Cysteinyl leukotrienes mediate lymphokine killer activity induced by NKG2D and IL-15 in cytotoxic T cells during celiac disease

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Celiac disease (CD) is a complex T helper 1 (Th1) cell–mediated immune disorder induced by dietary gluten that shares many common features with organ-specific autoimmune disorders, in particular type 1 diabetes and rheumatoid arthritis (Sollid and Jabri, 2013). IL-15 (Abadie and Jabri, 2014) and the activating natural killer receptor NKG2D have been implicated in these three organ-specific immune disorders. A key function played by NKG2D and IL-15 is to reduce the TCR activation threshold (Bauer et al., 1999; Meresse et al., 2004). In contrast to other activating NK receptors that signal through the immunoreceptor tyrosine activation motif (ITAM)–containing adapter DAP12, NKG2D exclusively associates with DAP10 in humans, which lacks ITAM sequences (Bauer et al., 1999; Wu et al., 1999; Rosen et al., 2004). Consequently, NKG2D cannot activate Zap70, and cytosis through this receptor has thus prompted extensive work to elucidate the signaling pathway involved. Work by Leibson and colleagues has shown that, in addition to phosphoinositide 3-kinase (PI3K; Wu et al., 1999), Vav, growth factor receptor–bound protein 2 (Grb2), and phospholipase C γ (PLCγ; Billadeau et al., 2003; Meresse et al., 2004), allowing for the killing of intestinal epithelial cells (IECs) expressing the stress-inducible molecule MICA (Hüe et al., 2004; Meresse et al., 2004). In contrast to other activating NK receptors that signal through the immunoreceptor tyrosine activation motif (ITAM)–containing adapter DAP12, NKG2D exclusively associates with DAP10 in humans, which lacks ITAM sequences (Bauer et al., 1999; Wu et al., 1999; Rosen et al., 2004). Consequently, NKG2D cannot activate Zap70, and cytosis through this receptor has thus prompted extensive work to elucidate the signaling pathway involved. Work by Leibson and colleagues has shown that, in addition to phosphoinositide 3-kinase (PI3K; Wu et al., 1999), Vav, growth factor receptor–bound protein 2 (Grb2), and phospholipase C γ (PLCγ; Billadeau et al., 2003; Meresse et al., 2004), allowing for the killing of intestinal epithelial cells (IECs) expressing the stress-inducible molecule MICA (Hüe et al., 2004; Meresse et al., 2004). In contrast to other activating NK receptors that signal through the immunoreceptor tyrosine activation motif (ITAM)–containing adapter DAP12, NKG2D exclusively associates with DAP10 in humans, which lacks ITAM sequences (Bauer et al., 1999; Wu et al., 1999; Rosen et al., 2004). Consequently, NKG2D cannot activate Zap70, and cytosis through this receptor has thus prompted extensive work to elucidate the signaling pathway involved. Work by Leibson and colleagues has shown that, in addition to phosphoinositide 3-kinase (PI3K; Wu et al., 1999), Vav, growth factor receptor–bound protein 2 (Grb2), and phospholipase C γ (PLCγ; Billadeau et al., 2003; Meresse et al., 2004), allowing for the killing of intestinal epithelial cells (IECs) expressing the stress-inducible molecule MICA (Hüe et al., 2004; Meresse et al., 2004).
Upshaw and Leibson, 2006; Upshaw et al., 2006; Segovis et al., 2009) are critically involved in NKG2D-mediated cytolysis. Our group has further dissected the downstream signaling events and shown that, in contrast to the TCR, NKG2D requires extracellular signal-regulated kinase (ERK), JNK, and type IV cytosolic phospholipase A2 (cPLA2) activation to mediate cytolysis (Meresse et al., 2004; Tang et al., 2009).

