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Granzyme H destroys the function of critical adenoviral proteins required for viral DNA replication and granzyme B inhibition

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Granzymes are key components of the immune response that play important roles in eliminating host cells infected by intracellular pathogens. Several granzymes are potent inducers of cell death. However, whether granzymes use additional mechanisms to exert their antipathogen activity remains elusive. Here, we show that in adenovirus-infected cells in which granzyme B (gzmB) and downstream apoptosis pathways are inhibited, granzyme H (gzmH), an orphan granzyme without known function, directly cleaves the adenovirus DNA-binding protein (DBP), a viral component absolutely required for viral DNA replication. We directly addressed the functional consequences of the cleavage of the DBP by gzmH through the generation of a virus that encodes a gzmH-resistant DBP. This virus demonstrated that gzmH directly induces an important decay in viral DNA replication. Interestingly, gzmH also cleaves the adenovirus 100K assembly protein, a major inhibitor of gzmB, and relieves gzmB inhibition. These results provide the first evidence that granzymes can mediate antiviral activity through direct cleavage of viral substrates, and further suggest that different granzymes have synergistic functions to outflank viral defenses that block host antiviral activities.

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Introduction

The host–virus relationship is a dynamic process in which the virus attempts to minimize its visibility while the host attempts to prevent and eradicate infection with minimal collateral damage to itself. Among human viruses, adenoviruses have evolved multiple mechanisms to control the host immune and inflammatory responses, and about a third of its genome is devoted to this purpose. Immediately upon infection, E1A blocks interferon-induced gene expression and the VA-RNA inhibits interferon-induced PKR activity (Burgert *et al*, 2002). At the same time, E1A reprograms the cell for DNA synthesis and induces the intrinsic cellular apoptosis program that is interrupted by E1B-19K, which inhibits apoptosis at the mitochondrial level (Cuconati and White, 2002). In addition, E1B-55K in concert with E4orf6 blocks p53-mediated apoptosis (Dobner *et al*, 1996). Various proteins encoded in the adenovirus E1, E3 and L4 transcription units protect cells from killing mediated by cytotoxic cells: (i) E3-14.7K seems to interfere with the cytolytic and proinflammatory activities of tumor necrosis factor (TNF), (ii) the receptor internalization and degradation complex (formerly E3-10.4 K/14.5K) removes Fas and TNF-related apoptosis-inducing ligand (TRAIL) receptors from the cell surface (Lichtenstein *et al*, 2004), (iii) E1B-19K and E3-14.7 can directly interact and inhibit members of the Fas signaling complex (Lichtenstein *et al*, 2004), (iv) the 100K assembly protein (100K) directly inhibits the granule cytotoxic protease granzyme B (gzmB) (Andrade *et al*, 2001, 2003) and (v) the E3-19K protein subverts antigen presentation by MHC class I molecules by inhibiting their transport to the cell surface, interfering with recognition of infected cells by cytotoxic T cells (Lichtenstein *et al*, 2004). In concert, these adenoviral countermeasures ensure prolonged survival in the infected host and, as a consequence, facilitate transmission. Interestingly, despite these extensive immunomodulatory and antiapoptotic functions (including an ability to induce malignant tumors in rodents (Trentin *et al*, 1962; Jonsson and Ankerst, 1977) and transform cultured cells (Gallimore, 1972)), adenoviruses are responsible for mild, self-limited illness in humans, suggesting the existence of potent unexplored antiviral pathways to which adenovirus has not evolved inhibitory activity.

Killer lymphocytes are key players in the effector arm of the immune response that eliminate tumor cells or cells infected with intracellular pathogens. Upon recognition by the cytotoxic effector cell, killing of the target cell is induced through pathways initiated by death receptors or through cytotoxic granule exocytosis (Russell and Ley, 2002). Findings in genetically manipulated mice, human genetic diseases and *in vitro* studies suggest that the granule exocytosis pathway has the dominant role in eliminating virus-infected cells, protecting against other intracellular pathogens (such as mycobacteria) and tumor surveillance (Kagi *et al*, 1994; Smyth and Trapani, 1998; Smyth *et al*, 2000; Balkow *et al*, 2001; Trapani and Smyth, 2002; Muller *et al*, 2003; Zelinskyy *et al*, 2004). Cytotoxic granules contain the membrane-perturbing protein perforin and a family of serine

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proteases known as granzymes, complexed with the proteoglycan serglycin (Russell and Ley, 2002). Perforin provides target cell access and/or trafficking signals for the granzymes, and the granzymes deliver the lethal hits (Keefe *et al*, 2005). Granzymes are closely related to one another structurally, and their genes are clustered in several loci on distinct chromosomes (Grossman *et al*, 2003). Humans express five granzymes; gzmA and gzmK, which are trypsin-like; gzmB, which cleaves after Asp residues; gzmM, which cleaves after Met and other long unbranched hydrophobic residues; and gzmH, which has chymotrypsin-like (chymase) activity (Edwards *et al*, 1999; Kam *et al*, 2000; Mahrus and Craik, 2005). A total of 10 granzymes (A–G and K–M) have been identified in mice and seven in rat (A, B, C, I, J, K and M) (Grossman *et al*, 2003). No human equivalents of mouse granzymes C–G are known, and gzmH appears to be specifically human. In humans and mice, the most studied granzymes are gzmA and gzmB, which induce cell death through different pathways in which targeted proteolysis of cellular substrates is the central mechanism (Lieberman and Fan, 2003; Andrade *et al*, 2004). Regarding the remaining granzymes, little is known about their function, hence their designation as 'orphans' (Grossman *et al*, 2003). Interestingly, gzmB-, gzmA- and gzmB/A-deficient mice retain cell-mediated cytotoxicity (Heusel *et al*, 1994; Ebnet *et al*, 1995; Simon *et al*, 1997; Waterhouse *et al*, 2006) and are susceptible only to selected viruses *in vivo* (Mullbacher *et al*, 1996, 1999). In contrast, perforin-deficient mice have a profound defect in eliminating multiple viral and nonviral pathogens (Kagi *et al*, 1994), and the defect of immune surveillance that results in the development of spontaneous aggressive cancer, especially B-cell lymphoma (Smyth *et al*, 2000). The considerable functional redundancy inherent in the remaining granule toxins (e.g. orphan granzymes) almost certainly reflects the need for multiple molecular strategies to outflank viral and tumor defenses designed to block target cell death.

