

Interleukin 15 and CD4⁺ T Cells Cooperate to Promote Small Intestinal Enteropathy in Response to Dietary Antigen

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BACKGROUND & AIMS: CD4⁺ T cells specific for dietary gluten and interleukin 15 (IL15) contribute to the pathogenesis of celiac disease. We investigated whether and how they interact to damage the intestine using mice that overexpress human IL15 in the intestinal epithelium and have CD4⁺ T cells specific for ovalbumin, a dietary antigen. **METHODS:** We crossed mice with CD4⁺ T cells specific for ovalbumin (OTII) with mice that overexpress human IL15 under an intestine-specific promoter (B6 × IL15Tge). The offspring (OTII × IL15Tge mice) received control or ovalbumin-containing diets until 3 months of age. Enteropathy was monitored by weight, ratio of villous:crypt length, and the number of intestinal lymphocytes. Phenotype, cytokine production, and degranulation of mucosal and spleen lymphocytes were analyzed by multicolor flow cytometry or enzyme-linked immunosorbent assay. Regulatory T-cell function and CD8⁺ T-cell activation were analyzed in co-culture assays. **RESULTS:** Exposure to ovalbumin reduced growth and led to enteropathy in OTII × IL15Tge mice but not in control OTII × B6 littermates. Enteropathy was associated with expansion of mucosal granzyme B⁺ CD8⁺ T cells, and developed despite increased frequency of functional ovalbumin-specific regulatory T cells. Ovalbumin-activated CD4⁺ T cells secreted IL2, which along with IL15 stimulated expansion of noncognate intestinal cytotoxic CD8⁺ T cells, which did not respond to regulatory T cells and induced epithelial damage. **CONCLUSIONS:** We observed that in mice given food antigen, cooperation between IL15 and CD4⁺ T cells is necessary and sufficient to activate CD8⁺ T cells and damage the small intestine. We propose that this process is involved in the development of celiac disease.

Keywords: Gluten Allergy; Treg Cells; Immune Regulation; Mouse Model.

In physiologic conditions, robust immunologic mechanisms avoid adverse responses to food antigens.¹ In contrast, in celiac disease (CD), abnormal activation of intestinal lymphocytes by dietary gluten leads to severe small intestinal enteropathy. In CD, intestinal lesions depend on the activation of lamina propria (LP) CD4⁺ T lymphocytes (TLs) by gluten peptides selectively presented by HLA-DQ2/8 molecules, the main genetic risk factor.² This mechanism is necessary, however, it is not sufficient to break tolerance to the dietary antigen and to induce

tissue damage.² Previous studies, including ours, also have implicated interleukin 15 (IL15), a cytokine up-regulated in the CD intestine. IL15 promotes the survival, accumulation, and activation of cytotoxic CD8⁺ intraepithelial lymphocytes (IEL), which can induce epithelial lesions.^{3–6} IL15 also might impair local immune regulation, either by making TLs resistant to suppression by transforming growth factor- β and Forkhead box P3 (FOXP3⁺) regulatory T cells (Tregs),^{7–9} or by hampering the generation of Tregs.¹⁰ Whether and how gluten-specific CD4⁺ TLs and IL15 interact to activate CD8⁺ IELs and to drive tissue damage, however, is unknown.

To address this question, we have developed a mouse model. Transgenic CD4⁺ T cells specific for ovalbumin (OTII) mice with CD4⁺ TL specific for an epitope of ovalbumin (OVA) were crossed with heterozygous B6 × IL15Tge mice, which express a secreted form of human IL15 in the intestinal epithelium.¹¹ We next compared the consequences of OVA-containing diet in the created OTII × IL15Tge mice and in OTII × B6 littermates, which also possess OVA-specific CD4⁺ TL but do not overexpress IL15. In OTII × IL15Tge mice, but not in OTII × B6 mice, early and chronic exposure to dietary OVA led to growth retardation, and to an enteropathy associated, as in CD, with expansion of Granzyme B⁺ CD8⁺ TL in LP and epithelium. We further show that IL15 cooperates with IL2 produced by OVA-activated CD4⁺ TLs to stimulate the expansion of cytotoxic CD8⁺ TL.

Materials and Methods

Mouse Models

B6 (C57BL/6j) mice, OTII mice, b2m^{-/-} mice, and major histocompatibility complex (MHC)II^{-/-} mice were purchased from

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Abbreviations used in this paper: CD, celiac disease; DC, dendritic cell; IEL, intraepithelial lymphocyte; IFN, interferon; IL, interleukin; iTreg, regulatory T cell induced in the periphery in response to exogenous antigens; LP, lamina propria; mAb, monoclonal antibody; MLN, mesenteric lymph nodes; nTreg, natural regulatory T cell generated in the thymus; OTII, CD4⁺ T cells specific for ovalbumin; OVA, ovalbumin (albumin from chicken egg white); PBS, phosphate-buffered saline; rhIL, recombinant human interleukin; TL, T lymphocyte; TCR, T-cell receptor; Treg, regulatory T cell; WT, wild type.

Charles River (L'Arbresle, France). OTII RAG2^{-/-} Thy1.1 mice and OTI RAG2^{-/-} mice were a gift from O. Lantz (Institut Curie, Paris, France). Heterozygous B6 × IL15Tge male mice, which express human IL15 under control of the intestine-specific T3b promoter, were crossed with female OTII or B6 mice.¹¹ A diet containing 10% OVA or an isoprotein/isocaloric control diet (Genestil, Royaucourt, France) was given to OTII or B6 mice during the last week of gestation, and to their offspring until 3 months of age. Daily intake of OVA was approximately 17 mg/g of mouse body weight. Animals were bred under specific pathogen-free conditions in accordance with European Guidelines. Experiments were approved by the local ethics committee.

Histology and Immunohistochemistry

Sections (5 μm) from formal-fixed, paraffin-embedded duodenum were stained with H&E. Granzyme B was detected using antimouse Granzyme B goat IgG (AF1865; R&D Systems, Lille, France), biotinylated rabbit antigoat IgG (E0466; Dako, Les Ulis, France), and streptavidin peroxidase (P0397; Dako).

Cell Isolation and Flow Cytometry

Isolation of LP, mesenteric lymph nodes (MLNs), and spleen cells, and staining for surface markers and intracellular Foxp3, Granzyme B, and cytokines, were performed as described.¹² Antibodies are listed in the [Supplementary Materials and Methods section](#). Cells were analyzed on a FACS Canto II using BD FACSDiva software (BD Biosciences, Le Pont-de Claix, France).

Cell Subset Purification and Functional Assays

T-cell subsets were enriched by an AutoMACS Pro Separator (Miltenyi Biotec, Paris, France) and further purified using a FACS Aria cell sorter II (BD Biosciences). To test Treg activity, 5 × 10⁴ OVA-specific responder T cells (Vβ5⁺CD25⁺CD4⁺) were co-cultured at a 1:1 ratio with OVA-specific Tregs (Vβ5⁺CD25⁺CD4⁺), or, as a control, with OVA-nonspecific TLs (Vβ5⁺CD4⁺CD25⁻), in 96-well, round-bottom plates with 0.5 μg/mL OVA₃₂₃₋₃₃₉ peptide (Covalab, Villeurbanne, France) and 5 × 10⁴ irradiated spleen cells from OTII mice fed the control diet. Proliferative responses were assessed in triplicate cultures by [³H] thymidine incorporation. To test T-cell response to Tregs, 5 × 10⁴ CD45⁺CD25⁻ B6 spleen cells were labeled using the Cell Trace Violet cell proliferation kit (Life Technologies, Saint Aubin, France) and co-cultured at a 1:1 ratio with unlabeled CD45⁺CD25⁺ cells or, as a control, with unlabeled CD45⁺CD25⁻ cells. To assess proliferation, cells were stimulated for 48–72 hours with 0.2 μg/mL CD3 monoclonal antibodies (mAbs) (145-2C11; BD Biosciences), and then analyzed by flow cytometry. To assess induction of interferon (IFN)-γ and Granzyme B, cells were stimulated with CD3 mAbs and 0.2 μg/mL CD28 mAbs (37.51; BD Biosciences), in the presence or absence of 20 ng/mL human IL15 (Miltenyi Biotec), and then analyzed by enzyme-linked immunosorbent assay (R&D Systems).

