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Phosphatidylinositol 3-Kinase-Dependent and -Independent Cytolytic Effector Functions

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Two distinct forms of short-term cytolysis have been described for CD8+ CTLs, the perforin/granzyme- and Fas ligand/Fas (CD95 ligand (CD95L)/CD95)-mediated pathways. However, the difference in signal transduction events leading to these cytolytic mechanisms remains unclear. We used wortmannin, an irreversible antagonist of phosphatidylinositol 3-kinase (PI3-K) activity, to investigate the role of PI3-K in influenza-specific CD8+ CTL cytolytic effector function. We found that the addition of wortmannin at concentrations as low as 1 nM significantly inhibited both the Ag/MHC-induced cytolysis of CD95+ target cells and serine esterase release. In strong contrast, W did not inhibit the Ag/MHC-induced CD95L expression or the CD95L/CD95-mediated cytolysis of CD95+ targets. A combination of wortmannin and blocking mAb against CD95L inhibited the cytolysis of CD95+ targets, indicating that the wortmannin-independent cytolysis was due to CD95L/CD95 mediated cytolysis. These findings suggest a differential role for PI3-K in mediating cytolysis and, thus far, the earliest difference between perforin/granzyme- and CD95L/CD95-dependent cytolysis. Our data reinforce the idea of a TCR with modular signal transduction pathways that can be triggered or inhibited selectively, resulting in differential effector function.

Cytotoxic T lymphocytes exert short-term, cell-mediated cytolysis via two distinct effector mechanisms, perforin/granzyme exocytosis and CD95 ligand (CD95L)/CD95-mediated cytolysis (1–3). Perforin-mediated cytolysis involves the exocytosis of preformed granules containing granzymes and perforin into the target cell leading to target cell apoptosis (4, 5). Fas ligand (FasL)/Fas-mediated cytolysis relies on the transcription and translation of the fasL gene (1, 6) and the resulting surface expression of FasL to engage the Fas expressed on the target to cause apoptosis. As more is understood about the signal transduction events leading to perforin/granzyme and CD95L/CD95 cytolysis, it has become increasingly clear that these two mechanisms may not simply be two outcomes of the same signal. Instead, the emerging picture is one in which different stimuli may preferentially induce one or both cytolytic mechanisms. Previous reports from our laboratory and others have suggested differential activation requirements for the induction of these two cytolytic effector functions following stimulation through the TCR (1–3, 7). We have shown that stimulation through the TCR with different stimuli (i.e., altered peptide ligands (8) or superantigens (9)) may selectively trigger one cytolytic mechanism. All of these data suggest that two distinct, although not necessarily exclusive, signal transduction pathways lead to perforin/granzyme- or CD95L/CD95-mediated cytolysis.

Phosphatidylinositol 3-kinase (PI3-K), a phospholipid-modifying enzyme that phosphorylates phosphoinositols at the 3′ position of the inositol ring, has been implicated as a key player in the mediation of signals transduced through the TCR, CD28, and the IL-2R (10–12). Stimulation of the TCR and activation of Lck allow the association of PI3-K with the newly phosphorylated CD3 ζ-chain (13–15), suggesting a potential for PI3-K to serve as an upstream regulator of several T cell activation events. The significance of PI3-K in CD8+ TCR activation and cytolytic effector functions (16) has not been fully addressed with regard to the perforin/granzyme and CD95L/CD95/CD95 pathways. Therefore, we investigated the role of PI3-K in the activation of cytolytic effector functions. Here, we report the effect of wortmannin, an irreversible inhibitor of PI3-K (17), on influenza-specific CD8+ murine CTL clones, and thus the role of PI3-K in Ag/MHC-induced cytolysis.