Because cPLA2 plays a key role in the synthesis of eicosanoids by catalyzing the release of arachidonic acid (AA) from membrane phospholipids (Funk, 2001; Peters-Golden and Henderson, 2007), we wanted to know which, if any, eicosanoids were involved in NKG2D-mediated cytolysis and CD pathogenesis. Eicosanoids are signaling molecules that are involved in multiple pathophysiological processes, including inflammation and immunity (Funk, 2001; Peters-Golden and Henderson, 2007). cPLA2 plays a key role in the synthesis of eicosanoids by catalyzing the release of AA from membrane phospholipids. AA serves as substrate for cyclooxygenase-2 (COX2) and 5-lipoxygenase (5-LO), enzymes that process AA into prostaglandins and leukotrienes, respectively (Funk, 2001; Peters-Golden and Henderson, 2007). The overproduction of leukotrienes is a major cause of inflammatory disorders (Samuelsson, 1983; Peters-Golden and Henderson, 2007; Funk, 2011). They are broadly divided into two categories: the cysteinyl leukotrienes (CystLTs), which require the enzyme leukotriene C4 (LTC4) synthase (LTC4S) for their synthesis and are involved in the pathogenesis of allergic disorders such as asthma and allergic rhinitis (Funk, 2011; Kanaoka and Boyce, 2014), and leukotriene B4 (LTB4), which requires the enzyme leukotriene A4 hydrolase (LTA4H) and is involved in the pathogenesis of organ-specific autoimmune disorders such as rheumatoid arthritis and psoriasis (Fig. 1 A; Peters-Golden and Henderson, 2007; Yokomizo, 2015).

In this study we investigated which pathway downstream of cPLA2 participated in NKG2D-mediated cytolysis and CD pathogenesis. Surprisingly, we uncovered a novel function for CystLTs as inflammatory mediators that promote NKG2D lymphokine killer activity in IE-CTLs and are up-regulated in the CD mucosa, suggesting that inhibitors of CystLTs used to treat asthma, such as montelukast, might be efficacious in the treatment of CD.

RESULTS AND DISCUSSION

5-LO plays a critical role in NKG2D-mediated cytolysis

Our previous work demonstrated that the function of NKG2D is critically regulated by the release of AA mediated by cPLA2 (Tang et al., 2009). To assess the potential role of leukotrienes in NKG2D-mediated cytolysis, we first sought to determine whether 5-LO was activated upon NKG2D stimulation. When active, 5-LO is found in the nucleus where it oxidizes AA released from phospholipids by cPLA2 into leukotrienes (Fig. 1 A; Peters-Golden and Henderson, 2007). IE-CTLs were stimulated with monoclonal antibodies against NKG2D or CD3, and nuclear fractions were separated and analyzed by Western blot using an anti–5-LO antibody (Fig. 1 B). We noted that stimulation through NKG2D but not CD3 resulted in rapid and significant increases of 5-LO within the nuclear fraction, suggesting that 5-LO is made active and is in close proximity to its substrate. In agreement, pretreatment of cells with MK886, a pharmacological inhibitor of 5-LO, blocked perinuclear increase of 5-LO in response to NKG2D. This suggests that 5-LO plays a specific role in transducing signals through NKG2D but not CD3 in IE-CTLs.

We next assessed whether 5-LO translocation was correlated with NKG2D-mediated cytolysis. IE-CTLs were incubated with the 5-LO inhibitor MK886, and their capacity to lyse MICA-expressing EL4 target cells (EL4-MICA) was determined. Inhibition of 5-LO resulted in significantly reduced cytolysis through NKG2D (Fig. 1 C). As we previously demonstrated the requirement for cPLA2 in NKG2D-mediated killing (Tang et al., 2009), we also used the pharmacological inhibitor of cPLA2 AACOCF3 (CF3) as a control, noting that the use of MK886 had a comparable effect on cytotoxicity. To further demonstrate a role for 5-LO in NKG2D-mediated cytolysis, we took advantage of the TALL-104 T cell line that is amenable to transfection. Using siRNA, we knocked down 5-LO or COX2, an enzyme that processes AA into prostaglandins but is dispensable for leukotriene synthesis (Fig. 1 A). After confirming knockdown by Western blot (not depicted), we performed cytotoxicity assays against EL4-MICA target cells. Ablation of 5-LO resulted in a significant decrease in NKG2D-mediated killing against MICA-expressing target cells compared with a scrambled control siRNA, whereas no significance was observed upon reduction of COX2 (Fig. 1 D).

