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Endogenous heat shock protein GroEL of *A. actinomycetemcomitans* preferentially targets primary human CD8+ T cells

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Abstract: Apoptosis can be used to manipulate host cells by bacterial products such as bacterial heat shock proteins (Hsp). One of the virulence factors of periodontal pathogen *Aggregatibacter actinomycetemcomitans* is heat shock protein GroEL (AaGroEL), which has been shown to interact with host cells. AaGroEL (Hsp64) also has the potential to modulate immune system cells. In this study we used endogenous AaGroEL protein as an antigen to study bacterial Hsp-induced apoptosis in different immune system cells. Human peripheral blood mononuclear cells and cell lines were cultured with different doses (50–1000 ng/mL) of endogenous AaGroEL at various time points. Apoptosis of the cells was measured by Annexin V and 7AAD labeling. Apoptotic cells were analyzed by flow cytometry. Our data suggested that AaGroEL-responding primary CD8+ T cells were more susceptible to apoptosis than CD4+ T cells. Furthermore, the magnitude of apoptosis in the Jurkat T cell line was higher than that in primary CD8+ T cells. There was no statistically significant level of apoptosis in the chronic myeloid leukemia (K562) cell line, which belongs to myeloid lineages. Thus, *A. actinomycetemcomitans* GroEL protein has more potent apoptotic effect on cells that are derived from a lymphoid progenitor.

Key words: CD4 T cells, CD8 T cells, bacterial heat shock proteins, GroEL, apoptosis

1. Introduction

Aggregatibacter actinomycetemcomitans (Aa) is one of the major etiologic agents implicated in some forms of periodontal disease in adolescents and adults (Slots and Ting, 1999). It has also been implicated in several nonoral infections such as brain abscess, endocarditis, lung infection, and endophthalmitis (van Winkelhoff and Slots, 1999). *A. actinomycetemcomitans* is a gram-negative, facultative, anaerobic, nonspore-forming, and nonmotile coccobacillus. Previous investigations have suggested that *A. actinomycetemcomitans* produces a variety of virulence factors including the leukotoxin (Ltx) (Lally et al., 1997; Korostoff et al., 1998), cytolethal distending toxin (Cdt) (Shenker et al., 1999, 2001), and lipopolysaccharide (Lps) (Kiley and Holt, 1980; Patil et al., 2006). *A. actinomycetemcomitans* also expresses heat shock protein GroEL (Hsp64) (Koga et al., 1993). Many of these factors appear to exert their pathogenic effects by immunomodulation.

We are interested in *A. actinomycetemcomitans* heat shock protein GroEL (AaGroEL) as a pathogenic virulence factor and its effects on immune systems cells, including human T cells. Heat shock proteins show highly conserved sequence similarities among differences species, including bacteria. Other bacterial GroEL proteins have been shown to initiate apoptotic signaling (Equil et al., 2006; Jha et al., 2011), but there are very limited data available on the apoptotic ability of the GroEL protein of *A. actinomycetemcomitans*. Previous studies showed that *A. actinomycetemcomitans* GroEL has a cytotoxic effect on epithelial cells in higher doses (Goulhen et al., 1998) and osteolytic activity on alveolar bone (Kirby et al., 1995). There is also a study conducted by Nalbant et al. showing that cell extract of ΔltxA/ΔcdtABC double knockout strain of *A. actinomycetemcomitans* causes cell death in T cells. That study also demonstrated that inhibition assays with anti-*E. coli* GroEL monoclonal antibodies decreased T cell apoptosis, suggesting that there might be a role for heat shock proteins in T cell apoptosis (Nalbant et al., 2003). T cells can be found in the inflammatory infiltrate of periodontal diseases characterized by infection in the presence of different gram-negative bacteria including *A. actinomycetemcomitans*. CD4+ and CD8+ T cells are present in periodontal lesions as activated/memory T cells (Taubman and Kawai, 2001). Thus, it is important to reveal whether there is selective killing of different T cell subtypes, such as CD4+ and CD8+ T cells, by *A. actinomycetemcomitans* GroEL protein.

