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Refractory celiac disease type II (RCDII) is a severe complication of celiac disease (CD) characterized by the presence of an enlarged clonal population of innate intraepithelial lymphocytes (IELs) lacking classical B-, T-, and natural killer (NK)-cell lineage markers (Lin$^-$IELs) in the duodenum. In ∼50% of patients with RCDII, these Lin$^-$IELs develop into a lymphoma for which no effective treatment is available. Current evidence indicates that the survival and expansion of these malignant Lin$^-$IELs is driven by epithelial cell-derived IL-15. Like CD, RCDII is strongly associated with HLA-DQ2, suggesting the involvement of HLA-DQ2–restricted gluten-specific CD4$^+$ T cells. We now show that gluten-specific CD4$^+$ T cells isolated from CD duodenal biopsy specimens produce cytokines able to trigger proliferation of malignant Lin$^-$IEL lines as powerfully as IL-15. Furthermore, we identify TNF, IL-2, and IL-21 as CD4$^+$ T-cell cytokines that synergistically mediate this effect. Like IL-15, these cytokines were found to increase the phosphorylation of STAT5 and Akt and transcription of antiapoptotic mediator bcl-x$_L$. Several small-molecule inhibitors targeting the JAK/STAT pathway blocked proliferation elicited by IL-2 and IL-15, but only an inhibitor targeting the PI3K/Akt/mTOR pathway blocked proliferation induced by IL-15 as well as the CD4$^+$ T-cell cytokines. Confirming and extending these findings, TNF, IL-2, and IL-21 also synergistically triggered the proliferation of freshly isolated Lin$^-$IELs and CD3$^-$CD56$^+$ IELs (NK-IELs) from RCDII as well as non-RCDII duodenal biopsy specimens. These data provide evidence implicating CD4$^+$ T-cell cytokines in the pathogenesis of RCDII. More broadly, they suggest that adaptive immune responses can contribute to innate IEL activation during mucosal inflammation.

celiac disease | refractory celiac disease | enteropathy-associated T-cell lymphoma | small-molecule inhibitor | intraepithelial lymphocyte

Lymphomas arising in refractory celiac disease type II (RCDII) are prime examples of inflammation-associated cancers. Celiac disease (CD) is caused by an inappropriate, inflammatory CD4$^+$ T-cell response to the common food protein gluten (1). Although most patients with CD improve on a gluten-free diet, a small fraction (2–5%) of patients with adult-onset CD do not and continue to experience symptoms caused by chronic inflammation of the upper small intestine. Many of these patients suffer from RCDII, characterized by an expanded lineage-negative (Lin$^-$) innate intraepithelial lymphocyte (IEL) population in the duodenum (2, 3). Even though these Lin$^-$ innate IELs (hereinafter Lin$^-$IELs) are defined by the absence of surface lineage markers, including several T-cell markers (CD3, CD4, CD8, CD14, CD19, and CD56), they do express intracellular CD3 (icCD3ε) and usually have incomplete or out-of-frame T-cell receptor (TCR) rearrangements, suggesting that they derive from early T-cell and/or natural killer (NK)-cell precursors (2–6). Thus, the diagnosis of RCDII is based on the detection of icCD3ε$^+$ Lin$^-$IELs (also known as aberrant IELs) by flow cytometry (7), immunohistochemistry (8), and/or PCR-based detection of clonal TCR γ-locus (*TRG*) rearrangements (5). RCDII can be defined as a low-grade intraepithelial lymphoma, because the Lin$^-$IELs expanded in RCDII often give rise to type I enteropathy-associated T-cell lymphoma (EATL).

The main treatment goal in RCDII is to eliminate the Lin$^-$IEL population before its transformation into a high-grade lymphoma. Cladribine (2-CDA) is thought to be especially active against low-grade malignancies with limited proliferative capacity, and reduces Lin$^-$IEL frequency in ∼40% of patients (9). In patients not responding to 2-CDA, autologous bone marrow transplantation does not reduce Lin$^-$IEL frequency (10). Approximately 33–52% of patients with RCDII develop a type I EATL within 5 y after diagnosis (11). This EATL is routinely treated with chemotherapy and corticosteroids (e.g., CHOP: cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone), but this treatment lacks efficacy, with an estimated 5-y survival rate of only 8–25% (12, 13). Thus, a more effective treatment for RCDII is urgently needed.

The common γ-chain cytokine interleukin-15 (IL-15) is essential for the survival, differentiation, and proliferation of NK cells and certain T cells and may be produced by tissue cells to act as a local danger signal that promotes the destruction of infected cells (14). IL-15 levels are also elevated in the duodenal epithelium of patients with active CD and RCDII (15). Although a gluten-free diet normalizes IL-15 levels in CD, IL-15 expression remains high in RCDII. IL-15 signaling via JAK3 and STAT5 increases

Significance

Refractory celiac disease type II (RCDII) is a severe variant of celiac disease, an autoimmune disorder of the small intestine caused by inflammatory T-cell responses to gluten, a common food protein. Typical of RCDII is the presence of aberrant lymphocytes in the duodenal epithelium, which often give rise to a lethal lymphoma. A single growth factor promoting the expansion of aberrant cells has been identified: epithelial cell-derived IL-15. The experiments described in this paper identify three additional growth factors—TNF, IL-2, and IL-21—produced by gluten-specific T cells. Thus, these findings suggest a potential mechanism for the contribution of gluten-specific T cells to RCDII.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE73599).

See Commentary on page 1220.

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anti-apoptotic factor bcl-x_L levels and induces the proliferation of IELs, thereby increasing IEL numbers (16). Aberrant Lin$^-$IEL lines from RCDII patients are more sensitive to IL-15 than nonaberrant T-cell IEL lines, which may explain the selective expansion of (pre)malignant Lin$^-$IELs in this disease (16). Therefore, IL-15 was proposed to be a key therapeutic target in CD and RCDII (16, 17). However, recent data show that epithelial IL-15 is up-regulated in only ∼40% of patients with active CD (18), suggesting that the contribution of IL-15 to disease varies and that other cytokines may play roles as well.

CD is strongly associated with the presence of *HLA-DQA1* and *HLA-DQB1* alleles encoding HLA-DQ2.5 (*DQA1*0501* and *DQB1*02*), present in ∼90% of CD patients vs. 25% of nonceliac controls (1). This is explained by the fact that, in contrast to other HLA-DQ variants, HLA-DQ2.5 binds many different gluten peptides with high affinity and low off-rates, thereby allowing efficient induction of a broad gluten-specific CD4$^+$ T-cell response (19, 20). Indeed, HLA-DQ2.5–restricted gluten-specific CD4$^+$ T cells can be isolated from the lamina propria of duodenal biopsy specimens from patients with CD, but not from individuals without CD (21). RCDII is more strongly associated with this HLA-DQ variant compared with uncomplicated CD. All RCDII patients carry HLA-DQ2.5, and ∼50–60% of RCDII and EATL patients are HLA-DQ2.5 homozygous, compared with 25% of CD patients without RCDII and 2% of nonceliac controls (22, 23). This double gene dose is associated with increased production of inflammatory cytokines by gluten-specific CD4$^+$ T cells (19). This indicates that the presence of a gluten-specific CD4$^+$ T-cell response may be essential for the development of RCDII. The presence of gluten-specific CD4$^+$ T cells in RCDII patients has not been established, however.

We hypothesized that cytokines produced by gluten-specific CD4$^+$ T cells in the lamina propria contribute to the survival and expansion of Lin$^-$IELs in RCDII. In support of this concept, we now show that cytokines TNF, IL-2, and IL-21 produced by gluten-specific T cells synergistically drive proliferation of cultured and freshly isolated Lin$^-$IELs from RCDII patients, indicating that not only IL-15 from epithelial cells, but also cytokines from lamina propria CD4$^+$ T cells, can contribute to lymphomagenesis in RCDII.

