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Role of PGRP-S/DnaK Complex in Pathogenicity of
Fusobacterium nucleatum

by

Babak Monzavi, DDS

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Oral and Craniofacial Sciences

in the

AKNOWLEDGEMENT

I would like to show my gratitude to my mentors, Dr. Tri Huynh, Dr. Ling Zhan, and Dr. Grayson Marshall, whose knowledge and assistance has made this study successful.

Finally, I express my deepest appreciation to my caring, loving, and supportive wife, Nazgol, and the new joy of our life, Neema.

Role of PGRP-S/DnaK Complex in Pathogenicity of *Fusobacterium nucleatum*

By Babak Monzavi, DDS

Introduction: *Fusobacterium nucleatum* (Fn) is one of the most prevalent pathogens found in various systemic and oral infections. It has been shown that Fn is able to induce apoptotic cell death and this ability appears to be mediated through the immune cells being aggregated (Jewett 2000, Huynh 2011). The exact mechanism of how Fn aggregates immune cells remains elusive. PGRP-S is a member of Peptidoglycan recognition proteins (PGRPs) on immune cells and an important host innate immunity arm capable of peptidoglycan and allied bacteria recognition. Hsp70 (heat shock-binding protein 70) is a member of the large stress-induced protein family in eukaryotic cells which makes stable complexes with PGRP-S and induces apoptotic cell death. DnaK homologues (prokaryotic Hsp70) has been identified and characterized in Fn with the Hsp70 family.

Objective: In this study, we investigated the function of PGRP-S/DnaK complex in the binding of immune cells (Jurkat T cells) to Fn and its role in cell death induction.

Material and method: Jurkat T cells were co-cultured with Fn (PK1594) with or without antibodies (Abs) to block DnaK and PGRP-S. Flow cytometry and Caspase-Glo3/7 assay were used to evaluate the levels of binding and apoptosis. Nonspecific IgG antibodies were used as controls. ANOVA with post-hoc Tukey's test was used for statistical analysis.

Results: Blockage of the DnaK and PGRP-S by antibody significantly decreased the binding and aggregation between Jurkat T cells and Fn in flow cytometry

(J+Fn=64.3$\pm$1.5, J+Fn+Abs=18.7$\pm$2.9, P<0.05). Apoptosis of Jurkat T cells also decreased significantly in caspase assays (J+Fn=1700$\pm$479, J+Fn+Abs=664$\pm$161, P<0.05). Furthermore, the addition of PGRP-S and DnaK monoclonal antibodies inhibited the up-regulation of FasL expression when Jurkats were co-cultured with *F. nucleatum* in Epi-fluorescence observation.

Conclusion: Our results suggested that PGRP-S/DnaK Complex may play a significant role in the aggregation and apoptosis of Jurkat T cell induced by *Fusobacterium nucleatum*.

Table of Contents

List of Tables

List of Figures

Introduction

The interaction between bacteria and the host defense is a key element to determine the status of health and disease in the mouth. *Fusobacterium nucleatum*, a gram negative anaerobic bacteria, is one of the most frequently isolated oral bacteria and has pathologic roles in several oral diseases such as alveolar bone abscesses (1), dental pulp infection (2,3), dentoalveolar abscesses (4), endodontic flare-ups (5), primary apical periodontitis and acute apical abscess (6), post-treatment disease (7).