Finally, we wanted to evaluate whether inhibition of 5-LO also inhibited TCR-mediated cytolysis in CTLs. To test this, we again pretreated IE-CTLs with MK886 or vehicle control and then assessed their cytotoxic capacity using an antibody-redirected lysis assay against target cells not expressing MICA. Upon cross-linking with an anti-NKG2D mAb, there was a significant decline in killing when 5-LO was inhibited (Fig. 1 E, left). However, and in accordance with previous findings showing no role for cPLA2 in TCR-mediated cytolysis (Tang et al., 2009), MK886 did not affect TCR-mediated lysis, even when baseline rates of cytotoxicity were roughly the same (Fig. 1 E, right). Overall, these data suggest that 5-LO is not only active in IE-CTLs, but that it drives NKG2D-mediated lysis of target cells specifically and does not play a role in TCR-mediated cytotoxicity.

CystLT synthesis and signaling in an autocrine manner plays a role in NKG2D-mediated cytotoxicity in IE-CTLs

Upon liberation of AA from the nuclear membrane by cPLA2, 5-LO catalyzes its oxidation to produce 5-hydroperoxyeicosatetraenoic acid (5-HPETE). It then converts 5-HPETE into prostaglandins and leukotrienes, respectively (Fig. 1 A). After confirming knockdown by Western blot (not depicted), we performed cytotoxicity assays against EL4-MICA target cells. Ablation of 5-LO resulted in a significant decrease in NKG2D-mediated killing against MICA-expressing target cells compared with a scrambled control siRNA, whereas no significance was observed upon reduction of COX2 (Fig. 1 D).

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LTA4H had no effect on cytotoxicity, blockade of LTC4S resulted in significant loss of cytolytic capacity (Fig. 2A). We then transfected TALL-104 cells with siRNA to knockdown BLT1 and CystLT receptor 1 (CystLTR1), receptors for LTB4 and the CystLTs, respectively (Fig. 2B). Ablation of CystLTR1 resulted in significant loss of NKG2D-mediated killing.

We wanted to determine whether LTB4 or the CystLTs were required for NKG2D-mediated killing or whether 5-LO was involved in a different pathway. To test this, we incubated IE-CTLs with pharmacological inhibitors against LTA4H (U75) or LTC4S (MK571) and then assessed their ability to kill MICA-expressing target cells. Although inhibition of LTA4H had no effect on cytotoxicity, blockade of LTC4S resulted in significant loss of cytolytic capacity (Fig. 2A). We then transfected TALL-104 cells with siRNA to knockdown BLT1 and CystLT receptor 1 (CystLTR1), receptors for LTB4 and the CystLTs, respectively (Fig. 2B). Ablation of CystLTR1 resulted in significant loss of NKG2D-mediated...
whether LTD4 alone was sufficient to increase NKG2D-mediated cytolysis. Treatment with LTD4 marginally increased NKG2D-mediated cytolysis, albeit not significantly (not depicted). Overall, these data demonstrate an essential role for the CystLT–CystLTR1 but not LTB4–BLT1 pathway in CTL killing mediated by NKG2D.

We then wanted to know whether CystLTs produced through 5-LO activation were capable of driving cytotoxicity compared with control siRNA and with BLT1 knockdown (Fig. 2 B).

To further establish a crucial role for the CystLTs specifically, we tested whether their addition could rescue cytotoxicity after pharmacological inhibition of 5-LO. We administered LTD4 or LTB4 to IE-CTLs pretreated with MK886 and found that the addition of LTD4 but not LTB4 resulted in total restoration of cytotoxicity (Fig. 2 C).