Results

Gluten-Specific T Cells Are Present in the Duodenum of RCDII Patients. Although RCDII is considered a complication of CD, the presence of gluten-specific T cells in RCDII patients has not been established. Thus, we attempted to expand gluten-specific T cells from RCDII patients by culturing duodenal biopsy specimens with gluten. Similar to biopsy specimens from CD patients without RCDII, most of the resulting lymphocyte cell lines proliferated in response to a gluten digest treated with tissue transglutaminase 2 (TG2) (Fig. 1 *A* and *B*). The TG2 enzyme is critical to the CD4$^+$ T-cell response in CD, because it enhances gluten peptide binding to HLA-DQ2.5 (24, 25). CD4$^+$ T-cell clones isolated from one of the gluten-reactive cell lines (Fig. 1*A*) displayed a diverse pattern of gluten reactivity, with individual clones differing in their degree of TG2 dependence (Fig. 1*C*). Many of these clones specifically recognized DQ2.5-glia-α1a (Fig. 1*C*), one of the immunodominant epitopes in HLA-DQ2–associated CD (26). In conclusion, gluten-specific CD4$^+$ T cells can be found in the duodenum of most RCDII patients and display diverse antigen specificities that include one of the immunodominant epitopes in CD.

TNF, IL2, and IL21 Produced by Gluten-Specific CD4$^+$ T Cells Induce Proliferation of Malignant Lin$^-$IEL Lines. We next tested the ability of cytokines produced by gluten-specific CD4$^+$ T cells to induce proliferation of malignant Lin$^-$IELs from RCDII patients (Fig. 2). To this end, cell-free supernatants were harvested from gluten-specific CD4$^+$ T-cell clones activated by gluten peptide-loaded, HLA-DQ2$^+$ peripheral blood mononuclear cells (PBMCs). These

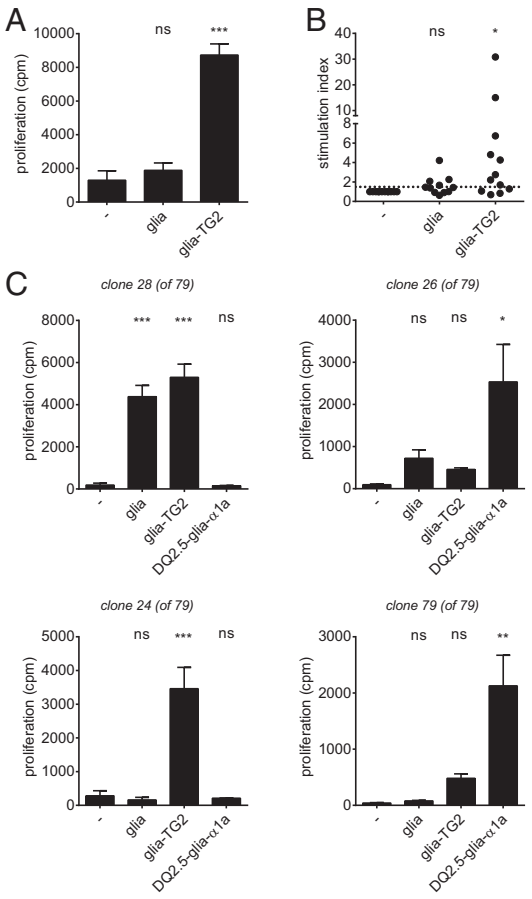

Fig. 1. Gluten-specific T cells are present in RCDII patient biopsy specimens. Duodenal biopsy specimens from 12 RCDII patients were cultured with gliadin, the water-soluble component of gluten, and the resulting lymphocyte cell lines were tested for their reactivity against a gliadin digest that had (glia-TG2) or had not (glia) been treated with the enzyme TG2, which enhances binding to HLA-DQ2. (*A*) Proliferation of a single biopsy line in response to HLA-DQ2$^+$ PBMCs loaded with glia or glia-TG2. (*B*) Proliferation of all 12 biopsy lines. Data were normalized based on the medium (–) control and expressed as the stimulation index. (*C*) The gliadin-reactive cell line from *A* was cloned by limiting dilution, and the resulting T-cell clones (n = 79) were tested individually for recognition of gliadin and DQ2.5-glia-α1a, one of the immunodominant T-cell epitopes in CD (26). Data for 4 of 44 gliadin-reactive clones are shown, of which 17 recognized the DQ2.5-glia-α1a peptide. Data are expressed as means, and the error bars in *A* and *C* represent SEM. Results of the statistical analyses are depicted as follows: ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

CD4$^+$ T-cell supernatants were subsequently incubated with Lin$^-$IEL lines from RCDII patients (27, 28), and Lin$^-$IEL proliferation was determined by measuring ^{3}H-thymidine uptake. Three out of four Lin$^-$IEL lines proliferated in response to CD4$^+$ T-cell supernatant, two to a similar degree as an optimal dose of IL-15 (Fig. 2*A*). Supernatants from seven out of nine CD4$^+$ T-cell clones, isolated from seven CD patients, induced Lin$^-$IEL proliferation (Fig. 2*B*). Control supernatants from CD4$^+$ T-cell clones incubated in the absence of peptide induced no proliferation (Fig. 2 *A* and *B*). Thus, most gluten-specific CD4$^+$ T cells produced cytokines able to induce the proliferation of malignant Lin$^-$IEL lines.

To identify the CD4$^+$ T-cell cytokines responsible for the proliferation of the Lin$^-$IEL lines, we took an unbiased approach, using a combination of transcriptomics and proteomics. First, we determined which gene transcripts were up-regulated on activation. To this end, CD4$^+$ T-cell clone L10, which recognizes DQ2-glia-α1, one of the immunodominant T-cell epitopes in CD (29), was

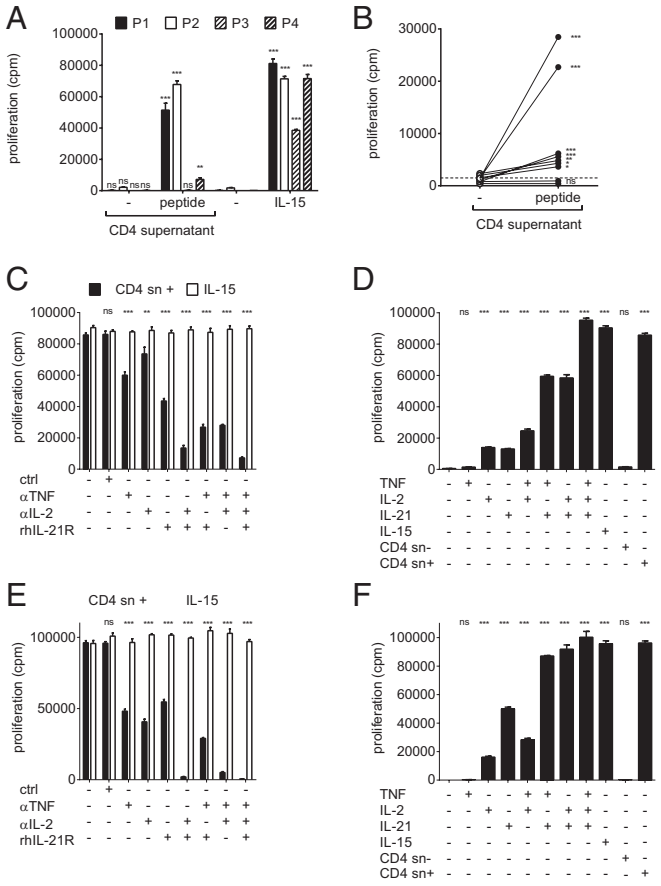

Fig. 2. TNF, IL-2, and IL-21 produced by gluten-specific CD4$^+$ T cells are necessary and sufficient to induce the proliferation of Lin$^-$IEL lines from RCDII patients. (*A*) Proliferation of malignant Lin$^-$IEL lines P1, P2, P3, and P4 was measured in response to a 1:1:1:1 mix of supernatants from four gluten-specific CD4$^+$ T-cell clones (including L10), stimulated in either the absence (–) or the presence (+) of cognate gluten peptide. For a control, the cells were cultured in the absence or presence of IL-15. Data are representative of three independent experiments. (*B*) Proliferation of Lin$^-$IEL line P2 in response to supernatants from nine gluten-specific CD4$^+$ T-cell clones (including L10) stimulated in the absence (–) or presence (+) of peptide. Similar results were obtained in another experiment using four additional CD4$^+$ T-cell supernatants and Lin$^-$IEL lines P1, P2, P3, and P4. (*C* and *E*) Proliferation of Lin$^-$IEL lines P2 (*C*) and P1 (*E*) in response to IL-15 or CD4$^+$ T-cell supernatant (clone L10) depleted of TNF, IL-2, and/or IL-21 using antibodies (TNF and IL-2) or a recombinant receptor (IL-21). Results are shown for supernatant stimulation (CD4 sn$^+$), but not for IL-15 [not significantly different from unblocked (–) control across the board]. (*D* and *F*) Proliferation of Lin$^-$IEL line P2 (*D*) and P1 (*F*) in response to combinations of recombinant IL-2 (1 IU/mL), IL-15 (10 ng/mL), IL-21 (100 ng/mL), and TNF (10 ng/mL) or CD4$^+$ T-cell supernatant (clone L10). Results of the statistical analyses are depicted as follows: ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