F. nucleatum attributes a lot as a potential periodontal and endodontic pathogen. *F. nucleatum* participates in both adhesion and coaggregation reactions of dental plaque bacteria and may play a key role in the multigeneneric coaggreagation network found in the periodontal pocket. During dental plaque maturation, there is a shift from early colonizers, mainly gram positive microflora, to late colonizers which are the gram negative bacteria. *Fusobacterium* acts as a bridge between early and late colonizers bacteria (8). During the late stages of plaque formation, there is a shift from a gram-positive to gram-negative microflora, in which, *F. nucleatum*, increases proportionately (9). Synergistic pathogenicity of *F. nucleatum* with other two important bacteria in dental plaque, *Prevotella intermedia* (10) and *Porphyromonas gingivalis* (11) were also reported. (Figure 1).
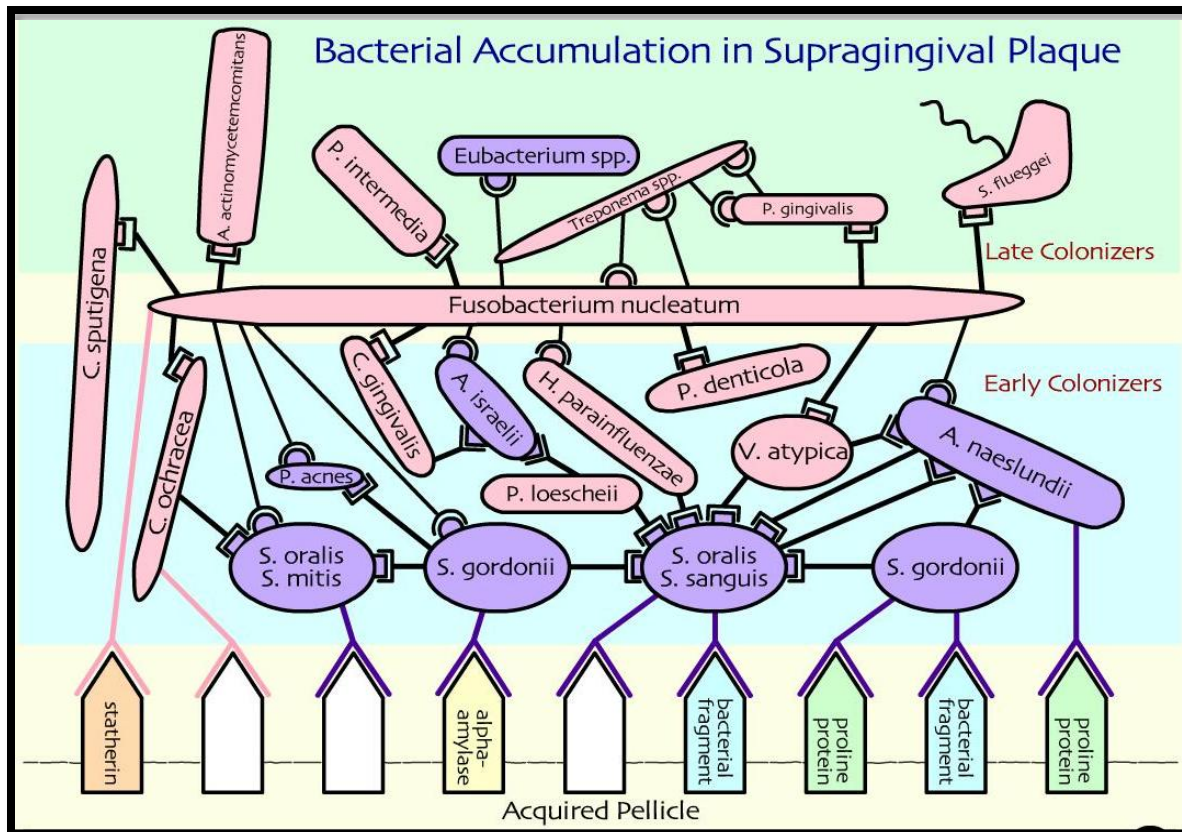
Figure 1: Spatiotemporal model of oral bacterial colonization, showing recognition of salivary pellicle receptors by early colonizing bacteria and coaggregations between early colonizers, *fusobacteria*, and late colonizers of the tooth surface (12).

In the root canals of untreated teeth, as well as root-filled teeth with periapical lesions, *F. nucleatum* constitutes one of the most frequently isolated bacteria, and its pathogenicity has been shown to be synergistic with other oral anaerobes (12, 13, 14). Matsuo et al (15) used immunohistologic techniques to demonstrate that *F. nucleatum* is the most abundant bacterium that invades dentinal tubules of the roots in untreated teeth with apical lesions. In addition, *F. nucleatum* was also identified as one of the few bacteria enduring endodontic treatment procedures (16).