To analyze interactions between CD8⁺ and CD4⁺ TLs, CD8⁺ and CD4⁺ TLs purified from indicated sources were stained using Cell Trace Violet, added with spleen CD11c⁺ dendritic cells (DCs) (DC/T ratio, 1:10), and stimulated with OVA₂₅₃₋₂₆₇, OVA₃₂₃₋₃₃₉ (2 μg/mL), or OVA protein (400 μg/mL) plus 20 ng/mL IL15. After 4 days, proliferation and activation

markers were assessed by flow cytometry. Degranulation of CD8⁺ TL was analyzed by stimulating cells for 4 hours with 1 μg/mL CD3 mAb, and then staining for CD107a-PE (1D4B; eBioscience, Paris, France).

Adoptive Cell Transfer

Spleen and MLN cells from OTII RAG2^{-/-} Thy1.1 transgenic mice (2 × 10⁶ cells in 100 μL phosphate-buffered saline [PBS]) were injected intravenously into B6 or B6 × IL15Tge mice. On days 1 and 2 after transfer, mice were gavaged with 50 mg OVA in 100 μL PBS, or with PBS alone. CD25⁺Foxp3⁺Thy1.1⁺ Tregs were assessed by flow cytometry in spleen and MLN on day 5.

Western Blot

Detection of Granzyme B in duodenal tissue was performed as described,⁷ using goat anti-Granzyme B (AF1865; R&D Systems). Granzyme B was quantified relative to actin, using a LAS-1000 CCD camera and Image Gauge software (Fujifilm Lifesciences, Bois d'Arcy, France).

Messenger RNA Expression

RNA was purified from duodenal biopsy specimens using an RNeasy Mini kit (Qiagen France S.A.S., Courtaboeuf, France) and reverse-transcribed.¹² Quantitative polymerase chain reaction was performed using TaqMan gene expression assays (Life Technologies). Data were normalized to Hypoxanthine-guanine phosphoribosyltransferase.

IL2 Treatment

B6 and B6 × IL15Tge mice were treated for 5 days by daily intraperitoneal injections of 200,000 IU recombinant human IL2 (rhIL2) (Proleukin, Novartis, France), generously provided by D. Klatzmann (CHU Pitié-Salpêtrière, Paris, France).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 6.00 (GraphPad Software, Inc., La Jolla). Weight curves were compared using repeated analysis of variance followed by the Tukey post hoc test. Other comparisons used the Mann-Whitney *U* test. A *P* value less than .05 was considered statistically significant.

Results

Chronic Oral Exposure to OVA Causes Small Intestinal Lesions in OTII × IL15Tge Mice

Whether fed with control diet or OVA diet, OTII × B6 mice showed similar weight gain and a similar villous/crypt length ratio ([Figure 1A and B](#)), indicating that the presence of many CD4⁺ TLs specific for the dietary antigen ([Supplementary Figure 1A](#)) is not sufficient to induce an enteropathy. In contrast, 3-month-old OTII × IL15Tge mice fed the OVA diet showed slower growth; increased cellularity in LP, epithelium, and submucosa; villous blunting; and crypt elongation in the proximal duodenum ([Figure 1A–F](#) and [Supplementary Figure 2A](#)). Epithelial lesions were patchy and their intensity varied. They were particularly severe in 7 of 11 OVA-fed

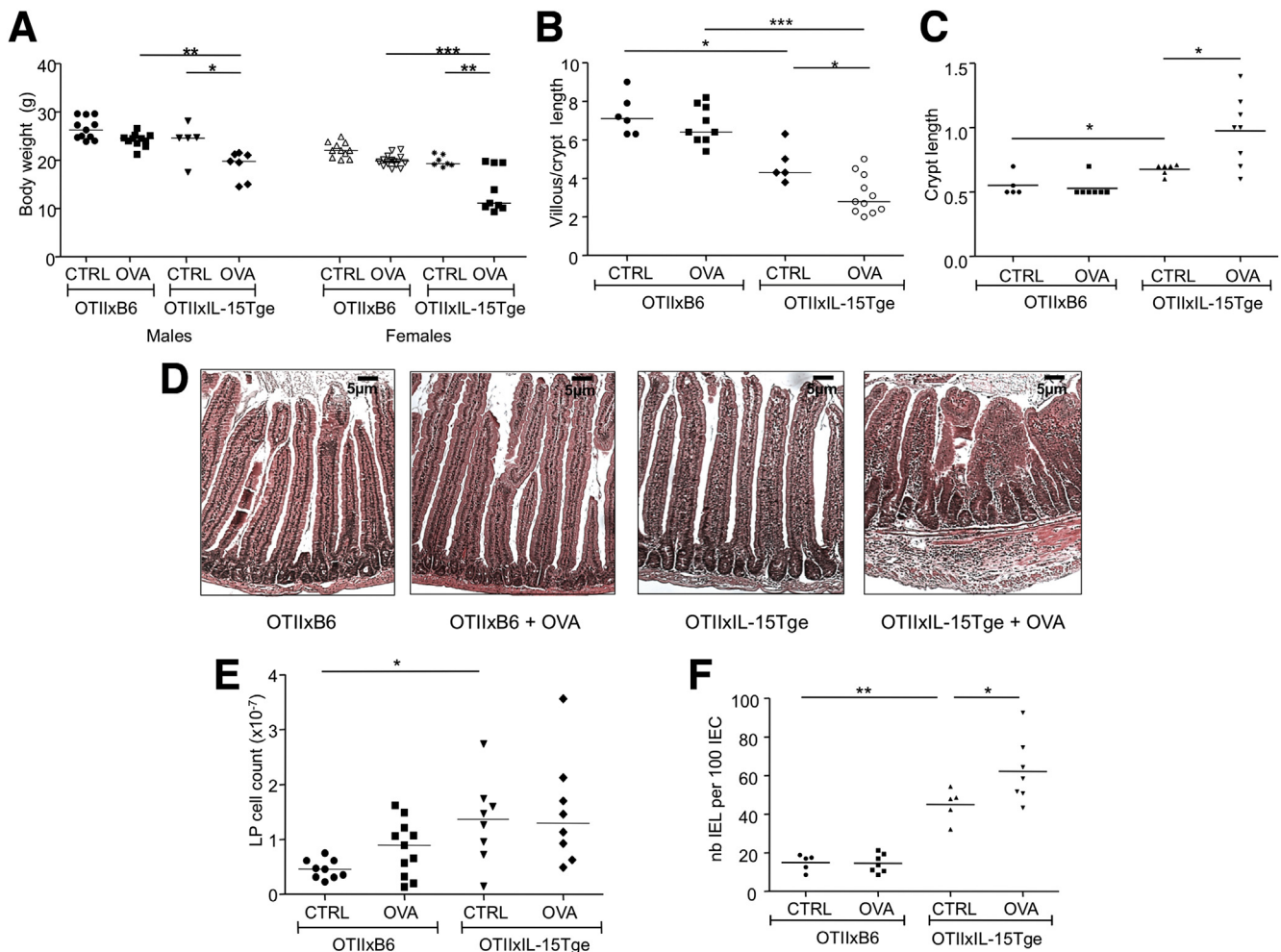


Figure 1. Enteropathy development in OVA-fed OTII $\times$ IL15Tge mice. (A) Comparison of body weight of 3-month-old OTII $\times$ B6 and OTII $\times$ IL15Tge mice fed OVA-containing (OVA) or control (CTRL) diets. Males and females were analyzed separately. (B, C, E, and F) Comparison of (B) villous/crypt length ratio, (C) crypt length, (E) absolute numbers of small intestinal LP cells, and (F) numbers of IELs per 100 intestinal epithelial cells (IECs) between indicated groups of mice. (D) Representative H&E-stained duodenal sections at 3 months in each group. Scale bars: 5 μ m. * P < .05, ** P < .01, *** P < .001.

OTII $\times$ IL15Tge mice, which displayed a hypotonic and enlarged duodenum and severe growth retardation (Figure 1A and Supplementary Figure 2A). Some changes were observed in 3-month-old OTII $\times$ IL15Tge mice on the control diet (Figure 1B, C, E, and F). However, epithelial lesions were mild, and growth was comparable with that of OTII $\times$ B6 littermates. Indeed, in our colony, the spontaneous enteropathy described in B6 $\times$ IL15Tge mice¹¹ was observed only in some mice after 10 months (Supplementary Figure 2B). Overall, these data indicated that IL15 and CD4⁺ T cells activated by the dietary antigen cooperated to induce tissue damage.

