nature publishing group

IL-15 positively regulates IL-21 production in celiac disease mucosa

M Sarra[1], ML Cupi[1], I Monteleone[1], E Franzè[1], G Ronchetti[1], A Di Sabatino[2], P Gentileschi[3], L Franceschilli[3], P Sileri[3], G Sica[3], G Del Vecchio Blanco[1], M Cretella[1], OA Paoluzi[1], GR Corazza[2], F Pallone[1] and G Monteleone[1]

Celiac disease (CD)-associated inflammation is characterized by high interleukin- 21 (IL-21), but the mechanisms that control IL-21 production are not fully understood. Here we analyzed IL-21 cell sources and examined how IL-21 production is regulated in CD. Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs), isolated from CD patients and non-CD controls, were analyzed for cell markers, cytokines, and transcription factors by flow cytometry. IL-21 was highly produced by CD4+ and CD4+/CD8+ IELs and LPLs in active CD. IL-21-producing cells coexpressed interferon-γ (IFN-γ) and to a lesser extent T helper type 17 (Th17) cytokines. Treatment of control LPLs with IL-15, a cytokine overproduced in CD, activated Akt and STAT3 (signal transducer and activator of transcription 3), thus enhancing IL-21 synthesis. Active CD biopsies contained elevated levels of Akt, and blockade of IL-15 in those samples reduced IL-21. Similarly, neutralization of IL-15 in biopsies of inactive CD patients inhibited peptic–tryptic digest of gliadin-induced IL-21 expression. These findings indicate that in CD, IL-15 positively regulates IL-21 production.

INTRODUCTION

Celiac disease (CD) is a gluten-sensitive enteropathy that occurs in genetically predisposed individuals after ingestion of gluten. Originally considered a malabsorption syndrome of childhood, CD is now recognized as a common condition that may be diagnosed at any age.[1] Ingestion of gluten in CD patients triggers a mucosal inflammatory response, which leads to epithelial tissue damage, villous atrophy, crypt cell hyperplasia, and increased number of intraepithelial lymphocytes (IELs).[2] The exact mechanism by which CD-associated inflammatory response is induced and sustained is not fully understood. There is evidence that gluten peptides are deamidated by the enzyme tissue transglutaminase 2, processed by antigen-presenting cells, and presented to lamina propria CD4+ T cells (T-LPLs) in the context of HLA-DQ2/DQ8 molecules.[2] This sequence of events leads to the activation and expansion of gluten-specific T cells.[1,2] It has also been shown that certain non-immunodominant gluten peptides derived from α-gliadin (e.g., peptides 31–43 and 31–49), which are distinct from those that bind DQ2 or DQ8 and activate gluten peptide–specific CD4+ T cells, induce the production of interleukin (IL)-15 by innate immune cells, thereby leading to epithelial cell death.[3] These findings correlate with the demonstration that IL-15 is overexpressed in both the lamina propria and intestinal epithelium of patients with active CD as compared with normal controls or inactive CD patients.[4,5] Studies examining the mechanisms by which IL-15 promotes epithelial injury have revealed that IL-15 upregulates the nonconventional HLA molecules MICA/B on enterocytes and enhances both NKG2D and DAP10 in IELs,[6] with the downstream effect of promoting IEL-mediated epithelial damage. IL-15 also controls the activation of adaptive immune responses, as substantiated by its ability to make mucosal T lymphocytes resistant to the transforming growth factor-β1-mediated immune-suppression and sustain the survival of such cells.[2,7–9]

Analysis of cytokines produced by T-LPLs in the gut of CD patients has shown that CD-associated inflammation is dominated by production of interferon-γ (IFN-γ) and IL-17A, the cytokine signatures of T helper (Th) 1 and Th17 cell responses, respectively.[10,11] In this context, we and others have recently demonstrated that in CD, gliadin-specific T cells produce high levels of IL-21,[12,13] a cytokine that is supposed to play a key role in the pathogenesis of CD. Indeed, IL-21 amplifies Th1 and Th17 cell responses,[14,15] enhances secretion of extracellular matrix-degrading proteases by stromal cells[16] and chemoattractants

[1]Department of Internal Medicine, University "Tor Vergata" of Rome, Rome, Italy. [2]Department of Internal Medicine, Fondazione IRCCS Policlinico, San Matteo, Università di Pavia, Pavia, Italy. [3]Department of Surgery, University "Tor Vergata" of Rome, Rome, Italy. Correspondence: G Monteleone (Gi.Monteleone@Med.uniroma2.it)

Received 13 July 2011; accepted 8 June 2012; published online 11 July 2012. doi:10.1038/mi.2012.65

by epithelial cells,[17] and counterregulates the immune-suppressive activities of regulatory T cells.[18] Chromosomal 4q27 region harboring the IL-2 and IL-21 genes is associated with CD,[19] but if these genetic alterations affect IL-21 protein remains to be ascertained. Similarly, the cell source of IL-21 and the basic mechanisms that control IL-21 expression in CD mucosa are not known. In this study, we performed a single-cell analysis of IL-21-producing CD4+ T cells to ascertain which cells produce IL-21 and to investigate the factors involved in the regulation of IL-21 production in CD.

RESULTS

IL-21 is produced by both CD4+ IELs and T-LPLs in CD

To evaluate cell sources of IL-21 in CD mucosa, we performed a single-cell analysis of IL-21 production by flow cytometry using IELs and T-LPLs isolated from patients with active and inactive CD and non-CD controls. The fraction of IL-21-producing CD3+ IELs was significantly higher in active CD patients as compared with inactive CD patients and normal controls (**Figure 1a**). There was no significant difference in the percentage of such cells between inactive CD and non-CD samples (**Figure 1a**). Similarly, the percentage of IL-21-producing T-LPLs was significantly higher in patients with active CD as compared with non-CD controls (**Figure 1c**). Moreover, IELs and LPLs isolated from active CD biopsies expressed elevated levels of IFN-γ as compared with cells isolated from inactive CD or non-CD control biopsies (**Supplementary Figure S1** online). In active CD, there was higher percentage of CD4+ and CD4+CD8+ T cells as compared with controls (**Supplementary Figure S2** online). In both epithelial and lamina propria compartments of active CD, IL-21 was produced by CD4+ and CD4+CD8+ T cells, whereas CD4−CD8+ cells and CD4−CD8− cells did not express IL-21 (**Figure 1b,d**). These data confirm precedent studies showing that IL-21 is produced by activated CD4+ but not CD4− T cells.[20]

To confirm the localization of IL-21-producing cells, duodenal sections of non-CD controls and active CD patients were stained with anti-IL-21 antibody. IL-21-positive cells were evident both in the epithelium and lamina propria of CD patients (**Figure 1e**). Western blotting of extracts of cells isolated from the epithelium and lamina propria and densitometric analysis of immunoreactive bands revealed that IL-21 expression was significantly increased in CD patients in comparison with non-CD controls (**Figure 1f,g**).

The γ/δ-positive IELs produce IL-21 in CD

A typical histological feature of CD is the increased infiltration of the epithelial layer by γ/δ-positive lymphocytes.[21] By flow cytometry we confirmed that the percentage of γ/δ-positive IELs was increased in duodenal biopsies of patients with active CD and patients with inactive CD as compared with non-CD controls (**Figure 2a**). Phenotypic analysis of γ/δ-positive IELs in the duodenal mucosa revealed that the majority of them were negative for both CD4 and CD8 with no difference between active CD and controls (**Supplementary Figure S3A** online). Less than 10% of γ/δ-positive IELs was CD4+CD8− in active CD and this percentage was significantly higher than that in controls (**Supplementary Figure S3A** online), thus confirming previous reports.[22] In contrast, the fraction of γ/δ-expressing CD8+CD4− IELs was significantly higher in controls than in active CD mucosa (**Supplementary Figure S3A** online). As CD4+ T cells produce IL-21, we next examined whether γ/δ-positive IELs expressed IL-21. **Figure 2b** shows that the percentage of IL-21-producing γ/δ-positive IELs was increased in active, but not inactive, CD mucosa in comparison with non-CD controls (**Figure 2b**). By flow cytometry we also showed that nearly half of γ/δ-expressing CD4+ IELs isolated from the duodenum of active CD patients were positive for IL-21 (**Supplementary Figure S3B** online, right panel).

CXCR5-positive T-LPLs are a source of IL-21 in CD

A subset of T cells, termed T follicular helper cells, provides help to B cells and represents one of the most numerous and important subsets of effector T cells in lymphoid tissues.[23] T follicular helper cells express the CXC-chemokine receptor 5 (CXCR5) and IL-21,[24] and have recently been involved in the development of immune-mediated diseases.[25,26] We next examined whether in the human duodenum, IL-21-expressing CD4+ cells were positive for CXCR5. CXCR5+CD4+ cells were present in lamina propria mononuclear cell (LPMC) samples of both controls and CD patients (**Figure 3a**). Immunofluorescence of duodenal sections taken from active CD patients showed that cells positive for both CD4 and CXCR5 were mostly confined in follicular structures, although some of them were seen in the lamina propria compartments outside the follicles (**Figure 3b**, right panel), thus corroborating previous data.[27] Interestingly, the percentage of IL-21-producing CXCR5+/CD4+ T cells was higher in CD than in non-CD controls (**Figure 3c**).

