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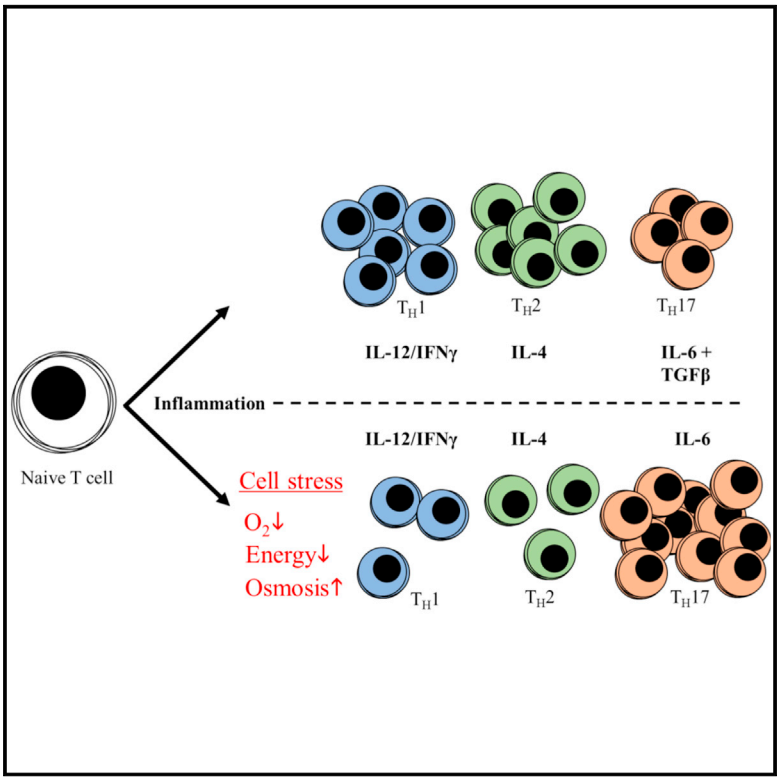
Cell Reports

Cellular Stress in the Context of an Inflammatory Environment Supports TGF-β-Independent T Helper-17 Differentiation

Graphical Abstract



Authors

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In Brief

Brucklacher-Waldert et al. show how environmental conditions resulting in cellular stress, such as low metabolite levels, specifically enhance Th17 cell differentiation. Under inflammatory conditions, this stress can substitute for TGF-β signaling resulting in de novo Th17 cell differentiation.

Highlights

- Limited metabolite availability results in cellular stress
- Cellular stress enhances Th17 cell polarization
- Cellular stress induces de novo Th17 cell differentiation
- Cellular stress can substitute for TGF-β in Th17 cell differentiation

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Cellular Stress in the Context of an Inflammatory Environment Supports TGF-β-Independent T Helper-17 Differentiation

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SUMMARY

T helper-17 (Th17) cells are associated with inflammatory disorders and cancer. We report that environmental conditions resulting in cellular stress, such as low oxygen, glucose, and isotonic stress, particularly enhance the generation of Th17 cells. Pharmacological inhibition of cell stress reduces Th17 cell differentiation while stress inducers enhance the development of Th17 cells. The cellular stress response results in Th17 cell development via sustained cytoplasmic calcium levels and, in part, XBP1 activity. Furthermore, in an inflammatory environment, conditions resulting in cell stress can bring about de novo Th17 cell differentiation, even in the absence of transforming growth factor β (TGF-β) signaling. In vivo, cell stress inhibition enhances resistance to Th17-mediated autoimmunity while stress-exposed T cells enhance disease severity. Adverse metabolic environments during inflammation provide a link between adaptive immunity and inflammation and may represent a risk factor for the development of chronic inflammatory conditions by facilitating Th17 cell differentiation.

INTRODUCTION

T cell function is tightly controlled by factors encountered in the micro-environment and specifically tailored to successfully orchestrate a response against invading microorganisms and their products. The set of instructions for antigen-inexperienced $CD4^+$ T cells to differentiate into several defined T helper (Th) lineages, each with specific job descriptions (Veldhoen, 2009), has largely focused on cytokines as well as strength of signaling from the T cell receptor and co-receptors (Zygmunt and Veldhoen, 2011). Several Th subsets are now well defined, including Th1, Th2, follicular helper T cells (Tfh), regulatory T cells (Treg), and Th17 cells. Although the strict divide between subsets has been blurred in recent years, each T cell subset is characterized by transcription factors important for lineage differentiation and maintenance as well as the production of particular cytokines, chemokines, and the expression of cell surface receptors.

Th17 cells are directly implicated in a variety of inflammatory diseases, autoimmune disorders, and cancers (Brucklacher-Waldert et al., 2014; Grivennikov et al., 2012; Kryczek et al., 2009; Veldhoen, 2009).They are characterized by the expression of interleukin (IL)-17 and transcription factors of the retinoic acid receptor (RAR)-related orphan receptor (ROR) family, RORα and RORγt. The differentiation of Th17 cells appears to uniquely require the presence of two cytokines—IL-6 and transforming growth factor β (TGF-β) (Veldhoen et al., 2006a). The pro-inflammatory cytokine and acute phase protein IL-6, although IL-21 can substitute in some cases (Korn et al., 2007), is required to initiate the Th17 differentiation program but only in the presence of TGF-β (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). IL-6, via its receptor and signal transducer and activator of transcription 3 (STAT3) signaling, is now well known to be important for murine and human Th17 differentiation (de Beaucoudrey et al., 2008; Harris et al., 2007; Ma et al., 2008; Stockinger and Veldhoen, 2007). Due to the largely anti-inflammatory and cell-cycle inhibitory nature of TGF-β, its role in Th17 cell differentiation remained controversial (Acosta-Rodriguez et al., 2007; Wilson et al., 2007), however, it can contribute to human Th17 cell differentiation as well (Manel et al., 2008; O'Garra et al., 2008; Volpe et al., 2008; Yang et al., 2008). The role of TGF-β in Th17 cell differentiation is not well understood. Although TGF-β signaling

via the TGF-βR-complex is important (Veldhoen et al., 2006a, 2006b), the downstream signaling components, SMAD2, SMAD3, and SMAD4 (Derynck and Zhang, 2003) have been shown to be non-essential for Th17 cell development (Ghoreschi et al., 2010; Hahn et al., 2011; Ichiyama et al., 2011). The description of Th17 cells generated in the absence of TGF-β signaling underscored that an alternative pathway for Th17 cell development, without the involvement of TGF-β, may exist (Ghoreschi et al., 2010).

Th17 cells are enriched at sites of chronic inflammation, such as arthritic synovia, and the local environment in the synovia has been reported to be able to enhance their differentiation (Egan et al., 2008; Nistala et al., 2010). Similarly, chronic inflammatory conditions are required in the Th17 cell-dependent mouse model of experimental autoimmune encephalomyelitis (EAE) to sustain autoimmunity (Veldhoen et al., 2006b). Th17 cells are also found in many tumor infiltrates (Grivennikov et al., 2012; Kryczek et al., 2009), suggesting that microenvironments with altered metabolite availability such as found in tumors (DeBerardinis et al., 2008), may preferentially recruit Th17 cells or support the Th17 cell lineage differentiation program. In line with this hypothesis, changes in the local environment have recently been shown to influence Th17 cells (Dang et al., 2011; Kleinewietfeld et al., 2013; Shi et al., 2011; Wu et al., 2013).

How alterations in the local environment may influence the differentiation of naive $CD4^+$ T cells into the Th17 cell lineage is not well understood. The rate of biomolecule synthesis in all cells, which impacts on cell differentiation, survival, and ability to secrete products, is known to be synchronized with nutrient availability (Kaufman et al., 2002). The coordination between metabolite availability and requirement takes place in the endoplasmic reticulum (ER). Imbalance between nutrient availability and biomolecule demand can result in cellular stress such as ER-stress. The ER-stress response, or unfolded protein response (UPR), is initiated to restore ER function, upon viral infection, hypoglycemia, hypoxia, and osmotic disturbance thereby enabling cell survival (Kaufman et al., 2002). In this paper, we show that reduction in availability of metabolites such as glucose and oxygen and increased ionic pressure can enhance Th17 cell differentiation. This is in stark contrast to other Th effector cell subsets as well as Treg cell differentiation, the polarization of which are either inhibited or not affected under these conditions. We show that the decrease in availability of metabolites ultimately results in cellular-stress and increased and sustained levels of cytoplasmic calcium. Inhibition of cellular stress signaling branches highlighted the importance of cell stress in enhancing Th17 cell differentiation under altered environmental conditions. Furthermore, we provide evidence that cellular stress can be responsible for de novo differentiation of Th17 cells in an inflammatory context, even in the absence of TGF-β signaling, with enhanced plasticity toward Th1-like cells. Our data indicate that inflammatory environments in combination with cellular stress are very conductive to the differentiation of Th17 cells, thereby potentially tipping the balance toward a more severe inflammatory response accompanied by aggravated immunopathology.

