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Coordinated Induction by IL15 of a TCR-Independent NKG2D Signaling Pathway Converts CTL into Lymphokine-Activated Killer Cells in Celiac Disease

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Summary

A major function of NKG2D linking innate and adaptive immunity is to upregulate antigen-specific CTL-mediated cytotoxicity in tissues expressing stress-induced NKG2D ligands, such as MIC, by coactivating TCR signaling. Here, we show that, under conditions of dysregulated IL15 expression in vivo in patients with celiac disease and in vitro in healthy individuals, multiple steps of the NKG2D/DAP10 signaling pathway leading to ERK and JNK activation are coordinately primed to activate direct cytolytic function independent of TCR specificity in effector CD8 T cells. These findings may not only explain previous reports of transformation of CTL into NK-like "lymphokine-activated killers" (LAK cells) under high doses of IL2 (a substitute for IL15) but may also have significant implications for understanding and treating immunopathological diseases.

Introduction

Recent studies demonstrating a broad expression of NK lineage receptors of the NKG2 family by $CD8^+$ $TCR\alpha\beta^+$ effector CTL have revealed the existence of novel mechanisms regulating cytolytic functions in the tissue microenvironment (Groh et al., 2001, 2003; Jabri et al., 2000, 2002; Roberts et al., 2001; Speiser et al., 1999). By upregulating the expression of MIC, ULBP, and HLA-E (Bauer et al., 1999; Braud et al., 1998; Cosman et al., 2001; Lee et al., 1998), stressed, transformed, or infected human tissues can coengage their cognate NKG2D and CD94/NKG2 receptors on the surface of CTL to modulate TCR signaling, effectively controlling antigen-specific killing (Groh et al., 2001, 2002, 2003; Jabri et al., 2000, 2002; Roberts et al., 2001). This important link between innate and adaptive immunity serves therefore to focus CTL killing on transformed targets, minimizing potential damage to unaltered cells expressing crossreactive self-antigens.

NKG2D is a potent costimulator of TCR-mediated effector functions but does not appear to function independently of TCR signaling (Billadeau et al., 2003; Groh et al., 2001; Jamieson et al., 2002). NKG2D signaling is mediated by the adaptor DAP10 (Wu et al., 1999) through a YXXM motif similar to that of CD28, a costimulator of naive T cells.

NK receptors, like immunoreceptor complexes on the surface of B and T cells, can generally be classified into two categories, one with an immunoreceptor tyrosine-based activation motif (ITAM) that can recruit and activate ZAP 70/Syk and one with a YXXM motif that binds and activates the p85 subunit of PI3 kinase. Four adaptor molecules essential for NK receptor surface expression and signaling have been identified: DAP12, DAP10, $CD3\zeta$, and $Fc\epsilon RI\gamma$. ITAM-bearing DAP12 can induce a full signaling cascade resulting in cytolysis, cytokine secretion, and proliferation (reviewed in Lanier, 2001; Raulet, 2003; Vivier et al., 2002; Yokoyama, 2000), whereas YXXM-bearing DAP10 seems to exert mainly costimulatory functions (Groh et al., 2001; Wu et al., 1999), although it is capable of directly activating NK cell cytolytic function (Billadeau et al., 2003; Zompi et al., 2003). Activated mouse NK cells express a splice variant of NKG2D that associates with DAP12 and induces both cytolysis and cytokines (Diefenbach et al., 2002; Gilfillan et al., 2002). However, a splice variant capable of associating with DAP12 appears to be lacking in humans (T.S., unpublished data).

The emerging diversity and plasticity of the NKG2D signaling pathways in NK cells suggested, therefore, that, under some conditions, T cells themselves could be directly activated. Of particular relevance are reports of TCR-independent cytolytic activity of CTL cultured with high doses of IL2 or IL15 (so-called LAK cells) published several years ago (Brooks, 1983; Gamero et al., 1995), as high doses of IL2 can substitute for IL15, a key cytokine for NK cell differentiation and effector/memory T cell survival and activation (Fehniger and Caligiuri, 2001; Waldmann and Tagaya, 1999). Furthermore, we previously reported that, in celiac disease, intraepithelial intestinal CTL expressed high levels of NK cell-associated receptors and expanded massively under high exposure to IL15 in the epithelial compartment (Jabri et al., 2000). Celiac disease is elicited by gluten intolerance

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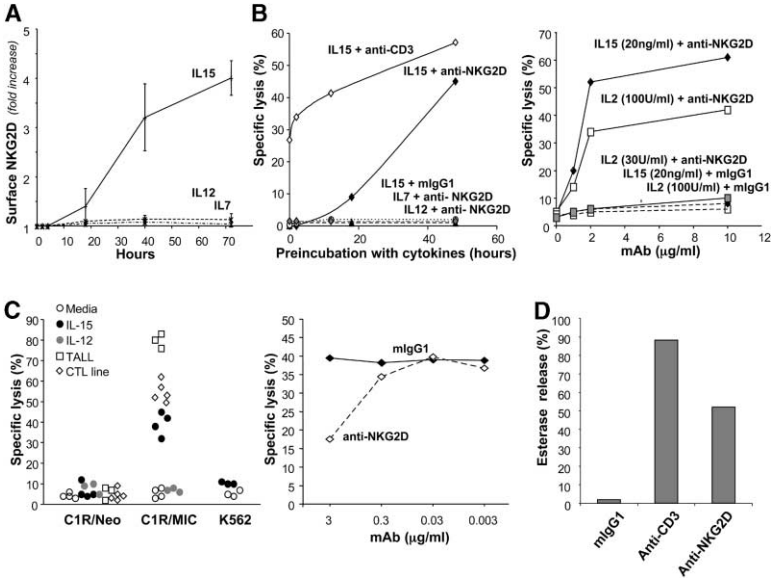

Figure 1. Induction of NKG2D Function in Normal Intestinal CTL by IL15 and High Doses of IL2

(A) Kinetics of NKG2D upregulation by IL15 in TCR$\alpha\beta^+$CD8$^+$ CTL. Surface levels of NKG2D in intraepithelial CTL freshly isolated from normal subjects and incubated with IL15, IL12, or IL7 as indicated were determined. Increased mean fluorescence intensity ($\pm$SEM) of TCR$\alpha\beta^+$CD8$^+$ gated cells is shown as ratio of cytokine-treated over medium alone. Data are representative of three independent experiments.

(B) Induction of NKG2D-mediated cytolysis by IL15 and IL2, measured with ^{51}Cr-labeled FcγR$^+$ P815 targets in the presence of anti-NKG2D or anti-CD3 mAb at E/T ratio 33:1. Data are representative of three independent experiments. In the right panel, different concentrations of anti-NKG2D mAb were used to elicit cytolytic activity from IEL pretreated with IL15 or IL2 as indicated.