Evidence of F. nucleatum modulating immunological system in pathological process

There is increasing evidence that bacteria can modulate the apoptotic signaling cascade of host cell, thereby play a major role in pathogenesis (17). This evidence indicates that apoptosis of the host immune cell may represent as a defense mechanism by pathogens. Certain bacteria have developed complex mechanisms to modulate the fate of the host cell, which include induction or blockage of apoptosis (18). Two main pathways for the induction of the apoptosis have been identified: the intrinsic and extrinsic pathway (receptor mediated). In the extrinsic pathway, typical death receptors include: Fas (CD 95), TNF receptor and Trail (19).

Several groups have reported the immune-suppressive activities of *F. nucleatum* (20, 21, 22). The exact mechanism of immune-suppression is still unclear. Sonicated extracts of *F. nucleatum* could inhibit human T-cell responses to mitogens and antigens (20). *F. nucleatum* could also induce apoptotic cell death in peripheral blood mononuclear cells (PBMCs) and Jurkat T-cells, a leukemic T cell line (22). The co-incubations of *F. nucleatum* with immune cells result in aggregation of the immune cells within minutes of the addition

of the bacteria (23, 24, 25). Also, the capability to induce cell death in immune cells appears to be mediated through its ability to induce immune cell aggregation as a part of pathogenesis of *F. nucleatum* (25). In addition, up-regulation of Fas death ligands and receptors play a significant role in immune cell apoptosis and periapical bone resorption (26).

Increased aggregation of cells by *F. nucleatum* can aid in signaling for cell death at several levels. It can provide a bacterial scaffold that could bring the cells to close proximity to each other for cross receptor signaling from one cell to another. *F. nucleatum* can also induce high levels of tumor necrosis factor-alpha (TNF-α) and interferon-γ secretion, which in combination could induce synergistic expression of adhesion molecules (22, 27). However, since the aggregation of cells occurs within minutes, it is plausible that this mechanism may play only a minor role.

Potential host recognition protein interaction with apoptotic pathway in response to F. nucleatum

Peptidoglycan is an essential and specific component of the bacterial cell wall, and therefore serves as an ideal recognition signature for the immune system. Peptidoglycan recognition proteins (PGRPs) are conserved from insects to mammals, and are able to bind PGN (non-catalytic PGRPs), and in some cases, to efficiently degrade it (catalytic PGRPs) (28). Human PGRP family consists of four members: PGRP-S, PGRP-L, PGRP-Iα and PGRP-Iβ. PGRP-L is expressed in the liver, PGRP-Iα and PGRP-Iβ in the esophagus (and to a lesser extent in the tonsils and thymus), and PGRP-S is in bone marrow (and to a lesser extent in neutrophils). In insects, PGRP-S triggers the activation of the prophenoloxidase cascade.

This cascade is an antimicrobial defense mechanism in insects that generates antimicrobial products and surrounds microorganisms with melanin. Because mammals do not have the prophenoloxidase cascade, mammalian PGRP-S must play a different role. It has recently been shown that mouse PGRP-S is present in neutrophils and inhibits growth of Gram-positive bacteria. However, PGRP-S may have another, as yet unidentified function, because in humans it is expressed in the bone marrow 50–100 times higher than in neutrophils (29).

Another protein which may also interact with apoptotic pathway is heat shock-binding protein 70. This multifunctional protein, Hsp70, is a member of the large stress-induced protein family. The main function of these proteins is to protect cells against damage produced by various stress factors (30). Hsp70 has recently been reported to prevent apoptosis and to act outside, as well as within the cell. Hsp70 can produce stable complexes with the proteins of the apoptotic-signaling pathway, including JNK, AIF, apoptotic protein activation factor 1, and granzyme B. This activity is mainly directed to promote survival of the tumor cells (31). Hsp70 readily interacts with various proteins, whereby its physiological role can change essentially. Hsp70 normally assists with the folding of nascent proteins and the refolding of partly denatured ones. In doing so, it supports cell and organism survival in stress exposure. Making stable complexes with protein components of the apoptotic signaling pathway in tumor cells, it supports their survival and thus works against the host (32). DnaK homologues (prokaryotic Hsp70) has been identified and characterized in *F. nucleatum* (ATCC 10953) with Hsp70 family. The N-terminal of 25 residues of DnaK homologues revealed up to 80% identity with members of the HSP70 family. (33).

