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Implication of a retrovirus-like glycoprotein peptide in the immunopathogenesis of Ebola and Marburg viruses

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ABSTRACT Ebola and Marburg viruses can cause hemorrhagic fever (HF) outbreaks with high mortality in primates. Whereas Marburg (MARV), Ebola Zaire (ZEBOV), and Ebola Sudan (SEBOV) viruses are pathogenic in humans, apes, and monkeys, Ebola Reston (REBOV) is pathogenic only in monkeys (1–3). Early immunosuppression may contribute to pathogenesis by facilitating viral replication (4–6). Lymphocyte depletion, intravascular apoptosis, and cytokine dysregulation are prominent in fatal cases (7). Here we functionally characterize a 17 amino acid domain in filoviral glycoproteins that resembles an immunosuppressive motif in retroviral envelope proteins (8, 9). Activated human or rhesus peripheral blood mononuclear cells (PBMC) were exposed to inactivated ZEBOV or a panel of 17mer peptides representing all sequenced strains of filoviruses, then analyzed for CD4+ and CD8+ T cell activation, apoptosis, and cytokine expression. Exposure of human and rhesus PBMC to ZEBOV, SEBOV, or MARV peptides or inactivated ZEBOV resulted in decreased expression of activation markers on CD4 and CD8 cells; CD4 and CD8 cell apoptosis as early as 12 h postexposure; inhibition of CD4 and CD8 cell cycle progression; decreased interleukin (IL)-2, IFN- γ , and IL12-p40 expression; and increased IL-10 expression. In contrast, only rhesus T cells were sensitive to REBOV peptides. These findings are consistent with the observation that REBOV is not pathogenic in humans and have implications for understanding the pathogenesis of filoviral HF.—Yaddanapudi, K., Palacios, G., Towner, J. S., Chen, I., Sariol, C. A., Nichol, S. T., Lipkin, W. I. Implication of a retrovirus-like glycoprotein peptide in the immunopathogenesis of Ebola and Marburg viruses. *FASEB J.* 20, 2519–2530 (2006)

Key Words: *filovirus* • *immunosuppression* • *lymphocyte depletion* • *apoptosis* • *cytokine*

THE FILOVIRUSES CAUSE hemorrhagic fevers with high levels of fatality. They are classified in two genera within

the family *Filoviridae*: *Ebola virus* (EBOV) and *Marburg virus* (MARV). Four species of Ebola virus are currently recognized: *Zaire*, *Sudan*, *Reston*, and *Ivory Coast*. Ebola virus species *Zaire* (ZEBOV) and *Sudan* (SEBOV) as well as *Marburg* (MARV) are highly pathogenic in human and nonhuman primates, with case fatality levels of up to 90%. Ebola virus species *Reston* (REBOV) is pathogenic in monkeys but does not cause disease in humans or great apes (1–3). Fatal outcome in filoviral infection is associated with an early reduction in the number of circulating T cells, failure to develop specific humoral immunity, and the release of proinflammatory cytokines (7, 10, 11). The membrane-anchored filoviral glycoprotein (GP) is present on the surface of virions and infected cells; GP mediates receptor binding and fusion. Filoviral GPs are considered to be major viral pathogenic determinants and contribute to both immunosuppression and vascular dysregulation (12–14).

The transmembrane glycoproteins of many animal and human retroviruses share structural features, including a conserved region that has strong immunosuppressive properties (15, 16). CKS17, a synthetic peptide corresponding to this domain in oncogenic retroviruses, has been used to dissect the pathophysiology of immunosuppression (17, 18). CKS17 causes an imbalance of human type-1 and type-2 cytokine production, suppresses cell-mediated immunity (19), and blocks the activity of protein kinase C, a cellular messenger involved in T cell activation (20, 21). During the course of establishing a microbial sequence database to support development of tools for surveillance and differential diagnosis of infectious diseases, we discovered a region of strong secondary structure conservation between the C-terminal domain of the envelope glycoprotein of filoviruses and CKS17 (Supplemental

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Fig. 1). An alignment of the filoviral glycoprotein and retroviral immunosuppressive domains illustrated primary sequence similarity between a wide range of retroviruses and filoviruses. Three cysteine residues implicated in disulfide bonding were also conserved, reinforcing similarities at the level of secondary structure. Here we report functional analysis of the putative immunosuppressive domain in various species of EBOV and MARV and demonstrate that the immunosuppressive effect of different species of GP peptides is consistent with pathogenicity observed in different animal hosts.

MATERIALS AND METHODS

Design of the synthetic peptides

Filoviral 17-mer peptides corresponding to the immunosuppressive domain were synthesized at the Northeast Biodefense Center Proteomics Core-Keck Laboratory, Yale University. Identification of the region with strong secondary structure similarity to the retrovirus glycoprotein was done using the program 3D-PSSM (22) (Supplemental Fig. 1). The ZEBOV peptide is ILNRKAIDFLLQRWGGT. ZEBOV and SEBOV differ by one residue at position 12 (ZEBOV, glutamine; SEBOV, arginine); both have isoleucine at position 1. REBOV differs from ZEBOV only in the presence of leucine at position 1.

Cell culture

Human PBMC were isolated from heparinized venous blood of healthy volunteers by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ, USA). Monkey PBMC were separated from heparin-treated peripheral blood collected from healthy adult rhesus (*Macaca mulatta*) macaques using a similar procedure. Human PBMC were suspended at 10^6 /ml in RPMI 1640 supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA, USA) and cultured in the presence of soluble anti-human CD28 at 2 μ g/ml on plates coated with anti-human CD3 antibody (Ab) at 10 μ g/ml (anti-CD3/CD28) alone (23); anti-CD3/CD28 and inactivated ZEBOV (inactZEBOV; equivalent of 25 infectious units per cell prior to γ -irradiation using 5×10^6 rads); or anti-CD3/CD28 and filoviral peptides at 40 μ M concentration. Cells were incubated at 37°C in 5% CO₂ for 12 or 48 h prior to analysis. Conditions were similar for experiments with rhesus PBMC except that cells were activated by culture on plates coated with anti-human CD3 epsilon Ab (anti-CD3 ϵ , clone: SP34, cross-reactive with rhesus CD3) (24) at 10 μ g/ml.

Cell surface phenotype

All monoclonal antibodies (mAbs) used in FACS analyses were generated using human antigens; some were human-specific (Caltag): Anti-CD4-APC (clone: S3.5, isotype: mouse IgG2a), anti-CD8-APC (clone: 3B5, isotype: mouse IgG2a), anti-CD25-FITC (clone: CD25-3G10, isotype: mouse IgG₁), anti-CD4-PE (clone: S3.5, isotype: mouse IgG2a) and anti-CD69-FITC (clone: CH/4, isotype: mouse IgG2a); others were cross reactive with macaque (25, 26): anti-CD4-PE (clone: L200, isotype: mouse IgG_{1,κ}), anti-CD8-APC-CY7 (clone: rNase protection assay (RPA)-T8, isotype: mouse IgG_{1,κ}) and anti-CD69-FITC (clone: FN50, isotype: mouse

IgG_{1,κ}) (BD PharMingen, San Diego, CA, USA). At 12 or 48 h, PBMC were stained for surface expression of CD4, CD8, CD25, and CD69 using the relevant mAbs. Cells were washed twice with RPMI 1640 medium supplemented with 0.5% FBS (wash medium). 1×10^6 cells were then incubated with fluorochrome-tagged primary Ab in a total volume of 0.1 ml for 30 min at 4°C. Cells were subsequently washed twice with 2 ml of wash medium to remove any unbound Ab and fixed in 0.5 ml of 1% paraformaldehyde solution. Cells were then analyzed by multicolor flow cytometry on a LSRII Analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). Data was obtained using FACS DiVa acquisition software (Becton Dickinson) and analyzed using FlowJo6.1 (Tree Star) after appropriate gating to exclude dead cells and debris based on forward scatter and side scatter. Fluorescent markers used were APC (allophycocyanin), FITC (fluorescein isothiocyanate), PE (phycoerythrin), and APC-CY7 (allophycocyanine 7).

5-bromo-2-deoxyuridine (bromodeoxyuridine) labeling and cell cycle analysis

Intracellular bromodeoxyuridine (BrdU) was measured using a commercial assay (BrdU Flow Kit, BD Biosciences, Bedford, MA, USA). Human PBMC were activated with anti-CD3/CD28 in the absence or presence of filoviral peptides for 48 h. Three hours before harvest, 10 μ M of BrdU was added to each well. Cells were resuspended in 50 μ l of staining buffer (PBS+3.0% FBS). Fluorescent antibodies specific to detect CD4 and CD8 were added. Cells were fixed, permeabilized, and treated with DNase (30 μ g per tube) to expose incorporated BrdU. Intracellular BrdU was stained with anti-BrdU-FITC Ab. Cells were washed and 20 μ l of 7-amino-actinomycin D (7-AAD) solution was added for staining of total DNA. Cells were resuspended in staining buffer and analyzed by flow cytometry.

