CYTOKINE EXPRESSION PATTERN OF HUMAN T LYMPHOCYTES IN RESPONSE TO *A. actinomycetemcomitans* GROEL PROTEIN

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by
Tahsin SAYGILI

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We approve the thesis of **Tahsin SAYGILI**

Assist. Prof. Ayten NALBANT
Supervisor

Assist. Prof. Bünyamin AKGÜL
Committee Member

Assist. Prof. Dr. Ali ÇAĞIR
Committee Member

02 July 2009

Assoc. Prof. Dr. Sami DOĞANLAR
Head of the Department of
Molecular Biology and Genetics

Prof.Dr. Hasan BÖKE
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

CYTOKINE EXPRESSION PATTERN OF HUMAN T LYMPHOCYTES IN RESPONSE TO *A.actinomycetemcomitans* GROEL PROTEIN

Actinobacillus (Aggregatibacter) *actinomycetemicomitans* is a bacterium that plays important role in Periodontitis. Recent publications have correlated AaGroEL (*A.actinomycetemcomitans* GroEL Protein) protein with periodontal pathologies however, how does this protein affect T lymphocytes and the profile of T lymphocytes' immune response, are not reported yet.

Within this thesis project, AaGroEL protein is produced as its recombinant form. The effects of AaGroEL protein, after it is obtained, have been investigated on naive T lymphocytes in peripheral blood.

In this research, the proliferative and activating effects of AaGroEL protein on T lymphocytes were determined by investigating the expression of CD25 and CD69 cell surface molecules. AaGroEL protein has an effect on CD4+ T lymphocytes and depending on time, it increases CD25 and CD69 expression. Additionally, it was found that, CD4+CD25+T cells are negative for FOXP3.

The cytokines of CD4+ T lymphocytes were profiled. Firstly, the cytokines that were released by PBMC's on culture supernatant were determined. Following AaGroEL stimulation, IL-6, IL-10, IFNg ve TNFα were secreted to the supernatant. Intracellular cytokine staining was applied to determine the source of those cytokines which was found that CD4+ T cells produce those cytokines as well as nonlymphocytes and B or/and CD8+ cells also secrete them. IL-2 was secreted by CD4+ T lymphocytes only. These findings have demonstrated us; AaGroEL protein activates naive CD4+ T cells towards Th1 response.

In conclusion, AaGroEL protein has an antigenic effect on T lymphocytes, regulates immune response and play important role on periodontal pathology.

ÖZET

İNSAN T LENFOSİTLERİNİN *A. actinomycetemcomitans* GroEL PROTEİNİNE VERDİKLERİ SİTOKİN ANLATIMININ PROFİLLENMESİ

Actinobacillus (Aggregatibacter) *actinomycetemicomitans* diş eti hastalıklarının patolojisinde rol oynayan bakteridir. Son yıllarda yapılan çalışmalar AaGroEL (*A.actinomycetemcomitans* GroEL Protein) proteininin periodontal hastalıkların patolojisiyle ilişkisini düşündürmekle beraber, bu proteininin T lenfositlerini nasıl etkilediği ve AaGroEL'e karşı oluşan T lenfosit immun yanıtının profili henüz bilinmemektedir.

Tez kapsamında, AaGroEL rekombinant olarak üretilmiştir. Elde edilen rAaGroEL proteininin etkileri periferal kanındaki naif T lenfositleri hücrelerinde araştırılmıştır.

Yapılan çalışmalarda, AaGroEL proteininin T lenfositlerini prolifere ve aktive ettikleri CD25 ve CD69 moleküllerinin eksprasyonuyla tespit edilmiştir. AaGroEL proteini CD4+T hücrelerini etkilemiş, bu hücrelerde zamana bağlı CD25 ve CD69 eksprasyonunu arttırmıştır. Ayrıca CD4+CD25+T hücrelerinin FOXP3- olduğu da gösterilmiştir.

Aktif hale geçen CD4+T hücrelerinin ürettikleri sitokin profilleri çalışılmıştır. Buna göre ilk olarak PBMC hücrelerinin kültür süpernatanına salgıladığı sitokinler saptanmıştır. IL-6, IL-10, IFNg ve TNFα sitokinlerinin AaGroEL stimülasyonunu takiben üretildikleri tesbit edilmiştir. Hücre içi sitokin işaretlemesinde ise bu sitokinlerin CD4+ T hücrelerinden geldiği saptanırken CD8+ T hücreleri ve/ veya B hücreleri ile lenfosit olmayan bir popülâsyonun da bu sitokokinleri sentezlediği tespit edilmiştir. Bunlara ilave olarak da IL-2 sitokininin CD4+ T hücreleri tarafından üretildiği de gösterilmiştir. Bu bulgular Th1 hücrelerinin aktive oldukları yönündedir.

Sonuç olarak, AaGroEL proteininin T lenfositleri üzerinde antijenik etkisi olduğu, immun yanıtı regüle edebileceği ve peridontal patolojide rol oynayacağı açıktır.

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ABBREVIATIONS

A.actinomycetemcomitans	Actinobacillus (Aggregatibacter) *actinomycetemicomitans*
AaGroEL	*A.actinomycetemcomitans* GroEL
bp	Base pair
CD	Cluster of differentiation
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
IL	Interleukin
IFNγ	Interferon gamma
kDa	kilo Dalton
LAL	Limulus amebocyte lysate
LB	Luria-Bertani
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rAaGroEL	Recombinant *A.actinomycetemcomitans* GroEL
TGFβ	Transforming growth factor beta
Th	Helper T cell
TNFα	Tumor necrosis factor alpha

CHAPTER 1

INTRODUCTION

Periodontitis is a chronic inflammatory disease of the supporting tissues of the teeth caused by groups of microorganisms, resulting in teeth loss (Armitage 1999). Pro-inflammotary cytokines are involved in this chronic inflammation process (Page 1991). There is an unbalanced host response against periodontopathogens whereby periodontopathogens trigger a destructive immune response for host (Seymour, et al. 1993). Periodontitis is not a localized rather a systemic disease because the periodontitis patients have increased susceptibility to cardiovascular diseases including atherosclerosis, myocardial infarction and stroke (Seymour, et al. 2007). Among others (*P.gingivalis, F.nucleatum* and *T.forsithia*), *A.actinomycetemcomitans* is one of the main periodontopathogens (Eley and Cox 2003). The natural habitat of this bacterium is the oral cavity of mammals. Leukotoxin and Cytolethal Distending Toxin are the known virulence factors of *A.actinomycetemcomitans* (Shenker, et al. 2001, Lally, et al. 1994). However when both are deleted, the bacteria still have toxic effect on lymphocytes which is the proof of a possible unknown virulence factor of the bacteria (Nalbant, et al. 2003). Moreover, an osteolytic protein of *A.actinomycetemcomitans* was identified as a member of the GroEL family of molecular chaperones (Kirby, et al. 1995). The proliferating and cytotoxic activities of *A.actinomycetemcomitans* GroEL on epithelial cells were also reported (Goulhen, et al. 1998, Paju, et al. 2000). However, how *A.actinomycetemcomitans* GroEL protein manipulates overall immune response has not been elucidated yet. Since T lymphocytes orchestrate the immune response in order to sustain pathogenic conditions, this gap in literature can be filled by reporting *A.actinomycetemcomitans* GroEL effects on T lymphocytes.