The peptidoglycan recognition protein PGRP-S is shown to form a stable 1:1 complex with the major stress protein Hsp70. Neither protein is cytotoxic by itself, but their complex induces apoptotic death in several tumor-derived cell lines. The PGRP-S -Hsp70 Complex induces cell death in culture (34). A single primary interaction of lymphocyte PGRP-S with the target Hsp70 initiates a chain or cascade of conformational and spatial shifts spreading over a sizable area of the cell surface. Such events are allowed by appreciable lateral mobility of surface components, are possibly driven by oligomerization of Mts1 (metastasin-1) between neighboring protein groups, and may of course involve other intramembrane proteins. Ultimately they create a series of similar PGRP-S -Hsp70 interface elements to reinforce the cell contact. The same or analogous events simultaneously promote FasL trimerization/activation and proper positioning to induce apoptosis through the target Fas receptor. In other words, here Mts1 may be an element that permits self-organization of the contact zone into a web-like structure, and at the same time transmits a signal to the death-triggering agent (32).

PGRP-S -Hsp70 binding is a prerequisite to the induction of cytolysis in half of FasL-responsive targets. They kill such cells through a FasL/Fas-mediated contact mechanism that, however, requires (prior) interaction between PGRP-S and Hsp70 on their respective surfaces (35). Mts1 always attends PGRP-S and Hsp70 at the Cell Interface. Multipoint binding is required by general considerations and is suggested, among other things, by confocal micrographs, showing that in the zone of lymphocyte–tumor cell contact a group of PGRP-S sites encircles a single Fas site. Still more interestingly, Mts1 may take part in

functional coupling between the recognition step (PGRP-S / Hsp70) and the death-inducing step (FasL/Fas) (32).

Recently, a study by our group indicates that PGRP-S has a role in *F. nucleatum*'s ability to induce apoptotic cell death in Jurkat T cells. PGRP-S expression in Jurkat-cells increased significantly when co-cultured with Fn (Evans et. al AADR 2011). There was a significant dose-dependent decrease in Jurkat T cell apoptosis upon co-culture with Fn in the presence of monoclonal anti-PGRP-S antibodies.

These evidences indicate that PGRP-S and DnaK may play a significant role in *F. nucleatum*'s ability to induce apoptotic cell death in Jurkat T cells. However, there is no study to verify the role of PGRP-S/DnaK complex in pathogensis of *F. nucleatum*. Therefore, further investigation on the interaction between PGRP-S/DnaK complex and apoptotic pathway is necessary.