stimulated with plate-bound CD3/CD28-specific or control antibodies, and transcripts present in RNA purified from these cells were quantified using whole-genome expression arrays (Affymetrix Human Gene 1.0 ST). Analysis of three biological replicates showed consistent activation-induced up-regulation of 141 transcripts, 31 of which encoded secreted proteins (Table 1). These included transcripts encoding cytokines previously reported for gluten-specific CD4$^+$ T cells, such as IFNγ, TNF, IL-10, and IL-21 (30, 31), as well as cytokines not commonly associated with CD, such as IL-22 (30) and amphiregulin (AREG), both of which are involved in the homeostasis of intestinal epithelial cells (IECs).

Next, we analyzed supernatants from CD4$^+$ T-cell clone L10 by mass spectrometry (MS). For optimal sensitivity, a large number of L10 CD4$^+$ T cells (>20 million) was activated by CD3/CD28-specific antibodies in the absence of serum, and a tryptic digest of the resulting cell-free supernatant was analyzed by online nano-HPLCy-MS/MS. Of the 31 cytokines induced at the transcript level, 19 were also detected in the supernatant of this clone, including IFN-γ, TNF, IL-10, IL-21, IL-22, and AREG (Table 1). Of note, IL-17A, which is present in CD biopsy specimens (32) but not produced by gluten-specific CD4$^+$ T-cell clones (30), was not detected at either the transcript level or the protein level. The same was true for IL-15, which is not produced by T cells. In short, these findings are consistent with previous reports on the cytokine profiles of CD4$^+$ T cells in CD (19, 30, 31, 33–40), and identify additional cytokines produced by a gluten-specific CD4$^+$ T-cell clone.

To determine which of these CD4$^+$ T-cell cytokines induce proliferation of malignant Lin$^-$IELs, we used expression array data (Illumina Human HT-12 V3) from three of our Lin$^-$IEL lines (3). These data (GEO accession no. GSE33078) were screened for the presence of transcripts encoding receptors for the cytokines made by the CD4$^+$ T-cell clone (Table 1), revealing that the Lin$^-$IEL lines expressed a receptor for 4 out of the 19 secreted CD4$^+$ T-cell cytokines: TNF, IL-2, IL-10, and IL-21 (Table 1). Because IL-10 inhibits rather than stimulates lymphocyte proliferation, we focused our attention on TNF, IL-2, and IL-21.

To test whether these three cytokines indeed mediate proliferation induced by CD4$^+$ T-cell supernatant, we measured Lin$^-$IEL cell line proliferation in response to L10 supernatant in the presence of antibodies that neutralize these cytokines. Whereas antibodies to TNF, IL-2, and recombinant IL-21R each partially inhibited proliferation, these three neutralizing agents in combination virtually abolished proliferation (Fig. 2 *C* and *E*). In line with this finding, recombinant IL-2, IL-21, and TNF separately induced limited proliferation (IL-2 and IL-21) or almost no proliferation (TNF), but when combined induced the same degree of proliferation as CD4$^+$ T-cell supernatant or an optimal dose of IL-15 (Fig. 2 *D* and *F*). Of note, the IL-2 concentration used (1 IU/mL) in this experiment was too low to activate the cells via CD122/CD132, a receptor with high affinity for IL-15 but low affinity for IL-2. These experiments demonstrate that in the absence of IL-15, the cytokine trio IL-2, IL-21, and TNF is necessary and sufficient for the induction of malignant Lin$^-$IEL proliferation by CD4$^+$ T-cell supernatant.

TNF, IL-2, and IL-21 Synergize to Induce Proliferation of Malignant Lin$^-$IEL Lines. The experiments described above indicate that TNF synergizes with IL-21 and, to a lesser extent, with IL-2 in the stimulation of Lin$^-$IEL proliferation. To more precisely determine the degree of synergy between these cytokines, proliferation was measured in response to combinations of a wide dosage range of two cytokines (Fig. 3). Consistent with the results obtained with fixed cytokine doses (Fig. 2*D*), IL-2 and IL-21 each induced significant proliferation, whereas TNF did not (Fig. 3 *A* and *B*); however, the presence of even low doses of TNF greatly enhanced the response to IL-21 (Fig. 3*A*). TNF also slightly enhanced IL-2 induced proliferation (Fig. 3*B*). In contrast, no synergy was observed between IL-2 and IL-21 (Fig. 3*C*). In conclusion, TNF, IL-2, and IL-21 produced by activated gluten-specific CD4$^+$ T cells synergistically induce the proliferation of malignant Lin$^-$IEL lines from RCDII patients, and this synergy involves mainly IL-21 and TNF.

TNF, IL-2, and IL-21 Together Induce Phosphorylated STAT5, Phosphorylated Akt, and bcl-x_L to a Similar Degree as IL-15. Common γ-chain cytokines deliver proliferation and survival signals via JAK1 and JAK3, which phosphorylate and activate STAT3 and STAT5 (41). In malignant Lin$^-$IEL lines, IL-15 induces phosphorylation of STAT3, STAT5, AKT, and ERK and increases levels of bcl-2 and bcl-x_L transcripts (16). Of these, phosphorylated STAT5 (pSTAT5) and bcl-x_L contribute to increased survival, and phosphorylated

Table 1. Activation-induced CD4 T-cell cytokines and corresponding receptors on Lin⁻IEL lines

Gene	FC	pfp	MS	Receptor genes	P1	P2	P3
IL21	**19.2**	**0.000**	**x**	***IL21R/IL2RG***	**x**	**x**	**x**
IL3	15.6	0.000	x	*IL3RA/CSF2RB*			
XCL1	17.9	0.000		*XCR1*			
CCL1	15.6	0.000	x	*CCR8*			
IL13	13.4	0.000	x	*IL4R/IL13RA1*			
CSF2	9.6	0.000	x	*CSF2RA/CSF2RB*			
IL8	9.4	0.000	x	*IL8RA/IL8RB*			
CCL4	9.1	0.000		*CCR5*			
IL2	**7.6**	**0.000**	**x**	***IL2RA/IL2RB/IL2RG***	**x**	**x**	**x**
CCL4L1	7.5	0.000	x	*CCR5*			
TNF	**6.6**	**0.000**	**x**	***TNFRSF1A***	**x**	**x**	**x**
				TNFRSF1B	**x**	**x**	**x**
CCL20	5.1	0.001		*CCR6*	x	x	x
CCL3L1	5.5	0.001	x	*CCR5/CCBP2*			
IL22	6.4	0.001	x	*IL10RA/IL22RA1*			
AREG	5.1	0.002	x	*EGFR*			
PDGFA	4.9	0.002		*PDGFRA/PDGFRB*			
IL10	**6.3**	**0.003**	**x**	***IL10RA/IL10RB***	**x**	**x**	**x**
IL1A	4.3	0.003		*IL1R1/IL1RAP*			
				IL1R2/IL1RAP			
HBEGF	4.0	0.003	x	*EGFR*			
XCL2	3.9	0.004		*XCR1*			
IFNG	4.1	0.004	x	*IFNGR1/IFNGR2*		x	
CD40LG	3.8	0.005	x	*TNFRSF5*			
LTA	3.7	0.006		*TNFRSF1A*	x	x	x
				TNFRSF1B	x	x	x
				TNFRSF14	x	x	x
FASLG	3.7	0.007		*TNFRSF6*	x	x	
CCL3	3.9	0.006	x	*CCR5/CCBP2*			
IL5	4.5	0.007		*IL5RA/CSF2RB*			
CSF1	3.5	0.011	x	*CSF1R*			
NAMPT	3.3	0.012		None			
F5	3.8	0.015		None			
IGF2	2.9	0.042		*IGF1R*			
				IGF2R	x	x	x
LIF	2.4	0.047	x	*LIFR/IL6ST*			