Materials and Methods

Cells

CD8+ CTL clones were maintained as described previously (7, 9). Briefly, clones were stimulated weekly with A/JAP/57 irradiated, infected, syngeneic splenocytes from BALB/c mice in the presence of 20 U/ml human rIL-2 (Chiron Pharmaceuticals, Emeryville, CA), 10% FCS, 2 mM glutamine, and 50 mM 2-ME in Iscove’s complete media (Life Technologies, Gaithersburg, MD). CTLs were used 4–7 days poststimulation and were washed before use in all assays. A20.2J CD95+ cells and A20.FO CD95− cells (an anti-CD95-resistant variant of A20.2J) (obtained from Dr. M. Sitkovsky, National Institutes of Health, Bethesda, MD) were used as target cells and APCs. Targets were maintained in culture in RPMI 1640 (Life Technologies) supplemented with 10% FCS, 2 mM glutamine, 1% nonessential amino acids, and 0.5% sodium pyruvate.

Cytotoxicity

The DNA fragmentation assay (18) was used for the analysis of perforin- and CD95L/CD95-mediated cytolysis. Targets were incubated overnight at 37°C with [3H]TdR, washed two times, and either mock-treated or sensitized with the influenza peptides hemagglutinin (HA)204-212 for CTL clone 11-1 or nucleoprotein (NP)147-155 for CTL clone 14-13 at a final concentration of 0.01 µg/ml or 0.1 µg/ml, respectively. CTLs were pre-treated for 30 min with 0.01–100 nM of wortmannin (Sigma, St. Louis, MO) at 37°C, where indicated. The anti-CD95L mAb MFL3 (19) (PharMingen, San Diego, CA) was added to inhibit the CD95L-CD95 interaction as described.

References

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3. Abbreviations used in this paper: CD95L, CD95 ligand; FasL, Fas ligand; PI3-K, phosphatidylinositol 3-kinase; HA, hemagglutinin; NP, nucleoprotein.
Diego, CA) was added to CTLs in the presence or absence of wortmannin and concurrently allowed to preincubate for 30 min. Targets were plated at 10^6 cells/well with CTLs at an E:T ratio of 5:1, unless otherwise indicated. Assays were conducted in quadruplicate as described previously (9). The percentage of DNA loss was calculated as follows: % DNA loss = 100% × (cpm retained without CTLs − cpm retained with CTLs)/[cpm retained without CTLs], where the cpm retained without CTLs was never <90% of the target cpm at t = 0.

Serine esterase release assay

Serine esterase assays were conducted as described previously (9). CTLs were pretreated for 30 min with 0.0–100 nM of wortmannin at 37°C and subsequently plated at 5 × 10^6 CTLs/well with target cells at an E:T ratio of 5:1. Targets were mock-stimulated with media or stimulated with influenza peptide Ag (HA204-212 for CTL clone 11-1 or NP147-155 for CTL clone 14-13 at a final concentration of 0.01 μg/ml or 0.1 μg/ml, respectively). Spontaneous and total release controls were as described previously (9). Granzyme A activity was determined as described previously (7, 9). Means and SDs of quadruplicates are shown. The percentage of serine esterase release was calculated as follows: % serine esterase release = 100% × ([experimental release − spontaneous release]/[total release − spontaneous release]).

Flow cytometry

CTLs were separated from splenocytes and dead cells by centrifugation over an Isopaque-Ficoll gradient. Subsequently, 5–7 × 10^6 cells/well were added to a 96-well plate in media containing 1–2.5% FCS. CTL clones were stimulated at an E:T ratio of 5:1 with the influenza peptide Ag (HA204-212 for CTL clone 11-1 or NP147-155 for CTL clone 14-13 at a final concentration of 0.01 μg/ml or 0.1 μg/ml, respectively) presented by the target cells. After 4–5 h of incubation at 37°C in a CO2 incubator, CTLs were stained on ice at 1:100 with a directly conjugated anti-CD3e (145.2C11) or with anti-CD95L (MFL3) (19) (PharMingen). Anti-CD95L mAb was used with a goat F(ab′)2 anti-hamster-FITC-conjugated secondary Ab (Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