RESULTS

Reduced Glucose Metabolism Enhances Th17 Cell Differentiation

To address how conditions of altered metabolite availability may affect Th cell generation and function, we cultured naive $CD4^+$ T cells under conditions of limited glucose metabolism with the help of the glucose analog and competitive inhibitor 2-deoxy glucose (2DG). Th1, Th2, and iTreg cell differentiation, analyzed by signature cytokine and transcription factor expression levels, was dose-dependently inhibited by 2DG, ultimately resulting in cell death at concentrations above 1 mM (Figures 1A, 1B, and S1A). In stark contrast, Th17 cell differentiation, analyzed via IL-17 and RORγt transcripts and protein, was dose-dependently enhanced in the presence of 2DG, without marked effects on survival or proliferation (Figures 1A, 1B, and S1B and data not shown). We did not observe Treg cell polarization under Th17 cell differentiation conditions in the presence of 2DG (Figure S1C) and confirmed the dose-dependent inhibition of glycolysis by 2DG via the inhibition of lactate production (Figure S1D). 3-Bromopyruvic acid (BrPA), an aerobic glycolysis inhibitor acting via inhibition of glyceraldehyde 3-phosphate dehydrogenase (Cardaci et al., 2012), had a very similar effect to 2DG on Th cell differentiation. It inhibited the polarization and cell viability of Th1 and Treg cells while robustly increasing Th17 cell polarization (Figure 1C).

IL-2 is a T cell autologous factor required for proliferation and survival of most T lymphocytes but inhibits Th17 cell polarization (Laurence et al., 2007). Its inhibition under adverse metabolic conditions could explain the inhibition of Th1, Th2, and Treg cells while enhancing Th17 cell polarization. However, upon Th cell subset polarization from naive precursors in the presence of 2DG or BrPA IL-2, transcript and protein levels were found boosted in all Th subsets compared with controls (Figures 2A, 2B, and S2A). IL-2 neutralization during Th17 cell differentiation did not alter 2DG's ability to enhance Th17 cell development (Figure 2C). A consequence of reduced glucose metabolism could be the surface expression level of the high-affinity IL-2 receptor (IL-2Rα, CD25) (Datema and Schwarz, 1978). The presence of IL-2Rα was dose-dependently inhibited by 2DG and BrPA in all Th subsets, in contrast to CD69 expression (Figures 2D and S2B). Conform reduced presence of the IL-2Rα on the cell surface upon T cell activation, the IL-2 response mediated by phosphorylation of signal transducer and activator of transcription (Stat)5 was 2DG dose-dependently reduced in Th17 cells (Figure S2C). Because IL-2 is an important factor in Th1, Th2, and iTreg cell differentiation as well as for T cell survival (Moriggl et al., 1999), the reduced IL-2 responsiveness could explain the high sensitivity to cell death upon co-culture with 2DG of these Th subsets.

Although the glycolysis inhibitor BrPA had similar Th17 cell enhancing effect as 2DG, we wished to establish if 2DG-induced reduction in glycosylation capacity alone was responsible for enhanced Th17 cell polarization. The addition of mannose, an alternative source for sugar residues and major component of sugar moieties in glycoproteins, during naive T cell polarization cultures improved T cell viability and IL-2Rα expression levels

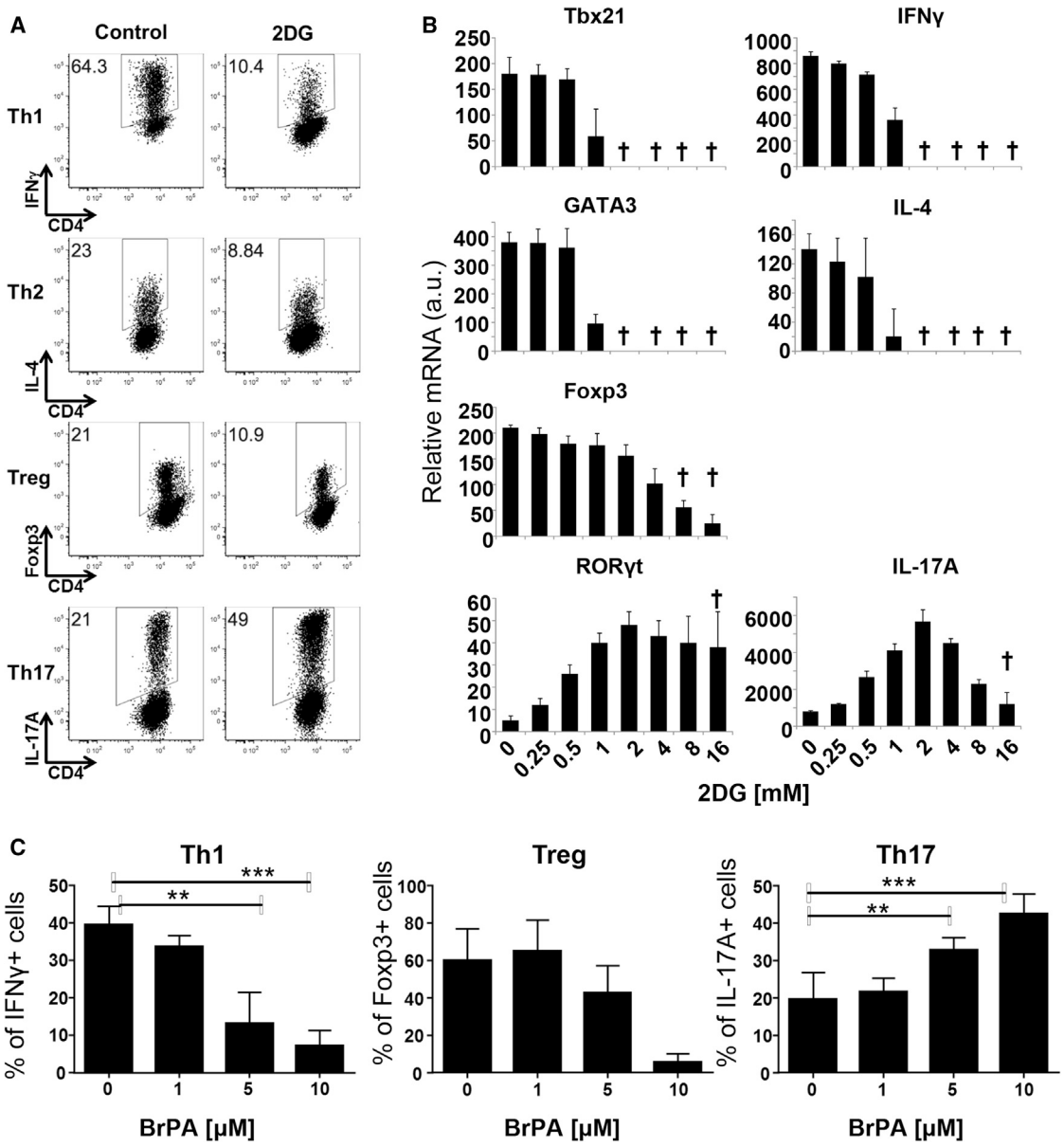

Figure 1. Reduced Glucose Accessibility Enhances Th17 Cell Differentiation

Naive mouse CD4$^+$ T cells were cultured on anti-CD3/CD28-coated wells for 3 days under indicated Th subset polarization conditions in the absence or presence of 2DG (A and B) or BrPA (C).

(A) Naive T cells were cultured under indicated Th cell subset conditions in the presence or absence of 1 mM 2DG and were stained intracellularly for indicated cytokines or Foxp3.

(B) mRNA expression analysis by real-time PCR after 72 hr of culture of indicated Th subsets with concentration range, 0.25–16 mM, of 2DG for indicated gene products. Ct values were normalized to Hprt and the relative expression is displayed. †Indicates over 50% cell death.

(C) Naive T cells were cultured under indicated Th cell subset conditions in the presence or absence of indicated concentrations of BrPA upon which polarization toward Th1 (left), Treg (middle), or Th17 (right) cells was assessed by intracellular flow cytometry. Data are representative of six (A) or three (B and C) experiments. Error bars represent SD of pooled data (n = 4–6).

but did not counteract the effects of 2DG on Th17 cell polarization (Figure S2D and data not shown). 2-Fluoro-2-deoxy-glucose (2FDG), a glucose analog with reduced effects on glycosylation compared with 2DG (Datema et al., 1980), was similarly able to increase cell viability as well as boost Th17 cell differentiation (Figure S2D). Furthermore, exogenous mannose did not reduce the inhibitory effect of 2DG on polarization of other Th subsets (Figure S2E). These data indicated that reduced glucose availability boosts Th17 cell differentiation and inhibits the polarization and survival of other known Th cell subsets.