In CD, the majority of IL-21-producing T cells coexpress IFN-γ

Although in mice IL-21 is mostly produced by Th17 cells,[28] studies in human systems indicate that additional T-cell subsets can make IL-21. For example, we have recently shown that in the inflamed gut of patients with CD, IL-21 is preferentially synthesized by IFN-γ-producing cells.[29] Therefore, we determined whether in CD mucosa IL-21-producing cells coexpress Th1- or Th17-related cytokines. Flow cytometry analysis of both IELs and T-LPLs isolated from active CD biopsies showed that the majority of IL-21-producing cells coexpressed IFN-γ and to a lesser extent IL-17A and IL-22 (**Figure 4a,b**). Consistently, more than one-third of IL-21-positive T cells coexpressed T-bet (**Figure 4c**), a transcription factor involved in the commitment of Th1 cells.[30]

IL-15 enhances IL-21 production through an Akt-dependent mechanism

As in CD, IL-15 targets CD4+ T lymphocytes,[8] we determined whether IL-15 controls IL-21 production. To this end, we cultured normal jejunal IELs and T-LPLs with or without IL-15 in the presence or absence of activating anti-CD3 antibody. IL-15 increased IL-21 and IFN-γ and this was evident at both RNA

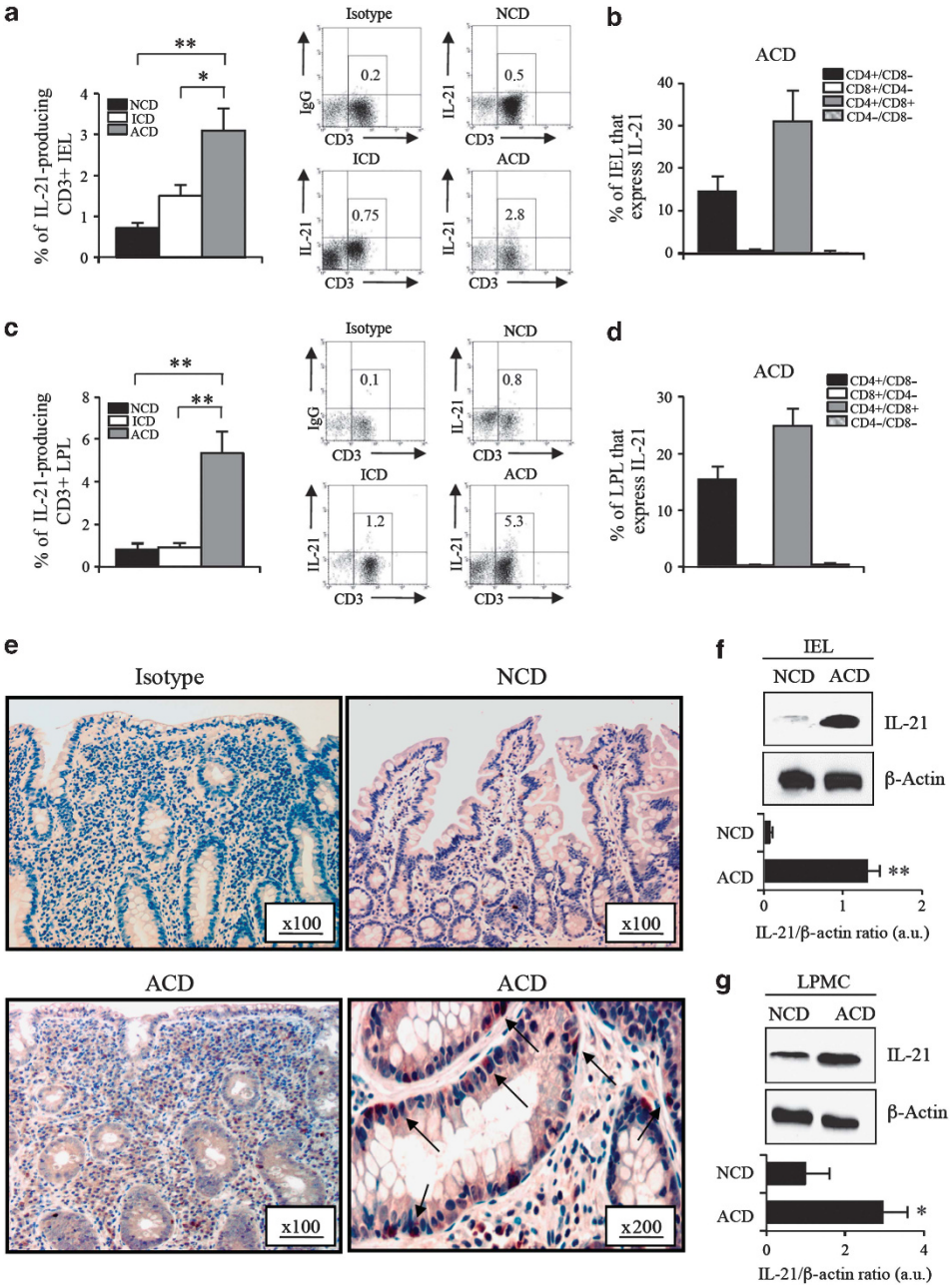

Figure 1 Interleukin- 21 (IL-21) is highly expressed in intraepithelial lymphocytes (IELs) and lamina propria T lymphocytes (T-LPLs) of active celiac disease (ACD) patients. (**a**–**d**) IELs (**a**) and lamina propria mononuclear cells (LPMCs) (**c**) were isolated from 7 non-CD controls (NCD), 5 inactive CD (ICD) patients, and 8 ACD patients, stimulated with phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin for 5 h, and then analyzed for the expression of CD3 and IL-21 by flow cytometry. Data indicate mean±s.e.m. of all experiments. Right panels of figures **a** and **c** show representative dot plots of IL-21-positive IELs and T-LPLs isolated from the duodenum of 1 NCD, 1 patient with ICD, and 1 patient with ACD. Numbers in the selected areas indicate the percentage of IL-21-expressing CD3-positive T cells. Staining with control immunoglobulin G (IgG) antibody is also shown. $^{*}P<0.05$; $^{**}P<0.01$. (**b**) IELs and (**d**) LPLs gated on CD3 and expressing CD4 and/or CD8 were also evaluated for IL-21 in three patients with ACD. Data indicate mean±s.e.m. of all experiments. (**e**) Representative photomicrographs showing IL-21 immunostaining in duodenal paraffin-embedded specimens from 1 NCD and 1 patient with ACD. Arrows indicate IL-21-positive IELs. Isotype control staining is also shown. (**f**, **g**) Representative western blots showing IL-21 and β-actin in proteins extracted from (**f**) IELs and (**g**) LPMCs isolated from NCD controls and ACD patients. One of four independent experiments is shown. Bottom insets show quantitative analysis of IL-21/β-actin protein ratio, as measured by densitometry scanning of western blots. Values are expressed in arbitrary units (a.u.) and are the mean±s.e.m. of IELs and LPMC isolated from 4 NCD and 4 ACD patients. $^{*}P<0.05$; $^{**}P<0.01$.

and protein levels (**Figure 5a–d**). The IL-15-mediated inducing effect on IL-21 and IFN-γ production was seen even when cells were stimulated with anti-CD3 (**Figure 5a–d**). By flow cytometry we next showed that IL-15 increased not only the percentages of IL-21- or IFN-γ-producing cells, but also the fraction of cells coexpressing both cytokines (**Figure 5e**).

