production. CD4+ T cells from IRE1α KO mice have reduced IL-4 protein expression, and this impaired IL-4 production is not due to the altered expression of Th2 lineage-specific transcription factors, such as GATA3. Instead, IL-4 mRNA stability is reduced in IRE1α KO T cells. Furthermore, treatment of T cells with an IRE1α-specific inhibitor, 4μ8C, leads to a block in IL-4, IL-5, and IL-13 production, confirming the role of IRE1α in the regulation of IL-4. This study identifies a regulatory function for IRE1α in the promotion of IL-4 in T cells.

Upon recognition of specific antigens by the T cell receptor (TCR)3 in the presence of co-stimulation, naïve T cells proliferate and undergo a number of changes that mark them as activated cells. Among these modifications is the ability to secrete large quantities of cytokines in response to pathogens. CD4+ T helper (Th) cells can be divided into Th1, Th2, Th17, and T regulatory (Treg) cells based on their cytokine expression profiles. Th1 cells express IFN-γ and protect against intracellular pathogens, Th2 cells produce IL-4 and protect against extracellular pathogens, Th17 cells produce IL-17 and protect against fungal and bacterial infections, and Tregs produce TGF-β and IL-10 and protect against overabundant immune responses (1, 2). Strength and length of TCR signal, proximal events downstream of the TCR, cytokine milieu, and gene transcription have been strenuously analyzed to understand how T cells differentiate into effector lineages and express cytokines. However, the effect of stress induced by the increased demand for protein synthesis and processing on T helper effector functions is not well understood.

Modifications that occur in cells such as activation and changes in structure can lead to a “backlog” of unfolded proteins, and the cell has to resolve this to avoid apoptosis. This process is called the unfolded protein response (UPR) (reviewed in Ref. 3). The UPR is a conserved pathway that allows the endoplasmic reticulum (ER), the major organelle responsible for folding proteins, to respond to the evolving cells. Three conserved pathways are activated during UPR in higher order organisms: IRE1, pancreatic ER kinase (PERK), and ATF6 (activating transcription factor 6). IRE1 has two forms: IRE1α and β; IRE1α is expressed throughout the body, and IRE1β is expressed in the gut (reviewed in Ref. 4). IRE1 is a protein kinase that promotes self-phosphorylation. Upon phosphorylation, IRE1 functions as a ribonuclease that is crucial for splicing X box binding protein 1 (Xbp-1) pre-mRNA. The splicing of Xbp-1 mRNA (Xbp-1s) is required for Xbp-1 protein to function as a transcription factor and mediate the up-regulation of ER chaperone proteins (5, 6). PERK activation initiates translational arrest via phosphorylation of EIF2α, and the activation of ATF6 is required to promote transcription of proteins involved in the UPR pathway, such as Xbp-1 (7–9).

Components of the UPR have been implicated in T cell development, activation, and differentiation. IRE1α is activated in T
were generated by crossing mice in all experiments. However, the functional roles of IRE1α in CD4 T cell differentiation remain uncharacterized. We report that although T cell-specific IRE1α conditional knock-out mice proliferate normally in response to TCR stimulation, they have diminished IL-4 production. This defect is not due to abnormalities at the transcriptional level, but instead is due to reduced IL-4 mRNA stability. In addition, treatment of CD4+ T cells with a potent and specific IRE1α antagonist, 4µ8C (14), suppresses IL-4 production. These data indicate that IRE1α contributes to the regulation of IL-4 in mouse CD4+ T cells.

**EXPERIMENTAL PROCEDURES**

Mice—The IRE1αfl/fl mice were generated as described previously (15, 16). Exons 16 and 17 are flanked by loxP sites in these mice, and upon deletion, a truncated, inactive form of IRE1α is expressed (15). The IRE1αfl/fl mice were backcrossed with C57BL/6 mice for >10 generations to maintain the C57BL strain background. The T cell specific IRE1α KO mice were generated by crossing IRE1αfl/fl mice with transgenic mice expressing Cre under the control of the Lck gene promoter (Lck-Cre), allowing for T cell specific IRE1α KO mice. In these studies, mice with the following genotypes, IRE1αfl/fl, Lck Cre0/0, and IRE1αfl/wt Lck Cre+, were used as controls because both the expression level and activity of IRE1α in the T cells from these mice were indistinguishable from the wild-type mice (data not shown). C57BL/6 (B6), IRE1αfl/fl Lck Cre+ (referred to as IRE1α mutant or knock-out (KO), IRE1αfl/fl Lck Cre0/0 and IRE1αfl/wt Lck Cre+ (referred to as IRE1α WT or control), and ER stress-activated indicator (17) mice were maintained and bred under pathogen free conditions in the Northwestern University animal facilities according to Institutional Animal Care and Use Committee regulations. All mice were maintained on the C57BL/6 background and used for experiments between ~6–12 weeks. Every effort was made to use littermates in all experiments.

