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The *Aggregatibacter actinomycetemcomitans* heat shock protein GroEL interacts directly with human peripheral blood T cells

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Abstract: Heat shock family protein GroEL of *Aggregatibacter actinomycetemcomitans* (Aa) has antigenic properties. We previously demonstrated that *A. actinomycetemcomitans* GroEL-like protein affects human CD4 T cells by converting them into IL-10 and IFNg double cytokine producing Tbet+ Th1 cells. The objective of this study was to investigate whether or not AaGroEL communicates with T cells directly. To do this, sorted cells from peripheral blood mononuclear cells were stimulated with AaGroEL for 48 h. Flow cytometry was used to measure soluble and intracellular cytokine expression in the cell cultures and detect TLR2 expression on the surface of T cells. Expression of six different soluble cytokines was evaluated by CBA assay. To determine whether AaGroEL affects CD3+ T cells directly or not, purified CD3+ T cells or CD14+ cells were cultured with AaGroEL separately, and the quantity of soluble cytokine was measured. Results showed that sorted CD3+ cells produced soluble IL-6, TNFα, and IFNγ cytokines. Additionally, the intracellular cytokine staining data showed that AaGroEL-stimulated CD3+ cells were also TNFα- and IFNγ-positive. Moreover, AaGroEL-responsive T cells slightly increased their TLR2 expression. These findings suggest that CD3+ T cells produce cytokines in response to AaGroEL protein without requirements for other cells, such as CD14+ monocytes.

Key words: T cells, *Aggregatibacter actinomycetemcomitans* GroEL, Hsp64, cytokines, TLR2

1. Introduction

Aggregatibacter actinomycetemcomitans is a gram-negative, facultative anaerobic, nonspore-forming, and nonmotile coccobacillus (Slots et al., 1999). *A. actinomycetemcomitans* is known to be the major cause of periodontal diseases, which are the most common inflammatory diseases affecting gums and teeth surrounding connective tissue worldwide. The bacterium is an etiologic agent of localized aggressive periodontitis (Herbert et al., 2016). This group of diseases is multifactorial, with components including pathogen effect, genetic background, and the environment (Haubek and Johansson, 2014; Åberg et al., 2015). Bacterial GroEL is a member of the heat shock protein family in prokaryotes. Its major role in the cell is to assist in proper protein folding. It is also suggested that bacterial heat shock proteins can play a role in pathology (Res et al., 1991). For instance, the 64-kDa GroEL is known to be an immunodominant antigen for humans (Koga et al., 1993; Tabeta et al., 2001; Nalbant et al., 2003; Tahsin et al., 2012).

The oral epithelial cell lining is the invading point of *A. actinomycetemcomitans* into the host tissue. The GroEL protein of *A. actinomycetemcomitans* stimulates epithelial cell proliferation. When GroEL is highly present, it causes cell death (Goulhen et al., 1998; Paju et al., 2000). Surface-associated material of *A. actinomycetemcomitans*, including GroEL, showed osteolytic activity on alveolar bone (Kirby et al., 1995). Additionally, sera from periodontitis patients reacted strongly with *A. actinomycetemcomitans* GroEL, indicating a humoral immune response to this protein (Koga et al., 1993). Furthermore, *A. actinomycetemcomitans* GroEL polarizes T cells into dual cytokine producing Th1 cells (Tahsin et al., 2012). Altogether, GroEL seems to have the capacity to be a potent antigen of *A. actinomycetemcomitans*.

Although GroEL of *A. actinomycetemcomitans* is a potential virulence factor, there are other virulence factors for this bacterium. Lipopolysaccharide (LPS) of *A. actinomycetemcomitans* has been shown to stimulate inflammatory cytokine IL-8 production in gingival epithelial cells, IL-6 production in periodontal ligament fibroblasts, and bone resorption (Nishida et al., 2001; Sfakianakis et al., 2001; Patil et al., 2006). Leukotoxin (Ltx) of *A. actinomycetemcomitans* has toxic effects on human lymphocytes, monocytes, and neutrophils by forming