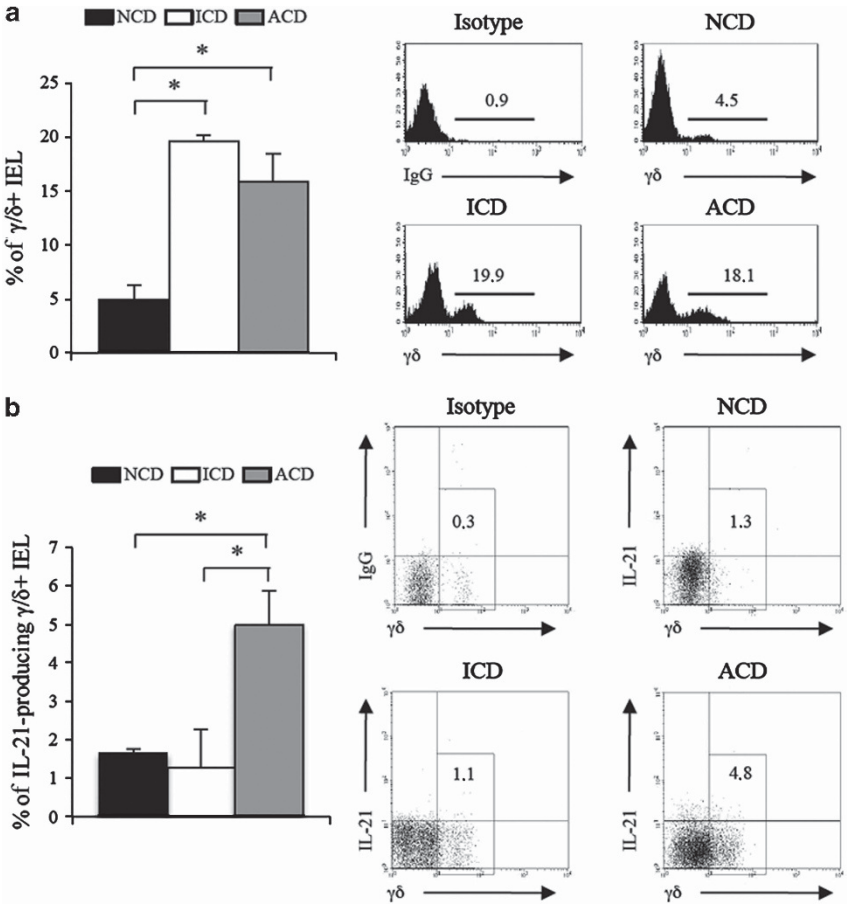

Figure 2 The γ/δ-positive intraepithelial lymphocytes (IELs) produce interleukin- 21 (IL-21) in celiac disease (CD). (**a**) IELs isolated from 6 non-CD controls (NCD), 4 inactive CD (ICD), and 6 active CD (ACD) patients were analyzed for the expression of CD3 and γ/δ by flow cytometry. Data indicate mean±s.e.m. of all experiments. *$P<0.001$. (**a**, right panels) Representative histograms of γ/δ-positive CD3+ cells in duodenal IEL samples of 1 NCD, 1 ICD patient, and 1 ACD patient. Numbers indicate the percentage of positive cells. Staining with a control immunoglobulin G (IgG) antibody is also shown. (**b**) Percentage of IL-21-producing γ/δ-positive IELs. Cells were isolated as indicated in **a**, stimulated with phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin for 5 h, and analyzed by flow cytometry. Data indicate mean±s.e.m. of all experiments. *$P<0.01$. (**b**, right panels) Representative dot plots showing IL-21-producing γ/δ-positive IELs in cell samples isolated from the duodenum of 1 NCD, 1 ICD patient, and 1 ACD patient. Numbers indicate the percentage of IL-21-producing γ/δ-positive cells. Staining with a control IgG antibody against γ/δ is also shown.

To determine whether IL-15 also regulates the expression of IL-21 in CXCR5+CD4+ cells, normal jejunal CD4+ LPLs were either left unstimulated or stimulated with IL-15 and then cultured with or without phorbol 12-myristate 13-acetate (PMA) and ionomycin. After 5 h, cells were assessed by flow cytometry. **Figure 5f** shows that nearly 10% of CD4+LPLs cultured with medium alone in the absence of PMA and ionomycin were positive for CXCR5, thus indicating that expression of CXCR5 on CD4+LPLs is not secondary to PMA/ionomycin stimulation. As expected, PMA/ionomycin stimulation doubled the fraction of CXCR5+CD4+ cells. Treatment of cells with IL-15 increased the percentage of IL-21-expressing CXCR5+ and CXCR5− CD4+ LPLs, without affecting the fraction of CD4+LPLs expressing CXCR5 (**Figure 5f**).

Next, we explored mechanisms by which IL-15 regulates IL-21 production. Analysis of signaling pathways activated in T cells has previously shown that IL-15 induces strong activation of STAT5 (signal transducer and activator of transcription 5), Akt, and STAT3.[31–33] As STAT5 does not regulate IL-21 production,[34] we initially focused our work on Akt. We monitored the phosphorylation of Akt in normal T-LPLs treated with IL-15 for different time points. By using an antibody that specifically recognizes Akt phosphorylation on serine 473 (S473) residue, we showed that IL-15 enhanced Akt activation (**Figure 6a,b**). To inhibit Akt, cells were treated with 100 nM wortmannin (WRT), as previous studies have documented the specificity of this compound when used at this concentration.[35] WRT abrogated IL-15-induced Akt phosphorylation (**Figure 6c**) and significantly reduced IL-15-driven IL-21 and IFN-γ synthesis (**Figure 6d,e**). Flow cytometric analysis revealed that WRT also reduced the fraction of IL-21/IFN-γ double-positive cells (**Figure 6f**). The fact that suppression of Akt by WRT was not accompanied by abrogation of IL-15-driven IL-21 production (**Figure 6d–f**) prompted us to examine the involvement of other signaling pathways. As we previously showed that IL-21 production in T cells is positively regulated by STAT3,[36] we examined

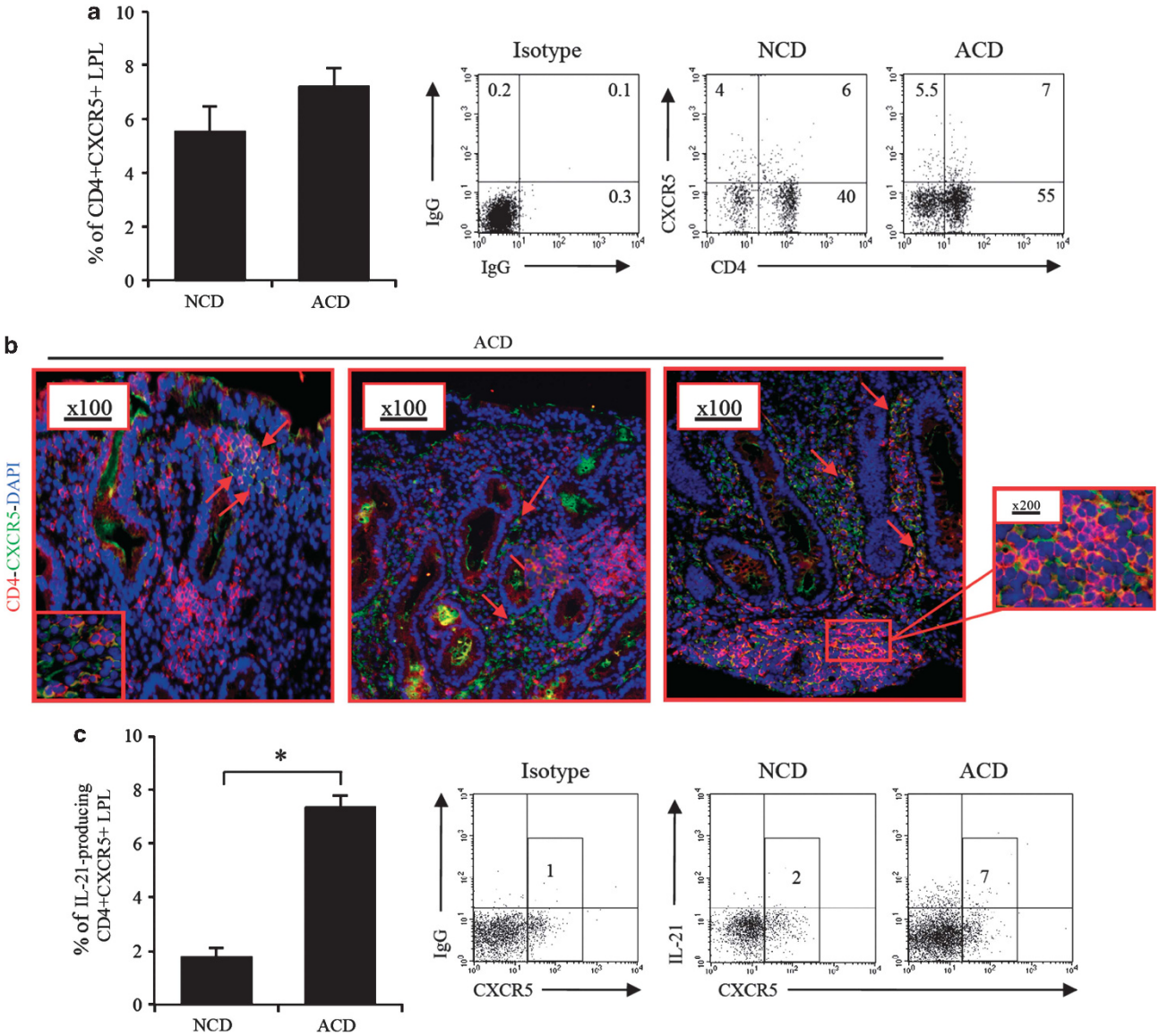

Figure 3 Interleukin- 21 (IL-21) is produced by CD4+CXCR5+ T cells in celiac disease (CD). (**a**) Lamina propria mononuclear cells (LPMCs) isolated from 6 active CD (ACD) patients and 6 non-CD controls (NCD) were gated on CD3 and analyzed for the expression of CD4 and CXCR5 (CXC-chemokine receptor 5) by flow cytometry. Data indicate mean±s.e.m. of all experiments. (**a**, right panels) Representative dot plots showing CXCR5+CD4+ cells in LPMC samples of 1 NCD and 1 ACD patient. Staining with control immunoglobulin G (IgG) antibodies is also shown. Numbers in the quadrants indicate the percentage of positive cells. (**b**) Representative microphotographs of CD4 (red)-, CXCR5 (green)-, and 4',6-diamidino-2-phenylindole (DAPI; blue)-stained sections of duodenal samples taken from 4 ACD patients. Arrows indicate the presence of CXCR5-expressing CD4-positive cells (yellow). (**b**, right panel) Inset shows a follicular structure (×200) with cells coexpressing CXCR5 and CD4. (**c**) LPMCs isolated as indicated in **a** were stimulated with phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin for 5 h and subsequently analyzed by flow cytometry. CD4+ cells were gated and analyzed for CXCR5 and IL-21. Data indicate mean±s.e.m. of all experiments. *$P<0.05$. (**c**, right panels) The percentage of CD4-positive cells expressing CXCR5 and IL-21 isolated from 1 ACD patient and 1 NCD are shown. Staining with a control IgG antibody against CXCR5 is also shown. Numbers indicate the percentage of IL-21-expressing CXCR5+CD4+ T cells.