**CD4+ T Cell Purification and in Vitro Differentiation of Th Cells**—CD4+ T cells from the spleens from control and IRE1α knock-out mice were positively selected using the CD4+ Dynabead kit (Invitrogen). Purity, as assessed by flow cytometry, was typically 92% or higher. Purified T cells were plated in 24-well dishes (1 × 105/well) that were precoated with 5 µl/ml of anti-CD28 (clone 2.43, rat IgG) and 3 µg/ml of anti-CD3 (145-2C11). The cells were cultured under Th0 (10 ng/ml of IL-2), Th1 (10 ng/ml of IL-2, 5 ng/ml IL-12 and 3.3 µg/ml anti-IL-4), Th2 (10 ng/ml of IL-2, 10 ng/ml IL-4, 0.12 µg/ml anti-IL-12, and 5 µg/ml anti-IFN-γ), or Th17 (5 µg/ml of anti-IL-17, 5 µg/ml of anti-IFN-γ, 1 ng/ml of TGFβ, and 20 ng/ml of IL-6) skewing conditions in 2 ml of RPMI complete T cell medium (RPMI 1640 +1-glutamine, 10% FBS, 50 µM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, and 0.05 mg/ml gentamycin) for 5 days or as indicated in the text. Recombinant mouse IL-6, TGFβ, IL-4, and IL-2 was purchased from eBioscience (San Diego, CA) or Biolegend (San Diego, CA). No azide/low endotoxin-grade anti-IL-12 (C17.8), anti-IFN-γ (XMG1.2), anti-CD3, anti-CD28, and anti-IL-4 (11B11) antibodies were purchased from eBiosciences or Bio X Cell (West Lebanon, NH). Secondary stimulations were performed by incubating cells with phorbol 12-myristate 13-acetate (5 ng/ml) and ionomycin (500 ng/ml) for 4 h or as indicated in the text. For the inhibitor experiments, cells were cultured for 4 days in the presence of dimethyl sulfoxide or IRE1α inhibitor (4µ8C) at a concentration of 2.4 µg/ml with anti-CD3/CD28 stimulation as above. 4µ8C was purchased from Ryan Scientific (Mount Pleasant, SC). Tunicamycin was purchased from Calbiochem (San Diego, CA).

**T Cell Enrichments**—Splenocytes were incubated on 10-cm dishes coated with anti-IgM (Jackson ImmunoResearch Laboratories) at a concentration of 1 µg/ml for 2 h. Non-adherent cells were harvested, and the percent cells were typically 65–70% CD3+.

**CFSE Staining**—T cells were purified using the CD4+ and CD8+ dyna-bead kits and stained with CFSE (eBioscience) as described (18). Briefly, the cells were brought to a concentration of 10 million cells/ml in PBS and incubated with an equal volume of 5 µM CFSE for 7 min to obtain a final concentration of 2.5 µM CFSE. The reaction was stopped by adding 10% calf serum, and cells were washed 2× in PBS. The cells were incubated at a concentration of 1 × 106/ml in 96-well dishes with anti-CD3 and anti-CD28 for 3 days (1 µg/ml) and then analyzed by flow cytometry.

**Cell Population Analysis**—All cells were analyzed on an Accuri flow cytometer (BD Biosciences). Splenocytes and thymocytes were isolated from mice, incubated with Fc block for 15 min, and stained with antibodies against CD4 (RM4-5), CD25 (PC6.2), CD62L (MLR-14), CD44 (IM-7), CD4 (RM-45), CD8 (53-6.7), NK1.1 (PK136), and B220 (RA3-6B2) for 20 min and then analyzed. Cells were also incubated with Foxp3 permeabilization buffer, incubated with Foxp3 (FJK-16S), and analyzed by flow cytometry.

**Analysis of Cytokine Production by Intracellular Cytokine Staining (ICCS) and ELISA**—Cells were incubated with 1× monensin (eBiosciences), 5 ng/ml of phorbol 12-myristate 13-acetate (Sigma-Aldrich), and 500 ng/ml of ionomycin (Sigma-Aldrich) for 4 h at 37 °C in complete T cell medium. The cells were then fixed and permeabilized using IC fixation buffer or the Foxp3 permeabilization kit (eBiosciences) following the manufacturer’s instructions. The cells were stained with fluorochrome-conjugated CD4 (RM4-5), IFN-γ (XMG1.2), IL-4 (11B1), and IL-17 (TC11-18H10.1) antibodies at 4 °C for 30 min, washed, resuspended in FACs buffer, and analyzed by flow cytometry. With regards to the GATA-3 and T-bet staining, cells were fixed and permeabilized using the eBioscience Foxp3 staining buffer kit (eBioscience); the cells were then incubated with T-bet (ebio4B10) and GATA-3 (TWA1) antibody for 30 min. All antibodies for ICCS were obtained from eBiosciences, Biolegend, or BD Biosciences.