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pores on their membranes and suppressing the immune cell response (Mangan et al., 1991; Lally et al., 1997; Korostoff et al., 1998). Cytolethal distending toxin (CDT) of *A. actinomycetemcomitans* is coded by the *cdtABC* operon. CDT has been shown to act as a holotoxin (Mayer et al., 1999). This toxin family causes cell distention and cell cycle arrest at the G0/G1 or G2/M phase in eukaryotic cells (Shenker et al., 2000). Although there are extensive studies of Ltx and CDT virulence factors, the antigenic property of *A. actinomycetemcomitans* GroEL protein is not well characterized. In one study, Nalbant et al. showed that *ltxA* and *cdtABC* double mutants still cause apoptosis in T cells. N-terminal protein sequencing and inhibition assay results indicated that GroEL heat shock protein was related to the induction of apoptosis in peripheral blood mononuclear cells (PBMCs) (Nalbant et al., 2003). Recently, it was shown that GroEL of *A. actinomycetemcomitans* differentiates human T cells into IFNγ and IL-10 dual cytokine expressing Tbet+ Th1 cells (Saygılı et al., 2012).

In the literature, the effects of *A. actinomycetemcomitans* GroEL (AaGroEL) protein were shown on epithelial cells, osteoclasts, and T cells; however, the mechanism of interaction between GroEL protein and human T cells is not known yet. Therefore, in this study, *A. actinomycetemcomitans* GroEL protein was used to investigate whether AaGroEL affects T cells directly or not.

2. Materials and methods

2.1. Human subjects and peripheral blood mononuclear cells

All blood donors were systemically and periodontally healthy adult volunteers. They were nonsmokers and under the age of 50. A total of 35 subjects were asked to sign an informed consent form that had been previously approved by the Bioethics Committee of Dokuz Eylül University Medical School, İzmir, Turkey. Venous blood was drawn from the volunteers at the İYTE Health Service by health professionals. PBMCs were isolated by the Ficoll–Hypaque density gradient centrifugation method (Böyum, 1968).

2.2. Preparation of recombinant AaGroEL (rAaGroEL)

Recombinant AaGroEL protein, previously prepared in our laboratory, was used in this study (Saygılı et al., 2012). Briefly, AaGroEL 64-kDa AaGroEL was first cloned into the pGEM T Easy (Promega) vector and then transferred to the pET28a+ (Novagen) expression vector. The confirmed pET/AaGroEL vector was transformed into *BL21* (*DE3*) *E. coli* cells for protein expression. After OD600 reached a value of 0.6, IPTG (1 mM) was added and incubated for 4 h. Protein purification from cell extract was carried out with TALON Cell Thru Resin according to the manufacturer's instructions (Clontech). The purity and concentration of the eluted protein was confirmed by 8% SDS-PAGE and the Bradford protein assay (Bio-Rad), respectively. Protein identity was further confirmed with western blotting and MS analysis. Possible LPS contamination of the purified protein sample was checked using the LAL chromogenic endpoint assay (Hycult Biotech). Possible LPS contamination was eliminated using polymyxin B loaded resin (Pierce).

2.3. Stimulation of PBMCs with rAaGroEL protein

The cultures were carried out in U-bottom cell culture tubes. Each tube contained 10^6 cells in a total volume of 500 µL. The negative control contained RPMI with 10% FBS and PBMCs. As a positive control, a phorbol ester, PMA, and a Ca++ ionophore, ionomycin, were used at final concentrations of 25 ng/mL and 1 µg/mL, respectively. rAaGroEL was added to the cultures at 20 µg/mL concentration, which is the optimal dose to activate PBMCs (Saygılı et al., 2012). The culture tubes were incubated at 37 °C and 5% CO_2. Immediately after the predetermined culture time, labeling was carried out. For statistical analysis each set was prepared in triplicate.