Gluten-specific CD4⁺ T-cell clone L10 was activated using plate-bound CD3/CD28 or control antibodies, and RNA isolated at 3 h after activation was analyzed using an Affymetrix human ST1.0 array (GEO accession no. GSE73599). The experiment was performed in triplicate and yielded a list of 141 genes significantly induced by CD3/CD28 cross-linking. Of these, 31 transcripts encoding secreted proteins are listed, with fold change (FC) and percentage of false-positive predictions. RT-qPCR experiments confirmed the induction of *IL10*, *IL13*, *IL21*, *IL22*, *IFNG*, *LIF*, and *AREG* transcription in L10 and four additional gluten-specific CD4⁺ T-cell clones, both on CD3/CD28 cross-linking and on recognition of cognate peptide (data not shown). Supernatants from this clone were analyzed by MS at 4 h after activation in serum-free medium. Three independent biological replicates were performed, and proteins (encoded by the genes in the first column) detected in supernatants from the CD3/CD28-activated T-cell clone are marked with an x. The corresponding receptor genes are listed, and the presence of their transcripts in three Lin⁻IEL lines from RCDII patients, previously determined using an Illumina array (3) (GEO accession no. GSE33078), is indicated. *IL21*, *IL2*, *TNF*, and *IL10*, shown in bold type, are the only cytokines for which activation detectably induced their transcription and secretion and for which the malignant Lin⁻IEL lines express a receptor.

AKT (pAKT) contributes to the proliferation of malignant Lin⁻IELs (16). To test whether CD4⁺ T-cell cytokines also initiate these signaling pathways in Lin⁻IEL lines, we measured the phosphorylation of STAT5 and AKT (Fig. 4), as well as bcl-x_L transcript and protein levels (Fig. 5), in response to TNF, IL-2, and IL-21.

Like IL-15, IL-2 and IL-21 increased the amounts of intracellular pSTAT5 (Fig. 4*A*). Consistent with the fact that the JAK/STAT pathway is not involved in TNF signaling, TNF did not induce pSTAT5 (Fig. 4*A*). Incubation with TNF did lead to increased levels of intracellular AKT phosphorylated at serine 473 (Fig. 4*B*). Although IL-2 and IL-15 also increased pAKT, IL-21 did not (Fig. 4*B*). Bcl-x_L showed yet another pattern; bcl-x_L transcript and protein levels were significantly increased by TNF and to a lesser extent by IL-21, but not by IL-2 (Fig. 5). Thus, TNF (pAKT, bcl-x_L), IL-2 (pSTAT5, pAKT), and IL-21 (pSTAT, bcl-x_L) each triggered a unique combination of signals. Although no synergy between IL-21 and TNF was observed in the induction of pSTAT5, pAKT, or bcl-x_L, the combination of TNF, IL-2, and IL-21 induced these signals to a similar degree as did IL-15.

Inhibition of Proliferation by Small-Molecule Inhibitors of Signal Transduction. To test which pathways contributed to proliferation of malignant Lin⁻IEL lines in response to CD4⁺ T-cell supernatant and combinations of TNF, IL-2, IL-21, and IL-15, we used small-molecule kinase inhibitors (Fig. 6). These inhibitors target known intermediates of pathways involved in common γ-chain cytokine signaling, including JAK (tofacitinib, baricitinib, ruxolitinib, fedratinib), PI3K (dactolisib, idelalisib, copanlisib, Ly294002), AKT (MK-2206), bcl (ABT-737), and MEK (U0126). In the first experiments, a single inhibitor per target was combined with cytokines used at a single dose (Fig. 4*A*). The proliferation induced by IL-2 and IL-15 was similarly affected by this panel of inhibitors and was virtually completely blocked by inhibitors of JAK (tofacitinib) and PI3K (dactolisib). Compared with the proliferation induced by IL-2 and IL-15, the proliferation induced by IL-21 was consistently less sensitive to tofacitinib, which targets JAK3 (Fig. 5*A*). Of note, the TNF/IL-2/IL-21 combination and CD4⁺ T-cell supernatant exhibited comparable inhibitor sensitivities (Fig. 6*A*), in line with our observation that these cytokines mediate proliferation induced by the supernatant (Fig. 2).

To more precisely gauge the contribution of the various pathways to the proliferation of malignant Lin⁻IEL lines, we titrated IL-15, IL-21, and CD4⁺ T-cell supernatants in the presence or

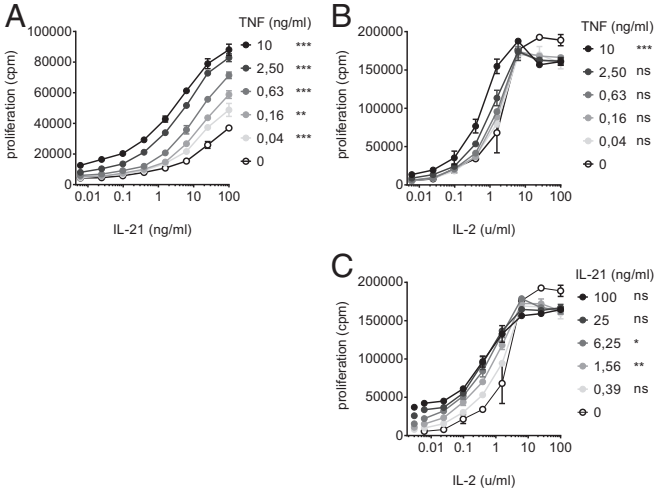

Fig. 3. TNF and IL-21 synergistically stimulate the proliferation of Lin⁻IEL lines. Proliferation of Lin⁻IEL cell line P2 was measured in the presence of cotitrations of IL-21 and TNF (*A*), IL-2 and TNF (*B*), and IL-2 and IL-21 (*C*). In *C*, the proliferation induced by IL-21 in the absence of IL-2 is shown at the leftmost part of the graph (unconnected dots). Data are from one experiment representative of five experiments with comparable results, using cell lines P1 ($n = 2$), P2 ($n = 4$), and/or P4 ($n = 1$). The area under the curve (AUC) of each curve was compared with that of the curve below it, i.e., with a one-step lower concentration of TNF (*A* and *B*) or IL-21 (*C*). Results of the statistical analyses are depicted as follows: ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

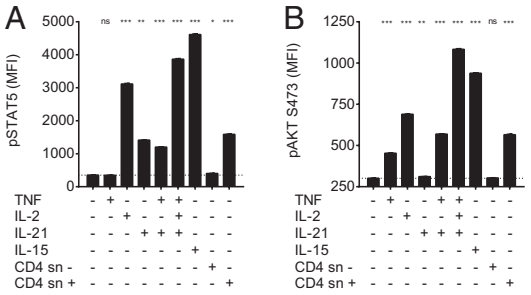

Fig. 4. TNF, IL-2, and IL-21 together induce pSTAT5 and pAKT to a similar degree as IL-15. Lin$^-$IEL cell line P2 was stimulated with cytokines (1 u/mL IL-2, 10 ng/mL IL-15, 100 ng/mL IL-21, 10 ng/mL TNF) or CD4$^+$ T-cell supernatant (clone L10) or supernatant from CD3/CD28-activated (CD4 sn$^+$) or nonactivated (CD4 sn$^-$) gluten-specific CD4$^+$ T-cell clone L10. The induction of STAT5-pY694 (*A*) and AKT-pS473 (*B*) was measured by Phosflow. Data in *A* and *B* are representative of a total of seven experiments with similar results, using cell lines P1 (n = 3), P2 (n = 6), and P4 (n = 1). Results of the statistical analyses are depicted as follows: ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

absence of inhibitors for each of the pathways (Fig. 5*B*). In line with the activation of STAT5, AKT and bcl-x$_L$ by IL-15 (Figs. 4 and 5), IL-15–induced proliferation was completely blocked by inhibitors of JAK3 (tofacitinib), bcl (abt-737), and PI3K (Ly494002) and partially blocked by AKT inhibition (MK2206), whereas MEK1/2 (U0126) inhibition had no impact (Fig. 6*B*). Compared with the proliferation induced by IL-15, that induced by IL-21 was less sensitive to the JAK inhibitor tofacitinib and more sensitive to the MEK1/2 inhibitor U0126 (Fig. 6*B*). These results confirm that IL-21 differs from IL-15 in terms of signal transduction (Fig. 4), and indicates the existence of JAK3-independent IL-21 signaling pathways leading to proliferation.