ELISA was used for the analysis of cytokine production in the culture supernatants as reported (19). The assay was performed for cytokines IL-2, IL-4, IL-5, IL-13, IFN-γ, and IL-17 according to the manufacturer’s instructions. The cells were cultured for 4 days in the presence of dimethyl sulfoxide or IRE1α inhibitor (4µ8C) at a concentration of 2.4 µg/ml with anti-CD3/CD28 stimulation as above. 4µ8C was purchased from Ryan Scientific (Mount Pleasant, SC). Tunicamycin was purchased from Calbiochem (San Diego, CA).
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to the eBiosciences protocol. All antibodies were obtained from eBioscience or Biolegend and used at 1 μg/ml.

**RNA Isolation and qRT-PCR—**RNA was isolated from day 5 and day 7 skewed cells using TRIzol® (Invitrogen) and reverse transcribed using the QScript cDNA Supermix (Quanta BioSciences, Gaithersburg, MD) as reported (20, 21). Day 7 skewed cells were restimulated prior to RNA isolation as explained above. qRT-PCR was performed using Sybr Green incorporation on an IQ2 machine (Bio-Rad). Target levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) or β-actin. The relative expression for the qRT-PCR samples is calculated as $2^{-\Delta Ct\text{sample}}$, where CT is the cycle number it took for the sample to reach the analysis threshold. $\Delta Ct$ is the CT of the sample for the gene of interest minus the CT of the normalizing gene, HPRT. The primer sequences (5′-3′) used in this study are as follows: Il4-F, AACTCCATGCCT-TGAAGAAGAACTC and Il4-R, CCAGGAAGTCTTGGGATGCT- GATGTG; Iifn-γ-F, ACAATGAAGCCTCAACACTGC and Iifn-γ-R, CTTCCCAACTATGTCCACTTGAG; Hprt-F, 5′ TGGGTCTACCTCTGCTGCTT and Hprt-R, CCTGTTCA- TCATCGCTAATCAC; Xbp-1-F, ACACGTGGGAAGATGGACAC and Xbp-1-R, CCATGGGAAGATGGTCTGGG; Xbp-1s-F, ACACGTGGGAATGGACAC and Xbp-1s-R, GTGTCAGA- GTCCATGGGA; and β-actin-F, GGCTCCTAGCACCATTG- AAG and β-actin-R, GAAAGGTGTAAAACGCAGC.

**Western Blotting Analysis of Protein Expression in T Cells—** Equivalent numbers of cells for the samples to be analyzed were lysed in radioimmune precipitation assay buffer (EMD Milli- pore), and debris was pelleted by centrifugation at 14,000 rpm for 10 min at 4 °C. The lysates were run on an 8 or 10% SDS gel, transferred to PVDF, and immunoblotted with the following antibodies: anti-rabbit IRE1α (14C10; Cell Signaling- Danvers, MA, USA), anti-mouse JNK (Cell Signaling), anti-mouse phospho-JNK (EMD Millipore), anti-mouse tubulin (Santa Cruz Bio- technology, Santa Cruz, CA), anti-β-actin rabbit (Sigma-Aldrich), anti-mouse EIF2α (Cell Signaling), anti-rabbit phospho-IRE1α (Novus Biologicals, Littleton, CO), anti-rabbit phospho-p38 (Sigma-Aldrich), anti-GAPDH (Santa Cruz Biotechnology), and anti-mouse phospho-EIF2α (Cell Signaling). For the phospho-p38 and phospho-JNK immunoblots, 2 × 10⁶ cells were stimulated as indicated in the text, and lysates were run on a 10% gel. The blots were stripped and reprobed with tubulin, GAPDH, or β-actin antibody for loading controls (22).

**RNA Stability Assays—**Cells were activated under Th0 conditions as above for 5 days and then harvested and incubated in Th0 medium for 2 days without anti-CD3 and anti-CD28. The cells were then restimulated for 90 min before being treated with actinomycin D (Sigma-Aldrich) at a concentration of 3 μg/ml. Briefly, the cells were incubated with biotinylated anti-CD3/anti-CD28 (Biolegend) at a concentration of 3 μg/ml for 20 min, washed briefly with PBS, resuspended in 20 μg/ml of strepavidin (Jackson ImmunoResearch Laboratories), and placed at 37 °C. RNA was isolated, and qRT-PCR was performed as indicated above. The data were normalized to the zero time point and shown on a semi-log graph (y = percent change; x = time). The $T_{1/2}$ was determined from the slope of the line of best fit.