Heat shock proteins (HSPs) are a group of proteins highly conserved among species and participate in crucial functions during both stress and normal conditions. Folding and unfolding of proteins (Gething and Sambrook 1992), degradation of damaged proteins (Parsell and Lindquist 1993) and protective effects during elevated

temperatures (Lindquist 1986) are some of the most vital roles of HSPs. In addition to these known functions, several studies have shown the antigenic nature of bacterial HSP60 – GroEL protein: PBMCs secrete proinflammatory cytokines IL-1β, TNF-α, IL-6, IL-8, and IL-12 after the stimulation of HSP60.1 or HSP60.2 of M. *tuberculosis* (Lewthwaite, et al. 2001). When *Δhsp60.1* mutant M. *tuberculosis* is prepared, it was observed that this mutant can survive although it cannot induce an inflammatory response after its infection in animal models. (Hu, et al. 2008). Intramuscular DNA transfection of *B.abortus* GroEL gene induces IFNγ (Leclerq, et al. 2002). Elevated levels of cytokine mRNAs of IL-1α, IL-1b, IL-6, TNFα were observed in macrophage cultures which were stimulated with HSP60 homologues from different bacteria such as *L.pneumophila, E.coli, M.leprae, and M.bovis* (Retzlaff, et al. 1994). PBMCs secrete cytokines IFN-γ and IL-10 in response to *H.pylori* HSP60 or mycobacterial HSP65 and secrete the lymphokines (Sharma, et al. 1997). *P.acnes* GroEL stimulates the secretion of IL-1α, TNF-α and GM-CSF from keratinocytes (Graham, et al. 2004).

Two decades ago, Mosmann and Coffman defines two T cell subsets based on their distinct cytokine profile, type 1 helper T cells (Th1) and type 2 helper T cells (Th2). (Mosmann, et al. 1986). The key cytokines of Th1 and Th2 were attributed to IFNγ and IL-4, respectively. Not only each cytokine favors its corresponding phenotype but also inhibits the other's development (Parronchi, et al. 1992). Th1 cells are responsible for eradicating extracellular pathogens and main players of autoimmunity. Th2 cells, in contrast, mediate humoral immunity, including production of IgE and activate mast cells, which drive immune responses to helminthes. Allergy is the pathogenic condition of Th2 cells. After almost a decade from that, Tr1 and Th3 regulatory T cell subset was discovered and their key cytokines are TGF-β and IL-10, respectively (Roncarolo, et al. 2001, Weiner 2001). Both cytokines suppress Th1 and Th2 response and thus maintain homeostasis. Recently, a new subset, Th17, was named based on its key cytokine IL-17 and their main role is positive regulation of tissue inflammation against extracellular pathogens (Park, et al. 2005).

The aim of this study is to profile the cytokines that T lymphocytes produce in response to *A.actinomeycetemcomitans* 64 kDa HSP – GroEL protein. We also speculate which T cell subset gains effector functions, which cell groups of PBMCs secrete the cytokines in a high level and how the cells interact to gain effector functions.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cloning and Expression of 64-kDa Heat Shock Protein GroEL of *A. actinomycetemcomitans*

64-kDa heat shock protein of *A. actinomycetemcomitans* was cloned using recombinant DNA technologies. Based on the gene sequence in GenBank code D28817, primers were designed as forward and reverse, GG**CATATG**GCAGCAAAAGACGT and GG**GAATTC**TTACATCATCCCGCCCA, respectively (MWG – Biotech Company). The bold nucleotides are the recognition sequences of NdeI/EcoRI restriction enzymes (Fermentas), respectively. The PCR reaction was carried out by using; 1X PCR buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.5µM each of primer mix, 100ng DNA, 1.0 unit Taq DNA Polymerase. The PCR product which is 1644bp length fragment was gel isolated using DNA Gel Extraction Kit (Fermentas) and the isolated fragment was cloned into pGEM T Easy (Promega) vector. Then the cloned fragment was ligated into pre-digested (with NdeI/EcoRI restriction enzymes) pET28a+ (Novagen) expression vector and transformed into *DH5a E.coli* cells. Ligation was done according to 1:3, vector:insert ratio and T4 DNA ligase (Fermentas) was used. The recombinant pET/AaGroEL vector isolated from *DH5a E.coli* cells was sent for sequencing in order to confirm the cloning was performed without any mutation. The confirmed pET/AaGroEL vector was transferred into *BL21(DE3)* host *E.coli* cells for protein expression. Overnight grown 50ml *BL21(DE3) E.coli* culture carrying pET/AaGroEL vector were transferred into 500ml fresh LB broth (Gibco) containing 30µg/mL Kanamycin (Applichem). After the OD600 reached to 0.6, IPTG was added as 1mM and incubated for further 4 hours (which was predetermined as the highest recombinant protein expression time, data not shown).

2.2. Purification of Recombinant 64-kDa Heat Shock Protein, GroEL of *A. actinomycetemcomitans* (rAaGroEL)

After incubation for 4 hours, the cells were harvested at 3000g for 15min at 4^0 C and the cell pellet was suspended into buffer containing sodium phosphate and NaCl (pH=7.0). The sonication was done for 30sec. 5 times with 30sec. intervals between bursts. Centrifugation was done at 3000g for 30min. to pellet the cell debris. Cell extract was loaded into Talon Cellthru Resin filled columns (Clontech). The protein purification was done according to the manufacturer's instructions. Briefly, 15ml cell extract and 3ml (bed volume) resin were incubated for 1hour and resin bounded proteins were washed for 10 minutes and 3 times using sodium phosphate buffer. To elute proteins, 10ml 200mM imidazole containing sodium phosphate buffer was used. The purity and concentration of the eluted protein was confirmed by %8 SDS-PAGE and Bradford protein assay. 64-kDa heat shock protein was further confirmed with western blotting and MS analysis. Possible LPS contamination was eliminated using Polymyxin B loaded resin (Pierce). The resulting protein sample's LPS content was measured using LAL assay (Hycult Biotech).

