Cytosolic PLA_2 is required for CTL-mediated immunopathology of celiac disease via NKG2D and IL-15

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IL-15 and NKG2D promote autoimmunity and celiac disease by arming cytotoxic T lymphocytes (CTLs) to cause tissue destruction. However, the downstream signaling events underlying these functional properties remain unclear. Here, we identify cytosolic phospholipase A_2 ($cPLA_2$) as a central molecule in NKG2D-mediated cytolysis in CTLs. Furthermore, we report that NKG2D induces, upon recognition of MIC^+ target cells, the release of arachidonic acid (AA) by CTLs to promote tissue inflammation in association with target killing. Interestingly, IL-15, which licenses NKG2D-mediated lymphokine killer activity in CTLs, cooperates with NKG2D to induce $cPLA_2$ activation and AA release. Finally, $cPLA_2$ activation in intraepithelial CTLs of celiac patients provides an in vivo pathophysiological dimension to $cPLA_2$ activation in CTLs. These results reveal an unrecognized link between NKG2D and tissue inflammation, which may underlie the emerging role of NKG2D in various immunopathological conditions and define new therapeutic targets.

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Abbreviations used: AA, arachidonic acid; BLT, benzyloxycarbonyl lysine thiobenzyl; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A2; ERK, extracellular signal–regulated kinase; IE-CTL, intraepithelial CTL; ITAM, immunoreceptor tyrosine-based activation motifs; JNK, c-Jun N-terminal kinase; LA, linoleic acid; MIC, MHC class I–related chain; PI3-K, phosphatidylinositol 3-kinase; PB-CTL, peripheral blood CTL; siRNA, small interfering RNA; ULPB, UL-16–binding protein.

NKG2D is a NK cell receptor expressed by all human CTLs (1) that recognizes MHC class I–related chain (MIC) and UL-16–binding protein (ULPB) molecules expressed on stressed and transformed target cells (1–3). NKG2D has been implicated in antitumor immunity mediated by both NK cells and CTLs (4, 5) and in T cell–mediated immune disorders such as celiac disease (6, 7), rheumatoid arthritis (8), and NOD mouse model for juvenile type 1 diabetes (9). In humans, NKG2D associates exclusively with DAP10 (6, 10–12), an adaptor with a YINM cytoplasmic tail motif that activates phosphatidylinositol 3-kinase (PI3-K) (10), but not the syk/ZAP-70 kinase family. Because of these similarities with the CD28 signaling pathway, NKG2D–DAP10 was initially thought to function mainly as a co-stimulator (1) and to play a role in autoimmunity by promoting activation of autoreactive T cells. However, several studies indicate that NKG2D–DAP10 in human can also mediate cytolysis independently of TCR engagement in effector CTLs exposed to IL-15 or high doses of IL-2 (6, 13, 14). This layer of effector T cell regulation by NKG2D at the level of cytolysis may serve to effectively and rapidly eliminate infected or transformed target tissue cells independently of antigen specificity, and may participate in aberrant tissue destruction in diseases in which IL-15 expression is dysregulated (for review see reference [15]).

The downstream co-stimulatory and cytolytic signaling pathways recruited by NKG2D in CTLs remain incompletely characterized. Intriguingly, several studies suggest that $cPLA_2$ may be involved in inflammatory and autoimmune diseases (16–18). However, how $cPLA_2$ drives immunopathological processes and whether it involves CTLs is poorly understood.

F. Tang and Z. Chen contributed equally to this paper.

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Interestingly, several reports point to a potential role of $cPLA_2$ in T cell proliferation (19, 20). Furthermore, $cPLA_2$ was shown to be involved in granule exocytosis by neuronal cells (21, 22), hormonal cells (23), and granulocytes (24–27), suggesting that it might also be implicated in granular release occurring in the context of cytolysis and cytokine secretion in T cells. Finally, $cPLA_2$ activation by surface receptors is dependent on phosphorylation at Ser^{505} by MAP kinases (28), and NKG2D induces c-Jun N-terminal kinase (JNK) and extracellular signal–regulated kinase (ERK) activation in CTLs (6). Together, these observations prompted us to examine a potential link between $cPLA_2$ and NKG2D effector function in CTLs and its relevance in celiac disease.

RESULTS

$cPLA_2$ plays a critical role in direct NKG2D-mediated cytolysis

NKG2D is licensed to mediate cytolysis independently of TCR activation in CTLs when they are in an effector stage and in the presence of IL-15 or high doses of IL-2 (6). Importantly, under these conditions it is possible to assess how $cPLA_2$ affects NKG2D effector functions independently from other receptors. To determine that our findings were not restricted to a particular subset of effector CTLs, we analyzed the effect of $cPLA_2$ inhibition in a variety of effector CTLs. Specifically, we studied freshly isolated effector intestinal intraepithelial CTLs (IE-CTLs) that were prestimulated in vitro with IL-15, normal IE-CTL clones, IE-CTL clones derived from celiac patients, peripheral blood effector CTL (PB-CTL) clones, and the leukemia TALL-104 CD8 T cell line. This latter cell line was previously used as a model to study the NKG2D cytolytic signaling pathway (6). All clones and cell lines were grown in the presence of a high concentration of IL-2, which is known to substitute for IL-15.

The $cPLA_2$ inhibitor AACOCF3 (CF3) impaired NKG2D-mediated cytolysis in antibody-redirected cytolytic assays (Fig. 1 A, left). Importantly, this finding could be extended to cytolytic assays using MIC-transfected C1R cells as targets (Fig. 1 A, right). Furthermore, arachidonic acid (AA) significantly restored cytolysis, strongly arguing against a nonspecific effect of the $cPLA_2$ inhibitor AACOCF3 (Fig. 1 A, right). Finally, AACOCF3 and AA had a dose-dependent effect on NKG2D-mediated cytolysis in freshly isolated IE-CTLs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071887/DC1).

To further assess the critical role of $cPLA_2$ in NKG2D-mediated cytolysis and granule release, $cPLA_2$ was "knocked down" in TALL-104 (Fig. 1, B and C) and PB-CTLs (Fig. 1 B, right) using $cPLA_2$ small interfering (si)RNA. Importantly, $cPLA_2$ siRNA specifically targeted $cPLA_2$ expression, as it affected neither ERK nor JNK expression (not depicted) and activation (Fig. 1 B, left), nor 5-lipoxygenase (5-LO) expression (Fig. 1 C, left). The effect of $cPLA_2$ knock down on NKG2D-mediated cytolysis was assessed in two ways. First, in redirected cytolytic assays, as expected, NKG2D-mediated cytolysis was inhibited by up to 50% in the presence of $cPLA_2$ siRNA, but not control siRNA. In contrast, TCR-mediated cytolysis remained essentially intact for this level of specific cytolysis (Fig. 1 B, middle). The level of inhibition observed for NKG2D-mediated cytolysis was highly significant considering that some residual $cPLA_2$ could be detected (Fig. 1 B, left). Second, similar results were obtained when using MIC^+C1R target cells (Fig. 1 D). Importantly, addition of AA (Fig. 1 D), but not a control polyunsaturated fatty acid, linoleic acid (LA), rescued NKG2D-mediated cytolysis (Fig. 1 D, right). The assays were performed either with global unsorted siRNA-transfected CTLs (Fig. 1, B and D, right), or with CTLs sorted into GFP^+-transfected and GFP^--untransfected CTLs (Fig. 1 D, middle).

Having shown that $cPLA_2$ played a role in NKG2D-mediated cytolysis, we wanted to more specifically demonstrate its role in the release of cytolytic granules. We previously reported, that ligation of NKG2D receptors expressed on CTLs with monoclonal anti-NKG2D mAb triggered granule release using a BLT esterase assay (6). As anticipated, knock down of $cPLA_2$ by siRNA (Fig. 1 C) blocked degranulation. Similar results were obtained in the presence of the pharmacological inhibitor CF3 (unpublished data). Importantly, $cPLA_2$ inhibition did not affect the level of granzyme and perforin expression in CTLs (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071887/DC1), further suggesting that $cPLA_2$ plays a role in NKG2D-mediated degranulation.

Interestingly, $cPLA_2$ inhibition by AACOCF3 or knock down by siRNA affected only marginally TCR-mediated cytolysis (Figs. 1 and 2).

Altogether, these results demonstrate that $cPLA_2$ and AA play a critical role in NKG2D-mediated, but not TCR-mediated, cytolytic granule exocytosis in effector CTLs.