**Statistical Analysis—**The data were analyzed using a two-tailed Student’s paired t test. Samples were considered to differ significantly if the result of the test is a p value of ≈ 0.05. The S.E. is graphed in all experiments unless indicated in the figure legends.

**RESULTS**

**IRE1α Expression and Activation Are Elevated During CD4⁺ Cell Activation and Differentiation—**We analyzed IRE1α expression and activation in WT CD4⁺ T cells to determine whether IRE1α plays a role in T cell activation or effector functions. IRE1α protein is elevated >3-fold, and its phosphorylated form can be detected in CD4⁺ T cells upon TCR/CD28 stimulation within 24 h but not in naïve T cells (Fig. 1, A and B). Similarly, the expression level of Xbp-1, the downstream target gene of IRE1α, is significantly increased upon TCR/CD28 stimulation (Fig. 1 C). These results suggest a role for the IRE1α/Xbp-1 pathway in T cell activation.

Next, we analyzed IRE1α and Xbp-1 expression and activation during T cell differentiation. CD4⁺ T cells were cultured under non-polarization condition (Th0) or under Th1, Th2, and Th17 differentiation conditions for 5 days. Intracellular staining confirmed that the cells were activated and differentiated into each effector population (Fig. 1D). We then analyzed both total and spliced Xbp-1 and Xbp-1s mRNA in differentiated CD4⁺ T cells. As indicated in Fig. 1E, the highest levels of Xbp-1 and Xbp-1s were detected in Th0 and Th2 cells (Fig. 1E). Because IRE1α is the only known upstream enzyme that mediates Xbp-1 mRNA splicing, it is not surprising that the levels of activated IRE1α (phosphorylated IRE1α) positively correlate to that of Xbp-1s levels in effector CD4⁺ T cells, the highest of which are detected in Th0 and Th2 cells (Fig. 1F). Expression levels of IRE1α, phospho-IRE1α, Xbp-1s, and Xbp-1 from cells treated with the pharmacological ER stress inducer tunicamycin were analyzed and served as positive controls (Fig. 1, A–C, and F).

We then isolated CD4⁺ splenocytes from ER stress-activated indicator mice to measure Xbp-1 spliced protein product under Th0, Th1, Th2, and Th17 conditions. The ER stress-activated indicator transgenic vector carries a fusion of the Xbp-1 gene with the *Venus* gene, a variant of GFP (17). Upon IRE1α activation, Xbp-1 mRNA is spliced to produce a functional protein, and the resulting Xbp-1-venus fusion protein can be detected by flow cytometry. The mean fluorescence intensity is elevated upon T cell activation, and it is highest in Th0 and Th2 and lowest in Th17 cells (Fig. 1G), corresponding with our qRT-PCR data (Fig. 1F). Similar to Xbp-1s mRNA and the activated IRE1α, expression of Xbp-1s protein appears to positively correlate with the ability of T cells to produce IL-4 (Fig. 1, D–G). These results show that the IRE1α/Xbp-1 pathway is activated during T helper cell activation and differentiation, implying a possible role of this pathway in regulating CD4⁺ T cell functions.

**IRE1α Is Required for Optimal IL-4 Production by CD4⁺ T Cells—** To determine the regulatory functions of IRE1α in T cell activation and differentiation, we generated T cell-specific IRE1α conditional knock-out mice by breeding IRE1α floxed mice (15) with Lck-Cre transgenic mice. As described previ-
ously, exons 16 and 17 of the \textit{IRE1}α gene are floxed in these mice, resulting in a truncated, inactive form of \textit{IRE1}α protein (15). The Cre DNA recombinase is mediated by the \textit{Lck} promoter, which drives gene expression during thymic development, and the gene is specific for T cells, allowing for a targeted deletion of \textit{IRE1}α gene during T cell development (23, 24).

Immunoblotting analysis detected the mutant form of \textit{IRE1}α protein in naïve T cells from the \textit{IRE1}α\textsuperscript{f/f}\textit{Lck-Cre}\textsuperscript{H11001} mice (Fig. 2A). However, the \textit{IRE1}α deletion in T cells is incomplete with an average of 80–90% reduction of \textit{IRE1}α protein in the T cells from the conditional knock-out mice as compared with control mice (Fig. 2A). This is possibly due to the level of expressed Cre being insufficient to completely delete the loxp-flanked \textit{IRE1}α gene.