2.3. PBMC (Peripheral Blood Mononuclear Cells) Isolation

The whole blood was taken from healthy volunteers (both systemically and periodontically) into blood collection tubes with heparin (Becton Dickinson) by the help of IZTECH medical personel under the consent of 9 Eylül Hospital. PBMC isolation from whole blood was carried out with the technique called "Ficoll – Hypaque Density Gradient Centrifugation (Boyum, et al. 1991)" [Ficoll (Biochrom) RPMI + 10% FBS (Invitrogen)]

2.4. Stimulation of PBMC with rAaGroEL Protein

The cultures were carried out in U bottom cell culture tubes. Each tube contained 10^6 cells in total 500µl. Negative control contained RPMI/10% FBS plus PBMC. As a positive control a phorbol ester PMA and a Ca++ ionophore, ionomycin were used at the final concentrations of 25ng/ml and 1µg/ml, respectively. rAaGroEL was put on culture at 20µg/ml concentration (which is the optimal dose to activate PBMC). The culture tubes were incubated at 37^0 C and 5% CO_2 at 37^0C. After the pre-determined culture time, immediately the labeling was carried out. For statistical analysis each set was prepared as triplicate.

2.5. Phenotypic Characterization of T Cells by Cell Surface Labeling

The antigen stimulated cells were harvested at 300g for 5min (all centrifugation at this step was done with these settings) and the supernatants were removed by vacuum system. The pellets were washed with 1XPBS/2%FBS and harvested. The determined combination of cell surface antibodies (CD3, CD4, CD14, CD25, CD69 (Becton Dickinson) with applicable conjugated fluorescence dyes) were added with pre-titrated dilution factors. The samples were incubated for 30min at dark and washed with 1XPBS/2%FBS. Washed cells were analyzed by BD FACSarray Flow Cytometry in 1XPBS/2%FBS.

2.6. Measurement of Soluble Cytokine Amounts by Cytometric Bead Array (CBA)

The secreted IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ cytokines in culture supernatants were analyzed by BD™ Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II. The manufacturer instructions were followed. Briefly, the standard cytokine mix that contains equal amounts of each 6 cytokines was serially diluted for 8 times. The beads are designed so that each bead can bind a specific cytokine. The six bead populations were mixed in an equal amount (10µl/cytokine/sample). The prepared bead mixture was put on the standards and the samples. Finally 50µl PE detection reagent was added and they are incubated for 3 hours in dark. The beads were washed carefully after incubation and analyzed by BD FACSarray Flow Cytometry. The data were analyzed using FCAP Array software.

2.7. Cytokine Profiling by Intracellular Cytokine Staining

For intracellular cytokine staining the culture was prepared same as above with the exception that before 4 hours from culture ends, Golgistop (Becton Dickinson) was added (final dilution was 1:1500). The cell surface labeling was done and 4% Paraformaldehyde (Applichem) was added for fixation. After vortexing each tube, they were incubated for 10min at dark. The cells were washed for 2 times, and for permeabilization, 0,5% Triton-X (Applichem) was added, gently mixed. The cells were harvested at 300g for 5min. and supernatant was removed as approximately 50µl solution was left on cells. Cytokines antibody was added directly and tubes were incubated for 30min. The cells were washed with 1XPBS/2%FBS and analyzed.

2.8. Analysis of Regulatory T Cells

After the cells were cell surface labeled with CD25 and CD4, FOXP3 labeling was carried out using Human FOXP3 Buffer Set (Becton Dickinson) according to the manufacturer instructions. Briefly cells were fixed and permeabilized as in intracellular cytokine staining procedure with the exception that, fixing and permeabilizing solutions of the Human FOXP3 Buffer Set were used instead of paraformaldehyde and triton-x buffers, respectively.

2.9. Data Analysis

Data analysis was carried out using FACSarray system software and FCAP software. A standard two-tailed t test was used for statistical analysis with *P* values of 0.05 or smaller is taken significant.

CHAPTER 3

RESULTS

3.1. Cloning, Expression and Purification of 64-kDa Heat Shock Protein GroEL of *A. actinomycetemcomitans*

To amplify HSP60 GroEL genes of *A.actinomycetemcomitans*, the designed primers were used which contain NdeI/EcoRI restriction sites before the start and after the stop codon of the gene, respectively. After amplification, the cloning was performed into pGEM T Easy and pET28a+ vectors, whose use was for cloning and expression, respectively. The confirmation of amplification and cloning was performed using 1% Agarose Gel Electrophoresis (Figure 3.1). pET28/AaGroEL recombinant vector was sequenced and confirmed that cloning was performed without mutation (Data not shown). IPTG induction was performed to overexpress recombinant protein in *E.coli BL21(DE3)* host cells. In order to determine the time point at which the protein expression level is the highest, a time kinetic assay was performed from 1 h to 12 h. From this assay, 4th hour was decided the most effective time for protein expression (Data not shown). The purification was done using Talon metal affinity columns. Imidazole was used to elute the protein. 8% SDS-PAGE was performed in order to confirm the purity (Figure 3.1). To confirm the protein specificity, western blotting was done using anti-GroEL antibody. As controls for western blot, heat – shocked A. *actinomycetemcomitans* and rEcoli GroEL (Stressgen) were used (Figure 3.1).

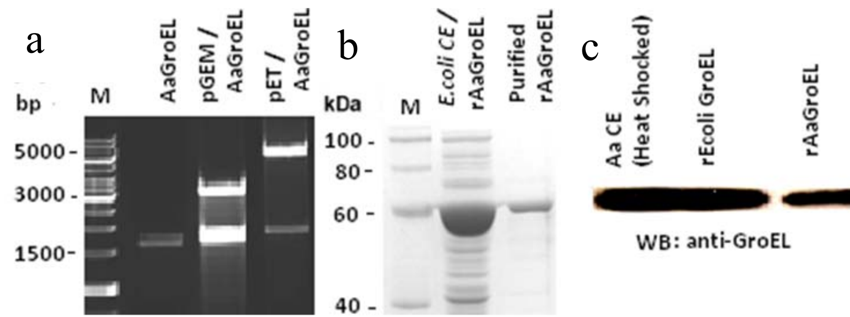


Figure 3.1. Cloning, expression, purification and confirmation of rAaGroEL protein. a) First lane (after marker) corresponds to; 1644bp AaGroEL fragment, amplified by PCR. Second lane shows the confirmation of the cloning process into pGEM T Easy vector. Third lane shows the confirmation of the cloning process into pET28a+ vector b) rAaGroEL was overexpressed in E.coli cells; First lane corresponds to rAaGroEL overexpressed E.coli lysate. Second lane corresponds to purification of rAaGroEL protein using Talon Resin. c) Purified rAaGroEL protein was confirmed using polyclonal anti-GroEL antibody.