(C) Left: Cytolysis of C1R/MIC transfectants and erythroleukemia K562 cell line. Freshly isolated intraepithelial CTL incubated for 48 hr with cytokines or medium as indicated, intraepithelial CTL lines derived from normal individuals, or CD8$^+$TCR$\alpha\beta^+$ TALL-104 leukemia line expressing high levels of NKG2D were used as effector T cells. Right: Blocking of C1R/MIC lysis by intraepithelial CTL lines with different concentrations of anti-NKG2D 1D11 mAb or isotype control. Data are representative of two independent experiments.

(D) Cytolytic granule release measured by BLT esterase assay. Receptor ligation with monoclonal anti-NKG2D or -CD3 mAb, but not with isotype control, triggered granule release in intraepithelial CTL lines. The data are representative of three independent experiments.

in HLA-DQ2 and DQ8 individuals and, while the presence of gluten-specific DQ2- or DQ8-restricted CD4 T cells is well established in the lamina propria (Koning, 2003; Sollid, 2000), a key pathologic event is a massive cell death in the epithelial compartment infiltrated by IL15-activated CTL, suggesting the contribution of aberrant TCR-independent NK-like killing (Green and Jabri, 2003).

Here, we show that IL15 elicits a coordinated series of biochemical changes in the NKG2D signaling pathway, ultimately arming TCR-independent NK-like killing through NKG2D and effectively converting CTL into LAK cells both in vitro in culture systems and in vivo in celiac patients. Because the NKG2D ligand MIC is markedly induced in celiac intestinal epithelial cells, our findings raise the possibility that IL15-induced LAK cells may significantly contribute to tissue damage in celiac disease as well as other pathological processes.

Results

IL15 Arms NKG2D-Mediated Cytolysis in Freshly Isolated Intraepithelial CTL

We previously showed that NKG2D was expressed at low levels on all intraepithelial CTL and could be upregulated by exposure to IL15 (Roberts et al., 2001). Upon 48 hr exposure of fresh intraepithelial CTL to IL15 (but not IL12 or IL7), NKG2D acquired the ability to mediate cytolysis of both P815 cells (anti-NKG2D redirected lysis) (Figure 1B) and C1R/MIC transfectants (Figure 1C). The level of cytolysis paralleled the level of NKG2D induction (compare Figures 1A and 1B), reaching up to 40% at an E:T of 33:1. High doses of IL2 (100 U/ml), which substitute for IL15, but not low doses (30 U/ml), were also effective in arming NKG2D-mediated killing (Figure 1B, right). In contrast, as expected from effector CTL, CD3 engagement was effective in inducing lysis ex vivo (Figure 1B). In addition, NKG2D mediated lysis of C1R/MIC but not control cells in TCR$\alpha\beta^+$CD8$^+$ CTL lines cultured with 100 U/ml IL2 or 20 ng/ml IL15 and the CD8 leukemia T cell line TALL-104 (Figure 1C, left). Importantly, intraepithelial CTL preincubated with IL15 did not kill K562, a common NK target (Figure 1C, left) that expressed very low levels of MIC and ULBP proteins, and C1R/MIC killing was inhibited in the presence of blocking anti-NKG2D 1D11 mAb (Figure 1C, right). Finally, ligation of NKG2D receptors with monoclonal anti-NKG2D mAb triggered granule release by intraepithelial CTL, as shown by the BLT esterase assay (Figure 1D), ruling out cryptic involvement of other receptor/ligand pairs for target killing by NKG2D.

IL15 Arms NKG2D-Mediated Cytolysis in Effector but Not in Naive or Resting Memory PBL

The above findings with fresh and cloned intraepithelial CTL contrasted with those reported with PBL-derived CTL clones where NKG2D engagement alone was insufficient to induce lysis (Billadeau et al., 2003), suggesting differential effects of IL15 based on the source or the activation status of the CTL. We therefore examined naive and resting memory CD8$^+$TCR$\alpha\beta^+$ cells obtained by sorting CD8β^+CD45RO$^-$ and CD8β^+CD45RO$^+$, respectively, from peripheral blood. As shown in Figure 2A, prestimulation with IL15 for up to 3 weeks was not sufficient to arm NKG2D-mediated lysis. However, short prestimulation by plate bound anti-CD3 mAb for 24 hr prior to incubation with IL15 (Figures 2A and 2B, "effector CD8 T-PBL cells") or with IL2 at high doses (Figure 2B) did arm NKG2D-mediated lysis in both naive and resting memory cells, suggesting that activation of the NKG2D pathway of cytolysis by IL15 is restricted to the

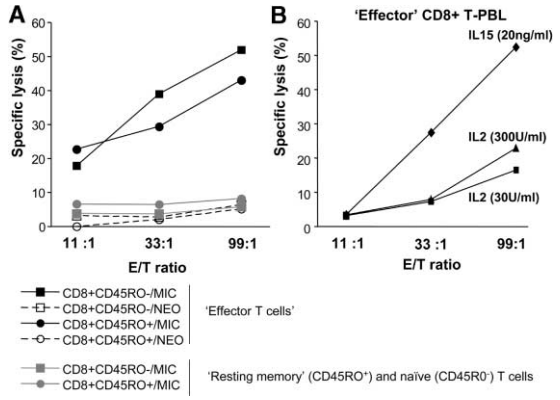

Figure 2. IL15 and High IL2 Can Arm NKG2D in Effector but Not in Naive or Resting Memory Peripheral Blood CTL

(A) Purified CD45RO$^+$ and RO$^-$ TCR$\alpha\beta^+$CD8$^+$PBL subsets were cultured with IL15 for 3 weeks with (effector T cells) or without (naive and resting memory T cells) overnight prestimulation with plate bound anti-CD3 mAb. NKG2D-mediated lysis was measured against C1R/MIC transfectants. Specificity controls included C1R/NEO cells as well as blocking with anti-NKG2D 1D11 mAb (data not shown).

(B) Purified TCR$\alpha\beta^+$CD8$^+$ PBL were prestimulated with plate bound anti-CD3 overnight (effector) prior to incubation with IL15 or IL2 at 30 or 300 U/ml for 3 weeks as indicated. Data are representative of two independent experiments.

effector stage of CTL. We extended these observations to cultured CTL lines by showing that IL15 activated the NKG2D cytolytic pathway only if the cells had been recently restimulated (<3 weeks) through their antigen receptor (data not shown).

Altogether, these results suggest that CD8 T cells need to be in an effector stage in order for IL15 to arm NKG2D-mediated lysis.