Apoptosis assays

PBMC were stained for surface expression of CD4 and CD8 using the relevant mAbs. Cells were washed twice with PBS, resuspended in 0.1 ml Annexin V binding buffer (BD Biosciences), and incubated with 5 μ l of FITC-conjugated Annexin V (BD Biosciences) and 10 μ l of propidium iodide (PI) for 15 min at room temperature. The cells were immediately analyzed by flow cytometry on a FACSCalibur (Becton Dickinson). Data were obtained using CellQuest acquisition software (Becton Dickinson) and analyzed using FlowJo6.1 (Tree Star, Ashland, OR, USA). Cells stained with Annexin V-FITC alone and PI alone were used as controls.

Cytokine assays

Cell-free supernatants from PBMC cultures were collected and analyzed using the Beadlyte Human 11-Plex Cytokine Detection System (Upstate Biotechnology, Lake Placid, NY, USA). The lyophilized mixed standard was resuspended in cell culture medium and serially diluted. Samples or standards were incubated with the 11-Plex cytokine capture bead suspension array in a 96-well filter plate for 2 h at room temperature. The beads were washed and biotinylated reporter 11-plex antibodies were added for 1.5 h. Streptavidin-PE was then added to each well. After a 30 min incubation, the beads were washed and resuspended in assay buffer. The median fluorescence intensity of 100 beads per cytokine was read using a Luminex 100 Instrument. Concentrations were interpolated from standard curves.

Statistical analysis

All statistical analyses were performed using InStat 3 (GraphPad Software). Data from all FACS assays (cell surface phenotype, BrdU incorporation, cell cycle analysis, apoptosis) were first tested for normal distribution by the Kolmogorov and Smirnov (K-S) test, then analyzed for significance using ANOVA and Dunnett's specialized multiple comparison test. Cytokine assays were analyzed using Kruskal-Wallis nonparametric ANOVA and the Dunn multiple comparison test. Cytokine data were fitted on a sigmoidal dose-response curve.

RESULTS

We assessed the effect of synthetic 17mer peptides corresponding to the candidate immunosuppressive domain in filoviral glycoproteins. Human PBMC were exposed for 48 h to either inactZEBOV or 40 μ M filoviral peptides in the presence of anti-CD3/CD28. Flow cytometric analysis revealed a significant decrease in the percentage of cells positive for CD4 and CD8

after treatment with inactZEBOV or ZEBOV, SEBOV, or MARV peptide, but not REBOV peptide (Fig. 1A, B).

ZEBOV peptide treatment also reduced the amount of CD4 and CD8 expressed on the cell surface of human PBMC. Exposure to ZEBOV peptide resulted in a 3.5-fold reduction in cell surface expression of CD4 and a 4.2-fold reduction in the cell surface expression of CD8 (CD4 expression with ZEBOV peptide, $n=5$: mean fluorescence intensity value for CD4 expression \pm SD of the mean, 961 ± 40 ; CD4 expression without ZEBOV peptide, $n=5$: $3,360 \pm 145$; $P<0.01$; CD8 expression with ZEBOV peptide, $n=5$, mean fluorescence intensity value for CD8 expression \pm SD: $4,025 \pm 75$; CD8 expression without ZEBOV peptide, $n=5$: $17,027 \pm 565$; $P<0.01$; Fig. 1C). A similar decrease in CD4 and CD8 expression was observed on PBMC treated with SEBOV or MARV peptides (data not shown). No decrease in the expression levels of CD4 or CD8 was observed with REBOV peptide treatment (Fig. 1C).

ZEBOV peptide caused a significant decline in the

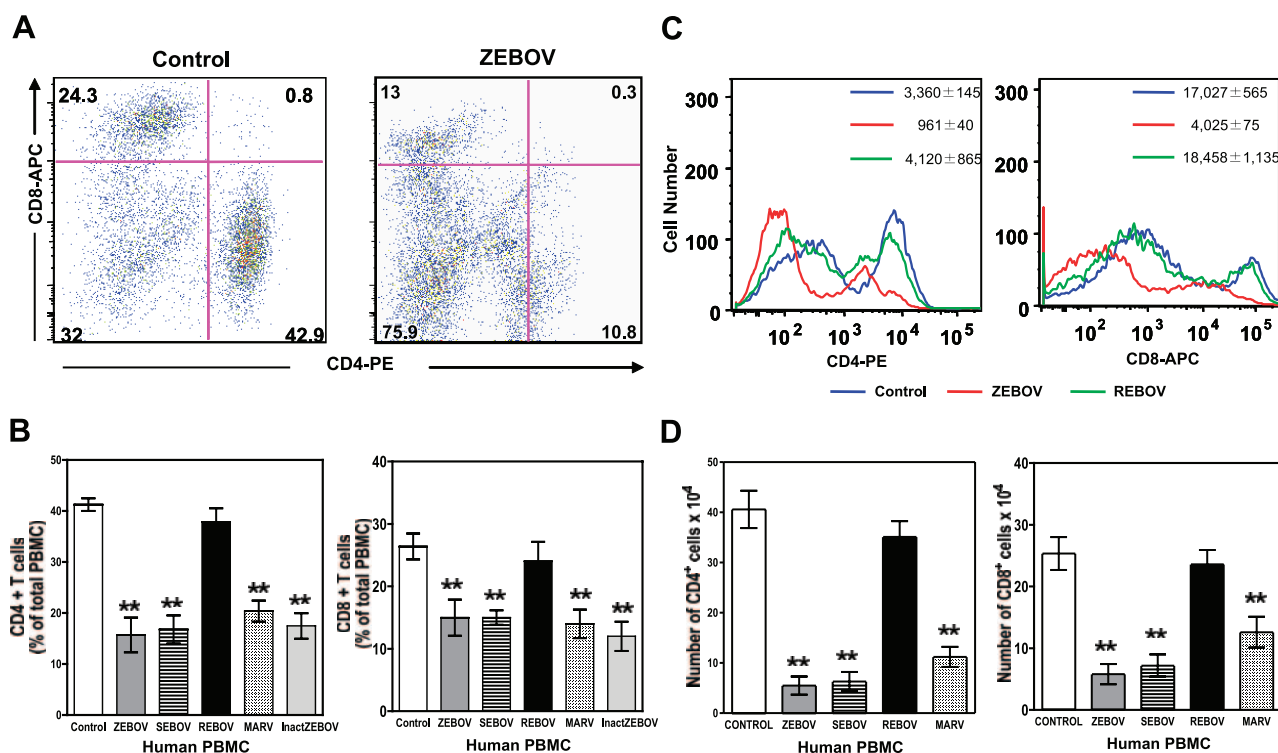


Figure 1. Depletion and inactivation of human T lymphocytes after exposure to inactivated filovirus or filoviral peptides. PBMC were exposed to filoviral peptides, inactZEBOV, or to neither peptide nor virus for 48 h in the presence of anti-CD3/CD28. After staining with antibodies to CD4 and CD8, cells were analyzed by flow cytometry. A) Dot plots of CD4⁺ vs. CD8⁺ lymphocytes after activation with anti-CD3/CD28 alone (control), or anti-CD3/CD28 and ZEBOV peptide. Experiments were performed with PBMC from 5 different donors; data from one representative donor are shown. Numbers in quadrants represent the percentages of each subpopulation. B) Percentages of CD4⁺ and CD8⁺ lymphocyte subsets in PBMC following activation with anti-CD3/CD28 antibodies alone or anti-CD3/CD28 and either filoviral peptides or inactZEBOV. Results are expressed as percentage of total PBMC. Values represent mean \pm SD calculated from 5 different donors in each sample group. C) Representative histogram showing cell surface expression of CD4 and CD8 markers on PBMC activated with anti-CD3/CD28 alone, or anti-CD3/CD28 and ZEBOV peptide. Numbers indicate the mean fluorescence intensity of CD4 or CD8 expression \pm SD. Values were derived from 5 donors in each sample group. D) Absolute numbers of CD4⁺ and CD8⁺ T cells following activation with anti-CD3/CD28 alone or anti-CD3/CD28 and filoviral peptide. Data represent mean \pm SD calculated from 5 different donors in each sample group. ** $P<0.01$ (relative to control samples; ANOVA and Dunnett's test for multiple comparisons).

absolute numbers of both CD4⁺ and CD8⁺ T cells. Exposure to ZEBOV peptide resulted in a 7.4-fold decrease in the number of CD4⁺ T cells and a 4.4-fold decrease in the number of CD8⁺ T cells (number of CD4⁺ T cells with ZEBOV peptide, $n=5$: $5.5 \pm 1.8 \times 10^4$; number of CD4⁺ T cells without ZEBOV peptide, $n=5$: $40.6 \pm 3.7 \times 10^4$; $P<0.01$; number of CD8⁺ T cells with ZEBOV peptide, $n=5$: $5.8 \pm 1.6 \times 10^4$; number of CD8⁺ T cells without ZEBOV peptide, $n=5$: $25.4 \pm 2.6 \times 10^4$; $P<0.01$; Fig. 1D). A similar decline in absolute T cell numbers was also observed with SEBOV or MARV peptide treatment. REBOV peptide exposure caused no significant depletion of T cells (Fig. 1D).