3.2. Confirmation of Retention of Function after Lipopolysaccharide (LPS) Removal and Polymyxin B Neutralization

The purified protein was used to stimulate PBMCs. Figure 3.2 shows the TNFα production of PBMCs in response to rAaGroEL. Unstimulated cells secreted TNFα in 3% level whose 1.9% came from CD4+ T cells. After stimulation, 16% of cells secreted TNFα and the contribution of CD4+ T cells was 4.3%. This means with rAaGroEL TNFα secretion was amplified by more than 2-fold by CD4+ T cells and more than 3-fold by total PBMCs. More importantly, this increase was not significantly decreased by LPS removal or Poly B neutralization of LPS. Consequently, the function was retained and did not come from LPS (Figure 3.2).

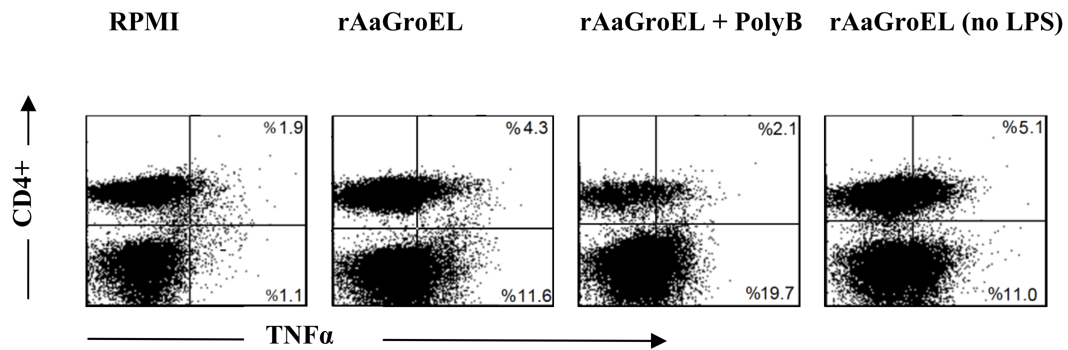


Figure 3.2. LPS removal and confirmation of function retention. In order to test if the function came from possible LPS contamination, PBMC was isolated from whole blood and stimulated with 20µg/ml rAaGroEL, 20µg/ml rAaGroEL + Polymyxin B, 20µg/ml rAaGroEL (LPS was removed using Poly B supported resin). After 48H stimulation cells were harvested washed and cell surface staining was performed. The cells, then, were fixed and permeabilized, incubated with anti-TNFα antibody. After a washing step, the cells were analyzed with BD FACSarray. (The data shows the representative of 3 biological replicas). Neither Polymyxin B nor LPS removal had an effect on the secretion of TNFα by PBMCs after stimulation with rAaGroEL.

3.3. Phenotypic Characterization of T Cells by Cell Surface Labeling after rAaGroEL Stimulation

In order to determine which subset of T cell, CD4+ or CD8+, gained effector function after stimulation with 20 µg/ml rAaGroEL, the expression of CD25 and CD69 cell surface markers were monitored in CD4+ T and CD8+ T cells. Although CD8+ T cells are significantly activated according to the CD69 marker, the main effector subset is CD4+ T cells against rAaGroEL. Whereas CD69 expressing CD8+ T cells were increased from 8% to 24%, CD69 expressing CD4+ T cells were increased from 14% to 32%. In addition, only 4% of CD8+ T cells were both CD25+ and CD69+ but as much as 15% of CD4+ T cells were positive for both markers (Figure 3.3).