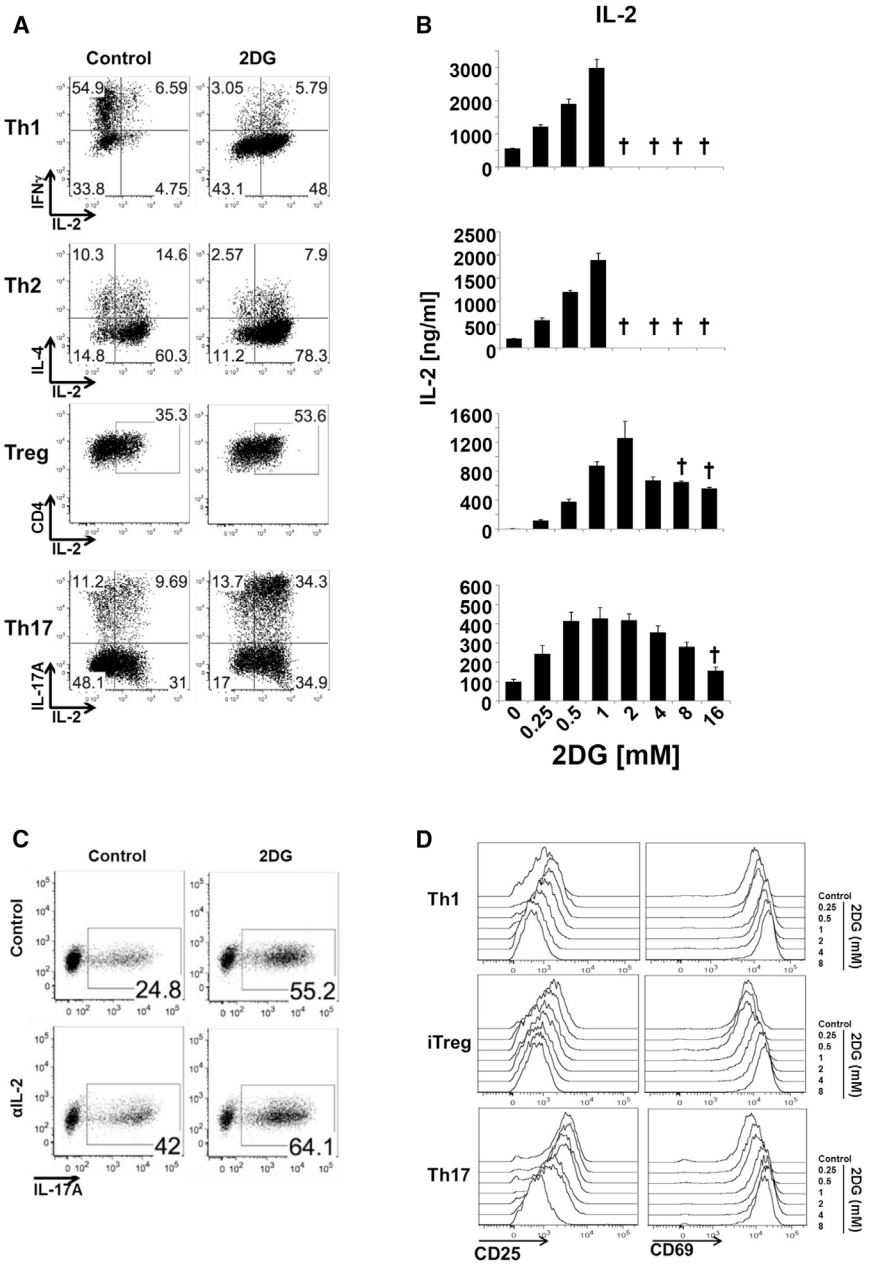

Figure 2. Limited Glucose Metabolism Reduces IL-2R Expression

Naive mouse CD4$^+$ T cells were cultured on anti-CD3/CD28-coated wells for 72 hr under indicated Th subset polarization conditions.

(A) Indicated Th cells cultured in the presence or absence of 1 mM 2DG were stained intracellularly for indicated cytokines and IL-2.

(B) IL-2 mRNA expression analysis by rea-time PCR of indicated Th subsets with concentration range, 0.25–16 mM, of 2DG for indicated gene products. Ct values were normalized to Hprt and the relative expression is displayed. †Indicates over 50% cell death.

(C) Naive T cells cultured under Th17 cell polarizing conditions and 2DG (left) and neutralizing anti-IL-2 (bottom row) were assessed for IL-17A expression by flow cytometry.

(D) Naive T cells cultured under indicated Th polarizing conditions and concentrations of 2DG for 20 hr were assessed for extracellular CD25 (left column) and CD69 (right column) intensity of staining by flow cytometry. Data are representative of at least three biological repeats. Error bars represent SD of pooled data (n = 6).

Cell Stress Enhances Th17 Cell Polarization

Reduced glucose metabolism induces the expression of glucose-regulated proteins (GRP), especially GRP78 required for the correct assembly of proteins in the ER (Wang and Kaufman, 2014). Any deregulation of protein assembly results in ER stress. Upon altering metabolite levels during culture conditions during Th17 cell differentiation, we noticed a marked increase in polarization of naive T cells toward the Th17 cell subset (Figures 3 and S3). Hypoxia resulted in the increase of Th17 cell differentiation, supported by HIF1α (Figure S3A) (Dang et al., 2011; Shi et al., 2011; Wang et al., 2014). Sodium chloride, previously shown to increase Th17 cell differentiation via serum glucocorticoid kinase (SGK)1 (Kleinewietfeld et al., 2013; Wu et al., 2013), an immediate early response gene induced following cellular stress (Leong et al., 2003), resulted in decreased levels of Th1 and Th2 cell polarization but increased levels of Th17 cell differentiation (Figure 3A). Changing ionic pressure with the sugar mannitol equally resulted in dose-dependent increased Th17 cell differentiation but no other Th subsets (Figure S3C). Furthermore, known ER stress inducers, such as the N-linked glycosylation inhibitor tunicamycin, had a marked stimulatory effect on Th17 cell differentiation but not on other Th cells (Figure 3B). Thapsigargin, an irreversible inhibitor of the sarco/endoplasmic reticulum calcium ATPase (SERCA) that prevents reabsorption of calcium into the ER resulting in a strong cell stress response (Kaufman, 1999), robustly enhanced the generation of Th17 cells (Figure 3B). Cyclopiazonic acid (CPA), a reversible selective inhibitor of SERCA (Moncoq et al., 2007), had a similar effect on Th cell polarization compared with thapsigargin (Figures 3C and S3D). Because the inhibitory effect of CPA on SERCA can be reversed, we cultured naive T cells in the presence of CPA for different lengths of time. This revealed the need for SERCA inhibition, resulting in increased levels of cytoplasmic calcium and cell stress, over at least a 48 hr period to enhance Th17 cell polarization (Figure 3D). Stress alone was insufficient to reduce IL-2Rα expression (Figures 3B and S3B). Thus, this set of data suggests that conditions resulting in cell stress, although not essential or required, can enhance Th17 cell differentiation.