Results

Role of PI3-K in Ag-induced cytolysis

The A/JAP/57 influenza-specific CTL clones 11-1 and 14-13 are well characterized with respect to their recognition of influenza Ag (20, 21). Both clones are capable of cell-mediated cytolysis via perforin and CD95L/CD95 pathways when induced by Ag/MHC. To specifically investigate the effect of wortmannin on perforin/granzyme cytolysis, we first performed cytolytic assays in which we tested various concentrations of wortmannin using CD95^+ target cells (Fig. 1A). Wortmannin concentrations of 0.001–100 nM (Fig. 1A and data not shown) revealed that even for concentrations as low as 1 nM, there was a substantial decrease in Ag/MHC-induced cytolysis for both clones. Although concentrations of >1 nM did increase the inhibition observed, we chose to use 1 nM (the ~50% inhibitory concentration in Fig. 1A) in subsequent experiments because of the high specificity of wortmannin for PI3-K in this concentration range (17, 22–26). Fig. 1B shows assays in which the cytolytic activity of both clones was monitored at various E:T ratios in the presence of 1 nM of wortmannin. Even at the higher E:T ratio, a significant inhibition of perforin/granzyme cytolysis for both clones was observed.

To further document the effect of wortmannin on perforin/granzyme granule exocytosis, we investigated the effect of wortmannin on serine esterase release (Fig. 2). Results for serine esterase release in the presence of various concentrations of wortmannin mirrored what was observed in cytolytic assays (Fig. 1), where exocytosis was greatly inhibited for similar concentrations of wortmannin. These data, along with the results seen in Fig. 1, suggest that perforin/granzyme-mediated cytolysis is wortmannin-sensitive and therefore likely to be PI3-K-dependent.

![FIGURE 1. Wortmannin inhibition of perforin/granzyme cytolysis of CD95^+ targets. A, Dose response for CTL clones 11-1 (■) and 14-13 (●) and cytolysis of CD95^+ targets at an E:T ratio of 5:1. B, Effect of a variable E:T ratio and wortmannin (1.0 nM) pretreatment on cytolysis. Open symbols (○, 11-1; □, 14-13) represent untreated CTL clones; filled symbols (■, 11-1; ●, 14-13) represent wortmannin-pretreated CTL clones. Means and SDs of quadruplicate cultures are shown.](http://www.jimmunol.org/)

Effect of PI3-K on CD95L/CD95-mediated cytolysis

We subsequently investigated the effect of wortmannin on CD95L/CD95-mediated cytolysis. In contrast to the inhibition seen for perforin-mediated cytolysis, cytolytic assays consistently revealed very little or no effect of wortmannin on Ag/MHC-induced cytolysis by either CD8^+ CTL clone on CD95^+ targets. Fig. 3 represents dose response assays in which concentrations of ≤100 nM of wortmannin failed to inhibit CD95L/CD95-mediated cytolysis. Concordantly, flow cytometric analysis revealed a lack of inhibition by wortmannin on Ag/MHC-induced CD95L expression (Fig. 4).

Next, we investigated whether CD95L/CD95-mediated cytolysis was solely responsible for the cytolysis of CD95^+ targets in the presence of wortmannin. We reasoned that treating with wortmannin in combination with an anti-CD95L mAb should significantly