To further characterize the immunosuppression observed with the filoviral peptides, we evaluated the phenotypic status of PBMC exposed to filoviral peptides. The interleukin (IL)-2 receptor α chain (IL-2R) is an essential component of high-affinity IL-2 receptors. Whereas resting T cells do not express high-affinity IL-2R, receptors are rapidly expressed on T cells after activation with antigen or mitogens (27). The interaction of IL-2 with IL-2R triggers proliferation. IL-2R expression (CD25) was measured on human PBMC activated with anti-CD3/CD28 in the presence or absence of filoviral peptides (Fig. 2). ZEBOV peptide treatment resulted in a reduction in the percentages of

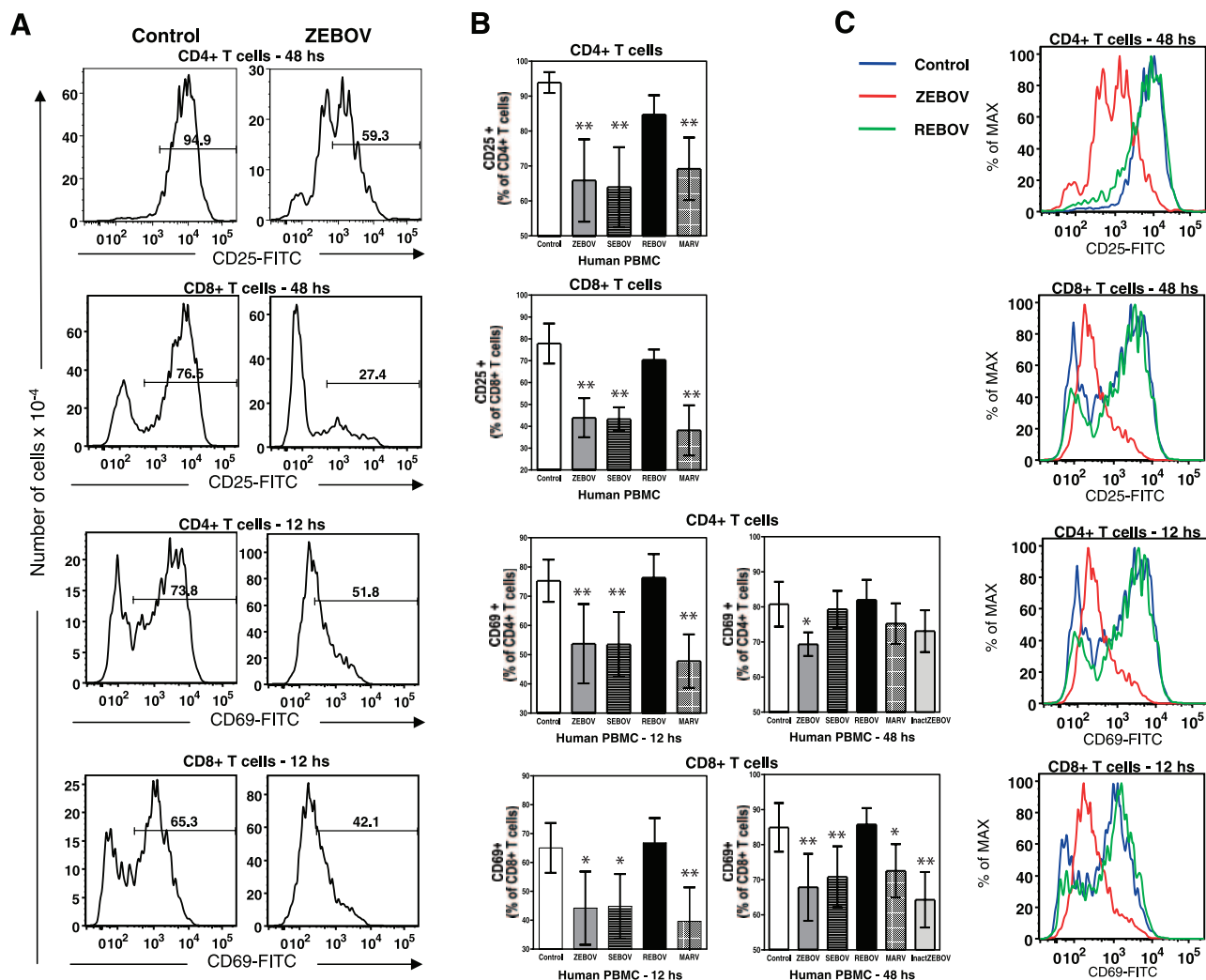


Figure 2. Inactivation of human T lymphocytes after exposure to inactivated filovirus or filoviral peptides. PBMC were exposed to filoviral peptides, inactZEBOV, or to neither peptide nor virus for 12 or 48 h in the presence of anti-CD3/CD28. After staining with antibodies to CD4, CD8, CD25, and CD69, cells were analyzed by flow cytometry. A) Histograms represent expression of activation markers in PBMC activated with anti-CD3/CD28 alone or anti-CD3/CD28 and ZEBOV peptide. Data were obtained from 5 different donors; data from one representative donor are shown. Numbers in gates represent the percentages of CD25 or CD69 positive cells in the CD4⁺ T or CD8⁺ T cell subpopulations, respectively. B) Percentages of CD25 or CD69 positive cells in CD4⁺ and CD8⁺ lymphocyte subsets following activation with anti-CD3/CD28 alone or anti-CD3/CD28 in the presence of inactZEBOV or filoviral peptide. Results are expressed as percentages of CD4⁺ or CD8⁺ T cells. Values represent mean \pm SD calculated from 5 different donors in each sample group. Data for CD69 expression were obtained 12 and 48 h after activation. * $P<0.05$; ** $P<0.01$ (relative to control samples; ANOVA and Dunnett's test for multiple comparisons). C) Representative histogram showing cell surface expression of CD25 and CD69 markers on PBMC activated with anti-CD3/CD28 alone, or anti-CD3/CD28 and ZEBOV peptide. The mean fluorescence intensity is represented as a percentage of the maximum expression. Data were obtained from 5 different donors; data from one representative donor are shown.

CD25+ cells in both CD4+ and CD8+ T cell populations (percentage of CD4+ T cells treated with ZEBOV peptide that are CD25+, $n=5$: $65.9 \pm 11.8\%$; without ZEBOV peptide, $n=5$: $93.9 \pm 3.0\%$; $P < 0.01$; percentage of CD8+ T cells treated with ZEBOV peptide that are CD25+, $n=5$: $43.9 \pm 9.0\%$; without ZEBOV peptide, $n=5$: $77.9 \pm 9.2\%$; $P < 0.01$; Fig. 2A, B). Similar effects on IL-2R expression were obtained in both CD4+ and CD8+ T cells after exposure to SEBOV or MARV peptides. No effect was observed with the nonpathogenic strain, REBOV (Fig. 2B). The mean fluorescent intensity of CD25 expression was also decreased on CD4+ and CD8+ T cells treated with ZEBOV peptide but not REBOV peptide (Fig. 2C).

Lymphocyte activation in response to polyclonal mitogens, antibodies, or antigens is characterized by co-ordinated surface expression of activation/adhesion molecules. CD69 expression was used as a marker for T cell activation (28) after exposure to anti-CD3/CD28 in the presence of inactZEBOV or filoviral peptides. Exposure for 48 h to ZEBOV peptide resulted in a decrease in the percentages of CD69+ cells in both CD4+ and CD8+ T cell populations (percentage of CD4+ T cells treated with ZEBOV that are CD69+, $n=5$: $69.4 \pm 3.4\%$; without ZEBOV, $n=5$: $80.8 \pm 6.4\%$; $P < 0.05$; percentage of CD8+ T cells treated with ZEBOV that are CD69+, $n=5$: $67.9 \pm 9.6\%$; without ZEBOV, $n=5$: $84.9 \pm 6.9\%$; $P < 0.05$; Fig. 2B). Exposure for 48 h to SEBOV or MARV peptides or inactZEBOV resulted in a significant reduction in the percentage of CD69+ CD8+ T cells; a trend toward reduction was observed in CD69+ CD4+ T cells that did not achieve statistical significance (Fig. 2B). Exposure for 12 h to ZEBOV, SEBOV, or MARV peptide resulted in a significant reduction in percentages of both CD69+ CD4+ T cells and CD69+ CD8+ T cells (Fig. 2A, B). No effect was observed with REBOV peptide at either 12 or 48 h (Fig. 2B). The mean fluorescent intensity of CD69 expression was also decreased on CD4+ and CD8+ T cells treated for 12 h with ZEBOV peptide but not REBOV peptide (Fig. 2C).