Hypothesis

We hypothesized that the binding of PGRP-S on Jurkat T cells to DnaK on *Fusobacterium nucleatum* play a significant role in *F. nucleatum*'s ability to induce apoptotic cell death in Jurkat T cells (Figure 2).
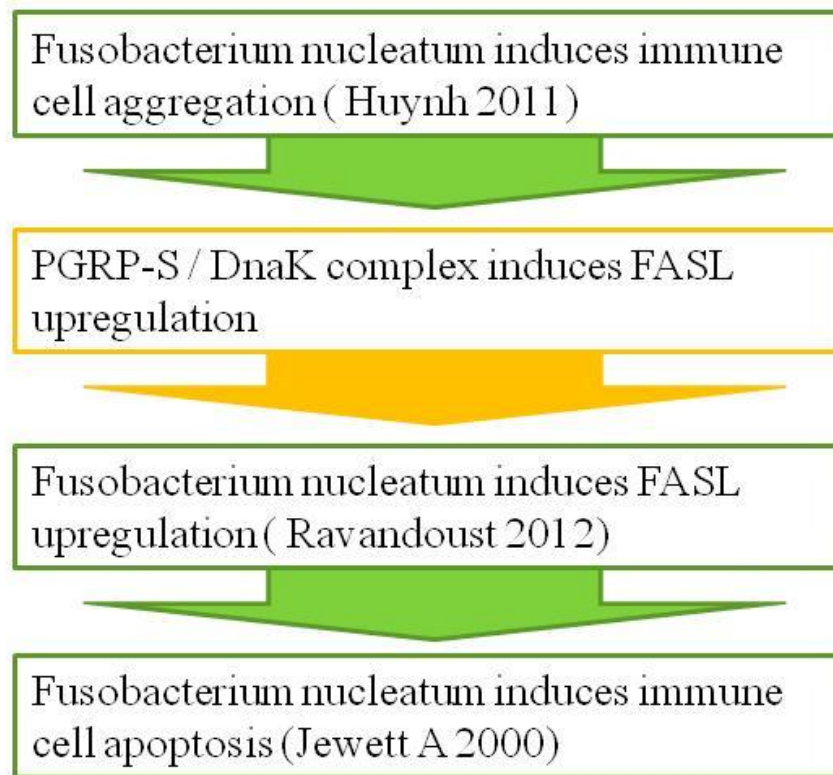
Figure 2: Proposed model for *Fusobacterium nucleatum* induction of immune cell apoptosis.

Our hypothesis is highlighted in orange.

Specific aim 1:

To investigate the function of PGRP-S - DnaK complex in cell death induction (apoptosis) by *F. nucleatum*.

Specific aim 2:

To study the role of PGRP-S - DnaK complex in aggregation of immune cells by *F. nucleatum*.

Specific aim 3:

To examine the role of PGRP-S - DnaK complex in the modulation of FasL expression upon the co-culture of F. nucleatum with Jurkat T cells.

Materials and Methods

Cell lines, bacterial strains, antibodies, and reagents

Jurkat cells (a Leukemic T cell line) were maintained in RPMI 1640 media (Sigma-Aldrich, St. Louis, MO) supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 1% penicillin-streptomycin, and 10% fetal calf serum. *F. nucleatum* (PK 1594) obtained from Dr. P. Kolenbrander of the National Institutes of Health and grown in Difco Columbia Broth (BD, Franklin Lakes, NJ) supplemented with 1% DTT (DL-Dithiothreitol; Clelands reagent)(Thermo Fisher Scientific, Waltham, MA) in an incubator with an atmosphere of 80% N2, 10% CO2 and 10% H2 at 37°C. *F. nucleatum* were then treated with 1%

paraformaldehyde for 1 h at room temperature. The bacteria were then washed three times with 1× phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) and used in the experiments (25).

The following antibodies and reagents were used in the current study:

Monoclonal PGRP-S antibody (Clone: 188C424) (Thermo Fisher Scientific, Waltham, MA)

Monoclonal Anti-DnaK antibody (ab69617) (Abcam, Cambridge, UK)

Mouse IgG1Isotype control (Clone: #11711) (R&D Systems, Minneapolis, MN)

Molecular Probes CellTracker fluorescent probes: CellTracker Green CMFDA (Life Technologies, Carlsbad, CA)

PMA/Ionomycin calcium salt (Sigma-Aldrich, St. Louis, MO)

Anti-Fas Antibody (human, activating), clone CH11 (EMD Millipore, Billerica, MA)

Mouse Anti-Human Fas Ligand-FITC (southern biotech, Birmingham, AL)

Apoptosis Assay

Jurkat cells ($2x10^4$ per well in 50 µl RPMI) were cultured for 18 hours: a) alone, and b)in the presence of PGRP-S antibody (10µl with 1 to 50 dilution ratio in PBS). *F. nucleatum* ($1x10^6$ per well in 50 µl Columbia Broth) were cultured for 18 hours: a) alone, and b) in the presence of DnaK antibody (10µl with 1 to 50 dilution ratio in PBS).