2.4. Detection of cytokines

The secreted IL-2, IL-4, IL-6, IL-10, TNFα, and IFNγ cytokines in culture supernatants were analyzed with the BD Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II. The manufacturer's instructions were followed. Briefly, the standard cytokine mix, which contained equal amounts of each of the six cytokines, was serially diluted 8 times. The beads are designed so that each bead can bind a specific cytokine. The six bead populations were mixed in equal amounts (10 µL/cytokine/sample). The prepared bead mixture was added to the standards and the samples. Samples were supernatants of AaGroEL-stimulated and RPMI-treated cells cultures at different time points. Finally, 50 µL of PE detection reagent was added, and samples were incubated for 3 h in the dark. The beads were washed carefully after incubation and analyzed with BD FACSArray flow cytometry. The data were analyzed using FCAP Array software.

To measure intracellular cytokine levels, CD3+ sorted cells were cultured in the presence of AaGroEL protein for 48 h, as described above. GolgistopTM (1:1500 dilution, BD Biosciences) was added to the cultures 4 h prior to the indicated culture times. Cells were fixed and permeabilized after the addition of CD3 monoclonal antibody to label T cells. Then cytokine antibodies, including IL-10, TNFα, and IFNγ, were added to the cells. Finally, cytokine-labeled cells were analyzed by flow cytometry.

2.5. Measurement of toll-like receptor (TLR) expressions

PBMCs were cultured with rAaGroEL (20 µg/mL) and LPS (100 ng/mL) for 48 to 72 h. RPMI and PMA (25 ng/mL)/ionomycin (1 µg/mL) were used as negative and positive controls, respectively. At indicated time points, cells

were labeled with anti-CD3 and anti-TLR2 antibodies. Cell surface labeling was carried out according to the manufacturer's instructions (BD Biosciences). Labeled cells were acquired by FACSArray flow cytometry and analyzed by FACSArray software or FlowJo. CD3-gated T cells were analyzed for TLR2 expression.

2.6. Statistical analysis

Cells were acquired by FACSArray flow cytometry and analyzed by FACSArray, FlowJo, or FCAP. For further analysis, data from flow cytometry were exported to Microsoft Office Excel. Experimental and control samples were compared for statistical analysis by a two-tailed Student's t-test, and P values smaller than 0.05 were accepted as statistically significant. Error bars represent the standard deviation and asterisks indicate that P was less than 0.05.

3. Results

3.1. GroEL protein interacts with purified CD3+ T cells and CD14+ monocytes

We investigated whether GroEL protein directly affects CD3+ T cells or not. Usually, T cells require antigen processing and presentation by antigen presenting cells (APCs) such as monocytes. To study this, isolated PBMCs were sorted using CD3 and CD14 magnetic beads to obtain pure cells of each type, respectively. Sorting efficiency was >98% for CD3+ cells and >95% for CD14+ cells. CD3+ and CD14+ cells were then cultured individually with 20 µg/mL rAaGroEL protein. In addition, CD3+ and CD14+ cells were also cocultured, based on the percentages of starting PBMCs (5:1, T cells to monocyte ratio), and stimulated with rAaGroEL. Unsorted PBMCs were used as controls. All culture combinations were carried out for 48 h. Following the stimulation of cells with AaGroEL, culture supernatants were collected at indicated time points. The simultaneous expression of six cytokines (IL-2, IL-4, IL-6, IL-10, TNFα, and IFNg) was measured. After CBA assay, cytokine concentrations were calculated based on standards. Figure 1A shows the representative flow data of the CBA assay. Cytokines are shown with an arrow on the left panel. The conditions of the experiment are shown with boxes on the right panel. The six bead populations are listed as IFNγ, TNFα, IL-10, IL-6, IL-4, and IL-2 from bottom to top, respectively. At the beginning of the culture, the six cytokines were aligned to the left. The shifting intensity of each population to the right implies an increase of its mean fluorescence intensity, which is used for the calculation of each cytokine's concentration (as pg/mL) in culture supernatants, based on the intensity of standard cytokine mixtures. Figure 1B shows the calculated concentrations of 6 cytokines at 48 h in a table. In all types of cultures, IL-2 and IL-4 were not detectable or stimulated. Although there were significant amounts of IL-10 and IFNγ stimulation in whole PBMCs (103 and 86 pg/mL, respectively), neither the sorted CD3+ T and CD14+ monocyte cells nor the cocultured CD3+ and CD14+ cells secreted IL-10 in the presence of rAaGroEL. Moreover, IFNγ levels increased 3-fold in the presence of rAaGroEL in CD3+ sorted T cells. IL-6 production was stimulated in all types of cultures, yet the whole PBMCs displayed the greatest increase in the presence of rAaGroEL (1482 pg/mL) (Figures 1A and 1B). TNFα was secreted in all culture combinations. In the purified CD3+ cell culture, TNFα cytokine expression increased 2.4-fold compared to RPMI-treated cells (25 to 61 pg/mL). Collectively, the presented data in Figures 1A and 1B show that rAaGroEL can cause CD3+ T cells to produce IFNγ, IL-6, and TNFα cytokines in the absence of antigen-presenting cells such as CD14+ monocytes.