Enteropathy Develops in OVA-Fed OTII $\times$ IL15Tge Mice Despite Expansion of Functional OVA-Specific Tregs

Exposure to dietary OVA increased the frequency of activated (CD44^{hi}CD62L^{lo}) OVA-specific V β 5⁺V α 2⁺CD4⁺ T cells in LP, MLN, and spleen (Supplementary Figure 1B), and that of memory (CD44^{hi}CD62L^{hi}) OVA-CD4⁺ T cells in MLN and spleen (Supplementary Figure 1C). There was more increase in OTII $\times$

IL15Tge mice than in OTII $\times$ B6 mice, indicating that IL15 bolsters the response of CD4⁺ T cells to OVA. T-cell activation, however, was associated with significantly fewer OVA-CD4⁺ T cells in spleen and MLN, likely reflecting activation-induced deletion¹³ (Supplementary Figure 1A). IFN- γ -producing CD4⁺ T cells were increased in OTII $\times$ IL15Tge mice on the control diet compared with OTII $\times$ B6 mice on the control diet. After OVA feeding, they did not increase further except in the spleen of OTII $\times$ IL15Tge mice (Supplementary Figure 3A and B). In contrast, many OVA-CD4⁺ T cells displayed a regulatory phenotype (CD4⁺CD25⁺Foxp3⁺), particularly in OVA-fed OTII $\times$ IL15Tge mice. In these mice, approximately 80% of OVA-CD4⁺ T cells in LP and 20% in MLN and spleen were CD25⁺Foxp3⁺ (Figure 2A). This was surprising because IL15 reportedly impaired the conversion of naive T cells into Tregs.¹⁰ Tregs comprise both natural Tregs generated in the thymus (nTregs) and Tregs induced in the periphery in response to exogenous antigens (iTregs).¹⁴ In OVA-fed OTII $\times$ B6 mice and OTII $\times$ IL15Tge mice, most OVA-specific Tregs in LP showed intracellular expression of Helios, a suggested but equivocal marker of nTregs^{15,16} (Supplementary Figure 4).

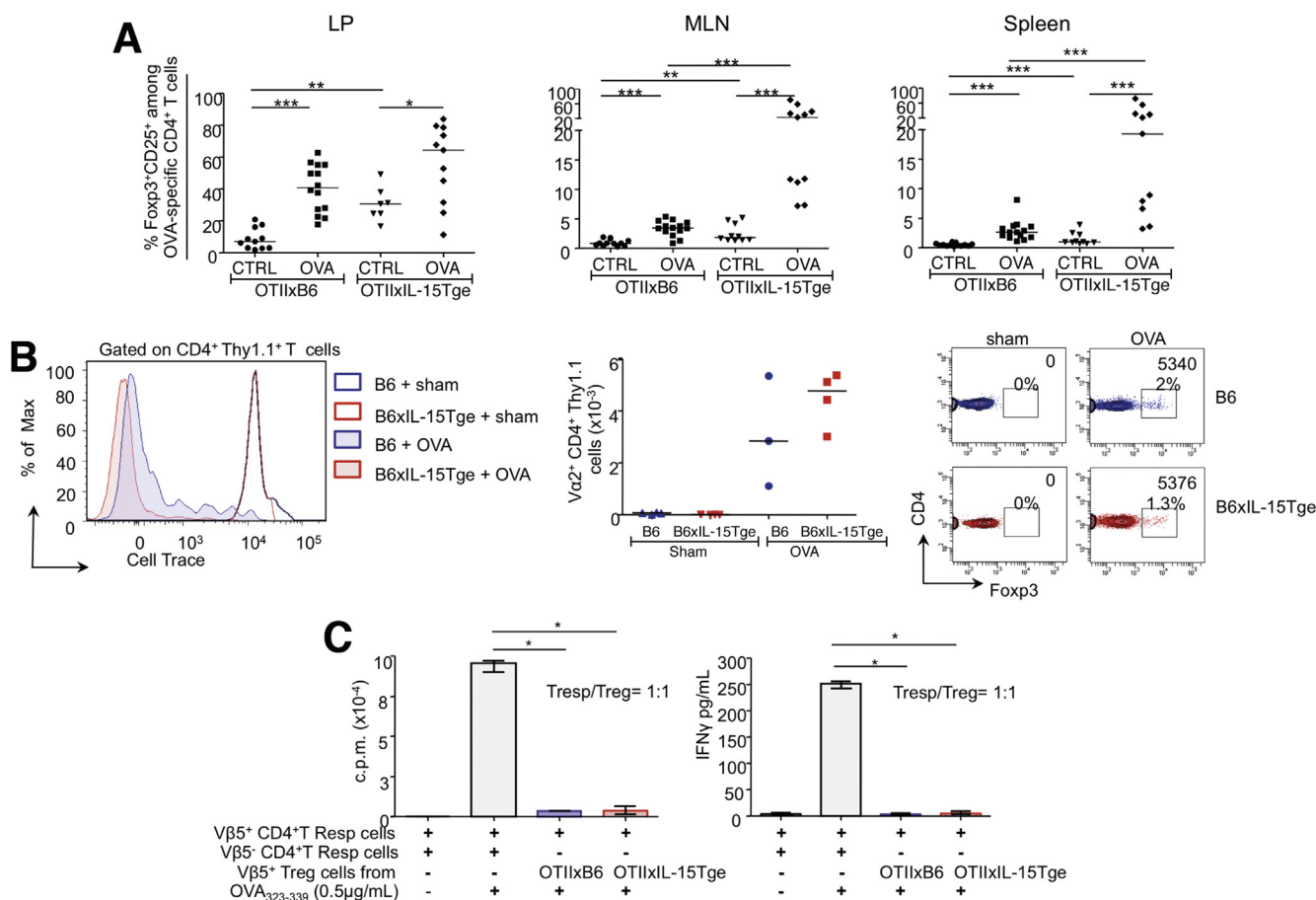


Figure 2. Expansion of functional Tregs in OVA-fed OTII × IL15Tge mice. (A) Frequencies of Foxp3⁺CD25⁺ cells (Tregs) among OVA-CD4⁺ T cells in LP, MLN, and spleen of OTII × B6 and OTII × IL15Tge mice fed with OVA or CTRL diets. (B) *Left panel:* proliferation of OVA-CD4⁺ T cells of donor origin assessed 5 days after adoptive transfer of 8 × 10⁶ Cell Trace-labeled cells from OTII RAG2^{-/-} Thy1.1⁺ mice. Recipient mice were gavaged with 50 mg OVA or PBS (sham) on days 1 and 2 after transfer. *Middle panel:* numbers of induced Foxp3⁺ Tregs among OVA-CD4⁺ T cells of donor origin (Thy1.1) in MLN of B6 or IL15Tge recipients (Thy1.2). Pooled results from 2 independent experiments. *Right panel:* representative dot plots showing percentages and numbers of induced Foxp3⁺ Tregs among OVA-CD4⁺ T cells of donor origin (Thy1.1) in MLN of B6, or of B6 × IL15Tge recipients (Thy1.2) as indicated. (C) Inhibition of proliferation (*left graph*) and of IFN-γ secretion (*right graph*) of OVA-CD4⁺ responder T cells (Vβ5⁺CD4⁺CD25⁺) isolated from control OTII mice by OVA-specific Tregs (Vβ5⁺CD4⁺CD25⁺) purified from OVA-fed OTII × B6 and OTII × IL15Tge mice. Pooled results from 3 to 4 independent experiments performed in triplicate. Bar graphs show medians ± range. **P* < .05, ***P* < .01, and ****P* < .001.

Conversion of naive OVA-CD4⁺ T cells into iTreg was therefore tested by transferring CD4⁺ T cells from OTII RAG2^{-/-} Thy1.1 mice (which lack nTregs) into wild-type (WT) B6 or into IL15Tge (Thy1.2) recipient mice. On days 1 and 2 after transfer, mice were gavaged with 50 mg OVA, or with PBS alone. On day 5, OVA-CD4⁺ donor T cells strongly proliferated in OVA-fed mice, but not in sham-fed mice (Figure 2B). OVA-CD4⁺ T cells, of donor origin (Thy1.1⁺Vα2⁺), and with a Treg phenotype, were observed in comparable numbers in MLN of OVA-fed WT mice and OVA-fed IL15Tge mice, but were undetectable in sham-fed mice. Therefore, in OVA-fed OTII × IL15Tge mice, the increase in Treg numbers may result from expansion of nTregs and also from conversion of naive T cells into iTregs.