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In this study we used purified, endogenous *A. actinomycetemcomitans* GroEL protein as an antigen to investigate the ability of GroEL to induce apoptosis among different cell types including primary CD4+ and CD8+ T subsets as well as the Jurkat T cell line and chronic myeloid leukemia cell line (K562). To this end, human peripheral blood mononuclear cells and cell lines were cultured with AaGroEL, and apoptosis of cells was measured. To the best of our knowledge, our data demonstrated for the first time that *A. actinomycetemcomitans* GroEL protein induces apoptosis preferentially in primary CD8+ T cells. Furthermore, purified *A. actinomycetemcomitans* GroEL protein kills a number of cells with different magnitude.

2. Materials and methods

2.1. Human subjects and peripheral blood mononuclear cells

The ethics committee of Dokuz Eylül University Medical School, İzmir, Turkey approved this study, and 21 systemically and periodontally healthy adult volunteers were included in this study. Subjects included 14 males and 7 females (15 donors were 20–25 years of age; 6 donors were 26–30 years of age). All donors were nonsmokers. Participants provided written informed consent to participate in this study. Venous blood was drawn from the volunteers at İYTE Health Service by health professionals. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation method (Böyum, 1968).

2.2. Bacterial culture, purification, and verification of GroEL protein

Aggregatibacter actinomycetemcomitans (29522) type strain was obtained from American Type Cell Culture (ATCC). *A. actinomycetemcomitans* was grown as previously described (Nalbant and Zadeh, 2000). Briefly, bacteria were grown on solid media plates containing trypticase soy broth (TSB) supplemented with trypticase soy agar (1.5%), yeast extract (0.6%), and 10% horse serum. A single colony was selected and confirmed by its morphology, Gram staining, catalase test, and PCR. The selected colony was then grown on selective medium TSBV (3% trypticase soy broth, 0.1% yeast extract, and 10% horse serum with 75 μg/mL bacitracin and 5 μg/mL vancomycin) for 48 h at 37 °C and in a 5% CO_2 incubator. To induce heat shock protein expression, bacteria cultures were incubated at 43 °C for 1 h in a water bath. Bacterial cells were disrupted by sonification (30 cycles), and the cell debris was removed by centrifugation (7500 rpm, 1 h). Protein concentration of *A. actinomycetemcomitans* cell extract (AaCE) was measured by Bradford protein assay. Aa-cell extracts were stored in aliquots at –80 °C until use (Nalbant et al., 2003).

GroEL protein was purified from AaCE by ATP affinity chromatography and electroeluted from SDS-PAGE (Hinode et al., 1996). Briefly, adenosine 5'-triphosphate (ATP) agarose (5 mL) (AppliChem, Darmstadt, Germany) was loaded on a gravity column, washed, and equilibrated. Then, 1 mL of AaCE (2 mg/mL) was incubated on the column for 2 h. Unbound proteins were washed. Proteins were eluted by 5 mM ATP in 5 bed volumes of elution buffer. Protein concentrations were determined by Bradford protein assay.

ATP affinity chromatography fractions were used for electroelution (Hinode et al., 1996). Briefly, fractions electrophoresed under cooling conditions and the protein bands were stained with 300 mM $CuCl_2$. The GroEL band was cut out and destained in buffer (pH 9.0) containing 250 mM Tris-250 mM EDTA for 10 min. Gel slices were washed with elution buffer containing 25 mM Tris, 125 mM glycine, and 0.1% SDS. Samples were electro-eluted for 3 h for 50 V in elution buffer. Electroeluted AaGroEL (data submitted for publication) protein was dialyzed overnight against 10 mM Tris-HCl buffer (pH 7.0). Furthermore, native AaGroEL protein was dialyzed against 1X PBS and concentrated against concentration buffer (Pierce Chemical Co., Rockford, IL, USA). To verify GroEL protein, AaGroEL protein was confirmed (data submitted for publication) by western blot (Saygılı et al., 2012). Following conformation by western blot, the relevant protein bands were cut from gel slices and sent to Proteome (Berlin, Germany) for analysis by LC-ESI-MS. Purified protein was confirmed as *Actinobacillus actinomycetemcomitans* GroEL protein (data not shown). Purified AaGroEL protein was used as an antigen.