It has been shown that the ER stress chaperone protein gp96 is critical for the early lymphopoiesis of both T and B cells (25). However, the partial deletion of the \textit{IRE1}α gene did not affect T cell development because the \textit{IRE1}α\textsuperscript{KO} mice have normal ratios of CD4\textsuperscript{H11001} and CD8\textsuperscript{H11001} T cells in their thymus and spleen (Fig. 2B and C). As expected, T cell-specific \textit{IRE1}α gene depletion does not affect the development of other immune cells such as B cells, NK cells, and NK T cells because their percentages and ratios are unaltered in the mutant mice (Fig. 2C). In addition, the expression of cell surface CD44 and CD62L on CD4\textsuperscript{H11001} and CD4\textsuperscript{+} T cells, as well as the percentages of CD25\textsuperscript{+}FoxP3\textsuperscript{+} Tregs in the spleens from knock-out mice and their littermates are indistinguishable (Fig. 2D). 

\textbf{FIGURE 1.} \textit{IRE1}α and downstream target of \textit{IRE1}α, \textit{Xbp}-1, and the spliced form of \textit{Xbp-1} (\textit{Xbp-1s}), are elevated upon activation in T cells. A, \textit{IRE1}α is up-regulated and activated upon T cell activation. Protein was isolated from naïve CD4\textsuperscript{H11001} cells and CD4\textsuperscript{H11001} cells stimulated with anti-CD3 and anti-CD28 for 24 h. Immunoblots were performed for \textit{IRE1}α and phosphorylated \textit{IRE1}α\textsuperscript{p} \textit{IRE1}α (p-\textit{IRE1}α). Cells treated with tunicamycin (TM) were used as a positive control. B, the band densities in A were quantified. The total \textit{IRE1}α data shown are representative of five experiments, and the phosphorylated \textit{IRE1}α data are representative of four experiments. C, \textit{Xbp-1} is up-regulated during T cell activation. RNA was isolated from naïve CD4\textsuperscript{H11001} cells and CD4\textsuperscript{H11001} cells stimulated with anti-CD3 and anti-CD28 for 8 and 24 h with cells treated 8 h in the presence of tunicamycin used as a control, and qRT-PCR was performed. The data graphed are an average of four experiments and are normalized to naïve cells. The error bars represent the S.E. Double asterisks indicate data are significant ($p$ ≈ to 0.01 for 8 and 24 h versus naïve (Student's paired t test)). D–F, CD4\textsuperscript{+} cells were activated under Th0, Th1, Th2, Th17, and Treg conditions for 5 days. Their differentiation into each Th population was confirmed by intracellular cytokine staining (D). Protein was isolated from each type of Th cells and immune-blotted for \textit{IRE1}α, phospho\textit{IRE1}α, and tubulin (E). The mRNA levels of \textit{Xbp-1} and its spliced form (\textit{Xbp-1s}) in the Th cells were analyzed by qRT-PCR (F). A control, RNA was isolated from cells activated in the presence of tunicamycin for 24 h and analyzed for \textit{Xbp-1} and \textit{Xbp-1s}. The data are representative of a minimum of three experiments. G, \textit{Xbp-1s} protein is elevated upon T cell activation, with a trend toward expression being highest in cells producing IL-4. CD4\textsuperscript{+} T cells from ER stress-activated indicator mice were placed under Th0, Th1, Th2, and Th17 conditions for 5 days. Mean fluorescence intensity (MFI) was measured in naïve cells and at day 5. The data are the average of three experiments. The error bars in all graphs represent the standard error.
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IL-2 in response to TCR stimulation at levels similar to control T cells (Fig. 2, E and F). Therefore, IRE1α does not appear to be important in T cell development or at the early stage of activation.

The percent of IL-4 producers and the production of IL-4 cytokine in the culture supernatants were significantly reduced in IRE1α KO CD4 T cells upon in vitro activation for 5 days compared with that of control cells as determined by flow cytometry and ELISA (Fig. 3). In addition, the amount of IL-4 produced by a single cell, as measured by mean fluorescence intensity, was reduced in the KO compared with the control (Fig. 3D). In contrast, the percent IFN-γ producers and cytokine expression was normal (Fig. 3, A, C, and E). These results indicate that genetic suppression of IRE1α function impairs IL-4 production by CD4 T cells.

The IL-4 gene is located at what is termed the Th2 cytokine gene locus, along with cytokines IL-13 and IL-5, and its expression is associated with the presence of cytokines IL-5 and IL-13 (26). Therefore, we analyzed the expression of Th2 cytokines IL-5 and IL-13 by ELISA. We found that these cytokines are produced at normal levels in the IRE1α KO Th0 cells (Fig. 3E), indicating that reducing IRE1α specifically impairs IL-4 production but not other Th2 cytokines by CD4+ T cells. However, given the fact that IRE1α protein is partially expressed in the mutant CD4 T cells, it is possible that low levels of IRE1α is sufficient for other IL-5 and IL-13 cytokine production but not IL-4. In addition, IRE1α KO T cells were able to produce cytokine IL-17, a hallmark cytokine of Th17 cells, appropriately.