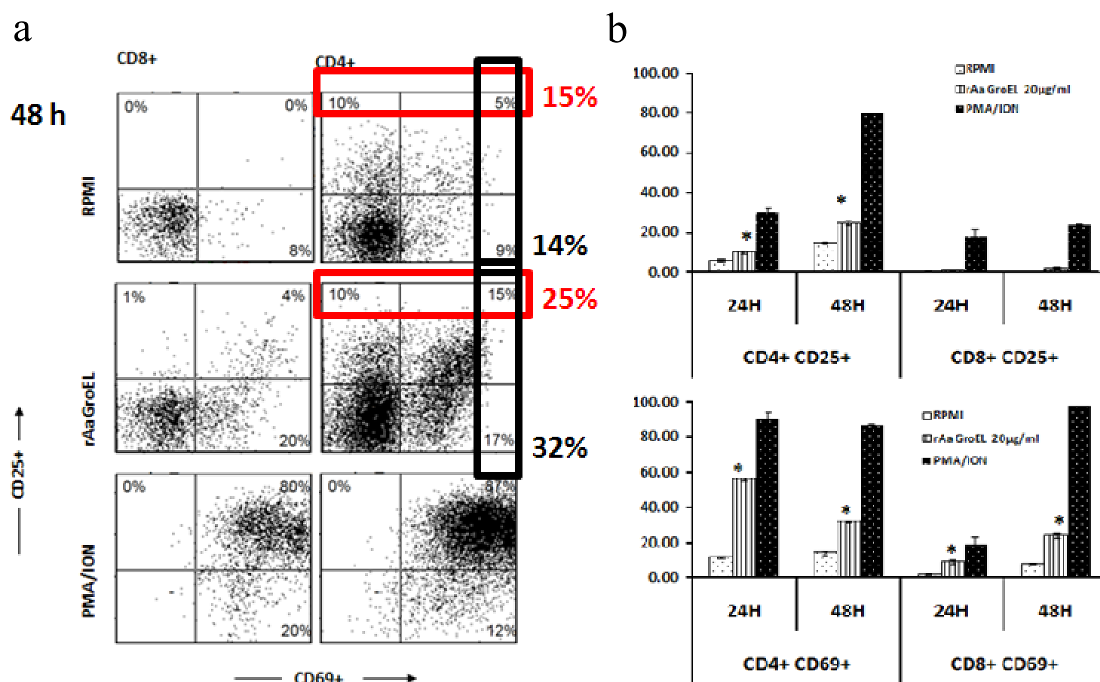


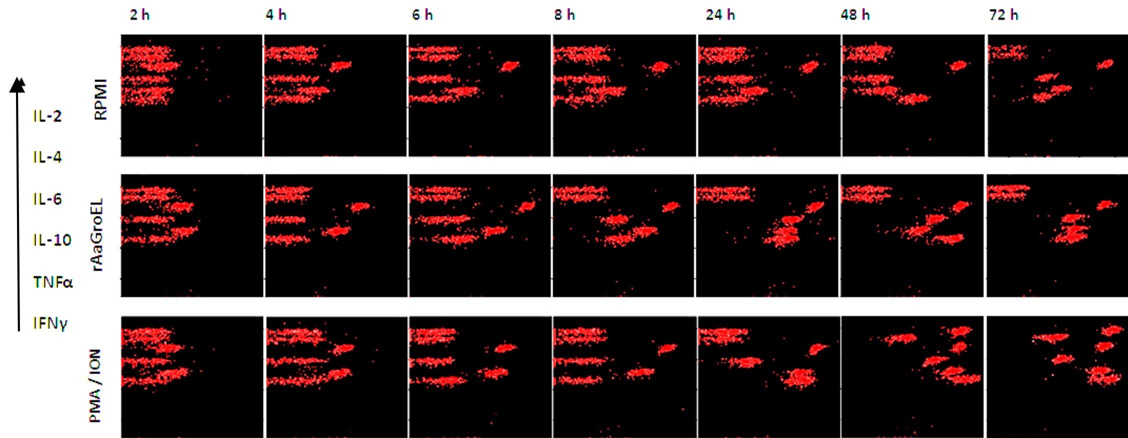
Figure 3.3. Phenotypic characterization of T cell subset, stimulated by rAaGroEL. Following 48 h stimulation of PBMCs with 20 µg/ml rAaGroEL, Cell surface labeling was performed using fluorescence conjugated anti - CD4, CD8, CD69 and CD25 antibodies. The labeled cells were analyzed with FACSarray flow cytometer (Becton, Dickinson). a) CD8+ gated and CD4+ gated T cells were monitored in terms of CD69+ and CD25+ (The data are representative data for 3 biological replicas). Whereas only 4% of CD8+ T cells are double positive for CD25 and CD69 cell surface markers, as much as 15% of CD4+T cells expressed CD25 and CD69. b) Graph representation of CD69 and CD25 expression levels by CD4+ and CD8+ T cells at 24 h and 48 h. Stars show $p<0.05$ and Error bars represent SD.

3.4. Determination of T Helper 1 and T Helper 2 Cytokines by Cytometric Bead Array

Following stimulation of PBMCs with 20 µg/ml rAaGroEL protein, culture supernatants were collected at indicated time points and subjected to Cytometric Bead Array (CBA assay). After CBA assay the cytokine concentrations were calculated based on standards. Figure 3.4 shows the plots of calculated concentrations of 6 cytokines at certain time points. According to this, there was no IL-2 and IL-4 at any time. In addition there were high amounts of secreted IFNγ, approximately 3 ng/ml at 48 h. Also, IL-10 was secreted upto 1.5 ng/ml at 24 h and decreased at 48 h where IFNγ secretion was increased. The secretion of these cytokines indicates that Th1 polarization

takes place. In addition, IL-6 amounts were increased up to 20 ng/ml from 6 h to 72 h and TNFα secretion peaked at 8 h and 24 h (8 ng/ml) (Figure 3.4).

a



b

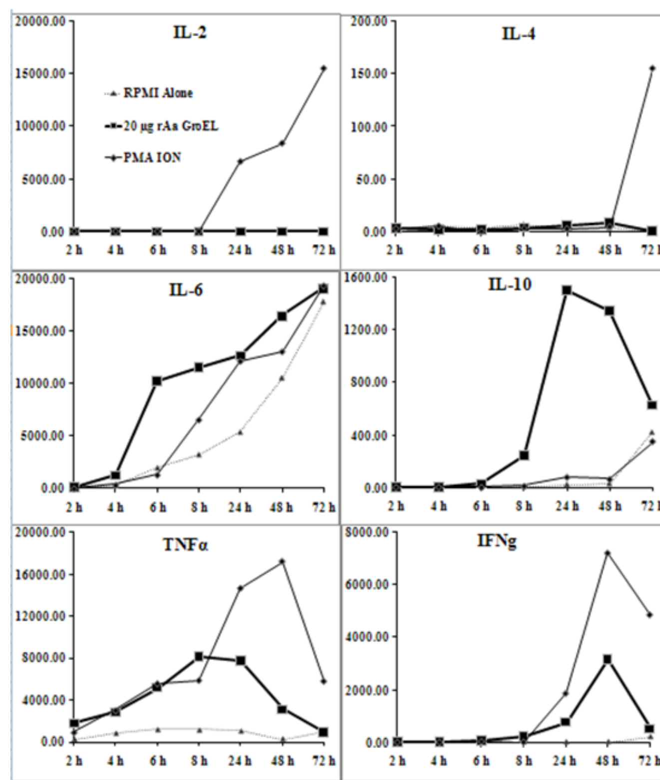


Figure 3.4. Measurement of soluble cytokines by using CBA assay. 20µg/ml rAaGroEL stimulated and unstimulated cells were put on culture. Cultures were stopped at various time points from 2 h to 72 h. Supernatants were collected and subjected to CBA assay as described at methods part. a) Flow data of CBA assay. 6 bead populations IFNγ, TNFα, IL-10, IL-6, IL-4 and IL-2 (from bottom to top) 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, relative to the intensities of standard cytokine mixtures. b) Each cytokine concentration was plotted vs. time.

3.5. Cytokine Profiling of T Cells and Monocytes by Intracellular Cytokine Staining

After monitoring the secreted cytokines, intracellular cytokine staining was performed to monitor further which cell type(s) secrete the cytokines. Also the cytokines that are not covered by CBA assay kit IL-17, TGFβ and IL-12 were also studied. Among the cytokines, IL-4, IL-17 and TGFβ were not detected at any time point (Figure 3.10).

IL-12 level was increased 3-fold in CD14+ monocytes (7% in unstimulated culture and 23% after stimulation) at 16 h (Figure 3.5) after stimulation with 20 μg/ml rAaGroEL. At 24 h there was a 3-fold increase in CD14+ monocytes (6% in unstimulated culture and 19% after stimulation).