Fresh Intraepithelial CTL from Active Celiac Patients Mediate TCR-Independent Lysis through NKG2D without the Need for IL15 Prestimulation

Because, in celiac disease, expanded intraepithelial CTL are constitutively exposed to very high amounts of IL15 (Jabri et al., 2000; Mention et al., 2003), we tested whether they expressed high levels of NKG2D and could mediate direct cytolysis through NKG2D. Intraepithelial CTL freshly isolated from the biopsies of active celiac patients (n = 15) consistently expressed NKG2D at 4–20 (average 6.5) times higher levels than normal intraepithelial CTL (n = 17) (Figure 3A). NKG2D levels decreased conspicuously under GFD (gluten-free) diet (n = 9), though they remained slightly elevated even after 1–2 years, in accordance with the finding that IL15 does not return to complete normalcy under GFD (Mention et al., 2003). Two representative experiments with multiple samples examined on the same day are shown in Figure 3A along with a summary display of data from all 41 samples. It is noteworthy that, in active celiac disease, NKG2D was expressed at levels comparable to those observed after prestimulation of normal intraepithelial CTL with IL15 for 48 hr (compare N+IL15 with AC in Experiment 1, Figure 3A). The higher level of NKG2D expression in fresh celiac intraepithelial CTL correlated with the observation that NKG2D mediated significant levels of cytolysis (>10% specific lysis) in 6/7 active celiac patients, but not in 4/4 celiac patients under GFD and 12/12 individuals with a normal intestine, either in redirected cytolysis assay (3/3) or with MIC-transfected targets (3/4) (Figure 3B, left). CTL cell lines derived from celiac intraepithelial CTL induced NKG2D-dependent killing of various intestinal epithelial cell lines (MIC4, LOVO, INT-407, HCT-116) expressing the NKG2D ligands MIC or ULBP, albeit with variable efficiency (see a representative experiment in Figure 3B, right). Importantly, intraepithelial celiac CTL did not kill the NK target K562, which expresses very low levels of MIC and ULBP ligands, suggesting that these celiac CTL lacked the activating NK receptors required to kill K562.

Altogether, these results support a scenario whereby, upon IL15 exposure in vivo, celiac intraepithelial CTL have turned on NKG2D-mediated NK-like cytolytic activity.

MIC Expression Is Upregulated in Active Celiac IEC

MIC is constitutively expressed on the surface of IEC (intestinal epithelial cells) in the colon (Groh et al., 1996; and unpublished data), but it is usually present at very low levels on normal small bowel IEC. We studied MIC expression by flow cytometry (n = 20) and immunohistochemistry (n = 18) in control, active celiac patients, and patients under GFD. As shown in Figure 3C, around 25% of IEC expressed high levels of MIC on their surface in 4/5 active celiac patients, but not in normal controls (n = 11) and in patients under GFD (n = 4). In frozen sections of the small intestine, as shown in a representative example in Figure 3D, IEC from normal (n = 6) and GFD (n = 6) individuals expressed little MIC, whereas, in marked contrast, 5/6 active celiac patients expressed very high levels of MIC in surface and crypt IEC. Altogether, these results show a strong correlation between MIC induction on small bowel IEC and the ingestion of gluten by celiac patients. This upregulation of MIC is particularly significant in the context of the upregulation of NKG2D-mediated cytolytic properties of intraepithelial CTL.

Human NKG2D Is Selectively Associated with DAP10

Because DAP12-dependent NKG2D-mediated killing by NK cells was shown in mice (Diefenbach et al., 2002; Gilfillan et al., 2002), we investigated whether NKG2D was associated with DAP12. We found that NKG2D coprecipitated with DAP10 but not with DAP12, even when DAP12 was expressed (Figure 4A). We also verified that NKG2D was not associated with other ITAM-bearing adaptors, such as TCR CD3ζ and FcϵRIγ chains (data not shown). Finally, unlike CD3, NKG2D did not induce ZAP70 phosphorylation, further supporting the notion that NKG2D signaling does not involve an ITAM-bearing adaptor molecule such as DAP12 (Figure 4B). These results are in agreement with the findings that NKG2D could not mediate proliferation and cytokine secretion in CTL (Roberts et al., 2001; data not shown).

ERK and JNK Activation Are Critical for NKG2D-Mediated Lysis in CTL

In order to determine the signaling pathways involved in NKG2D-mediated cytolysis in human CTL, we first

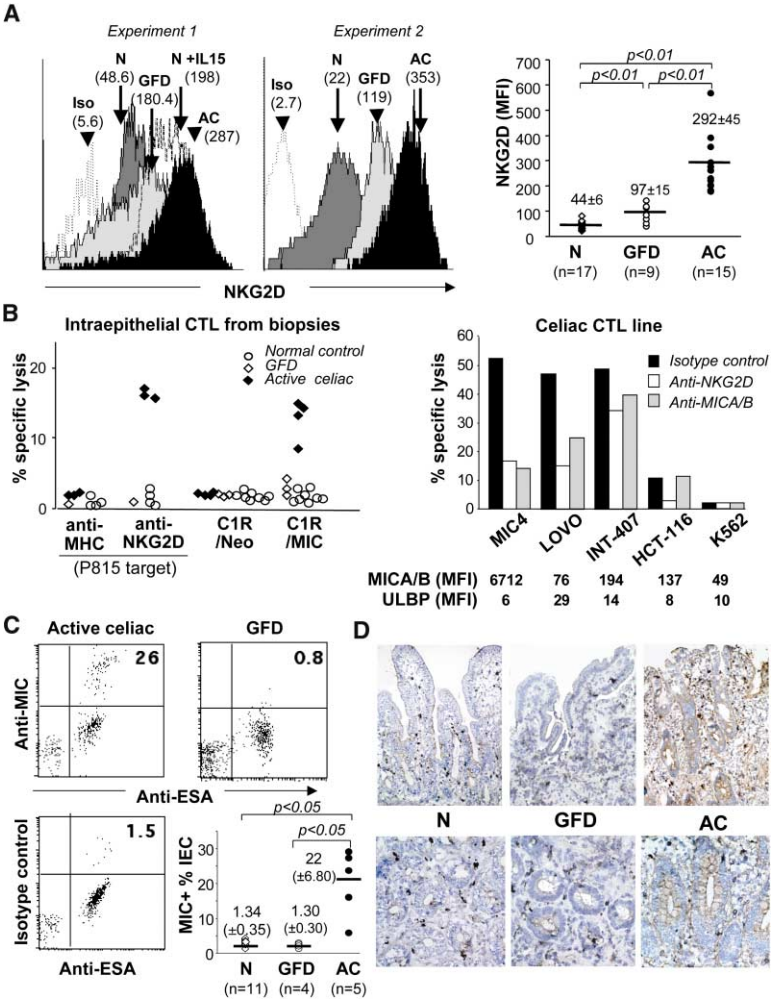

Figure 3. Constitutive Activation of NKG2D in Intraepithelial CTL and Expression of MIC in IEC from Active Celiac Patients

(A) Surface NKG2D levels on freshly isolated TCR$\alpha\beta^+$CD8$^+$ intraepithelial CTL from active celiac patients, celiac patients under GFD, and healthy individuals. Two representative experiments are shown. Note that in Experiment 1, normal (N), GFD, and active celiac patient (AC) were stained the same day with the same antibody mix. Normal intraepithelial CTL incubated with IL15 for 48 hr are shown for comparison. In Experiment 2, GFD and AC were stained the same day. A dot plot summarizing all samples investigated is shown in the right panel. Statistical significances of the differences in NKG2D mean fluorescence intensity (MFI) between the three groups were calculated by analysis of variance (ANOVA), followed by Tukey's adjustment for multiple comparisons.

