Dual Regulation by Apurinic/Apyrimidinic Endonuclease-1 Inhibits Gastric Epithelial Cell Apoptosis during Helicobacter pylori Infection

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Abstract

Human apurinic/apyrimidinic endonuclease-1 (APE-1), a key enzyme involved in repair of oxidative DNA base damage, is an important transcriptional coregulator. We previously reported that Helicobacter pylori infection induces apoptosis and increases APE-1 expression in human gastric epithelial cells (GEC). Although both the DNA repair activity and the acetylation-mediated transcriptional regulation of APE-1 are required to prevent cell death, the mechanisms of APE-1-mediated inhibition of infection-induced apoptosis are unclear. Here, we show that short hairpin RNA–mediated stable suppression of APE-1 results in increased apoptosis in GEC after H. pylori infection. We show that programmed cell death involves both the caspase-9–mediated mitochondrial pathway and the caspase-8–dependent extrinsic pathway by measuring different markers for both the pathways. Overexpression of wild-type APE-1 in APE-1–suppressed GEC reduced apoptosis after infection; however, overexpression of the DNA repair mutant or the nonacetylable mutant of APE-1 alone was unable to reduce apoptosis, suggesting that both DNA repair and acetylation functions of APE-1 modulate programmed cell death. We show for the first time that the DNA repair activity of APE-1 inhibits the mitochondrial pathway, whereas the acetylation function inhibits the extrinsic pathway during H. pylori infection. Thus, our findings establish that the two different functions of APE-1 differentially regulate the intrinsic and the extrinsic pathway of H. pylori–mediated GEC apoptosis. As proapoptotic and antiapoptotic mechanisms determine the development and progression of gastritis, gastric ulceration, and gastric cancer, this dual regulatory role of APE-1 represents one of the important molecular strategies by H. pylori to sustain chronic infection. Cancer Res; 70(7); 2799–808. ©2010 AACR.

Introduction

Helicobacter pylori infects half of the world’s population and is a causative agent of gastritis, peptic ulcer, gastric cancer, and lymphoma (1, 2). The host response to H. pylori provides an environment in which epithelial cells may be damaged by mediators of inflammation, including cytokines (3), proteases, and reactive oxygen and nitrogen species (4, 5). Infection with H. pylori results in apoptosis of gastric epithelial cells (GEC) due to multiple mechanisms, including the Fas/FasL system (6), MHC class II (7), the mitochondrial pathway (8), as well as the p53 protein family (9).

Apoptosis is executed by the activation of caspases that act as effector molecules (10). Caspase activation is initiated at different points including tumor necrosis factor receptor superfamily members such as Fas/CD95 (representing the extrinsic pathway) or at the mitochondrial level (the intrinsic pathway; ref. 11). Both the intrinsic (8, 12) and the extrinsic apoptotic pathways (13) are important in H. pylori–induced GEC death.

Mammalian apurinic/apyrimidinic endonuclease-1 (APE-1) is a multifunctional protein that regulates apoptosis and is induced by reactive oxygen species (ROS; refs. 14, 15). It plays a central role in the base excision repair pathway to correct DNA damaged by ROS and alkylating agents (16, 17). We have shown that APE-1 expression is increased in the gastric mucosa during H. pylori infection (18) and that APE-1 controls infection-mediated chemokine expression in GEC (19). Another distinct transcriptional regulatory role of APE-1 is mediated by the NH2-terminal Lys6/Lys7 acetylation of APE-1, which represses certain promoters (20, 21). Recently, we established that acetylated APE-1 represses bax expression by binding to the negative calcium response element present in the promoter of this gene (22) and inhibits H. pylori–mediated GEC apoptosis.

APE-1 downregulation inhibits cell proliferation and activates apoptosis in cell lines of diverse origin (23, 24). It has been shown that both the repair activity and the acetylation-mediated transcriptional regulatory functions of APE-1 are required to prevent apoptosis (25). However, how the diverse functional properties of APE-1 differentially regulate apoptosis, including the intrinsic and extrinsic apoptotic pathways,
has not been examined. Because H. pylori infection regulates apoptosis via both pathways, we sought to define the role of the different functional regions of APE-1 in controlling apoptosis. Using gastric epithelial AGS cells with stably downregulated APE-1, we observed increased apoptosis after H. pylori infection. APE-1 suppressed apoptosis through its effects on both the mitochondrial pathway and the extrinsic pathway. Furthermore, we found that the DNA repair activity of APE-1 regulated the intrinsic pathway, whereas the acetylation function regulated the extrinsic pathway, thereby showing that APE-1 has distinct functions that differentially inhibit apoptosis during H. pylori infection, which may affect the development of gastric cancer and other clinical consequences of infection.