$cPLA_2$ plays a critical role in NKG2D-mediated cytolytic co-stimulatory functions

NKG2D was shown to function as a co-stimulatory receptor that enhances TCR-dependent cytolytic responses (13, 29). We therefore investigated whether $cPLA_2$ was also implicated in NKG2D cytolytic co-stimulatory functions. This study had to be performed in CTLs grown in medium with low IL-2, in which NKG2D was not licensed to mediate direct killing. As previously reported (29), NKG2D significantly increased TCR-mediated cytolysis in two distinct PB-CTL clones (Fig. 2). Importantly, $cPLA_2$ inhibition prevented NKG2D enhancement of TCR-mediated cytolysis, and AA significantly rescued NKG2D co-stimulation (Fig. 2). In contrast, $cPLA_2$ inhibition had little or no significant effect on TCR-mediated cytolysis (Fig. 2).

In summary, these observations also support a role for $cPLA_2$ in NKG2D cytolytic co-stimulatory functions.

NKG2D induces $cPLA_2$ activation and AA release in effector CTLs

To establish a biochemical basis for $cPLA_2$ functions in CTLs, we determined NKG2D-induced $cPLA_2$ phosphorylation and AA release in several different CTL subsets, including in IE-CTL clones derived from celiac patients. Expression of $cPLA_2$ in

mature T cells has been controversial (19, 30). However, using Western blot analysis, we demonstrated $cPLA_2$ expression in different effector CTLs (Fig. 3, left). Furthermore, as shown in Fig. 3, NKG2D cross-linking induced $cPLA_2$ phosphorylation in a dose-dependent manner (Fig. 3, left).

To further assess the ability of NKG2D to activate $cPLA_2$, we studied perinuclear translocation of $cPLA_2$ and $cPLA_2$ activity in IE- and PB-CTLs. As anticipated, NKG2D engagement induced perinuclear translocation of $cPLA_2$ (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20071887/DC1)

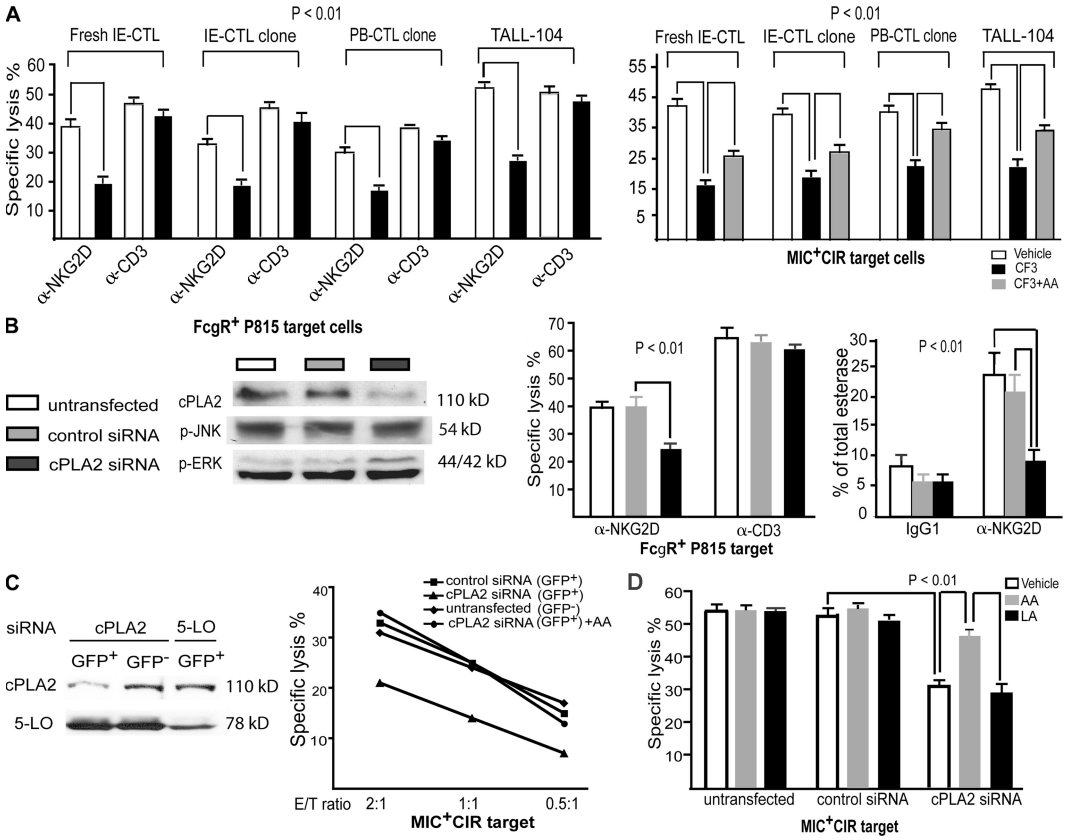

Figure 1. NKG2D-mediated cytolysis in CTL is $cPLA_2$ dependent. (A) $cPLA_2$ inhibition by the pharmacological inhibitor AACOCF3 (CF3) blocks NKG2D-mediated cytotoxicity. Fresh IE-CTL prestimulated with IL-15 for 48 h, celiac IE-CTL clones, PB-CTL clones, and TALL-104 leukemia CTLs were pretreated for 30 min with vehicle control or CF3 before the cytolysis assay. Depending on the nature of the CTLs studied, distinct E/T ratios were used to achieve similar levels of specific lysis (50:1 for fresh IE-CTLs and PB-CTLs, 15:1 for celiac IE-CTLs, and 5:1 for TALL-104 leukemia CTLs). Data are means ± SD of three independent experiments for each cell line. P values reported are from a Tukey-adjusted pairwise comparison. (left) Cytolysis of FcγR+ P815 cells was significantly blocked by 20 μM CF3 in the presence of anti-NKG2D, but not anti-CD3, antibody. (right) Cytolysis of C1R/MIC transfectants was blocked upon pretreatment of CTLs with 20 μM CF3 and restored when 100 μM AA was added 1 h after the beginning of the cytolysis assay. Importantly, CF3 did not affect the level of intracellular granzyme and perforin expression (Fig. S2). (B) Knockdown of $cPLA_2$ in TALL-104 CTLs significantly impairs NKG2D-mediated cytolysis, but not TCR-mediated cytolysis under optimal conditions of stimulation. Untransfected $cPLA_2$ and control siRNA-transfected TALL-104 CTLs were studied 72 h after transfection. (left) $cPLA_2$ siRNA significantly decreases $cPLA_2$ expression without affecting JNK and ERK activation. Untransfected and transfected TALL-104 were cultured for 72 h, serum-starved overnight, and stimulated for 5 min (p-ERK) or 15 min (p-JNK), respectively, with anti-NKG2D. Cell lysates were analyzed by Western blot with anti-$cPLA_2$, then stripped and blotted with anti-phospho-ERK (p-ERK) or -JNK (p-JNK) mAbs. *Right panel:* $cPLA_2$ siRNA inhibited significantly NKG2D-mediated cytolysis in a redirected cytolytic assay using ^{51}Cr-labeled FcγR+ P815 targets. (C) $cPLA_2$ knocked down in PB-CTLs blocked the release of cytolytic granule. An esterase assay was performed 36 h after transfection. Data are means ± SD of six experimental points from two independent experiments. P values are from a Tukey-adjusted pairwise comparison. (D) AA reconstitutes NKG2D cytolytic function against C1R/MIC+ target cells in TALL-104 transfected with $cPLA_2$ siRNA. Rescue experiments were performed by adding 50 μM of AA or a control polyunsaturated fatty acid LA after 1 h co-culture with target cells. (left) Knock-down of $cPLA_2$ did not affect 5-LO expression, whereas 5-LO siRNA decreased significantly 5-LO expression without affecting $cPLA_2$ expression. Cells were sorted into transfected (GFP+) and untransfected cells (GFP-) cell populations 24 h after transfection with $cPLA_2$ siRNA, control siRNA or 5-LO siRNA in addition to GFP plasmids. Cell lysates were analyzed 12 h later by Western blot with anti-$cPLA_2$ antibody, then stripped and immunoblotted with anti-5-LO antibody. (middle) Cytolysis assays were performed 36 h after transfection at the indicated E:T ratios in transfected (GFP+) and untransfected (GFP-) cells. One representative experiment out of two independent experiments is shown. (right) Cells were cultured for 72 h after transfection with $cPLA_2$ or control siRNA and cytolysis assays were performed in absence of cell sorting on the total cell population. The transfection efficiency, determined by GFP expression, was ~60%. Data are means ± SD of six experimental points from two independent experiments. P values are from a Tukey-adjusted pairwise comparison.

and up-regulated $cPLA_2$ activity (Fig. S3 B), which were blocked in the presence of the pharmacological inhibitor CF3 (Fig. S3).