2.3. Cell cultures and stimulants

PBMCs were cultured at a concentration of 2×10^6 cells per milliliter in a volume of 500 μL. Cells were incubated at different time points (0–96 h) with or without stimulants at 37 °C in a humidified incubator with 5% CO_2. The medium consisted of RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin, 100 pg/mL streptomycin (Biochrom AG), and 25 mM HEPES buffer (Invitrogen, Carlsbad, CA, USA). RPMI alone was used as a negative control, and camptothecin (4 μM) (Sigma-Aldrich, St Louis, MO, USA) as a positive control. Electroeluted AaGroEL protein was used in cell cultures as a stimulant at various concentrations (50–1000 ng/mL). Each culture condition in all experiments was carried out in triplicate.

2.4. Detection of apoptosis

Cells were washed with PBS and labeled first with CD4 and CD8 and then with Annexin V and 7AAD in the presence of Ca^{++} binding buffer (Becton Dickinson, Mountain View, CA, USA) for 30 min and analyzed by flow cytometry. For

data analysis, cells were gated on cells of interest; %Annexin V+ only cells are early apoptotic, %Annexin V+7AAD+ double positive cells are late apoptotic, and %7AAD+ only cells are dead cells. In this paper we summed Annexin V+ only cells and Annexin V+7AAD+ double positive T cells and refer to it as %total-apoptotic T cells.

2.5 Statistical analysis

The samples were acquired by FACSArray, and data were analyzed by FACSArray system software and FlowJo software. Flow data were exported to MS Office Excel for further analysis. All the experiments were performed in triplicate, and the test samples were compared to negative control by using Student's t-test. A 2-tailed Student's t-test was used for statistical analysis, and $P < 0.05$ was accepted as statistically significant. Error bars represent the standard deviation, and * indicates $P < 0.05$.

3. Results

3.1. GroEL is not a strong apoptosis inducer on CD4+ T cells

A. actinomycetemcomitans GroEL is known to stimulate epithelial cell proliferation and cause cell death and tissue destruction under high concentrations in periodontitis (Goulhen et al., 1998). However, its apoptotic effect on human T cell subsets is unknown. Thus, we monitored the rate of apoptosis in CD4+ T cells by incubating the endogenous AaGroEL with PBMC in a time- and dose-dependent manner. In a 48-h dose kinetics experiment, the rate of apoptosis in AaGroEL-stimulated CD4+ T cells ranged from 5.8% to 41% at doses of 50–1000 ng/mL (Figure 1A). The extension of the incubation time from 48 h to 72 h resulted in a proportional increase in the apoptosis rate as expected. We also studied the time kinetics of plasma membrane changes of CD4+ T cells by culturing PBMCs with 100 ng/mL AaGroEL for 0–96 h. There was a time-dependent elevation in the rate of apoptosis ranging from 8.6% at 24 h to 14.9% at 96 h (Figure 1B). At 48 h there was a 2-fold, statistically significant difference between the antigen-treated and control T cells ($P < 0.05$; Figure 1B). On the other hand, an apoptosis rate of 4.5% at 24 h remained nearly constant throughout the time points in negative culture cells. We interpret these data to mean that AaGroEL has mild apoptotic effects on human CD4+ T cells.

3.2. GroEL preferentially targets CD8+ T cells to induce apoptosis

To evaluate the sensitivity of different T cell sub-populations to AaGroEL, the apoptosis rate of CD8+ T cells was monitored concurrently with that of CD4+ T cells. Interestingly, CD8+ T cells displayed nearly 2-fold higher sensitivity to AaGroEL-mediated apoptosis at all doses