(B) Left: Cytolytic activity of freshly isolated normal and celiac intraepithelial CTL against ^{51}Cr-labeled FcγR$^+$ P815 targets in the presence of anti-NKG2D or isotype-matched control mAbs or against ^{51}Cr-labeled C1R/MIC versus C1R/Neo targets. Right: Celiac CTL lines killed ^{51}Cr-labeled intestinal MIC$^+$ cell lines (MIC4, LOVO, INT-407) but not MIC/ULBP-negative/low K562 cells. Killing was significantly blocked by anti-NKG2D 1D11 mAb or by anti-MIC 6D4 mAb. Data are representative of three independent experiments.

(C) Flow cytometry analysis of freshly isolated IEC (ESA$^+$) from active celiac patients shows surface MIC. The dot plot summarizes the % of MIC$^+$ IECs in normal, GFD, and active celiac patients. Statistical significances were evaluated using a nonparametric, Kruskal-Wallis test.

(D) Immunohistochemical analysis of MIC expression in IEC of active celiac patients, celiac patients under GFD, and controls. Upper panels, intestinal villi; lower panels, intestinal crypts.

studied the effects of a panel of kinase inhibitors on NKG2D- versus TCR-mediated killing in celiac and normal intraepithelial CTL lines. Redirected cytolytic assays were performed using concentrations of anti-CD3 and anti-NKG2D that elicited comparable levels of cytotoxicity (Figure 5A). All the results obtained with anti-NKG2D were confirmed in cytolytic assays using C1R/MIC transfectants (data not shown). Pretreatment of CTL with the PLC-γ inhibitor U73122 (but not with the inactive analog U73343) and the PI3-kinase inhibitors wortmannin and Ly294002 suppressed NKG2D and TCR-mediated lysis. In contrast, the MEK1/2 inhibitor PD98059 and the JNK inhibitor SP600125 inhibited NKG2D but not CD3-mediated lysis. The p38 inhibitor SB203580 had no effects. The combination of JNK and MEK1/2 inhibitors had additive but not synergistic effect on NKG2D-mediated lysis (Figure 5A, right), suggesting that the two pathways were distinct. These results were further confirmed in five separate experiments using celiac CTL lines, normal intraepithelial CTL lines, and the TALL-104 leukemia T cell line (data not shown).

We then investigated whether, as suggested by the pharmacological experiments, NKG2D/DAP10 activated ERK and JNK. As shown in Figure 5B (upper left), ERK phosphorylation peaked at 5 min after NKG2D stimulation and returned to basal level after 30 min. Likewise, JNK was activated 15 min after NKG2D stimulation using a JNK kinase assay (Figure 5B, upper right) and returned to basal levels after 30 min. Similar results were observed with an antibody directed against the phosphorylated form of JNK (data not shown). ERK phosphorylation was dramatically reduced in cells pretreated with wortmannin and PD98059, but not with the p38 inhibitor SB203580 (Figure 5B, lower left). In contrast, wortmannin did not block JNK activation, whereas the JNK inhibitor SP600125 did (Figure 5B, lower right).

Altogether, these results suggest that NKG2D can mediate ERK phosphorylation and JNK activation and that the catalytic function of PI3-kinase is essential for ERK, but not for JNK activation.

Coordinated Priming of the NKG2D Pathway by IL15

Semiquantitative PCR analysis showed >10-fold increase in both NKG2D and DAP10 transcripts upon IL15

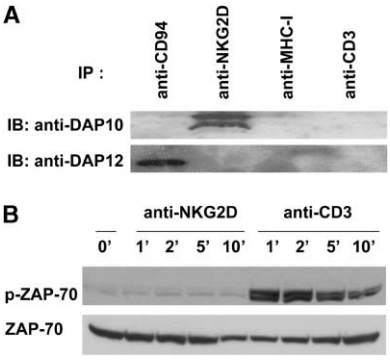

Figure 4. NKG2D Associates with DAP10 and Does Not Mediate ZAP70 Phosphorylation in CTL

(A) NKG2D$^+$CD94/NKG2C$^+$ cells from a TCR$\alpha\beta^+$ line derived from active celiac intraepithelial CTL were surface labeled with anti-CD94, -NKG2D, -MHC class I and -CD3 mAbs prior to immunoprecipitation and immunoblotting with antibodies against DAP10 and DAP12. Note that this CTL line expresses DAP12, which coprecipitates with CD94, but not NKG2D.

(B) Ligation of CD3, but not NKG2D, induced ZAP70 phosphorylation. Staining with total anti-ZAP70 mAb demonstrates equal loading.

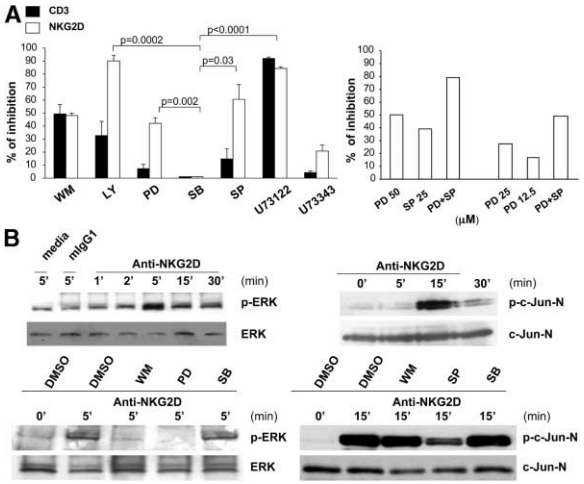

Figure 5. Role of ERK and JNK in NK62D-Mediated Lysis

(A) Suppression of NKG2D mediated lysis by ERK and JNK inhibitors. NKG2D^{high} TCR$\alpha\beta^+$ CTL lines derived from celiac patients were pretreated for 30 min with various kinase inhibitors prior to a cytolysis assay against FcγR$^+$ P815 targets in the presence of anti-CD3 mAb (black) or anti-NKG2D mAb (white). Data represent three independent experiments. The percentage of inhibition was calculated by reference to DMSO-treated CTL. Similar results were obtained using normal subjects' intraepithelial CTL and TALL-104 (data not shown). Note that MEK1/2 inhibitor and JNK inhibitors block NKG2D but not CD3-mediated lysis and have an additive effect on NKG2D-mediated cytolysis (right).