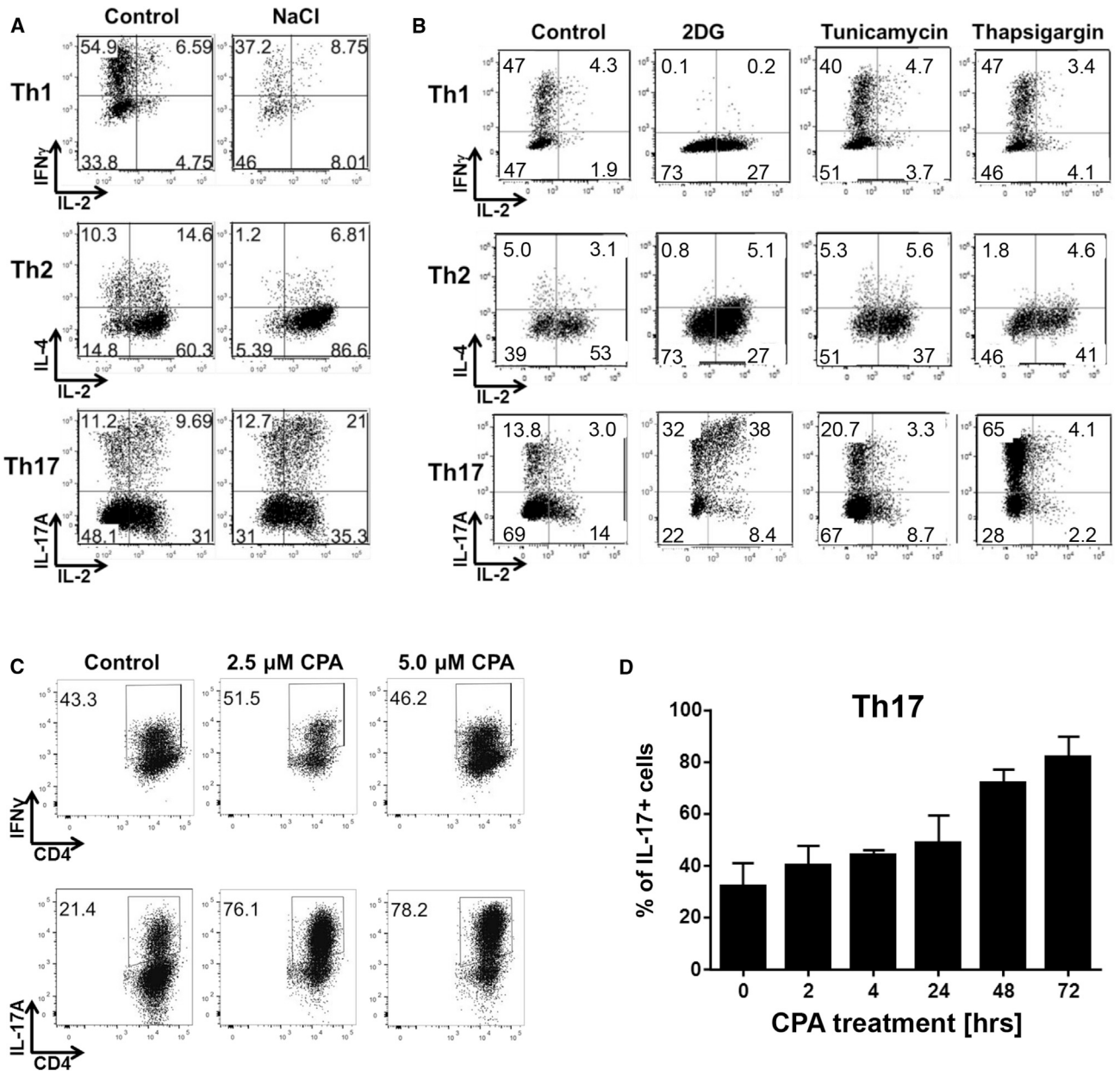

Figure 3. Cellular Stress Specifically Enhances Th17 Cell Differentiation

Naive mouse CD4$^+$ T cells were cultured on anti-CD3/CD28-coated wells for 72 hr under indicated Th subset polarization conditions.

(A–C) Naive T cells were cultured under indicated Th cell polarization conditions in the presence of 50 mM NaCl (A), 1 mM 2DG, 100 ng/mL tunicamycin, or 0.5 nM thapsigargin (B), or 2.5 or 5 μM CPA (C), and stained intracellularly for indicated cytokines.

(D) Naive T cells were cultured under Th17 cell polarization conditions in the presence of 2.5 μM CPA for indicated time points after which CPA was removed. Average percentage of IL-17-producing cells from four biological repeats is shown (n = 4). All data are representative of at least four independent biological repeats.

Cell Stress Inhibitors Reduce Th17 Cell Differentiation

Several pharmacological compounds have been shown to be able to alleviate cell stress. However, the stress response consists of many pathways. The ER-stress response alone consists of three interconnected branches: the double-stranded RNA-activated protein kinase-like ER kinase (PERK), the activation transcription factor (ATF)6, and the inositol requiring protein (IRE)1, collectively increasing ER-resident chaperones, enlarging the ER, reducing gene transcription, and increasing ER-associated degradation (ERAD). We tested known inhibitors, affecting particular cell- and ER-stress branches, for their ability to influence Th17 cell polarization. The PERK inhibitor GSK2606414

dose-dependently counteracted enhanced Th17 cell differentiation, most strongly when cell stress was induced with limited glucose availability (Figures 4A, 4B, and S4H), without affecting the polarization of other Th cells (Figures S4A and S4H). The IRE1 inhibitor 4μ8c (Cross et al., 2012), inhibited Th17 cell polarization under conditions of hypoglycemia and osmotic stress (Figures 4A, 4B, and S4I), without adversely affecting the polarization of other Th cell subsets (Figures S4B and S4I). The bile acid tauroursodeoxycholic acid (TUDCA), an inhibitor of cell stress (Keestra-Gounder et al., 2016; Xie et al., 2002), inhibited Th17 cell differentiation and counteracted the effect of hypoglycemia, osmotic stress, and hypoxia (Figures 4A, 4B, and S4G). TUDCA did not adversely affect the polarization of other tested effector T cell subsets (Figures 4A, 4B, S4B, and S4G). In order to assess if cell stress may play a role at the early stages of T cell differentiation, we added stress inducers or inhibitors at different times after T cell activation and polarization. Both inducers and inhibitors influenced Th17 cell polarization within the first hours of culture (Figure S4C). This indicated the immediate-early enhancing effect of cell stress on Th17 cell polarization in addition to the required maintenance of stress levels for at least the first 48 hr to efficiently boost Th17 cell polarization (Figure 3D). These results highlight that situations ultimately resulting in cell stress can skew T cell responses toward the Th17 cell subset.

XBP1 Influences Th17 Cell Differentiation

The ER-stress response results in transcriptional activity in which several factors are implicated (Kaufman, 1999). CCAAT/enhancer binding homologous protein (CHOP), downstream of the PERK branch, has been implicated in cytokine secretion in dendritic cells (Goodall et al., 2010). However, naive $CD4^+$ T cells deficient in CHOP did not show altered Th subset polarization potential compared with controls upon in vitro polarization (Figure 4C, data not shown). X-box-binding-protein (XBP) 1 mRNA, the most conserved ER-stress pathway regulating the expression of a subset of ER chaperone genes (Cox and Walter, 1996), was recently identified in a genome-wide temporal analysis of genes involved in Th17 cell differentiation (Yosef et al., 2013). Naive T cells isolated from $XBP1^{fl/fl}$ or $Rag1^{cre}$ $XBP1^{fl/fl}$ (from now on referred to as $XBP1^{ko/ko}$) mice did not differ under Th1, Th2, or Treg cell differentiation conditions and remained sensitive to stress-induced cell death (Figure S4D). Under Th17 cell polarization, reduced levels of IL-17 and RORγt were observed in the absence of *Xbp1* (Figures 4D, S4E, and S4F). Conditions of osmotic stress and hypoglycemia were able to enhance Th17 cell differentiation in the absence of XBP1, albeit with reduced levels compared with controls, while stress inhibitors reduced Th17 cell development in $XBP1^{ko/ko}$ more efficiently compared with $XBP1^{fl/fl}$ controls (Figure S4F). XBP1 was, however, required for enhanced Th17 cell polarization under hypoxic conditions (Figures 4D and S4F). The reduced response to conditions of hypoxia in the absence of *Xbp1* is in line with its requirement to form a transcriptional complex with HIF1α that regulates the expression of hypoxia response genes in tumors (Chen et al., 2014). Collectively, these results highlight that XBP1 plays a supporting role in enhancing Th17 cell differentiation under cell stress conditions.

Cell Stress Results in Sustained Levels of Intracellular Calcium

Cellular stress is characterized by calcium release from the ER into the cytoplasm leading to a cellular response (Brickley et al., 2013). In T cells, calcium signals are required to recruit and retain nuclear factor of activated T cells (NFAT) in the nucleus for the expression of cytokines such as IL-2 and IL-17 (Hermann-Kleiter and Baier, 2010). In line with the requirement of calcium for TCR signaling and T cell activation, blocking calcium release-activated channels (CRAC) with YM-58483 (BTP2) showed a reduction in polarization of all Th subsets tested (Figure S5A). However, it did indicate a heightened requirement for calcium signaling for Th17 cell differentiation compared with other Th cells. We observed that T cells polarized in the presence of TGF-β, namely Th17 and Treg cells, show a sustained high intracellular calcium level compared with Th1 cells after 20 hr of activation (Figure 5A). Furthermore, we confirmed that cytoplasmic calcium levels were increased upon co-culture with compounds enhancing Th17 cell differentiation during Th cells cultures (Figures 5A, S5B, and S5C), and the calcium ionophore ionomycin markedly increases Th17 cell polarization (Figures S5D and S5I). These data indicate that environmental changes in metabolite levels or ionic pressure can result in increased cytoplasmic calcium levels via induction of cell stress, thereby enhancing Th17 cell polarization.