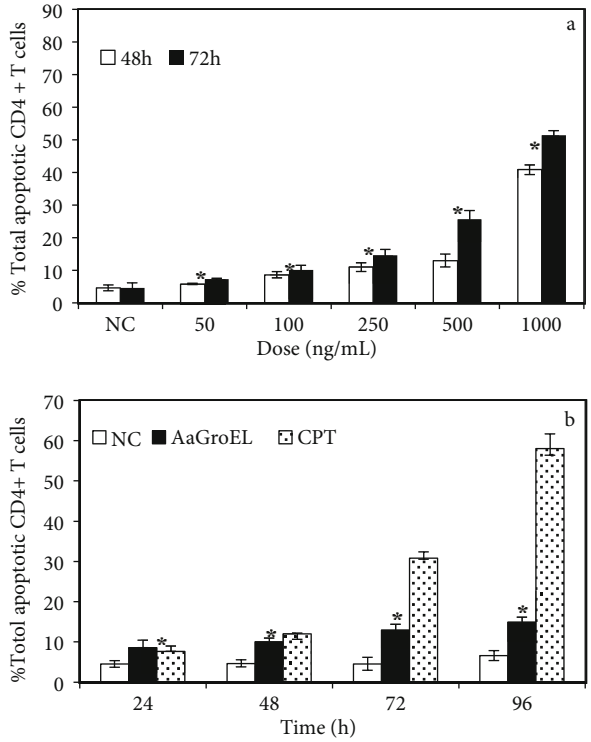

Figure 1. GroEL effect on CD4+ T cells. A. Dose response of AaGroEL. Human PBMCs were cultured with different concentration of AaGroEL protein (50–1000 ng/mL) at 48 h and 72 h. B. Time kinetics of AaGroEL. AaGroEL protein (100 ng) was cultured for various intervals from 0 to 96 h. RPMI (NC) and camptothecin (CPT) were used as negative and positive controls, respectively. CD4+ apoptotic T cells were measured by flow cytometry. Error bars represent the standard deviation, and * indicates $P < 0.05$.

tested (Figure 2A). Following both 48- and 72-h cultures there was a dose-dependent increase in the percentage of CD8+ T cells with plasma membrane changes. More significantly, the rate of apoptosis in CD8+ T cells was more prominent under shorter treatments (24 h versus 48 h; Figure 2A), further pointing to the potential sensitivity of CD8+ T cells to AaGroEL-mediated apoptosis. Although AaGroEL at a dose of 1000 ng/mL induced 41% apoptosis in CD4+ T cells at 48 h, a dose of 500 ng/mL was sufficient to induce apoptosis in 51% of CD8+ T cells (Figure 2A). The kinetics of plasma membrane changes of CD8+ T cells was also measured for 0–96 h at a dose of 100 ng/mL. There was a statistically significant difference in the apoptosis rate of AaGroEL-treated CD8+ T cells (3-fold difference compared to negative control at 48 h, $P < 0.05$; Figure 2B). The time-dependent increase in the apoptosis rate reached 22% at 96 h. However, in negative control cultures, the rate of apoptosis stayed nearly the same throughout the cultures, up to 96 h.

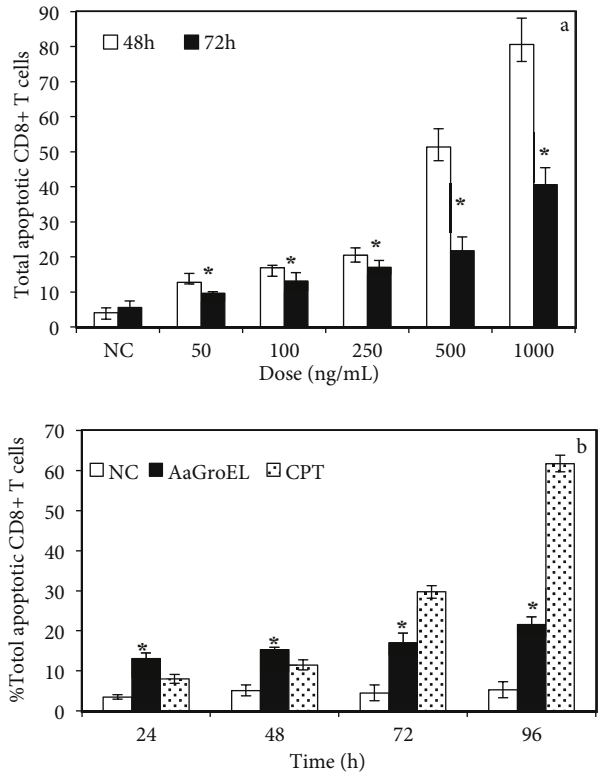

Figure 2. GroEL effect on CD8+ T cells. A. Dose response of AaGroEL. Human PBMCs were incubated with various concentration of AaGroEL protein (50–1000 ng/mL) from 48 h to 72 h. B. Time kinetics of AaGroEL. AaGroEL protein (100 ng) was cultured at 0–96 h. RPMI (NC) and camptothecin (CPT) were used as negative and positive controls, respectively. CD8+ apoptotic T cells were measured by flow cytometry. Error bars represent the standard deviation, and * indicates $P < 0.05$.