Materials and Methods

Cell culture and bacterial strains. AGS cell line is a human gastric adenocarcinoma line obtained from the American Type Culture Collection (ATCC). Empty vector (pSIREN), APE-1 short hairpin RNA (shRNA)—expressing cells, or non-transfected AGS cells were harvested and cultured as previously described (22). The effectiveness of shRNA suppression was periodically tested, and on average, a 60% reduction in APE-1 protein level was observed in shRNA cells compared with AGS and pSIREN cells. H. pylori 26695, a cag PAI (+) strain (ATCC), was maintained on blood agar plates (Becton Dickinson). Bacteria were cultured overnight at 37°C in Brucella broth (Life Technologies) with 10% fetal bovine serum (Life Technologies). Bacteria were washed, and clarified at 13,000 × g for 10 min at 4°C. Cell lysates were measured by fluorescence (excitation, 400 nm/emission, 505 nm) using a Gemini plate reader.

Transfection. AGS, pSIREN, or shRNA cells (1 × 10⁶) were seeded into 6-well plates 18 to 24 h before transfection. For overexpression studies, shRNA cells were transfected with 2 μg of plasmid DNA and 6 μL of Lipofectamine 2000 reagent (Invitrogen) as per manufacturer’s protocol. Cells were infected with H. pylori 40 h after transfection.

Immunoprecipitation. Cells (5 × 10⁶) were plated in 10-cm plates. Following infection, cells were washed with PBS, lysed with 250 μL of RIPA buffer [150 mmol/L NaCl, 50 mmol/L Tris-Cl (pH 7.4), 1% NP-40, 0.1% sodium deoxycholate, 1 mmol/L EDTA with protease inhibitor cocktail] on ice for 30 min. Lysates of two wells treated identically were pooled and clarified at 13,000 × g for 10 min at 4°C. Cell lysates (500 μg) were incubated with either 7.5 μL of p300 antibody or 7.5 μL of Fas antibody (Santa Cruz Biotechnology) at 4°C overnight. Fifteen microliters of 50% A/G plus agarose (Santa Cruz Biotechnology) were added and incubated for additional 3 h. The agarose-bound immunocomplex was washed thrice with the same lysis buffer, boiled, and resolved by SDS-PAGE followed by Western blot to detect associated proteins.

Mitochondrial membrane potential assay. JC-1 is a cationic dye that accumulates in negatively charged mitochondria of healthy cells in a membrane potential–dependent manner, where it forms aggregates and fluoresces red. In apoptotic cells where the mitochondrial membrane potential (MMP) has collapsed, JC-1 exists only as monomers throughout the cell and fluoresces green. Using a JC-1 MMP detection kit (JC 100, Cell Signaling Technology), cells were analyzed by flow cytometry and the percentage of cells that shifted to green (FL-2) was calculated for control and infected groups according to the manufacturer’s protocol.

Statistical analysis. Results are expressed as the mean ± SEM. Results were compared using two-tailed Student’s t test and considered significant if P < 0.05.

Results

APE-1 inhibits H. pylori–mediated apoptosis. To determine the effect of the level of APE-1 on infection-induced apoptosis in GECs, we used AGS, pSIREN, and shRNA cells as described in Materials and Methods. Western blot analysis of whole-cell lysates showed a significant time-dependent increase in the level of active caspase-3 and cleaved PARP after H. pylori infection (MOI 100) in APE-1–suppressed shRNA
cells compared with vector control (Fig. 1A). Elevated caspase-3 activity in shRNA cells compared with control or uninfected cells further corroborated these findings (Fig. 1B). These data suggest that APE-1 is important for the inhibition of cell death during *H. pylori* infection. Given that active caspase-3 generation requires both caspase-8 and caspase-9, their specific inhibitors were used to show partial reduction of active caspase-3 level as detected by Western blot analysis after *H. pylori* infection. The magnitude of inhibition by either inhibitor was less in cells with reduced levels of APE-1 (Fig. 1C), suggesting that APE-1 is involved in both caspase-8–mediated and caspase-9–mediated apoptotic pathways.