Cell Stress in a Pro-inflammatory Environment Is Sufficient for De Novo Th17 Cell Differentiation

Although we and others previously reported that a combination of cytokines, IL-6, and TGF-β is sufficient to generate Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a), the signaling requirements downstream of the TGF-β receptor remain unknown. We observed that the Th17 cell-enhancing effect of cell stress inducers is most apparent under Th17 cell differentiation conditions with reduced levels of TGF-β (data not shown). Strikingly, in vitro stimulation of naive T cells in the presence of IL-6 and stress inducers, but not cell stress inducers alone, was sufficient to induce Th17 cell development, even when TGF-β was neutralized (Figures 5B and S5E). Furthermore, the presence of additional pro-inflammatory cytokines, IL-1β, TNF, or IL-23, increased the efficiency of Th17 cell development under conditions of ER-stress while neutralizing TGF-β (Figure 5C). In addition, we show that IL-6 in combination with cell stress is sufficient for the expression of RORγt (Figure S5F). To further assess the requirement for TGF-β, we used naive $CD4^+$ T cells sourced from dominant-negative TGF-βRII (dnTGF-βRII) mice, in which signaling via TGF-βRII is abrogated (Gorelik and Flavell, 2000; Veldhoen et al., 2006b). We confirmed that naive $CD4^+$ T cells sourced from dnTGF-βRII mice, in contrast to wild-type animals, were not able to polarize to Th17 cells in the presence of IL-6 and TGF-β, but generated Th1 cells similarly or better to controls (Figures 5D, 5E, S5F, and S5G). TGF-βRII signaling was, however, not essential for stress-induced de novo Th17 polarization under inflammatory conditions that could be efficiently inhibited by TUDCA (Figure 5D). Furthermore, we show that XBP1 facilitates stress-induced Th17 cell polarization, is essential under conditions of hypoxia, and can be efficiently inhibited with TUDCA (Figure S5H). Collectively, these data

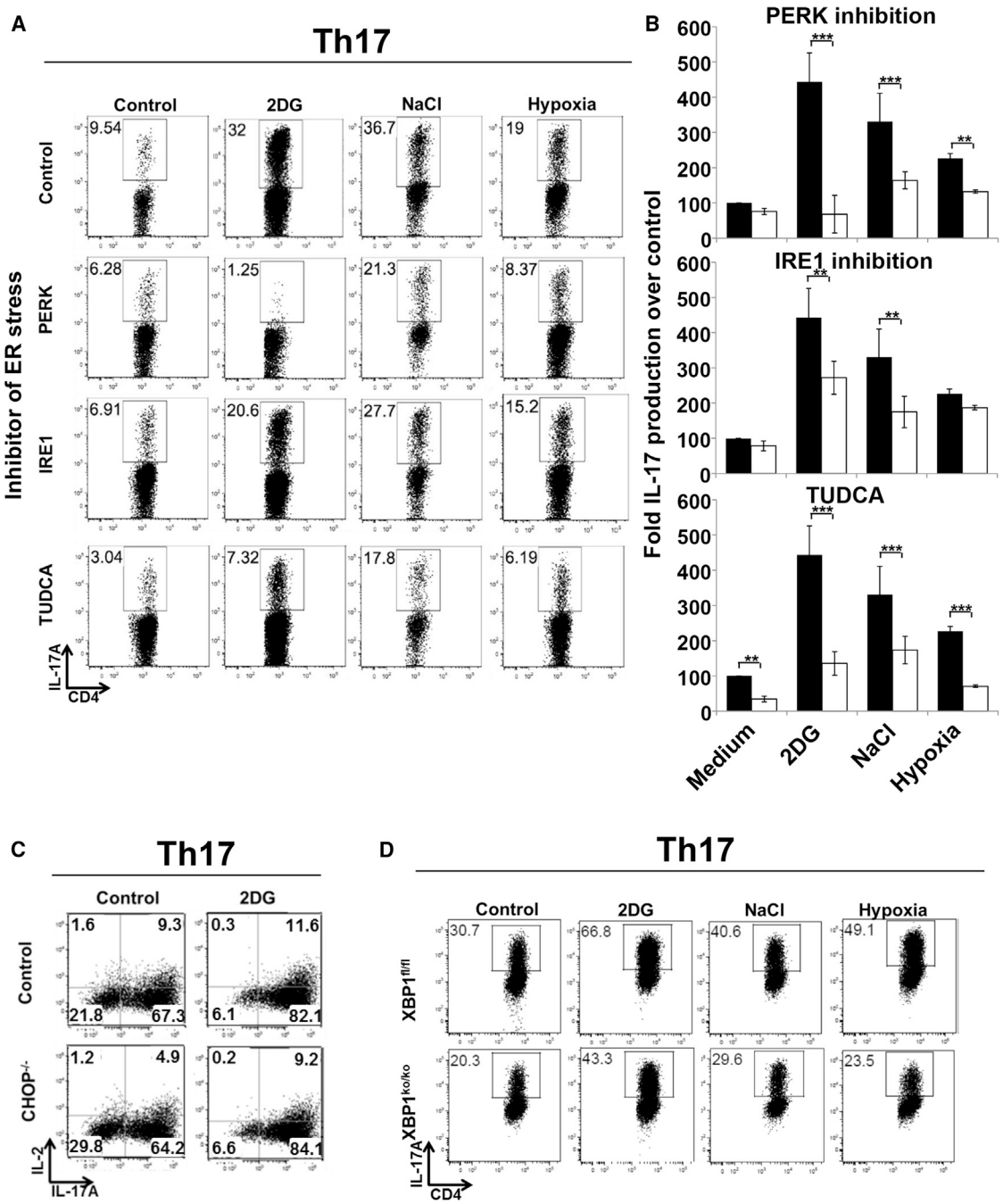

Figure 4. Cellular Stress Enhances Th17 Cell Differentiation

Naive mouse CD4$^+$ T cells were cultured on anti-CD3/CD28-coated wells for 72 hr under Th17 cell polarization conditions.

(A) T cells cultured under indicated ER stress-inducing conditions, 2DG (second column), NaCl (third column), and hypoxia (fourth column), were co-cultured with known inhibitors of cell stress-response pathways, PERK inhibitor GSK2606414 (second row), IRE1 inhibitor 4μ8c (third row), and tauroursodeoxycholic acid (TUDCA) (fourth row).

(B) Average level of IL-17 production compared with control (= 100) of conditions described under (A) from six independent biological repeats (black bars control, white bars with inhibitor).

(C) Naive T cells isolated from controls (top) or CHOP$^{-/-}$ (bottom) mice were cultured under control (right column) or reduced glucose metabolism (right column) and assessed for IL-17 production by flow cytometry.

(D) Naive T cells isolated from control XBP1$^{fl/fl}$ (top row) or Rag1^{Cre} XBP1$^{fl/fl}$ (XBP1$^{ko/ko}$) (bottom row) mice were cultured under control (first column), limited glucose metabolism (2DG, second column), osmotic stress (NaCl, third column), or limited oxygen pressure (hypoxia, fourth column) and assessed for IL-17 production by flow cytometry. **$p < 0.01$, ***$p < 0.001$.

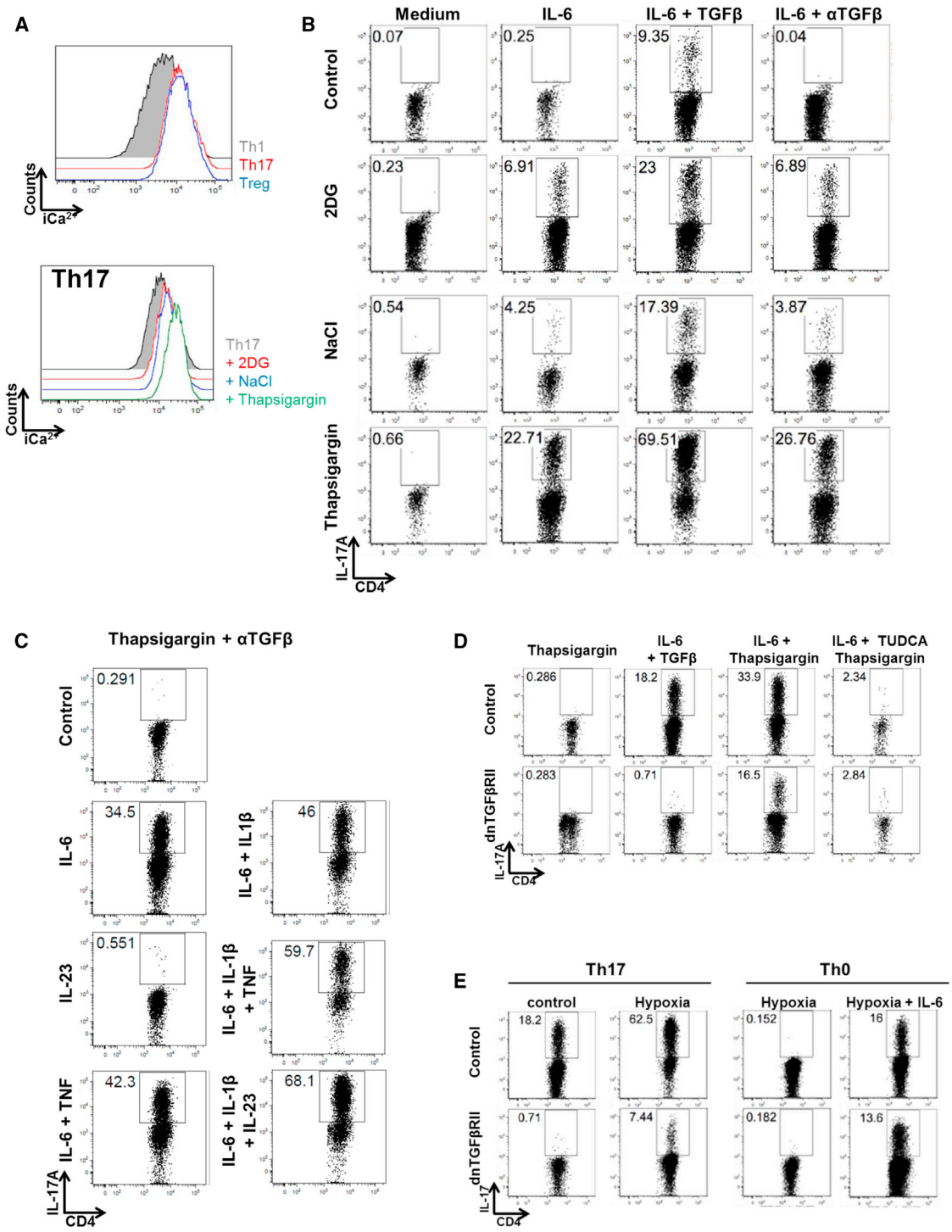

Figure 5. Inflammatory and Cellular Stress Environment Can Drive Th17 Polarization

Naive mouse $CD4^+$ T cells were cultured on anti-CD3/CD28-coated wells under indicated Th subset polarization conditions.