Then the apoptosis assay groups (n=3/group) were co-cultured for another 18 hours as follows:

1. RPMI (Negative control), 2. Jurkat cells (J) (Negative control: baseline apoptosis), 3. Jurkat cells + *F. nucleatum* (J+F) , 4. Jurkat cells with PGRP-S antibody + *F. nucleatum* with DnaK antibody (J-Ab+F-Ab), 5. Jurkat cells with PGRP-S antibody + *F. nucleatum* (J-Ab+F), 6. Jurkat cells + *F. nucleatum* with DnaK antibody (J+F-Ab), 7. Jurkat cells + *F. nucleatum* with Mouse IgG1Isotype control (J+F -IgG) (Positive control).

Cells were harvested and assessed for levels of apoptosis using the Caspase Glo 3/7 assay Kit (Promega, Madison WI) with a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale CA) following the manufacturer's instructions using Softmax Pro software. The Caspase-Glo 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in purified enzyme preparations or cultures of adherent or suspension cells. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light.

Binding Assay

Observation:

F. nucleatum (15×10^6 per well in 250 μl Columbia Broth) were cultured for 18 hours in the presence of CellTracker Green CMFDA following the manufacturer's instructions. Then, they were cultured for 18 hours: a) alone, and b) in the presence of of DnaK antibody (10μl with 1 to 25 dilution ratio in PBS). Jurkat cells (25×10^4 per well in 250 μl RPMI) were

cultured for 18 hours: a) alone, and b) in the presence of PGRP-S antibody (10µl with 1 to 25 dilution ratio in PBS).

Aggregations of immune cells by *F. nucleatum* were assessed with Nikon Eclipse TS100F (Nikon, Tokyo, Japan). Eclipse TS100F is an inverted microscope for Epi-fluorescence observation which were used with NIS- Elements Imaging software to evaluate binding in the following groups (n=3/group) after 4 hours of co-culture:

1. Jurkat cells (J) (Negative control), 2. Jurkat cells + *F. nucleatum* with CellTracker Green (J+F), 3. Jurkat cells with PGRP-S antibody + *F. nucleatum* with DnaK antibody and CellTracker Green (J-Ab+F-Ab), 4. Jurkat cells + *F. nucleatum* with Mouse IgG1Isotype control and CellTracker Green (J+F -IgG) (Positive control).

Epi-fluorescence microscope is an optical microscope that uses fluorescence. The specimen is illuminated with light of a specific wavelength which is absorbed by the fluorophores, causing them to emit light of longer wavelengths. The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. The observation were conducted in three light conditions: the high-intensity LED light and under fluorescence light to observe the aggregation of the cells, and with closed shutter (no light) to observe the emitted fluorescence of binding sites for each group.

Quantitative evaluation:

Jurkat cells (25×10^4 per well in 250 µl RPMI) were cultured for 18 hours: a) alone, and b) in the presence of PGRP-S antibody (10µl with 1 to 25 dilution ratio in PBS). *F. nucleatum* (15×10^6 per well in 250 µl Columbia Broth) were cultured for 18 hours: a) alone, and b) in

the presence of CellTracker Green CMFDA following the manufacturer's instructions, c) in the presence of DnaK antibody (10µl with 1 to 25 dilution ratio in PBS) and, d) in the presence of both DnaK antibody (10µl with 1 to 25 dilution ratio in PBS) and CellTracker Green CMFDA.

The binding of immune cells by *F. nucleatum* were quantified with LSR II flow cytometer (BD, Franklin Lakes, NJ) using FACSDiva software version 6.1.3 in the following groups (n=3/group) after 18 hours of co-culture:

1. Jurkat cells (J) (Negative control), 2. *F. nucleatum* (F) (Negative control), 3. Jurkat cells + *F. nucleatum* (JF) (Negative control), 4. Jurkat cells + *F. nucleatum* with CellTracker Green (JF-dye), 5. Jurkat cells with PGRP-S antibody + *F. nucleatum* with DnaK antibody and CellTracker Green (JFAb- dye), 6. Jurkat cells + *F. nucleatum* with Mouse IgG1Isotype control and CellTracker Green (JF IgG- dye) (Positive control).