(B) NKG2D signaling activates PI3 kinase, ERK, and JNK. Celiac TCR$\alpha\beta^+$ CTL lines were stimulated with anti-NKG2D, isotype-matched control Ig, or medium followed by crosslinking with F(ab')$_2$ goat anti-mouse IgG for the indicated duration before lysis. Upper panels: Time course of ERK and JNK activation. Phosphorylation of ERK is shown by direct immunoblotting with an anti-phospho ERK mAb. After JNK pull-down by c-Jun-N-GST fusion protein, active JNK was tested in a kinase assay by immunoblotting with anti-phospho-c-Jun. Equal loading shown by antibody to total ERK and c-Jun. Lower panels: Lysates from cells incubated for 30 min with the indicated kinase inhibitors or DMSO were treated as specified for the upper panels. PI3 kinase inhibitor WM blocked ERK but not JNK activation. These experiments are representative of six independent experiments using different celiac and normal intraepithelial TCR$\alpha\beta^+$ CTL line, and the TALL-104 CTL leukemia cell line.

stimulation of normal fresh intraepithelial CTL (Figure 6A). This finding was confirmed by real-time PCR showing 9.5-fold $\pm$ 0.67-fold increase in NKG2D mRNA and 12.3 $\pm$ 0.48 increase in DAP10 mRNA over CTL in medium alone. We have shown increased surface NKG2D in Figure 1A and found here that DAP10 protein was markedly induced as well, whereas the total amount of CD3ζ remained unchanged (Figure 6B). Because the PI3 kinase→ERK signaling pathway is critical for NKG2D-mediated cytolysis, we investigated whether IL15 could induce ERK phosphorylation in normal freshly isolated intraepithelial CTL. As shown in Figure 6C, ERK phosphorylation occurred within 5 min of stimulation by IL15, but not IL7. Furthermore, pretreatment of CTL with the PI3-kinase inhibitor wortmannin or the MEK1/2 inhibitor PD98059 inhibited ERK phosphorylation, whereas the p38 inhibitor SB203580 had no effect (Figure 6D).

Altogether the results indicate that IL15 promotes NKG2D-mediated signaling and cytolysis by coordinately increasing the levels of NKG2D and DAP10 expression and by inducing the phosphorylation of ERK in a PI3 kinase-dependent manner.

Upregulation of Phospho-ERK in Celiac IEC and Intraepithelial CTL

Because IL15 is highly upregulated in celiac epithelial cells (Jabri et al., 2000; Mention et al., 2003) and because IL15 induces ERK phosphorylation in intraepithelial CTL (Figure 6) and in epidermal cell lines (Yano et al., 2003), we investigated whether phospho-ERK1/2 was upregulated in celiac intraepithelial CTL and IEC. Expression of phospho-ERK1/2 was studied by immunohistochemistry in fixed biopsy samples of three normal, three GFD, and three active celiac patients using anti-phospho-ERK1/2 mAb. In normal gut epithelium, some phospho-ERK1/2 could be detected in a few crypt epithelial cells but not in the surface epithelium or in intraepithelial CTL (Figure 7A), whereas active celiac samples exhibited a dramatic upregulation of phospho-ERK1/2 in all IEC and intraepithelial CTL (Figures 7C and 7D). After GFD, the pattern of phospho-ERK1/2 expression returned to normal (Figure 7B). Altogether, these results suggest that ERK is activated in celiac CTL and that this activation might be, at least in part, promoted by IL15.

Discussion

There have been many reports that CD8$^+$ TCR$\alpha\beta^+$ T cells in long-term culture with high concentrations of IL2 or IL15 become so-called lymphokine-activated killers (LAK cells) with NK-like cytolytic function, i.e., the ability to kill tumor targets seemingly independent of TCR specificity (Brooks, 1983; Gamero et al., 1995). These observations conflicted with the dogma that TCR specificity is the primary driving force during peripheral activation and target killing. Because this conversion into so-called LAK cells might have resulted from aberrant changes induced in vitro, neither the mechanism(s) nor the in vivo relevance of this transformation into LAK have

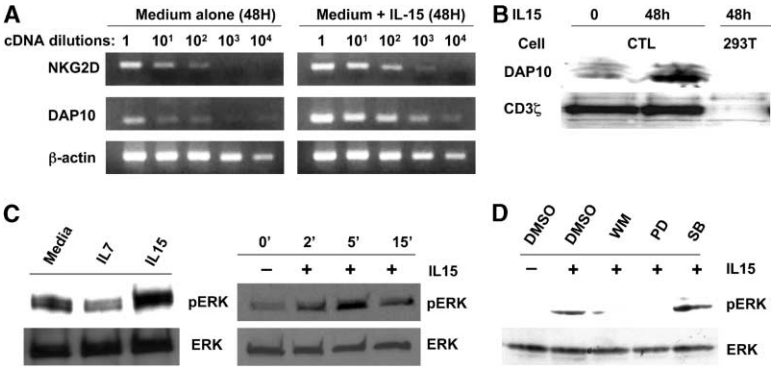

Figure 6. Coordinated Induction by IL15 of NKG2D and DAP10 Expression and ERK Phosphorylation in Normal Freshly Isolated Intraepithelial CTL

(A) Semiquantitative RT-PCR analysis of freshly isolated cells cultured with medium alone or with 20 ng/ml IL15 for 48 hr.

(B) DAP10 and CD3ζ immunoblot on total cell lysates of fresh normal intraepithelial CTL and control 293T fibroblast. (A) and (B) are representative of two separate experiments.

(C) Total lysates of freshly isolated intraepithelial CTL prestimulated for the indicated duration with IL15 or IL7 were analyzed by immunoblotting with anti-phospho-ERK and reprobed with antibody to ERK to assess equal loading. One experiment representative of three is shown.

(D) Total lysates from cells incubated with the indicated kinase inhibitors or control DMSO prior to IL15 stimulation were treated as in (C).

been decisively pursued. The present study of normal intestinal CTL exposed to IL15 in vitro and of celiac CTL that are naturally exposed to IL15 in vivo provides both a mechanism and a physiopathological correlate for the LAK cell conversion involving specifically the NKG2D/MIC receptor/ligand system. It is also possible, but remains to be directly tested, that additional activating NK receptor/ligand systems (Lanier, 2001) might be involved in other cases, allowing LAK cells to kill NKG2D ligand-negative target cells as well.

While both human and mouse NK cells were previously found to induce cytolysis upon NKG2D engagement (Billadeau et al., 2003; Zompi et al., 2003), the same effect could not be documented in human TCR$\alpha\beta^+$ CTL, which nonetheless constitutively express NKG2D (Bauer et al., 1999; Billadeau et al., 2003). In contrast, studies looking at effector T cells (Roberts et al., 2001) or tumoral leukemia TCR$\alpha\beta^+$ CTL (Verneris et al., 2003) suggested that IL15 or high concentrations of IL2 could arm NKG2D-mediated lysis in TCR$\alpha\beta^+$ CTL. Our study provides potential explanations for these seemingly contradictory findings. First, the history of exposure to IL15 in vivo or to high doses of IL2 as a substitute in vitro, may be a determining factor that is likely to differ widely with the source of T cells and the protocols of cell culture and cloning. Second, we have shown that the activation status of the CD8 T cells, e.g., naive versus memory versus effector, is a critical factor as well, since only effector CTL (as defined by the ability to induce target cytolysis immediately upon TCR engagement), whether directly isolated from human tissues or generated in vitro after TCR-mediated activation of resting CTL, could be turned into LAK cells. Another particularly relevant example of such differences is illustrated by the report that IL15 in absence of TCR stimulation could upregulate noninhibitory CD94 receptors in effector CTL but not in resting memory CD45RO$^+$CD8$^+$ PBL (Jabri et al., 2000). Finally, it is possible that NKG2D-mediated cytolysis requires the coengagement of other unidentified receptors by killer cells themselves through homotypic interactions.

