



# Ebola virus infection of human PBMCs causes massive death of macrophages, CD4 and CD8 T cell sub-populations *in vitro*<sup>☆</sup>

Manisha Gupta<sup>\*</sup>, Christina Spiropoulou, Pierre E. Rollin

Special Pathogens Branch, DVRD, Centers for Disease Control and Prevention, Mailstop G14, 1600 Clifton Road, Atlanta, GA 30333, USA

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## Abstract

Ebola virus causes an often fatal disease characterized by poor immune response and high inflammatory reaction in the patients. One of the causes for poor immunity is virus-mediated apoptosis of lymphocytes in the host. In this study, we infected human PBMCs with Ebola Zaire virus and study apoptosis of different cell types using flow cytometry. We have shown that Ebola virus causes bystander death of CD4 and CD8 T cells. Cells infected with virus had 30–40% active caspase 3<sup>+</sup>, annexin-V<sup>+</sup> and Bcl2<sup>low</sup> phenotype by day 8 PI as compared to inactivated virus-treated cells. 60–70% of the macrophages were also dead by day 8 PI and had similar phenotype. Our data also showed that virus may induce death signals in Fas<sup>+</sup>/FasL<sup>+</sup> T lymphocytes and macrophages but did not upregulate Fas/FasL expression in these cells. Lastly, CD4, CD8 and CD14 cells were purified after infection and were studied for death signals by RNase protection assay. We found an upregulation of TRAIL mRNA in CD4 and CD8 T cells on day 7 PI. A two-fold increase in CD4 T cells and three-fold increase in CD8 T cells were observed in TRAIL mRNA levels as compared to uninfected controls and inactive virus-treated cells. Surprisingly, we did not find any difference in TRAIL mRNA levels between infected macrophages and uninfected controls. These data suggest that Ebola virus evades the immune response by causing massive lymphocyte death. In addition, they may give an explanation on why the host is unable to produce a good antibody response in the absence of CD4 T cells.

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**Keywords:** Ebola virus; Apoptosis; Fas; FasL; TRAIL

## Introduction

Ebola virus infection causes severe and often fatal disease in humans and non-human primates. Disease is characterized by high levels of inflammatory cytokines that result in septic shock and multiorgan failure (Bray and Mahanty, 2003). Ebola virus replicates in a variety of cell types including macrophages, epithelial cells, endothelial cells and hepatocytes and rapidly spreads in the vital organs of the host. Poor immune response and lymphopenia are among the hallmark features of the disease (Baize et al., 1999; Sanchez et al., 2004). *In vitro* infection of

dendritic cells with Ebola virus also suggested that T cell function could be impaired (Mahanty et al., 2003). Previous reports on samples collected from Ebola patients have shown a decrease in the total number of peripheral blood mononuclear cells (PBMCs) (Mahanty and Bray, 2004; Sanchez et al., 2004). In Ebola patients' PBMCs, Baize et al. demonstrated a decrease in mRNA levels of CD8, CD3 and IFN- $\gamma$ , IL-4 and IL-2, suggesting a lack of anti-viral immune response during infection (Baize et al., 1999). Fatal patients do not develop anti-Ebola antibodies before death and the appearance of anti-Ebola IgG is also delayed in survivors (Busico et al., 1999). These reports indirectly suggest that Ebola virus might cause apoptosis of various cell populations.

In this study, we use an *in vitro* system in which human PBMCs were infected with Ebola virus. Various cell types such as CD4 T cells, CD8 T cells, B-cells, NK cells and macrophages were studied for activation and apoptosis signals on various days after infection. Our data indicate that 20–30% of CD4 and

**Abbreviations:** FSC, forward scatter; GFP, green fluorescent protein; MOI, multiplicity of infection; PBMCs, peripheral blood mononuclear cells; PI, post-infection; TRAIL, tumor necrosis factor related apoptosis inducing ligand.

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<sup>\*</sup> Corresponding author. Fax: +1 404 639 1118.

E-mail address: [mgupta@cdc.gov](mailto:mgupta@cdc.gov) (M. Gupta).

CD8 T cells died during the course of infection and 70–80% macrophages underwent apoptosis by day 8 post-infection (PI). Ebola virus induced apoptotic signals in Fas<sup>+</sup>/FasL<sup>+</sup> CD4 and CD8 T cells. We also found an upregulation of TRAIL mRNA in CD4 and CD8 T cells after Ebola virus infection, suggesting that TRAIL may be playing a role in the induction of apoptosis in T cells.

## Results

### *Ebola virus causes massive CD4 T cell death*

PBMCs from healthy volunteers were isolated and inoculated with 0.5 MOI of either Ebola virus or gamma-irradiated inactivated virus and studied for apoptosis using flow cytometric methods. PBMCs were first studied for CD3 levels, CD4 levels, forward and side scatter. We found a 21% decline in CD3 levels on day 4 and a 44% decline on day 8 post-infection as compared to uninfected controls and inactivated virus-treated cells (Table 1). Similarly a 30% decline in CD4 levels on day 4 and a 61% decline on day 8 post-infection as compared to the controls (Table 1). We also found an increase in the number of FSC<sup>low</sup> CD3<sup>+</sup>CD4<sup>+</sup> cells (mean channel value <200) after virus infection (27% in infected cultures versus 17–18% in controls on day 4; 41% in infected cultures versus 21% on controls on day 8 post-infection).

To further confirm that the FSC<sup>low</sup> cells were undergoing apoptosis, we studied CD3<sup>+</sup>CD4<sup>+</sup> T cells for Bcl2, active caspase 3 and annexin-V markers. On day 4 PI, 13–17% cells were Bcl2<sup>low</sup> (Fig. 1) and remained the same on day 8 PI in both controls. In virus-infected cells, there was a dramatic increase in Bcl2<sup>low</sup> cells. The Bcl2<sup>low</sup> population increased from 31% on day 4 PI to 44% on day 8 PI (Fig. 1). These Bcl2<sup>low</sup> cells were also FSC<sup>low</sup>. About twice as many cells were dying by day 4 and three times more cells were dying by day 8 PI compared to controls. Similar

Table 1

Data in table shows mean fluorescent intensity of different markers on uninfected, inactivated virus-treated and live virus-infected PBMCs on days 4 and 8 post-infection