IFNγ, TNFα, IL-10 levels were monitored at 5 time points (16 h, 24 h, 48 h, 72 h, 96 h). rAaGroEL stimulated TNFα levels among CD4+ T cells were significant at all time points: 5-fold increases at 24 h relative to negative control from 3% to 15%. At 48 h, there were approximately 2-fold increases relative to negative control from 5% to 11%. 2-fold increases occurred at 72 h from 7% to 16% and 4-fold increases occurred relative to negative control, from 4% to 16% at 96H. Among CD14+ cells, TNFα was secreted at 24 h and 48 h in a highest level, where there were 5.5-fold increases relative to negative control from 10% to 55% at 24 h and there were 6-fold increases from 10% to 64% at 24 h. At 72 h and 96 h, the percentage was decreased due to the loss of monocytes although the p value is < 0.05 at all time points and for both CD4+T cells and CD14+ monocytes, when compared to negative control (Figure 3.6).

rAaGroEL stimulated IL-10 levels among CD4+ T cells were highest at 16 h (from 0% to 5%, p value is < 0.05), at 24 h IL-10 was not significantly increased but at 48 h, 72 h and 96 h there were 3-fold (from 3% to 9%), 2-fold (from 4% to 8%), and 3.5 fold increases (from 4% to 14%), occurred among CD4+ T cells, respectively; p value is < 0.05. rAaGroEL stimulated IL-10 levels among CD14+ monocytes, at 16 h there were more than 5.5-fold increases from 11% to 57%, p value is < 0.05. At 24 h nearly 8-fold increases occurred from 6% to 44%, at 48 h 9-fold increases occurred from 4% to 35%, 2-fold increases occurred at 72h from 10% to 23%, and at those times p value is < 0.05. No significant increases were observed among CD14+ monocytes at 96 h. (Figure 3.7). Next, in order to understand whether that IL-10 secreted CD4+ T cells are also positive

for FOXP3 or not, FOXP3 staining was carried out and it was observed that CD4+T lymphocytes are induced to secrete IL-10 but they are negative for FOXP3 (Figure 3.8).

IFNγ stimulation among CD4+ T lymphocyte was highest at 72 h and 96 h where there were 9% and 8% IFNγ stimulation, respectively. At other time points no IFNγ was detected. At all time points except 96 h, IFNγ was secreted by CD14+ monocytes in the presence of rAaGroEL (Figure 3.9).

Finally, IFNγ and IL-10 cytokines were analyzed simultaneously. It was observed that at 72 h, those cytokines were secreted from same population of CD4+T Lymphocytes.

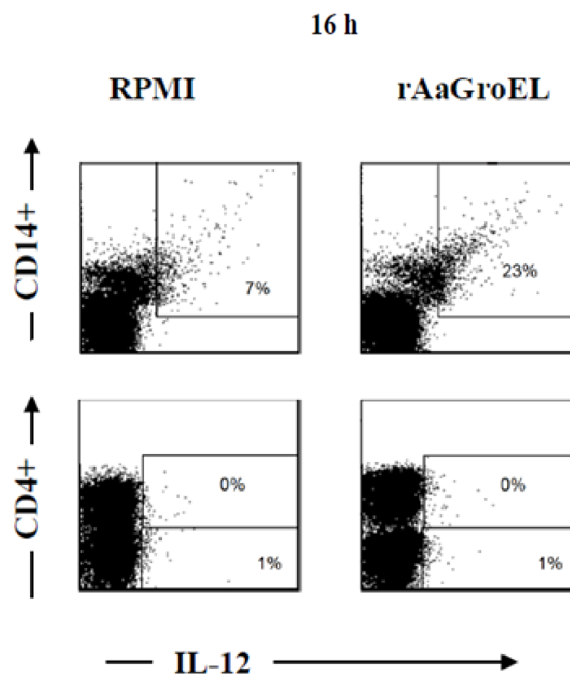


Figure 3.5. Intracellular cytokine staining to detect the CD14+Monocyte specific IL-12 expression. 20 µg/ml rAaGroEL stimulated and unstimulated cells were put on culture. Cultures were stopped at various time points. Cells were washed and cell surface stained with CD4+ and CD14+ antibodies. After fixation and permeabilization, IL-12 antibody was put on cells and analyzed by BD FACSarray flow cytometry. IL-12 was stimulated among CD14+Monocytes at 16 h by rAaGroEL.

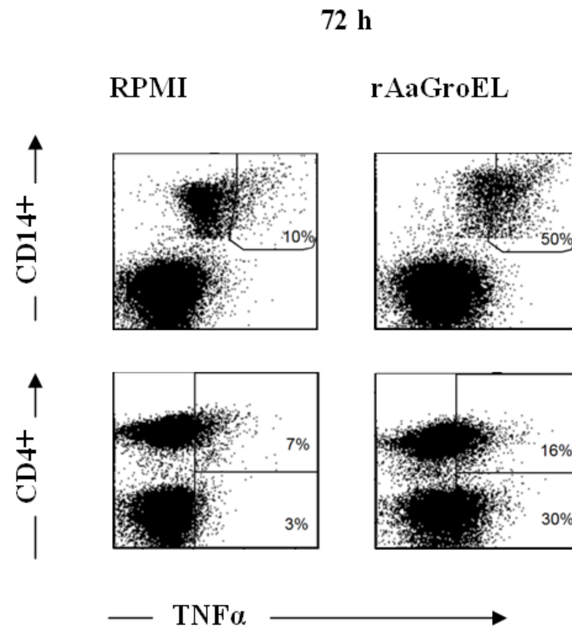


Figure 3.6. Intracellular cytokine staining to detect the CD4+T Lymphocyte specific and CD14+Monocyte specific TNFα expression. 20 µg/ml rAaGroEL stimulated and unstimulated cells were put on culture. Cultures were stopped at various time points. Cells were washed and cell surface stained with CD4+ and CD14+ antibodies. After fixation and permeabilization, TNFα antibody was put on cells and analyzed by BD FACSarray flow cytometry. TNFα was stimulated among CD4+T Lymphocytes and CD14+Monocytes at 72 h by rAaGroEL.