the role of this transcription factor in IL-15-driven IL-21 synthesis. Stimulation of CD4+ LPLs with IL-15 enhanced phosphorylation of STAT3 on 705 tyrosine residue, a phenomenon that associates with activation of STAT3 (**Supplementary Figure S4A** online). Inhibition of STAT3 activation with AG490 reduced IL-15-mediated IL-21 induction (**Supplementary Figure S4B** and **C** online). Simultaneous inhibition of AKT and STAT3 suppressed almost completely IL-15-driven induction of IL-21 (**Supplementary Figure S4C** online). Interestingly, the prosurvival effect of IL-15 on CD4+ T-LPLs was not affected by WRT or AG490 (**Figure 6g** and **Supplementary Figure S4D** online), suggesting that the inhibitory effect on IL-15-induced IL-21 synthesis was not due to a toxic effect of these compounds.

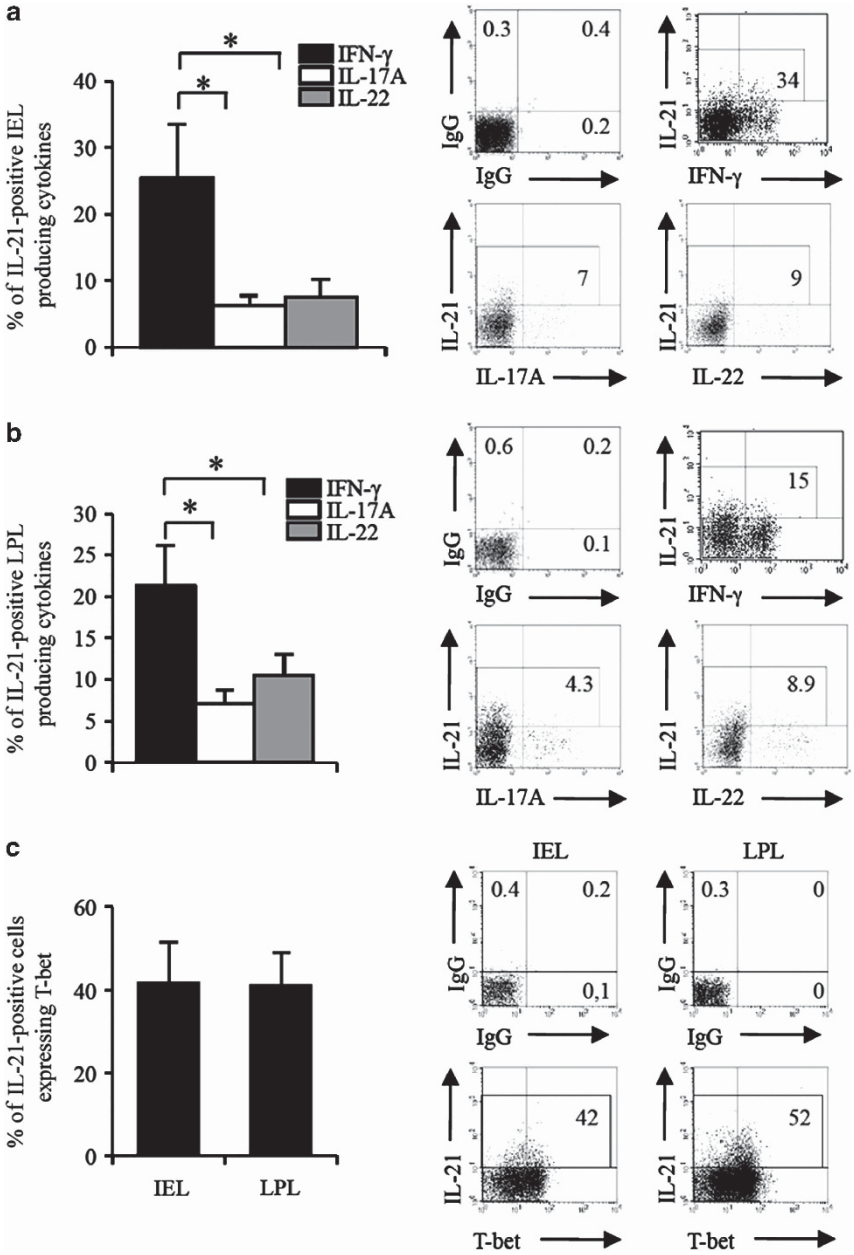

Figure 4 Interleukin- 21 (IL-21)-positive intraepithelial lymphocytes (IELs) and lamina propria T lymphocytes (T-LPLs) expressed interferon-γ (IFN-γ) and T-bet in celiac disease (CD). (**a**, **b**) IELs (**a**) and T-LPLs (**b**) isolated from 9 patients with active celiac disease (ACD) were stimulated with phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin for 5 h and subsequently analyzed by flow cytometry for IFN-γ, IL-17A, and IL-22. Data indicate mean±s.e.m. of all experiments. (**a**, **b**, right panels) Representative dot plots showing the expression of IL-21, IFN-γ, IL-17A, or IL-22 in IELs and T-LPLs isolated from the duodenum of 1 patient with ACD. Numbers indicate the percentage of IL-21-positive cells expressing IFN-γ or IL-17A or IL-22. Stainings with control immunoglobulin G (IgG) antibodies are also shown. *$P<0.001$ **$P<0.0006$. (**c**) IELs and T-LPLs isolated from 5 patients with ACD were stimulated as above and analyzed by flow cytometry for the expression of IL-21 and T-bet. Data indicate mean±s.e.m. of all experiments. (**c**, right panels) Representative dot plots showing the expression of IL-21 and T-bet in IELs and T-LPLs isolated from the duodenum of 1 patient with ACD. Numbers indicate the percentage of IL-21-positive cells expressing T-bet. Staining with control IgG antibodies is also shown.

Activation of Akt in CD mucosa

To confirm that Akt is involved in the control of IL-21 in CD, we evaluated p-Akt in biopsies taken from active CD patients and non-CD controls. A more pronounced expression of p-Akt was seen in CD as compared with controls (**Figure 7a**). Treatment of explants, taken from active CD patients, with WRT reduced IL-21 expression (**Figure 7b**). In line with the above data, the addition of a neutralizing anti-IL-15 to cultures of CD duodenal explants reduced p-Akt and IL-21 protein and RNA expression (**Figure 7c,d**). In a final set of experiments we assessed whether inhibition of endogenous IL-15 negatively regulated IL-21 induction in biopsies of inactive CD patients stimulated *in vitro* with a peptic–tryptic digest of gliadin (PT). Initially, we determined the variation in the response of individual biopsies