![Graph A](image)

**Figure 1.** Increased apoptosis in APE-1–suppressed GEC after *H. pylori* infection. A, pSIREN and shRNA cells were treated with *H. pylori* (MOI 100) for 6, 12, or 24 h or left uninfected. Western blot analysis was performed for active caspase-3 (left) or cleaved PARP (right). Corresponding densitometry data normalized to α-tubulin are depicted as the mean ± SEM of three separate experiments. *, *P* < 0.05, shRNA cells compared with corresponding pSIREN cells. B, caspase-3 activity was measured in pSIREN and shRNA cells with or without *H. pylori* infection (MOI 100) for 6, 12, and 24 h. Columns, mean normalized data (n = 3); bars, SEM. *, *P* < 0.05, shRNA cells compared with corresponding pSIREN cells. C, pSIREN and shRNA cells were treated with 10 or 20 μmol/L of caspase-8 or caspase-9 inhibitor 1 h before *H. pylori* infection (MOI 100) for 24 h or left uninfected and analyzed by immunoblotting for active caspase-3.
APE-1 inhibits the intrinsic pathway of apoptosis. We next examined the contribution of APE-1 in modulating apoptosis resulting from the perturbation of mitochondria due to *H. pylori* infection. A significant increase in active caspase-9 (Fig. 2A) and enhanced release of cytochrome c (Fig. 2B) into the cytosol after infection were observed in APE-1 downregulated cells relative to the vector control. To determine if APE-1 regulates Bcl-2 family members, cell lysates of *H. pylori*-infected GEC were assayed for expression of antiapoptotic Bcl-xL and proapoptotic Bcl-xS. *H. pylori* decreased Bcl-xL and increased Bcl-xS in a time-dependent manner, and this effect was greater in APE-1-deficient cells (Fig. 2C). To directly measure mitochondrial damage, the loss of MMP was analyzed at 1 hour after *H. pylori* infection. Infection increased the loss of MMP in both cell types but the loss was greater in APE-1-deficient cells, further indicating an inhibitory role of APE-1 in the mitochondrial pathway of apoptosis (Fig. 2D).