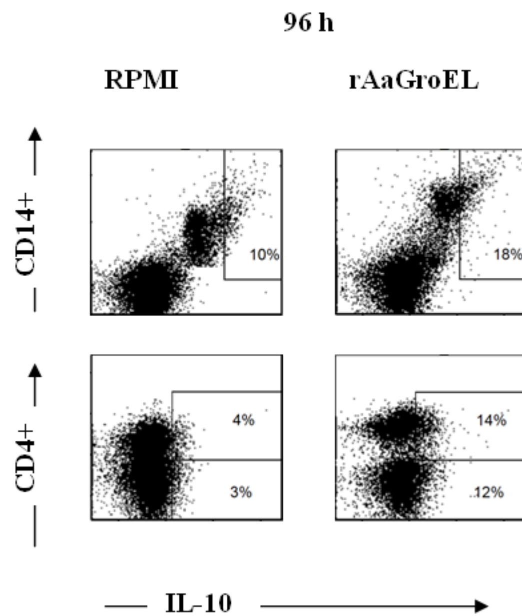


Figure 3.7. Intracellular cytokine staining to detect the CD4+T Lymphocyte specific and CD14+Monocyte specific IL-10 expression. 20 µg/ml rAaGroEL stimulated and unstimulated cells were put on culture. Cultures were stopped at various time points. Cells were washed and cell surface stained with CD4+ and CD14+ antibodies. After fixation and permeabilization, IL-10 antibody was put on cells and analyzed by BD FACSarray flow cytometry. IL-10 was stimulated among CD4+T Lymphocytes and CD14+Monocytes at 96 h by rAaGroEL.

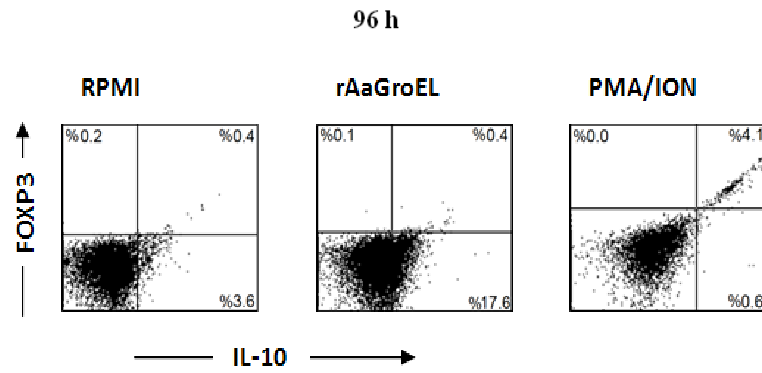


Figure 3.8. Intracellular cytokine staining and FOXP3 staining to search for Treg phenotype. 20 µg/ml rAaGroEL stimulated and unstimulated cells were put on culture. Cultures were stopped at various time points. Cells were washed and cell surface stained with CD4+ and CD14+ antibodies. After fixation and permeabilization, IL-10 and FOXP3 antibodies were put on cells and analyzed by BD FACSarray flow cytometry. IL-10 expressing cells were negative for FOXP3.

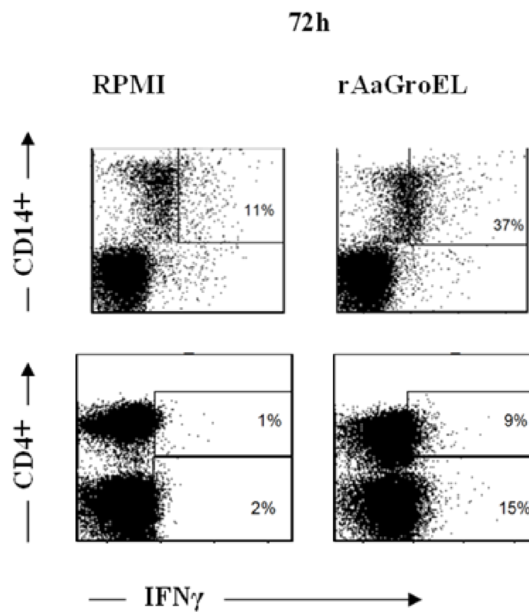


Figure 3.9. Intracellular cytokine staining to detect the CD4+T Lymphocyte specific and CD14+Monocyte specific IFNγ expression. 20 µg/ml rAaGroEL stimulated and unstimulated cells were put on culture. Cultures were stopped at various time points. Cells were washed and cell surface stained with CD4+ and CD14+ antibodies. After fixation and permeabilization, IFNγ antibody was put on cells and analyzed by BD FACSarray flow cytometry. IFNγ was stimulated among CD4+T Lymphocytes and CD14+Monocytes at 72 h by rAaGroEL.

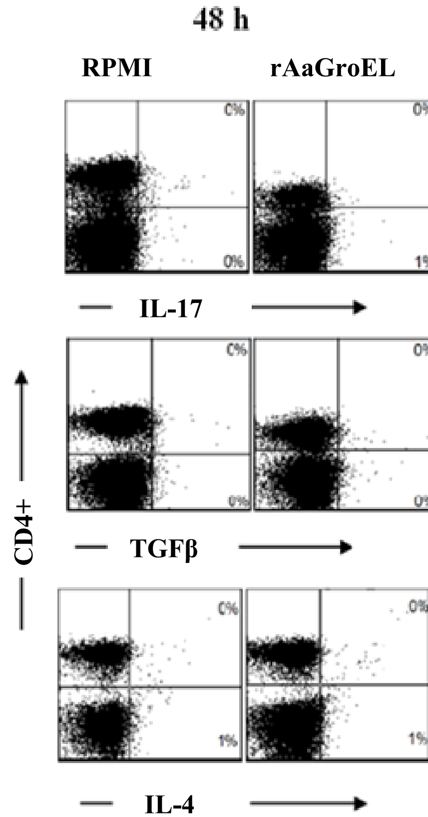


Figure 3.10. Intracellular cytokine staining to detect the CD4+T Lymphocyte specific and CD14+Monocyte specific IL-17, TGFβ, IL-4 expressions. IL-17, TGFβ, IL-4 were not secreted by CD4+ T lymphocytes and CD14+ Monocytes after stimulation with rAaGroEL

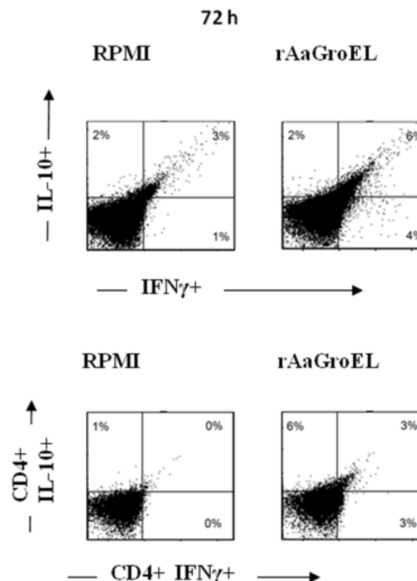


Figure 3.11. Intracellular cytokine staining to detect the CD4+T Lymphocyte specific and CD14+Monocyte specific IL-10 and IFNγ expression simultaneously. 20 µg/ml rAaGroEL stimulated and unstimulated cells were put on culture. Cultures were stopped at various time points. Cells were washed and cell surface stained with CD4+ and CD14+ antibodies. After fixation and permeabilization, IL-10 and IFNγ antibodies were put on cells and analyzed by BD FACSarray flow cytometry. IFNγ and IL-10 were secreted with the same CD4+T Lymphocytes popularion at 72 h by rAaGroEL.