![FIGURE 2. Wortmannin inhibition of serine esterase release by CTL clones. Clones (■, 11-1; ●, 14-13) were pretreated for 30 min with the indicated dose of wortmannin and plated with CD95^+ target cells for 5.5 h. Serine esterase release was determined by colorimetric assay. Means and SDs of quadruplicates are shown.](http://www.jimmunol.org/)
affect the cytolysis of CD95\(^+\) targets if the wortmannin-insensitive cytolysis observed previously (Fig. 3) was attributable to CD95L/CD95 cytolysis. Consistent with previous data, wortmannin alone inhibited the cytolysis of CD95\(^+\) targets (Fig. 5, A and B) but not CD95\(^-\) targets (Fig. 5, C and D). As expected, the anti-CD95L mAb had no effect on CD95\(^+\) or CD95\(^-\) target cytolysis when used alone (Fig. 5, A–D), as perforin cytolysis was still intact. This is also seen when cytolysis is measured by the \(^{51}\)Cr release assay (Ref. 7 and our unpublished data). Again, as expected, when used in conjunction with wortmannin on CD95\(^+\) targets (Fig. 5, A and B), anti-CD95L mAb caused no further diminution of cytolysis. In sharp contrast, wortmannin and the anti-CD95L mAb together greatly reduced cytolysis to levels close to background for CD95\(^-\) targets (Fig. 5, C and D). This finding confirmed that CD95L was responsible for the remaining cytolytic effector function seen in the presence of wortmannin. Thus, although wortmannin strongly inhibits the perforin/granzyme-mediated lysis of target cells, it does not inhibit CD95L up-regulation or subsequent CD95L/CD95-mediated cytolysis.

**Discussion**

PI3-K has been shown to be recruited to the cell membrane during the initial signaling events of TCR/CD3 and CD28 stimulation.

Several groups have reported PI3-K binding to the TCR, IL-2R, and CD4, among other cell surface molecules (10). This observation, along with evidence of phosphorylation by the Src family tyrosine kinase Lck (27) and association with TCR \(\zeta\) and another Src family member, Fyn, imply a role for PI3-K in the very earliest of signal transduction events. Because of the strong specificity of wortmannin for PI3-K at the concentrations used here, we believe that PI3-K activity is the likely target of wortmannin inhibition. Therefore, the data suggest that the differential activation of the perforin/granzyme and CD95L/CD95 mechanisms of cytolysis is already present at this early point in signal transduction from the TCR.

Although the precise role of PI3-K in perforin/granzyme cytolysis in CD8\(^+\) CTLs is still unclear, there are several strong possibilities. PI3-K functions upstream of several proteins (Rho family GTP-binding proteins) that are believed to be responsible for cytoskeletal rearrangement and polarization (11, 28), both of which are essential for perforin/granzyme cytolysis. Recent reports for NK and mast cells (29–31) have found that the effect of wortmannin on cytolysis in these cells is due to the inhibition of granule exocytosis. The inhibition of perforin granule release by wortmannin (Fig. 2), and thus perforin-mediated cytolysis (Figs. 1 and 5), could be the result of an inhibition of the cytoskeletal rearrangement and/or polarization of the granules. Another likely explanation is that wortmannin prevents docked perforin granules from being released. Work in our lab is currently underway to address these possibilities.

Our laboratory has reported a qualitative difference in the intracellular calcium mobilization requirements that signal perforin- and CD95L/CD95-mediated cytolysis (7, 9, 20). Although the Ca\(^{2+}\) mobilization events required for perforin cytolysis occur...
within seconds of TCR stimulation (32), they are the result of a cascade of upstream signaling events. As stated earlier, PI3-K is implicated in the signaling events that transpire shortly after TCR stimulation. Because \( \text{Ca}^{2+} \) mobilization is known to be downstream of these early events, such as \( \zeta \)-chain phosphorylation and ZAP-70 recruitment, there is a strong possibility that PI3-K is actually upstream of \( \text{Ca}^{2+} \), and that its activity may be involved in the mobilization of intracellular \( \text{Ca}^{2+} \). Therefore, the wortmannin-mediated inhibition of perforin release could be due to impaired calcium mobilization during Ag/MHC recognition by the CTLs.

In summary, we report that wortmannin specifically inhibits perforin/granzyme-mediated cytolysis and granule exocytosis. In contrast, neither induction of CD95L expression nor CD95L/CD95-mediated cytolysis is impaired by wortmannin concentrations of \( \leq 100 \) times that required to significantly inhibit perforin/granzyme cytolysis. To date, this is the earliest signal transduction difference between the perforin and CD95L/CD95 cytolytic mechanisms.

References