3.3. GroEL is more effective at inducing apoptosis in Jurkat T cells

The Jurkat cell line is an immortalized cell line of human T lymphocyte cells that are used for a variety of T cell properties. To substantiate the AaGroEL-mediated apoptosis of human T cells, Jurkat cells were cultured with endogenous AaGroEL at a dose of 100 ng/mL for 24 h and 48 h. Data showed that AaGroEL-mediated apoptosis increased from 10% to 30% in Jurkat T cells at 24–48-h time intervals, respectively. Compared to negative controls, the AaGroEL apoptotic activity in Jurkat T cells was 15-fold higher in AaGroEL-stimulated Jurkat T cells at 48 h ($P < 0.005$; Figure 3). These results clearly suggest that AaGroEL induces Jurkat T cell apoptosis. Surprisingly, the magnitude of apoptosis in Jurkat T cells was higher than that in the primary human T cells.

3.4. GroEL cannot induce apoptosis on myeloid progenitor derived cells

Chronic myeloid leukemia (K562) cells stimulated with AaGroEL showed that there was 11% of apoptosis at 48 h. When it was compared with the negative control there was no significant level of apoptosis ($P = 0.06$). It was clear from the data that K562 cells did not undergo apoptotic changes in response to AaGroEL (Figure 4).

4. Discussion

The apoptotic process can be one of the tools that virulence factors of bacteria utilize to manipulate host cells to gain benefit of invasion towards bacteria. *A. actinomycetemcomitans* is a potent etiologic agent of periodontal diseases, which are chronic inflammation of the oral cavity. In addition to other pathogenic determinants, *A. actinomycetemcomitans* expresses heat shock protein GroEL. GroEL homolog of other bacteria has been shown to interact with different host cells with different functional ability. There are studies (Kirby et al., 1995; Goulhen et al., 1998) about the effect of AaGroEL heat shock protein on human host cells, including epithelial cells, but there are no studies on immune system cells, including T cell subsets. For example, the long-term effects of *Actinobacillus actinomycetemcomitans* heat shock protein 60 on skin keratinocyte (HaCaT cell line) increased the rate of epithelial cell death. In this work we used previously purified, endogenous *A. actinomycetemcomitans* GroEL protein (data submitted for publication) as an antigen to investigate the ability of GroEL to induce preferential apoptosis among different cell types. To this end, human peripheral blood mononuclear cells and cell lines were cultured with AaGroEL, and apoptosis of cells was measured. Our data suggested that primary CD8+ T cells were more susceptible to AaGroEL-mediated apoptosis than CD4+ T cells. Interestingly, the magnitude of apoptosis in Jurkat T cells was higher than in the primary human T cells. Furthermore, K562 cells did not undergo apoptotic changes in response to AaGroEL.

Plasma membrane changes due to antigenic stimulation can be monitored as an indication of apoptosis in eukaryotic cells, including phosphatidylserine exposure on the cell surface. Annexin V is used to measure the presence of phosphatidylserine because it has a high binding affinity to phosphatidylserine in the presence of Ca++. Annexin is usually used with 7AAD, a DNA binding dye, to distinguish apoptotic cells from necrotic ones. In this study we used both AnnexinV and 7AAD to measure apoptosis. First, we measured apoptosis of CD4+ and CD8+ T cells. AaGroEL-induced apoptosis was dose and time dependent in CD4+ and CD8+ T cells (Figures 1 and 2). Interestingly, 500 ng/mL AaGroEL protein induced 13% apoptosis in CD4+ T cells compared to 51% apoptosis in CD8+ T cells at 48 h, indicating that CD8+ T cells are more susceptible to AaGroEL-induced apoptosis. Our findings suggest that AaGroEL might affect primary T cell subsets differently. This finding is notable