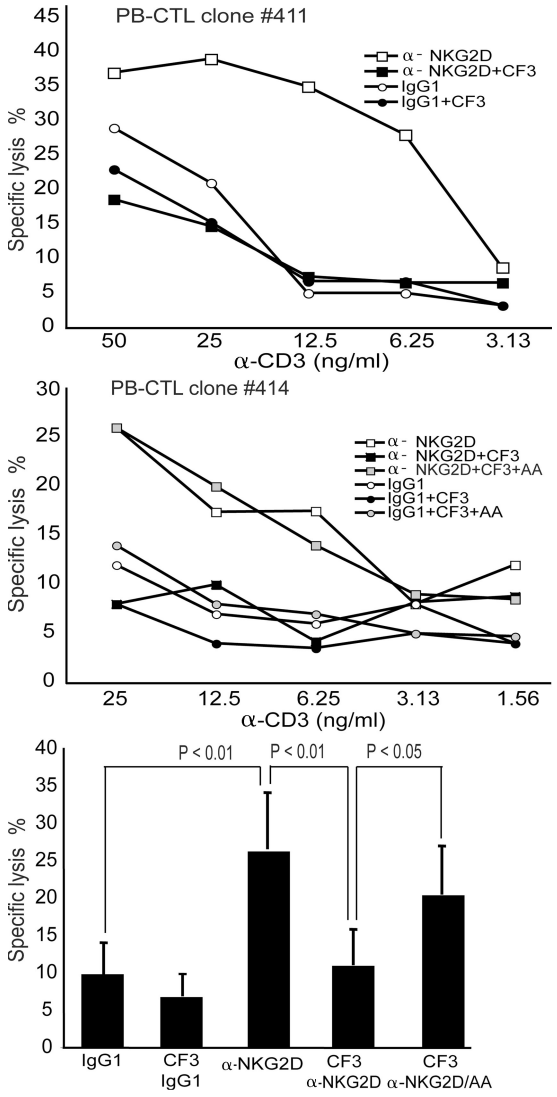

Figure 2. $cPLA_2$ activation is necessary for NKG2D cytolytic co-stimulatory functions. Inhibition of $cPLA_2$ blocks NKG2D augmentation of cytolytic T cell functions and AA reverses this blockage. Co-stimulation of cytolytic T cell functions by NKG2D was studied using three different PB-CTL clones (#411, #414, and #348) that were generated and cultured with low IL-2 (5 U/ml), in which NKG2D does not mediate direct cytolysis. Representative experiments of clone 411(top) and 414 (middle) are shown. PB-CTLs were pretreated with vehicle or CF3. Redirected cytolysis was performed against ^{51}Cr-labeled $FcgR^+$ P815 targets (E/T ratio of 50:1) in the presence of anti-CD3 mAbs at the indicated concentration in combination with either anti-NKG2D mAbs or isotype control IgG1 at fixed concentration (1 μg/ml). (bottom) A rescue experiment was performed by adding AA to CF3-pretreated cells 1 h after co-culture with target cells. A summary of three independent experiments for an anti-CD3 mAb concentration of 12.5 ng/ml with means ± SD is shown. P values are from a Tukey-adjusted pairwise comparison.

To determine whether the capacity of NKG2D to induce $cPLA_2$ phosphorylation in CTLs was dependent on their activation status, we sorted freshly purified resting $CD8\beta^+$ peripheral blood T cells that were used either immediately for biochemical studies or to establish a short-term effector CTL line by allogenic stimulation, as previously described (31). Interestingly, NKG2D and IL-15 failed to induce $cPLA_2$ phosphorylation in resting $CD8^+TCR\alpha\beta^+$ peripheral blood lymphocytes, but not in the short-term derived effector CTL line (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20071887/DC1). These findings are in accordance with previously reported data (6) showing that licensing of NKG2D by IL-15 to mediate cytolysis was restricted to CTLs in the effector stage.

Finally, we investigated whether induction of $cPLA_2$ phosphorylation was associated with AA release (Fig. 3, right). This was investigated under conditions of stimulation resulting in

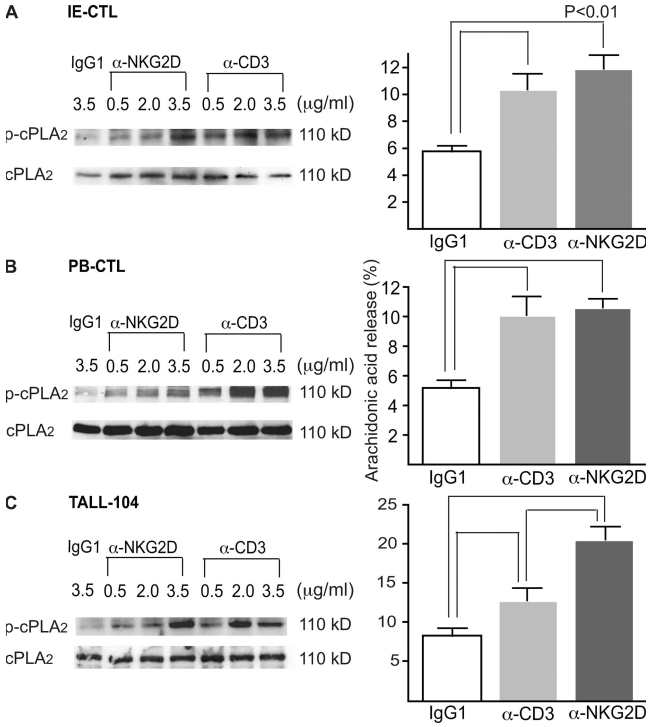

Figure 3. NKG2D and TCR engagement induce $cPLA_2$ phosphorylation and AA release in CTLs. Celiac IE-CTL clone (A), PB-CTL clone (B), and TALL-104 CTLs (C) were studied. (left) Concentration-dependent $cPLA_2$ phosphorylation upon NKG2D and TCR engagement. CTLs were serum starved for 16 h, and then stimulated for 15 min as indicated. Cell lysates were analyzed by Western blot using anti–phospho-$cPLA_2$ ($p\text{-}PLA_2$)–specific antibody. The membrane was stripped and reblotted with anti-$cPLA_2$ antibody to assess equal loading. (right) AA release upon NKG2D and TCR engagement. CTLs were incubated with ^3H-AA for 10 h, washed to remove free ^3H-AA, and stimulated with the indicated antibody or isotype control for 1 h. Supernatants and cell pellets were separately harvested. Total and released ^3H-AA were measured to determine the percentage of released ^3H-AA. Mean value of triplicates was used for each of the three experiments. Data are means ± SD of three independent experiments. P values are from a Tukey-adjusted pairwise comparison.

maximal levels of $cPLA_2$ phosphorylation. TCR stimulation induced AA release. However, importantly, NKG2D could induce AA release independently of TCR engagement.

Collectively, these results demonstrate that NKG2D can induce $cPLA_2$ activation and mediate AA release independently of TCR stimulation. This unexpected function of NKG2D suggests that in tissues expressing stress-induced ligands, CTLs could participate in the activation of granulocytes sensitive to the presence of AA.

ERK and JNK regulate NKG2D-mediated cytolysis through $cPLA_2$ activation in CTLs

Previous studies had shown that NKG2D activated ERK and JNK through distinct pathways and that both MAP kinases regulated NKG2D-mediated cytolysis in CTLs in an additive manner (6). However, how ERK and JNK played a role in cytolysis remained elusive. Interestingly, $cPLA_2$ activation by surface receptors was shown to be dependent on MAP kinase activation (for review see reference [28]). This prompted us