In OVA-fed OTII × IL15Tge mice, the massive expansion of OVA-CD4⁺ T cells with a Treg phenotype contrasted with early onset enteropathy, suggesting that Tregs were not functional. Vβ5⁺CD25⁺CD4⁺ T cells therefore were purified from MLN and spleen and their immunosuppressive function

was tested against spleen Vβ5⁺CD25⁺CD4⁺ responder T cell (Tresp) stimulated with OVA₃₂₃₋₃₃₉ peptide. As shown in Figure 2C, Vβ5⁺CD25⁺ Tregs from OVA-fed OTII × B6 mice and from OVA-fed OTII × IL15Tge mice, at a 1:1 Tresp/Treg ratio, completely blocked proliferation and IFN-γ production. At a 2:1 Tresp/Treg ratio, inhibition of proliferation by Tregs from OTII × IL15Tge mice was more potent than inhibition by Tregs from OTII × B6 controls, indicating that IL15 does not impair but stimulates Treg function (Supplementary Figure 5).

In OTII × IL15Tge Mice, the OVA Diet Promotes a Massive Expansion of CD8⁺ T Cells Expressing Granzyme B

In OTII × IL15Tge mice, in duodenum, interferon gamma transcripts were strongly up-regulated (not shown) and there were more IFN-γ-producing CD8⁺ T cells in LP, MLN,

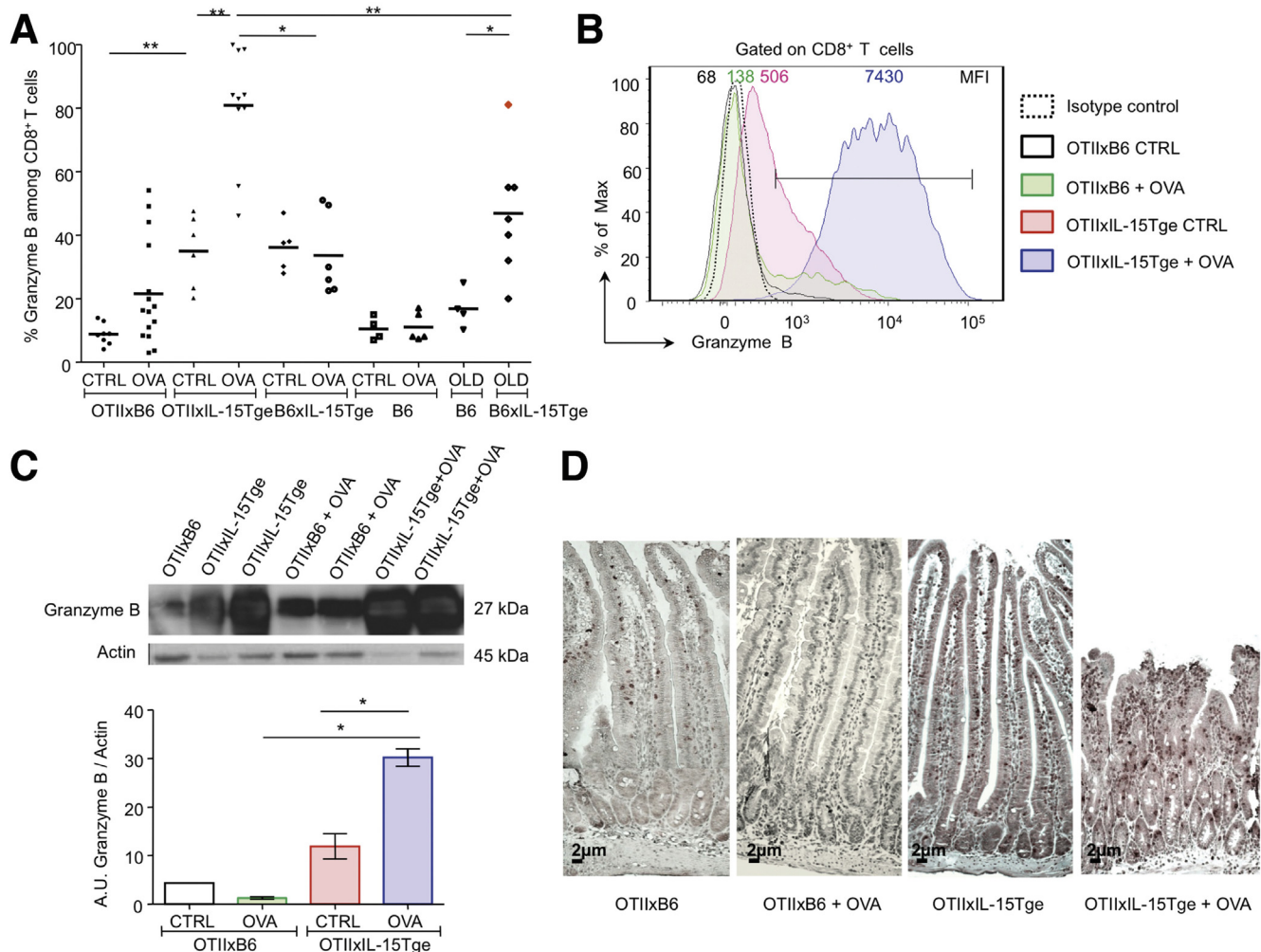


Figure 3. Expansion of Granzyme B⁺ CD8⁺ T cells in OVA-fed OTII $\times$ IL15Tge mice. (A) Frequency of LP Granzyme B–producing CD8⁺ T cells in 3-month-old OTII $\times$ B6, OTII $\times$ IL15Tge, B6, and B6 $\times$ IL15Tge mice fed with OVA or control (CTRL) diets and in old B6 or B6 $\times$ IL15Tge mice (≥ 10 mo) on a CTRL diet. Red dot indicates the old B6 $\times$ IL15Tge mouse with enteropathy. (B) Granzyme B intracellular staining of LP CD8⁺ T cells in 1 representative experiment of 3 experiments. Mean fluorescent intensities are shown. (C) Western blot detection of Granzyme B in duodenal biopsy specimens of indicated mice. Granzyme B/actin ratios (arbitrary units [AU]) are shown. (D) Immunohistochemical staining of Granzyme B on paraffin duodenal sections of indicated mice. Scale bar: 2 μ m. * P < .05, ** P < .01, and *** P < .001.

and spleen than in OTII $\times$ B6 controls. Absolute numbers of natural killer cells also were increased in spleen, LP, and epithelium of OTII $\times$ IL15Tge mice compared with OTII $\times$ B6 littermates. These changes, however, were independent of diet (Supplementary Figures 6 and 7). In contrast, CD8⁺ T cells expressing Granzyme B were not only more numerous in LP of control diet OTII $\times$ IL15Tge mice than of OTII $\times$ B6 mice, but they increased further after OVA feeding (Figure 3A), and flow cytometry showed their enhanced content in Granzyme B (Figure 3B). These results were confirmed in duodenal biopsy specimens by Western blot (Figure 3C). In addition, immunohistochemistry showed many Granzyme B⁺ cells close to epithelial lesions in OVA-fed OTII $\times$ IL15Tge mice (Figure 3D). Granzyme-B⁺ CD8⁺ T cells were more numerous in the LP of 3-month-old B6 $\times$ IL15Tge mice than of B6 littermates, but frequency was comparable in mice fed control or OVA-containing diet (Figure 3A). Overall, these results suggested that IL15 and

activated OVA-CD4⁺ T cells cooperated to expand intestinal cytotoxic CD8⁺ T cells. In B6 $\times$ IL15Tge mice, the frequency of LP Granzyme B⁺ CD8⁺ T cells was the same at 3 months and 10 months, except in 1 old mouse displaying duodenal epithelial lesions and massive IEL infiltration, further implying a link between Granzyme B⁺ cells and enteropathy (Figure 3A and not shown).