(A) Upon 20 hr of culture, Th1, Th17, and Treg cells were assessed for cytoplasmic calcium levels by flow cytometry (top). Naive T cells cultured under Th17 cell differentiation conditions in the presence of indicated ER-stress inducers were assessed for cytoplasmic calcium levels by flow cytometry (bottom).

(B and C) Naive T cells were cultured under (B) neutral conditions (first column), IL-6 only (second column), Th17 conditions (TGF-β, IL-6, anti-IFNγ, and anti-IL-4) (third column), or IL-6 and neutralizing anti-TGF-β (fourth column), and in the presence of indicated ER-stress inducers (rows) or (C) with thapsigargin and neutralizing anti-TGF-β in the presence of indicated cytokines. Cells were assessed on day 3 for Th17 cell differentiation by intracellular staining for IL-17.

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demonstrated that inflammatory conditions and a microenvironment inducing cellular stress can be sufficient to skew the T cell response in favor of Th17 cell differentiation in a TGFβ-independent manner.

Cellular Stress Can Contribute to Immunopathology

TUDCA is used to reduce the cellular stress caused by chemotherapeutic agents, reperfusion, and steroids in humans. We confirmed it does not affect T cell proliferation or cell viability (Figure S6A and data not shown). This and its water solubility make TUDCA an ideal compound for in vivo dosing to test its potential to reduce Th17 cell-mediated pathology. Using the Th17 cell-dependent multiple sclerosis mouse model, experimental autoimmune encephalomyelitis (EAE) (Veldhoen et al., 2006b), daily dosing of TUDCA was able to significantly delay the onset of signs of EAE compared to controls (Figures 6A, 6B, and S6B). The dosing of TUDCA significantly reduced the number of T cells found in the CNS at day 12 after immunization, while at that point in time the lymph node draining the immunization site showed a trend to lower Th17 cell numbers and proportions and an increase in Th1 cells (Figures 6C, 6D, and S6C). However, clinical signs of EAE did develop from day 14 and T cell cytokine production from TUDCA-treated mice was indistinguishable from controls at late time points (data not shown). In line with in vitro obtained results that cell stress has a marked influence on early Th17 cell differentiation, the dosing of mice at the start of clinical manifestations of EAE with TUDCA had no effect on disease progression compared with control animals or IL-17 or IFNγ producing $CD4^+$ T cells in the CNS (Figures 6E and S6D). Because the administration of TUDCA has the potential to reduce cellular stress experienced by any cell type, we made use of mice in which we conditionally excised Xbp1 in lymphocytes; $Rag1^{Cre}$ $Xbp1^{fl/fl}$. Compared to wild-type and $Xbp1^{fl/fl}$ controls, which responded very similar to EAE induction, the conditional excision of Xbp1 in lymphocytes resulted in a delayed onset and clinical score EAE symptoms (Figure 6F).

Subsequently, we analyzed the transcriptional profile of Th17 cells generated from naive precursors with help of classic IL-6 and TGF-β or with IL-6 and the ER stress-inducer CPA (Figure 2D). To avoid polarization differences in total cell cultures, we made use of IL-17A reporter mice and sorted fluorescent positive cells prior to analysis. Of note, despite similar levels of *Il17* and *Rorc*, we observed marked reduction of *Ahr* and *Il10* as well as increase of *Tbx21* and *Ifng* (Figures 6G and S6E). The observed gene expression profile observed in cell stress-generated T cells resembles that observed in Th17 cells differentiated with IL-1β, IL-6, and IL-23 in the absence of TGF-β, which were shown to be highly pathogenic (Ghoreschi et al., 2010). Mice adoptively transferred with T cells generated in the presence of ER-stress inducer CPA showed delayed signs of classic EAE, starting with tail paralysis, compared with T cells generated in the presence of TGF-β (Figure 6H). However, transfer of Th17 cells generated under stress resulted in atypical EAE caused by exacerbated brain inflammation whereby mice exhibit a variety of clinical symptoms ranging from ataxia to unilateral paralysis (Figure 6I). Atypical EAE was observed despite the observed increase in Th1-associated transcripts but is in line with the increased Th17 cell ratio as previously reported (Stromnes et al., 2008) (Figure S6) and observed under ER stress-inducing conditions. Collectively, these data show that stress can facilitate the generation of Th17 cells in vivo and aggravate (auto) immunity.

DISCUSSION

The present study shows that de novo Th17 cell differentiation can be initiated under combined conditions of inflammation and those inducing cellular stress, such as restricted metabolite availability or ionic pressure and describes an unexpected role for the stress response in skewing T cell polarization toward a Th17 cell response and contributing to autoimmunity in the EAE mouse model.

Th17 cells have evolved to offer protection against potential threats at epithelial barrier sites, as suggested by the enrichment of IL-17 expressing lymphocytes at these sites observed in cyclostomes to vertebrates (Hirano et al., 2013). Trauma or tissue damage, conserved microbe- or danger-associated molecular patterns trigger receptors on innate immune cells resulting in the production of a range of pro-inflammatory cytokines. IL-6 is produced upon inflammation as well as injury as part of the acute-phase response and has context-dependent pro- and anti-inflammatory properties (Hunter and Jones, 2015). Recently, pathogen recognition receptor triggering was shown to be an important mediator of stress-induced inflammation, triggering the production of IL-6 (Keestra-Gounder et al., 2016), connecting cellular stress with innate immunity. We showed that the presence of TGF-β provides the context required to generate Th17 cells (Veldhoen et al., 2006a). However, the essential requirement for TGF-β in this process has been questioned (Ghoreschi et al., 2010). Here, we reconcile these seeming discrepancies by showing that metabolic disturbances in the local environment facilitate the polarization of Th17 cells and can be sufficient for de novo Th17 cell differentiation in the absence of TGF-β via induction of the cells' stress response. Furthermore, we found a high correlation in transcripts found in purified Th17 cell generated in the presence of stress with those described by Ghoreschi et al. (2010) in the absence of TGF-β, such as reduced expression of *Ahr*, *Il22*, *Il10*, and *Il17f* and increased expression of the Th1 cell-associated genes *Tbx21* and *IFNγ*. Disturbances in the local environment link with the fact that invading pathogens or products will often disrupt cellular pathways as well as compete for finite resources, while cellular stress and malignancy alters metabolite availability, altering the microenvironment (Cairns et al., 2011). Although it

(D and E) Naive T cells derived from dnTGF-βRII (bottom rows) or controls (top rows) were cultured with (D) indicated cytokines, thapsigargin or TUDCA, or (E) cultured under Th17 or Th0 cell polarization conditions in normoxia or hypoxia as indicated. Cells were assessed on day 3 for Th17 differentiation by intracellular staining for IL-17. Results are representative of three (A, C, D, and E) or six (B) experiments.