	Isotype control	Uninfected control	Inactivated virus	Live virus
<i>Day 4</i>				
CD3	30±4	434±34	431±35	342±40
CD4	30±10	596±40	631±30	419±20
CD8	40±5	1965±120	2165±120	1203±120
CD14	30±7	1443±40	1195±35	220±20
<i>Day 8</i>				
CD3	30±5	503±30	525±40	277±77
CD4	30±6	1079±40	1202±50	419±29
CD8	40±8	2590±120	2788±200	1051±45
CD14	30±4	2806±130	3315±150	169±55

Data are represented as mean±SD from five different experiments. Approximately 50,000 cells were counted in each sample. The numbers of CD3<sup>+</sup>CD4<sup>+</sup> in uninfected and inactivated virus-treated cells were 20,540±2200 and 21,613±2400 respectively and 18,540±500 in infected cultures. Similarly CD3<sup>+</sup>CD8<sup>+</sup> cells were 12,500±590 and 12,200±400 in uninfected control and inactivated virus-treated PBMCs and 12,800±540 in virus-infected cultures. Similar numbers were found on day 8 post-infection.

numbers were found with active caspase 3 markers. Quadrant statistics was done for FSC and active caspase 3 on CD3<sup>+</sup>CD4<sup>+</sup> T cells. We found that in infected population 6–15% cells were FSC<sup>high</sup> and active caspase 3<sup>high</sup> as compared to 14–19% in the uninfected control and the inactivated virus-treated cell populations on days 4 and 8 PI (Fig. 1). In the virus-infected group, 33% of the cells were FSC<sup>low</sup> and active caspase 3<sup>high</sup> as compared to 16% in controls on day 4 PI (middle panel, Fig. 1). Also in this group, the percentage of caspase 3<sup>high</sup> FSC<sup>low</sup> cell population increased from 33% on day 4 PI to 52% on day 8 PI as compared to 12–18% in control groups (lower right square, Fig. 1). We next studied annexin-V as another apoptotic marker. We found annexin-V markers on both FSC<sup>high</sup> and FSC<sup>low</sup> cells. On day 4 PI, in two control groups, 18% cells were FSC<sup>high</sup> and annexin-V<sup>+</sup> as compared to 2% in live virus-infected cells (top right square, Fig. 1) whereas most of the annexin-V<sup>+</sup> cells (21%) were FSC<sup>low</sup> cells in infected cell population (lower right square, Fig. 1). Similarly on day 8 PI, 42% of the cells were FSC<sup>low</sup> annexin-V<sup>+</sup> in infected cell population as compared to 1% in the controls. In the control groups, all annexin-V<sup>+</sup> cells were FSC<sup>high</sup> cells. These data together suggested that 15–30% CD3<sup>+</sup>CD4<sup>+</sup> T cells were dying during the course of Ebola virus infection. They also showed that active caspase 3 and annexin-V markers are expressed in both blast cells (FSC<sup>high</sup>) and small sized (FSC<sup>low</sup>) cells. However increase in the frequency of active caspase 3<sup>high</sup> and annexin-V<sup>+</sup> cells was observed on FSC<sup>low</sup> cell population and decrease in frequency of active caspase 3<sup>high</sup> and annexin-V<sup>+</sup> in FSC<sup>high</sup> cells infected with Ebola virus compared to uninfected controls.

### *Ebola virus causes massive death of CD8 T cells*

Infected PBMCs from healthy patients were inoculated with 0.5 MOI of Ebola Zaire or inactivated virus and studied for apoptosis of CD3<sup>+</sup>CD8<sup>+</sup> T cells. We first studied CD8 levels on T cells. We found a 40% decline in CD8 levels in infected cultures on day 4 and a 60% decline on day 8 post-infection compared to uninfected control and inactivated virus-treated cells (Table 1). Similar reduction was found in CD3 levels on CD8 T cells as seen on CD3<sup>+</sup>CD4<sup>+</sup> T cells. We also found an increase in the number of FSC<sup>low</sup> cells in virus-infected cultures as compared to controls (mean channel value <200; 20–22% in controls versus 31% infected cells on day 4 and 20% in controls versus 42% in infected cultures on day 8 post-infection). We tried next to determine whether the FSC<sup>low</sup> population was undergoing apoptosis by staining for Bcl2, active caspase 3 and annexin-V markers. Fig. 2 shows that 40% of the CD3<sup>+</sup>CD8<sup>+</sup> T cells were FSC<sup>low</sup> and Bcl2<sup>low</sup> (top left panel) as compared to 20–21% Bcl2<sup>low</sup> in control and inactivated virus-treated cells on day 4 PI. The percentage of Bcl2<sup>low</sup> cells increased to 50% following Ebola infection as compared to 16–21% Bcl2<sup>low</sup>FSC<sup>low</sup> cells in controls on day 8 PI. Similar to the Bcl2 results, 37% of the cells were FSC<sup>low</sup> cells and active caspase 3<sup>high</sup> versus 21–23% in controls and inactivated virus-treated cells on day 4 PI. The percentage of FSC<sup>low</sup> active caspase 3<sup>high</sup> cells in virus-infected cultures increased to 55% as compared to 15–21% in controls on day 8