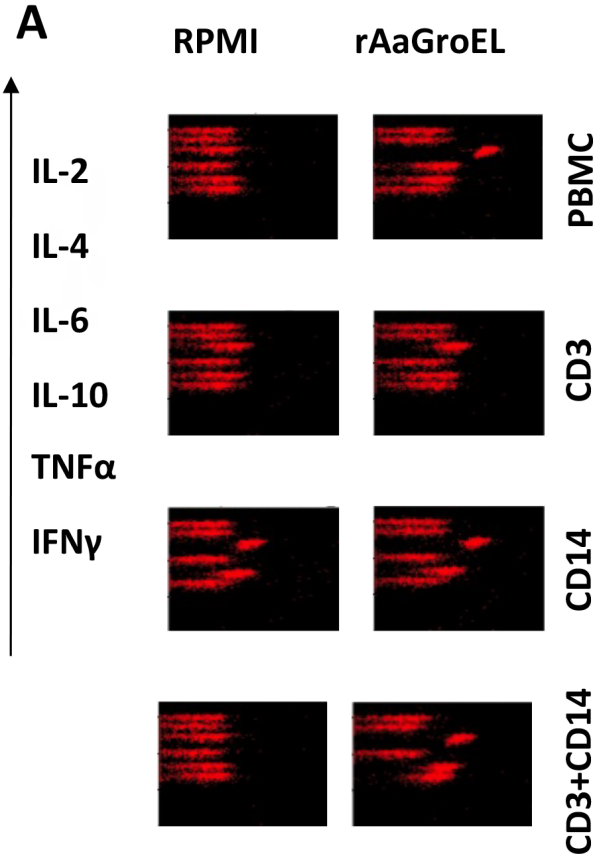
3.2. AaGroEL stimulates expression of TNFα and IFNγ cytokines

Next, rAaGroEL-stimulated sorted CD3+ T cell cultures were carried out for 48 h to measure intracellular cytokine levels. We confirmed secreted cytokines as measured by CBA assay, with intracellular cytokine production as detected by flow cytometry. For intracellular staining, the IFNγ, IL-10, and TNFα cytokines were measured. Data showed that AaGroEL protein-responsive CD3-positive T cells had increased their IFNγ expression (5.2%) compared to the negative control cells (1.3%). There was a statistically significant difference (4-fold difference when AaGroEL-treated cells were compared to the negative control at 48 h, $P < 0.05$, Figures 2A and 2B). IL-10 expression did not change in rAaGroEL-stimulated sorted CD3+ T cells (1.56%) compared to the negative controls (1.36%, $P = 0.53908$, Figures 2A and 2B). Furthermore, there was a 5-fold increase in TNFα cytokine levels in response to AaGroEL protein as compared to the negative controls ($P < 0.05$, Figures 2A and 2B). Overall, TNFα and IFNγ cytokines were detected with different expression patterns in rAaGroEL-stimulated T cell cultures. The expression of these cytokines indicates that AaGroEL stimulates T cells to express pro- and antiinflammatory cytokines.