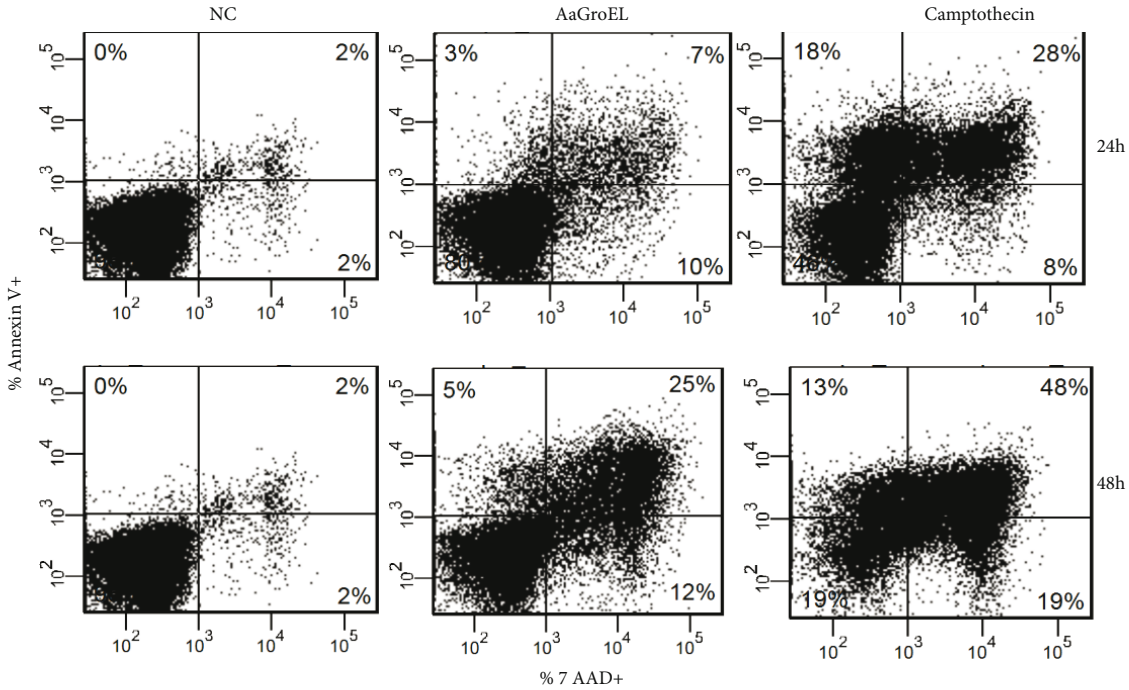

Figure 3. GroeL effect on Jurkat T cell line. Purified AaGroEL protein was cultured with Jurkat T cells for 24 h and 48 h. Cells were labeled with Annexin V and 7AAD to measure apoptotic changes. RPMI (NC) and camptothecin (CPT) were used as negative and positive controls, respectively. Representative flow data shows %Annexin V+ versus %7AAD+ Jurkat T cells. Data are representative of 3 experiments with samples from different donors.