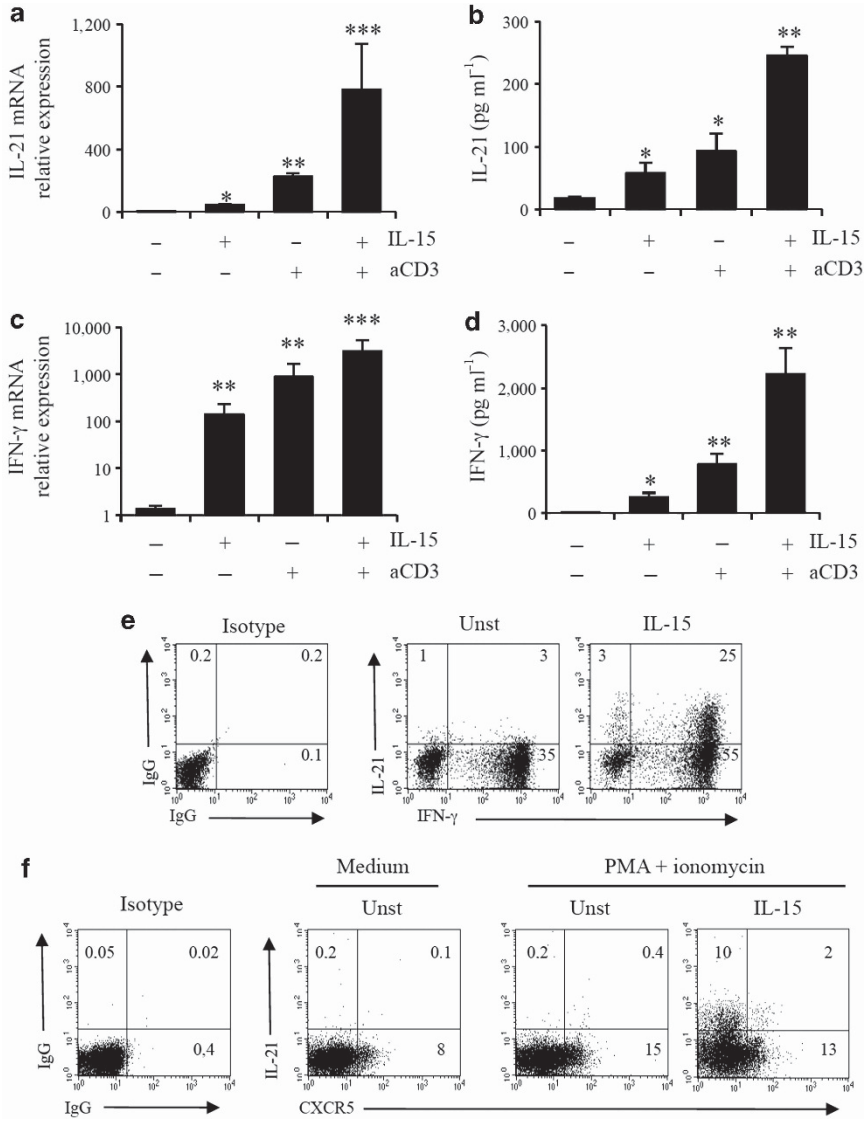

Figure 5 Interleukin- 15 (IL-15) enhances IL-21 and interferon-γ (IFN-γ) production in CD4+ lamina propria T lymphocytes (T-LPLs). CD4+ T cells were isolated from jejunal mucosa and cultured with or without (unstimulated (Unst)) activating anti-CD3 antibody in the presence or absence of IL-15 for 24 or 48 h. (**a**, **b**) IL-21 and IFN-γ RNA expression was analyzed by real-time PCR after 24 h of culture, (**c**, **d**) whereas supernatants obtained after 48 h of culture were measured for IL-21 and IFN-γ by enzyme-linked immunosorbent assay. Data indicate mean±s.e.m. of three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.005$. (**e**) CD4+ cells were cultured with or without IL-15 for 48 h. Phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin were added in the last 5 h of culture, and cells were analyzed for the expression of IFN-γ and IL-21 by flow cytometry. (**f**) CD4+ cells were cultured as in **e**. Cultures were then either left untreated (medium) or treated with PMA, ionomycin, and monensin for 5 h and cells were analyzed for the expression of CXCR5 (CXC-chemokine receptor 5) and IL-21 by flow cytometry. Representative dot plots show one of three experiments in which cells isolated from three normal controls were used and similar results were obtained. Numbers indicate the percentage of positive cells in the designed gates.

taken from the same patient to PT stimulation in terms of IL-21 induction. To this end, mucosal explants taken from the duodenum of inactive CD patients were cultured in the presence or absence of PT. Cultures were performed in duplicate. After 24 h, IL-21 expression was evaluated by real-time PCR. Biopsies taken from the same patient and cultured with medium alone contained similar levels of IL-21 and responded to PT stimulation with enhanced expression of IL-21 (**Supplementary Figure S5** online). However, there was a variation in the response to PT, probably reflecting the grade of T-cell infiltration in the single biopsies (**Supplementary Figure S5** online). In these cultures, blockade of IL-15 significantly reduced PT-induced IL-21 RNA expression (**Figure 7e**).

DISCUSSION

CD is a disorder characterized by excessive immune response to ingested wheat gluten and related cereal proteins.[1,2] Although innate immune response seems to be sufficient to elicit epithelial damage, a considerable amount of work has been produced to indicate that gluten-reactive CD4+ T cells synthesize large

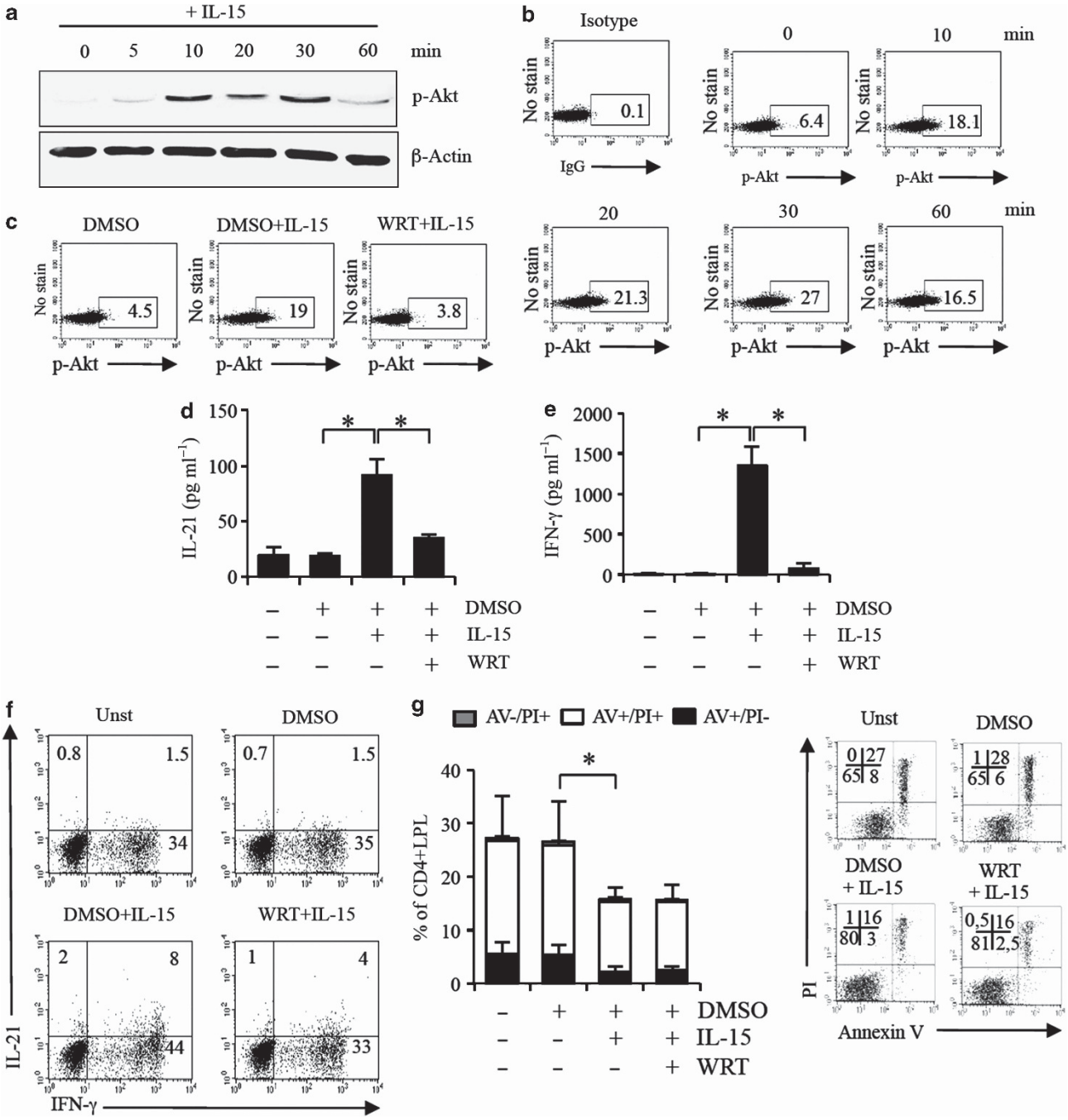

Figure 6 Interleukin- 15 (IL-15) enhances IL-21 production in CD4+ lamina propria T lymphocytes (T-LPLs) through a mechanism involving the activation of AKT. (**a**) Representative western blots showing p-Akt and β-actin in proteins extracted from CD4+ T-LPLs isolated from the jejunum of normal controls and stimulated with IL-15 (50 ng ml^{-1}) for the indicated time points. (**b**) CD4+ T-LPLs were isolated and cultured as indicated in **a** and analyzed for p-Akt by flow cytometry. Staining with a control immunoglobulin G (IgG) is also shown. Numbers in the quadrants indicate the percentage of p-Akt-expressing T-LPLs. One of three experiments in which similar results were obtained is shown. (**c**) Cells were stimulated with or without IL-15 (50 ng ml^{-1}) for 30 min in the presence of wortmannin (WRT; 100 nM) or dimethyl sulfoxide (DMSO) and then analyzed for p-Akt by flow cytometry. Numbers indicate the percentage of p-Akt-expressing T-LPLs. One of three experiments in which similar results were obtained is shown. (**d**, **e**) CD4+ T-LPLs isolated from normal jejunal specimens were stimulated with or without IL-15 (50 ng ml^{-1}) in the presence or absence of WRT (100 nM) or DMSO for 48 h. (**d**) IL-21 and (**e**) IFN-γ were measured in the culture supernatants by enzyme-linked immunosorbent assay (ELISA). Data indicate mean±s.e.m. of three independent experiments; *$P<0.05$. (**f**) CD4+ T-LPLs were isolated and cultured as indicated in **d** and **e**. Phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin were added in the last 5 h of culture, and cells were analyzed for the expression of IL-21 and IFN-γ. Numbers in the dot plots indicate the percentages of IL-21- and/or IFN-γ-positive cells. One of four experiments, in which similar results were obtained, is shown. Unst, unstimulated. (**g**) Percentage of AnnexinV (AV)+/propidium iodide (PI)−, AV+/PI+, and AV−/PI+ CD4+ T-LPLs. Cells were cultured as indicate in **f**. Data indicate mean±s.e.m. of five separate experiments. *$P=0.04$. Right inset shows representative dot plots showing the percentage of AV+/PI−, AV+/PI+, and AV−/PI+ CD4+ T-LPLs isolated from the jejunum of 1 control and stimulated with or without IL-15 in the presence of DMSO or WRT.