Because JAK3 is considered essential for all common γ-chain cytokine signaling (42), we used a large panel of JAK inhibitors to rigorously test the JAK dependence of IL-21 signaling (Fig. 6*C*). Ruxolitinib (JAK1/2), baricitinib (JAK1/2), and tofacitinib (JAK3) were all highly effective in blocking the proliferation induced by IL-15 across a wide range of cytokine doses, but consistently less effective in blocking IL-21–induced proliferation (Fig. 6*C*). Proliferation in response to CD4$^+$ T-cell supernatant was also less sensitive to these inhibitors than to IL-15 (Fig. 6*C*). Blockade of JAK2 (fedratinib), which is not involved in common γ-chain cytokine signaling, did not impair proliferation in response to any of the stimuli. Thus, even though IL-21 belongs to the common γ-chain cytokine family, IL-21–induced signal transduction in malignant Lin$^-$IELs is partially resistant to inhibitors targeting JAK1 and/or JAK3.

Because the PI3K inhibitors dactolisib and Ly494002 were the most effective against all cytokines tested (Fig. 5 *A* and *B*), we investigated which p110 PI3K chain is involved in driving proliferation. Dactolisib (p110α/β/γ/δ) and Ly494002 (p110α/β/δ) efficiently blocked proliferation induced by IL-15, IL-21, and CD4$^+$ T-cell supernatant (Fig. 6*D*). In contrast, idelalisib (p110δ) and copanlisib (p110α/β) were considerably less effective against IL-21 and almost completely ineffective against IL-15 and supernatant (Fig. 6*D*). These findings suggest that whereas p110γ is more important to IL-15–induced proliferation, p110α/β/δ makes a greater contribution to IL-21–induced proliferation.

Using specific kinase inhibitors, these experiments show that the JAK/STAT and PI3K/AKT pathways both contribute to the proliferation induced by IL-15 and CD4$^+$ T-cell cytokines. Our results indicate that JAK1 and JAK3 are essential for the effects of IL-15, but less so for IL-21. In contrast, PI3K subunits p110α/β/δ and MEK appear to contribute more to IL-21 signaling than to IL-15 signaling for proliferation. Finally, the PI3K inhibitor dactolisib, which has undergone phase 1 and phase 2 clinical trials for multiple solid malignancies, most efficiently blocked proliferation for all cytokines tested.

TNF, IL-2, and IL-21 Synergistically Induce Proliferation of Freshly Isolated Innate IELs, Including Malignant Lin$^-$IELs from RCDII Patients. We next determined the cytokine sensitivity of freshly isolated duodenal IELs from patients with RCDII and those without RCDII. To this end, IELs were isolated from duodenal biopsy specimens and immediately cultured with cytokines for 2 d, after which proliferating Ki-67$^+$ cells were identified by multiparameter flow cytometry (Fig. 7*A*). This allowed the simultaneous analysis of Lin$^-$IELs (CD45$^+$SSC^{lo}CD7$^+$CD3$^-$CD56$^-$), conventional NK cells (cNK; CD45$^+$SSC^{lo}CD7$^+$CD3$^-$CD56$^+$) and T cells (CD45$^+$SSC^{lo}CD7$^+$CD3$^+$CD56$^{-/+}$). In the majority of patients, fresh peripheral blood lymphocytes (PBLs) were also available and were included in the experiment.

Whereas TNF, IL-2, and IL-21 individually did not induce significant proliferation of Lin$^-$IELs, together they did induce Ki-67 expression in Lin$^-$IELs in 12 out of 32 biopsy specimens (Fig. 7*B*). In line with the results obtained with RCDII cell lines (Fig. 2*A*), the TNF/IL-2/IL-21 mixture triggered Lin$^-$IEL proliferation in 5 out of 10 RCDII patients (Fig. 6*D*). This cytokine mixture also triggered proliferation of local cNK cells, but did not cause detectable proliferation of local T cells or peripheral blood cNK or T cells (Fig. 7 *C* and *D*). In contrast, all innate and adaptive lymphocyte subsets in IELs and PBLs proliferated in response to IL-15, albeit to greatly divergent degrees, ranging from just above medium background in peripheral T cells to up to 70% dividing cells in Lin$^-$IELs (Fig. 7 *B*–*D*). These data indicate that TNF, IL-2, and IL-21 synergistically and selectively induce proliferation of innate IELs. Importantly, the responses of Lin$^-$IELs from RCDII patients and non-RCDII patients were indistinguishable (Fig. 7 *C* and *D*), suggesting that CD4$^+$ T-cell cytokines and IL-15 can contribute to the expansion of Lin$^-$IELs already before their malignant transformation in RCDII.

Discussion

Epithelial IL-15 is an important factor in CD pathogenesis (43, 44) and is currently the sole cytokine proposed to contribute to the selective expansion of malignant Lin$^-$IELs in RCDII (6, 16). We now show that in the absence of IL-15, CD4$^+$ T-cell cytokines TNF, IL-2, and IL-21 are also able to drive the expansion of nonmalignant and malignant Lin$^-$IEL. Using malignant Lin$^-$IEL lines from RCDII patients, we show that TNF, IL-2, and IL-21, produced by gluten-specific CD4$^+$ T cells from the lamina propria of CD patients, synergistically stimulate pro-

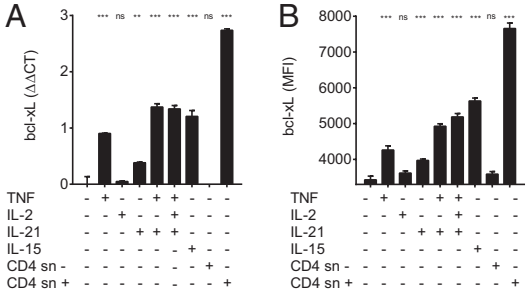

Fig. 5. TNF, IL-2, and IL-21 together induce bcl-xL mRNA and protein. Lin$^-$IEL cell line P2 was stimulated with cytokines (1 IU/mL IL-2, 10 ng/mL IL-15, 100 ng/mL IL-21, and 10 ng/mL TNF) or supernatant from CD3/CD28-activated (CD4 sn$^+$) or nonactivated (CD4 sn$^-$) gluten-specific CD4$^+$ T-cell clone L10. The expression of bcl-xL mRNA after 3 h of incubation (*A*) and protein after 24 h of incubation (*B*) was measured by RT-qPCR and FACS, respectively. Data are representative of two experiments using cell lines P1 and P2. Results of the statistical analyses are depicted as follows: ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