In this report, we demonstrate that the adenovirus type 5 (Ad5)-DBP, an essential component for viral replication, is cleaved by gzmB and gzmH. However, in adenovirus-infected cells in which viral apoptosis inhibitors have been expressed, including a potent gzmB inhibitor, gzmH is the prominent protease responsible for the cleavage of DBP and the decrease in viral DNA replication during cytotoxic killing. Interestingly, gzmH also targets the adenovirus 100K protein (a critical component required for virus assembly and gzmB inhibition), allowing the recovery of gzmB activity, which further targets the same viral products at unique sites. The direct cleavage of essential viral proteins by granzymes identifies a novel mechanism by which cytotoxic cells rapidly and directly block viral replication. The functional redundancy in the cleavage of essential viral proteins by granzymes with distinct cleavage specificity constitutes a potent antiviral effector mechanism that overcomes viral defenses against host antiviral activities.

Results

DBP is cleaved during cytotoxic-mediated cell death

During the characterization of a gzmB inhibitor encoded by Ad5 (Andrade *et al*, 2001), we serendipitously found that the adenovirus DBP was cleaved in Ad5-infected K562 cells killed by lymphokine-activated killer (LAK) cells, generating a 47 kDa fragment (Figure 1A, lanes 2 and 3). As the K562 cell line is resistant to apoptosis induced by FasL, TNF-α and TRAIL (Hietakangas *et al*, 2003), and additionally, Ad5 infection downregulates Fas and TRAIL receptors (Lichtenstein *et al*, 2004), it was very unlikely that the proteolytic activity against DBP was mediated through death receptors. Indeed, when the activity of perforin was blocked by the presence of EGTA in the LAK-mediated killing assay, generation of the 47 kDa fragment was entirely abolished (Figure 1A, lanes 5 and 6). Therefore, components of the granule exocytosis pathway must be directly responsible for this proteolytic activity.

DBP is cleaved by gzmB

To further dissect the components involved in the cleavage of DPB, we cloned and expressed DBP in target K562 cells. Interestingly, when DBP-expressing target cells were killed with LAK cells (Figure 1B, lane 4), DBP was cleaved generating the 47 kDa fragment and two additional fragments that were not observed during killing of Ad5-infected cells of approximately 60 and 50 kDa (Figure 1B, compare lanes 2

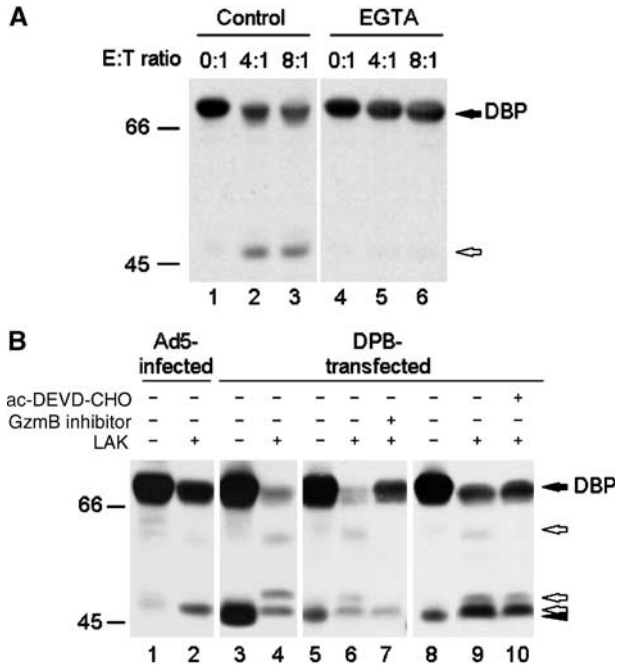

Figure 1 DBP is cleaved during LAK-mediated cell death. (**A**) Ad5-infected K652 cells were coincubated without or with LAK cells at increasing E:T ratios, in the absence (lanes 1–3) or presence of 8 mM EGTA (lanes 4–6) for 4 h at 37°C. (**B**) K562 cells were Ad5-infected (lanes 1 and 2) or pcDNA3.1-DBP vector-transfected (lanes 3–10). At 48 h post-infection or 24 h post-transfection, cells were incubated alone (lanes 1, 3, 5 and 8) or coincubated (lanes 2, 4, 6, 7, 9 and 10) with LAK cells (E:T ratio 8:1) for 4 h at 37°C. In some experiments, LAK and K562 cells were incubated with compound 6 (GzmB inhibitor) (lane 5) or Ac-DEVD-CHO (lane 10) before coincubation. After terminating the reactions, the samples were electrophoresed and immunoblotted with anti-DBP antibody 37-3. The data shown (B, lanes 8–10) were from a single autorad with intervening lanes containing data irrelevant to this figure spliced out. The solid arrow denotes intact DBP, unfilled arrows mark DBP fragments and the solid arrowhead denotes a 46 kDa species that is generated when DBP is expressed by transfection (lanes 3, 5 and 8) (Asselbergs *et al*, 1983). Note that the 46 kDa species is absent on lanes 4, 6, 7, 9 and 10, suggesting that it is also cleaved during cytotoxic killing.

and 4). As Ad5 encodes multiple apoptosis inhibitors, including a potent gzmB inhibitor (Andrade *et al*, 2001), we addressed whether these 60 and 50 kDa fragments result from the activity of apoptotic proteases possibly inhibited by Ad5. When effectors and DBP-expressing target cells were coincubated in the presence of compound 6, a potent cell-permeable gzmB inhibitor (Willoughby *et al*, 2002), generation of the 60 and 50 kDa fragments was entirely abolished (Figure 1B, lane 7). As the caspase inhibitor ac-DEVD-CHO only affected the production of the 60 kDa fragment (Figure 1B, lane 10), these data suggest that the 50 kDa fragment is directly induced by gzmB cleavage and that generation of the 60 kDa fragment requires caspase activation. Interestingly, the 47 kDa fragment was not affected either by compound 6 or ac-DEVD-CHO. Generation of the 47 kDa fragment of DBP in infected target cells killed by cytotoxic lymphocytes therefore results from the activity of a cytotoxic granule protease whose activity is not affected by Ad5.