Proliferative responses of T lymphocytes exposed to filoviral peptides were assessed by flow cytometric measurement of BrdU incorporation. Human PBMC were treated with anti-CD3/CD28 in the presence or absence of ZEBOV peptide or REBOV peptide for 48 h. BrdU was added for the final 3 h of culture. ZEBOV peptide treatment resulted in decreased BrdU labeling of CD4+ and CD8+ T cells (percentage of BrdU labeled CD4+ cells treated with ZEBOV peptide, $n=5$: $3.9 \pm 0.6\%$; without ZEBOV peptide, $n=5$: $14.4 \pm 2.3\%$; $P < 0.01$; percentage of BrdU labeled CD8+ cells treated with ZEBOV peptide, $n=5$: $4.8 \pm 1.8\%$; without ZEBOV peptide, $n=5$: $9.4 \pm 0.9\%$; $P < 0.01$; Fig. 3A, B). No significant change in BrdU labeling was observed with REBOV peptide (Fig. 3A, B). Cell cycle analysis of PBMC treated with ZEBOV peptide and anti-CD3/CD28 revealed an increase in the hypodiploid population together with decreased cell cycle progression (Fig. 3C). ZEBOV-peptide treated PBMC showed an

8.8-fold increase in the number of cells with hypodiploid DNA content (percentage of peptide-treated cells with hypodiploid DNA, $n=5$: $22.0 \pm 2.2\%$; untreated cells, $n=5$: $2.5 \pm 0.1\%$; $P < 0.01$) consistent with an induction in apoptosis (29) (Fig. 3C). A 3.5-fold decrease in the percentage of cells in the S phase was observed with ZEBOV peptide treatment (percentage of peptide-treated cells in S phase, $n=5$: $8.0 \pm 0.5\%$; untreated cells, $n=5$: $28.1 \pm 2.4\%$; $P < 0.01$), suggesting a decrease in the numbers of actively cycling cells. No change in the cycling pattern was observed with PBMC treated with REBOV peptide (Fig. 3C). These results suggest that filoviral peptide treatment may reduce the numbers of T cells by depression of proliferative responses as well as by induction of apoptosis.

Profound lymphopenia and lymphoid depletion due to apoptosis are characteristic features of fatal filoviral infections (7). Apoptosis may be independent of viral replication (30, 31). Treatment of human PBMC with inactZEBOV for 48 h in the presence of anti-CD3/CD28 resulted in a 2.9-fold increase in apoptotic cells in the CD4+ population and a 2.1-fold increase in the CD8+ population (percentage of Annexin V+ PI- CD4+ exposed to inactZEBOV, $n=5$: $41.0 \pm 3.3\%$; untreated cells, $n=5$: $14.3 \pm 2.3\%$, $P < 0.01$; percentage of Annexin V+ PI- CD8+ exposed to inactZEBOV, $n=5$: $30.1 \pm 1.9\%$; untreated cells, $n=5$: $14.2 \pm 2.1\%$, $P < 0.01$; Fig. 4A). ZEBOV peptide treatment also resulted in induction of apoptosis in both CD4+ and CD8+ T cells (Fig. 4B–D). Human PBMC were exposed to ZEBOV peptide in the presence of anti-CD3/CD28 for 12 h and subjected to flow cytometric analysis. Viable PBMC were gated according to forward scatter (FSC) and side scatter (SSC) profile (R1 gate, Fig. 4B). Live (R1) cells were further gated on CD4+ cells according to CD4 expression and FSC and on CD8+ cells according to CD8 expression and FSC (R5 gate; Fig. 4C, D). The percentages of apoptotic cells in CD4+ and CD8+ T cell populations were determined by Annexin V/PI staining. Cells positive for Annexin V and negative for PI were considered apoptotic (Fig. 4C, D). ZEBOV peptide treatment resulted in a 3.6-fold increase in apoptotic CD4+ cells and a 2.0-fold increase in apoptotic CD8+ cells (percentage of Annexin V+ PI- CD4+ treated with ZEBOV peptide, $n=5$: $43.6 \pm 5.8\%$; untreated cells, $n=5$: $12.2 \pm 1.9\%$; $P < 0.01$; percentage of Annexin V+ PI- CD8+ treated with ZEBOV peptide, $n=5$: $29.7 \pm 3.7\%$; untreated cells, $n=5$: $14.9 \pm 2.1\%$; $P < 0.01$; Fig. 4C, D). Effects were similar with human PBMC exposed to SEBOV or MARV peptides (Fig. 4E). No significant induction of apoptosis was observed after treatment with REBOV peptide (Fig. 4B, C–E). Taken together, these data implicate apoptosis in T cell depletion after filoviral peptide exposure and are consistent with the observation that whereas ZEBOV, SEBOV, and MARV are pathogenic for humans, REBOV is not.

Cytokines and chemokines play important roles in immunopathological processes and normal immune response. In addition, there is evidence for the involvement of inflammatory mediators in the pathogenesis of