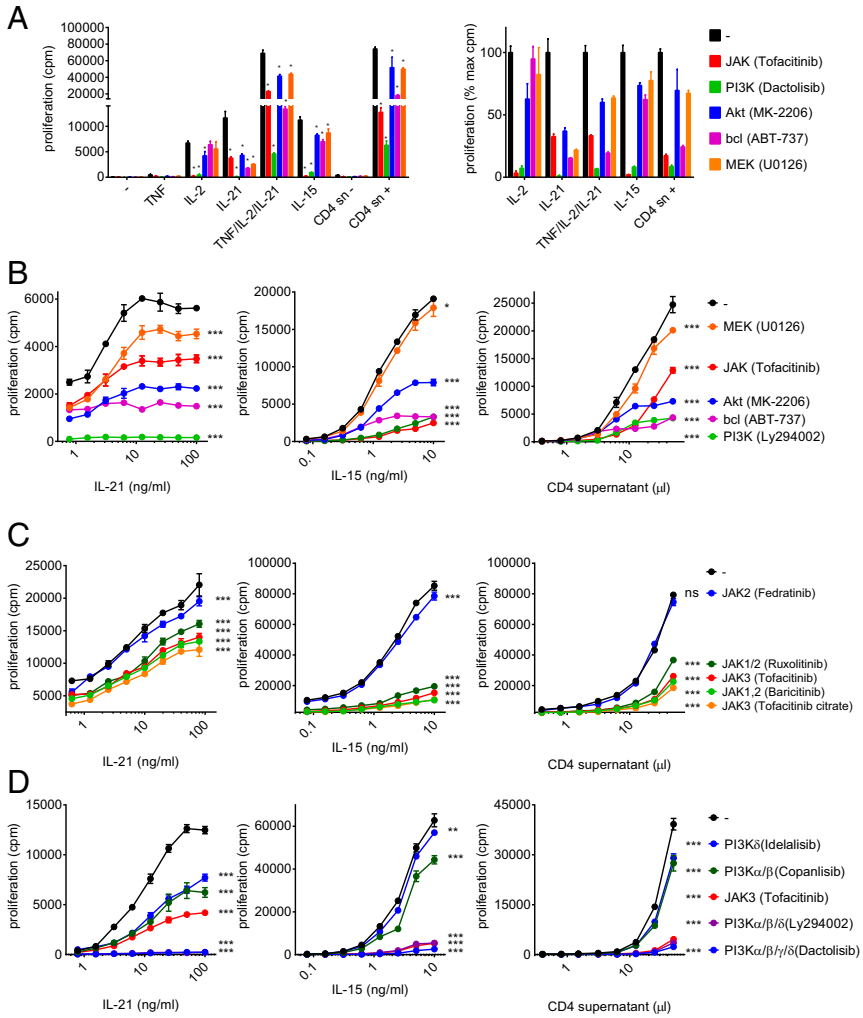

Fig. 6. Proliferation induced by TNF, IL-2, and IL-21 is sensitive to small-molecule inhibitors of the JAK/STAT and PI3K/AKT pathways. The proliferation of Lin$^-$IEL cell line P2 in response to combinations of cytokines (1 IU/mL IL-2, 10 ng/mL IL-15, 100 ng/mL IL-21, and 10 ng/mL TNF or the indicated concentrations) or to control-activated (CD4 sn$^-$) or CD3/CD28-activated (CD4 sn$^+$) CD4$^+$ T-cell supernatant (clone L10) was measured. Before exposure to the indicated stimuli, P2 was preincubated with inhibitors of JAK3, PI3K, AKT1-3, bcl-xL/bcl-2/mcl-1, or MEK1,2 (*A* and *B*); JAK inhibitors targeting specific JAKs (*C*); or PI3K inhibitors targeting specific p110 chains (*D*). Data in *A* are representative of two experiments, both using cell lines P1 and P2. Furthermore, *A* depicts proliferation both as raw cpm (left graph) and as a percentage of the maximum cpm response for each stimulus. Data in *B* are representative of two experiments using P1 and/or P2. Data in *C* and *D* are each representative of one experiment using both P1 and P2. Results of the statistical analyses are depicted as follows: ns, $P > 0.05$; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. For clarity, in *A* asterisks indicate any *P* value <0.05.

liferation. Together, these cytokines can induce pSTAT5, pAKT, bcl-x_L, and proliferation to a similar extent as IL-15. Thus, in line with the strong HLA-DQ2 association of RCDII, these findings suggest that not only IL-15, but also cytokines produced by gluten-specific CD4$^+$ T cells, contribute to the expansion of Lin$^-$IELs in RCDII (Fig. 8).

TNF, IL-2, and IL-21 together induced the proliferation of freshly isolated duodenal Lin$^-$IEL and cNK cells irrespective of disease status. This finding not only confirms our observations in malignant Lin$^-$IEL cell lines from RCDII patients, but also raises the possibility that the activation of lamina propria CD4$^+$ T cells promotes innate IEL expansion during infection or in inflammatory conditions such as CD. RCDII is usually diagnosed late in life, and thus gluten-specific CD4$^+$ T cells may contribute to the expansion of nonmalignant Lin$^-$IELs well before RCDII is diagnosed and a gluten-free diet is initiated. Of note, approximately one-half of RCDII patients are HLA-DQ2.5 homozygous (22, 23). This double HLA-DQ2.5 gene dose allows more efficient gluten presentation to CD4$^+$ T cells, resulting in increased cytokine production by these T cells (19). Although initiation of a gluten-free diet after the diagnosis of RCDII should halt this process, duodenal inflammation persists. With the exception of IL-15 (15, 16), data on the cytokines up-regulated in RCDII are extremely limited. In fact, a recent report on four RCDII patients on a gluten-free diet suggests a unique cytokine profile characterized by elevation of TNF but not of IL-15, IL-21, or IFN-γ (40). Therefore, it will be essential to determine whether TNF, IL-2, IL-15, and IL-21 are produced before and after the initiation of a gluten-free diet in a larger group of patients, which will determine their relevance as therapeutic targets in RCDII.

The identification of TNF, IL-2, and IL-21 was based on in vitro expanded gluten-specific T-cell clones, whose cytokine production profile may have been altered on in vitro culture. However, analyses of biopsy specimens support the idea that TNF, IL-2, and IL-21 are indeed produced in active CD. Experiments examining the expression of CD4$^+$ T-cell cytokine transcripts and proteins in patient biopsy specimens and biopsy-derived gluten-specific CD4$^+$ T cells focused on IFNG, TNF, IL2 (31, 33, 35, 36), and, more