DBP cleavage by caspases and gzmB was further analyzed using purified proteases and [^{35}S]methionine-labeled DBP generated by IVTT (*in vitro*-coupled transcription/translation). In the presence of gzmB, DBP was efficiently cleaved ($k_{cat}/K_m = 2.2 \times 10^5\,M^{-1}\,s^{-1}$), generating multiple fragments. The most prominent fragments generated migrated around 50 and 45 kDa (Figure 2A, lane 2). In contrast, none of several caspases (caspase-1, -3, -4, -5, -6, -7, -8 and -9) cleaved DBP *in vitro* (Figure 2B). Using the tetrapeptide specificity of gzmB (Thornberry *et al*, 1997) to predict potential gzmB cleavage sites in DBP, relevant P_1 aspartic acids were mutated to alanine (D→A), and susceptibility of wild-type and mutated forms to cleavage by gzmB was then assessed. The two most efficiently cleaved sites are located at SSQD^{62}↓A and VIVD^{106}↓S (Figure 2C, lanes 3 and 4, respectively); additional minor sites were also defined at IEMD^{300}↓V, LDAD^{429}↓L and LISD^{433}↓K (data not shown and Figure 4A).

DBP is a substrate for gzmH

To further address the component(s) of the granule exocytosis pathway responsible for the cleavage of DBP in Ad5-infected cells, cell lysates generated from Ad5-infected cells were incubated in the absence or presence of purified gzmA, gzmH, gzmK and gzmM. In addition, DBP-expressing target cells were killed by LAK cells in the absence or presence of increasing concentrations of FUT-175, a potent gzmA inhibitor (Poe *et al*, 1991). GzmA, gzmK and gzmM failed to cleave DBP *in vitro*, and FUT-175 did not modify the generation of the 47 kDa fragment during LAK-mediated killing of DBP-expressing K562 cells (data not shown). In contrast, DBP was cleaved by gzmH generating a 47 kDa fragment (Figure 3A, lane 4), which was identical to the DBP fragment generated during LAK-induced death of Ad5-infected K562 cells (Figure 3A, compare lanes 2 and 4). Even though gzmH is constitutively expressed by NK cells (the largest component of LAK cell activity in human peripheral blood; (Ortaldo *et al*, 1986; Phillips and Lanier, 1986), we confirmed the presence of gzmH in LAK cells using a recently described gzmH-specific monoclonal antibody (4G5) (Sedelies *et al*, 2004). Indeed, as well as gzmB, gzmH is expressed by LAK cells (Figure 3B, lanes 1 and 2, respectively). Direct cleavage of DBP by gzmH was further confirmed using [^{35}S]methionine-labeled DBP generated by IVTT. In the presence of gzmH,

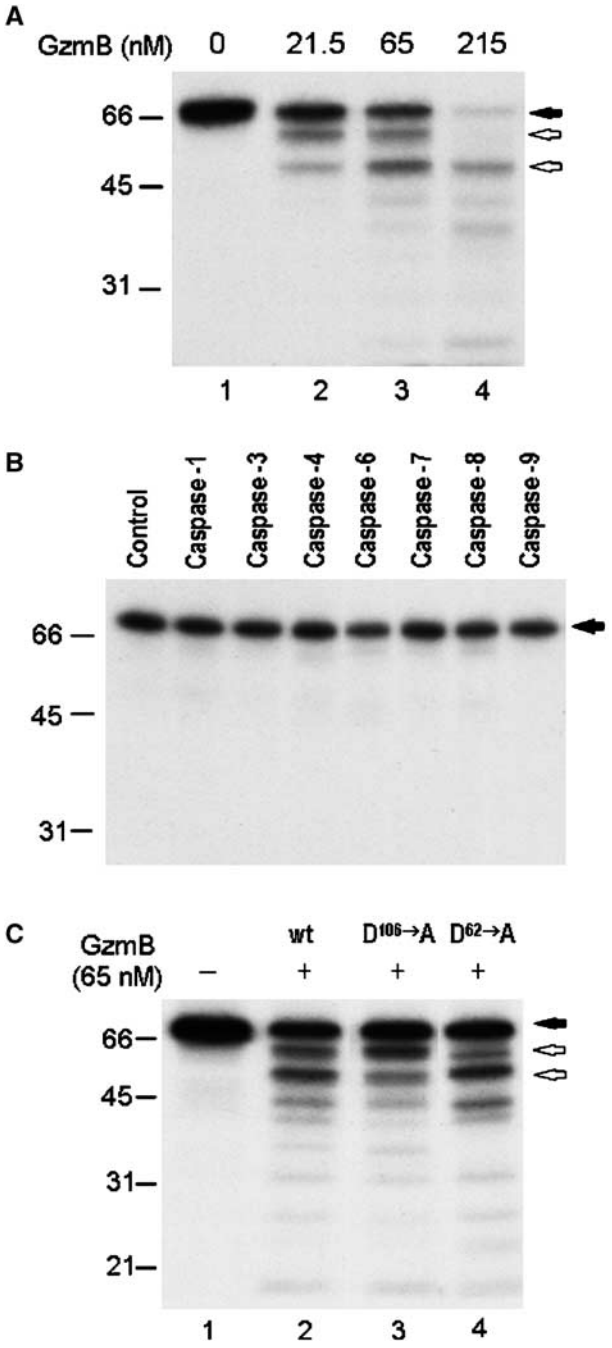

Figure 2 DBP is directly cleaved by gzmB. (**A**) DBP is efficiently cleaved by gzmB. [^{35}S]methionine-labeled DBP generated by IVTT was incubated in buffer B with increasing amounts of purified gzmB for 30 min at 37°C. (**B**) [^{35}S]methionine-labeled DBP was incubated in buffer B with 5 mM DTT and 41 U of caspase-1, 42 pM caspase-3, 2 μM caspase-4, 1.5 U caspase-6, 62.5 nM caspase-7, 52.8 mM caspase-8 or 88 mM caspase-9, for 60 min before SDS–PAGE analysis. (**C**) GzmB cleavage sites in DBP were defined by mutating P_1 Asp residues to Ala (D→A) and incubating wild-type (wt) and mutated [^{35}S]methionine-labeled products in buffer B with purified gzmB for 30 min at 37°C. The data shown (A, C) were from a single autorad with intervening lanes containing data irrelevant to this figure spliced out. Solid arrows denote intact DBP and unfilled arrows mark prominent gzmB cleavage fragments.