3.3. GroEL stimulates TLR2 expression on T cells

TLRs are expressed by the immune system cells, such as macrophages, dendritic cells, and B and T cells. It is known in the literature that bacterial heat shock proteins interact with TLRs. To investigate the role of TLR2 in AaGroEL-mediated T cell function, PBMCs were cultured with rAaGroEL protein. PMA/I- and LPS-treated cells were used as positive controls. At the indicated time point, cells were labeled with CD3 and TLR2 antibodies and analyzed by flow cytometry. Data showed that at 48 h, AaGroEL protein-responsive CD3-positive T cells had slightly increased their TLR2 expression (9.2%) compared to negative control cells (4%, $P < 0.05$) (Figure 3).

A



B

Conc. pg/ml	IL-2		IL-4		IL-6	
	RPMI	rAaGroEL	RPMI	rAaGroEL	RPMI	rAaGroEL
PBMC	3	6	5	6	4	1482
CD3	6	7	5	6	40	170
CD14	4	4	5	7	184	647
CD3+CD14	7	30	5	6	-	634
	IL-10		TNFα		IFNγ	
	RPMI	rAaGroEL	RPMI	rAaGroEL	RPMI	rAaGroEL
PBMC	9	103	-	69	14	86
CD3	9	10	25	61	4	13
CD14	7	11	120	140	3	4
CD3+CD14	7	13	11	102	2	3

Figure 1. GroEL affects purified CD3+ T cells and CD14+ monocytes. CD3- and CD14-positive cells were sorted from PBMCs using CD3 and CD14 magnetic beads to obtain purified cells of each type. After sorting, CD3+ and CD14+ cells were cultured individually with 20 µg/mL rAaGroEL protein. In addition, CD3+ and CD14+ cells were cocultured based on their presence in donor PBMCs and were stimulated with the same protein. Unsorted PBMCs were used as the control. All cell culture combinations were carried out for 48 h. Then culture supernatants of different cell culture combinations were subjected to CBA assay. A) Representative flow data showing localization of six populations of cytokine binding beads at 48 h. The arrow indicates the order of the bead populations from top to bottom (IL2 is first and IFNγ is last). At 0 h, beads were aligned to the left of the histogram. The shifting intensity of each population to the right implies an increase of the mean fluorescence intensity (MFI) of a particular bead carrying a specific cytokine. B. Shows studied cytokine's concentration (pg/mL) in table.