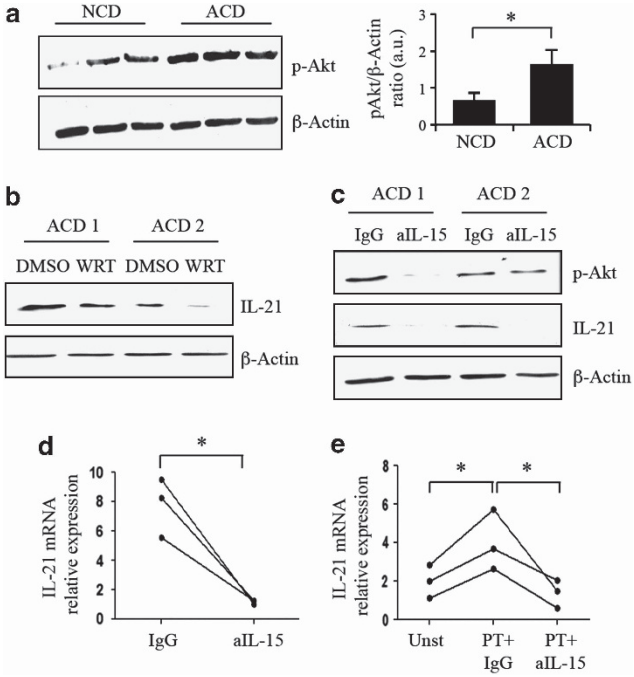

Figure 7 Activation of Akt in celiac disease (CD). (**a**) Representative western blots showing p-Akt and β-actin in proteins extracted from of 3 non-CD controls (NCD) and 3 patients with active celiac disease (ACD). Right inset shows quantitative analysis of p-Akt/β-actin protein ratio, as measured by densitometry scanning of western blots. Values are expressed in arbitrary units (a.u.) and are the mean±s.e.m. of 9 NCD and 9 ACD patients; $P=0.03$. (**b**) Western blots showing the content of interleukin- 21 (IL-21) and β-actin in duodenal biopsies of two CD patients, cultured with dimethyl sulfoxide (DMSO) or wortmannin (WRT; 100 nM) for 24 h. (**c**) Western blots showing p-Akt, IL-21, and β-actin in duodenal biopsies of two CD patients, cultured with a neutralizing anti-human IL-15 antibody or isotype control immunoglobulin G (IgG) for 24 h. (**d**) Duodenal biopsies isolated from three patients with active CD patients were treated with a neutralizing anti-human IL-15 or control IgG. After 24 h, tissues were analyzed for IL-21 RNA expression by real-time PCR. Data indicate mean±s.d. of all experiments. *$P<0.01$. (**e**) Duodenal biopsies isolated from three patients with inactive CD were treated with a peptic–tryptic digest of gliadin (PT, 1 mg ml^{-1}) in the presence or absence of a neutralizing anti-human IL-15 (aIL-15) or control IgG. After 24 h, tissues were analyzed for IL-21 RNA expression by real-time PCR. Data indicate mean±s.d. of all experiments. *$P<0.05$.

amounts of cytokines, which amplify the ongoing mucosal inflammation and contribute to the duodenal villous atrophy.[2,37] In this context, we have recently shown that in active CD, mucosal T lymphocytes produce IL-21 and that this cytokine sustains the synthesis of both IFN-γ and IL-17A.[11,12] Our data are consistent with the recent work by Bodd *et al.*,[13] showing that in CD, IL-21 and IFN-γ are made by gluten-specific T cells, whereas IL-17A is produced by nongluten-specific T cells. Altogether, these observations strongly suggest a role for IL-21 in CD-associated inflammatory response.

The present study was undertaken to assess which cells produce IL-21 and how IL-21 production is regulated in CD. By performing a flow cytometry analysis at single-cell level, we showed that IL-21 production is more pronounced in patients with active CD as compared with patients with inactive CD and non-CD controls and that in CD mucosa, both CD4+ T cells and CD4/CD8 double-positive cells express IL-21. However, it is likely that the contribution of CD4+CD8+ cells to IL-21 production is modest as the duodenal mucosa is infiltrated with a small number of these cells. This was evident both in the epithelium and lamina propria, as confirmed by immunohistochemistry and western blotting. We also showed that γ/δ-expressing CD4+ IELs were another source of IL-21 in the human duodenum. Increased numbers of γ/δ-positive cells were observed in IEL preparations of both active and inactive CD patients, but the percentage of IL-21-expressing γ/δ-positive IELs was upregulated only in active CD. As most γ/δ-positive IELs do not seem to recognize peptides in association with major histocompatibility complex molecules,[38] it is likely that induction of IL-21 in this cell subset is driven directly by cytokines/molecules produced within the inflamed intestine following gluten ingestion rather than gluten peptides. As discussed below, IL-15 could accomplish this function.

The exact role of γ/δ-positive IELs in the pathogenesis of CD remains unclear. It has been recently suggested that CD8+γ/δ+NKG2A+ IELs exert regulatory functions in CD patients on a gluten-free diet, as these cells secrete transforming growth factor-β1 and suppress the IL-15-stimulated cytotoxic activity of CD8+ IELs.[39] We were not able to purify sufficient numbers of IL-21-producing γ/δ-positive IELs to carry out functional studies. However, the fact that the majority of IL-21-expressing γ/δ-positive IELs coexpressed the inflammatory cytokine IFN-γ (Sarra *et al.*, unpublished observations) would suggest a proinflammatory role of IL-21-producing γ/δ-positive IELs. This hypothesis well fits with the demonstration that γ/δ-positive T cell-derived IL-21 enhances the production of IL-17A and IL-17F by CD4+ T cells and contributes to the pathogenesis of experimental autoimmune pathologies in mice.[40] It is thus conceivable that distinct subsets of γ/δ-positive IELs can accumulate during the various stages of CD. In the gut of active CD patients, where the excessive amount of IL-15 and IL-21 renders T cells refractory to the suppressive action of transforming growth factor-β1,[9,18] effector γ/δ-positive IELs could predominate. In contrast, when gluten is withdrawn from the diet and IL-15 and IL-21 levels diminish, γ/δ-positive IELs could become regulatory under the stimulus of transforming growth factor-β1 and contribute to the recovery from gluten-driven epithelial damage and maintenance of mucosal homeostasis.

The majority of IL-21-producing IELs and T-LPLs coexpressed IFN-γ, and to a lesser extent IL-17A or IL-22, supporting the hypothesis that in the human gut, IL-21 is preferentially made by Th1 rather than Th2/Th17 cells.[12,29,41] Indeed, in active CD, nearly 40% of IL-21-producing cells were positive for T-bet, a Th1-related transcription factor.[30] These data are in line with our previous report in which blockade of IL-21 in mucosal sample taken from active CD patients reduced T-bet expression.[12] Moreover, these findings confirm and expand on our own previously published studies showing that IL-21 is highly produced in other Th1-related gastrointestinal inflammatory disorders, such as CD and *Helicobacter pylori*-related gastritis.[29,41,42]

IL-21 is also produced by T follicular helper cells, a newly defined effector CD4+ T-cell subset that expresses the chemokine receptor CXCR5, preferentially localizes to lymphoid tissue, and provides help to B cells.[27] The gut is a lymphoid tissue-rich environment and inevitably, cells from lymphoid follicles are present in LPMC preparations. CXCR5+CD4+ cells isolated from the duodenum expressed IL-21, and the fraction of IL-21-producing CXCR5+CD4+ LPLs was higher in active CD samples than in control samples. We do not yet know what role is played by IL-21-producing CXCR5+CD4+ LPLs in CD, although it is conceivable that these cells could contribute to the enhanced production of auto-antibodies and promote CD8+ T cell-driven tissue damage in line with data generated in other systems.[26,43]

Data of the present work show that IL-15 positively regulates IL-21 expression in T-LPLs, and that IL-15-mediated IL-21 induction is enhanced by T cell receptor signaling. The positive effect of IL-15 on IL-21 synthesis was seen in both CXCR5-positive and CXCR5-negative T cells. Analysis of mechanisms by which IL-15 controls IL-21 production revealed that IL-15 activates both Akt and STAT3 in intestinal CD4+ LPLs and that simultaneous inhibition of Akt and STAT3 suppresses almost completely IL-15-induced IL-21 synthesis. In contrast, the antiapoptotic effect of IL-15 on intestinal CD4+ lymphocytes does not seem to rely on Akt, because preincubation of T-LPLs with WRT did not revert the prosurvival effect of IL-15. These later findings are consistent with data published by Malamut *et al.*,[44] showing that the pro-survival effect of IL-15 on IELs is not inhabitable by WRT. Duodenal biopsies of active CD patients displayed high levels of active Akt, and treatment of such samples with WRT reduced IL-21 production, thus confirming the role of Akt in driving IL-21 synthesis. Consistent with the above data, blockade of IL-15 in *ex vivo* cultures of CD biopsies reduced both Akt phosphorylation and IL-21 production. In line with our data is the demonstration that Akt drives inflammatory signals in other immune-mediated diseases such as systemic lupus erytemathosus, rheumatoid arthritis, multiple sclerosis, and type 1 diabetes.[45–49] Studies are now in progress to dissect the basic mechanisms by which Akt regulates IL-21 production in CD. Further work would also be necessary to ascertain whether IL-21 positively regulates IL-15 production, thereby facilitating a positive feedback loop that contributes to amplify the ongoing mucosal inflammation.