DBP was cleaved ($k_{cat}/K_m = 1 \times 10^4\,M^{-1}\,s^{-1}$), generating a prominent 47 kDa fragment, a 20 kDa complementary fragment (Figure 3C) and two minor fragments of approximately 35 and 30 kDa, which can be observed in longer film ex-

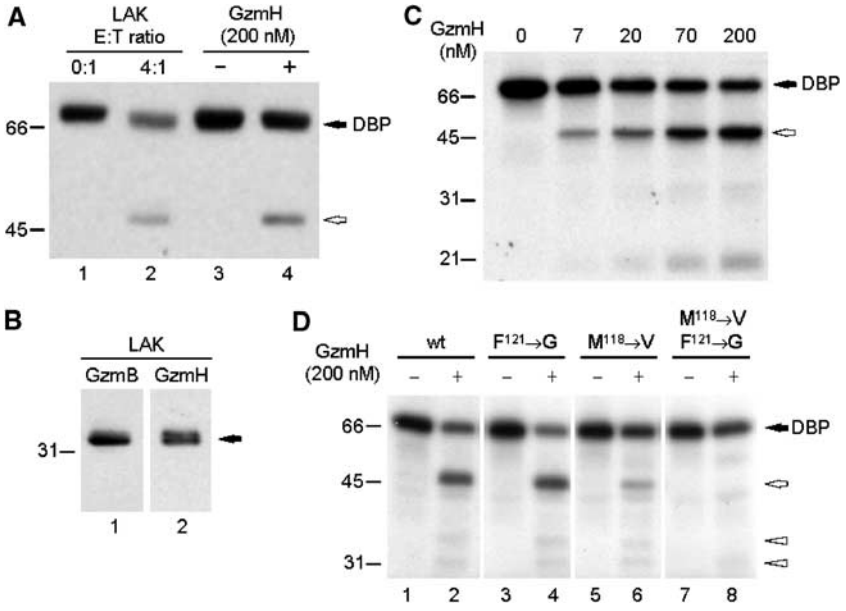

Figure 3 DBP is cleaved by gzmH *in vitro*. (**A**) Ad5-infected K562 cells were incubated alone (lane 1) or coincubated with LAK cells (lane 2) for 4 h at 37°C. Cell lysates from Ad5-infected K562 cells were incubated in the absence (lane 3) or presence (lane 4) of purified gzmH for 30 min at 37°C. After terminating the reactions, the samples were electrophoresed and immunoblotted with anti-DBP antibody 37-3. (**B**) LAK cells express gzmH. Cell lysates from LAK cells were electrophoresed and immunoblotted with a rabbit polyclonal against gzmB (lane 1) or the anti-gzmH monoclonal antibody 4G5 (lane 2). (**C**) [^{35}S]methionine-labeled DBP generated by IVTT was incubated in buffer A in the presence of increasing amounts of purified gzmH for 60 min at 37°C. (**D**) GzmH cleavage sites in DBP were defined by mutating P1 residues Phe^{121}→Gly, Met^{118}→Val and Phe^{121}→Gly/Met^{118}→Val, and incubating wild-type and mutated [^{35}S]methionine-labeled products in buffer A with or without gzmH for 60 min at 37°C. The solid arrow denotes intact proteins, the unfilled arrow marks the prominent gzmH cleavage fragment and the unfilled arrowheads mark minor DBP fragments.

posure (Figure 3D). Taken together, these data demonstrate that gzmH, a granule protease expressed by cytotoxic cells, directly cleaves DBP *in vitro*, and the fragment generated is identical to the DBP fragment observed during cytotoxic-induced death of Ad5-infected cells.

GzmH cleavage sites in Ad5-DBP

The specificity of gzmH has been defined using a panel of synthetic peptides (Edwards *et al*, 1999; Mahrus and Craik, 2005). The protease has a preference for Phe, Met and Tyr at the P_1 site. Based on the cleavage specificity and the size of the fragment generated by gzmH, the cleavage site was predicted. Using site-directed mutagenesis, we initially changed Phe-121 to Gly (F^{121}→G). This amino-acid substitution did not affect cleavage of DPB by gzmH (Figure 3C, lane 4). When Met-118 was changed to Val (M^{118}→V), gzmH cleavage of DBP decreased significantly, but it was not abolished (Figure 3D, lane 6). Interestingly, when a double mutant containing F^{121}→G and M^{118}→V was used in the gzmH cleavage assay, the generation of the 47 and 35 kDa fragments was abolished, with minimal generation of the 30 kDa fragment (Figure 3D, lane 8). DPB is therefore cleaved by gzmH at multiple sites, but the most efficiently cleaved is likely located at Met-118 (Figure 4A), and when this site is mutated, the enzyme instead cleaves at Phe-121, but less efficiently. Alternatively, it might be a unique cleavage site spanning residues 118 and 121.

GzmH cleavage of DBP: functional outcome in adenovirus DNA replication

DBP is a multifunctional protein that is essential for DNA replication and early and late gene expression (Klessig and Grodzicker, 1979; Nevins and Winkler, 1980; Babich and Nevins, 1981; Rice and Klessig, 1985; de Jong *et al*, 2003). As the activity of granzymes is highly dependent on extended and specific interactions with their substrates (Mahrus and Craik, 2005), we generated a mutant adenovirus (Ad5^{dbp}) that encodes a gzmH-resistant DBP containing the amino-acid changes F^{121}→G and M^{118}→V, to address directly the functional consequences of DBP cleavage by gzmH during cytotoxic-mediated cell death. The mutant virus was viable, and although it generated smaller plaques than the wild-type virus, its DBP expression, viral replication and late gene expression in infected cells were as efficient as the wild-type form (data not shown). Coincubation of LAK cells with Ad5 wild-type (Ad5wt)-infected target cells resulted in characteristic DBP cleavage, the level of which correlated with the effector:target (E:T) ratio used in the assay (Figure 4B, lanes 2–4). In contrast, when Ad5^{dbp}-infected cells were killed with LAK cells, cleavage of DBP was decreased even at the highest E:T ratio (Figure 4B, lanes 6–8). DNA viral replication was determined by quantifying the viral DNA load in infected target cells at 12 and 24 h post-cytotoxic killing (Figure 4C). At 12 h post-killing, Ad5wt-infected cells showed more than 60% decrease in DNA viral load. In contrast, the amount of viral DNA was minimally affected when Ad5^{dbp}-infected cells were killed by LAK cells ($P=0.046$, Student's *t*-test; one-tailed). Interestingly, at 24 h post-killing both Ad5wt- and Ad5^{dbp}-infected cells showed a dramatic and similar decay in the amount of viral DNA ($P=0.23$, one-tailed *t*-test). Taken together, these data demonstrate that direct cleavage of DBP by gzmH is a critical component of the cytotoxic antiviral response against adenovirus, which slows down DNA viral replication. Interestingly, additional antiviral mechanisms