IL15 Renders CD8⁺ T Cells Less Sensitive to Immunosuppression by Tregs

In OVA-fed OTII $\times$ IL15Tge mice, the up-regulation of Granzyme B in LP CD8⁺ T cells after OVA feeding, and the expansion of functional OVA-specific Tregs, seemed contradictory. This result, together with the expansion of effector CD4⁺ and CD8⁺ T cells observed in the same mice regardless of diet, suggested that effector cells exposed to IL15 might escape Treg control. This hypothesis was consistent with our

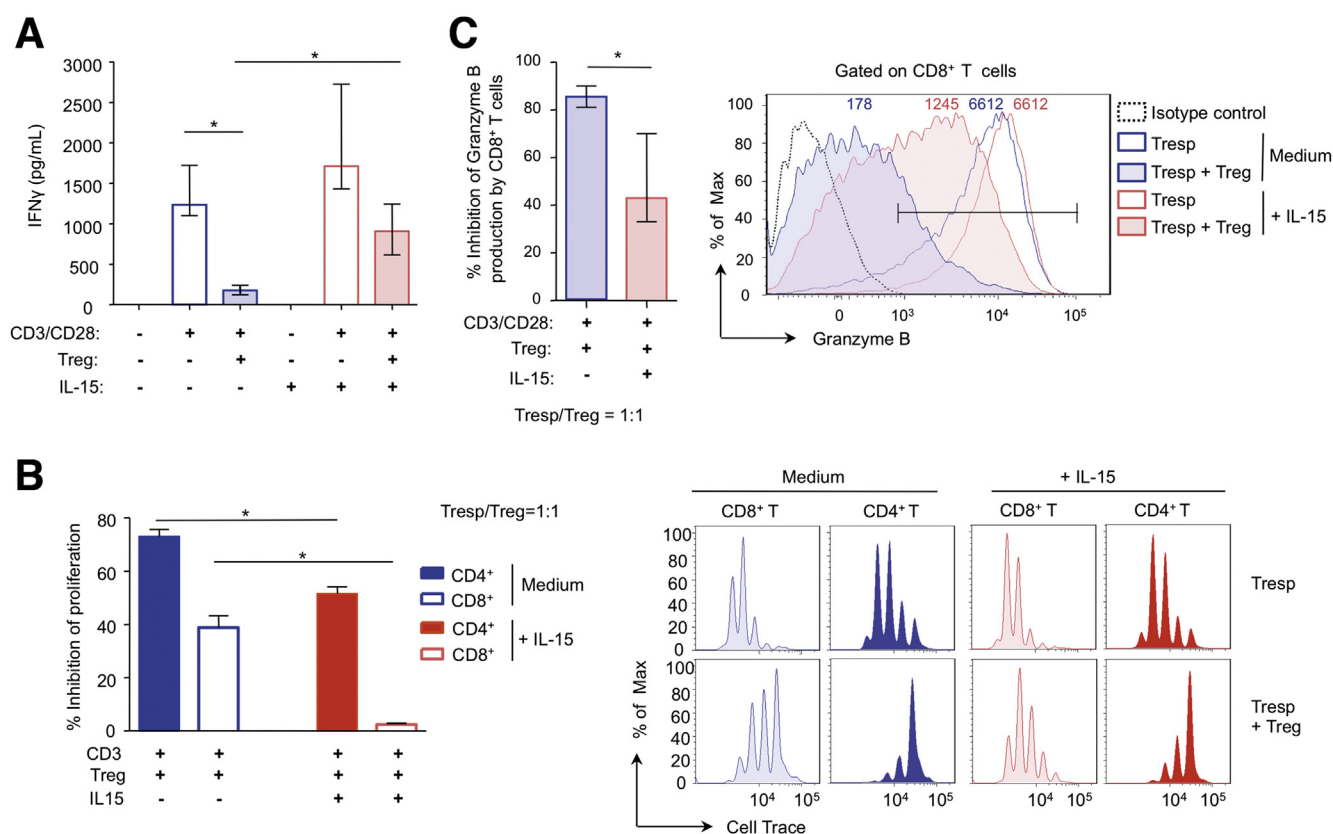


Figure 4. Impaired response of CD8⁺ T effector cells to Tregs in the presence of IL15. (A–C) Cell Trace-labeled CD45⁺CD25[−] spleen cells from B6 mice (responder cells [Tresp]) were co-cultured at a 1:1 ratio with unlabeled CD45⁺CD25[−] B6 spleen cells (>90% CD4⁺, Tregs) or unlabeled CD45⁺CD25[−] spleen cells for control and stimulated with 0.2 μg/mL (A and C) soluble anti-CD3/CD28 or (B) anti-CD3 alone in the presence or absence of 20 ng/mL human IL15. (A) Inhibition of IFN-γ secretion by Tregs determined by enzyme-linked immunosorbent assay in culture supernatants after 48 hours. (B) Proliferation was determined by dilution of Cell Trace in CD4⁺ and CD8⁺ TL cells after 48 hours. *Left panel*: percentage of inhibition of proliferation of responder cells in the presence of Tregs compared with control co-cultures. Results are pooled from 4 independent experiments. *Right panel*: analysis of proliferation in 1 representative experiment. (C) Granzyme B staining of Cell Trace-labeled responder CD8⁺ TLs after a 48-hour culture. *Left graph*: percentages of inhibition of Granzyme B expression in CD8⁺ TL by Tregs. *Right graph*: Granzyme B staining in indicated conditions in 1 representative experiment. Bar graphs show medians ± range. Results are pooled from 4 independent experiments. **P* < .05.

in vitro observations showing that, IL15 partially reversed Treg-mediated inhibition of proliferation and IFN-γ production (Figure 4A and B). In keeping with the known selective effect of IL15 on CD8⁺ TLs, and with the preferential in vivo expansion of activated CD8⁺ TLs in OTII × IL15Tge mice, reversal of Treg-mediated inhibition by IL15 was more efficacious in CD8⁺ than in CD4⁺ TLs. IL15 also weakened the inhibition exerted by Tregs on Granzyme B induction in spleen CD8⁺ TLs stimulated by CD3 mAb (Figure 4C), further suggesting that cytotoxic CD8⁺ TLs can escape Treg control in OTII × IL15Tge mice.

IL15 and OVA-Specific CD4⁺ T Cells Synergize to Stimulate T-Cell Receptor–Independent Activation of Granzyme B⁺ Cytotoxic CD8⁺ T Cells

In B6 mice, OVA immunization can stimulate specific CD8⁺ TLs directed against a dominant OVA_{257–263} epitope presented by MHC class I H2-K^b.¹⁷ However, we observed that the presence of OVA in the diet did not change

the numbers of CD8⁺ TLs stained with high-avidity H2-K^b/OVA_{257–263} dextramers in the epithelium, LP, MLN, and spleen of OTII × B6 or OTII × IL15Tge mice (Supplementary Figure 8). In addition, LP lymphocytes from OVA-fed OTII × B6 or OTII × IL15Tge mice also failed to proliferate when stimulated by the dominant MHC class I OVA_{257–263} peptide, even in the presence of IL15 (Figure 5A). In contrast, both CD4⁺ and CD8⁺ LP TLs proliferated in response to OVA protein and, strikingly, even more in response to the MHC class II restricted OVA_{323–339} peptide, the cognate target of OVA-CD4⁺ TLs (Figure 5A). Of note, CD4⁺ TLs proliferated in response to OVA and OVA_{323–339} peptide, without IL15 (data not shown), whereas CD8⁺ TLs only proliferated when OVA-CD4⁺ TLs and IL15 were added to the cultures (Figure 5A). Overall, these results suggested that IL15 and OVA-CD4⁺ TLs synergized to activate non-cognate LP CD8⁺ TLs.