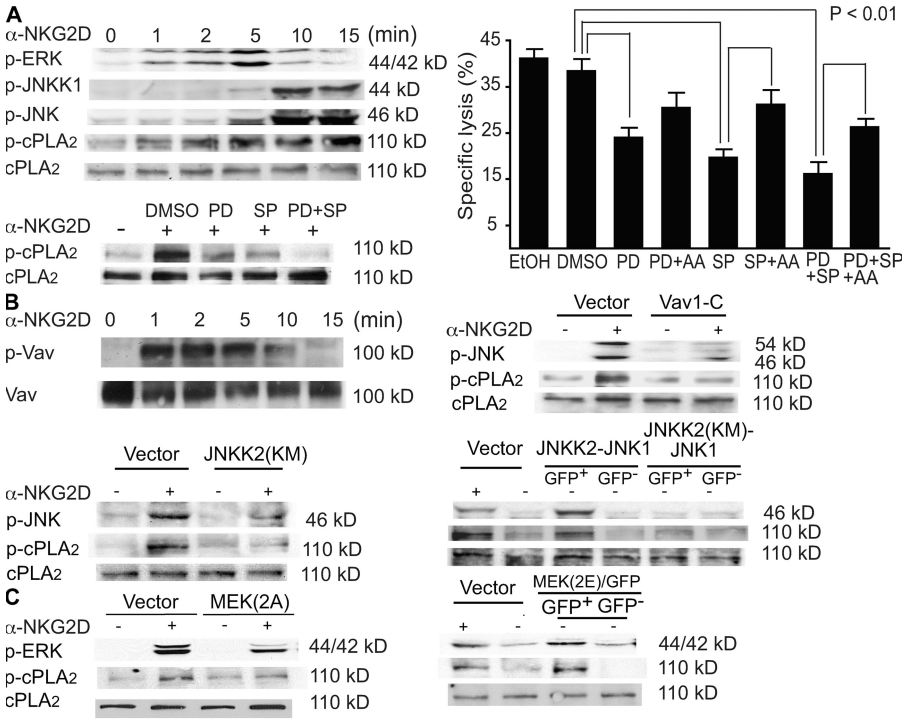

Figure 4. ERK and JNK control $cPLA_2$ activation and NKG2D-mediated cytolysis. (A) JNK and ERK both regulate $cPLA_2$ phosphorylation and cytolysis. Results shown were obtained with TALL-104 CTLs. Similar results were obtained with IE-CTLs. (top left) Phosphorylation time course. TALL-104 CTLs were serum-starved for 16 h before stimulation with anti-NKG2D antibodies for the indicated time. Cell lysates were subjected to Western blot analysis by anti–phospho-ERK, -JNKK1, -JNK, and -$cPLA_2$–specific antibodies. (bottom left) ERK and JNK inhibitors block, in an additive manner, $cPLA_2$ phosphorylation. TALL-104 CTLs were pretreated with vehicle or suboptimal doses (10 μM) of specific kinase inhibitors. PD 98059 (PD) and SP600125 (SP) are MEK1/2 and JNK inhibitors, respectively. $cPLA_2$ phosphorylation was determined by immunoblot with anti-phospho $cPLA_2$ after 15 min of stimulation with anti-NKG2D mAbs. Total $cPLA_2$ is shown as loading control. (right) AA significantly restored NKG2D-mediated cytolysis against C1R/MIC transfectants upon JNK, but not ERK, inhibition. TALL-104 CTLs were pretreated with vehicle and inhibitors as indicated in the top right graph. The rescue assay was done by addition of AA 1 h after co-culture of TALL-104 with ^{51}Cr-labeled C1R/MIC transfectants. Data are means ± SD of three independent cytolytic assays. (B) The Vav→JNKK1/2→JNK pathway controls $cPLA_2$ phosphorylation. Phosphorylation experiments were performed on cells starved overnight and stimulated for the indicated time with anti-NKG2D antibodies. Western blots for phospho-JNK and -$cPLA_2$ were performed on total cell lysates using anti–phospho-JNK and -$cPLA_2$ antibodies. Vav phosphorylation was evaluated after immunoprecipitation with anti-Vav1 mAb and immunoblotting with anti-phosphotyrosine mAb. Equal loading for total lysates and for Vav 1 immunoprecipitation were assessed using anti-$cPLA_2$ and -Vav antibody, respectively. (top left) Time course of anti-NKG2D–induced Vav phosphorylation. (top right) Dominant-negative Vav-1 (Vav 1-ΔC) blocked JNK and $cPLA_2$ activation. TALL-104 CTLs were transfected with control vector and Vav-ΔC and cultured for 48 h. Cells were then analyzed by Western blot as indicated. (bottom left) Dominant-negative JNKK2 blocks $cPLA_2$ phosphorylation. TALL-104 CTLs transfected with control vector and dominant-negative JNKK2(KM) were cultured for 48h and analyzed by Western blot. (bottom right) Constitutive JNK activation induced $cPLA_2$ phosphorylation. TALL-104 CTLs were transfected with JNKK2-JNK1 (which encodes a constitutively active form of JNK) or with JNKK2(KM)-JNK1 (which encodes a kinase-deficient mutant), in addition to GFP plasmids. After 40 h of transfection, cells were sorted by flow cytometry into GFP^+ and GFP^- cells. Presence of p-$cPLA_2$ was evaluated by immunoblot in the absence of NKG2D stimulation. NKG2D-stimulated and nonstimulated TALL-104 CTLs, transfected with a control vector, served as positive and negative control for JNK and $cPLA_2$ phosphorylation. Equal loading was assessed by immunoblotting with total $cPLA_2$. (C) ERK pathway regulates $cPLA_2$ phosphorylation. Dominant-negative and overexpression experiments were performed as in B. ERK and $cPLA_2$ phosphorylation were determined by Western blot. (left) Dominant-negative MEK-1 (MEK[2A]) significantly blocked $cPLA_2$ phosphorylation. (right) Constitutively active MEK-1 (MEK[2E]) induced $cPLA_2$ phosphorylation in the absence of NKG2D stimulation.

to investigate whether there was a link between ERK, JNK, $cPLA_2$ activation, and NKG2D-mediated cytolysis.

We first determined the kinetic of ERK, JNK, and $cPLA_2$ phosphorylation upon NKG2D stimulation. The peak of $cPLA_2$ phosphorylation occurred at 10–15 min after ERK and concomitant with JNKK1 and JNK phosphorylation (Fig. 4 A, top left). These results suggested that $cPLA_2$ phosphorylation was downstream of ERK and JNK.

To further evaluate the function of JNK and ERK in $cPLA_2$ activation and NKG2D-mediated cytolysis, we performed studies with pharmacological inhibitors targeting specific MAP kinases. As anticipated, $cPLA_2$ phosphorylation (Fig. 4 A, bottom left) and AA release (not depicted) were blocked in an additive manner upon ERK (PD98059) and JNK (SP600125) inhibition. In contrast, the p38 inhibitor SB 203580 had no effect (unpublished data), a result that is in agreement with its lack of function in NKG2D-mediated cytolysis (6). Finally, addition of AA reversed JNK inhibition, more significantly than ERK inhibition of NKG2D-mediated cytolysis (Fig. 4 A, right).

We next dissected the signaling pathway leading to JNK activation and further assessed its critical role in $cPLA_2$ activation using a dominant-negative and overexpression approach. Vav1, which regulates JNK activation through a Rho/Rac1→MAPK kinase kinase 1 pathway (32), was also reported to play an essential role in the NKG2D cytolytic pathway in NK cells (33, 34). These observations suggested that Vav1 could control JNK activation. In agreement, our kinetic analysis indicated that Vav1 activation preceded JNKK1 and JNK phosphorylation (compare Fig. 4, A and B, top left). In addition, a dominant-negative of Vav1, Vav1-C (33), blocked both JNK and $cPLA_2$ activation after NKG2D cross-linking in CTLs (Fig. 4 B, top right). To further assess the role of JNK in $cPLA_2$ phosphorylation, TALL-104 CTLs were transfected with a dominant-negative JNKK2, which was shown to prevent JNK activation. As anticipated, blocking JNK activation resulted in inhibition of $cPLA_2$ phosphorylation upon NKG2D engagement (Fig. 4 B, bottom left). Conversely, simple overexpression of constitutive active JNK (JNKK2-JNK1), but not inactive JNK (JNKK2[KM]-JNK1) (35), induced similar levels of $cPLA_2$ phosphorylation than NKG2D stimulation in CTLs (Fig. 4 B, bottom right).

Finally, because the upstream events leading to ERK activation were defined by a previous study (6) (see also Fig. 7), we focused on assessing the role of ERK in $cPLA_2$ activation. As predicted by the pharmacological studies, the dominant-negative of MEK-1 (MEK-2A) (36, 37), which is located upstream of ERK, blocked ERK and $cPLA_2$ phosphorylation (Fig. 4 C, left), whereas overexpression of constitutive active MEK-1 (MEK-2E) (36, 37), induced $cPLA_2$ phosphorylation (Fig. 4 C, right).

Altogether, these observations suggest that Vav is upstream of JNK, and that JNK and ERK control NKG2D-mediated $cPLA_2$ activation in CTLs in an independent and additive manner.