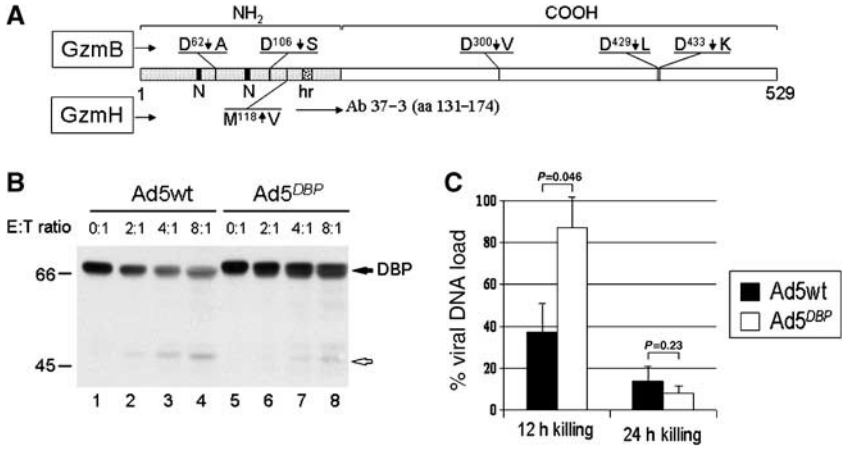

Figure 4 Adenovirus DNA replication and gzmH cleavage of DBP during cytotoxic-mediated cell death. (**A**) Schematic representation of the functional domains and the gzmB and gzmH cleavage sites in DBP. The protein is shown as consisting of an N-terminal domain (NH_2) containing residues 1–173 and a C-terminal domain (COOH) consisting of residues 174–529. The N-terminal domain contains two nuclear localization sequences (N) located at positions 42–46 and 84–89, and the host range (hr) region encompassing residues 130–148. The anti-DBP monoclonal antibody 37-3 (Ab 37-7) recognizes residues 131–174 (Cleghon *et al*, 1993). The gzmB and gzmH cleavage sites are marked above or below the diagram, respectively. (**B**) Ad5wt- or Ad5^{dbp}-infected K562 cells were coincubated with or without LAK cells at increasing E:T ratios. After 4 h, the samples were electrophoresed and immunoblotted with anti-DBP antibody 37-3. The solid arrow denotes intact DBP and the open arrow marks the gzmH cleavage fragment. (**C**) Ad5wt- or Ad5^{dbp}-infected K562 cells were coincubated in the absence (control) or presence of LAK cells at E:T ratio 8:1. After 12 and 24 h, genomic DNA was purified and the DNA viral load was quantified by real-time PCR as described in Materials and methods. Values are shown as percentages in which 100% represents the total viral DNA load in control non-killed cells at 12 or 24 h. The error bars represent the s.d.

that are independent of the cleavage of DBP by gzmH are recruited later to amplify the antiviral process already started by gzmH.

Adenovirus 100K assembly protein is cleaved by gzmH

As a consequence of the striking decrease in the viral DNA load observed in Ad5^{dbp}-infected cells at 24 h post-killing, we addressed the status of DBP at this time point. Surprisingly, the gzmH-resistant DBP was almost completely cleaved in Ad5^{dbp}-infected cells at 24 h post-killing, but the fragments generated instead corresponded to the gzmB-induced cleavage fragments (Figure 5A, compare lanes 2 and 3, and 5 and 6) suggesting that gzmB inhibition by Ad5 and specifically the 100K inhibitory activity against gzmB was abolished at later time points. As predicted, 100K was completely cleaved at 24 h in both Ad5wt- and Ad5^{dbp}-infected cells (Figure 5B, lanes 2 and 3, and 5 and 6, respectively). As gzmH is a very close relative of gzmB and both proteases cleave DBP, we wondered whether both granzymes might share substrate preferences and therefore, 100K might also be a gzmH substrate. Indeed, in the presence of purified gzmH, IVTT-generated, [^{35}S]methionine-labeled 100K was cleaved ($k_{cat}/K_m = 1.8 \times 10^4\ M^{-1}\ s^{-1}$) generating a prominent 90 kDa fragment (Figure 6A).

GzmH cleavage of 100K and recovery of gzmB activity

To address the contribution of gzmH-mediated cleavage of 100K to the recovery of gzmB activity, we used [^{35}S]methionine-labeled human procaspase-3 generated by IVTT as a gzmB substrate for *in vitro* studies using purified components. GzmB efficiently cleaves caspase-3, generating the characteristic 21 and 17 kDa fragments (Casciola-Rosen *et al*, 2006) (Figure 6B, lanes 2, 7 and 12). Purified 100K inhibited the activity of gzmB significantly, with decreased generation of the 21 kDa procaspase-3 fragment, and abolition of the 17 kDa fragment, whose production requires gzmB-dependent caspase-3 activation (Casciola-Rosen *et al*, 2006) (Figure 6B. lanes 4, 9 and 14). Interestingly, when 100K was preincubated with gzmH, 100K could still inhibit gzmB activity at early time points (Figure 6B, compare lanes 4

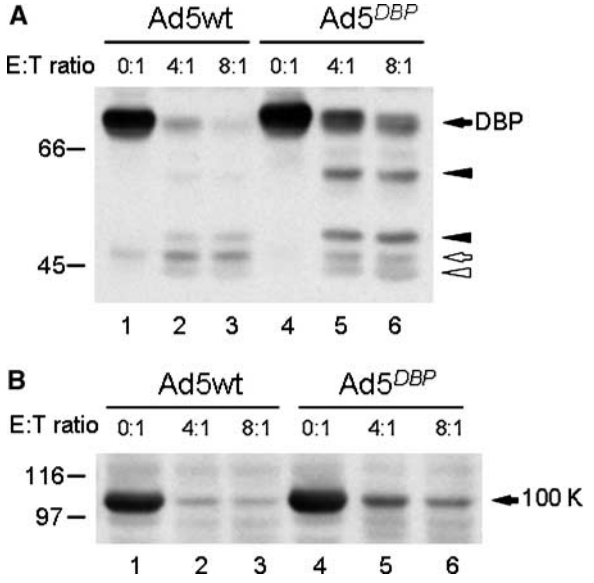

Figure 5 Adenovirus 100K assembly protein is cleaved during cytotoxic-mediated cell death. Ad5wt- or Ad5^{dbp}-infected K562 cells were coincubated in the absence or presence of LAK cells at increasing E:T ratios. After 24 h, the samples were electrophoresed and immunoblotted with anti-DBP antibody 37-3 (**A**) or anti-100K antibody (**B**). Solid arrows denote intact proteins, the open arrow marks the gzmH cleavage fragment, the solid arrowheads denote gzmB-induced fragments and the unfilled arrowhead marks a novel fragment likely generated from the co-cleavage of DBP by gzmH and gzmB.