To further confirm these results, CD8⁺ TLs expressing a single T-cell receptor (TCR) specific for the MHC class I-restricted OVA_{257–263} epitope were purified from OTI RAG2^{−/−} mice and cultured with B6 DCs and IL15 with or

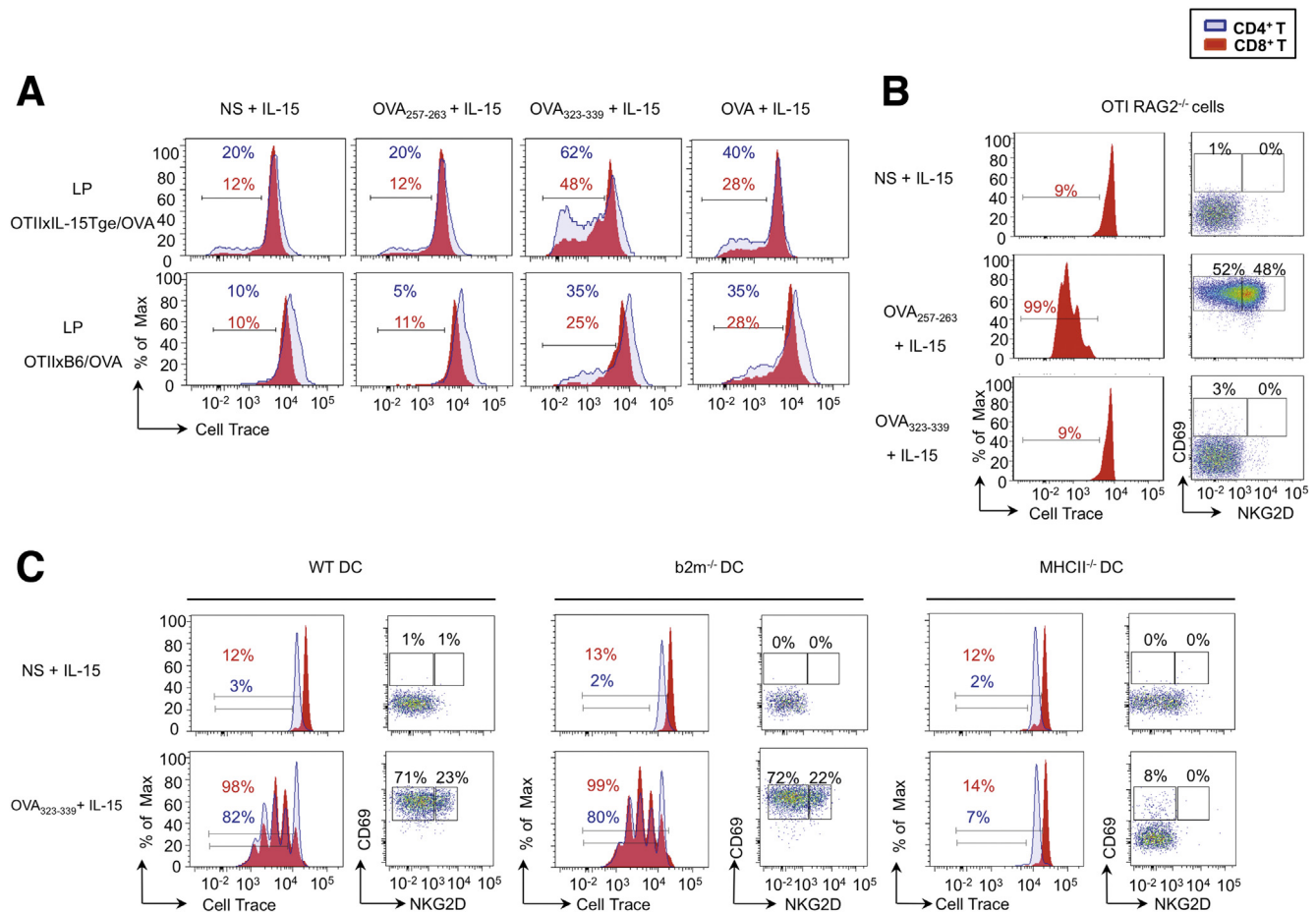


Figure 5. Activation of noncognate CD8⁺ T cells through cooperation between OVA-CD4⁺ T cells and IL15. (A) Flow cytometry analysis of Cell Trace–stained LP cells from OVA-fed OTII × IL15Tge mice or OTII × B6 mice, stimulated with OVA₂₅₇₋₂₆₃ (2 μg/mL) peptide, OVA₃₂₃₋₃₃₉ (2 μg/mL) peptide, or OVA protein (400 μg/mL) in the presence of IL15 (20 ng/mL) for 96 hours. (B) Proliferation (left panel) and CD69 and NKG2D expression (right panel) of Cell Trace–stained CD8⁺ TLs from OTI RAG2^{-/-} mice after a 72-hour stimulation with OVA₂₅₇₋₂₆₃ peptide (2 μg/mL) or OVA₃₂₃₋₃₃₉ (2 μg/mL) peptide, in the presence of IL15 (20 ng/mL). (C) Proliferation of CD4⁺ and CD8⁺ TLs and CD69 and NKG2D expression by CD8⁺ TLs after 72 hours co-culture of Cell Trace–stained CD4⁺ TLs from OTII RAG2^{-/-} mice, and CD8⁺ TLs from OTI RAG2^{-/-} mice, stimulated by OVA₃₂₃₋₃₃₉ peptide (2 μg/mL) plus IL15 (20 ng/mL); and either WT, major histocompatibility complex class I-deficient (b2m^{-/-}), or major histocompatibility complex class II-deficient (MHCII^{-/-}) DCs. One representative experiment of 3–4 independent experiments is shown. Blue histograms, CD4⁺ TLs; red full histograms, CD8⁺ TLs.

without CD4⁺ TLs from OTII RAG2^{-/-} mice. These mice have a single TCR specific for the MHC class II-restricted OVA₃₂₃₋₃₃₉ epitope. OTI-derived CD8⁺ TL alone did not respond to the OVA₃₂₃₋₃₃₉ peptide (Figure 5B), but they did respond when added with OVA-CD4⁺ TLs (Figure 5C). Furthermore, activation of OTI-derived CD8⁺ TLs was comparable in the presence of DCs from WT mice, or from MHC class I-deficient (b2m^{-/-}) mice, but was abolished when DCs derived from MHC class II-deficient mice. This confirmed that, in the presence of IL15, CD4⁺ TLs stimulated by their cognate antigen could activate CD8⁺ TLs without engaging their TCR (Figure 5C).

To extend these results to normal CD8⁺ TLs with a polyclonal repertoire, naive (CD62^{hi}CD44^{lo}) CD8⁺ TLs were purified from spleen and effector/memory (CD62^{lo}CD44^{hi}) CD8⁺ TLs from small intestinal LP and epithelium of B6 mice that had never been exposed to OVA. Then the CD8⁺ TLs were mixed with B6 DCs and OVA-specific CD4⁺ TLs

from OTII RAG2^{-/-} mice, and stimulated with OVA₃₂₃₋₃₃₉ peptide or with OVA protein. No proliferation was observed in spleen CD8⁺ TLs (Supplementary Figure 9). In contrast CD8⁺ TLs derived from LP (not shown) and from epithelium (Figure 6A), proliferated when co-cultured with activated OVA-CD4⁺ TLs and IL15. Such in vitro-activated intestinal CD8⁺ TLs showed a moderate increase in the expression of the Natural killer group 2, member D (NKG2D) expression, but they strongly up-regulated intracellular Granzyme B (Figure 6A and not shown). Accordingly, they showed more degranulation in response to CD3 stimulation than CD8⁺ TLs cultured without CD4⁺ TLs (Figure 6B and not shown). Overall, these results indicated that, in the presence of IL15, OVA-CD4⁺ TLs can provide noncognate help to intestinal CD8⁺ TLs that stimulates their expansion and cytotoxicity, irrespective of their specificity. These in vitro results were in keeping with vivo observations in OVA-fed OTII × IL15Tge mice, in which LP CD8⁺ TLs lacking any reactivity