**Figure 2.** Reduction of APE-1 level enhances *H. pylori*-mediated apoptosis via the mitochondria-dependent pathway. pSIREN and shRNA cells were treated with *H. pylori* (MOI 100) for 6, 12, or 24 h or left uninfected. Western blot analysis was performed for active caspase-9 (A), cytosolic cytochrome c (cyt c; B), or Bcl-xL and Bcl-xS (C). For Bcl-xL and Bcl-xS, normalized data are shown as a ratio. D, loss of MMP in pSIREN and shRNA cells with or without *H. pylori* infection (MOI 100) for 1 h. A to D, columns, mean normalized data (n = 3); bars, SEM. *, *P* < 0.05, infected shRNA cells compared with corresponding pSIREN cells.
Figure 3. Reduction of APE-1 level enhances H. pylori–mediated apoptosis via the extrinsic pathway. pSIREN and shRNA cells were treated with H. pylori (MOI 100) for 4, 8, or 12 h or left uninfected. Western blot analysis was performed for active caspase-8 (A) and FLIP<sub>L</sub> and FLIP<sub>S</sub> (B). Corresponding densitometry data, normalized to α-tubulin, are depicted as the mean ± SEM of three separate experiments. *, P < 0.05, shRNA cells compared with corresponding pSIREN cells. C, caspase-8 activity was measured in AGS, pSIREN, and shRNA cells with or without H. pylori infection (MOI 100) for 4, 8, or 12 h. Columns, mean normalized data (n = 3); bars, SEM. *, P < 0.05, shRNA cells compared with corresponding pSIREN cells. D, immunoprecipitation of DISC with Fas antibody at 2 and 4 h after H. pylori infection in pSIREN and shRNA cells. Western blot analysis was performed to detect procaspase-8, FADD, FLIP<sub>L</sub>, and FLIP<sub>S</sub>. IgG was used as the loading control. Corresponding densitometry data normalized to IgG are depicted as the mean ± SEM of three separate experiments. *, P < 0.05, shRNA cells compared with corresponding pSIREN cells.
Figure 4. Acetylation and DNA repair functions of APE-1 both contribute to the inhibition of apoptosis. A, schematic diagram showing WT human APE-1 (top), the repair mutant (middle), and the acetylation mutant (bottom). B, shRNA cell extracts were prepared after transient transfection followed by H. pylori infection and analyzed by Western blot for active caspase-3. FLAG antibody was used to assess transfected APE-1 expression. Corresponding densitometry data, normalized to α-tubulin, are depicted as the mean ± SEM of three separate experiments. *, P < 0.05, compared with WT APE-1. C, dose dependence of H. pylori infection in the induction of APE-1 acetylation. Densitometric analysis was performed to normalize AcAPE-1 to total APE-1 with an arbitrary value of 1 assigned to uninfected pSIREN cells. D, H. pylori dose-specific response of the acetylation function of APE-1 in inhibiting the intrinsic apoptotic pathway. shRNA cells were transiently transfected and infected with H. pylori (MOI 100 and 300) for 8 h (cytochrome c and Bax) and 12 h (caspase-9). Cells were lysed and analyzed by Western blot for cytosolic cytochrome c, Bax, and active caspase-9.
APE-1 inhibits the extrinsic pathway of apoptosis. The regulatory role of APE-1 in the extrinsic pathway of apoptosis was assessed by measuring active caspase-8. Western blot analysis showed an increase in active caspase-8 within 4 hours of *H. pylori* infection in APE-1–downregulated cells relative to the control cells (Fig. 3A). Similar results were obtained by measuring activation of caspase-8, further confirming the inhibitory role of APE-1 (Fig. 3B). FADD-like interleukin-1–converting enzyme-inhibitory proteins (FLIP), which counteract caspase-8 activation, were subsequently measured. The shorter protein FLIP<sub>S</sub> is exclusively a caspase inhibitor, whereas the longer FLIP<sub>L</sub> has dual functions as either a caspase inhibitor or an activator (27). A rapid decrease of FLIP<sub>S</sub> expression over 12 hours of *H. pylori* infection was observed in both cell types, but the decrease was greater in APE-1–deficient cells (Fig. 3C).

Caspase-8 is an integral part of the death-inducing signaling complex (DISC) along with Fas, FADD, or FLIP (28). Within 4 hours of *H. pylori* infection, DISC pulldown using Fas antibody showed enhanced binding of apoptotic procaspase-8 and FADD in shRNA compared with pSIREN cells, and the binding of antiapoptotic FLIP<sub>L</sub> was significantly decreased in shRNA cells (Fig. 3D). These results show that DISC association during *H. pylori* infection is regulated by APE-1.

Both repair and regulatory functions of APE-1 are involved in inhibiting apoptosis. To determine the relative contribution of the DNA repair versus the regulatory function of APE-1 in the inhibition of *H. pylori*–induced apoptosis, we used functional mutants of APE-1. A schematic diagram of APE-1 (Fig. 4A) illustrates that the NH<sub>2</sub>-terminal Lys<sup>6</sup> and Lys<sup>7</sup> are potential sites for acetylation, whereas the COOH-terminal His<sup>309</sup> is a key residue required for DNA repair activity (25). Along with FLAG-tagged wild-type (WT) APE-1, we constructed a DNA repair mutant by replacing His<sup>309</sup> with asparagine (H309N; Fig. 4A, middle) and an acetylation mutant by replacing Lys<sup>6</sup> and Lys<sup>7</sup> with arginines (K6R/K7R; Fig. 4A, bottom).

shRNA cells were separately transfected with an empty vector or the WT, K6R/K7R, or H309N APE-1 constructs. Subsequent Western blot analyses at 12 and 24 hours after *H. pylori* infection showed significantly reduced caspase-3 at both time points in WT APE-1–transfected cells compared with vector control (Fig. 4B). In contrast, active caspase-3 expression in cells transfected with either the DNA repair mutant or the acetylation mutant construct was not significantly different from cells not transfected with APE-1 (Fig. 4B). This finding underscores the importance of both functions of APE-1 in controlling apoptosis during *H. pylori* infection.