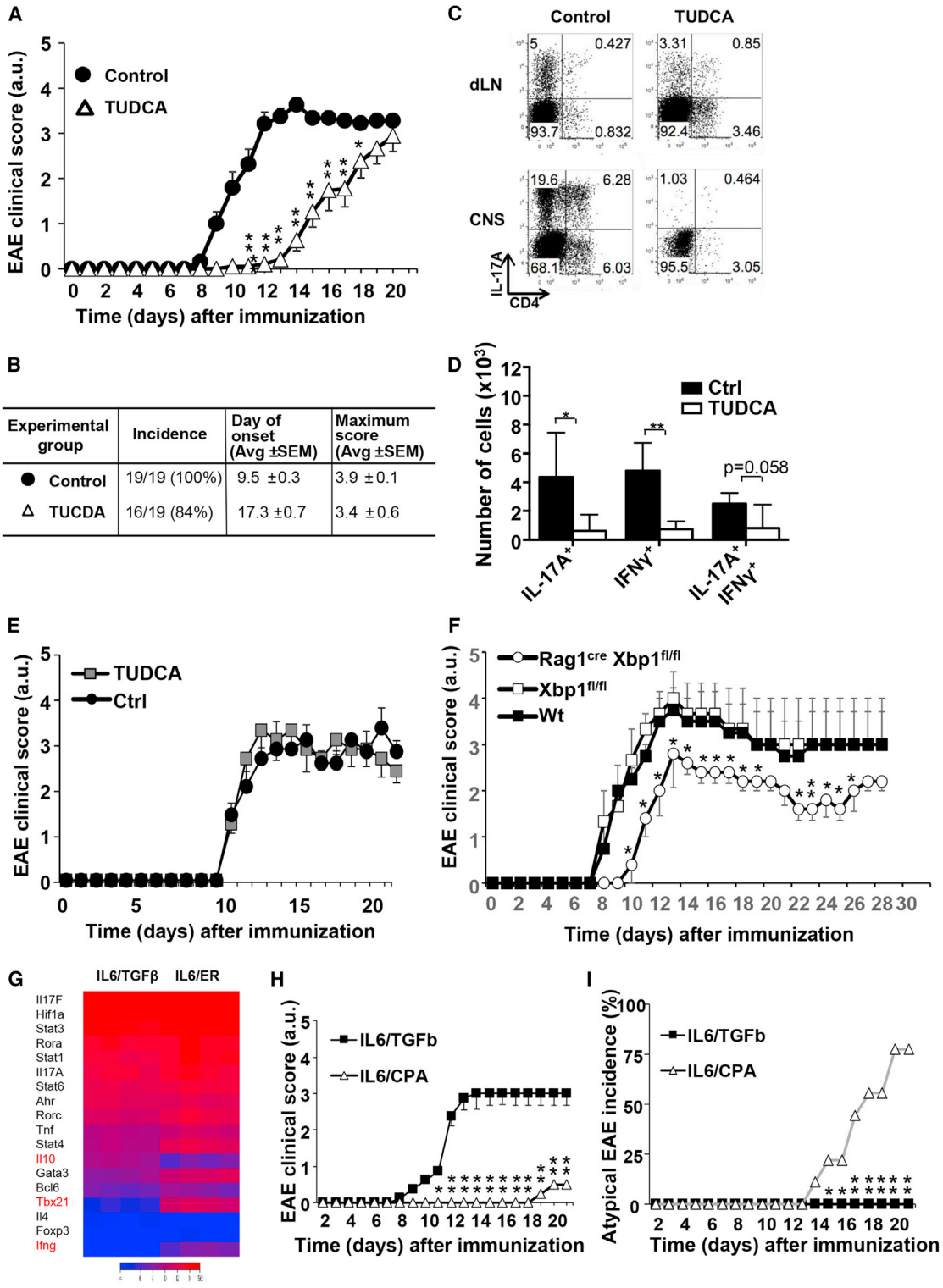

Figure 6. Inhibition of Stress Reduces Onset of EAE

C57BL/6 mice were subjected to MOG/CFA administration to induce EAE and daily PBS or 250 mg/kg TUDCA dosing.

(A and B) Clinical EAE scores (A) and incidence (B), average day of onset and maximum score data from mice immunized with MOG/CFA and PBS (filled symbols) or TUDCA (open symbols) (n = 19 from three biological repeats).

(C) Flow cytometry for IFNγ and IL-17 of $CD4^+$ T cells harvested from inguinal draining lymph node (iLN) or CNS 12 days after EAE induction.

(legend continued on next page)

is clear that T cell function is intimately linked to metabolic programs, whether and how environmental cues and cellular metabolism influence the outcome of T cell-mediated immune responses remains to be fully understood. We confirmed the importance of glucose metabolism for efficient polarization toward the Th1, Th2, and Treg cell lineages. The reduction in glycosylation and the surface levels of IL-2Rα may explain the sensitivity of Th1, Th2, and Treg cells to 2DG and BrPA with all three subsets depending on IL-2 signaling for their differentiation, proliferation, and survival (Moriggl et al., 1999). Although Th17 cell polarization is negatively affected by IL-2 (Laurence et al., 2007), and hence reduced IL-2Rα may contribute to enhanced Th17 differentiation, this seems only minor contributing factor for increased Th17 cell polarization. We did not observe reduced IL-2Rα expression levels under conditions with exogenous mannose, nor other stress conditions in which enhanced Th17 cell polarization was observed. Furthermore, although stress inhibitors reduced Th17 cell polarization, IL-2Rα expression was not restored. Of note, our observations with respect to reduced Treg and enhanced Th17 cell differentiation in the presence of similar concentrations of 2DG are in contrast to a recent report (Shi et al., 2011). We did note that reduced cell density at the start of the cultures can have a significant effect on Treg differentiation and the inhibitory efficiency of 2DG but consistently observed enhanced Th17 cell polarization upon co-culture with 2DG or BrPA.

Cellular stress response can be initiated by GPR78 via mechanisms not fully understood (Kaufman et al., 2002). Stress has a substantial influence on immunity, but efforts have largely concentrated on non-lymphoid cells and B cells, but not T lymphocytes (Todd et al., 2008). The expression of the inflammatory cytokines IL-6 and IL-23 by DCs, strongly associated with the IL-17 response (Stockinger and Veldhoen, 2007), is enhanced by the stress response (Goodall et al., 2010; Keestra-Gounder et al., 2016). In addition to conditions of limited glucose availability, we show that other environmental disturbances such as changes in sodium and oxygen levels enhance Th17 cell polarization. This is in agreement with previous reports highlighting a role for hypoxia, via HIF1α (Dang et al., 2011; Shi et al., 2011; Wang et al., 2014) and sodium pressure (Kleinewietfeld et al., 2013; Wu et al., 2013), known stress inducers, in supporting Th17 cell differentiation. Furthermore, we use the prototypical ER-stress inducers, thapsigargin and CPA, which markedly enhanced Th17 cell polarization. Our data indicate the stress response as a common, but heterogeneous, molecular mechanism underlying these observations. Furthermore, we show that depending on the identity of the stress inducer, at least two main branches of the stress response, IRE1 and PERK, play a role in supporting Th17 polarization.

One of the most conserved elements of the stress pathway, the transcription factor XBP1, has been associated with Th17 cell biology (Yosef et al., 2013). We show now that XBP1 is a mediator of Th17 cell differentiation. The generation of Th17 cells, via IL-6 and TGF-β, was reduced in the absence of XBP1 and mediators of stress-induced Th17 cells in the presence of IL-6 markedly less efficient without XBP1. This suggests that TGF-β, known to inhibit the activation and proliferation of lymphocytes, upon opposite instructions from IL-6, may result in cellular stress and that XBP1 transcriptional activity may directly regulate *Rorc*. This is in line with the reduced response to conditions of hypoxia in the absence of XBP1 that requires the formation of a transcriptional complex with HIF1α that regulates the expression of hypoxia response genes in tumors (Chen et al., 2014). HIF1α is known to be able to bind the *Rorc* promoter and enhance Th17 cell differentiation (Dang et al., 2011).

Th17 cells have been closely associated with immunopathology in disorders such as rheumatoid arthritis (RA), multiple sclerosis (MS), lupus erythematosus, psoriasis, graft versus host disease, and inflammatory bowel disease (IBD) and hence are a major therapeutic target (Miossec and Kolls, 2012). We show, in a mouse model of MS, that reducing cellular stress with daily dosing of TUDCA at the start of EAE induction, but not at clinical onset, can markedly reduce EAE. This is in line with the effects on enhancing Th17 cell differentiation we observed in vitro. However, clinical disease did ultimately manifest itself and reached similar levels as those observed in control animals. Excision of *Xbp1* in all lymphocytes during development resulted in a delayed onset and reduced clinical score of EAE. The enhancement of Th17 cell differentiation under conditions of environmental stress may, however, have direct implications in the establishment and maintenance of autoimmunity and chronic inflammatory conditions. Induction of cellular stress prior to T cell transfer in a model of passive EAE resulted in marked differences in EAE with Th17 cell-dominated atypical EAE observed only with conditions of added ER stress induction. This is in agreement with previous reports on different roles of mixtures of Th1/Th17 cells and Th17 cell-dominant populations in the EAE model (Stromnes et al., 2008). IBD disorders, strongly linked with the IL-17/IL-23 inflammatory axis, are associated with loss of barrier integrity, enabling bacterial translocation, inflammation, and metabolic alterations, as well as tissue damage, resulting in inflammation and tissue stress (Maloy and Powrie, 2011). Equally, the microenvironment in the synovia of arthritic joints may constitute a niche for Th17 cell development with high levels