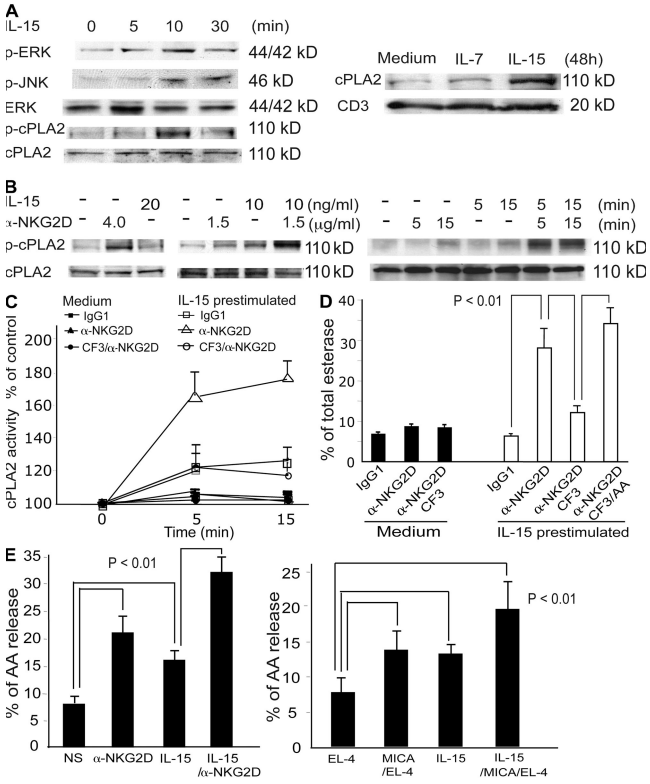

Figure 5. IL-15 promotes NKG2D–mediated cytolytic degranulation, $cPLA_2$ activation, and AA release in primary IE-CTLs. (A) IL-15 up-regulates $cPLA_2$ expression, and induces ERK, JNK, and $cPLA_2$ activation in fresh IE-CTLs. (left) IL-15 induces ERK, JNK, and $cPLA_2$ phosphorylation in a time-dependent manner. Fresh IE-CTLs were starved overnight and stimulated with 20 ng/ml IL-15 for indicated time periods. Cell lysates were immunoblotted with anti-p-ERK, -p-cPLA, and -p-$cPLA_2$ antibodies. Total ERK and total $cPLA_2$ are shown to assess equal loading. (right) Fresh IE-CTLs were cultured with IL-15 or IL-7 for 48 h. $cPLA_2$ expression was determined by Western blot. Equal loading was assessed using an anti-CD3ζ mAb. (B) IL-15 synergizes with NKG2D to induce $cPLA_2$ phosphorylation in a dose- (left) and time-dependent (right) manner. Fresh IE-CTLs (left) or TALL-104 CTLs (right) were stimulated with IL-15 alone, anti-NKG2D mAb alone, or IL-15 in combination with anti-NKG2D mAb for 15 min at the indicated concentration (left), or for the indicated time at saturating concentrations (4 μg/ml anti-NKG2D mAb and 20 ng/ml IL-15) (right). For each panel one out of 2 representative experiments is shown. (C and D) IL-15 stimulation plays a critical role in NKG2D-mediated $cPLA_2$ activity (C) and cytolytic granule release (D) in primary IE-CTLs. Freshly purified IE-CTLs were cultured with or without IL-15 (20 ng/ml) for 24–48 h and pretreated for 30 min with vehicle control or CF3 before stimulation with anti-NKG2D mAb or IgG1 isotype control. Rescue experiments were performed by adding 50 μM of AA after 1 h of stimulation. Data are means ± SD of six experimental points from two independent experiments. P values are from a Tukey-adjusted pairwise comparison. (E) IL-15 enhances NKG2D-mediated AA release. Fresh IE-CTLs were cultured with or without IL-15 (10 ng/ml) for 24 h and analyzed for AA release upon 1 h stimulation. (left) IE-CTLs were not stimulated (NS) or stimulated with plate-bound anti-NKG2D mAb (4 μg/ml). (right) IE-CTLs were stimulated with control EL-4 cells or MICA-transfected EL4 cells (MICA/EL-4) at a IE-CTLs to target cell ratio of 1:0.5. Data are means ± SD of three independent experiments. P values are from a Tukey-adjusted pairwise comparison.

IL-15 arms NKG2D to mediate cytolysis and AA release in primary IE-CTLs by up-regulating cPLA$_2$ expression and activation

We had previously demonstrated that IL-15 or high levels of IL-2 (which can substitute for IL-15) licensed NKG2D to mediate cytolysis independently of TCR activation in effector CTLs (6, 13). Furthermore, we showed that IE-CTLs exposed in vivo to IL-15 in active celiac disease killed MIC$^+$ target cells (6). Here, we investigated whether the licensing of NKG2D to mediated direct cytolysis in freshly purified IE-CTLs involved cPLA$_2$ activation by IL-15. We first determined whether IL-15 could activate cPLA$_2$. Interestingly, IL-15 induced not only ERK and JNK phosphorylation, as previously shown (6, 38), but also cPLA$_2$ phosphorylation (Fig. 5 A, left). The time course was similar to that of NKG2D (compare Fig. 5 A, left, and Fig. 4 A, left). In addition, cPLA$_2$ expression was increased in primary IE-CTLs stimulated for 48 h with IL-15, but not with IL-7 (Fig. 5 A, right). Furthermore, IL-15 synergized with NKG2D to induce cPLA$_2$ phosphorylation in a dose- (Fig. 5 B, left) and time-dependent manner (Fig. 5 B, right). cPLA$_2$ activity was significantly up-regulated only in freshly isolated IE-CTLs that were prestimulated with IL-15 for 48 h. Importantly, induction of cPLA$_2$ activity paralleled the ability of NKG2D to mediate degranulation (compare Fig. 5, C and D). Conversely, cPLA$_2$ inhibition blocked cPLA$_2$ activity (Fig. 5 C) and degranulation (Fig. 5 D), which was restored in the presence of AA (Fig. 5 D).

We next investigated whether NKG2D could induce AA release in primary effector IE-CTLs. As shown in Fig. 5 E, IL-15 and NKG2D independently induced AA release. The release was maximal when IE-CTLs were stimulated simultaneously by IL-15 and NKG2D. Importantly, AA release was also observed upon engagement of NKG2D by plate-coated monoclonal anti-NKG2D mAb (Fig. 5 E, left) and MIC-expressing EL4 fibroblasts (Fig. 5 E, right).

Altogether, these observations suggest that priming of the NKG2D cytolytic pathway by IL-15 in primary effector CTLs entails up-regulation of cPLA$_2$ expression and activation. Furthermore, IL-15 and NKG2D can induce AA release.

Expression of phosphorylated cPLA$_2$ in IE-CTLs of celiac patients

NKG2D and IL-15 were shown to play a critical role in celiac disease by licensing IE-CTLs to kill enterocytes (6). Furthermore, previous studies in active celiac patients reported that ERK (6) and JNK (38) phosphorylation were induced in IE-CTLs of active celiac patients. Together, these observations suggested that cPLA$_2$ might be activated in IE-CTLs of celiac patients.

In situ analysis of immunohistochemical staining with anti-CD3 antibody in combination with anti–phospho-cPLA$_2$ antibody or control antibody revealed that the frequency of IE-CTL–expressing phospho-cPLA$_2$ was highly increased in celiac patients (n = 6; 79 ± 8%) compared with controls (n = 4; 13 ± 4.6%; P = 0.001) (Fig. 6 B). In addition, to control for evaluation bias inherent to subjective morphological inspection and background staining, automatic image analysis was performed using the ACIS software (see Materials and methods) as previously described (39, 40). Specific cPLA$_2$ staining appears in red when staining is above background threshold values after overlay (Fig. 6 A). Importantly, the