It is reported that *H. pylori* MOI 100 is optimal for inducing apoptosis (29, 30), whereas APE-1 acetylation occurs maximally with a MOI of 300 (22). Thus, in this study, we confirmed the induction of APE-1 acetylation by *H. pylori* before embarking on the role of acetylated APE-1 in regulating different pathways of apoptosis. A significant increase in acetylated APE-1 at MOI 300 and 600 compared with MOI 100 was observed (Fig. 4C). To determine the contribution of the acetylation function of APE-1, we measured cytosolic cytochrome c, Bax, and active caspase-9 expression in shRNA cells as shown in Fig. 4B after infection with *H. pylori* MOI 100 and 300. Whereas WT APE-1 overexpression reduced the protein level of cytochrome c and Bax, overexpression of the acetylation mutant significantly increased their expression with MOI 300 compared with MOI 100 (Fig. 4D, top and middle, compare lanes 6 and 7). This suggests that the APE-1 acetylation inhibits the intrinsic apoptotic pathway when a higher dose of

**Figure 5.** DNA repair activity of APE-1 is required to inhibit mitochondria-mediated apoptosis during *H. pylori* infection. A, shRNA cells were transiently transfected as in Fig. 4B and infected with *H. pylori* for 6 or 12 h before performing Western blot analysis with active caspase-9 and Bcl-x<sub>L</sub> antibody. B, caspase-9 activity assay of shRNA cells identically transiently transfected as in Fig. 4B and infected with *H. pylori* (MOI 100). Bars depict fold change of each *H. pylori*–infected group relative to their respective uninfected group (mean ± SEM, *n* = 3), which is set to an arbitrary value of 1. *, *P* < 0.05, WT APE-1 or K6R/K7R compared with empty vector or H309N. C, measurement of loss of MMP in shRNA cells first transfected and then infected for 1 h with *H. pylori* (MOI 100). Bars depict fold change for each *H. pylori*–infected group relative to uninfected controls (mean ± SEM, *n* = 4), where the uninfected vector control is set to an arbitrary value of 1. *, *P* < 0.05, WT APE-1 or K6R/K7R compared with empty vector or H309N.
acetylation mutant compared with vector control after infection with both MOI 100 and 300 (Fig. 4D, middle, compare lanes 2 with 6 and lanes 3 with 7). These data implicate an inhibitory role of the DNA repair function of APE-1 downstream of mitochondrial membrane damage in the setting of *H. pylori* infection.

**The DNA repair function of APE-1 inhibits mitochondria-mediated apoptosis.** As *H. pylori* MOI 300 increased necrotic death of GEC over time, we used MOI 100 to examine the relative roles of the two different functions of APE-1 in subsequent experiments. Western blot analysis of whole-cell lysates at 6 and 12 hours after *H. pylori* infection showed increased levels of active caspase-9 and increased levels of antiapoptotic Bcl-xL (Fig. 5A) in cells transfected with the DNA repair mutant. In contrast, transfection with WT APE-1 or the acetylation negative mutant showed reduced levels of active caspase-9 and increased levels of Bcl-xL. Assays of caspase-9 activity corroborated the Western blot data (Fig. 5B). We also found a significant increase in MMP loss in DNA repair mutant–transfected APE-1–deficient cells after *H. pylori* infection; however, this was not observed in APE-1–deficient cells overexpressing WT or the acetylation negative mutant of APE-1 (Fig. 5C). This suggests that the DNA repair activity of APE-1 is required to inhibit the mitochondrial pathway of apoptosis.

**Acetylation function of APE-1 inhibits the extrinsic pathway of apoptosis.** Similar approaches were used to assess the relative roles of the repair and acetylation functions of APE-1 in the extrinsic pathway of apoptosis. In contrast to what was shown for the intrinsic pathway, the *H. pylori*–mediated increase in active caspase-8 was inhibited in both WT and DNA repair mutant APE-1–transfected shRNA cells. Overexpression of the acetylation mutant of APE-1 did not inhibit active caspase-8 generation (Fig. 6A) or caspase-8 activity (Fig. 6B). Finally, reduced expression of antiapoptotic FLIPs after infection in acetylation mutant–transfected cells compared with those transfected with WT or DNA repair mutant APE-1 (Fig. 6C) confirmed the previous observations, indicating that impaired acetylation of NH2-terminal lysines inhibits the ability of APE-1 to attenuate the caspase-8–mediated extrinsic pathway of apoptosis.