It is relevant to note, however, that even though NKG2D was able to trigger target cytolysis by effector CTL independently of their TCR specificity, it could not induce functions with a systemic effect such as cytokine secretion or proliferation (data not shown; Roberts et al., 2001). This is likely due to the fact that cytokine secretion and proliferation are mediated by ITAM-containing immunoreceptors such as DAP12 or CD3ζ in lymphocytes (Lanier, 2001; Raulet, 2003; Vivier et al., 2002; Zompi et al., 2003) and that NKG2D in human CTL exclusively associates with DAP10 but not DAP12 or CD3ζ.

The main signaling pathways controlling NK lysis have been proposed to include a PI3 kinase→Rac I→MEK1/2→ERK pathway that effects tumor lysis (Jiang et al., 2000) and a Syk-independent pathway involving PI3 kinase, PLC-γ2, Vav1, and Rho family GTPases that controls NKG2D-mediated lysis (Billadeau et al., 2003). While there is no evidence for Vav upstream of PI3 kinase, NKG2D stimulation can directly recruit PI3 kinase by DAP10 via its PI3 kinase binding-motif YNIM (Wu et al., 1999). Our studies have not only extended the importance of these signaling pathways in TCR$\alpha\beta^+$ CTL, but importantly also brought ERK and JNK activation into the general picture. The results obtained with the pharmacological inhibitors of kinases further suggested that ERK was downstream of PI3 kinase while JNK activation was independent of PI3 kinase. The involvement of two signaling pathways, one leading to Vav and JNK activation and the other to PI3 kinase and ERK phosphorylation, is further supported by the fact that JNK activation is independent from ERK phosphorylation and that ERK and JNK seem to act in an additive rather than synergistic manner to mediate NKG2D lysis. However, because cytolysis is a complex effector function, it is possible that additional signaling pathways may be necessary.

A key factor in arming the NKG2D-mediated cytolysis pathway of TCR$\alpha\beta^+$ effector CTL appears to be IL15. This observation is supported by the notion that IL15 plays a critical role for the survival of memory/effector T cells (Fehniger and Caligiuri, 2001; Waldmann and Tagaya, 1999) or in pathological conditions associated with high IL15 (Fehniger et al., 2001; Groh et al., 2003; Jabri et al., 2000; Mention et al., 2003; Roberts et al., 2001). In our study, the hyperinduction by IL15 of NKG2D and DAP10 seemed to be a determining factor in the ability of NKG2D to mediate cytolysis. This is supported by the finding that there is a threshold in NKG2D engagement that triggers lysis and that a reduction in the level of NKG2D by tumor-derived soluble MIC drastically impaired NKG2D-mediated killing (Groh et al., 2002). However, this is probably not sufficient, and the coordinated

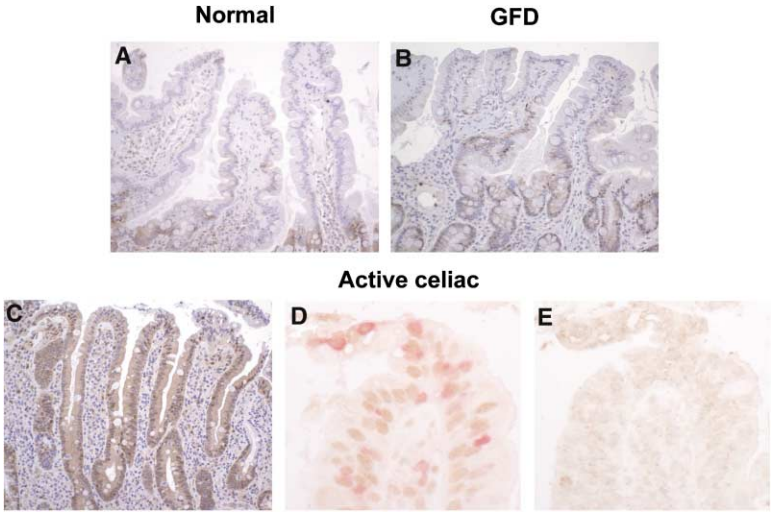

Figure 7. Phospho-ERK 1/2 Expression in IEC and Intraepithelial CTL of Active Celiac Patients

Single staining with anti-Phospho-ERK1/2 mAb shows only scarce expression in crypt epithelial cells of normal controls (A) and patients under GFD (B). In contrast, phospho-ERK is very highly expressed in surface and crypt IEC and intraepithelial CTL of active celiac patients (C). Double staining with anti-phospho-ERK1/2 (brown) and anti-CD3 (red) mAbs shows phospho-ERK1/2 in intraepithelial CTL (D). Isotype IgG2a control staining is shown in (E). (A), (B), and (C), ×20 magnification; (D) and (E), ×40. One representative experiment out of three is shown.

activation of PI3 kinase and ERK, and possibly of other components of the NKG2D pathway, provide another level of functional cooperation required to initiate the cytolytic program.

These findings have significant implications for our general understanding of how signaling through the same surface receptor can be modulated in different cell types or in different activation stages of the same cell type. Our observations in celiac disease suggest a new physiopathological dimension in vivo to the acquisition of in vitro LAK activity by these T cells. The pathology of celiac disease is the consequence of a massive epithelial cell death leading to widespread villous atrophy. While gluten-specific DQ2- and DQ8-restricted CD4 T cells are restricted to the lamina propria (reviewed in Koning, 2003; Sollid, 2000), the epithelium is the site of a massive TCR$\alpha\beta^+$ CTL expansion, which likely contributes to epithelial damage in an antigen-nonspecific manner (Green and Jabri, 2003). Our study supports a model in which intraepithelial CTL kill MIC expressing IEC through NKG2D upon stimulation by IL15 in active celiac disease. NKG2D activation by IL15 requires the CTL (fresh cell or cultured line) to be in an effector stage when it encounters IL15. These conditions are naturally achieved in the high antigenic environment of the gut when IEC express inordinately high levels of IL15 on their cell surface (Jabri et al., 2000; Mention et al., 2003), likely associated with IL15Rα (Dubois et al., 2002), during gluten exposure. Intraepithelial TCR$\alpha\beta^+$ CTL of active celiac patients exhibit the hallmarks of IL15 exposure, e.g., high levels of CD94 (Jabri et al., 2000) and NKG2D, the accumulation of phospho-ERK, and the ability to kill MIC-expressing targets independently from TCR engagement. Closing the loop is the observation that MIC is induced on intestinal epithelial cells during active celiac disease, providing the target for NKG2D-mediated killing. Although the early mechanisms causing MIC and IL15 overexpression by IEC and their link to CD4 T cell activation in the lamina propria remain to be understood, it is significant that exposure to gluten in vitro was reported to induce innate immune reactions including IL15 (Maiuri et al., 2003), perhaps initiating the deleterious series of events leading to uncontrolled destruction of IEC.