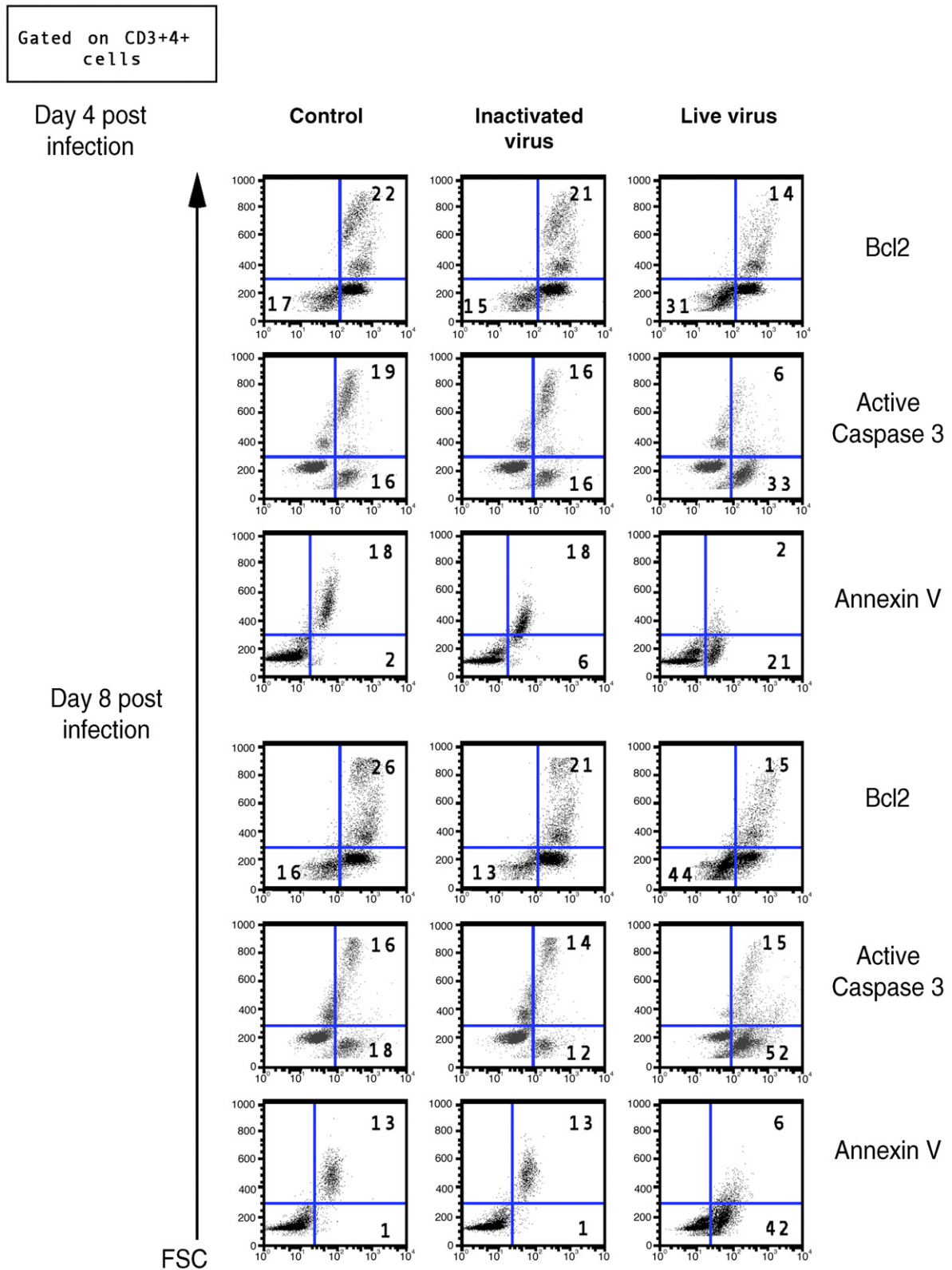


Fig. 1. CD4 T cells undergo massive cell death following viral infection. PBMCs from infected, uninfected control and inactivated virus-treated cultures were stained for Bcl2, active caspase 3 and annexin-V markers at day 4 and day 8 PI. The percentages of cells are indicated in each quadrant. *x* axis shows Bcl2, active caspase 3 and annexin-V staining and *y* axis shows FSC population on days 4 and 8 post-infection. Mean fluorescent intensity for isotype control for Bcl2 was  $20 \pm 4$  (mean  $\pm$  SD), for active caspase 3 was  $40 \pm 10$  and for annexin-V was  $30 \pm 10$  in five different experiments. Percentage of annexin-V<sup>+</sup> cells on FSC<sup>low</sup> population matches with Bcl2<sup>low</sup>FSC<sup>low</sup> and FSC<sup>low</sup> active caspase 3<sup>high</sup> numbers on day 4 and day 8 PI. These data together suggest that 30–40% CD4 T cells are undergoing apoptosis following virus infection by day 8 PI. This figure is a representative of five independent experiments.