CHAPTER 4

DISCUSSION

The only functions of heat shock proteins were thought to be folding and unfolding of proteins (Gething and Sambrook 1992), degradation of damaged proteins (Parsell and Lindquist 1993) and their protective effects during elevated temperatures (Lindquist 1986). However the reports in the last two decades have demonstrated that, bacterial HSPs have the ability to induce inflammatory responses by triggering the production of certain cytokines such as TNFα or IFNγ.

In this study, we demonstrated the antigenic effect of *A.actinomycetemcomitans* GroEL protein on peripheral blood mononuclear cells. In addition, we also generated some hypotheses regarding the type of immune response against this pure protein. Immediately after the pure protein was obtained, cell surface activation molecules, CD69 and CD25 was monitored in order to understand which subset of T lymphocytes was activated (CD4+ or CD8+). The expression of those molecules on CD4+ T cells clearly demonstrated that CD4+T cells were much more activated and proliferated than CD8+ T lymphocytes in response to rAaGroEL protein, although there was a small but still significant increase of CD69 molecule on CD8+T lymphocytes (Figure 3.3). But we have focused on CD4+T lymphocytes in this project due to the fact that their primary role is to regulate immune response with the help of cytokines that they produce.

Since the main effector function of CD4+T helper cells is to produce cytokines, the secreted cytokine types and levels were monitored. The secretion of 4 out of 6 cytokines was clearly stimulated in response to rAaGroEL protein. According to this assay there were no IL-2 and IL-4, but IFNγ, TNFα, IL-10 and IL-6 levels were increased by rAaGroEL (Figure 3.4). Since IL-6 and TNFα expressions were stimulated from the beginning, it is likely that the response started with inflammation. Thereafter, IL-10 secretion was induced at 6 h followed by IFNγ at 8 h. The presence of those two cytokines and also the absence of IL-4 were the signs of Th1 phenotype. Therefore, IL-12, main cytokine to initiate Th1 phenotype was monitored. It was observed that its

secretion by CD14+ monocytes was stimulated with rAaGroEL (Figure 3.5). As a consequence the Th1 polarization takes place with the stimulation of rAaGroEL.

After determination of the Th1 vs Th2 type of polarization among CD4+T helper lymphocytes, intracellular cytokine staining was performed in order to find out the cell types that secrete cytokines. And as a second purpose, in order to look for cytokines that were not able to monitor by CBA assay, Th17 and TGFβ. According to the results, after stimulation with rAaGroEL, IFNγ was secreted significantly by CD4+T lymphocytes at 16 h. Although no detectable levels of IFNγ were monitored at 24 h and 48 h, its secretion by CD4+T lymphocytes was significantly increased at 72 h and 96 h (Figure 3.9). In addition, there was continuous secretion of IFNγ by CD14+ monocytes although rAaGroEL significantly decreased the number of CD14+ monocytes (Data not shown). When rAaGroEL stimulated secretion of IL-10 was monitored intracellularly, an initial secretion of this cytokine by CD4+T lymphocytes at 16 h was detected significantly and also at 48 h there was a more significant secretion of IL-10 than 16 h by CD4+T lymphocytes. At other time points stimulated and unstimulated levels were almost equal for CD4+T lymphocytes. CD14+ monocytes secreted IL-10 was boosted at 16 h and decreased slowly although significant at 24, 48, 72 h. At 96 h, stimulated and unstimulated levels of IL-10 were almost equal for CD14+ monocytes (Figure 3.7). Intracellular double staining, IL-10 and IFNγ, results support the presence of Th1 polarization (Figure 3.11). Regarding inflammation related cytokine, TNFα was significantly secreted at all time points from 16 h to 96 h by rAaGroEL stimulation via both CD4+T lymphocytes and CD14+ monocytes (Figure 3.6). No detectable levels of IL-4, IL-17 and TGFβ, were detected at any time points (Figure 3.10).

CHAPTER 5

CONCLUSION

According to the above data, an overall scheme can be constructed: Since TNFα is a pro-inflammotary cytokine (Idriss and Naismith 2000), its presence activates monocytes, and in turn, activated monocytes can present rAaGroEL to naïve T lymphocytes to activate them. IL-6 has a costimulatory effect on T cells, independently of IL-2. Moreover it protects CD4+T lymphocytes from activation induced cell death by downregulating FasL molecule (Teague, et al. 1997, Ayroldi, et al. 1998). IL-6 is crucial for activation, proliferation and recruitment of CD4+T lymphocytes to the site of infection (Rochman, et al. 2005). Furthermore, the co-presence of IL-6 and TNFα was correlated with chronic inflammation (Trzonkowski, et al. 2003).

The induction of IFNγ by rAaGroEL is a direct indication of Th1 polarization since it enhances Th1 differentiation and facilitates antigen presentation by activating monocytes (Mosmann, et al. 1986). In addition, the absence of IL-4, which is a strong Th2 indicator, supports this hypothesis since IL-4 and IFNγ inhibit the production of each other (Mosmann, et al. 1986).

After the regulatory T cell subset was discovered, the fact that IL-10 is an indicator of Th2 was disproved. In addition, recent studies suggest the presence of Th1 cells which secrete IL-10 and IFNγ cytokines simultaneously (O'Garra and Vieira 2007). Since our findings indicate both cytokines are produced by CD4+T lymphocytes and in addition IL-10 positive cells are negative for FOXP3 transcription factor, the above information is further confirmed. Moreover, according to our results, IL-10 might be secreted by monocytes or B lymphocytes. As a result, IL-10 induction following rAaGroEL stimulation is important for the regulation of activated Th1 lymphocytes.

rAaGroEL protein directs Th0, naïve T cells into Th1 phenotype. As a future perspective, transcription factors such as t-bet and STAT4 expressions are needed to be confirmed with western blotting or real time PCR analysis.

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