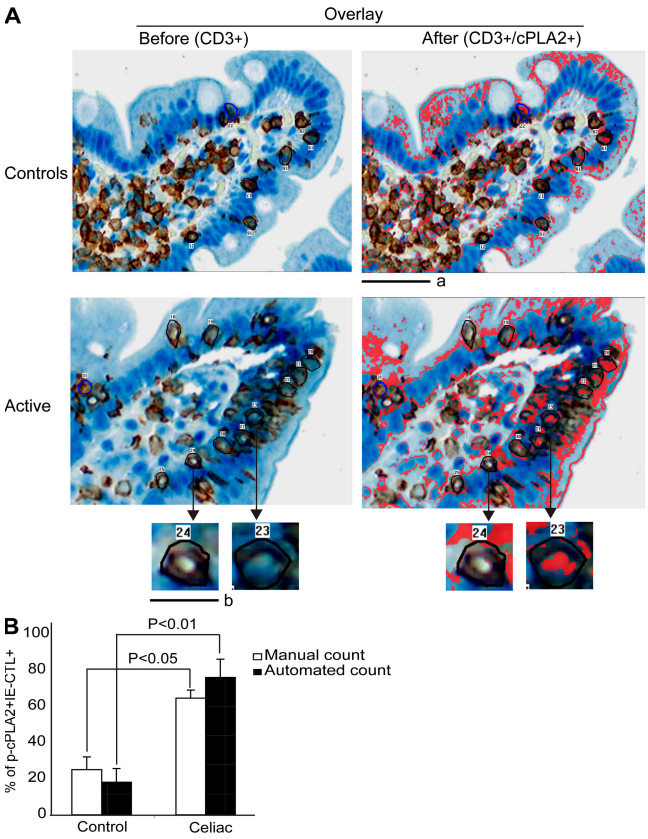

Figure 6. Phospho-cPLA$_2$ expression in IE-CTLs of active celiac patients. (A) Representative examples of double staining immunohistochemistry of CD3 (brown)/phospho-cPLA$_2$ (blue) in duodenal biopsies from patients with active celiac disease and controls (magnification ×200). Intraepithelial lymphocytes were randomly selected from the pool of CD3$^+$ cells and automatically numbered as shown. Expression of phospho-cPLA$_2$ was measured within each outlined CD3$^+$ IE-CTLs. In the left images, before overlay, phospho-cPLA$_2$–positive appears in blue. After overlay, in the right images, specific phospho-cPLA$_2$ staining as determined by threshold values is highlighted in red. (insets) IE-CTL #23 and #24 before and after applying the overlay tool. Note that double-positive IE-CTL #23 falls within the threshold values for phospho-cPLA$_2$–positive staining and thus is covered with solid red in overlaid image. In contrast, IE-CTL #24 falls below the threshold values for phospho-cPLA$_2$–positive staining and thus remains covered with white in overlaid image. Finally, increased phospho-cPLA$_2$–positive epithelial cells (colored in red after overlay) are present in active celiac patients compared with controls. Bars: (a) 40 µm; (b) 10 µm. (B) A summary with mean percentage of phospho-cPLA$_2^+$ IE-CTLs ± SD is presented after manual and automated count. For manual count, four control and six celiac patients were analyzed. For automated count, >100 IE-CTLs in two control and two celiac slides were analyzed. P value was determined with the exact Wilcoxon rank sum test. Note that manual and automated count give similar percentage of phospho-cPLA$_2^+$IE-CTLs.

automatic count confirmed the manual count by showing a similar significant increase in the percentage of $cPLA_2$-positive IE-CTLs (Fig. 6 B). This result is even more striking in regard to the augmentation in the number of IE-CTLs in celiac disease, reaching classically up to 10 times the value of that observed in normal controls (41). The total number of IE-CTL–expressing phospho-$cPLA_2$ in celiac patients can therefore be estimated to be 40–60-fold higher than in controls. This finding is particularly relevant in the context of our observations that $cPLA_2$ plays a critical role in NKG2D cytolytic functions of celiac and IL-15–stimulated IE-CTLs (Figs. 1 and 5).

Furthermore, phospho-$cPLA_2$, similar to phospho-ERK and JNK (6, 38), was also highly up-regulated in enterocytes of celiac patients (Fig. 6 A), especially in the nuclear compartment (not depicted) where accumulation correlates with activation (for review see reference [28]).

Altogether, these results illustrate that $cPLA_2$ is activated in IE-CTLs of active celiac patients and support, in association with other present (Figs. 1 and 5) and past (6) observations, an in vivo role for $cPLA_2$ in celiac disease pathogenesis.

DISCUSSION

Our study establishes an unexpected connection between Il-15– and CTL-mediated immunopathology in celiac disease and inflammatory lipid biology. Our observations also suggest that NKG2D effector functions, particularly cytolysis, are critically regulated by $cPLA_2$ in CTLs. Furthermore, we identify an unexpected role for Vav, JNK, and ERK in NKG2D-mediated cytolysis by showing that they mediate $cPLA_2$ activation. Finally, our study suggests that NKG2D and IL-15 could contribute to tissue inflammation and granulocyte activation by promoting the release of AA in CTLs (Fig. 7).

The molecular basis for exocytosis is a field of high investigation. It involves trafficking of granules to the plasma membrane and membrane fusion events. There is increasing evidence that exocytosis can be triggered, either under conditions of high-level calcium mobilization sufficient to activate snare-mediated membrane fusion (for review see references [42]), or under conditions of low levels of calcium mobilization (43) involving AA and its eicosanoids metabolites, which are known as fusogens (44). This latter mechanism is thought to play a role in neutrophil and neuroendocrine cell degranulation (45, 46). It may be particularly relevant for surface receptors that, similar to NKG2D, signal through an adaptor molecule lacking an ITAM motif (10), and induce relatively low levels of calcium (34, 47) (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20071887/DC1). Pharmacological studies performed in the 1980s (48–52) suggested that inflammatory lipid mediators may play a role in NK cell–mediated lysis in human and rodents without defining the receptors involved (48–52). More recently, $cPLA_2$ was proposed to contribute to NKR-P1A–induced cytolysis in rat NK cells (53). However, recent findings in human indicate that engagement of NKR-P1A by its ligand LLT1 (54, 55) inhibited rather than induced NK cell–mediated lysis (54). Furthermore, the nature of the adaptor molecule associated with NKR-P1A remains undefined. Thus, the role of $cPLA_2$ could not be ascribed to particular cytolytic signaling pathways and to individual NK receptors in human. Our study unambiguously identifies the NKG2D–DAP10 in human CTLs as an immunoreceptor complex that requires $cPLA_2$ activation and AA release as integral components of its cytolytic machinery. In addition, our preliminary data indicate that this finding is not limited to CTLs, because the NKG2D cytolytic pathway in NK cells also involves $cPLA_2$ activation (unpublished data). Interestingly, our data suggest that $cPLA_2$ is not significantly involved in TCR-mediated cytolysis. Future studies will determine whether, more generally, ITAM-signaling receptors mobilizing high levels of calcium mediate granule exocytosis independently of $cPLA_2$. In agreement, NK cell receptors associated with ITAM-bearing adaptor molecules induce high levels of calcium flux (47) and $cPLA_2$-independent cytolysis (56). Importantly, however, even though $cPLA_2$ is not directly involved in TCR-mediated cytolysis, it plays a critical role in the co-stimulation of cytolytic T cell responses. Notably,

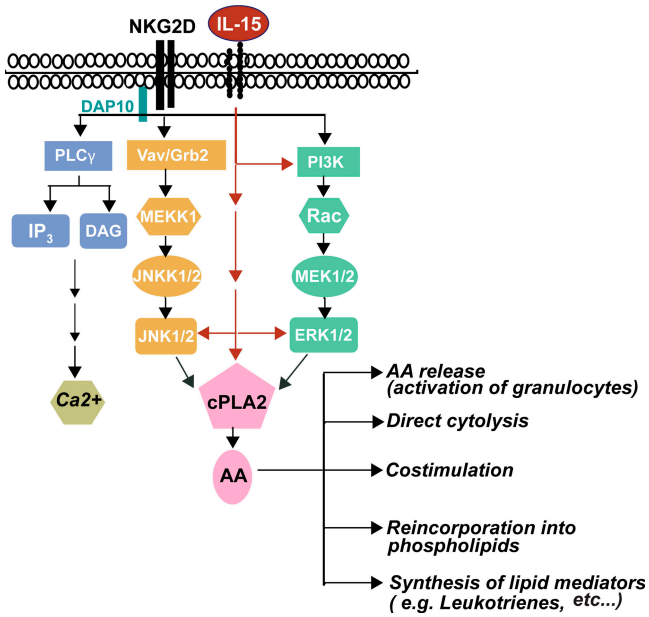

Figure 7. Model of the NKG2D signaling pathway in CTLs. The NKG2D cytolytic signaling pathway involves PLC-γ2, Vav1, and PI3-K activation (60). How these upstream signaling events are interconnected in CTLs remains to be determined. NKG2D-mediated cytolysis requires activation of $cPLA_2$ and AA release, suggesting that the level of calcium mobilization achieved upon NKG2D cross-linking is insufficient to allow membrane fusion events and exocytosis. Cytosolic PLA_2 activation itself is mediated through two independent pathways, Vav-1→JNKK1/2→JNK1/2→$cPLA_2$ and PI3-K→MEK1/2→ERK→$cPLA_2$. It is likely that these pathways also promote granule polarization (62, 63, 67). In addition, $cPLA_2$ activation is also required for NKG2D cytolytic co-stimulatory functions. Interestingly, IL-15, which licenses NKG2D killing in CTLs (6), enhances NKG2D-mediated JNK, ERK and $cPLA_2$ phosphorylation, and AA release. Once released, AA can potentially be reincorporated into phospholipids, mediate the biosynthesis of eicosanoids and induce granulocyte activation.

NKG2D was unsuccessful to induce $cPLA_2$ phosphorylation in resting $CD8^+TCR\alpha\beta^+$ peripheral blood lymphocytes. This finding may explain why NKG2D fails to exert co-stimulatory function in resting noneffector CD8 T cells (57). Together, these observations are in agreement with the general concept, reported by Bryceson et al. (58, 59) that receptors using different adapters and signaling pathways contribute distinctively and synergistically to cytolysis.