The recent reports that NKG2D and IL15 reduced the TCR activation threshold of CTL have raised the possibility that they might play a role in autoimmunity as well (Groh et al., 2003; Roberts et al., 2001). The present study further suggests that NKG2D might promote deleterious autoimmune responses by mediating direct killing of stressed tissue cells independently of TCR engagement. However, our findings also indicate that periodic reactivations of TCR in an IL15-rich environment are required to maintain this dangerous property of NKG2D. One would envision, therefore, that induction of NKG2D/MIC in chronic disease might reveal latent (low-affinity) crossreactivity to self-antigens and initiate an aberrant positive feedback loop resulting in the amplification and propagation of deleterious cytolytic LAK cells.

In normal conditions in the gut, where IEC are exposed to pathogens, the crosstalk between IEC and intraepithelial CTL mediated by NKG2D and its ligands might be critical to maintain a healthy epithelium by helping to eliminate infected cells. However, in conditions of uncontrolled IL15 expression by IEC, such as seen in celiac disease, arming NKG2D-mediated killing independent of TCR specificity might lead to indiscriminate epithelial cell destruction and tissue atrophy. These findings may provide the basis for novel therapeutic approaches in celiac disease aiming at suppressing uncontrolled CTL activation and conversion into LAK by blocking IL15 or NKG2D. Conversely, given the widespread expression of NKG2D ligands by transformed cells, administration of IL15 might be useful to promote tumor eradication in conditions when antigen-specific rejection is limiting, as well as prevent NKG2D down-modulation by soluble MIC secreted by tumor cells.

Experimental Procedures

Patients and Controls

Sixty adult patients (age: 16–78 years) with active celiac disease were investigated. Diagnosis of celiac disease was based on detection of anti-transglutaminase antibodies, presence of HLA DQ2 or DQ8, villous atrophy, and clinical and histological response to gluten-free diet. At the time of study, 35 had active celiac disease with partial villous atrophy whereas 25 had been under strict gluten-free diet (GFD) for 1–3 years. The GFD patients had become negative for anti-transglutaminase antibodies and recovered a normal or sub-

normal villous architecture. Twenty-five individuals undergoing either gastric bypass for morbid obesity or endoscopies and biopsies for functional intestinal disorders of nonceliac origin, as described previously (Jabri et al., 2000; Taunk et al., 1992), were studied as controls. Lymphocytes and epithelial cells were isolated from biopsies or surgical specimens. All subjects gave written informed consent and research was approved by institutional review boards.

Antibodies

Biotin or fluorochrome conjugated anti-CD3, -CD8, -TCR$\alpha\beta$, -TCR$\gamma\delta$, -CD103, -CD16, -CD56, -CD45RO, unconjugated anti-CD3 (clone UCTH1, IgG1), and mouse isotype-matched control Ig were purchased from PharMingen (San Diego, CA); anti-CD8β mAb was from Coulter-Immunotech (Miami, FL); anti-epithelial-specific antigen (ESA) conjugated to FITC was from Biomeda (Foster City, CA); anti-NKG2D mAb 1D11 (IgG1) was used unconjugated or biotinylated (Bauer et al., 1999); anti-MIC mAb 6D4 and N2DL-1, -2/ULBP-1, -2 were unconjugated (Groh et al., 1996; Steinle et al., 2001); PE-conjugated goat anti-mouse IgG1 and IgG2a sera were obtained from Southern Biotechnology (Birmingham, AL); and biotinylated antibodies were revealed with PE-conjugated streptavadin (PharMingen). Anti-DAP12 (DX37) (Lanier et al., 1998) and rabbit anti-DAP10 (Diefenbach et al., 2002; Wu et al., 2000) were generated as previously described; anti-CD3-ζ mAb, anti-phospho-ERK, and anti-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Vav, anti-ZAP70, and anti-phospho-ZAP70 were from Cell Signaling Technology (Beverly, MA); anti-phospho-JNK and anti-JNK2 polyclonal antibodies were purchased from Promega (Madison, WI) and Santa Cruz, respectively; anti-c-Jun-N antibody was from Cell Signaling Technology; anti-phosphotyrosine mAb 4G10 was from Upstate Biotechnology (Lake Placid, NY); and goat anti-mouse F(ab')$_2$ was from Jackson Immunoresearch Laboratories (West Grove, PA).

Recombinant Cytokines

IL15, IL12, IL7, and IL2 were from PharMingen.

Kinase Inhibitors and SAPK/JNK Assay Kit

The PI3 kinase inhibitor wortmannin and LY294002 were purchased from Sigma (St. Louis, MO) and Calbiochem (La Jolla, CA), respectively; MEK1/2 inhibitor PD98059, JNK2 inhibitor SP600125, and p38 inhibitor SB203580 were from Calbiochem; the SAPK/JNK assay kit was from Cell Signaling Technology.

Cell Isolation, Cell Line Generation, and Cell Culture

Intraepithelial CTL and Intestinal Epithelial Cell Isolation

Intraepithelial CTL and intestinal epithelial cell (IEC) were purified from jejunal biopsies as described previously but without using dithiothreitol (DTT) in order to better preserve the viability of IEC (Jabri et al., 2000). Normal intraepithelial CTL were isolated from healthy individuals undergoing gastric bypass for morbid obesity as described previously (Taunk et al., 1992). All freshly isolated intraepithelial CTL studied contained less than 5% CD3 negative (which correlated with the absence of killing of NK targets such as K562, which do not express NKG2D ligands) and TCR$\gamma\delta^+$ lymphocytes.

NKG2D$^+$ Intraepithelial CTL Lines and Clones

NKG2D$^+$TCR$\alpha\beta^+$CD8$^+$ intraepithelial CTL lines and clones were obtained and cultured as previously described (Jabri et al., 2002).

CD45RO$^+$ and CD45RO$^-$ TCR$\alpha\beta^+$CD8$^+$ PBL

Peripheral blood lymphocytes (PBL) were isolated from whole blood of healthy volunteers after Ficoll density gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ). Cells were stained with anti-CD8β, -CD45RO, -TCR$\gamma\delta$ to FACS-sort TCR$\alpha\beta^+$CD8$^+$CD45RO$^+$ and RO$^-$ PBL. Indeed, CD8β staining discriminates against NK cells, and TCR$\gamma\delta$ staining allows exclusion of $\gamma\delta$ T cells.

C1R-MICA and Control Transfectants

C1R cell transfectants expressing MICA (alleles *001 or *004) C1R-MIC and control C1R-Neo have been described (Groh et al., 2001).

LOVO, INT-407, and HCT-116

These intestinal cell lines have been described (Groh et al., 2001).

K562 Erythroleukemia Cell Line

This was obtained from the American type culture collection (ATCC, Manassas, VA) and cultured in RPMI-1640 medium with 10% FCS and antibiotics.

TALL-104

The TALL-104 line is a CD8$^+$ TCR$\alpha\beta^+$ cytotoxic cell line established from the blood of a child in relapse of acute lymphoblastic leukemia (ATCC). It is cultured in Iscove's modified Dulbecco's medium with 100 U/ml recombinant human IL2 and 20% FCS.