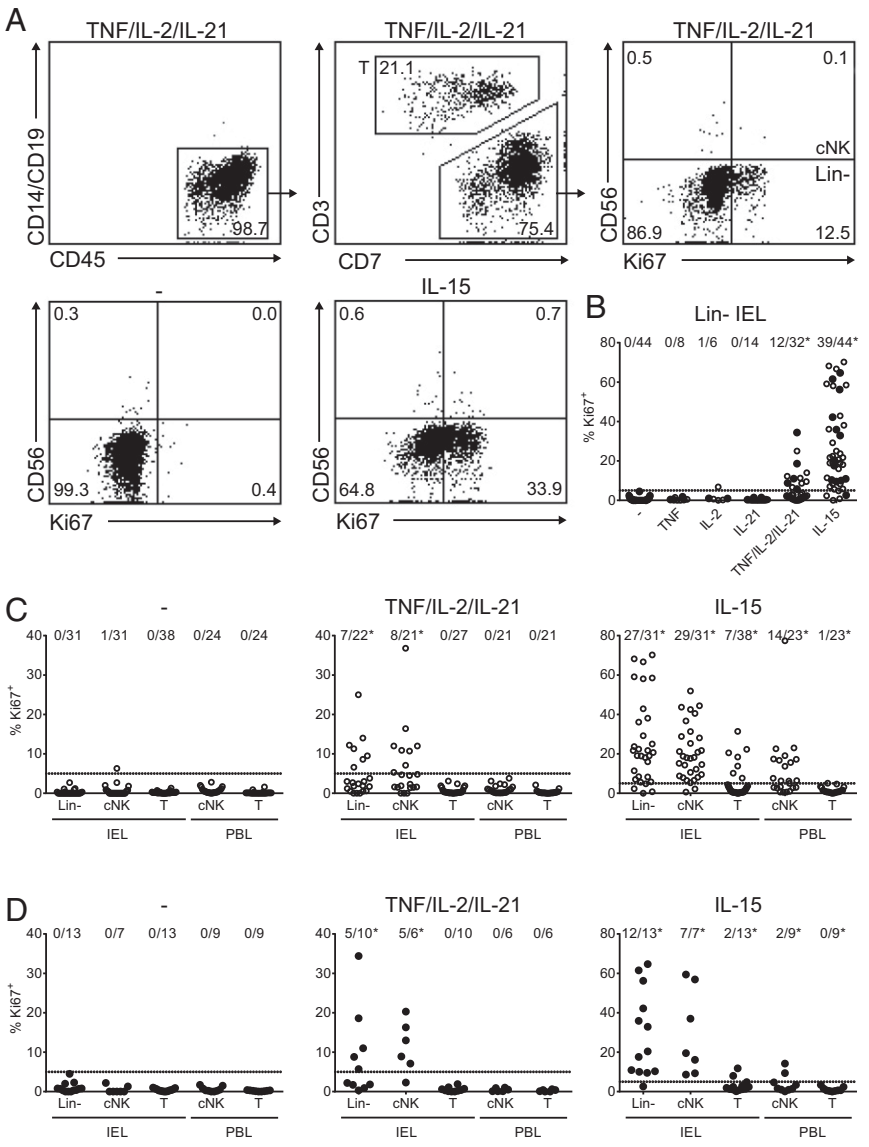

Fig. 7. TNF, IL-2, and IL-21 synergistically induce proliferation of freshly isolated innate IELs, including Lin$^-$IELs from RCDII patients. IELs were isolated from duodenal biopsy specimens, cultured for 2 d in the presence of the indicated cytokines (1 IU/mL IL-2, 10 ng/mL IL-15, 100 ng/mL IL-21, and 10 ng/mL TNF) and then analyzed by FACS for intracellular Ki-67 expression. IELs from 51 biopsy specimens from non-CD ($n = 24$), CD ($n = 12$), RCDI ($n = 2$), and RCDII ($n = 13$) patients were incubated with TNF ($n = 8$), IL-2 ($n = 7$), IL-21 ($n = 16$), and TNF/IL-2/IL-21 ($n = 37$), with negative (medium) and positive (IL-15) controls included for each sample ($n = 51$). For 33 biopsy specimens, fresh PBLs from the same patient were also included in the experiment; these did not contain a detectable Lin$^-$ population. (*A*) Gating strategy and Ki-67 staining of Lin$^-$IELs. Live, single CD45$^+$ cells, gated based on forward scatter, side scatter, and CD45 values, were separated based on their expression of CD14, CD19, CD3, CD7, and CD56. T cells were defined as CD45$^+$CD14$^-$CD19$^-$CD3$^+$CD7$^+$ cells, cNK cells as CD45$^+$CD7$^+$CD14$^-$CD19$^-$CD3$^-$CD56$^+$, and Lin$^-$ cells as CD45$^+$CD7$^+$CD14$^-$CD19$^-$CD3$^-$CD56$^-$. The top row shows the results of TNF/IL-2/IL-21 stimulation of IELs from an RCDII patient, and the bottom row shows the results of negative (–) and positive (IL-15) control stimulations of the same patient sample, gated on CD45$^+$CD14$^-$CD19$^-$CD3$^-$CD7$^+$ cells. The graphs in *B–D* show the percentages of Ki-67$^+$ cells among Lin$^-$, cNK, and T cells. Note that in RCDII patients, virtually all Lin$^-$IELs are aberrant IELs expressing intracellular CD3ε and all intracellular CD3ε$^+$ aberrant IEL are Lin$^-$IEL (3). Only measurements containing more than 50 Lin$^-$, cNK, or T cells were used. Closed circles depict the results from RCDII patient biopsy specimens, and open circles depict non-RCDII biopsy specimens. Asterisks indicate responses significantly greater than the medium control, according to the Kruskall–Wallis test and after Dunn's correction for multiple testing. P values <0.05 were considered significant. The fraction of biopsy specimens responding to a particular cytokine (mix) is indicated at the top of each graph, using 5% Ki-67$^+$ cells as an arbitrary cutoff value. (*B*) Ki-67 responses of Lin$^-$IELs from all biopsy specimens to TNF, IL-2, IL-21, IL-15, and combinations thereof. (*C*) Ki-67 responses of IEL and PBL subsets from non-RCDII patients to TNF/IL-2/IL-21 and IL-15. (*D*) Ki-67 responses of IEL and PBL subsets from RCDII patients to TNF/IL-2/IL-21 and IL-15.

recently, on IL17, IL21, and IL22 (30, 37–40). TNF, IL-2, and IL-21 were reported to be produced by gluten-activated CD4$^+$ T-cell clones on activation (19, 30, 31) and to be elevated in CD biopsy specimens exposed to gluten in vivo or ex vivo (33, 34, 39). In contrast, IL-17A is not produced by gluten-specific clones (30), but some studies have shown that it is elevated in gluten-exposed biopsy specimens (32, 45). This finding suggests that cytokines produced by gluten-specific CD4$^+$ T cells trigger IL-17 production by other immune cells (32). Our extensive transcriptomic and proteomic analysis of a single CD4$^+$ T-cell clone confirm and extend these findings, suggesting many additional cytokines that may contribute to the pathogenesis of (refractory) CD, such as IL-22, AREG, and HB-EGF, all of which are involved in the homeostasis of IECs.

The magnitude of the responses of primary IELs to cytokines varied among IEL subsets and patients. Whereas all Lin$^-$IEL

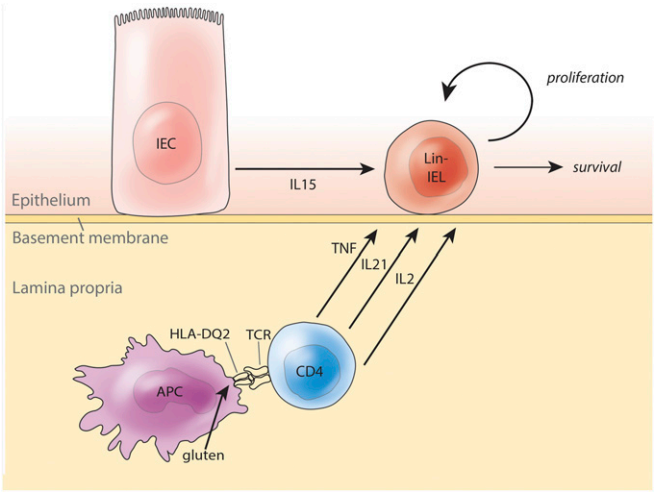

Fig. 8. Proposed mechanisms driving Lin$^-$IEL expansion in RCDII. During the development of RCDII, IL-15 is produced by IECs, and TNF, IL-2, and IL-21 are produced by gluten-specific CD4$^+$ T cells in the lamina propria. IL-15 is bound to IL-15Rα on the surface of IECs and presented in trans to nearby Lin$^-$IEL, whereas TNF, IL-2, and IL-21 reach Lin$^-$IEL by diffusion through the basement membrane. Both IL-15 (16) and the CD4$^+$ T-cell cytokines have the capability of contributing to Lin$^-$IEL proliferation and survival.

samples responding to TNF/IL-2/IL21 also responded to IL-15, not all samples that responded to IL-15 also responded to TNF/IL-2/IL21. This finding is in line with the results obtained with malignant Lin$^-$IEL lines, one of which did respond to IL-15 but not to the CD4$^+$ T-cell cytokines. The Lin$^-$IEL population in RCDII patients as well as controls consists of multiple subsets (46) that potentially differ in their responsiveness to these cytokines (4). Thus, differences in the composition of the Lin$^-$IEL population may explain the heterogeneity in TNF/IL-2/IL-21 responses among individuals.

Although IL-15, TNF, IL-2, and IL-21 are all well known for their antitumor effects, this is different in the case of some cancers. TNF can act as a tumor-promoting factor for solid malignancies, for example, by causing genetic damage and by promoting proliferation, survival, or angiogenesis (47). TNF is known to induce bcl-x_L in neuronal cells (48) and in a T-cell line (49), and our data suggest that a similar mechanism may operate in malignant Lin$^-$IELs in RCDII. The induction of the antiapoptotic bcl-x_L by TNF might be mediated via NF-κB, because the promoter of BCL2L1, the gene encoding bcl-x_L, contains a functional κB consensus site (49, 50). Furthermore, IL-2 can act as a growth factor for malignant T lymphocytes in the case of adult T-cell leukemia, a CD4$^+$ T-cell leukemia caused by the human T lymphotropic virus type I (51). Finally, IL-21 acts as an autocrine factor that uses STAT3 to promote IL-2–driven proliferation of the CD4$^+$ T cells that constitute Sézary lymphoma (52) and uses STAT5 to promote Hodgkin lymphomagenesis (53). Our experiments suggest that RCDII-associated type I EATL should be added to the list of malignancies supported by TNF, IL-2, and IL-21.