IRE1α-specific Inhibitor 4µ8C Suppresses IL-4 and IL-13 Production—To further confirm the role of IRE1α in IL-4 production, we investigated the effects of an IRE1α inhibitor, 4µ8C, on IL-4 expression in an in vitro culture system. This inhibitor specifically binds to IRE1α and blocks its RNase activity without affecting its auto-phosphorylation, resulting in reduced Xbp-1 splicing (14). On average, an ~60% reduction in Xbp-1 splicing was confirmed by qRT-PCR (Fig. 4A) upon treatment of CD4 T cells with the inhibitor. We found that 4µ8C treatment dramatically inhibits IL-4 production by CD4 T cells under Th0 conditions because both the IL-4 levels in the culture supernatant and the percentage of IL-4 positive cells were reduced by 4µ8C treatment (Fig. 4, B–D). In contrast, the expression of IFN-γ did not appear to be affected by IRE1α inhibitor 4µ8C (Fig. 4, B–D). These data confirmed our discovery using T cells from IRE1α conditional knock-out mice and led us to conclude that the suppression of IRE1α functions in T cells blocks IL-4 production. In addition, both IL-5 and IL-13 production were significantly reduced upon treatment with 4µ8C (Fig. 4D). In contrast, cytokines IFN-γ and IL-17 appeared to be normal (Fig. 3D). Therefore, the pharmacological suppression of IRE1α inhibits the production of all Th2 cytokines analyzed in CD4 T cells, whereas diminished expression of IRE1α leads to reduced IL-4 but not IL-5 and IL-13.

Because IRE1α is partially expressed in the conditional knock-out mice, we determined whether 4µ8C could increase inhibition of the Th2 cytokine production by IRE1α mutant CD4 T cells. As shown in Fig. 4E, IL-4 production is further repressed in IRE1α KO T cells when treated with 4µ8C, presumably through suppressing IRE1α. Consistent with the data in Fig. 3, decreasing IRE1α expression (IRE1α KO) did not achieve a statistically significant reduction in IL-5 production (Fig. 4F), but treatment of IRE1α KO T cells with the inhibitor results in a significant reduction of IL-5. In contrast, the production of IFN-γ KO T cells was not affected by treatment with 4µ8C (Fig. 4G). As a control Xbp-1s was measured in KO and control mice, and a statistically significant reduction in Xbp-1s expression was detected in IRE1α KO T cells (Fig. 4H). However, the Xbp-1s mRNA levels remain relatively high in IRE1α KO T cells, presumably catalyzed by the low level pres-
IRE1α Functions Required for IL-4 Production by CD4 T Cells under Th2 Conditions—T helper cell differentiation is induced by signals from the TCR and cytokines in the environment, and because IL-4 production is reduced in IRE1α KO CD4+ T cells under non-polarization conditions, we asked whether IRE1α is required for Th cell differentiation. CD4+ T cells from IRE1α KO mice and wild-type littermates were activated under Th0, Th1, Th2, and Th17 conditions. IL-4 production was impaired in IRE1α KO Th0 and Th2 cells, confirming that IRE1α function is required for IL-4 production by activated and differentiated CD4 T cells (Fig. 5, A and B). In contrast, the hallmark cytokines of Th1 and Th17 cells, IFN-γ and IL-17, were expressed normally under all conditions analyzed (Fig. 5A). In addition, the partial IRE1α gene deletion in CD4 T cells did not affect in vitro Treg differentiation (Fig. 5A). Therefore, we conclude that although IRE1α is required for the production of IL-4, IRE1α knock-out cells maintain the capacity to differentiate into the Th1, Th17, and Treg effector lineages.

The transcription factors GATA3 and T-bet play an important role in regulating IL-4 production. GATA-3 is required for the production of IL-4, and it can be inhibited by transcription factor T-bet, which promotes Th1 cells (27–29). Therefore, we measured GATA-3 and T-bet protein expression in IRE1α KO Th0 cells and found them to be normal (Fig. 5, C and D), excluding the possibility that IRE1α regulates IL-4 production through these transcription factors. In addition to GATA-3 and T-bet, MAP kinases JNK1 and -2 play an important role in regulating IL-4 production (30–33), and IRE1α has been implicated in the activation of JNK (34). To measure JNK activation and expression in IRE1α KO T cells, T cells were enriched from splenocytes of control and IRE1α KO mice and stimulated with anti-CD3 and CD28. We found JNK activation to be normal, indicating that the JNK pathway was not adversely affected by diminished IRE1α in T cells (Fig. 5E).