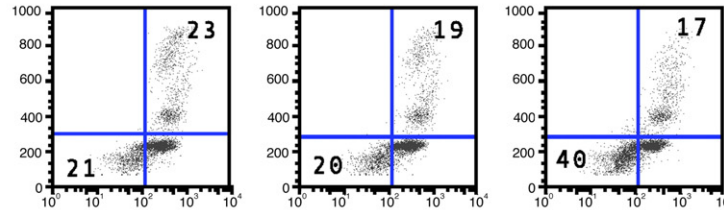
Gated on CD3+8+ cells

Day 4 post infection

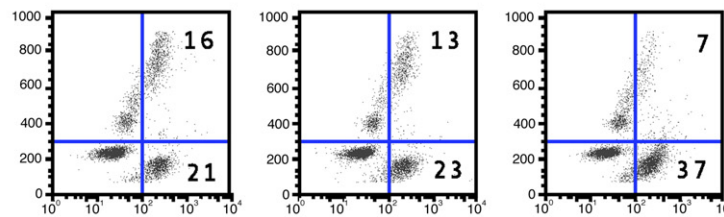
Control

Inactivated virus

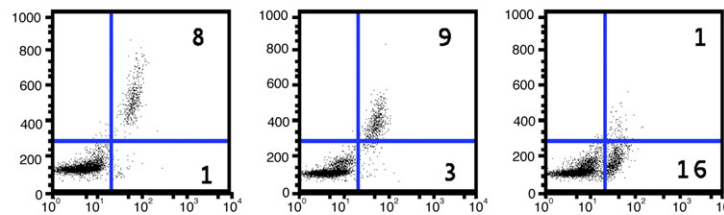
Live virus



Bcl2

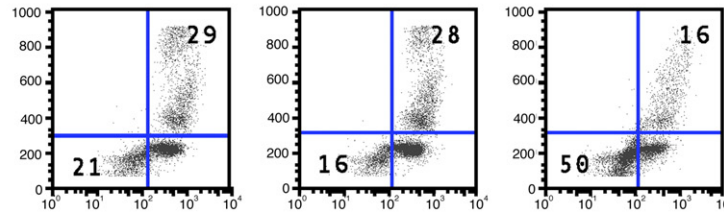


Active Caspase 3

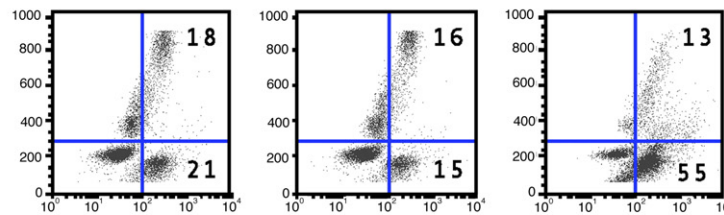


Annexin V

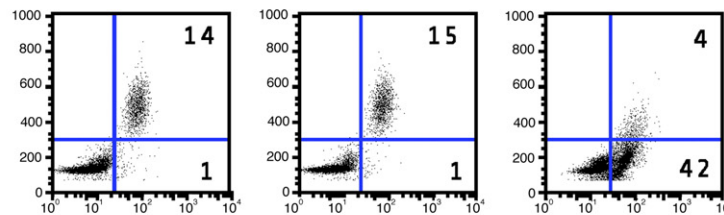
Day 8 post infection



Bcl2



Active Caspase 3



Annexin V

FSC

Fig. 2. Ebola virus causes CD8 T cell death. PBMCs were harvested on days 4 and 8 PI and stained for CD8, Bcl2 and active caspase 3 and annexin-V markers. The percentages of cells are indicated in each quadrant. Isotype control values were the same as shown in Fig. 1. x axis shows Bcl2, active caspase 3 and annexin-V levels and y axis represents FSC levels in controls, inactivated virus-treated and live virus-infected CD3<sup>+</sup>8<sup>+</sup> cells on days 4 and 8 PI. These data together indicate that there is a two to three-fold increase in Bcl2<sup>low</sup>, active caspase 3<sup>high</sup> and annexin-V<sup>+</sup> cells following viral infection as compared to two other populations. This figure is a representative of five independent experiments.

PI. Furthermore, annexin-V staining showed that 16% of cells infected with Ebola virus were  $FSC^{low}$  (right panel; Fig. 2) as compared to 1–3% cells in controls on day 4 PI. Numbers of annexin-V<sup>+</sup> cells were increased to 42% on day 8 following virus infections whereas remained unchanged (1%) in controls in the  $FSC^{low}$  population. It is important to note that nearly all annexin-V<sup>+</sup> cells in controls and inactivated virus-treated populations are blasts ( $FSC^{high}$  cells) and not the apoptotic cells ( $FSC^{low}$ ) unlike in the virus-infected group. Together, these results suggest that 30–40% of CD8 T cells were undergoing cell death by day 8 PI after viral infection.

#### *Ebola virus kills the majority of macrophages*

PBMCs infected with live virus or treated with inactivated virus or left in media alone were stained for CD14. PBMCs were analyzed for forward scatter on  $CD14^+$  cells and CD14 levels in all three cultures. Table 1 shows that there is a 85% reduction in CD14 levels in virus-infected cultures on day 4 and a 94% reduction on day 8 post-infection as compared to uninfected controls and inactivated virus-treated cells. Panel A in Fig. 3 shows forward scatter of  $CD14^+$  cells on days 4 and 8 PI. On day 4 PI, the numbers of  $FSC^{low}$  cells were lower in controls and inactivated virus-treated populations (27–33%) and increased 2.5 times after virus infection (77%). The percentage of  $FSC^{low}$  cells remained the same at day 8 PI (30–35%) in controls and inactivated virus-treated populations whereas it increased to 86% in the virus-infected population. To further confirm that  $FSC^{low}$  cells were apoptotic, we stained them for Bcl2, active caspase 3 and annexin-V markers. We found that 18–25% of the cells were  $FSC^{low}$  Bcl2<sup>low</sup> in controls and inactivated virus-treated populations on day 4 PI (Fig. 3B) whereas 70% of the virus-infected population was Bcl2<sup>low</sup> $FSC^{low}$ . The percentage of Bcl2<sup>low</sup> cells was increased to 82% in virus-infected populations by day 8 PI whereas it remained unchanged in controls and inactivated virus-treated groups (Fig. 3B). We next studied the frequency of active caspase 3<sup>high</sup> cells in  $FSC^{high}$  and  $FSC^{low}$  populations. We found that both  $FSC^{high}$  and  $FSC^{low}$  populations of  $CD14^+$  cells had high levels of active caspase 3 (Fig. 3C). In controls and inactivated virus-treated groups, 65–71% of the cells were  $FSC^{high}$  and active caspase 3<sup>high</sup> whereas 71% of virus-infected cell population were caspase 3<sup>high</sup> and  $FSC^{low}$ . A similar pattern was found on day 8 PI, 62–68% of the cells were  $FSC^{high}$  active caspase 3<sup>high</sup> in controls and inactivated virus-treated populations and 76% of the cells were  $FSC^{low}$  active caspase 3<sup>high</sup> in the Ebola virus-infected population (Fig. 3C). Fig. 3D shows that 63–72% annexin-V<sup>+</sup> cells were  $FSC^{high}$  in controls and inactivated virus-treated group whereas 73% annexin-V<sup>+</sup> cells were  $FSC^{low}$  in the virus-infected group on day 4 PI. This percentage of annexin-V<sup>+</sup> $FSC^{low}$  cells was increased to 90% at day 8 PI in virus-infected population while it remained unchanged in the controls. In addition, using Ebola-GFP virus, we further confirmed that 95–98% of  $CD14^+$  cells were infected on day 4 PI as shown previously (Towner et al., 2005) suggesting that most of the macrophages that were undergoing apoptosis were also infected with Ebola virus.