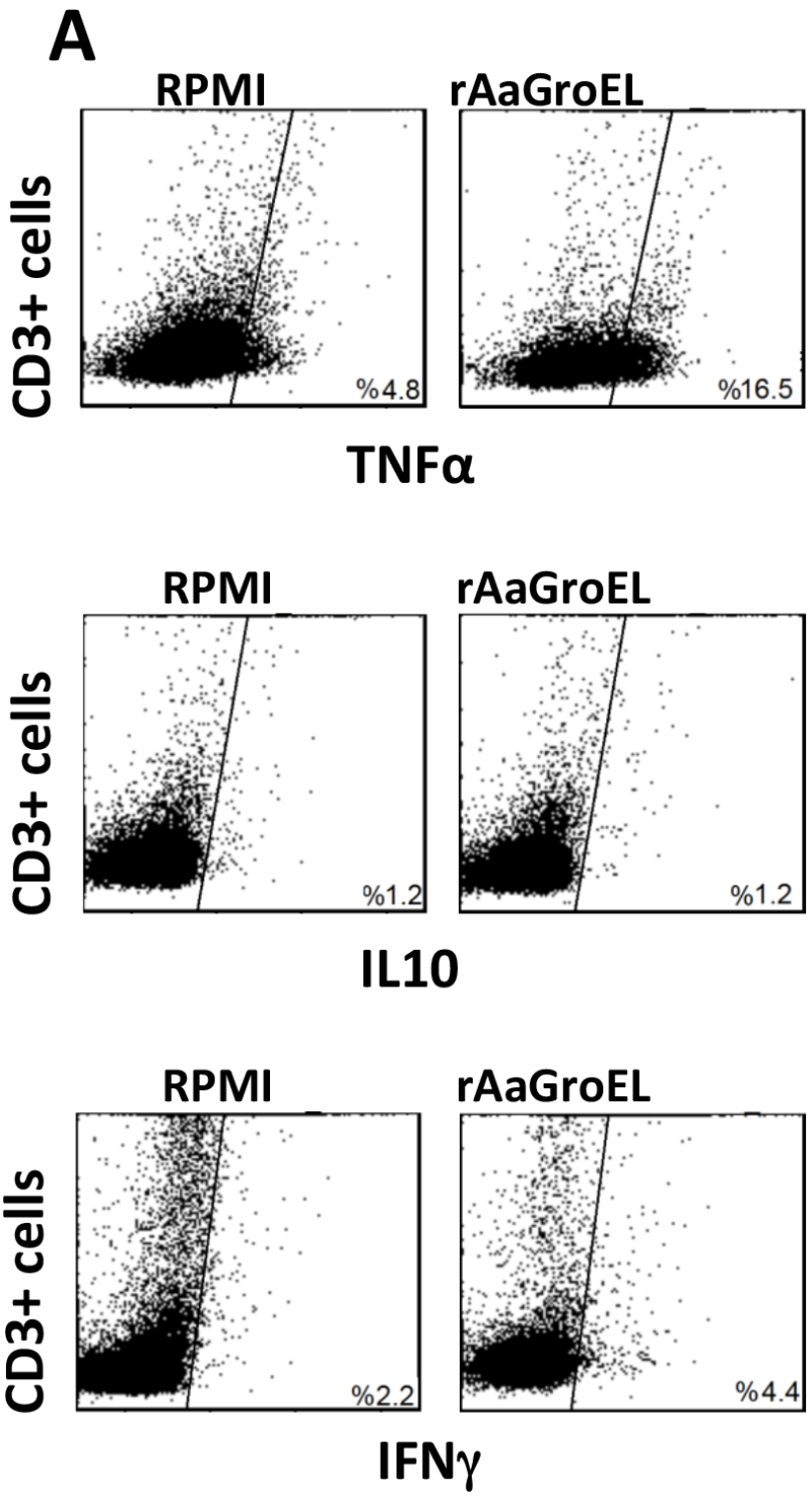


Figure 2. GroEL triggers cytokine expression. Sorted CD3 T cells were stimulated with 20 μg/mL rAaGroEL for 48 h. RPMI and PMA/I were used as negative and positive controls, respectively. Cultures were stopped at the indicated time points. Cultured cells were collected and subjected to intracellular cytokine staining, described in Section 2. A) Representative flow data show intracellularly detected TNFα, IL-10, and IFNg cytokines of sorted CD3+ T cells. Cells were gated on T cells for analysis. B) Means and standard deviations of the proportions of cytokine-positive T cells are shown. The data presented are representative of three experiments with different donors. Error bars represent standard deviation and asterisk indicates that P is less than 0.05.