UPR can be induced by PERK, IRE1α, and ATF6, and it is conceivable that when one of these pathways is inhibited, one or both of the other pathways is up-regulated (35). PERK has been proposed to regulate IL-4 expression by promoting inhibition of IL-4 translation via enhanced phosphorylation of EIF2α (13). Therefore, we measured EIF2α and phosphorylated EIF2α protein expression in IRE1α KO cells to test whether the
reduced IL-4 expression is due to the altered EIF2α phosphorylation. We find total EIF2α and phospho-EIF2α expression to be relatively normal (Fig. 5F), excluding the possibility that the reduction in IL-4 was due to the enhanced PERK/EIF2α activity in IRE1α KO T cells.

mRNA Stability and Control of Protein Translation Contributes to Post-transcriptional Regulation. Sequences in the 3′-UTR of mRNA can affect mRNA stability and protein translation, and the UTRs of cytokine mRNA contain adenylate-uridylate-rich sequences that promote mRNA instability and rapid degradation (reviewed in Ref. 36). To determine whether IL-4 mRNA stability was reduced in the absence of IRE1α, CD4+ splenocytes were activated for 5 days under Th0 conditions, removed from TCR stimulation, and rested in the pres-
ence of IL-2 for 2 days. They were then restimulated and treated with actinomycin D for various lengths of time, allowing us to block IL-4 transcription and measure the loss of IL-4 mRNA over time. The average of four experiments is graphed on a semi-log graph where x = time and y = percent change. We found that IL-4 mRNA was less stable in the KO cells compared with the controls after treatment with actinomycin D (Fig. 6B) as determined by calculating the slope of the line of best fit (con $t_{1/2}$ 234 min; KO $t_{1/2}$ 66 min). Addition of the pharmacological ER stress inducer tunicamycin did not alter IL-4 mRNA stability, indicating that the reduced stability is due to disruption of the ER stress response pathway. As expected, IFN-γ mRNA stability was similar between control and KO cells. These data indicate that IRE1α plays a role in promoting IL-4 by enhancing IL-4 mRNA stability.

p38 plays an important role in cytokine stability (37, 38), and in addition to its role in cleaving Xbp-1, IRE1α activates MAP kinase p38 (39). MAP kinase p38 activates mRNA binding protein HuR, and HuR promotes IL-4 mRNA stability (40, 41). Therefore, we measured p38 phosphorylation upon TCR activation in IRE1α KO T cells to determine whether reduced IL-4 mRNA stability is due to reduced p38 activation. We find reduced expression of the active form of p38 in IRE1α KO T cells upon stimulation (Fig. 6C), suggesting that the reduced IL-4 stability in IRE1α KO T cells is caused by diminished p38 activation.

DISCUSSION

This work demonstrates that IRE1α promotes IL-4 production by CD4+ T cells by stabilizing IL-4 mRNA (Fig. 7). This conclusion is supported by the following: first, TCR activates IRE1α in CD4+ T cells, as both IRE1α phosphorylation and Xbp-1 mRNA splicing are detected in CD4+ T cells upon TCR stimulation; second, targeted deletion of the IRE1α gene, although only resulting in a partial suppression of IRE1α protein expression, impairs IL-4 production both under non-polarizing and Th2 polarizing conditions; third, treatment of CD4+ T cells with an IRE1α-specific inhibitor 4μC suppresses IL-4, as well as IL-5 and IL-13 production; and fourth, we show that IRE1α functions are required for IL-4 mRNA stability because the half-life of IL-4 mRNA is significantly shortened in IRE1α knock-out T cells. These findings illuminate a previously unknown function for IRE1α in CD4 T cells.

Prior to this study, the IRE1α/Xbp-1 UPR pathway was found to be important for CD8+ T cell differentiation (11); however, it was unknown whether this pathway played a role in T helper activation/effector function. T helper cells up-regulate and activate the IRE1α/Xbp-1 UPR pathway early in response to TCR stimulation (Fig. 1). Previously, it was found that TCR stimulation induced ER stress response protein GRP78, which is located downstream of the IRE1α/Xbp-1 pathway, as early as 17 h (12). Moreover, we show that TCR stimulation can activate IRE1α and mediate Xbp-1 transcription within 24 h (Fig. 1a).
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**FIGURE 6.** IL-4 mRNA stability is reduced in IRE1α KO cells. Primary CD4 T cells were isolated from control and IRE1α knockout mice and were stimulated under Th0 condition for 5 days. The mRNA levels of IL-4 were analyzed by qRT-PCR. The data were normalized to HPRT and are shown relative to the control Th0 cells. The data graphed are the average of nine experiments. The error bars represent the S.E. A, B, and C, the Th0 cells were re-stimulated with anti-CD3 and anti-CD28. Protein was isolated, and immunoblotting was performed on the lysates for phospho-p38 and total p38. The data are representative of four experiments, and the relative expression of phospho-p38 to total p38 is graphed below. Student's t test was used for the statistic analysis; *, p = 0.05 for control versus KO-activated cells. N, naive; A, activated.