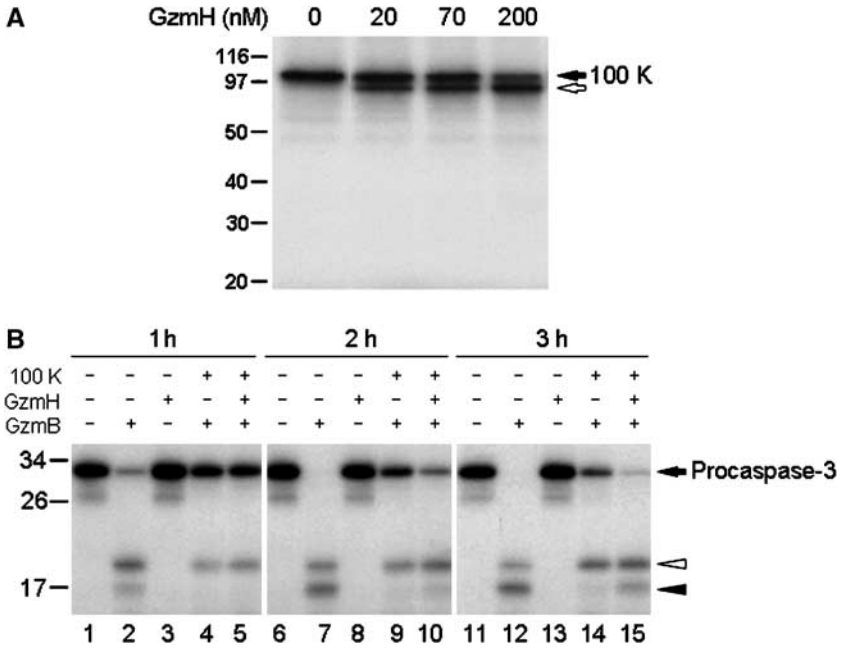

Figure 6 100K is directly cleaved by GzmH. (**A**) [^{35}S]methionine-labeled 100K generated by IVTT was incubated in buffer A in the presence of increasing amounts of purified gzmH for 60 min at 37°C. Samples were analyzed by electrophoresis on 10% SDS–polyacrylamide gels. (**B**) GzmH cleavage of 100K and gzmB reactivation. Purified gzmH (150 nM) or purified 100K (150 mM) were incubated alone or coincubated for 15 min at 37°C in buffer A. Then, aliquots containing 50 nM gzmH (lanes 3, 8 and 13), 100K (lanes 4, 9 and 14) or gzmH plus 100K (lanes 5, 10 and 15) were preincubated in the absence (lanes 3, 8 and 13) or presence (lanes 4, 5, 9, 10, 14 and 15) of 50 nM gzmB for 15 min at 4°C, before adding [^{35}S]methionine-labeled procaspase-3. As controls, [^{35}S]methionine-labeled procaspase-3 was incubated alone (lanes 1, 6 and 11) or in the presence of 50 nM gzmB (lanes 2, 7 and 12). After 1 h (lanes 1–5), 2 h (lanes 6–10) or 3 h (lanes 11–15) at 37°C, the reactions were terminated and gel samples electrophoresed on 12% SDS–polyacrylamide gels. Intact proteins and their cleaved fragments were visualized by fluorography. The solid arrows denote intact proteins, the open arrow marks the gzmH cleavage fragment and the unfilled arrowhead and the solid arrowhead denote the 21 and 17 kDa procaspase-3 fragments, respectively.

and 5). At later times, inhibition by gzmH-treated 100K is lost, allowing recovery of gzmB activity and full caspase-3 activation (Figure 6B, compare lanes 9 and 10, and 14 and 15). Finally, gzmH alone had no effect on the processing of procaspase-3 (Figure 6B, lanes 3, 8 and 13). Taken together, these data demonstrate that cleavage of 100K by gzmH induces gradual inactivation of the inhibitor, allowing recovery of gzmB activity.

Discussion

The granule exocytosis pathway plays a critical role in host defense against intracellular pathogens (Kagi *et al*, 1994; Smyth and Trapani, 1998; Balkow *et al*, 2001; Trapani and Smyth, 2002; Muller *et al*, 2003; Zelinskyy *et al*, 2004). Although several granzymes are potent inducers of cell death, whether granzymes use additional mechanisms to exert their anti-pathogen activity remains elusive. In this report, we have shown that gzmH and gzmB directly and efficiently cleave essential viral proteins. These studies identify the first two biological substrates of gzmH (DBP and 100K), the first non-serpin viral substrate of gzmB (DBP), and the first evidence that granzymes can mediate direct antiviral activity independently of the induction of cell death.

Viruses frequently use multiple mechanisms to evade immune attack, implying susceptibility to multiple antiviral pathways *in vivo*. Interestingly, although viruses have evolved pathways to block granzyme activity, the number of granzymes and their unique catalytic specificities provide significant combinatorial potential. In this regard, although both gzmH and gzmB target the same viral substrates, their unique and different cleavage specificity allows a distinct substrate processing, and the opportunity for combinatorial and synergistic antiviral effects against viral substrates and/or granzyme-specific inhibitors. In addition, the caspase-independent ability of granzymes to cleave viral proteins constitutes a rapid, direct and efficient antiviral mechanism that is insensitive to inhibitors of the signaling or execution components of the apoptotic cascade.