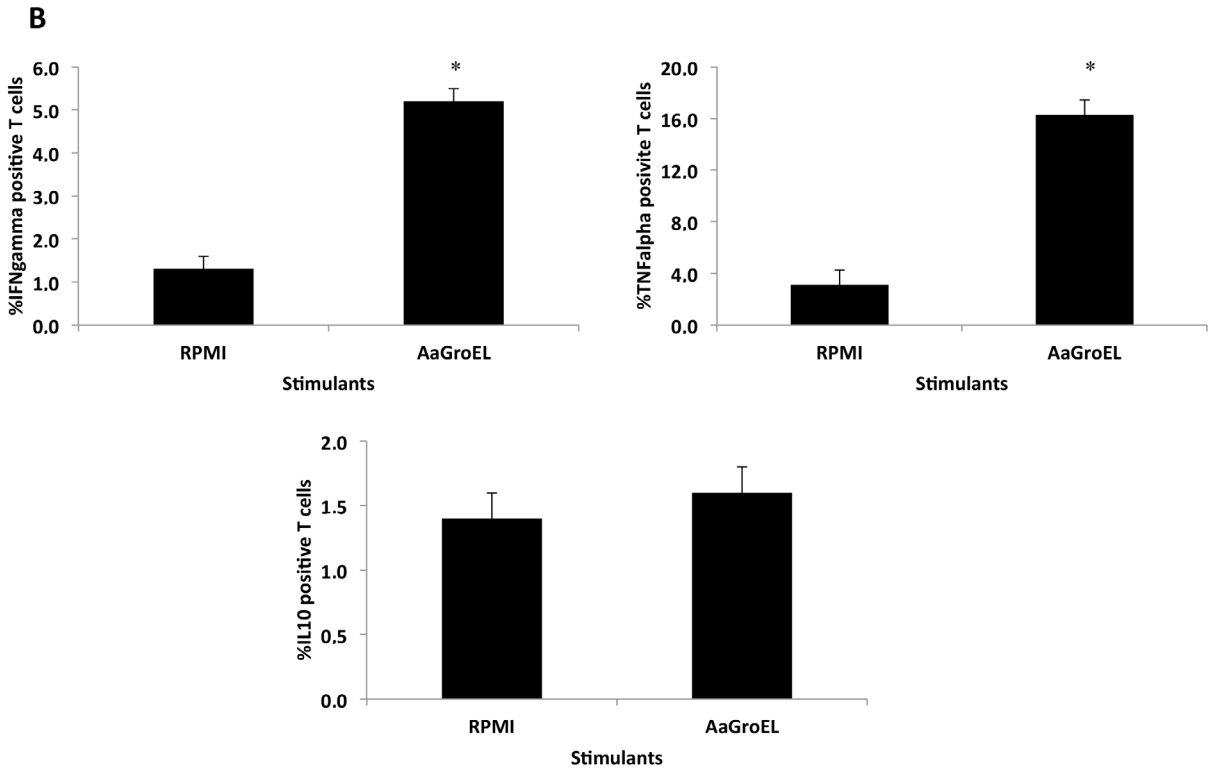


Figure 2. (Continued).

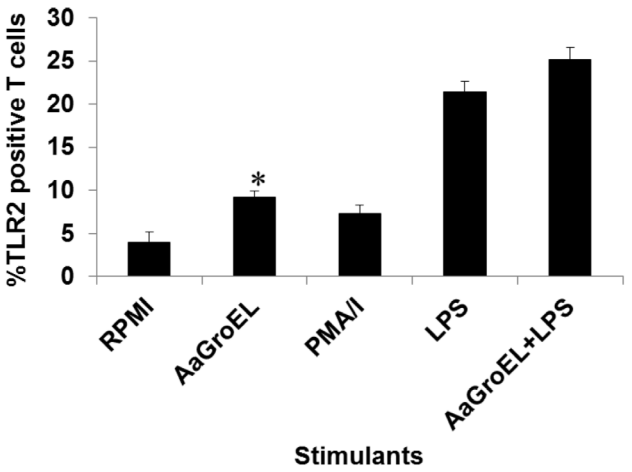


Figure 3. GroEL stimulates TLR2 expression. PBMCs were cultured with rAaGroEL (20 µg/mL) and LPS (100 ng/mL) for 72 h. RPMI and PMA (25 ng/mL) concurrently with ionomycin (1 µg/mL) were used as negative and positive controls, respectively. At the indicated time point, cells were labeled with anti-CD3 and anti-TLR2 antibodies. Cell surface labeling was carried out according to the manufacturer's instructions. Labeled cells were acquired by FACSArray and analyzed by FACSArray software. CD3 gated T cells were analyzed for TLR2 expression. A graph of TLR2-expressing T cells is shown. Data are representative of three experiments with different donors. Error bars represent standard deviation and asterisk indicates that P is less than 0.05.

4. Discussion

Heat shock proteins are chaperon proteins, expressed in both prokaryotic and eukaryotic cells at low levels and responsible for correct folding and movement of proteins in the cell. It is also known that bacterial heat shock proteins have antigenic capacities to affect human host cells. Recently it was shown that *A. actinomycetemcomitans* GroEL protein converts CD4 T cells into double cytokine producing Th1 cells (Tahsin et al., 2012). In this study, recombinant AaGroEL protein was used to investigate whether AaGroEL affects human peripheral blood T cells directly or not. To the best of our knowledge, our data demonstrated for the first time that AaGroEL protein causes cytokine production by sorted CD3+ T cells, in the absence of any antigen-presenting cells such as monocytes.