In conclusion, our study provides a detailed analysis of IL-21-producing T cells in CD mucosa and shows that IL-15 positively regulates IL-21 production. As IL-21 enhances IL-17A and IFN-γ synthesis in CD,[11,12] our findings contribute to delineate a scenario in which several cytokines produced by different T-cell populations help amplify the gluten-driven inflammation.

METHODS

Mucosal samples. Duodenal biopsies were taken from 38 patients with active CD during upper gastrointestinal endoscopy. The histopathological diagnosis of CD was based on typical mucosal lesions with crypt cell hyperplasia and villous atrophy. All active CD patients were positive for anti-endomysial and anti-transglutaminase antibodies at the time of diagnosis. Biopsies were also obtained from 15 inactive CD patients who adhered to a strict gluten-free diet and were in clinical and histological remission and negative for anti-endomysial and anti-transglutaminase antibodies. Non-CD controls ($N=28$) were under investigation for gastrointestinal symptoms, but had normal histology and no increase in inflammatory cells. Normal jejunal surgical specimens were available from eight patients who underwent gastro-jejunal bypass for obesity. Informed consent was obtained from all patients and controls and the study protocol was approved by the local ethics committee.

Cell isolation and culture. All reagents were from Sigma-Aldrich (Milan, Italy) unless specified. LPMCs and IELs were isolated as previously described[11] and resuspended (1×10^6 per ml) in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and gentamycin (50 μg ml^{-1}; complete medium; Lonza, Milan, Italy).

LPMCs were also isolated from jejunum surgical specimens of eight normal controls. LPMCs were used to purify CD4+ T-LPLs using a CD4+ cell isolation kit (Miltenyi Biotec, Bologna, Italy) according to the manufacturer's instruction. The resulting cell preparations (>95% CD4+) contained >85% of viable cells as assessed by flow cytometry.

IELs (2×10^5) and T-LPLs (2×10^5) were cultured in complete medium with PMA (40 ng ml^{-1}), ionomycin (1 mg ml^{-1}), and monensin (2 μM, eBioscience, San Diego, CA) for 5 h, and then assessed by flow cytometry. Additionally, T-LPLs (1×10^6 cells per ml) were cultured in complete medium with recombinant human IL-15 (50 ng ml^{-1}, R&D systems, Minneapolis, MN) in the presence or absence of anti-CD3 (aCD3) activating antibody (MiltenyiBiotec) WRT (100 nM), AG490 (50 μm, DBA Italia, Milan, Italy), and/or dimethyl sulfoxide (0.1%), harvested at the indicated time points and analyzed for active Akt and STAT3 by flow cytometry or western blotting. In parallel, T-LPLs (1×10^6 cells per ml) were cultured in complete medium with or without recombinant human IL-15, AG490, WRT, and/or dimethyl sulfoxide for 24 and 48 h, and analyzed for the content of IL-21 and IFN-γ by real-time PCR, enzyme-linked immunosorbent assay, and flow cytometry.

Flow cytometry. Cells were stained with the following monoclonal anti-human antibodies: fluorescein isothiocyanate (FITC) and allophycocyanin anti-IFN-γ, allophycocyanin anti-CXCR5, allophycocyanin anti-IL-21, anti-T-bet, allophycocyanin, PercP anti-CD4, PercP anti-CD3, FITC anti-γ/δ (clone 11F2; all from Becton Dickinson, Milan, Italy), PE anti-IL-21, Alexa Fluor 647 anti-IL-17A, Alexa Fluor 647 anti-IL-22 (from eBioscience), FITC anti-CD4 and allophycocyanin anti-CD8 (from Miltenyi Biotec), and allophycocyanin anti-p-Akt (from Cell Signaling, Danvers, MA). In all experiments, appropriate isotype control IgG (Becton Dickinson) was used. All antibodies were used in a 1:50 final dilution. For intracellular staining, cells were fixed and permeabilized using IC Fixation buffer and the permeabilization buffer (both from eBioscience) according to the manufacturer's instruction.

Immunohistochemistry and immunofluorescence. Tissue sections from three non-CD controls and three patients with active CD were cut, deparaffinized, dehydrated through xylene and ethanol, and incubated with a rabbit anti-human IL-21 antibody (Millipore, Milan, Italy) for 1 h at room temperature. Immunoreactive cells were visualized using MACH4 Universal HRP-Polymer kit with DAB (Biocare Medical, CA), according to the manufacturer's instructions, and lightly counterstained with hematoxylin. Isotype control sections were prepared under identical immunohistochemical conditions, as described above, replacing the primary antibody with a purified, normal rabbit IgG control antibody (Dako, Glostrup, Denmark).

Frozen sections of four active CD patients were also stained with anti-human CD4 (Santa Cruz Biotechnology, DBA, Milan, Italy) and CXCR5 (1:100 final dilution, R&D Systems) followed by incubation with a highly sensitive biotinylated secondary antibody (Dako) and Tyramide signal amplification kit (Perkin Elmer, Waltham, MA) or with cy3-conjugated secondary antibody (1:200 final dilution, Jackson Immunoresearch, Starfish, Milan, Italy).

Organ culture. Duodenal biopsies taken from five active CD patients were cultured in Aqix medium (Aqix, London, UK) supplemented with 1% of L-glutamine, penicillin (100 U ml^{-1}), streptomycin (100 mg ml^{-1}), and gentamycin (50 μg ml^{-1}). Biopsies were treated with a neutralizing monoclonal anti-human IL-15 (R&D Systems) or control IgG (both used 20 μg ml^{-1} final concentration), WRT (100 nM), or dimethyl sulfoxide. Organ cultures were performed as previously described.[11] After 24 h of culture, mucosal explants were examined for IL-21 and p-Akt. Duodenal biopsies were also taken from six patients with inactive CD, and cultured as described above with or without PT (1 mg ml^{-1}) in the presence or absence of a neutralizing monoclonal anti-human IL-15 (R&D Systems) or control IgG (both used 20 μg ml^{-1} final concentration). To assess the variability in the response of biopsies to gluten stimulation, paired mucosal explants taken from the duodenum of three patients with inactive CD were cultured with or without PT; each of these cultures was performed in duplicate. After 24 h of culture, tissue explants were used for examining IL-21 RNA expression by real-time PCR.

Protein extraction and western blotting. Proteins were extracted from freshly isolated IELs and LPMCs, biopsies, and mucosal explants using a buffer containing 10 mM HEPES (pH 7.9), 1 mM EDTA (pH 8.0), 60 mM KCl, Nonidet P40 0.2%, 1 mM DTT, 1 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM Na3VO4, and 1 mM NaF. Equal amounts of total proteins (50 μg per sample) were separated on a 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk and then incubated with anti-p-Akt, anti-p-Tyr (705)-Stat3 (all used 1:500 final dilution, Santa Cruz Biotechnology), and anti IL-21 (1:1,000 final dilution, ProSci, Poway, CA), followed by horseradish peroxidase-conjugated secondary antibodies (Dako, Milan, Italy). Bound antibodies were visualized with the use of enhanced chemiluminescence (Pierce, S.I.A.L., Rome, Italy). After detection of IL-21 and p-Akt, blots were stripped and incubated with a mouse anti-human β-actin antibody (1:5,000 final dilution) followed by a goat anti-mouse antibody conjugated to horseradish peroxidase. Computer-assisted scanning densitometry (Total Lab, AB.EL S.r.l., Rome, Italy) was used to analyze the intensity of the immunoreactive bands.