(D) Total T cell numbers expressing indicated cytokines harvested from the CNS 12 days after EAE induction are shown in control (black bars) and TUDCA-treated (open bars) mice. Data were obtained from six mice of each condition.
(E) Clinical EAE scores upon control (black circles) or TUDCA (gray squares) dosing at day 9 after EAE induction (n = 12 from two biological repeats).
(F) Clinical EAE scores from control C57BL/6 (closed squares), $Xbp1^{fl/fl}$ (open squares), and $Rag1^{-Cre}$ $Xbp1^{fl/fl}$ (open circles) mice (n = 11 from two biological repeats).
(G) Gene expression profile of indicated transcripts in Th17 cells generated from naive precursors with help of IL-6/TGF-β (left four columns) or IL-6/CPA (right four columns). Each column represents a biological repeat.
(H) Clinical classic EAE scores upon adoptive transfer of 2D2 T cells restimulated with MOG peptide and indicated cytokines prior to cell transfer (n = 9 from two biological repeats).
(I) Incidence of atypical EAE from the same experiment as shown under (H). Error bars represent SD of pooled data. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$; student's t test. EAE clinical scores and day of onset were assessed by Mann-Whitney test on each separate day.

of ER-stress (Park et al., 2014). Of note, in connective tissues between tendon or ligament and bone, the entheses, known to be under constant (mechanical and inflammatory) stress, a resident population of IL-17 producing T cells can be found that contributes to rheumatic diseases (Sherlock et al., 2012). Furthermore, our data may have implications for tumor immunology where tumor cells consume more metabolites than their requirement to proliferate and grow (Vander Heiden et al., 2009), actively altering their microenvironment and thereby influencing immunity (Chang et al., 2015; Cubillos-Ruiz et al., 2015; Ho et al., 2015). DCs have been shown to undergo ER-stress in a tumor environment (Cubillos-Ruiz et al., 2015). In line with this, Th17 cells are enriched in tumor environments and associated with cancer promoting processes such as angiogenesis (Numasaki et al., 2003). Grivennikov et al. (2012) revealed that established tumors facilitate microbial entry, which leads to an exacerbated tumor growth through the production of IL-17 by $CD4^+$ T cells.

The ability of ER-stress and sustained cytoplasmic calcium levels to enhance or induce Th17 cell-mediated responses may offer additional insights in the development and maintenance of immunopathology and autoimmunity such as resulting from infections or tissue insults.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6J and $Rag1^{Cre}$ $XBP1^{fl/fl}$ were bred at the Babraham Institute and Instituto de Medicina Molecular. The $XBP1^{fl}$ mice (Hetz et al., 2008) were kindly provided by Drs. L.H. Glimcher and A. Kaser. $Chop^{-/-}$ (*Ddit3*) (Zinszner et al., 1998) spleens were kindly provided by Dr. A. Kaser, $Vav1^{Cre}$ $HIF1\alpha^{fl/fl}$ and controls spleens were kindly provided by Dr. D. Cantrell. Dominant negative TGF-βR2 (dnTGF-βRII) mice were maintained in AniCan, a specific pathogen-free animal facility of the Centre Léon Bérard, Lyon, France. 2D2 mice were maintained at the Instituto Gulbenkian de Ciência and Instituto de Medicina Molecular Lisbon. All animals were bred and maintained under specific pathogen-free conditions, male and female mice were used between the ages of 8–12 weeks. All experiments were performed in accordance with the United Kingdom home office, Direção-Geral de Alimentação e Veterinária Portugal, and local ethical boards and guidelines.

In Vitro T Cell Cultures

For mouse T cell differentiation, naive $CD4^+CD62L^+$ T cells were isolated from spleens by magnetic beads following manufacturer's instructions (Miltenyi Biotec, Naive mouse T cell isolation kit II) or by $CD4^+CD62L^+CD25^-CD44^{lo}$ flow cytometry as described before (Veldhoen et al., 2006a). The culture medium used was Iscove's Modified Dulbecco's Media (IMDM) supplemented with 2×10^{-3} M L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5×10^{-5} M β-mercaptoethanol, and 5% FBS. A total of 5×10^5 Th cells were differentiated on plates coated with 2 μg/mL anti-CD3 (clone 2C11, BioXcell) and 2 μg/mL anti-CD28 (clone 37.51, BioXcell) in presence of: Th1, 2 ng/mL IL-12 (PeproTech); Th2, 10 ng/mL IL-4 (PeproTech) and 5 μg/mL anti-IFNγ (clone XMG1.2, BioXcell); Treg, 5 ng/mL TGF-β (PeproTech), 5 μg/mL anti-IL-4 (clone 11B11, BioXcell), and 5 μg/mL anti-IFNγ (BioXcell); and Th17, 20 ng/mL IL-6 (PeproTech) and 0.2 ng/mL TGF-β (Peprotech), 5 μg/mL anti-IFNγ (BioXcell) and 5 μg/mL anti-IL-4 (BioXcell). For indicated conditions, 5 ng/mL IL-2 (PeproTech) or 5 μg/mL neutralizing IL-2 antibodies (clone JES6-1A12, BioLegend), or 10 μg/mL neutralizing anti-TGF-β1,2,3 anti-body (clone 1D11, R&D) were added. For T cell proliferation, naive $CD4^+CD62L^+$ T cells were loaded with 2.5 μM CFSE (LifeSciences). Hypoxia experiments were performed using 1% oxygen in a Galaxy 48R incubator (New Brunswick).

Cells were pre-treated for 1 hr with indicated ER inhibitors and concentrations; 4μ8c (5 μM, Tocris), GSK2606414 (0.5μM, Tocris), TUDCA (0.1–0.5 mM, Sigma), or KN-93 (1 μM, Sigma) after which they were cultured on antibody coated plates in the presence or absence of indicated ER-stress inducers and concentrations; 2DG (0.25–16 mM), BrPA (1–10 μM), NaCl (40–50 mM), tunicamycin (100 ng/mL), or thapsigargin (0.5 nM), CPA (2.5–5 μM) (all Sigma).

Flow Cytometry

Single-cell suspensions were prepared from spleens, lymph nodes, and spinal cord. CNS-infiltrating immune cells were isolated from the spinal cord by isolating the soft tissue from the spine and mashing the spinal cord through 70-μm mesh filter followed by 36.5% Percoll separation. The proportion and absolute numbers of T cells were determined with help of counting beads (Spherotech). For cytokine profiles, cells were re-stimulated for 3 hr with 500 ng/mL of PdBU (Sigma) and 500 ng/mL of ionomycin (Sigma) in the presence of 1 μg/mL Brefeldin A (Sigma) and were stained with anti-CD4, anti-CD25, anti-IL-17A, anti-IFNγ, anti-IL-4, and Foxp3 (all Biolegend). A viability dye was used to exclude dead cells. All samples were analyzed on a Fortessa 4 or 5 cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

Calcium Detection

Intracellular calcium was determined by using the PBX Calcium Assay (BD). Briefly, cultured cells were mixed with an equal volume of 1× dye-loading solution containing the calcium indicator (according to the manufacturer's instructions). After 1 hr incubation at 37°C, cells were washed twice with Hank's balanced salt solution (HBSS), stained with antibodies, and analyzed on a BD LSRFortessa flow cytometer.

EAE Induction

EAE was actively induced by subcutaneously injecting 250 μL MOG_{35-55} peptide (ProImmune Ltd.) emulsified in IFA (Sigma) supplemented with 250 μL mycobacterium tuberculosis extract H37Ra (Difco). Additionally, the animals received intraperitoneally (i.p.) 200 ng pertussis toxin (List Biological Laboratories) on days 0 and 2. Clinical signs of EAE were assessed according to the following scores: 0, no signs of disease; 1, flaccid tail; 2, impaired righting reflex and/or gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; and 5, total hind limb paralysis with partial forelimb paralysis. TUDCA treatment commenced at 500 mg/kg at the day prior to MOG/CFA immunization and was followed with daily i.p. administration at 250 mg/kg. Passive EAE was induced by harvesting lymph nodes and spleens from MOG/CFA immunized 2D2 mice. Single cells suspensions were restimulated with 50 μg/mL MOG peptide for 4 days with IL-6 (20 ng/mL) and either TGF-β (0.5 ng/mL) or CPA (2 μM). Subsequently, cells were transferred intravenously (i.v.) into sublethally irradiated C57BL/6 mice, and daily scoring for clinical signs of EAE began from day 5 after transfer.

Transcript Analysis

RNA isolation was performed using QIAGEN's RNeasy Mini Plus Kit following the manufacturer's instructions. Real-time PCR was performed using Life Technologies' TaqMan Gene Expression Assays combined with a TaqMan Universal PCR Master Mix (Applied Biosystems). The comparative CT method was applied for relative quantification of mRNA levels. Data are presented as the fold change in expression normalized to internal control gene *Hprt*, which was found to be stably expressed under all experimental conditions tested.

Statistical Analysis

The p values were calculated with a two-tailed Student's t test. A p value <0.05 was considered significant with p values considered significant as follows: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Error bars denote ± SEM.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is ArrayExpress: E-MTAB-5692.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.052.

AUTHOR CONTRIBUTIONS

V.B.-W., C.F., M.S., O.F., S.I., and M.V. performed the experiments, interpreted the data, and assisted with the manuscript. J.C.M. and M.V. designed and coordinated the research. M.V. wrote the manuscript.

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