Figure 2. CystLT synthesis and signaling in an autocrine manner plays a role in NKG2D-mediated cytotoxicity in IE-CTLs. (A) IE-CTLs were pre-treated for 30 min with pharmacological antagonists of CystLTR1 or BLT1 at 10 µM (MK571 and U75, respectively) or vehicle control (DMSO) before the cytolysis assay performed against 51Cr-labeled EL4-MICA target cells at an effector/target ratio of 18:1. Data are means ± standard deviation of three independent experiments using three different CTL lines. (B) TALL-104 cells were transfected with siRNA against CystLTR1 or BLT1 or with a scrambled control siRNA by electroporation. Efficacy of knockdown is shown by a representative Western blot at the top. Cells were allowed to recover for 24 h before incubation with 51Cr-labeled targets at an effector/target ratio of 3:1. Data are means ± standard deviation of five independent experiments. (C) Human IE-CTLs were pretreated for 30 min with 10 µM MK886 or vehicle control, and rescue experiments were performed by adding 1 µM LTD4 or LTB4 after 1 h of co-culture of effector cells with EL4-MICA targets at an effector/target ratio of 18:1. The cytolytic assay was performed after an additional 3 h of incubation. Data are means ± standard deviation of three independent experiments using two different cell lines. (D and E) Schematic diagram of experimental design. Human IE-CTLs were stimulated for 4 h with plate-bound antibodies against NKG2D or an IgG control. Supernatants were collected and used to stimulate TALL-104 cells transfected with siRNA against LTC4S or CystLTR1 or a scrambled control siRNA in the presence of 51Cr-labeled EL4-MICA targets at the indicated effector/target ratios. Two different human IE-CTL lines were used. Data are representative of three independent experiments. (F) Experimental design is as in E, but here the effector/target ratio is 12.5:1 only. Data are normalized to the cytotoxic capacity measured in TALL cells that were transfected with control siRNA and are presented as means ± standard deviation of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
and IL-15 are involved in NKG2D-mediated cytolysis in CTLs and CD pathogenesis (Tang et al., 2009). The mechanism by which CysLTR1 promotes cytolysis remains elusive. One possibility may involve the ability of CysLTR1 to drive activation and expression of integrins (Massoumi et al., 2003; Meliton et al., 2007; Boehmler et al., 2009).

IL-15 is a proinflammatory cytokine up-regulated in various tissues under conditions of stress and inflammation (Abadie and Jabri, 2014), including in IECs of CD patients (Jabri et al., 2000; Mention et al., 2003). It is thought to play an important role in tissue immunity by directing immune responses toward T\(_{\text{H}1}\) immunity and promoting CTL responses (Abadie and Jabri, 2014). Our previous work has shown that IL-15 acts on multiple levels within the NKG2D–DAP10 signaling pathway, priming IE-CTLs to mediate cytolysis and release AA upon binding of NKG2D to stress ligands (Meresse et al., 2004; Tang et al., 2009). We wanted to determine whether IL-15 also directly promotes the CysLT pathway. Additionally, we wanted to test whether signaling through NKG2D would amplify CysLT production and/or responsiveness. Western blot analysis was used to test expression of CysLTR1 and LTC4S at the protein level after stimulation of IE-CTLs with the indicated doses of IL-15 or a cross-linking anti-NKG2D mAb (Fig. 3). We noted robust up-regulation of both CysLTR1 and LTC4S are up-regulated by IL-15 and NKG2D in a dose-dependent manner. (A) Fresh IE-CTLs were cultured overnight and stimulated with the indicated concentration of IL-15 for 3 h. Cell lysates were immunoblotted with antibodies against CysLTR1 or LTC4S. Blots are representative of four independent experiments. Bottom panels show mean fold change ± standard deviation of four independent experiments. (B) Fresh IE-CTLs were cultured as in A but were stimulated with an antibody against NKG2D for 3 h in lieu of IL-15. Lysates were isolated and immunoblotted as in A. \(\beta\)-Actin is shown as a loading control. Blots are representative of four independent experiments, and the bottom panels present means ± standard deviation of four independent experiments.
CystLTs mediate killing via NKG2D and IL-15 in CD | Tang et al.

Avenue because they would disrupt the end effector response

The CystLT pathway might represent a novel therapeutic target in organ-specific autoimmune disorders where IL-15 and/or CystLTs play a role, such as rheumatoid arthritis (Groh et al., 2003) and type-1 diabetes (Ogasawara et al., 2004; Chen et al., 2013). It has become increasingly appreciated that the functions exerted by CystLTs and their receptors are much more complex than previously thought (Bäck et al., 2011; Kanaoka and Boyce, 2014). Further uncovering how these receptors are regulated and what physiopathological functions they mediate will yield further therapeutic insights and contribute to our understanding of how effector T cells are regulated in tissues.