Studies had identified PLC-γ2, PI3-K, and Grb2/Vav1 as key upstream signaling molecules of the NKG2D cytolytic pathway (Fig. 7) (33, 60, 61). Our previous analysis of the human NKG2D–DAP10 cytolytic signaling pathway suggested that ERK and JNK, activated through independent pathways, played critical roles in NKG2D- but not TCR-mediated cytolysis (Fig. 7) (6). The role of PI3K in cytolysis is complex and remains incompletely understood; among its potential roles, PI3K is thought to be involved in ERK activation (6, 62, 63). A critical role for Vav in natural killer signaling and cytotoxicity has been reported in mouse and human by several groups (33, 64–68). Vav is thought to play a role similar to that of ERK (62, 63) in cytolysis by promoting granule polarization (67). This study reveals that Vav1 is upstream of JNK in the NKG2D cytolytic pathway (Fig. 7). More importantly, it sheds a new light on the role of the PI3K–ERK and Vav–JNK pathways in NKG2D-mediated cytolysis by showing that ERK and JNK critically regulate $cPLA_2$ activation, which in turn critically regulates NKG2D-mediated degranulation and cytolysis (Fig. 7). Whether AA, which is released upon $cPLA_2$ activation, plays a role in granule exocytosis as fusogen (22, 44, 45) or indirectly through its role in the biosynthesis of eicosanoids remains to be determined. AA serves as a substrate to 5-LO and cyclooxygenase (COX), which drive the leukotriene and prostaglandin synthesis pathways, respectively (28). Interestingly, although NKG2D engagement induces 5-LO and COX activation, only 5-LO is involved in NKG2D-mediated cytolysis (unpublished data). These observations are in agreement with reports suggesting that leukotrienes, but not prostaglandins, play a role in lymphokine-activated killer activity (51, 52) and NK-mediated cytolysis (69, 70).

Convergent observations suggest that IL-15 and NKG2D may coordinately regulate CTL effector functions and mediate organ-specific immunopathology (for review see reference [15]). For instance, the role of NKG2D as a direct mediator of cytolysis was shown in celiac disease (6). Celiac disease is an inflammatory intestinal disorder with an autoimmune component triggered by dietary gluten in genetically susceptible individuals (71, 72). Although the role of gluten-specific, DQ2- or DQ8-restricted CD4 T cells in the lamina propria was accepted early on (73), the role of IE-CTLs was questioned because no gluten-specific IE-CTLs could be identified (for review see reference [71]). Our finding that IL-15 primed NKG2D in CTLs to kill MIC-expressing enterocytes in celiac disease (6, 13) provided an explanation as to how IE-CTLs could cause intestinal damage and malabsorption, despite their inability to recognize gluten. Licensing by IL-15 was shown to involve up-regulation of NKG2D and DAP-10 expression, and activation of the PI3-K→ERK signaling pathway (6). We now show that IL-15 is also involved in the activation of JNK. More importantly, we demonstrate that IL-15–mediated licensing of the NKG2D cytolytic pathway requires $cPLA_2$ activation, and that $cPLA_2$ is highly activated in IE-CTLs of active celiac patients. Interestingly, $cPLA_2$ is concomitantly activated in the intestinal epithelial cells. This activation of $cPLA_2$ may be secondary to the activation of enterocytes by IL-15 (74) and/or AA released by IE-CTLs.

Finally, our study reveals that NKG2D and IL-15 signaling in effector CTLs causes CTLs to release AA and potentially eicosanoids, such as leukotrienes and prostaglandins. Eicosanoid synthesis by CTLs is supported by our findings that NKG2D induces AA production and activates 5-LO and COX (unpublished data). Interestingly, AA and eicosanoids favor granulocyte recruitment and activation (75–79). These findings establish an unrecognized link between CTLs, NKG2D, IL-15, and inflammation, which may have as of yet unrecognized pathological implications. In particular, it may be relevant in immune-mediated diseases, such as rheumatoid arthritis and celiac disease, where NKG2D and IL-15 play a pathogenic role (for review see reference [15]) and where granulocytes are activated (80–84).

Collectively, our findings invite us to reexamine the role of IL-15 and NKG2D in inflammation and autoimmunity, and develop therapeutic strategies aimed at blocking $cPLA_2$ in diseases associated with dysregulated IL-15 expression and NKG2D activation. Future studies will determine whether 5-LO and COX should also be targeted. Our preliminary observations suggest that 5-LO, but not COX, is involved in NKG2D-mediated immunopathology. In addition, in view of observations suggesting that prostaglandins inhibit NK cell cytolytic responses (85, 86), blocking COX activation may even be deleterious.

MATERIALS AND METHODS

Human subjects. For immunohistochemical studies, six adult patients with active celiac disease and four control aged-matched individuals undergoing biopsies for functional intestinal disorders of nonceliac origin were studied. Diagnosis of celiac disease was based on detection of antitransglutaminase antibodies, the presence of HLA DQ2 or DQ8, villous atrophy, and clinical and histological response to a gluten-free diet. In addition, IE-CTLs were isolated from surgical specimens of individuals undergoing gastric bypass for morbid obesity, as previously described (6). All subjects gave written informed consent and research was approved by the University of Chicago Institutional Review board.

CTL isolation and cell culture. IE-CTLs were purified from jejunal biopsies and surgical specimens, as previously described (31). Intraepithelial and peripheral blood $NKG2D^+TCR^+CD8^+$ CTL lines and clones were obtained and cultured as previously described (31). PB-CTL clone #348 and #414 (cultured with 5 U/ml of IL-2) in which NKG2D exerts co-stimulatory cytolytic functions but cannot induce direct cytolysis were obtained and cultured, as previously described (1, 8). Resting peripheral blood $CD8^+TCR\alpha\beta^+$ T lymphocytes were purified and an effector CTL line was generated according to a previously described protocol (6, 31). In brief, peripheral blood lymphocytes were isolated from whole blood of healthy volunteers after Ficoll density gradient centrifugation (GE Healthcare). Cells were stained with anti-CD8β APC-conjugated mAb (BD) and purified by AutoMACS (Miltenyi

Biotec) using anti-APC magnetic beads (Miltenyi Biotec). Purity (>95%) of isolation was confirmed by flow cytometry. Purified $CD8^+TCR\alpha\beta^+$ T cells were divided into two parts. One part was directly used for $cPLA_2$ signal transduction experiments. The other part was used to generate a short-term effector CTL line by allogenic stimulation, as previously described (31), to investigate whether the effector status impacted on the ability of NKG2D to induce $cPLA_2$ phosphorylation.

MICA-transfected (C1R-MIC) or control vector–transfected (C1R-Neo) C1R cells and $CD8^+TCR\alpha\beta^+$ cytotoxic leukemia TALL-104 cell line (American Type Culture Collection) were cultured, as previously described (6). EL4 (American Type Culture Collection TIB-39) are a murine T lymphoma cell line. MICA-transfected EL4 (EL4-MICA) and control EL4 cells were grown in RPMI 1640 supplemented with 10% FCS, glutamine, antibiotics, and G 418.

Reagents, antibodies, and recombinant cytokines. $cPLA_2$ inhibitor AACOCF3, MEK1/2 inhibitor PD98059, and JNK inhibitor SP600125 were obtained from Calbiochem. AA and LA were obtained from Sigma-Aldrich. Anti-CD3 (clone UCHT1, IgG1) and anti-NKG2D mAbs (clone 1D11, IgG1) and isotype-matched control IgG1 were purchased from BD; PE-conjugated anti-NKG2D 1D11 was purchased from eBioscience; and anti-Vav1, -$cPLA_2$, -ERK, -JNK-phospho-$cPLA_2$, -phospho-JNKK1, -phospho-PLCγ2, -phospho-ERK, and -phospho-JNK antibodies were purchased from Cell Signaling Technology. Antiphosphotyrosine mAb 4G10 was obtained from Millipore. Antiactin mAb was purchased from Sigma-Aldrich. Anti–5-LO mAb was obtained from Fitzgerald Industries Int. $F(ab')_2$ goat anti–mouse antibodies were obtained from Jackson ImmunoResearch Laboratories. Human IL-15 and IL-2 were purchased from BD.

Plasmids, siRNA, and transfection. Dominant-negative mutant Vav1 plasmid (pcDNA3.flag.h.Vav1-C) that contains only the C-terminal portion of Vav1 was a gift from P. Leibson and D. Billadeau (Mayo Clinic College of Medicine, Rochester, MN,) (33). Dominant-negative MEK1(S218a, S222a, MEK[2A]), and constitutively activated MEK1(MEK[2E]) expression plasmid were gifts from M.R. Rosner (University of Chicago, Chicago, IL) (36, 37). Expression vectors encoding JNKK2-JNK1 (which encodes a constitutively active form of JNK), JNKK2(K149M)-JNK1 (a kinase-deficient mutant), and JNKK2(KM) have been previously described (35). $cPLA_2$, 5-LO, and control synthesized siRNA oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. TALL-104 CTL cells were electroporated with the Amaxa Nucleofector (Amaxa, Inc.), using Amaxa Cell Line nucleofection solution V and program T-20. Cells were cultured for 24–72 h before being used for experiments. For overexpression experiments involving HA-tagged JNKK2-JNK1, HA-tagged JNKK2(KM)-JNK1, or MEK(2E), TALL-104 CTLs were cotransfected with the indicated plasmids and GFP plasmid at a 5:1 ratio. 40 h after transfection, cells were sorted by flow cytometry into GFP^+ and GFP^- cells with FACSAria (BD) before being analyzed for p-$cPLA_2$, p-JNK, and p-ERK expressions by Western blot. For siRNA experiments, 4×10^6 cells were transfected with 120 pmol siRNA and 30 pmol GFP plasmid. On average, 40% of cells were transfected based on GFP expression. Efficiency and specificity of protein knock-down was assessed by Western blot with the appropriate antibodies.