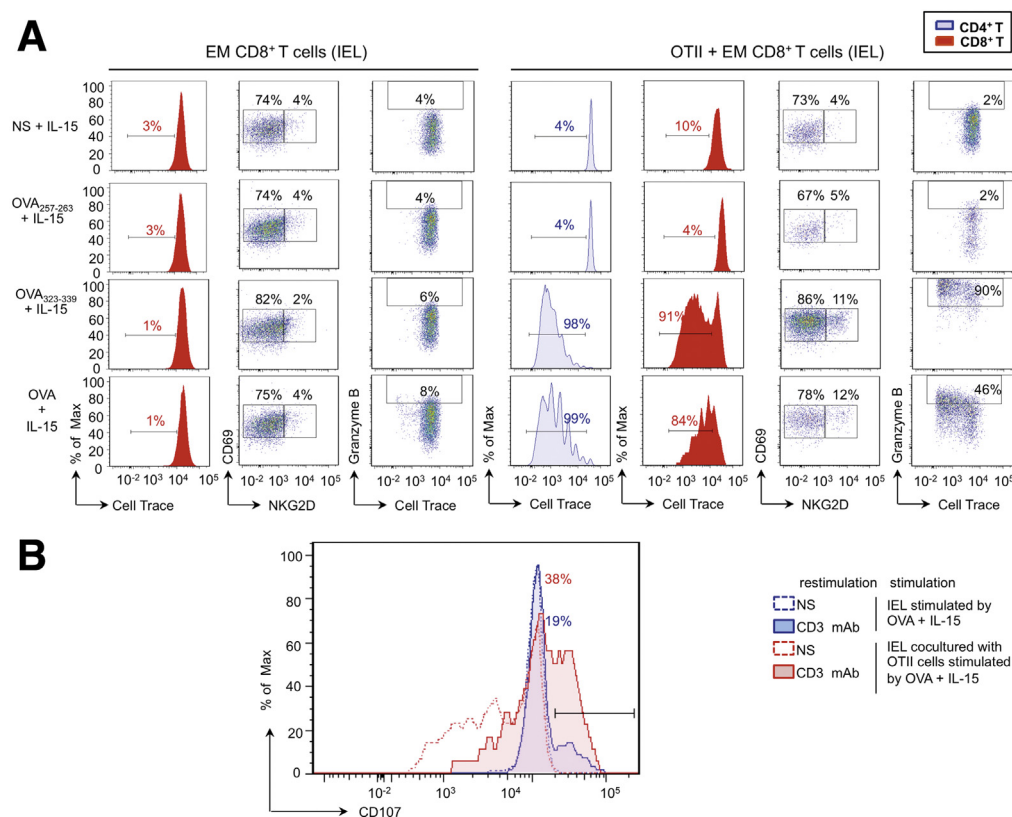


Figure 6. Activation of cytotoxic CD8⁺ T-IELs through cooperation between OVA-CD4⁺ T cells and IL15. Proliferation and expression of Granzyme B, CD69, and NKG2D in B6-derived CD8⁺ T-IELs after 96 hours of stimulation with OVA₂₅₇₋₂₆₃ peptide (2 μ g/mL), OVA₃₂₃₋₃₃₉ (2 μ g/mL) peptide, or OVA protein (400 μ g/mL), and 20 ng/mL IL15 in the absence (*left panel*) or presence of CD4⁺ TLs from OTII RAG2^{-/-} mice (*right panel*). The threshold for Granzyme B staining was set on nonstimulated cells. (*B*) CD107a staining of IELs activated as in panel A and stimulated by CD3 mAb (1 μ g/mL) for 4 hours. One representative of 3 independent experiments is shown.

against OVA showed moderate NKG2D expression, but massive amounts of Granzyme B (Figure 3A and Supplementary Figures 8 and 10).

CD4 Help to Cytotoxic CD8⁺ T Cells Depends on OVA-Induced IL2 Secretion

We next examined the mechanism of CD4 help. Suggesting the role of a soluble factor, Granzyme B⁺ CD8⁺ TLs (from OTI RAG2^{-/-} mice) were comparably activated whether cultured in contact with OVA-stimulated CD4⁺ TLs or cultured separately from the latter cells by a Transwell membrane (Corning Costar, Sigma Aldrich, Lyon, France) (Supplementary Figure 11). In addition, supernatants of OVA₃₂₃₋₃₃₉-stimulated CD4⁺ TLs from OTII RAG2^{-/-} mice activated both OTI Rag^{-/-}-derived CD8⁺ TLs and B6-derived IELs (Figure 7A and Supplementary Figure 12). Finally, help delivered by supernatants depended, as did CD4 help, on the presence of IL15 (not shown). One soluble factor that can mediate CD4 help is IL2.¹⁸ Consistent with this hypothesis, proliferation and up-regulation of CD69 and Granzyme B in CD8⁺ TLs were abrogated when supernatants were depleted of IL2 (Figure 7A and Supplementary Figure 12), and induced when supernatants were replaced by rhIL2 (not shown).

The hypothesis that IL2 mediates CD4 help and synergizes with IL15 in vivo in our model was supported by 2 pieces of evidence. First, *Il2* transcripts were increased significantly in the duodenum of OTII $\times$ IL15Tge mice on the

OVA diet, compared with mice on a control diet (Figure 7B). Second, in 4-month-old B6 $\times$ IL15Tge mice but not in age-matched B6 mice, intraperitoneal injections of rhIL2 for 5 days reduced the villous/crypt length ratio and increased the number of Granzyme B⁺ IELs (Figure 7C-E). Intestinal changes occurred despite expansion of Tregs, confirming that IL15 interferes with Treg-mediated suppression (Supplementary Figure 13). Interestingly, *Il2* messenger RNA tended to increase with age in the duodenum of B6 $\times$ IL15Tge mice. It was particularly increased in a 10-month-old B6 $\times$ IL15Tge mouse that developed epithelial damage (Figure 7B), suggesting that, in these mice, accumulation of IL2-producing CD4⁺ TLs also participates in enteropathy.

Discussion

Herein we show that, when mice are chronically exposed to OVA in their diet, IL15 and OVA-specific CD4⁺ TLs cooperate to induce gut damage. Confirming that the presence of numerous cognate CD4⁺ TLs cannot by itself break immune tolerance to a food protein,¹⁹ feeding large doses of OVA to OTII $\times$ B6 mice with transgenic OVA-CD4⁺ TLs did not induce enteropathy. In contrast, in OTII $\times$ IL15Tge mice, chronic exposure to OVA diet led to growth retardation and enteropathy, which was characterized, as in CD, by patchy lesions of villous blunting in the duodenum. The spontaneous enteropathy previously described in mice overexpressing IL15 in the gut epithelium¹¹ did not develop in our colony before 10 months. Thus, at 3 months of age,