CystLTs are dysregulated in active CD patients

Knowing that NKG2D and IL-15 both induce the CystLT pathway and are up-regulated in CD, we hypothesized that CystLTs and their receptors were up-regulated in CD. To test this hypothesis, we stained intestinal sections of active CD, gluten-free diet (GFD), and control patients undergoing biopsies for unrelated intestinal disorders by immunohistochemistry with antibodies for phospho–5-LO, LTC4S, or CystLTR1 (Fig. 2 E). These data support the notion that CystLTs can act in an autocrine manner in IE-CTLs upon NKG2D stimulation. However, we do not exclude the possibility that CystLTs produced by other cell types may also play a role in driving IE-CTL activation. Furthermore, these data suggest that IL-15, a cytokine associated with Th1 immunity (Abadie and Jabri, 2014), can up-regulate LTC4S and CystLTR1 in an analogous manner to Th1,2 cytokines (Jiang et al., 2006). Notably, this finding suggests that CystLTs may have deleterious roles in Th1-mediated autoimmune diseases, in particular disorders where IL-15 and NKG2D have been shown to contribute to pathogenesis.

The CystLT pathway might represent a novel therapeutic target for treatment of CD and Th1,1-mediated autoimmune disorders

Although CystLTs have previously been implicated in allergic diseases, in particular asthma (Kanaoka and Boyce, 2014), here we demonstrate an unexpected role for these molecules in CD, a Th1,1-mediated disorder. Given that NKG2D plays an important role in IEC destruction and the development of villous atrophy in CD, we believe that drugs targeting the CystLT pathway would constitute a very attractive therapeutic avenue because they would disrupt the end effector response responsible for tissue damage. It is possible to specifically target this pathway using drugs such as montelukast, which is FDA approved for the treatment of asthma and allergic rhinitis (Kanaoka and Boyce, 2014). Indeed, we were able to assess the potential of montelukast to significantly suppress cytotoxicity through NKG2D in human IE-CTL lines in vitro (Fig. 4 C). Such a drug might be able to accelerate mucosal healing after introduction of a GFD in active CD patients. Indeed, in a significant subset of adult CD patients, it is known that the GFD is not sufficient to fully promote normal mucosal healing (Rubio-Tapia et al., 2010). Intriguingly, there have been case reports of patients suffering from both asthma and CD who were prescribed CystLT inhibitors for the treatment of their lung disease for whom an unexpected positive additive effect was the resolution of their CD symptoms poorly controlled on a GFD (Fees, 2002). Conversely, our findings also suggest that cyclooxygenase inhibitors may exacerbate the disease by enhancing the production of leukotrienes. More generally, it is possible that the CystLT pathway could also be a therapeutic target in organ-specific autoimmune disorders where IL-15 and/or NKG2D play a role, such as rheumatoid arthritis (Groh et al., 2003) and type-1 diabetes (Ogasawara et al., 2004; Chen et al., 2013). It has become increasingly appreciated that the functions of CystLTs and their receptors are much more complex than previously thought (Bäck et al., 2011; Kanaoka and Boyce, 2014). Further uncovering how these receptors are regulated and what physiopathological functions they mediate will yield further therapeutic insights and contribute to our understanding of how effector T cells are regulated in tissues.

MATERIALS AND METHODS

Human subjects. For immunohistochemical experiments, the total number of patients (% female) was 15 (66.7%), 6 (83.3%), and 12 (66.7%) for active CD, GFD, and control subjects, respectively. The mean age was 32.1 yr for active CD, 37.5 yr for GFD, and 28.3 yr for control subjects. Additionally, biopsies were obtained from four active CD and three control patients to generate seven NKG2D+TCRαβ+CD8+ IE-CTL cell lines. Diagnosis of CD was based on the presence of elevated anti-transglutaminase antibodies in serum, the expression of HLA DQ2 or DQ8, the presence of increased intraepithelial lymphocytes (IELs), partial or total duodenal villous atrophy, crypt hyperplasia on duodenal biopsy, and clinical response to a GFD. Villous atrophy was found in 100% of active CD patients, 0% of GFD patients, and 0% of controls. The mean time on a GFD was 3 yr for the GFD group. Control individuals were undergoing endoscopies and biopsies for functional intestinal disorders of nonceliac origin. All subjects gave written informed consent, and all protocols were approved by the University of Chicago Institutional Review Board.