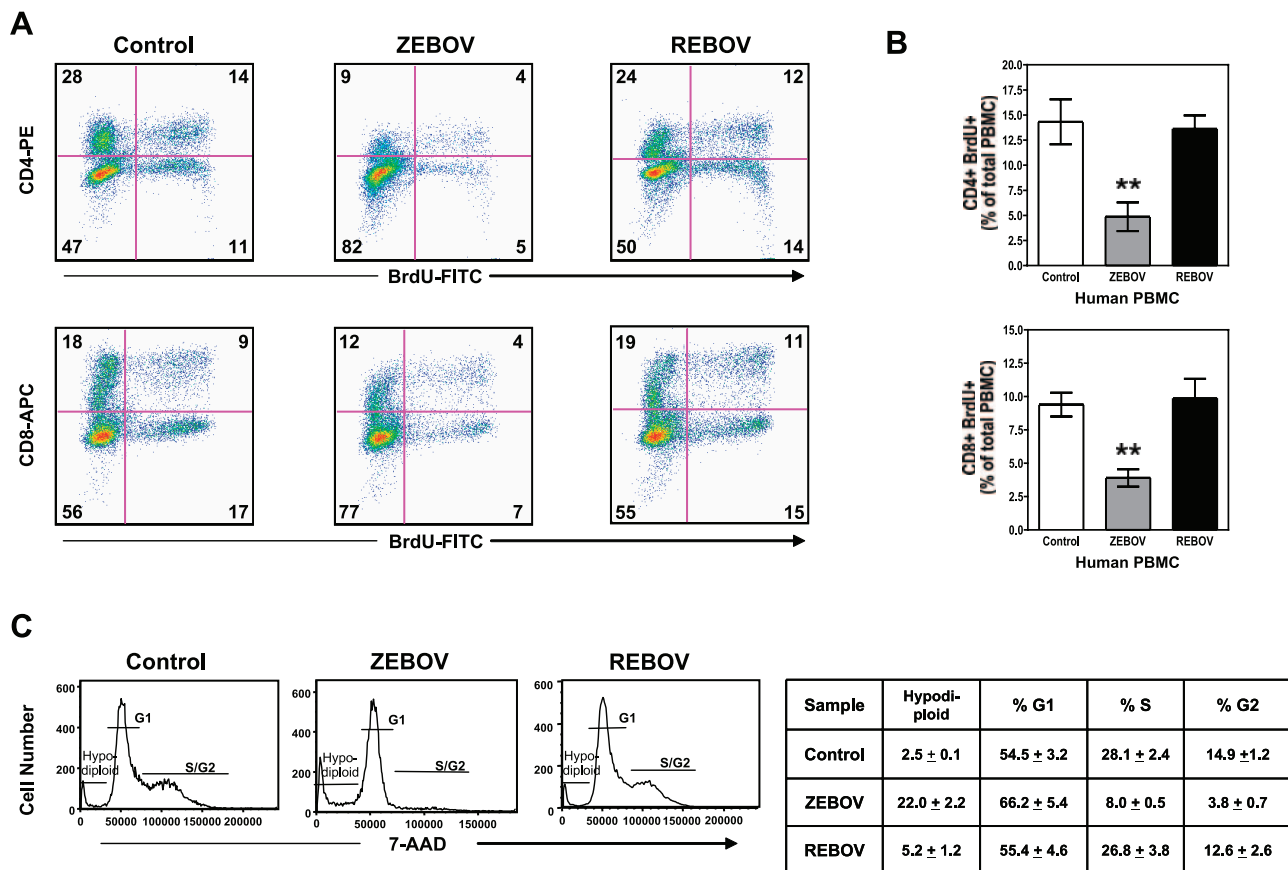


Figure 3. Defective proliferation and cell cycle progression in human T lymphocytes after exposure to filoviral peptides. PBMC were exposed to ZEBOV peptide, REBOV peptide, or no peptide for 48 h in the presence of anti-CD3/CD28. After staining with antibodies to CD4, CD8, and BrdU, cells were analyzed by flow cytometry. *A*) Dot plots of PBMC activated with anti-CD3/CD28 alone or anti-CD3/CD28 and ZEBOV peptide. Data were obtained from 5 different donors; data from one representative donor are shown. Numbers in quadrants represent the percentages of each subpopulation. *B*) Percentage of BrdU+ cells in CD4+ and CD8+ lymphocyte subsets following activation with anti-CD3/CD28 alone or anti-CD3/CD28 and either ZEBOV peptide or REBOV peptide. Results are expressed as percentages of total PBMC. Values represent mean \pm SD for 5 different donors in each sample group. *C*) Cell cycle analysis of PBMC activated with anti-CD3/CD28 or anti-CD3/CD28 and either ZEBOV peptide or REBOV peptide. Cells were stained with 7-AAD and analyzed by flow cytometry. Percentages of cells in G1, S, G2, and hypodiploid phases of the cell cycle are represented as a table. Values indicate mean \pm SD calculated from 5 different donors in each sample group. * $P < 0.01$ (relative to control samples; ANOVA and Dunnett's test for multiple comparisons).

EBOV infection from previous studies wherein infected individuals had elevated levels of circulating TNF- α , IL-1 β , IL-6, MIP1- α , and MCP-1 (10, 11, 32). We studied the influence of ZEBOV peptide on cytokine production by stimulated human PBMC. At 40 μ M concentration, ZEBOV peptide suppressed anti-CD3/CD28-induced production of the Th1 cytokines IFN- γ ($P < 0.05$; relative to control values) and IL-12p40 ($P < 0.05$; relative to control values) (Fig. 5A). ZEBOV peptide also suppressed production of the proliferative and differentiation factor IL-2 ($P < 0.05$; relative to control values) and induced a dose-dependent reduction in TNF- α ($P < 0.05$; relative to control values), IL-1 β ($P < 0.01$; relative to control values), and MCP-1 ($P < 0.01$; relative to control values) (Fig. 5B). There was no effect on MIP1- α (Fig. 5B). ZEBOV peptide effects on Th2 cytokines were less consistent. ZEBOV peptide exposure resulted in an increase of IL-10 ($P < 0.01$; relative to control values); a trend toward decrease was observed with IL-4; no pattern was observed with IL-6 (Fig. 5C).

Cytokine data were fitted on a sigmoidal dose-response curve (variable slope) with R^2 values ranging from 0.8885 to 0.9748 (IL-2, IFN- γ , IL-12, TNF- α , and IL-1 β). The R^2 value for IL-10 was 0.7922. No effects were observed when human PBMC were exposed to REBOV peptide (Fig. 5A–C).

The observation that REBOV peptide had no effect on human PBMC in multiple assays was consistent with its lack of pathogenicity in humans. Given, however, that REBOV is pathogenic in monkeys, we predicted that an immunosuppressive REBOV effect would be seen with monkey PBMC. To test this hypothesis, rhesus macaque (*Macaca mulatta*) PBMC were incubated with REBOV peptide in the presence of anti-CD3 epsilon Ab. ZEBOV is pathogenic in monkeys as well as apes and humans; thus, ZEBOV peptide was used as a positive control. Significant depletion of CD4+ T cells and CD8+ T cells was observed with exposure to REBOV peptide or ZEBOV peptide (Fig. 6A, B). REBOV peptide exposure for 48 h resulted in a 4.5-fold

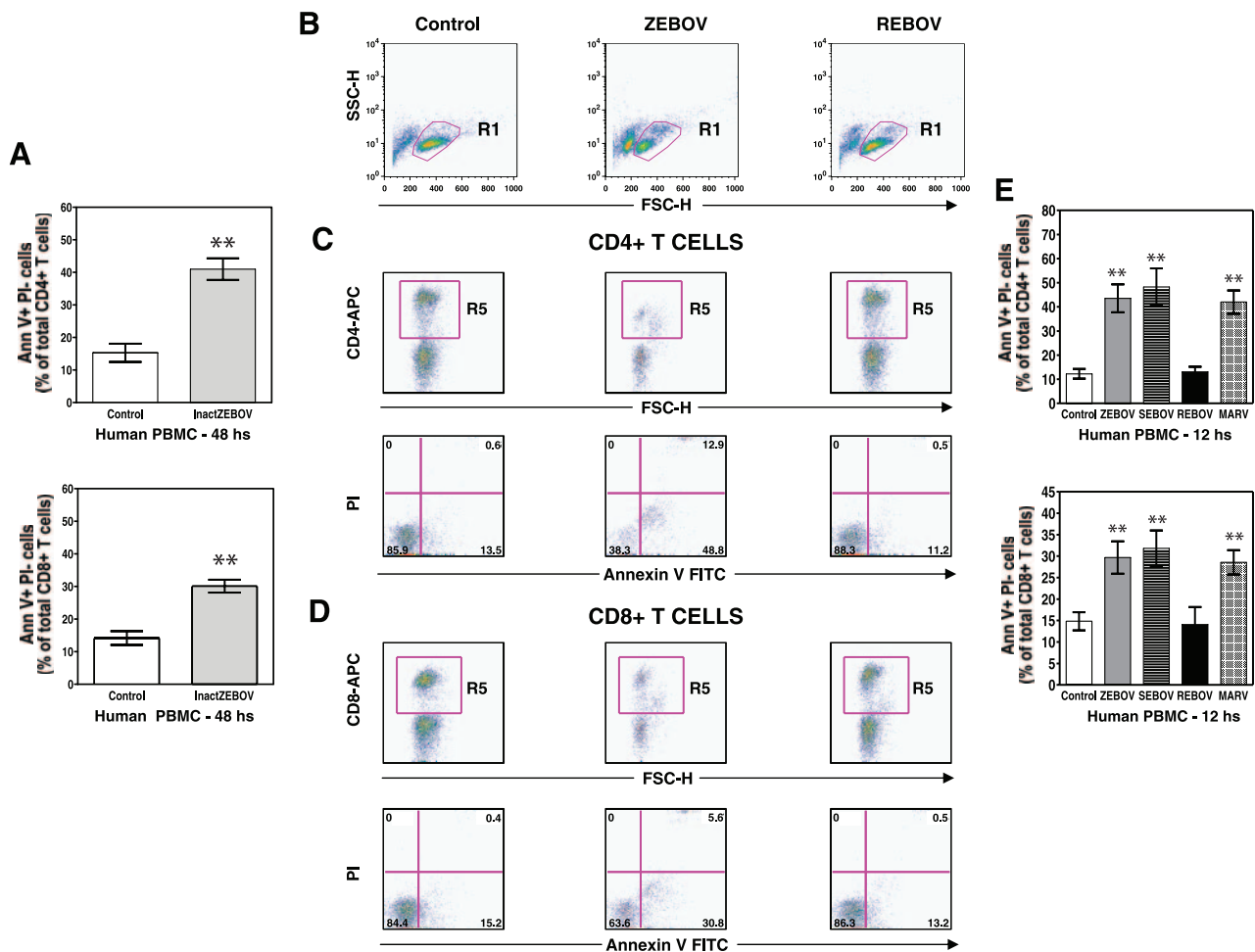


Figure 4. Filoviral peptide exposure induces human T cell apoptosis. PBMC were exposed to filoviral peptides, inactZEBOV, or to neither peptide nor virus in the presence of anti-CD3/CD28. After staining with antibodies to CD4, CD8, AnnexinV-FITC (marker for apoptosis) and PI (marker for apoptosis or necrosis), cells were analyzed by flow cytometry. **A)** Percentages of AnnexinV-FITC+ PI- cells in gated CD4+ or CD8+ cells after treatment with anti-CD3/CD28 antibodies alone or anti-CD3/CD28 and inactZEBOV for 48 h. Values represent mean \pm SD calculated from 5 different donors in each sample group. **B)** Strategy for gating PBMC. Viable PBMC were gated according to forward scatter (FSC) and side scatter (SSC) profile (R1 gate). **C)** Live (R1) cells were further gated according to CD4 expression and FSC (R5). Dot plots of AnnexinV-FITC and PI fluorescence on gated CD4+ cells. **D)** Live (R1) cells were further gated according to CD8 expression and FSC (R5). Dot plots of AnnexinV-FITC and PI fluorescence on gated CD8+ cells. **C, D)** Numbers in quadrants represent the percentages of each subpopulation. Experiments were performed with PBMC from 5 different donors; results obtained from one donor are shown. **E)** Percentages of AnnexinV-FITC+ PI- cells in gated CD4+ or CD8+ cells after treatment with anti-CD3/CD28 antibodies alone or anti-CD3/CD28 and filoviral peptide for 12 h. Values represent mean \pm SD calculated from 5 different donors in each sample group. $**P < 0.01$ (relative to control samples; ANOVA and Dunnett's test for multiple comparisons).