RNA extraction, complementary DNA preparation, and real-time PCR. RNA was extracted by using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Milan, Italy). A constant amount of RNA (0.5 μg per sample) was retro-transcribed into complementary DNA, and this was then amplified using a SYBR-green Master mix (Bio-Rad, Milan, Italy) with the following conditions: denaturation for 1 min at 95 °C, annealing for 30 s at 60 °C for β-actin and 58 °C for 18S, IL-21 and IFN-γ followed by 30 s of extension at 72 °C. Primer sequence was as follows: β-actin FWD: 5′-AAGATGACCCAGATCAT GTTTGAGACC-3′ and REV: 5′- AGCCAGTCCAGACGCAGGAT-3; 18S FWD 5′-TTCTTAGAGGGACAAGTGGC-3′ and REV: 5′-AATGGGGTTCAACGGGTTAC-3′; IL-21 5′-GGAGAGGATT GTCATCTGTC-3′ and REV: 5′-CACAGTTTGTCTCTACATCTTC-3′; IFN-γ FWD: 5′-TGGAGACCATCAAGGAAGAC-3′ and REV: 5′-GCGTTGGACATTCAAGTCAG-3′. β-Actin was used as housekeeping gene in the experiments performed with biopsies, whereas 18S was used for studies in which RNA was extracted from cells. Gene expression was calculated using the ΔΔCt algorithm.

Statistical analysis. Differences between groups were compared using Student's *t*-test, Wilcoxon test, or the Mann–Whitney *U*-test.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGMENTS
This work was supported by "Fondazione Umberto Di Mario" (Rome, Italy) and Giuliani Spa (Milan, Italy).

DISCLOSURE
G.M. has filed a patent entitled "A treatment for inflammatory diseases" (patent no. 08154101.3). The other authors declared no conflict of interest.

REFERENCES

1. Green, P.H. & Cellier, C. Celiac disease. *N. Engl. J. Med.* **357,** 1731–1743 (2007).
2. Jabri, B. & Sollid, L.M. Tissue-mediated control of immunopathology in coeliac disease. *Nat. Rev. Immunol.* **9,** 858–870 (2009).
3. Maiuri, L. *et al.* Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet* **362,** 30–37 (2003).
4. Mention, J.J. *et al.* Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. *Gastroenterology* **125,** 730–745 (2003).
5. Maiuri, L. *et al.* Interleukin 15 mediates epithelial changes in celiac disease. *Gastroenterology* **119,** 996–1006 (2000).
6. Meresse, B. *et al.* Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* **21,** 357–366 (2004).
7. De Nitto, D., Monteleone, I., Franze, E., Pallone, F. & Monteleone, G. Involvement of interleukin-15 and interleukin-21, two gamma-chain-related cytokines, in celiac disease. *World J. Gastroenterol.* **15,** 4609–4614 (2009).
8. Di Sabatino, A. *et al.* Epithelium derived interleukin 15 regulates intraepithelial lymphocyte Th1 cytokine production, cytotoxicity, and survival in coeliac disease. *Gut* **55,** 469–477 (2006).
9. Benahmed, M. *et al.* Inhibition of TGF-beta signaling by IL-15: a new role for IL-15 in the loss of immune homeostasis in celiac disease. *Gastroenterology* **132,** 994–1008 (2007).
10. Nilsen, E.M. *et al.* Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* **115,** 551–563 (1998).
11. Monteleone, I. *et al.* Characterization of IL-17A-producing cells in celiac disease mucosa. *J. Immunol.* **184,** 2211–2218 (2010).
12. Fina, D. *et al.* Interleukin 21 contributes to the mucosal T helper cell type 1 response in coeliac disease. *Gut* **57,** 887–892 (2008).
13. Bodd, M. *et al.* HLA-DQ2-restricted gluten-reactive T cells produce IL-21 but not IL-17 or IL-22. *Mucosal. Immunol.* **3,** 594–601 (2010).
14. Strengell, M., Sareneva, T., Foster, D., Julkunen, I. & Matikainen, S. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. *J. Immunol.* **169,** 3600–3605 (2002).
15. Korn, T. *et al.* IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* **448,** 484–487 (2007).
16. Monteleone, G. *et al.* Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. *Gut* **55,** 1774–1780 (2006).
17. Caruso, R. *et al.* A functional role for interleukin-21 in promoting the synthesis of the T-cell chemoattractant, MIP-3alpha, by gut epithelial cells. *Gastroenterology* **132,** 166–175 (2007).
18. Peluso, I. *et al.* IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes. *J. Immunol.* **178,** 732–739 (2007).
19. van Heel, D.A. *et al.* A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat. Genet.* **39,** 827–829 (2007).
20. Monteleone, G., Pallone, F. & Macdonald, T.T. Interleukin-21: a critical regulator of the balance between effector and regulatory T-cell responses. *Trends Immunol.* **29,** 290–294 (2008).
21. Halstensen, T.S., Scott, H. & Brandtzaeg, P. Intraepithelial T cells of the TcR gamma/delta+ CD8− and V delta 1/J delta 1+ phenotypes are increased in coeliac disease. *Scand. J. Immunol.* **30,** 665–672 (1989).
22. Rust, C. *et al.* Phenotypical and functional characterization of small intestinal TcR gamma delta + T cells in coeliac disease. *Scand. J. Immunol.* **35,** 459–468 (1992).
23. Crotty, S. Follicular helper CD4 T cells (T(FH)). *Annu. Rev. Immunol.* **29,** 621–663 (2011).
24. Bryant, V.L. *et al.* Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J. Immunol.* **179,** 8180–8190 (2007).

25. Vinuesa, C.G. *et al.* A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* **435,** 452–458 (2005).
26. Linterman, M.A. *et al.* Follicular helper T cells are required for systemic autoimmunity. *J. Exp. Med.* **206,** 561–576 (2009).
27. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* **192,** 1545–1552 (2000).
28. Wei, L., Laurence, A., Elias, K.M. & O'Shea, J.J. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J. Biol. Chem.* **282,** 34605–34610 (2007).
29. Sarra, M. *et al.* Interferon-gamma-expressing cells are a major source of interleukin-21 in inflammatory bowel diseases. *Inflamm. Bowel Dis.* **16,** 1332–1339 (2010).
30. Szabo, S.J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100,** 655–669 (2000).
31. Marzec, M. *et al.* Differential effects of interleukin-2 and interleukin-15 versus interleukin-21 on CD4+ cutaneous T-cell lymphoma cells. *Cancer Res.* **68,** 1083–1091 (2008).
32. Kim, H.R., Hwang, K.A. & Kang, I. Dual roles of IL-15 in maintaining IL-7Ralphalow. *J. Immunol.* **179,** 6734–6740 (2007).
33. De Totero, D. *et al.* The opposite effects of IL-15 and IL-21 on CLL B cells correlate with differential activation of the JAK/STAT and ERK1/2 pathways. *Blood* **111,** 517–524 (2008).
34. Zeng, R. *et al.* The molecular basis of IL-21-mediated proliferation. *Blood* **109,** 4135–4142 (2007).
35. Manning, B.D. & Cantley, L.C. AKT/PKB signaling: navigating downstream. *Cell* **129,** 1261–1274 (2007).
36. Caprioli, F. *et al.* Autocrine regulation of IL-21 production in human T lymphocytes. *J. Immunol.* **180,** 1800–1807 (2008).
37. Schuppan, D., Junker, Y. & Barisani, D. Celiac disease: from pathogenesis to novel therapies. *Gastroenterology* **137,** 1912–1933 (2009).
38. Kronenberg, M. Antigens recognized by gamma delta T cells. *Curr. Opin. Immunol.* **6,** 64–71 (1994).
39. Bhagat, G. *et al.* Small intestinal CD8+TCRgammadelta+ NKG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. *J. Clin. Invest.* **118,** 281–293 (2008).
40. Sutton, C.E. *et al.* Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* **31,** 331–341 (2009).
41. Monteleone, G. *et al.* Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease. *Gastroenterology* **128,** 687–694 (2005).
42. Caruso, R. *et al.* IL-21 is highly produced in Helicobacter pylori-infected gastric mucosa and promotes gelatinases synthesis. *J. Immunol.* **178,** 5957–5965 (2007).
43. King, C., Tangye, S.G. & Mackay, C.R. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu. Rev. Immunol.* **26,** 741–766 (2008).
44. Malamut, G. *et al.* IL-15 triggers an antiapoptotic pathway in human intraepithelial lymphocytes that is a potential new target in celiac disease-associated inflammation and lymphomagenesis. *J. Clin. Invest.* **120,** 2131–2143 (2010).
45. Di Cristofano, A. *et al.* Impaired Fas response and autoimmunity in Pten+/− mice. *Science* **285,** 2122–2125 (1999).
46. Borlado, L.R. *et al.* Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. *FASEB J.* **14,** 895–903 (2000).
47. Barber, D.F. *et al.* PI3Kgamma inhibition blocks glomerulonephritis and extends lifespan in a mouse model of systemic lupus. *Nat. Med.* **11,** 933–935 (2005).
48. Barber, D.F. *et al.* Class IB-phosphatidylinositol 3-kinase (PI3K) deficiency ameliorates IA-PI3K-induced systemic lupus but not T cell invasion. *J. Immunol.* **176,** 589–593 (2006).
49. Wu, T. & Mohan, C. The AKT axis as a therapeutic target in autoimmune diseases. *Endocr. Metab. Immune Disord. Drug Targets* **9,** 145–150 (2009).