Cell Staining and Flow Cytometric Analysis

For surface staining, cells were incubated with fluorochrome or biotin-conjugated antibodies according to standard protocols. Biotinylated antibodies and unconjugated antibodies were revealed with PE-conjugated streptavadin (PharMingen) and appropriate conjugated F(ab')$_2$ goat anti-mouse IgG isotype, respectively. Fluorescence was analyzed on a 4-color FACSCalibur (Becton Dickinson), with quadrants set to score as negative $>$99% of control Ig-stained cells.

Functional Assays

Cytotoxicity Assay

^{51}Chromium-release assay was performed as previously described using P815 cells (a FcγR+ mouse mastocytoma, ATCC), C1R-MICA *001 and *004 or control C1R transfectants, various epithelial cell lines, and the NK target cell line K562 at the indicated effector:target (E:T) ratio in duplicate (fresh cells) or triplicate (cell lines and clones) wells. For Fc-dependent redirected cytotoxicity, effectors and targets were incubated in the presence of soluble anti-NKG2D and/or anti-CD3. Control mouse mAbs were MOPC-21 (IgG1) or an IgG1 mAb against human HLA class I (PharMingen). Chromium release was measured using a scintillation counter (Packard, Meriden, CT). The percentage of specific cytotoxicity was calculated using the formula 100 $\times$ (cpm experimental $-$ cpm spontaneous)/(cpm maximum $-$ cpm spontaneous). When indicated, effector cells were treated for 30 min prior to the cytotoxic assay with the 500 nM Wortmannin, 50 μM Ly294002, 50 μM PD98059, 5 μM SB203580, 25 mM SP600125, or equivalent concentrations of the DMSO diluent. Blocking experiments with anti-NKG2D (1D11 mAb) and anti-MIC (6D4) were performed as described previously (Bauer et al., 1999).

Cytolytic Granule Release Assay

Cytolytic granule release was measured as previously described (Billadeau et al., 2003; Groh et al., 2001; Jamieson et al., 2002). Maximum granule release was determined using 0.2% Triton X_{100}. Esterase release was measured against a standard N-benzyloxycarbony lysine thiobenzyl ester (BLT) esterase.

Cell Signaling Biochemistry

To look at ERK and JNK phosphorylation, cells were serum starved for 30 hr. To test the effects of kinase inhibitors, cells were incubated for 30 min at 37°C with the indicated inhibitors prior to stimulation. To test cytokines, cells were incubated with cytokines for the indicated duration and washed prior to being lysed. To crosslink immunoreceptors, cells were incubated for 1 min at 37°C with isotype-matched control Ig, anti-CD3, or anti-NKG2D before adding goat anti-mouse IgG F(ab')$_2$ antibodies for the indicated duration. Cells were lysed for 20 min in a lysis buffer containing fresh protease and phosphatase inhibitors (50 mM Tris-Hcl [pH 7.5]; 150 mM NaCl; 1% Triton-X_{100}; 1 mM EDTA; 1 mM Na_3VO_4; 1 mM NaF; and protease inhibitor cocktail tablets). For detection of ERK1/2, JNK, phospho-ZAP70, and total ZAP70, total lysates were subjected to SDS-PAGE electrophoresis. JNK kinase assays were done according to kit instructions (Cell Signaling Technologies) using c-Jun-N-GST fusion protein coupled to glutathione beads. Phosphorylated ERK1/2, JNK, c-Jun, and ZAP70 were revealed with anti-phospho-ERK1/2 (Thr202/Tyr304) and phospho-JNK and anti-phospho-c-Jun-N (Ser63) and anti-phospho-ZAP70 (Tyr319), respectively, followed by HRP-conjugated goat anti-mouse or donkey anti-rabbit Abs (Jackson Immunoresearch Laboratories) using the enhanced chemiluminescence ECL kit from Amersham Pharmacia Biotech. To assess variations in loading gel lanes, membranes were stripped according to standard procedures and stained with antibodies to ERK, JNK, and c-Jun.

Immunohistochemistry

Immunohistochemical staining for MIC A/B was performed as described (Groh et al., 2001) on 4 μm cryostat sections from small intestinal biopsies. Antibody binding was detected by using second-

ary anti-mouse antibody conjugated to HRP-labeled polymer (EnVision$^+$ system, DAKO, Carpinteria, CA) and DAB chromogen. Sections were counterstained with Gill's 3 hematoxylin.

Immunohistochemical staining for phospho-ERK1/2 was performed on paraffin sections after antigen retrieval (DAKO). Slides were stained with anti-phospho-ERK (10 μg/ml, Santa Cruz) followed by biotinylated anti-mouse IgG secondary antibody (Vector Laboratories), followed by an avidin/horseradish peroxidase conjugate (ABC reagent, Vector Laboratories). Signals were visualized using diaminobenzidine (DAB) as the substrate (DAKO), and sections were then counterstained with hematoxylin. Double staining was performed using the double staining blocking kit (DAKO). Rabbit anti-CD3 antibody was used at 1:200 dilution (Oncogen, Seattle, WA). Negative controls included anti-phospho-ERK antibody (10 μg/ml) preincubated with a blocking peptide (100 μg/ml, Santa Cruz) for 2 hr at RT or mouse IgG2a isotype control (DAKO).

Semiquantitative PCR and Real-Time PCR

Semiquantitative PCR analysis was performed using primers specific for β-*actin* (5′-GGGTCAGAAGGATTCCTATG-3′; 5′-GGTCTCAAACATGATCTGGG-3′), *DAP10* (5′-ATCATCACTCCCTGCCTTTTACCC-3′; 5′-CAGGAACACCGCCCCCACGATGG-3′), and *NKG2D* (5′-ACACGATGGCAAAAGCAAAGAT-3′; 5′-TAGGACATGGGCCACAGTAAC3′).

Quantitative real-time PCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) and SYBR green amplification kit (PE Biosystems, Warrington, UK) with the same PCR conditions as for semiquantitative PCR (30 cycles, denaturation at 94°C for 30 s and primer annealing/elongation for 15 s at 55°C using a GeneAmp PCR sytem 9700 (Applied Biosystems). NKG2D and DAP10 expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. Specific primers: 5′-CATGTTCCAATATGATTCCACC-3′; 5′-CCTGGAAGATGGTGATGG-3′.

Relative levels of GAPDH, DAP10, and NKD2D PCR products were calculated by comparing the threshold cycle (CT) values. The range given for NKG2D or DAP10 expression in IL15-treated relative to nontreated intraepithelial CTL was determined by evaluating the expression of $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = \Delta CT_{IL15} - \Delta CT_{med}$ and $\Delta CT = CT - CT_{GAPDH}$.

Statistical Analysis

Mean fluorescence intensities of NKG2D were compared between normal, active celiac, and GFD patients using analysis of variance (ANOVA) followed by Tukey's adjustment for multiple comparisons. Since the variability within groups increased with the mean, a logarithmic transformation was applied, which stabilized the variances. For comparison of percentage of MIC-positive IEC between the different groups, a nonparametric Kruskal-Wallis test, followed by Dunn's test for pairwise contrasts, was performed.

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