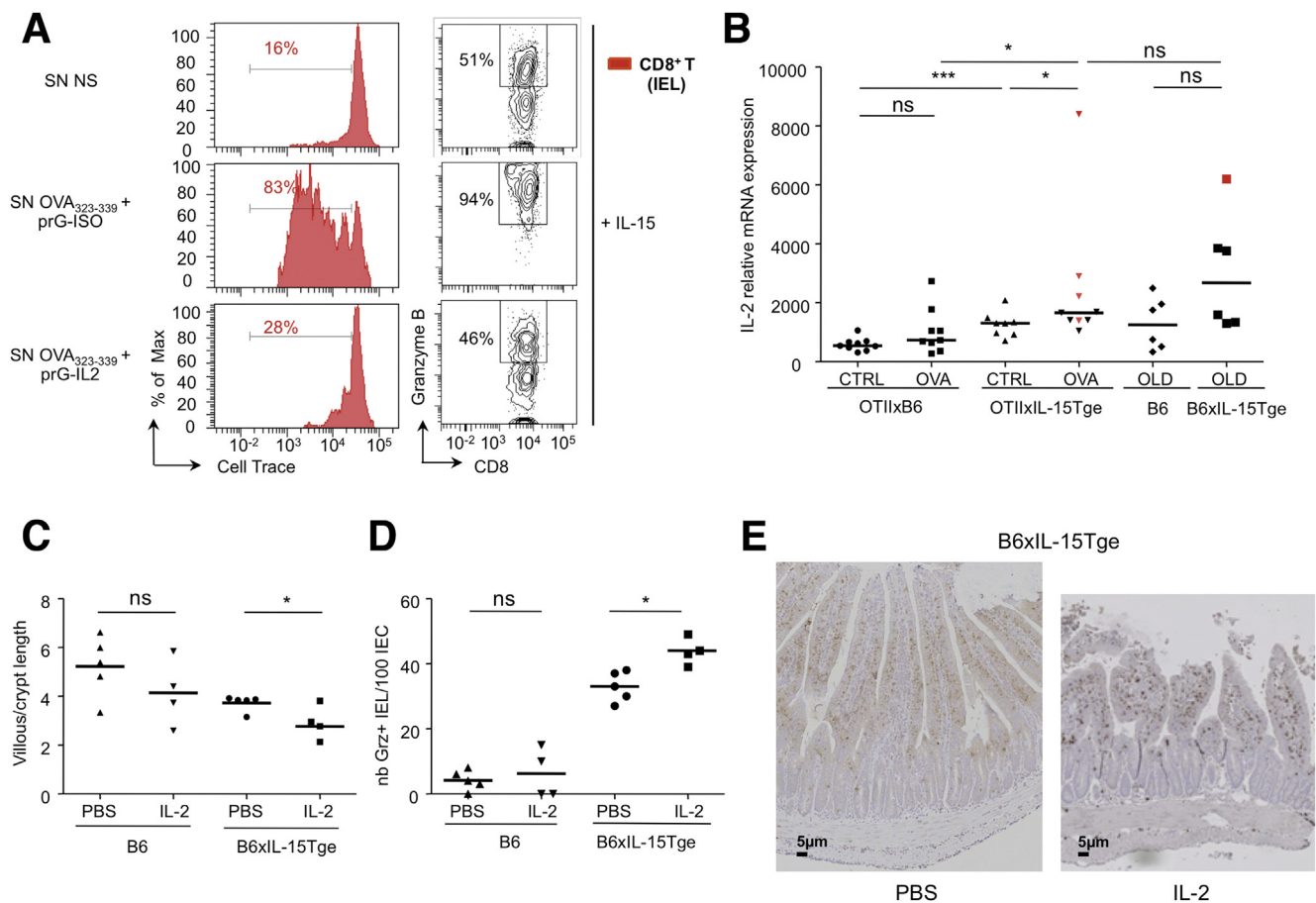


Figure 7. Expansion of cytotoxic CD8⁺ T cells through synergy between IL2 and IL15. (A) Proliferation and Granzyme B expression of CD8⁺ T-IELs after 96 hours of culture with IL15 (20 ng/mL), and supernatants of OVA-CD4⁺ TLs stimulated by OVA₃₂₃₋₃₃₉ (2 μg/mL), with or without IL2 depletion. One representative experiment of 3 independent experiments is shown. (B) *Il2* messenger RNA (mRNA) expression normalized to *Hprt1* mRNA in 3-month-old OTII × B6 and OTII × IL15Tge mice fed OVA or CTRL diets; and in old B6 or B6 × IL15Tge mice (≥10 mo) on a CTRL diet. Red dots indicate mice with enteropathy. (C and D) Comparison of (C) villous/crypt length ratios and (D) number of IELs per 100 intestinal epithelial cells (IECs) in B6 and B6 × IL15Tge mice after intraperitoneal injections of rIL2 (200,000 IU/day) or PBS for 5 days. (E) Immunohistochemical staining of Granzyme B in paraffin duodenal sections of B6 × IL15Tge mice treated with rIL2 or PBS. **P* < .05, ****P* < .001

epithelial lesions remained very mild, both in OTII × IL15Tge mice fed a control diet, and in B6 × IL15Tge mice fed an OVA diet. This indicates that IL15 and activated CD4⁺ TLs must interact to break local immune tolerance and to induce intestinal tissue damage. In OTII × IL15Tge mice, OVA diet accelerates the accumulation of intestinal activated CD4⁺ TLs and, thereby, the onset of enteropathy.

In OVA-fed OTII × IL15Tge mice, epithelial damage was associated with an increase in Granzyme B⁺ CD8⁺ TLs, in both epithelium and LP, despite a massive expansion in functional OVA-specific Tregs. This situation resembles CD, in which epithelial lesions are thought to depend on cytotoxic CD8⁺ TLs and also occur despite the presence of many Tregs in LP.^{9,20} Consistent with previous results in human beings,^{8,9} IL15 reduced the in vitro immunosuppression of effector T cells by Tregs, resulting in impaired control of IFN-γ and Granzyme B production. In vivo, more IFN-γ-producing CD4⁺ and CD8⁺ TLs were seen in the intestine and in the periphery of OTII × IL15Tge mice, regardless of diet. OVA feeding significantly increased IFN-γ-producing OVA-specific TLs in spleen, but not in MLN and intestine.

These results are consistent with the loss of peripheral tolerance already described in mice overexpressing IL15,¹¹ but suggest that IFN-γ-producing OVA-specific TL cells are not sufficient to cause epithelial damage. In contrast, Granzyme B⁺ CD8⁺ TLs, already increased in mice fed a control diet, further increased in OVA-fed mice.

The intestinal CD8⁺ TLs, which expanded in response to OVA feeding, were not directed against the dietary antigen. Thus, no reactivity could be shown against the dominant H2-K^b-restricted epitope OVA₂₅₇₋₂₆₃, using either high-avidity dextramers or proliferation tests. In CD, whether CD8⁺ TLs react against gluten-derived peptides is debated (reviewed by Meresse et al²). Results in this mouse model may not be directly translatable to CD. Nevertheless, they show that intestinal cytotoxic CD8⁺ TLs can expand independently of cognate TCR recognition of antigen in the diet. Moreover, our in vitro data indicate that cooperation between IL15 and IL2 released by CD4⁺ TLs activated by the dietary antigen is necessary and sufficient to stimulate cytotoxic CD8⁺ TLs independently of their antigen specificity. The synergistic effect of IL2 and IL15 was

restricted to memory/effector CD8⁺ TLs and was not observed in naive CD8⁺ TLs. This mechanism is thus not sufficient to activate naive CD8⁺ TLs, but may rapidly mobilize many effector CD8⁺ TLs at tissue sites independently of their specificity. Suggesting that this mechanism may operate in vivo in our model, *Il2* messenger RNA was enhanced significantly in the duodenum of OVA-fed OTII × IL15Tge compared with mice fed a control diet. Moreover, injection of IL2 into B6 × IL15Tge but not into WT B6 mice increased Granzyme B⁺ CD8⁺ IELs, and resulted in villous shortening. As expected, in vivo injection of IL2 induced an expansion of Tregs.²¹ However, as discussed earlier, IL15 may stop Tregs from controlling effector T cells, notably CD8⁺. The molecular mechanism(s) underlying the synergistic effects of IL2 and IL15 remain to be deciphered. Signals delivered to lymphocytes by IL2 and IL15 are overlapping because their receptors share 2 chains, which form a signaling module. However, differences in downstream signaling events have been reported both in vitro^{22,23} and in vivo.²⁴ Interestingly, a polymorphism in the *IL2/IL21* locus²⁵ conferred increased risk for CD and type 1 diabetes in human beings and in non-obese diabetic mice.²⁶ How this polymorphism affects the expression of either cytokine remains unclear in human beings. In human CD, IL21 is produced by gluten-activated CD4⁺ TLs (reviewed by Meresse et al²). In our mice, we did not observe any significant IL21 production (not shown). IL21, able to synergize with IL15 to expand cytotoxic CD8⁺ TLs,²⁷ may play a similar or complementary role to IL2 in human CD.

In CD, cytotoxic CD8⁺ TLs are thought to induce epithelial lesions, although the exact mechanism that triggers their cytotoxicity remains under discussion. Signals via activating natural killer receptors such as NKG2D were notably shown to stimulate the degranulation of human CD8⁺ T IELs independently of their specificity.⁶ In contrast with human CD8⁺ TLs, mouse CD8⁺ TLs do not constitutively express NKG2D and only 10%–20% of intestinal CD8⁺ TLs expressed NKG2D in OVA-fed OTII × B6 or OTII × IL15Tge mice. Similarly, 10%–20% of intestinal CD8⁺ TLs stimulated in vitro by OVA-stimulated CD4⁺ TLs and IL15 expressed NKG2D. In the latter CD8⁺ TLs, degranulation could be induced by a CD3 antibody used to mimic TCR stimulation (Figure 7) but not by an NKG2D antibody (data not shown). We do not exclude that in vitro conditions were not adequate to show activation via NKG2D and that this receptor plays a role in vivo. Thus, its ligand Rae-1e was detected by Western blot in intestinal tissue in all groups of mice (Supplementary Figure 14). Alternatively, degranulation of intestinal cytotoxic CD8⁺ TLs might be triggered via their TCR. In our model, intestinal memory/effector CD8⁺ TLs expanded and acquired Granzyme B regardless of their specificity. However, effector TLs migrate into the intestine only after a step of activation by cognate antigens in gut-associated lymphoid organs. A re-encounter with the same exogenous or perhaps also self-antigens in the small intestine might trigger their degranulation and induce tissue damage. This hypothesis, however, needs to be substantiated by further studies.

In conclusion, this study in a mouse model shows that interactions between IL15, produced in excess in the intestine, and CD4⁺ TLs, chronically activated by a dietary antigen, are necessary and sufficient to stimulate the expansion of intestinal cytotoxic effector CD8⁺ TLs, and to induce intestinal damage. A comparable scenario, illustrated in Supplementary Figure 15, may operate in celiac disease. Deciphering the mechanisms that control intestinal expression of IL15 in celiac patients may help to prevent disease in HLA-DQ2/8 individuals who are predisposed to develop gluten-specific CD4⁺ TLs.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2013.12.023>.

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Conflicts of interest

The authors disclose no conflicts.

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