decrease in the number of CD4+ T cells and a 4.6-fold decrease in the number of CD8+ T cells (number of CD4+ T cells with REBOV peptide, $n=5$: $4.8 \pm 0.7 \times 10^4$; number of CD8+ T cells without REBOV peptide, $n=5$: $26.6 \pm 2.7 \times 10^4$; $P < 0.01$; number of CD8+ T cells with REBOV peptide, $n=5$: $2.3 \pm 0.4 \times 10^4$; number of CD8+ T cells without REBOV peptide, $n=5$: $14.7 \pm 1.2 \times 10^4$; $P < 0.01$; Fig. 6B). REBOV peptide exposure resulted in a decrease in the percentages of CD69+ cells in both CD4+ and CD8+ T cell populations (percentage of CD4+ rhesus T cells treated with REBOV peptide that are CD69+, $n=5$: $63.6 \pm 1.9\%$; without REBOV peptide, $n=5$: $89.1 \pm 2.6\%$; $P < 0.01$; percentage of CD8+ rhesus T cells treated with REBOV peptide that are CD69+, $n=5$: $60.7 \pm 3.1\%$; without REBOV peptide, $n=5$:

$84.3 \pm 2.7\%$; $P < 0.01$; Fig. 6D). The mean fluorescent intensity of CD69 expression was also decreased in CD4+ and CD8+ T cells treated with REBOV peptide (Fig. 6E). REBOV treatment for 12 h resulted in a 3.1-fold increase in apoptotic CD4+ cells and a 2.6-fold increase in apoptotic CD8+ cells (percentage of Annexin V+ PI-rhesus CD4+ treated with REBOV peptide, $n=5$: $46.0 \pm 2.1\%$; untreated cells, $n=5$: $14.0 \pm 3.0\%$; $P < 0.01$; percentage of Annexin V+ PI-CD8+ treated with REBOV peptide, $n=5$: $31.8 \pm 3.3\%$; untreated cells, $n=5$: $12.3 \pm 3.0\%$; $P < 0.01$; Fig. 7A). Decreased T cell activation and increased apoptosis were also observed with ZEBOV peptide and inactZEBOV (Fig. 6C-E, Fig. 7A). Both ZEBOV peptide and REBOV peptide at a dose of 40 μ M cause a significant

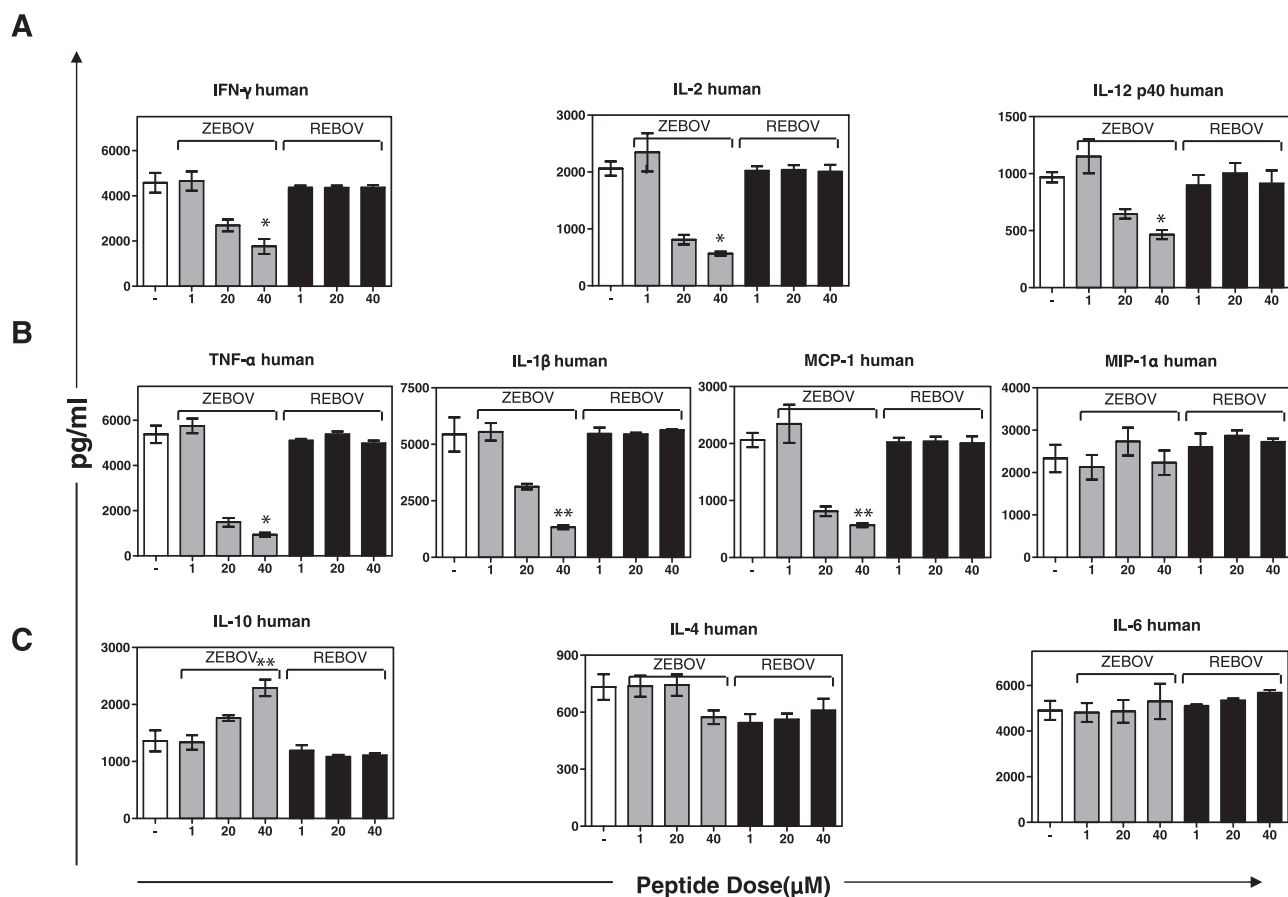


Figure 5. Exposure of human PBMC to ZEBOV peptide results in decreased release of IFN- γ , IL-2, IL-12p40, TNF- α , IL-1 β , and MCP-1, and increased release of IL-10. PBMC were exposed to 1, 20, or 40 μ M of ZEBOV peptide, REBOV peptide, or no peptide for 48 h in the presence of anti-CD3/CD28. Cytokines were assayed in cell supernatant using Luminex technology. Results indicate mean concentration (pg/ml) \pm SD. Values were obtained from 5 different donors in each sample group. * P < 0.05; ** P < 0.01 (relative to control samples; Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparison test).

decrease in Th1 (IFN- γ and IL-12p40; P < 0.05) and inflammatory cytokines (TNF- α and IL-1 β ; P < 0.05) (Fig. 7B) compared with control values. Levels of IL-8 and MIP1- α did not alter with exposure to either peptide (Fig. 7B).

DISCUSSION

Ebola and Marburg viruses can cause hemorrhagic fever (HF) outbreaks with high mortality in primates. Whereas MARV, ZEBOV and SEBOV are pathogenic in humans, apes, and monkeys, REBOV is pathogenic only in monkeys. Early immunosuppression may contribute to pathogenesis by facilitating viral replication. The filoviral proteins VP35 and VP24 have immunomodulatory effects. VP35 inhibits induction of IFN- α and - β by blocking phosphorylation and nuclear translocation of IFN regulatory factor-3 (4, 33). ZEBOV VP24 interacts with karyopherin α 1, the nuclear localization signal receptor for PY-STAT1 (34). Active virus replication is prerequisite for the immunosuppressive effects of VP35 and VP24. In contrast, the immunosuppressive effects

we have observed with filoviral GP sequences are independent of viral replication.

The 17mer filoviral peptides ZEBOV, SEBOV, or MARV had a strong immunosuppressive influence on anti-CD3/CD28-activated human PBMC. Furthermore, activated CD4 $^{+}$ and CD8 $^{+}$ T cells failed to up-regulate activation markers on their surface and exhibited reduced cell cycle progression. CD4 $^{+}$ and CD8 $^{+}$ T cell dysfunction may stem from immune inactivation after direct contact with the peptide. Alternatively, the effect may be the indirect result of inadequate stimulation by the antigen-presenting cells. *In vitro* studies of ZEBOV have revealed suppression of immune responses within infected monocyte/macrophages and endothelial cells (35, 36). Dendritic cells infected with ZEBOV are functionally impaired and only poorly stimulate T cells (37, 38). IFN- α / β production has been shown to influence dendritic cell functions. VP35 protein of ZEBOV suppresses the induction of IFN- α / β and may indirectly contribute to inhibition of dendritic cell functions (33).