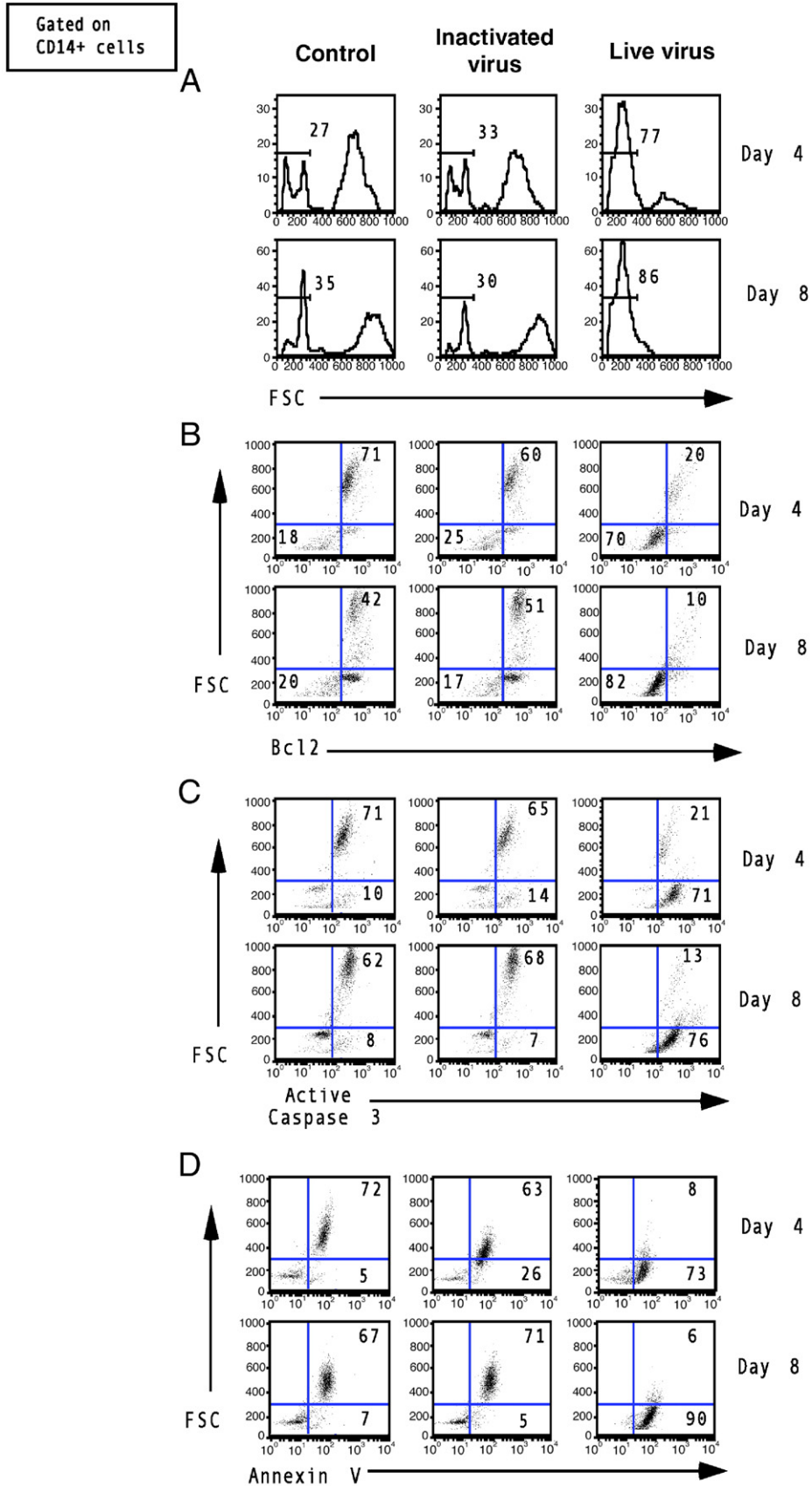
#### *Virus induces apoptosis in Fas and FasL positive T cells and macrophages*

We next determined which population within the CD4, CD8 or CD14 cells was undergoing apoptosis by day 4 PI. To study this, CD4, CD8 or CD14 cells were stained for Fas or FasL and gated on  $FSC^{high}$  and  $FSC^{low}$  populations. Fig. 4A shows that 13% of the  $CD3^+4^+$  cells were Fas<sup>+</sup> and large ( $FSC^{high}$ ) and 4% Fas<sup>+</sup> cells were small ( $FSC^{low}$ ) in controls and inactivated virus-treated populations. This percentage was reversed in the virus-infected group, i.e. 14% cells were Fas<sup>+</sup> $FSC^{low}$  and 4% were Fas<sup>+</sup> $FSC^{high}$ . A similar pattern was found in  $CD3^+8^+$  cells: 8–13% of the cells were  $FSC^{high}$  Fas<sup>+</sup> in control and inactivated virus-treated populations as compared to 5% following virus infection; 11% of the  $CD3^+8^+$  cells were  $FSC^{low}$ Fas<sup>+</sup> as compared to 1 to 2% in controls. Similarly in macrophages, 53–54% cells were Fas<sup>+</sup> $FSC^{high}$  in controls as compared to 11% in the virus inoculated cell populations. The percentage of Fas<sup>+</sup> $FSC^{low}$  cells was 43% in virus-infected populations as compared to 2% in the controls.

Fig. 4B shows the frequency of FasL<sup>+</sup> cells in  $FSC^{high}$  and  $FSC^{low}$  populations in different cell types. The percentage of FasL<sup>+</sup> $FSC^{low}$  cells was 17% in CD4 T cells after viral infection as compared to 7% in the controls (Fig. 4B). At the same time the frequency of FasL<sup>+</sup> $FSC^{high}$  cells was 3% following virus infection as compared to 17% in the controls. Similarly in CD8 T cells, there were a two-fold higher numbers of FasL<sup>+</sup> $FSC^{low}$  cells and a two to three-folds lower numbers of FasL<sup>+</sup> $FSC^{high}$  cells were found in virus-infected population as compared to the controls and inactivated virus-treated populations (Fig. 4B). In the macrophage population, the percentage of FasL<sup>+</sup> $FSC^{low}$  was four times higher in the viral infection group (69%) as compared to the controls (14–19%). At the same time the percentage of  $FSC^{high}$  cells was 21% after virus exposure as compared to 63–65% in the controls. Furthermore we found that Fas<sup>+</sup> $FSC^{low}$ /FasL<sup>+</sup> $FSC^{low}$  cells were also Bcl2<sup>low</sup> (data not shown) in CD4, CD8 and CD14 cells, confirming that these cells are undergoing cell death. These data together suggest that Ebola virus induces apoptosis in already activated cells and in particular targets Fas/FasL positive cells.