DBP is a multifunctional protein with at least 10 million copies per infected cell, which plays a variety of roles in the viral life cycle; it is essential for DNA replication and it is implicated in early and late gene expression as well as virion assembly (Klessig and Grodzicker, 1979; Nevins and Winkler, 1980; Babich and Nevins, 1981; Nicolas *et al*, 1983; Rice and Klessig, 1985; de Jong *et al*, 2003). DBP can be separated into two domains by limited chymotrypsin treatment (Figure 4A): (i) a highly phosphorylated N-terminal domain that contains two nuclear localization sequences and (ii) a C-terminal domain that is non-phosphorylated, contains two zinc atoms and is sufficient for all DNA replication functions *in vitro*. *In vivo*, DBP-deficient Ad5 mutants are not viable, as are deletion mutants of the C-terminal domain or the first 2–40 amino acids in DBP (Rice and Klessig, 1985; Vos *et al*, 1989). Given the relevance of DBP in the adenovirus life cycle, it is not surprising that cytotoxic cells have developed a strategy to target this molecule. Thus, during cytotoxic-mediated target killing, gzmH cleaves DBP at Met-118, separating the N-terminal (20 kDa minor large fragment) and C-terminal (47 kDa major large fragment) domains (Figure 3C),

decreasing adenoviral DNA replication. The analysis of a virus encoding a gzmH-resistant DBP made the surprising discovery that although DBP cleavage was prevented early, this was only transient, and was followed by cleavage with different fragments consistent with gzmB cleavage. Moreover, we identified that the 100K inhibitor of gzmB was cleaved and inactivated allowing the recovery of gzmB activity. 100K is a nonstructural protein, which has essential functions in the adenovirus life cycle, including virus assembly, activation of late viral protein synthesis and inhibition of cellular protein synthesis (Cepko and Sharp, 1982; Riley and Flint, 1993; Xi *et al*, 2005). In addition, 100K is a gzmB substrate that potently inhibits gzmB activity (Andrade *et al*, 2001). Interestingly, gzmH also cleaves 100K but different from gzmB, gzmH is not inhibited. Instead, cleavage of 100K by gzmH decreases the inhibitory efficiency of 100K against gzmB, allowing recovery in gzmB activity. Direct cleavage of DBP and 100K by gzmH and the further reactivation of gzmB are therefore potent anti-adenoviral mechanisms that are amplified during the process of cytotoxic killing.

Similar to gzmB, gzmH is taken up into the target cell likely through cell surface receptors, and after internalization, remains confined to endocytic vesicles (Edwards *et al*, 1999). Effecting release of the gzmH to the cytosol likely requires the presence of perforin. Whether gzmH is able to induce target cell death is still unknown. Interestingly, at least for the early decay in viral DNA replication observed during cytotoxic-induced target cell death, the analysis of the adenovirus encoding a gzmH-resistant DBP ($Ad5^{dbp}$) strongly suggests that only the direct cleavage of DBP by gzmH but not other gzmH-mediated activities (e.g. cell death) is responsible for this antiviral outcome. In addition, these results also suggest that other granzymes contained in LAK cells have no effect on the early decrease in viral DNA replication. LAK cells are potent killer cells mainly derived from activated NK and T cells (Ortaldo *et al*, 1986; Phillips and Lanier, 1986), which contain all granzyme activities at different ratios. Whether adenovirus encodes inhibitors for other granzymes in addition to gzmB still needs to be explored, as well as the antiviral effects that each granzyme might have on adenovirus either directly or through the induction of target cell death. With the exception of gzmB, which is expressed at much lower levels, all human granzymes are constitutively expressed at high levels in NK cells (Sayers *et al*, 2001; Sedelies *et al*, 2004). The efficient and direct cleavage of viral proteins by granzymes might therefore represent a critical pathway used by the innate immune system to initiate a cell-death-independent, rapid and specific response against granzyme-susceptible viruses. The discovery of novel substrates for orphan granzymes as well as potent and specific inhibitors against them will provide useful tools to better understand the mechanism of granzyme–pathogen interaction. Although gzmH appears to be specifically human, gzmC is the murine orphan granzyme most closely related to gzmH (Pham *et al*, 1996). As granzymes likely evolved to meet species-specific immune challenges (Andrade *et al*, 2003; Casciola-Rosen *et al*, 2006; Kaiserman *et al*, 2006), and similar to humans, mouse adenovirus also produces a mild disease in mice, it is possible that gzmC or other granzymes in mice might have a similar antiviral effect as gzmH in humans.

Antiretroviral therapy in HIV has demonstrated that major strides in antiviral treatment can come from combinatorial approaches, targeting multiple steps in the viral life cycle simultaneously and preventing the emergence of resistance (Bailey *et al*, 2004). In a parallel manner, we propose that the combination of different and unique catalytic activities in granzymes has evolved to effectively eliminate viruses and other intracellular agents, and prevent their acquisition of resistance to granzyme-mediated anti-pathogen pathways. As the functional consequences of genetic variation in granzymes are defined, as well as the granzyme targets in different pathogens are revealed, we anticipate that specific polymorphisms across the spectrum of granzymes might have a critical impact on the outcome in particular chronic infections, like tuberculosis, HIV, EBV and HCV.

Materials and methods

Human recombinant granzymes, caspases and Ad5-recombinant 100K

Granzymes K and M were expressed as inclusion proteins, refolded and converted into the active mature form as described (Hink-Schauer *et al*, 2002). GzmM was amplified from bone marrow cDNA and subcloned into the *Nde*I and *Pst*I sites of the pET24c vector (Novagen) using the forward primer DJ781 5′-GAGATATA CATATGGAAATCATCGGCGGCCGCGAAGTGATC-3′ (with an *Nde*I site) and the backward primer DJ725 5′-ATCACCCCAATGCAT CAGGCC-3′ (with an *Nsi*I site). GzmH was expressed similarly and prepared as will be described elsewhere (E Fellows, manuscript in preparation). Caspase-1, -3, -4, -5, -6, -7, -8, -9 and gzmB were gifts from Nancy Thornberry (Merck Research Labs, Rahway, NJ). GzmA was a gift from Judy Lieberman (CBR Institute for Biomedical Research, Boston, MA). His-tagged 100K was purified as described previously (Andrade *et al*, 2001).

Cell culture, viral infection and cell transfection

K562 cells were cultured using standard procedures. Unless otherwise specified, cells were infected at 20–40 PFU/cell, followed by incubation for 48 h at 37°C in a 5% CO_2 humidified incubator, before performing killing and/or biochemical analysis. Cell lines were transfected using Lipofectamine 2000 (Gibco BRL) as recommended by the manufacturer.