T cells do not support filoviral replication (7). The observation that inactZEBOV can induce T cell apoptosis in PBMC cultures is consistent with earlier studies

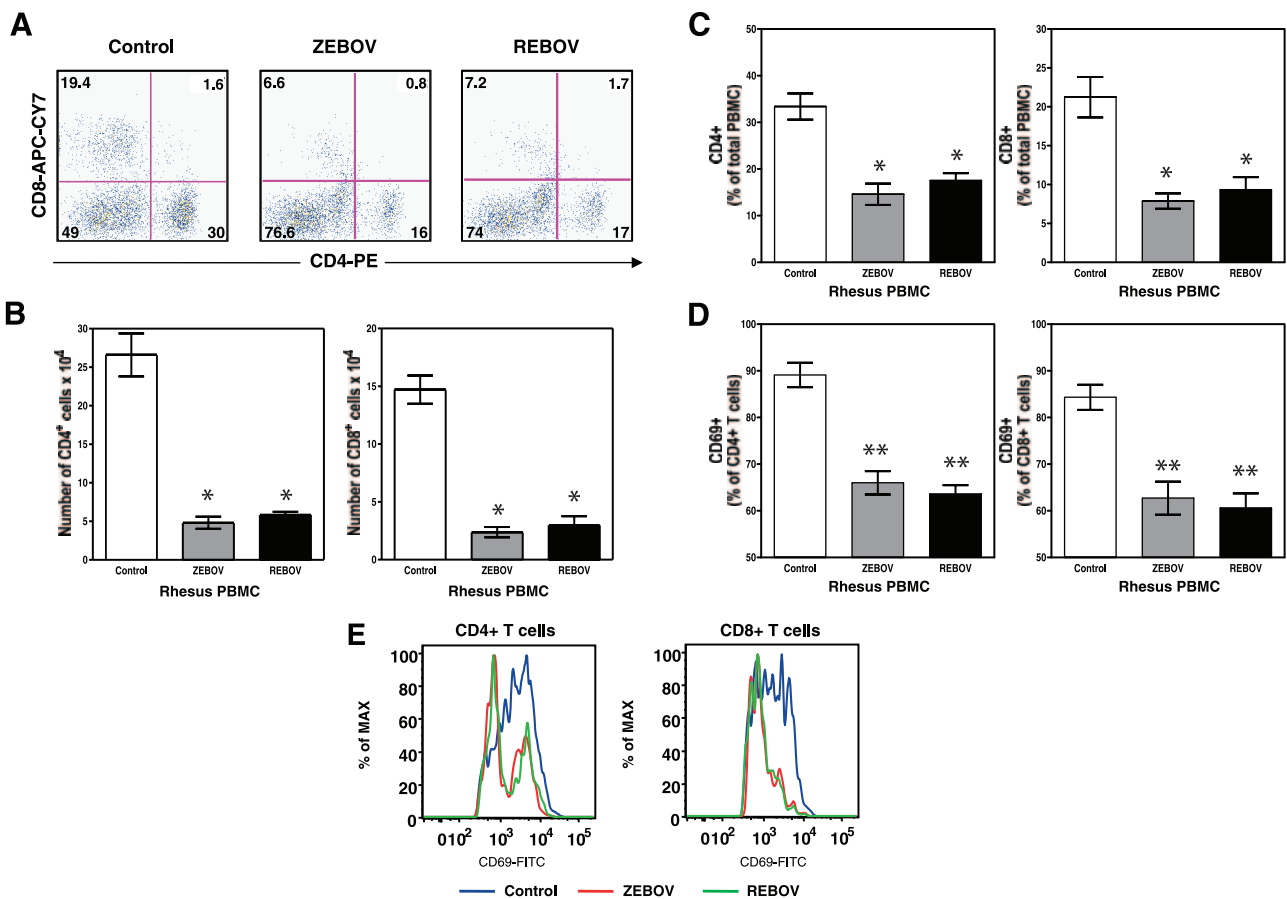


Figure 6. Exposure of rhesus macaque PBMC to REBOV peptide results in depletion and inactivation of CD4⁺ and CD8⁺ T cells. Rhesus PBMC were exposed to ZEBOV peptide, REBOV peptide, or no peptide for 48 h in the presence of anti-human CD3ε. **A)** Dot plots of CD4 *vs.* CD8 lymphocytes in activated PBMC exposed to ZEBOV peptide or REBOV peptide. Experiments were performed with PBMC from 5 different donors; results obtained from one donor are shown. Numbers in quadrants represent the percentages of each subpopulation. **B)** Absolute numbers of CD4⁺ and CD8⁺ T cells following activation of PBMC with anti-CD3ε alone or anti-CD3ε and either ZEBOV peptide or REBOV peptide. Data represent mean ± SD calculated from 5 macaques in each sample group. **C)** Percentages of CD4⁺ and CD8⁺ lymphocyte subsets in PBMC following activation with anti-CD3ε Ab alone or anti-CD3ε and either ZEBOV peptide or REBOV peptide. Results are expressed as percentages of total PBMC. Values represent mean ± SD calculated from 5 macaques in each sample group. **D)** Percentages of CD69⁺CD4⁺ and CD69⁺CD8⁺ subsets in PBMC following activation with anti-CD3ε or anti-CD3ε and either ZEBOV peptide or REBOV peptide. Results are expressed as percentages of CD4⁺ or CD8⁺ T cells. Values represent mean ± SD calculated from 5 macaques in each sample group. **P* < 0.05; ***P* < 0.01 (relative to control samples; ANOVA and Dunnett's test for multiple comparisons). **E)** Representative histogram showing cell surface expression of CD69 markers on rhesus PBMC activated with anti-CD3ε or anti-CD3ε and either ZEBOV peptide or REBOV peptide. Mean fluorescence intensity is represented as a percentage of the maximum expression. Data were obtained from 5 different donors; data from one representative donor are shown.

indicating that virus replication is not a prerequisite for T cell apoptosis (30, 31). Potential mechanisms for T cell apoptosis in PBMC cultures treated with filoviral peptides include direct interaction of peptides with the cell surface or indirect effects mediated by soluble factors released from monocytes exposed to these peptides. Studies of purified human CD4⁺ and CD8⁺ T cells indicate that ZEBOV peptide alone is sufficient to induce activation and cell death in either population. It is conceivable that direct and indirect mechanisms may both be implicated in T cell apoptosis.

We examined the influence of ZEBOV peptide on Th1- and Th2-related cytokine production by stimulated PBMCs using Luminex technology. Whereas T

helper type 1 cells predominantly produce IFN-γ, T helper type 2 cells secrete IL-4, IL-5, and IL-10.

IL-12, a cytokine produced by monocytes/macrophages, enhances cell-mediated immunity (39, 40). IL-10 is mainly produced by monocytes/macrophages and T cells; it inhibits activation of T-helper lymphocytes either directly (41) or by suppressing activation of antigen presenting cells (42). High plasma levels of IL-10 are reported in filovirus-infected patients with a fatal outcome (11). Our data show that the 17mer ZEBOV peptide suppresses expression of the type 1 cytokines IL-12 and IFN-γ while enhancing expression of the type 2 cytokine IL-10. Enhanced expression of IL-10 and reduced expression of IL-12 may imbalance Th1- and Th2-related cyto-