Cell signaling. To look at $cPLA_2$, JNK, and ERK phosphorylation, cells were serum-starved for 30 h. To test the effects of kinase inhibitors, cells were preincubated for 30 min at 37°C with the indicated inhibitors before stimulation. To cross-link immunoreceptors, cells were incubated for 4 min at 4°C with the indicated monoclonal antibody before adding $F(ab')_2$ GAM for the indicated duration at 37°C. Cells were lysed for 20 min in ice-cold lysis buffer containing fresh protease and phosphatase inhibitors (50 mM Tris-Hcl [pH 7.5]; 150 mM NaCl; 1% Triton-X100; 1 mM EDTA; 1 mM Na_3VO_4; 1 mM NaF; and protease inhibitor cocktail tablets). Cellular debris was removed by centrifugation at 15,000 rpm for 20 min at 4°C. Total lysates were subjected to SDS-PAGE electrophoresis and transferred to PVDF membranes (Bio-Rad Laboratories). Proteins were then detected by using the indicated antibodies, followed by HRP-conjugated goat anti–mouse (HRP-GAM) or donkey anti–rabbit (HRP-DAR) antibodies (Jackson Immunoresearch Laboratories) using the enhanced chemiluminescence (ECL) kit from GE Healthcare. Tyrosine-phosphorylated Vav1 was revealed by antiphosphotyrosine (4G10) antibody after immunoprecipitation of the cell lysates with rabbit polyclonal anti-Vav1 antibody.

Cytotoxicity assay. Chromium release assays were performed as previously described using P815 cells (a $Fc\gamma^+$ mouse mastocytoma; American Type Culture Collection), C1R-MICA1, EL4-MICA1, control C1R transfectants, and control EL4 cells at the indicated E:T ratio in triplicate wells (6). For Fc-dependent redirected cytotoxicity, effectors and targets were incubated in the presence of soluble anti-NKG2D or anti-CD3 mAbs at the indicated concentration. Chromium release was measured using a scintillation counter (Packard). Maximum release was determined by the addition of detergent (10% SDS) and spontaneous release 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). When indicated, effector cells were treated for 30 min before and during the cytotoxic assay with different inhibitors, lipid mediators, or equivalent concentrations of the DMSO or ethanol vehicles.

AA release assay. 10^6 cells/ml IE-CTLs, PB-CTLs, and TALL-104 CTLs were labeled with 0.2 μCi ^3H AA (specific activity 62.5 Ci/mmol; Perkin-Elmer) in RPMI 1640 or IMDM with 0.2% fetal bovine serum at 37°C 5% CO_2 for 1 or 8 h. After labeling, cells were washed at least three times to remove free ^3H AA and, when indicated, pretreated with inhibitors or vehicles for 30 min in medium with 0.2% bovine serum albumin (BSA). Cells were then incubated with anti-NKG2D, anti-CD3 mAbs, or mouse IgG1 control at 37°C for 4 min followed by $F(ab')_2$ goat anti–mouse IgG for 1 h in medium with 0.2% BSA. Supernatants and cell pellets were separately collected by centrifugation. ^3H AA was measured with a scintillation counter. Percentage of ^3H AA release was calculated as supernatant ^3H/(supernatant ^3H + pellet ^3H) × 100.

Flow cytometric analysis. For surface staining, cells were incubated with fluorochrome-conjugated antibodies according to standard protocols. Fluorescence was analyzed on a six-color FACSCanto (Becton Dickinson) with quadrants set to score as negative >99% of control Ig-stained cells.

Measurement of granule release by BLT esterase assay. Granule release was evaluated as previously described (6). In brief, CTLs were suspended in RPMI medium containing 2% FCS, incubated in 96-well with 4 μg/100 μl of anti-NKG2D mAb or isotype control for 4 min, and then stimulated for 4 h with goat anti–mouse IgG $F(ab')_2$ at 37°C. Maximum granule release was determined using 1% Triton X-100. The supernatants were evaluated for esterase secretion using a standard N-benzyloxycarbonyl lysine thiobenzyl ester (BLT; Calbiochem). The percentage of BLT esterase activity was calculated using the following equation: (experimental BLT esterase release − spontaneous BLT esterase release)/(maximum BLT esterase release − spontaneous BLT esterase release) × 100.

$cPLA_2$ enzyme activity assay. $cPLA_2$ activity was assessed in lysates of primary IE-CTL, PB, and IE-CTL lines according to the manufacturer's procedure (Cayman Chemical), as reported (87), using arachidonoyl thio-PC as a synthetic substrate. In brief, 4×10^6 cells were stimulated with anti-NKG2D or isotype control, and the pellets were lysed by three cycles of freeze thawing in lysis buffer (50 mM Hepes, pH 7.4, containing 1 mM EDTA, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 2 mM Na_3VO_4, 2.5 mM NaF, and 1 mM DTT). 10 μl sample supernatant and 5 μl assay buffer were well mixed and the reaction was initiated by adding 200 μl substrate solution to the wells. After 1 h incubation at room temperature, 10 μl of DTNB/EGTA was added to each well to stop the reaction. Absorbance was measured at 414 nm. $cPLA_2$ activity was expressed as the percentage of

nonstimulated control (absorbance of stimulated cells/absorbance of nonstimulated cells) × 100.

Immunohistochemistry and image analysis. Immunohistochemical double staining for CD3 and phospho-cPLA$_2$ was performed on 4 μM 10% formalin-fixed paraffin sections using the double staining blocking kit (DAKO). Monoclonal anti-CD3 antibody was used at 1:200 dilution (BioGenex), and rabbit polyclonal anti–p-cPLA$_2$ and isotype antibody were used at 1:50 dilution.

To control for evaluation bias inherent to subjective morphological inspection and background staining, automatic image analysis with the pixel and cell/object-based image analysis was performed using ACIS software from Clarient, as previously described (39, 40). For each slide, 5–6 areas were scanned at 200× magnification to yield high-resolution digital images. The ACIS software applies the color-specific thresholds configured by an experienced user-pathologist (MT) to differentiate specific blue phospho-cPLA$_2$ staining within lymphocytes and epithelial cells from the surrounding blue background staining. The same threshold values for specific blue staining were applied to both control and active celiac biopsy sections. The overlay tool was used to highlight phospho-cPLA$_2$–positive areas (defined by blue staining above background) in red color within the CD3$^+$ (brown) intraepithelial lymphocyte population (Fig. 6). From each digital image, at least 100 IE-CTLs were selected and numbered for analysis of phospho-cPLA$_2$ staining. All cells were classified as either double positive or only CD3 positive (phospho-cPLA$_2$ negative). Results obtained by software analysis were compared with cell counts obtained by manual morphological inspection.

Statistical analysis. Mixed effects models were constructed for all bar graphs in the figures. The treatment variable was included as a fixed predictor. Experimental plate was included as a random predictor in the model. The outcome variable (specific lysis) was log transformed after checking normality of the residuals from the mixed model. Tukey-adjusted pairwise comparisons are reported unless otherwise noted. The main effect of treatment was statistically significant for all conditions tested ($P < 0.001$).

For analysis of immunohistochemical data, celiac cases were compared with controls using exact Wilcoxon rank sum tests.

Online supplemental material. Fig. S1 shows dose-dependent inhibition by AACOCF3 (CF3) and rescue by AA of NKG2D-mediated cytolysis. Fig. S2 shows the effect of cPLA2 inhibition by AACOCF3 (CF3) on granzyme and perforin expression in IE- and PB-CTLs. Fig. S3 shows that NKG2D stimulation induces perinuclear cPLA2 translocation and cPLA2 activity in IE- and PB-CTLs in a time-dependent manner. Fig. S4 shows that NKG2D and IL-15 induce cPLA2 phosphorylation in effector, but not resting, peripheral blood CTL. Fig. S5 shows that NKG2D induces notably lower levels of calcium release than the TCR in CTLs. A supplemental materials and methods is also provided. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071887/DC1.

This paper is dedicated to the memory of Paul J. Leibson who pioneered the field of NK cell signaling. His work, in particular on NKG2D, has been a source of inspiration.

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