The molecular mechanisms underlying the synergistic induction of malignant Lin$^-$IEL line proliferation by TNF, IL-2, and IL-21 remain unresolved, with no clear synergy observed in the induction of pSTAT5, pAkt S473, or bcl-x_L. In proliferation assays, the strongest synergy was detected between TNF and IL-21, which use distinct signaling pathways. TNF promotes cell survival via TRAF2, which activates three pathways: IKK/NF-κB, RIP/MKK3/MAPK, and MEKK1/MKK7/JNK/AP1 (47). Although IL-21 is known to signal via the JAK/STAT pathway like other common γ-chain cytokines, its unique partial resistance to JAK1 and JAK3 inhibitors revealed by our study indicates that it also activates a JAK/STAT-independent pathway. Thus, TNF might selectively boost JAK-independent IL-21 signaling. The fact that the PI3K inhibitor dactolisib efficiently impairs proliferation induced by CD4$^+$ T-cell cytokines indicates that the PI3K pathway is involved; however, this is normally linked to the phosphorylation of AKT, and IL-21 did not detectably induce pAKT S473, and the AKT inhibitor MK-2206 did not completely block the proliferation induced by any of the cytokine mixtures. It possible that IL-21 activates Akt by phosphorylation at T308 rather than S473, and that T308 phosphorylation is sufficient to drive proliferation. In short, elucidation of a JAK-independent IL-21 signaling pathway suggested by our findings may be key to understanding its synergy with TNF. In addition, recent data show that in a subset of RCDII patients, the malignant Lin$^-$IELs have gain-of-function mutations in STAT3 (6). Unlike IL-15, which primarily activates STAT5, IL-21 preferentially activates STAT1 and STAT3 (54, 55); thus, the JAK-dependent IL-21 pathway may be especially affected in these cells.

The contribution of epithelial IL-15 to CD and RCDII is well accepted (56, 57); however, recent data show that IL-15 is upregulated in only a subset of CD patients (18). In addition, a sizeable subset of malignant Lin$^-$IELs in RCDII patients express low amounts of CD122, suggesting poor IL-15 responsiveness (4). A growing body of evidence (reviewed in ref. 58) indicates that CD4$^+$ T cells can directly regulate the function of innate lymphocytes. A recent study using TCR-transgenic mice shows that cytokines produced by lamina propria CD4$^+$ T cells responding to a food protein, most likely including IL-2, contribute to IEL activation (59). Our findings support this concept by demonstrating that CD4$^+$ T-cell cytokines TNF, IL-2, and IL-21 together can be as powerful as IL-15 in the induction of pSTAT5, pAkt, bcl-xL, and proliferation in malignant Lin$^-$IEL lines from RCDII patients. Furthermore, in CD, gluten-specific CD4$^+$ T cells reside in the duodenal lamina propria (60) and thus in close proximity to IELs. The relative contribution of cytokines produced by lamina propria CD4$^+$ T cells and epithelial IL-15 to the expansion of Lin$^-$IEL in RCDII patients is currently unclear and may vary among patients and according to disease stage. Thus, treatment with monoclonal antibodies neutralizing IL-15 (16) or its receptor (17, 61) might not be effective in all patients, and our findings suggest that oral administration of small-molecule inhibitors more broadly targeting cytokine signaling may be an attractive alternative strategy to counter the expansion of malignant Lin$^-$IELs in RCDII patients.

Materials and Methods

Biopsy Specimens. This study was approved by the Medical Ethical Committees of VU University Medical Center and Leiden University Medical Center and executed in accordance with the local ethical guidelines and the Declaration of Helsinki. After informed consent was obtained, large spike forceps biopsy specimens (Medi-Globe) were taken from the second part of the duodenum during upper endoscopy and stored at room temperature in HBSS (Life Technologies) until use.

Cell Lines and Culture. Malignant RCDII Lin$^-$IEL lines and gluten-specific CD4$^+$ T-cell lines and clones were isolated and cultured as described previously (25, 27, 62). To generate CD4$^+$ supernatants for Lin$^-$IEL stimulation, the clones were activated via their T-cell receptors, by stimulation with either gluten peptide-loaded PBMCs or plate-bound antibodies to CD3 and CD28.

Transcriptomic and Secretomic Analysis of a Gluten-Specific CD4$^+$ T-Cell Clone. CD4$^+$ T-cell clone L10, which recognizes DQ2-glia-α1, one of the immunodominant T-cell epitopes in CD (29), was stimulated with plate-bound CD3/CD28-specific (2.5 μg/mL each) or control antibodies. After 3–4 h of incubation, either cells (3 h) or supernatants (4 h) were harvested. Cellular transcriptomes were determined using Human Gene 1.0 ST arrays (Affymetrix), and proteins in the supernatants (secretomes) were identified by nano-HPLCy-MS/MS analysis of a tryptic digest of the supernatant. For both transcriptomic (GEO accession no. GSE73599) and secretomic analyses, three independent biological replicates of each condition were performed.

Proliferation Assays. At 2–4 wk after restimulation with feeder cells, Lin$^-$IEL lines were washed and rested in the absence of cytokines for 1–3 d. Lin$^-$IEL were preincubated [37 °C, 5% (vol/vol) CO_2] with small-molecule inhibitors for 30 min (or not) and then cultured for 2 d in 96-well plates in the presence of cytokines or CD4$^+$ T-cell supernatant (50 μL/well) in a total volume of 150 μL, after which 0.5 μCi ^{3}H-thymidine was added to each well. In experiments using antibodies or a recombinant receptor to deplete specific cytokines, IL-15 or CD4$^+$ T-cell supernatant was preincubated with anti-TNF, anti–IL-2, or recombinant human IL21R for 30 min before addition of the cells. After overnight incubation at 37 °C, cells were harvested (Tomtec Harvester; Tomtec), and ^{3}H-thymidine incorporation was determined.

Phosflow Experiments. Lin$^-$IEL lines, starved of IL-15 for 1 or 2 d, were incubated for 15 min with combinations of TNF, IL-2, IL-21, IL-15, or CD4$^+$ T-cell supernatant (50 μL/well) in a total volume of 100 μL. Immediately after incubation, cells were fixed using Phosflow Fix Buffer I (BD Biosciences) for 10 min. Cells were permeabilized using Phosflow Perm Buffer III (BD Biosciences) and then incubated for 30 min on ice. Then the cells were washed with FACS buffer and stained with fluorochrome-labeled antibodies recognizing AKT-pS473 or STAT5-pY694 (BD Biosciences) for 1 h at room temperature. Cells were washed and analyzed by flow cytometry using an LSRII flow cytometer and FACSDiva software (BD Biosciences).

RT-qPCR Detection of bcl-x_L. Lin$^-$IEL lines, starved of IL-15 for 1 or 2 d, were incubated for 3 h with combinations of TNF, IL-2, IL-21, and IL-15 or CD4$^+$ T-cell supernatant. After stimulation, ice-cold PBS was added to the cells, which were then centrifuged twice in ice-cold PBS. cDNA synthesized from total RNA was used as template for RT-qPCR detection of bcl-x_L transcripts, using GAPDH as a reference.

Ki-67 Induction in Freshly Isolated IELs. IEL isolated from two to four duodenal biopsy specimens were incubated for 2 d with combinations of TNF, IL-2, IL-21, and IL-15 or CD4$^+$ T-cell supernatant in a total volume of 100 μL in 96-well round-bottom plates. Harvested cells were surface-stained with antibodies to CD3, CD7, CD8, CD14, CD19, CD45, and CD56 before intracellular staining for Ki-67, and subsequently analyzed by flow cytometry using an LSRII flow cytometer and FACSDiva software.

Statistical Analysis. All data were analyzed using built-in analysis methods in GraphPad Prism version 7.00. Multiplicity-adjusted P values are depicted as follows: $*P \leq 0.05$, $**P \leq 0.005$, and $***P \leq 0.0005$.

More details on all aspects of this study are provided in *SI Materials and Methods*.

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