**Discussion**

*H. pylori* pathogenesis depends on the balance between proliferation and cell death of the gastric epithelium (8, 31), and both bacterial factors and host signaling pathways determine the outcome of infection. *H. pylori* infection has been shown to exert antiapoptotic effects to promote gut epithelium self-renewal in response to bacterial factor CagA (31). Our results indicate that molecular events within host cells also play a role in inhibiting apoptosis due to Cag PAI-bearing *H. pylori* strain, 26695. Specifically, we showed that APE-1 has a key inhibitory role in programmed cell death resulting from *H. pylori* infection of GECs. We also showed that APE-1 inhibited both the intrinsic and the extrinsic pathways of cell death and that the DNA repair activity and the acetylation function of APE-1 differentially inhibited these two pathways.
Thus, our results enhance the current understanding of programmed cell death during *H. pylori* infection and advance our knowledge of the multifunctional role of APE-1 in modulating apoptosis. Given the role apoptosis and alterations of cell cycle may play in carcinogenesis, our findings also have implication for understanding *H. pylori*–associated disease pathogenesis.

The data presented in this report indicated that suppression of endogenous APE-1 in GEC rendered the cells more susceptible to apoptosis after *H. pylori* infection as evidenced by increased caspase-3 activity. To further define the basis for caspase-3 activation, we first investigated the intrinsic pathway of apoptosis (32, 33). Our findings of increased cytosolic cytochrome c, together with elevated caspase-9 in APE-1–suppressed GEC compared with vector control cells, provided evidence for APE-1 as a regulator of the intrinsic apoptotic pathway. The increase in the Bcl-xL/Bcl-x ratio in shRNA cells indicated a shift toward proapoptotic Bcl-2 family members when APE-1 is reduced, presumably because of damaged mitochondrial genome due to the accumulation of ROS in GEC, which we and others have shown occurs during *H. pylori* infection (4, 12).

Our work also established a role of APE-1 in the extrinsic pathway of apoptosis, which is dependent on Fas-mediated DISC activation (34). FLIPs binding to the DISC complex prevents apoptosis by inhibiting the DISC-mediated activation of procaspase-8 (35, 36). We showed that APE-1 suppression not only resulted in a decrease in antiapoptotic FLIPs expression but also diminished the DISC binding of FLIPs. Downregulation of APE-1 led to an increase in DISC-associated caspase-8 and FADD, possibly due to a decrease in DISC-associated FLIPs. In our study, the increase in caspase-8 activation occurred earlier than the increase in caspase-9 similar to previously published observations (37, 38). This pattern of caspase-activation was not affected by cellular levels of APE-1. Many of the effects of APE-1 in suppressing *H. pylori*–induced apoptosis show similar patterns as reported for epidermal growth factor receptor (EGFR) activation (39). Thus, examining the possible relationship between APE-1 and EGFR transactivation will be an important area for future research.

APE-1 exerts its many effects via DNA repair, reductive activation, and acetylation-mediated gene regulation. However, it has been difficult to establish the relative contribution of these various properties of APE-1 to regulate apoptosis. Although we showed that APE-1 separately inhibited the intrinsic and extrinsic pathways of apoptosis, links between these two pathways are known to exist at different levels. On death receptor signaling, activation of caspase-8 results in translocation of truncated Bid to mitochondria with subsequent activation of caspase-9 (40). We observed that the DNA repair activity of APE-1 was critical in inhibiting the intrinsic pathway, as overexpression of the acetylation mutant of APE-1 inhibited caspase-9 activation but had no effect on caspase-8. Conversely, overexpression of the DNA repair mutant affected only the intrinsic pathway including caspase-9 activation and suppression of antiapoptotic Bcl-xL, whereas caspase-8 activity remained low, presumably because of functional NH2-terminal Lys6 and Lys9.

In summary, our findings show APE-1 as a critical host response molecule that may promote persistence of *H. pylori* infection by inhibiting apoptosis. This is the first report showing the simultaneous inhibition of mitochondrial damage and Fas-mediated apoptosis by APE-1. Moreover, the intrinsic and extrinsic pathways of apoptosis are shown to be inhibited by two different functional domains of APE-1. The role of APE-1 in *H. pylori*–mediated disease pathogenesis remains to be elucidated. Our findings also have implications for understanding programmed cell death in other infections and host cell populations.

**Disclosure of Potential Conflicts of Interest**

The authors have disclosed that there are no potential conflicts of interest.

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