Analysis of LAK-induced cell death

LAK cells were prepared as described (Andrade *et al*, 1998). LAK cells correspond to a mixed population of cytotoxic lymphocytes in which the NK cells form the largest component of LAK cell activity in human peripheral blood (Ortaldo *et al*, 1986; Phillips and Lanier, 1986). Target cells were coincubated in the absence or presence of LAK effector cells at 37°C. The time and E:T ratios are specified in the figure legends. When required, target cells were preincubated for 1 h or 30 min in the presence of 5–100 μM FUT-175 or 100 μM Ac-DEVD-CHO, respectively, or effectors and targets were preincubated for 1 h in the presence of 20 μM compound 6, followed by effector/target cell coincubation. After washing, cells were immediately lysed in SDS sample buffer and boiled. Samples were analyzed by electrophoresis on 10% SDS–polyacrylamide gels. 100K and DBP were visualized by immunoblotting as described (Andrade *et al*, 1998), using a rabbit polyclonal antibody (Andrade *et al*, 2001) and the monoclonal antibody 37-3 (Cleghon *et al*, 1993), respectively.

DBP cleavage in lysates of Ad5-infected cells

After washing three times in PBS, K562-infected cells were lysed in buffer A (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40). Cell lysates were incubated at 37°C for 60 min in the presence of increasing concentrations (0–500 nM) of gzmK, gzmM or gzmH. GzmA cleavage was performed in buffer C (1 mM $CaCl_2$/1 mM $MgCl_2$/50 mM Tris–HCl, pH 7.5) as described above. The reactions were stopped by boiling in SDS sample buffer and samples were analyzed by immunoblotting as above.

Cloning of DBP, hexon and 100K from Ad5

Total RNA was purified from Ad5-infected HeLa cells 24 h post-infection using Trizol (Gibco BRL) as described by the manufacturer. First strand cDNA synthesis was performed with SuperScript

II (Gibco BRL) according to the manufacturer's directions. cDNA was used as template in a PCR with *Pfu*/*Taq* DNA polymerase (1:1), with oligonucleotides containing restriction enzyme adapters. The PCR-generated cDNA fragment containing the entire hexon open reading frame (ORF) was cloned into pcDNA3.1(+) (Invitrogen) and DBP was cloned into pcDNA3.1(+) and N-terminal FLAG-tagged-pcDNA3.1(+). Cloning of 100K has been described previously (Andrade *et al*, 2001).

Cleavage of in vitro-generated DBP and 100K

[^{35}S]methionine-labeled proteins were generated by IVTT (Promega). Cleavage reactions were performed in buffer A or B (10 mM Hepes/KOH, 2 mM EDTA, 1% Nonidet P-40, pH 7.4) as denoted in figure legends. The amount of proteases and length of incubation are also specified. After terminating the reactions by adding SDS sample buffer and boiling, samples were electrophoresed on 10% SDS–polyacrylamide gels. Intact proteins and their cleaved fragments were visualized by fluorography. k_{cat}/K_m values were determined as described (Andrade *et al*, 1998).

Determination of gzmB and gzmH cleavage sites in DBP by P_1 mutagenesis

DBP cDNA were used as template for site-directed mutagenesis by overlap-extension PCR (Strategene). [^{35}S]methionine-labeled polypeptides were generated by IVTT and used as substrates for cleavage assays containing gzmB or gzmH and analyzed as described above. To facilitate the mapping of the gzmH cleavage site, we tagged DBP with FLAG at the N-terminus and FLAG-DBP generated by IVTT was cleaved *in vitro* by gzmH. Based on the cleavage pattern, we concluded that gzmH cleaves DBP at the N-terminal side (data not shown).

Generation of a gzmH-resistant DBP-adenovirus

The E2A cDNA encoding the double mutant DBP ($F^{121}\rightarrow G/M^{118}\rightarrow V$) was exchanged with the wild-type sequence present in pPF446, a derivative of pBR322 that contains the right hand terminal *Bam*HIB fragment of Ad5 (P Freimuth, unpublished). A set of marker rescue experiments was performed with viral DNA from the temperature-sensitive mutant H5*ts*125 and the two pPF446 plasmids. Briefly, parallel cotransfections with either pPF446 or pPF446 mutant ($F^{121}\rightarrow G/M^{118}\rightarrow V$) and H5*ts*125 DNA were set up in 293 cells, incubated at 32°C and the yields titrated at 39°C on A549 cells to select for ts^+ recombinant virus. Transfer of the granzyme-resistant site was expected to occur at a frequency dependent on the distance from the ts^+ site (Volkert *et al*, 1989), provided the amino-acid changes of the former were compatible with viral viability. Plaques arising from cotransfection with pPF446 ($F^{121}\rightarrow G/M^{118}\rightarrow V$) exhibited two morphologies, one identical to that from the parallel transfection with pPF446 and a second type in which the plaques were smaller and the infected cells were less bi-refringent. The virus in the latter proved to have the desired mutations, as determined by sequencing of PCR-amplified DNA from the DBP coding region. Following a single round of plaque purification in A549 cells, high titer stocks were prepared using standard virological procedures.

Quantification of the adenovirus DNA load

K562-infected cells were coincubated in the presence or absence of LAK cells at an E:T ratio of 8:1. After 12 or 24 h, additional LAK cells were added to each reaction to reach the same cell number and cell ratio per sample (i.e. 8×10^5 effectors and 1×10^5 targets) and 1×10^6 non-infected K562 cells were also added as carriers. Then, genomic DNA was immediately purified by using the QIAamp DNA mini kit (Qiagen). We then performed real-time PCR as previously described (Claas *et al*, 2005) with primers ADVs (5′-CATGACTTTT GAGGTGGATC-3′) and ADVas (5′-CCGGCCGAGAAGGGTGTGCG CAGGTA-3′), which amplify a PCR product of 137 bp of the adenovirus hexon gene, using the MX3000P system (Stratagene) and Brilliant SYBR Green QPCR Master Mix (Stratagene). To dilution series of a plasmid containing the hexon ORF were applied to real-time PCR in the presence of DNA from K562 non-infected cells. The obtained C_T values were used to establish a standard curve, and subsequently the viral load of the experimental samples was determined by entering the C_T value of the sample in the standard curve. As negative control, DNA from non-infected K562 cells was used.

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