Generation of CTL lines and cell culture. Intraepithelial and peripheral blood NKG2D+TCRαβ+CD8+ CTL lines and clones were isolated as previously described from biopsies of four active CD and three control patients (Jabri et al., 2000). After isolation, IE-CTLs were stained with anti-NKG2D, anti-TCRαβ, anti-CD8α, and anti-CD103 antibodies, and NKG2D+TCRαβ+CD8+ IE-CTLs were purified using a FACSAria (BD). After sorting, cell lines were generated and cultured as previously described (Jabri et al., 2002). Importantly, NKG2D signaling and NKG2D-mediated cytolysis are similar in control and CD NKG2D+TCRαβ+CD8+ IE-CTL lines, as long as these IE-CTLs are in an effector state and cultured in the presence of IL-15 or high concentrations of IL-2, which mimick the effects of IL-15 (Meresse et al., 2004). These conditions reflect the environment of IE-CTLs in CD (Meresse et al., 2004).
Figure 4. The CystLT pathway is dysregulated in active CD patients. (A) Immunohistochemical staining was performed on duodenal paraffin-embedded intestinal sections for the following markers: phospho–5-LO, LTC4S, and CystLTR1. The top panels are representative images from stainings of sections from patients with active CD, defined as those with elevated levels of serum anti–tissue transglutaminase antibodies, partial or total duodenal villous atrophy, crypt hyperplasia, and increased presence of IELs. The middle panels are representative images from control biopsies, defined as patients undergoing upper endoscopy for unrelated disorders who did not meet the criteria for CD. The bottom panels are representative images from patients with CD on a GFD for a mean of 3 yr who lacked villous atrophy. Bar, 100 µM. (B) The number of IELs stained positive for phospho–5-LO, LTC4S, and CystLTR1 per 100 IECs were counted and plotted. Slides from 15 active CD patients, 6 GFD patients, and 12 control patients were stained with each antibody. Data are presented as means ± standard deviation. P-values were determined by Wilcoxon rank sum test. (C) After pretreatment for 30 min with 10 µM montelukast, IELs were incubated with ^51Cr-labeled effector cells expressing MICA at the indicated effector to target ratio, and specific lysis was measured. Data are presented as means ± standard deviation of three independent experiments using two different cell lines. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Indeed, what differentiates CD and controls in vivo is that both IL-15 and the NKG2D ligand MICA/B are up-regulated in the epithelium of CD patients, thus providing both signals to IE-CTLs that enable NKG2D to mediate direct cytolyis (Meresse et al., 2004).

The TALL-104 line is a CD8+ TCRαβ+ cytotoxic cell line established from the blood of a patient with acute lymphoblastic leukemia (ATCC). It was cultured in Iscove’s modified Dulbecco’s medium plus 20% FCS, antibiotics, and 100 U/ml recombinant human IL-2.

MICA-transfected EL4 (EL4-MICA) and control vector EL4 (ATCC TIB-39) are mouse T lymphoma cell lines. They were grown in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics, and MICA-expressing cells were maintained using G418. As a target cell for the antibody-redirected lysis experiments, the P815 (ATCC TIB-64TM) mouse mastocytoma cell line was used. It was grown in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics.

Reagents, antibodies, and recombinant cytokines. Human IL-15 and IL-2 were purchased from BD, LT4B, LT4D, BLT1 antagonist U73502, polyclonal antibodies specific for 5-LO, polyclonal antibodies specific for CysLTR1, polyclonal antibodies specific for BLT1, and monothiocet (sodium salt) were obtained from Cayman Chemical. Anti–TATA-binding protein (TBP) antibody was obtained from Biodisc International Anti-CD3 (clone UCHT1, IgG1) and anti-NKG2D (clone 1D11, IgG1) monoclonal antibodies with corresponding IgG1 isotype-matched control were purchased from BD. Polyclonal antibodies against human LTC4S were purchased from Arviva Systems Biology and Cell Signaling Technology. A monoclonal antibody specific for COX2 (clone D5H9) was purchased from Cell Signaling Technology. Anti-phosphotyrosine monoclonal antibodies (clone 4G10) were obtained from EMD Millipore. The chemical antagonists for 5-LO (MK886) and CysLTR1 (MK571), along with anti–β-actin monoclonal antibodies, were purchased from Sigma-Aldrich. F(ab’)2 goat anti–mouse antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc.

siRNA and transfection. siRNAs specific for human 5-LO, COX2, CysLTR1, BLT1, LTC4S, and a scrambled control siRNA were purchased from Santa Cruz Biotechnology, Inc. TALL-104 cells were electroporated using a Lonza Amazac Nucleofector in Lonza cell line nucleofection solution V and program T20. Cells were allowed to recover in culture for 24 h after transfection before use in experiments. Efficiency and specificity of knockdown were assessed at the protein level by Western blot.