First we showed that when only sorted CD3+ T cells were treated with AaGroEL, they were able to produce soluble TNFα, IL-6, and IFNγ cytokines (Figures 1A and 1B). Our conclusion is that cultures of sorted CD3+ T cells treated with AaGroEL resulted in the production of cytokines. Since the purity of CD3+ sorted T cells was greater than 98%, we interpreted these data to mean that GroEL causes CD3+ T cells to produce cytokines in the absence of other antigen-presenting cells such as CD14+ monocytes. The results indicate that this is a direct effect of GroEL on human T cells. As we are not interested in

which T cell subset was activated by GroEL in this study, we did not examine whether IFNγ or other cytokines are produced by CD4+ or CD8+ T cells. Moreover, in sorted CD14+ monocyte cultures, IL-6 and TNFα levels were detected, while in coculture, the level of IL-6 did not increase further. In coculture experiments, the same number of cells (1×10^6 in 500 µL) in each sample was maintained to ensure a 5:1 T cell-to-monocyte ratio. This was the ratio calculated in the whole PBMCs of the donor. Because T cells produce low levels of IL-6 compared to CD14+ monocytes (170 pg/mL vs. 647 pg/mL), the higher percentage of T cells in the coculture samples should result in approximately 551 pg/mL (5:1 ratio, meaning 80% T cells + 20% monocytes). Furthermore, sorted CD3+ T cells expressed TNFα and IFNγ cytokines in response to AaGroEL, which is detected by intracellular cytokine staining (Figures 2A and 2B).

Previous studies indicated that bacterial heat shock proteins activate APCs via TLRs and trigger cytokine expression. For example, distinct *Mycobacterium tuberculosis* heat shock proteins may interact with different members of the TLR family on APCs (Bulut et al., 2005; Kim et al., 2015). Additionally, Argueta et al. showed that *P. gingivalis* GroEL protein interacts with macrophage-like THP-1 cells by binding to TLR2 and TLR4 molecules (Argueta et al., 2006). Furthermore, *F. nucleatum*- and *A. actinomycetemcomitans*-infected macrophages in mice used TLR2/4 receptors to produce cytokines (Park et al., 2014). Recently it was also shown that *A. actinomycetemcomitans*-infected THP-1 cells increased the TLR2 expression and apoptosis of these cells (Kato et al., 2013). These findings suggest that multiple TLRs may participate in the cytokine production of macrophages against bacteria or bacterial products. It is important to point out that these studies did not investigate the TLRs on human T cells in response to bacterial antigens. However, in the present study, the effect of AaGroEL protein on T cells was measured by TLR2 expression. Data showed that AaGroEL-responsive CD3+ T cells slightly increased their TLR2 expression (Figure 3). Although the TLR2 signaling is interesting, the purpose of this study was to investigate whether rAaGroEL affects T cells directly or not. This finding only suggests that TLR2 might play a role in this communication. Thus, the components of the TLR2 signaling pathway were not a focus of this study. It is also known in the literature that there are different ways in which the bacterial virulence factor interacts with host target cells. For instance, both CdtC and CdtB subunits of *A. actinomycetemcomitans* CDT interact with membrane cholesterol within lipid rafts (Boesze-Battaglia et al., 2009, 2015). Moreover, the active subunit CdtB of CDT also functions as a phosphatidylinositol-3,4,5-triphosphate (PIP3) phosphatase (Shenker et al., 2014). Thus, the molecular mechanism of AaGroEL recognition by T cells should be investigated in future studies.

In this study, it was shown for the first time that the GroEL protein of *A. actinomycetemcomitans* can cause pure/sorted human T cells to produce cytokines. Furthermore, AaGroEL as an antigen may not require any APCs, such as monocytes, to have an effect on human peripheral T cells or to mediate T cell immune response. Characterization of molecular mechanisms of AaGroEL-mediated immune response will not only aid in understanding this organism's pathogenic capacity, but may also offer additional experimental tools for manipulating T cell immune responses.

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