#### *Virus induces TRAIL mRNA in CD4 and CD8 T cells but not in macrophages*

We isolated CD4, CD8 and macrophages using magnetic bead columns on day 7 PI. Cells were checked for purity by staining and cell numbers were normalized before lysis. RNase protection assay (RPA) was performed. From the multiple proapoptotic genes included in the multi-probe hApo-3d used, only TRAIL mRNA showed a significant induction. Fig. 5 shows the levels of TRAIL mRNA for purified CD4 and CD8 T cells in uninfected controls, inactivated virus-treated cells and Ebola virus-infected cells. The mRNA levels of housekeeping gene L32 from the same RPA experiment are shown in the bottom panel. It was apparent that in CD4 and CD8 T cells L32 mRNA in virus-infected cells was much less than in control and inactivated virus-treated cells. In spite of that, TRAIL mRNA in



both CD4 and CD8 T cells was higher in virus-infected cells than in the two experimental controls. Quantitative densitometric measurements indicated that in virus-infected CD4 T cells there was approximately a two-fold increase in TRAIL mRNA levels compared to the two control populations (Fig. 5B). In CD8 T cells, TRAIL mRNA levels were approximately 3 times higher than in uninfected control and inactivated virus-treated cells (Fig. 5B). Surprisingly, we did not find any difference in TRAIL mRNA levels in virus-infected macrophages on day 2 and day 7 PI (data not shown) despite previous report on human monocytes using Ebola Zaire 95' subtype by Hensley et al. (Hensley et al., 2002). These data together suggest that T cells probably used the TRAIL pathway for the induction of apoptosis whereas macrophages might be using a pathway other than TRAIL to undergo apoptosis.

## Discussion

In this study, we have provided direct evidence of apoptosis in lymphocytes caused by Ebola virus infection. Our findings suggest that Ebola virus induces apoptosis in CD4 and CD8 T cells. Previous reports have suggested a decrease in CD4 and CD8 T cell numbers in patients with a fatal outcome around day 6 or 8 post-exposure (Baize et al., 1999; Sanchez et al., 2004). Our data clearly indicate that, on day 8 following infection, 30–40% of the CD4 and CD8 T cells are dead. This observation may help to explain why fatal cases do not have anti-Ebola antibodies at death and survivors have a delay in the production of anti-Ebola IgG. Our findings show that Ebola virus causes massive death of CD4 T cells which are required for IgM to IgG switching. Other immune cells like CD8 T cells involved in the control of Ebola infection are also undergoing cell death and cannot control virus levels in the host. A number of other factors like IFN- $\alpha$  could play an important role in controlling infection. We and others have found a decrease in IFN- $\alpha$  levels during infection ((Baize et al., 1999; Gupta et al., 2001). High levels of TNF- $\alpha$ , MCP-1, MIP1- $\alpha$  and other cytokines/chemokines were present between day 1 and 7 post-infection (Bray and Mahanty, 2003). These cytokines may play an important role in recruiting macrophages and T cells at the site of infection and may cause cell death. We have also studied but not reported in this study apoptosis in NK cells and B cells. We did not find any significant difference in Bcl2 and active caspase 3 levels in these populations compared to controls until day 8 PI (data not shown); suggesting that B cells themselves are not affected and may not be responsible for the delay in appearance or low levels of anti-Ebola antibodies. A previous report in macaques has shown similar results on B cell following infection over a period of time (Reed et al., 2004).

FSC<sup>high</sup> T cells have activated phenotype and are dividing, which have been shown previously (Bonnevier and Mueller, 2002; Schmidt and Mescher, 1999). Data in Figs. 1 and 2 were divided in FSC<sup>high</sup> and FSC<sup>low</sup> populations. To confirm that FSC<sup>high</sup> cells are activated, we marked FSC<sup>high</sup> CD3<sup>+</sup>4<sup>+</sup>/FSC<sup>high</sup> CD3<sup>+</sup>8<sup>+</sup> cells for CD25. We found that only FSC<sup>high</sup> cells have higher levels on CD25 as compared to FSC<sup>low</sup> cells (data not shown). Our data indicate that there are two to three-fold higher numbers of Bcl2<sup>low</sup>, active caspase 3<sup>high</sup> and annexin-V<sup>+</sup> CD4 and CD8 T cells present following virus exposure compared to controls. Of note, active caspase 3 and annexin-V are present in both blasts (FSC<sup>high</sup>) and in apoptotic cells (FSC<sup>low</sup>) however there is a significantly higher numbers of apoptotic cells showing active caspase 3 and annexin-V in virus-infected populations than the controls. The numbers of blast cells showing higher levels of active caspase 3 and annexin-V after virus infection do not significantly increase, in fact the staining for these markers is reduced on FSC<sup>high</sup> cells as compared to the controls, suggesting that more death than activation is going on in virus-infected cultures. Our data show that 60–70% of the infected macrophages had lower levels of Bcl2 and were positive for annexin-V. We also found that most of the Ebola-infected macrophage population lost adherence by day 4 PI and 99% of the cells were infected with Ebola virus {determined by Ebola-GFP} suggesting that the virus may directly induce death in macrophages. There are no previous reports of Ebola virus infecting T cells. Recently published study has shown that immunosuppressive glycopeptide in Ebola virus can cause cell death without infection (Yaddanapudi et al., 2006). The mechanisms of apoptosis in macrophages and T cells are still unknown.

Our findings show that Ebola virus induces apoptosis in Fas<sup>+</sup> and FasL<sup>+</sup> T cells and macrophages. In all the cell types studied, we saw a significant reduction in the numbers of blast cells positive for Fas/FasL. All blast cells which were positive for Fas/FasL in the infected cultures on the top right panel in Figs. 4A and B moved down in the quadrant (Figs. 4A and B, top right panel to bottom right panel) in virus challenged group and were much smaller in size. These small cells (FSC<sup>low</sup>Fas<sup>+</sup>) also have low levels of Bcl2 (data not shown) suggesting that they were undergoing cell death following virus challenge. Of note the total numbers of Fas<sup>+</sup>/FasL<sup>+</sup> cells did not change i.e. the number of Fas<sup>+</sup>FSC<sup>low</sup> plus Fas<sup>+</sup>FSC<sup>high</sup> cells in virus-infected cultures was not very different from total Fas<sup>+</sup> cells in the controls. This indicates that there is no induction of Fas/FasL on T cells and macrophages. Previous reports have shown upregulation of Fas mRNA levels in infected patients (Baize et al., 1999) and macaques (Reed et al., 2004). Another report mentions no difference in Fas levels in infected patients as compared to controls (Sanchez et al., 2004). By using the flow

Fig. 3. Ebola virus kills the majority of macrophages by day 8 PI. PBMCs, either infected with Ebola virus or treated with gamma-irradiated killed virus or uninfected cells, were stained for CD14, Bcl2, active caspase 3 and annexin-V markers at day 4 and day 8 PI. The percentages of cells are indicated in each quadrant. Panel A shows forward scatter for CD14<sup>+</sup> cells. Panel B shows Bcl2 levels in FSC<sup>low</sup> and FSC<sup>high</sup> cells on CD14<sup>+</sup> cells. Panel C shows staining for active caspase 3 in FSC populations. Panel D shows annexin-V staining in different FSC populations. Isotype control values were the same as mentioned in Fig. 1. Data together indicate that 70% of macrophages are Bcl2<sup>low</sup>, active caspase 3<sup>high</sup> and annexin-V<sup>+</sup> by day 4 PI after virus challenge and are FSC<sup>low</sup>. It also indicates that most of the macrophages are shrinking after virus exposure. This experiment is repeated five times.