Western blot analysis. Before stimulation with IL-15 and/or MK820, human IELs were starved of IL-2 in culture for 20–24 h. To examine CysLTR1 and LTC4S expression, cells were treated with IL-15 at the indicated concentrations for 3 h at 37°C. Immunoceptor cross-linking was performed by incubating cells for 4 min with the indicated receptor-specific antibody before the addition of F(ab’)2 GAM for 3 h at 37°C. Cells were then harvested and lysed for 20 min in cold lysis buffer containing fresh protease inhibitors (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, and a protease inhibitor cocktail tablet [Thermo Fisher Scientific]). Cellular debris was removed by centrifugation at 15,000 RPM in a table-top centrifuge for 20 min at 4°C. 80 μg lysate protein was subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Detection was performed using the indicated primary antibodies, followed by an HRP-conjugated secondary IgG and the LumIGLO chemiluminescent substrate kit (Cell Signaling Technology). Scanning densitometry was performed using ImageJ software (National Institutes of Health).

To investigate perinuclear translocation of 5-LO in response to stimulation, nuclear fractions were extracted using the NE-PER nuclear extraction kit (Thermo Fisher Scientific). Samples were then run on a 10% polyacrylamide gel in the presence of SDS and were subjected to electrophoresis. Separated proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) and blocked with 5% nonfat dry milk in TBS-T for 1 h, followed by overnight incubation with primary antibody at 4°C. Membranes were washed and then incubated for 45 min with an HRP-linked goat anti–rabbit or goat anti–mouse antibody at room temperature. Development was performed by enhanced chemiluminescence, and membranes were exposed to films. Equal loading and the nuclear nature of the extract were confirmed using an anti–TBP antibody that recognizes a DNA-binding protein specific for the TATA box.

Cytotoxicity assay. Chromium release assays were performed as previously described (Tang et al., 2009). In brief, EL4-MICA and control EL4 cells were radiolabeled with 51Cr before incubation with effector cells in duplicate wells at the indicated effector/target ratios. Chromium release into the supernatant was measured using a scintillation counter (Packard). Maximum release was determined by the addition of 10% SDS to target cells, while spontaneous release was also assessed and ranged from 5–10% of the maximum. The percentage of specific cytotoxicity was calculated using the formula 100 × (CPM experimental − CPM spontaneous)/(CPM maximum − CPM spontaneous), Where indicated, effector cells were treated for 30 min before and during the cytotoxic assay using various inhibitors or lipid mediators or, alternatively, equivalent concentrations of either DMSO or ethanol vehicle control. Alternatively, antibody-redirected cytotoxicity assays were performed, wherein effector cells were activated through a specific cross-linking antibody and directed to lyse target cells lacking stress ligands. Finally, cytotoxicity was also assessed after stimulation with conditioned supernatants. In brief, IELs were stimulated with plate-bound NGK2D-specific cross-linking antibodies for 4 min before the addition of GAM for 3 h, and supernatants were harvested and applied to TALL cells 24 h after being transfected with siRNA specific for LTC4S or CysLTR1. Subsequent cytotoxicity of these TALL cells against MICA-expressing targets was then evaluated.

Immunohistochemical staining. Immunohistochemical staining was performed on paraffin sections after antigen retrieval and peroxidase block (EnVision+ HRP system; Dako). Slides were stained with anti-phospho–5-LO (1:50, L1168; Sigma-Aldrich), anti–CysLTR1 (5 μg/μl, C93491; Sigma-Aldrich), and anti–LTC4S (0.005 ng/μl, AP94035_T100; Aviva) antibodies, followed by HRP-conjugated anti–rabbit secondary antibody (Dako). Antibody revelation was performed with DAB chromogen (Dako), and counterstain was completed with hematoxylin (Gill 3 Hematoxylin; Thermo Fisher Scientific). Slides were evaluated on a DM2500 microscope (Leica) using an HC PlanAPOchromat 20×/0.70 lens (Leica) at 22°C. Images were acquired with a Retiga EXI FAST 1394 camera (QImaging), processed by Image-Pro Plus 7.0 software, and exported in TIF format. Counting of positive IELs was performed by two independent readers in a double-blinded manner.

Statistical analysis. Wilcoxon rank sum testing was used to assess statistical significance, denoted as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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