Interestingly, T cells that make significant amounts of IL-4 have the greatest IRE1α/Xbp-1 UPR pathway response. Iwakoshi et al. (42) observed a similar effect with regard to IL-4 inducing Xbp-1 transcription in B cells. We speculate that in addition to TCR signaling, IL-4 further promotes the activation of IRE1α/Xbp-1 in T cells. Although we find increased Xbp-1 splicing and protein in Th0 and Th2 cells, we cannot disregard that this may be a consequence of the presence of enhanced Xbp-1 transcript. Future studies need to be embarked upon to definitively determine whether TCR stimulation can induce IRE1α to cleave Xbp-1 independent of its effects on Xbp-1 transcript.

IRE1α appears to regulate Th cytokine production in a specific manner because only IL-4 production was affected by the partial loss of IRE1α in CD4+ T cells (Figs. 3 and 5). However, treatment of WT cells with a robust IRE1α inhibitor results in reduction of cytokines IL-4, IL-5, and IL-13 (Fig. 4). One possible explanation is that IL-4 is more sensitive to the partial loss of IRE1α in T cells. The IRE1α KO expresses 80–90% less of the wild-type IRE1α protein, and the remaining IRE1α could be sufficient for IL-5 and IL-13 but not IL-4 expression in T cells. The IRE1α inhibitor significantly suppressed Xbp-1 splicing, leading to a more robust reduction of IL-4 production compared with the KO mice (a 50% reduction compared with a 37% reduction of IL-4 producers).

**FIGURE 7.** A model for how loss of IRE1α affects IL-4 expression. Upon T cell stimulation, IRE1α is activated. IRE1α promotes IL-4 mRNA stability, and in its absence, IL-4 mRNA is degraded, resulting in subsequent reduction of IL-4 protein.
mRNA stability (38, 41). In addition, p38 has been shown to promote Xbp-1 mRNA stability and Xbp-1 protein nuclear translocation (44). Moreover, EIF2α appears to promote the translation of proteins involved in the UPR response (45). However, because p38 is important in the production and stability of Th2 cytokines IL-5 and IL-13 (46), one would expect that all Th2 cytokines would be reduced in IRE1α KO T cells. However, we do not know the extent to which active p38 is required for the various cytokines. Although p38 activation is reduced, it is still present, and the amount of active p38 required for IL-4 stability in T cells may be greater than for the other cytokines measured. Therefore, additional studies are needed to determine how IRE1α specifically regulates the production of IL-4, and possibly IL-5 and IL-13, by CD4+ T cells.

How Does T Cell Stimulation Induce IRE1α Expression and Activation?—In addition to misfolded protein, accumulated evidence indicates that specific physiological stimuli can activate the UPR pathway, including the IRE1α/Xbp-1 branch. For example, we and others (16, 48) have shown that Toll-like receptors can activate the IRE1α/Xbp-1 pathway to induce inflammatory cytokine production during bacterial infection. We show that TCR/CD28 stimulation triggers IRE1α phosphorylation, Xbp-1 transcription, and subsequently Xbp-1 mRNA splicing in CD4+ T cells. Moreover, this is likely enhanced by IL-4 because the highest levels of IRE1α and Xbp-1 are in CD4+ T cells that produce IL-4. The underlying molecular mechanism of how TCR/CD28 signaling promotes activation of the IRE1/Xbp-1 pathway is still unknown. PKC plays a critical role in ER stress response because inhibition of PKC blocks ER stress response upon TCR stimulation (12). Therefore, we speculate that one of the possible mechanisms behind TCR/CD28-mediated IRE1/Xbp-1 activation is through PKC activation.

The PKC family of kinases consists of 15 isoforms; however, the contributions of family member PKCθ in T cell functions are numerous. PKCθ plays a crucial role in mediating early T cell responses, IL-2 cytokine production, TCR/MHC synapse, and early transcription factor activation (reviewed in Ref. 49). Moreover, PKCθ promotes Th2 cytokine expression (47, 50). It is conceivable that IRE1α contributes to the TCR-induced PKCθ activation of IL-4. A model for how IRE1α may promote IL-4 is indicated in Fig. 7.

In summary, our results indicate that IRE1α promotes the expression of IL-4. These findings have important implications for the treatment of IL-4-mediated diseases using IRE1α-specific inhibitors.

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