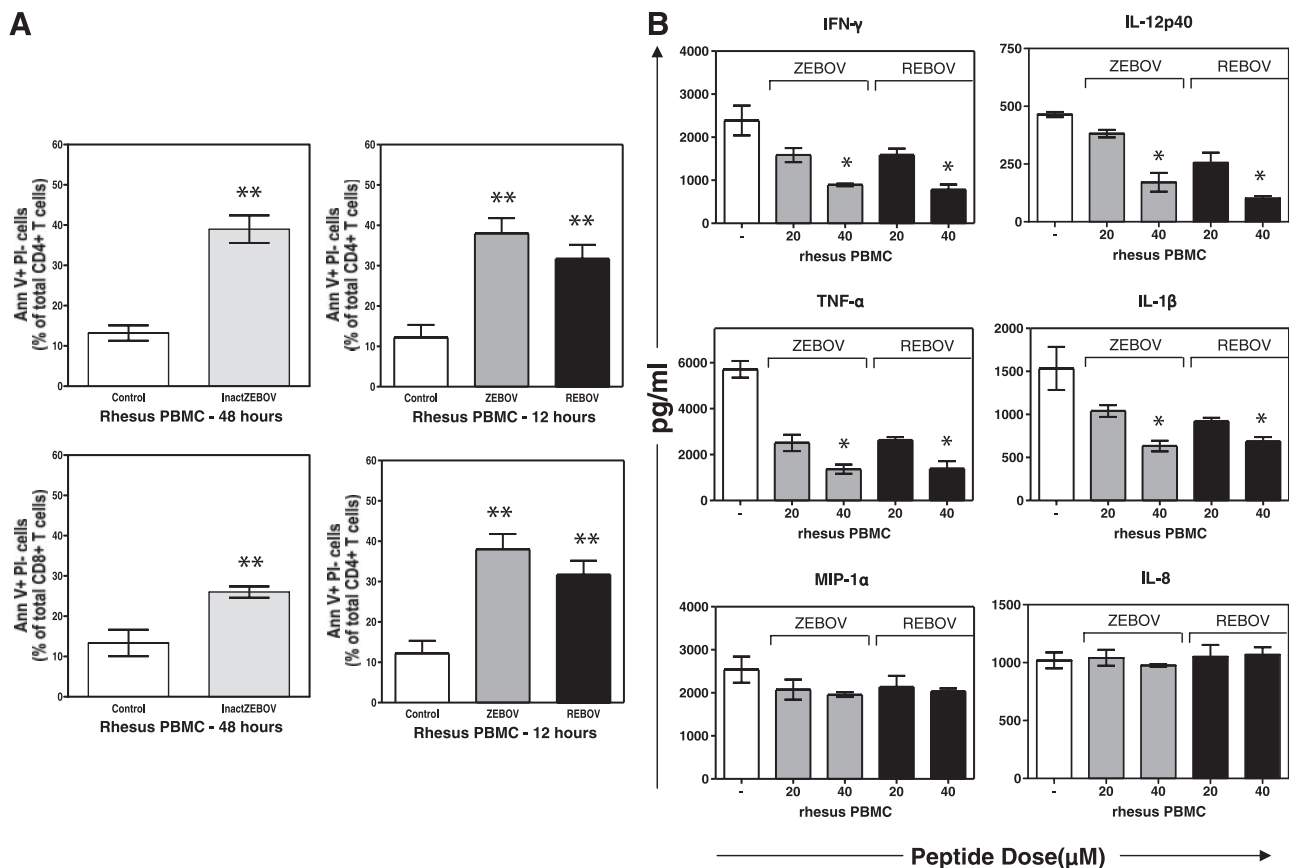


Figure 7. Exposure of rhesus macaque PBMC to REBOV peptide results in apoptosis of CD4+ and CD8+ T cells and in decreased release of IFN- γ and IL-12p40. **A)** PBMC were exposed to filoviral peptide, inactZEBOV, or to neither peptide nor virus in the presence of anti-CD3 ϵ . After staining with antibodies to CD4, CD8, AnnexinV-FITC (marker for apoptosis), and PI (marker for apoptosis or necrosis) cells were analyzed by flow cytometry. Percentages of AnnexinV-FITC+ PI- cells in gated CD4+ or CD8+ T cells after treatment with anti-CD3 ϵ alone or anti-CD3 ϵ and inactZEBOV, ZEBOV peptide, or REBOV peptide. Values represent mean \pm SD calculated from 5 macaques in each sample group. * P < 0.01 relative to control samples (ANOVA and Dunnett's test for multiple comparisons). **B)** PBMC were exposed for 48 h to 1, 20, or 40 μ M of ZEBOV peptide, REBOV peptide or no peptide in the presence of anti-CD3 ϵ . Cytokines in cell supernatants were assayed by flow cytometry (Luminex). Results indicate mean concentration (pg/ml) \pm SD. * P < 0.05; ** P < 0.01 (relative to control samples; Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparison test).

kine production and suppress cell-mediated immunity. Haraguchi *et al.* (19) have demonstrated that CKS-17, a retroviral peptide, acts directly on monocytes/macrophages and differentially modulates the production of IL-10 and IL-12. Furthermore, a neutralizing anti-human IL-10 monoclonal antibody (mAb) blocks the peptide-mediated inhibition of IFN- γ , supporting the hypothesis that inhibition of IFN- γ production may be secondary to an increase in IL-10 and depression in IL-12 levels produced by the retroviral peptide. Similar cytokine-mediated cross-regulation may be implicated in filoviral immunosuppression.

Proinflammatory cytokines and chemokines play a vital role in one of the earliest phases of the host resistance to viral and microbial infections by participating in various cellular and inflammatory processes. In our study, 17mer filoviral peptides decreased secretion in PBMC cultures of proinflammatory cytokines TNF α and IL-1 β and chemokine MCP-1. These defective inflammatory responses may be associated with impaired T cell activation observed in peptide-treated lymphocytes. Nonfatal ZEBOV infection

is associated with early inflammatory responses (32). The observed peptide-mediated cytokine inhibition suggests that filoviral transmembrane glycoprotein may be involved in suppressing the onset of early inflammatory responses that are crucial for controlling viral spread in filoviral infections.

All African EBOV subtypes (ZEBOV, SEBOV, and Ivory Coast) cause a severe hemorrhagic disease in humans and nonhuman primates with extraordinarily high fatality rates. The fourth subtype, REBOV, initially isolated from cynomolgus monkeys, is nonpathogenic in humans and appears to be a lethal pathogen only for nonhuman primates (2). Exposure of human PBMC to REBOV peptides had no effect on markers of CD4+ or CD8+ activation, viability, or cytokine levels in cell supernatants. Whereas human PBMC were sensitive to ZEBOV but not REBOV, monkey PBMC were sensitive to both ZEBOV and REBOV. These findings demonstrate that strain-specific differences in peptide sequence determine immunological effects on PBMC *in vitro* and correlate with the pathogenic potential of

ZEBOV, SEBOV, and MARV viruses *vs.* REBOV virus in human and nonhuman primates (12).

The rapidly progressing high-fatality hemorrhagic fever associated with EBOV and MARV infections is accompanied by profound immunosuppression and vascular dysfunction. Several factors likely contribute to the severity of disease. These viruses quickly replicate and cause cytotoxicity in a wide range of cells and tissues within the body, and the viral glycoprotein (particularly the mucin-like domain) has been implicated in this cytotoxicity (12). Recent studies have also demonstrated an immunosuppressive effect of the viral VP35 protein in inhibiting IFN regulatory factor 3 (IRF-3) activation and induction of IFN- α and - β as well as other antiviral responses (33, 43). Our findings show that in addition to contributing to HF pathogenicity through cytotoxicity, filoviral glycoproteins also have a potent immunosuppressive effect. The 17 amino acid motif described here dysregulates Th1 and Th2 responses and depletes CD4 and CD8 T-cells through apoptosis. Focused investigation of interactions between filoviral glycoproteins and the host immune system may enable development of specific strategies to reduce the extreme morbidity and mortality associated with HF due to EBOV and MARV infections. **[F]**

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Sequence of the 17AA Immunosuppressive Domain

Filovirus

ZEBOV strain KIKWIT (1995)	ILNRKAIDFLLQRWGGTC	Ebola-like virus
ZEBOV strain GABON (1994)	ILNRKAIDFLLQRWGGTC	
ZEBOV strain MAYINGA (1976)	ILNRKAIDFLLQRWGGTC	
ZEBOV strain EKRON (1976)	ILNRKAIDFLLQRWGGTC	
CIEBOV strain IVORY COAST (1994)	ILNRKAIDFLLQRWGGTC	
REBOV strain RESTON (1989)	LLNRKAIDFLLQRWGGTC	
REBOV Strain SIENA (1992)	LLNRKAIDFLLQRWGGTC	
SEBOV strain BONIFACE (1976)	ILNRKAIDFLLRWGGTC	Marburg-like virus
SEBOV strain MALEO (1979)	ILNRKAIDFLLRWGGTC	
MARV strain OZOLIN (1975)	LINRHAIDFLLTRWGGTC	
MARV strain POPP (1967)	LINRHAIDFLLTRWGGTC	
MARV strain MUSOKE (1980)	LINRHAIDFLLTRWGGTC	
MARV strain RAVN (1987)	LINRHAIDFLLTRWGGTC	
MARV strain ANGOLA (2005)	LINRHAIDFLLRWGGTC	

Retrovirus (selected)

MMLV (CKS17)	LQNRRLDLLFLKEGGGLC
HTLV-2	AQNRRLDLLFWEQGGGLC
HERV-R	YQNRLALDYLLAQEEGVC
HERV_K	MNNRLALDYLLAEQGGVC
Friend MLV	LQNRRLDLLFLKEGGGLC
Avian Sarcoma Virus	LQNRAAIDFLLLAHGHGC
Zebrafish ERV	IQNRLALDMLLSERGGVC
	** : * * :

Blue residues: Species characteristics residues

Red residues: Particular residues change for the specie

* Conserved residue