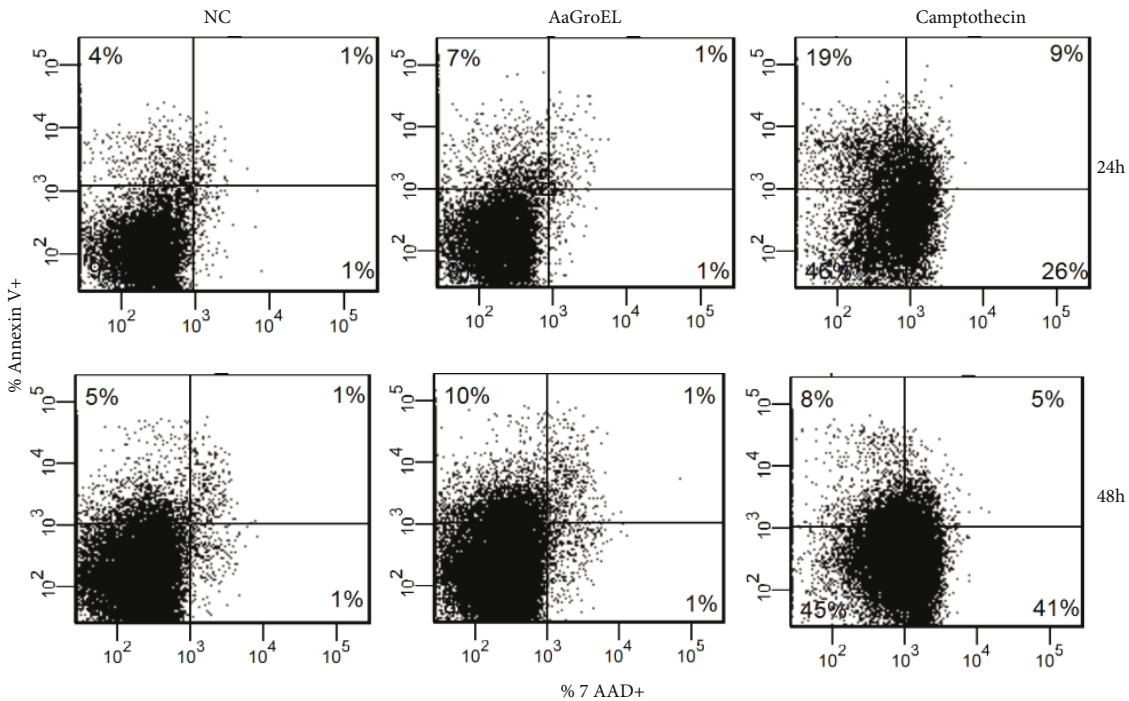

Figure 4. GroeL effect on K562 cell line. Purified AaGroEL protein was cultured with the K562 cell line for 48 h. Cells were labeled with Annexin V and 7AAD to measure apoptotic changes. RPMI (NC) and camptothecin (CPT) were used as negative and positive controls, respectively. Representative flow data shows %Annexin V+ versus %7AAD+ K562 cells. Data are representative of 3 experiments with samples from different donors.

considering reports that there are infiltrated activated/memory CD4+ and CD8+ T cells in the inflamed tissues of periodontal diseases (Taubman and Kawai, 2001). It is possible that the killing of CD8+ T cells with the GroEL of *A. actinomycetemcomitans* skews immune response towards CD4+ T-cell–mediated immunity.

Next we measured apoptosis in the Jurkat T cell line at 100 ng/mL AaGroEL protein. At 48 h, there was 30% apoptosis in Jurkat T cells. When we compared the apoptotic rate of Jurkat T cells with primary CD4+ T cells at the same time point and dose, there was a 3-fold increase in the apoptotic rate of Jurkat T cells compared to a 2-fold increase in CD8+ T cells (Figures 1–3). It appears that the GroEL protein of *A. actinomycetemcomitans* has a more potent apoptotic effect on Jurkat T cells. This finding parallels an observation made in LtxA, one of the virulence factors of *A. actinomycetemcomitans*. It was shown that LtxA kills human malignant white blood cell lines and primary leukemia cells from acute myeloid leukemia patients, whereas healthy peripheral blood mononuclear cells are relatively resistant to LtxA-mediated apoptosis (Kachlany et al., 2010). Interestingly, our findings point to the differing sensitivity of healthy human peripheral blood CD4+ and CD8+ T cells to GroEL-mediated apoptosis.

Previous studies of *A. actinomycetemcomitans* GroEL showed that it was cytotoxic to host cells (Kirby et al., 1995; Goulhen et al., 1998; Paju et al., 2000; Zhang et al., 2004). However, it is not yet known how GroEL can kill human primary T cells. Leukotoxin of *A. actinomycetemcomitans* interacts with lipid rafts of the plasma membrane to stimulate leukocyte-function–associated antigen-1 (LFA-1) signaling to induce apoptosis of target cells (Fong et al., 2006). Thus, the molecular mechanisms of *A. actinomycetemcomitans* GroEL-induced apoptotic pathways in human T cells need to be investigated.

Overall, data demonstrated that purified, endogenous *A. actinomycetemcomitans* GroEL protein preferentially induces apoptosis in primary CD8+ T cells compared to CD4+ T cells. Furthermore, Jurkat T cells are more susceptible to *A. actinomycetemcomitans* GroEL apoptosis than primary CD4+ and CD8+ T cells. AaGroEL was not able to trigger apoptotic changes in K562 cells, suggesting that AaGroEL is a more potent apoptotic inducer in cells of a lymphoid progenitor. Taken together, apoptotic activity of endogenous GroEL of *A. actinomycetemcomitans* indicates that AaGroEL protein is a virulence factor of this bacterium that utilizes apoptosis to interact with immune system cells to manipulate the host.

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