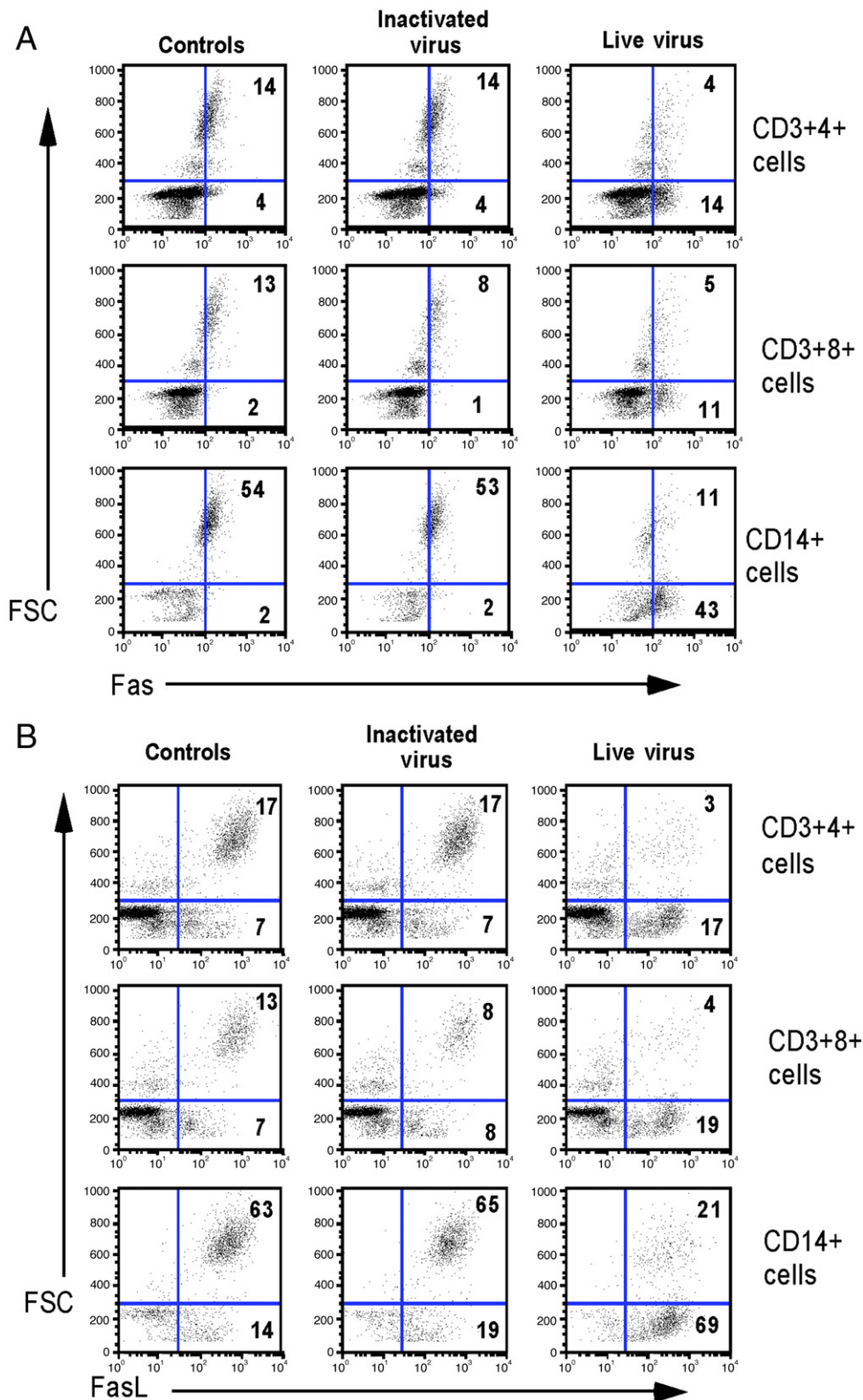


Fig. 4. Ebola virus induces apoptosis in Fas<sup>+</sup>/FasL<sup>+</sup> cells. Panel A shows Fas expression in CD4, CD8 T cells and in macrophages. Respective cell populations were screened for Fas levels in FSC<sup>low</sup> and FSC<sup>high</sup> cells. The percentages of positive cells are indicated in each quadrant. Mean fluorescent intensity of isotype control for Fas was 50±10 (mean±SD) and for FasL was 30±10 from three different experiments. Panel B indicates FasL levels in CD4, CD8 T cells and macrophages. FasL levels plotted against forward scatter. This figure is a representative of three independent experiments. Data suggest that Ebola virus might target Fas<sup>+</sup>/FasL<sup>+</sup> T cells and macrophages and may induce death signal in these populations.



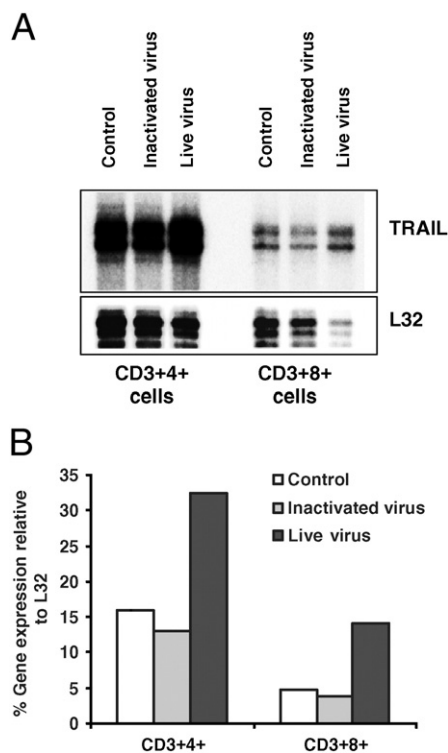


Fig. 5. Induction of TRAIL mRNA in Ebola virus-infected CD4 and CD8 T cells. RNase protection assay was performed in total RNA isolated from CD3<sup>+</sup>4<sup>+</sup> and CD3<sup>+</sup>8<sup>+</sup> cells treated with media, gamma-irradiated inactivated Ebola virus or infected with Ebola virus at day 7 PI using hApo-3d multi-probe (A). Quantitative densitometric measurements of TRAIL gene expression shown in panel B presented as a percentage of housekeeping L32 gene expression. The levels of TRAIL mRNA among samples were determined by using the phosphorImager system. The data are representative of two independent experiments.

cytometry our data clearly show that there was no difference in protein levels of Fas and FasL on various cell types.

TRAIL has been shown to play an important role in apoptosis in HIV-infected CD4 T cells (Miura and Koyanagi, 2005) and in primary small airway and tracheal–bronchial cells by respiratory syncytial virus (Kotelkin et al., 2003). Our study clearly shows a significant increase in TRAIL mRNA levels in CD4 and CD8 T cells on day 7 PI. A significant number of cells were dying after virus exposure suggesting that TRAIL might be responsible for apoptosis in these cells. Surprisingly, we did not find any difference in TRAIL levels in infected macrophages from that of controls suggesting that TRAIL is not responsible for apoptosis in these cells. We know that virus infection in macrophages upregulates TNF- $\alpha$  and many other cytokines (Gupta et al., 2001). The possible role of TNF- $\alpha$ , Fas, FasL or TRAIL in the induction of apoptosis in PBMCs infected with Ebola virus needs further study.

Our data clearly indicate that Ebola virus causes massive death of human CD4 and CD8 T cells and clarifies why there is a delay in the production of anti-Ebola IgG in infected patients. In addition these data show that macrophages which are the reservoir for the virus are also dying early in infection. Further studies are needed to elucidate the details of the mechanisms of virus-mediated apoptosis in T cells and macrophages.

## Materials and methods

### Isolation of PBMCs and infection

A blood sample from healthy individuals was collected and layered on ficoll gradient medium (ICN Biomedicals, Aurora, OH). Purified PBMCs were counted and cultured on 6-well plates at  $12 \times 10^6$  cells/well in RPMI medium with 10% FCS supplemented with antibiotics and L-glutamine. Cells were incubated for 1 h to allow macrophage adherence. After removing the non-adherent cells, adherent cells were either infected with the Mayinga strain of Zaire Ebola virus (passed twice in Vero E6 cells) at a multiplicity of infection (MOI) of 0.5 or treated with same amount of inactivated virus (gamma irradiation:  $5 \times 10^6$  rads) for 1 h, along with uninfected controls as previously described (Gupta et al., 2001). After incubation, non-adherent cells were added back to the inoculated cells; activation and apoptosis marker staining was done on specific days PI. Using the same MOI of an Ebola-GFP virus we found, in a separate experiment, that 90% of the macrophages were infected by day 4 PI as shown previously (Towner et al., 2005). All the virus manipulations were done in a biosafety level (BSL)-4 laboratory at the Centers for Disease Control and Prevention in Atlanta.

### Surface and intracellular staining

On days 4 and 8 PI, adherent and non-adherent cells were resuspended and distributed in 96 well plates at  $1 \times 10^6$  cells/well. Cells were labeled as previously described (Gupta et al., 2005). For surface markers, cells were incubated with 50  $\mu$ l of anti-CD3-PerCP, CD4-APC, CD8-APC, CD19-APC, CD14-APC, CD56-APC, Fas-FITC, FasL-PE and isotype controls for 30 min on ice. All antibodies were purchased from BD Pharmingen (San Diego, CA). After surface staining, cells were washed and permeabilized using a cytofix kit (BD Pharmingen, CA) according to the manufacturer's instructions. After permeabilization, cells were incubated with active caspase-3-PE, Bcl2-PE and isotype controls for 30 min on ice. Un-adsorbed antibodies were washed away by centrifugation and cells were fixed with 0.2% paraformaldehyde and stored at 4  $^{\circ}$ C until processing. Approximately 50,000 cells were counted in each sample. Cells were gated on CD3, CD4, CD8 and CD14 markers above MFI values of their isotype controls (MFI of 20, 30, 30 and 40 respectively).

To further study apoptosis, cells were also stained for annexin-V in conjunction with CD3-PE, CD4-APC, CD8-APC, CD19-APC, CD14-APC and CD56-APC markers. All cell staining was performed in the BSL-4 laboratory. Cell signals were acquired on FACScalibur inside the BSL-4 laboratory and analyzed on FACS FlowJo (BD Biosciences) software.

### Isolation of CD4, CD8 and macrophages using magnetic beads

100–120  $\times 10^6$  PBMCs from infected, uninfected and killed virus-treated cultures were incubated with CD14-magnetic beads (Milteneyi Biotech, Auburn, CA) for 15 min on ice.

After incubation, the cells were run through a pre-washed column according to the manufacturer's instructions. The unbound fraction was collected in separate tubes. The column was removed from the magnet and the retained fraction was washed out. Bound and unbound fractions were pelleted and then counted. CD14 negative cells were then incubated with CD4 beads following the same procedure. The CD14/CD4 negative cells were incubated with CD8 beads and the same procedure was repeated. An aliquot of each fraction was tested for purity by staining with their respective marker antibodies. Cell signals were acquired on the flow cytometer (FACScalibur) and analyzed on FlowJo software. Each fraction was 90–95% pure using a flow cytometry analysis. Cells were normalized and lysed using Tri-pure reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN) for further study of apoptotic signals.

#### *RNase protection assay*

Total RNA from each experimental group was extracted using Tri-Pure reagent (1 ml per  $5 \times 10^6$  cells) followed by chloroform extraction and matrix purification (Rnaid kit Bio-101, La Jolla, CA). The levels of proapoptotic gene transcripts were then determined using RiboQuant RPA kit and probe hApo-3d (PharMingen, San Diego, CA) following instructions provided by the manufacturer. Quantitative measurements of the levels of mRNA were made using the phosphoImager system. The values obtained were normalized against the mRNA of the housekeeping gene L32 loaded in the same lane.

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