FasL expression up-regulation:

Observation:

F. nucleatum (15x106 per well in 250 µl Columbia Broth) were cultured for 18 hours: a) alone, and b) in the presence of DnaK antibody (10µl with 1 to 25 dilution ratio in PBS). Jurkat cells (25x104 per well in 250 µl RPMI) were cultured for 18 hours: a) alone, and b) in the presence of PGRP-S antibody (10µl with 1 to 25 dilution ratio in PBS).

All samples were covered with 5-10 µg/mL anti-Fas (human, activating), clone CH11 in 1% BSA in PBS and incubated for 2 hour s at room temperature. Cells were washed twice with

PBS for 5 minutes following the manufacturer's instructions. Cells were incubated in the dark, with a 1:100 dilution of Mouse Anti-Human Fas Ligand-FITC secondary antibody in 1% BSA in PBS for 1.5 hours at room temperature. Cells were washed twice with PBS, for 5 minutes.

Upregulation of FasL in immune cells by *F. nucleatum* was assessed with Nikon Eclipse TS100F (Nikon, Tokyo, Japan). Eclipse TS100F is an inverted microscope for Epi-fluorescence observation which was used with NIS- Elements Imaging software in the following groups (n=3/group) after 4 hours of co-culture:

1. Jurkat cells (J) (Negative control), 2. Jurkat cells + *F. nucleatum*(J+F), 3. Jurkat cells with PGRP-S antibody + *F. nucleatum* with DnaK antibody(J-Ab+F-Ab), 4. Jurkat scells +PMA/Ionomycin (positive control).

The observation were conducted in three light conditions: the high-intensity LED light and under fluorescence light to observe the aggregation of the cells, and with closed shutter (no light) to observe the emitted fluorescence of FasL expression for each group.

Data Analysis:

All experiments were performed in triplicate and were repeated a minimum of 3 times. Results from both the caspase assay and binding assay underwent one-way analysis of variance (ANOVA) ($p < 0.05$), followed by the post-hoc Tukey's test with the use of SPSS Statistical Editor Software (SPSS, Chicago, IL).

RESULTS

Aim 1: To investigate the function of PGRP-S - DnaK complex in cell death induction (apoptosis) by *F. nucleatum* :

The caspase levels (apoptotic marker) in different groups were presented in Table 1.

The results showed that the co-culture of Jurkat cells with *F. nucleatum* increased caspase activity compared to Jurket cells culture alone. Addition of either PGRP-S and/or DnaK monoclonal antibodies decreased caspase activity of the co-culture of Jurkats and *F. nucleatum* but the caspase activity of these groups were still higher than mono-culture of Jurket. Among all co-culture groups, J-Anti-PGRP-S + F-Anti DnaK showed lowest Caspase activity which is significantly different from all other co-culture groups (P<0.05) (Figure 3 and Table 1).

Table 1: The caspase levels (apoptotic marker) in different groups.

Groups	Caspase levels (mean $\pm$ SD)
RPMI (Negative control)	60.8 $\pm$3.2[a*]
Jurkat cells (Negative control: baseline apoptosis)	288.1 $\pm$ 33.2 [b]
Jurkat cells + *F. nucleatum*	1700.07 $\pm$ 479.41 [c]
Jurkat cells with PGRP-S antibody + *F. nucleatum* with DnaK antibody (J-Ab+F-Ab)	663.8 $\pm$ 160.8 [d]
Jurkat cells with PGRP-S antibody + *F. nucleatum* (J-Ab+F)	1061.2 $\pm$ 89.1 [e]
Jurkat cells + *F. nucleatum* with DnaK antibody (J+F-Ab)	1301.1 $\pm$ 298.2 [e]
Control (J+F -IgG) (Positive control)	1035.8 $\pm$ 130.7 [e]

* Results with different superscript letter indicates that the results are statistically significantly different from the other group (Student T test, P<0.05)
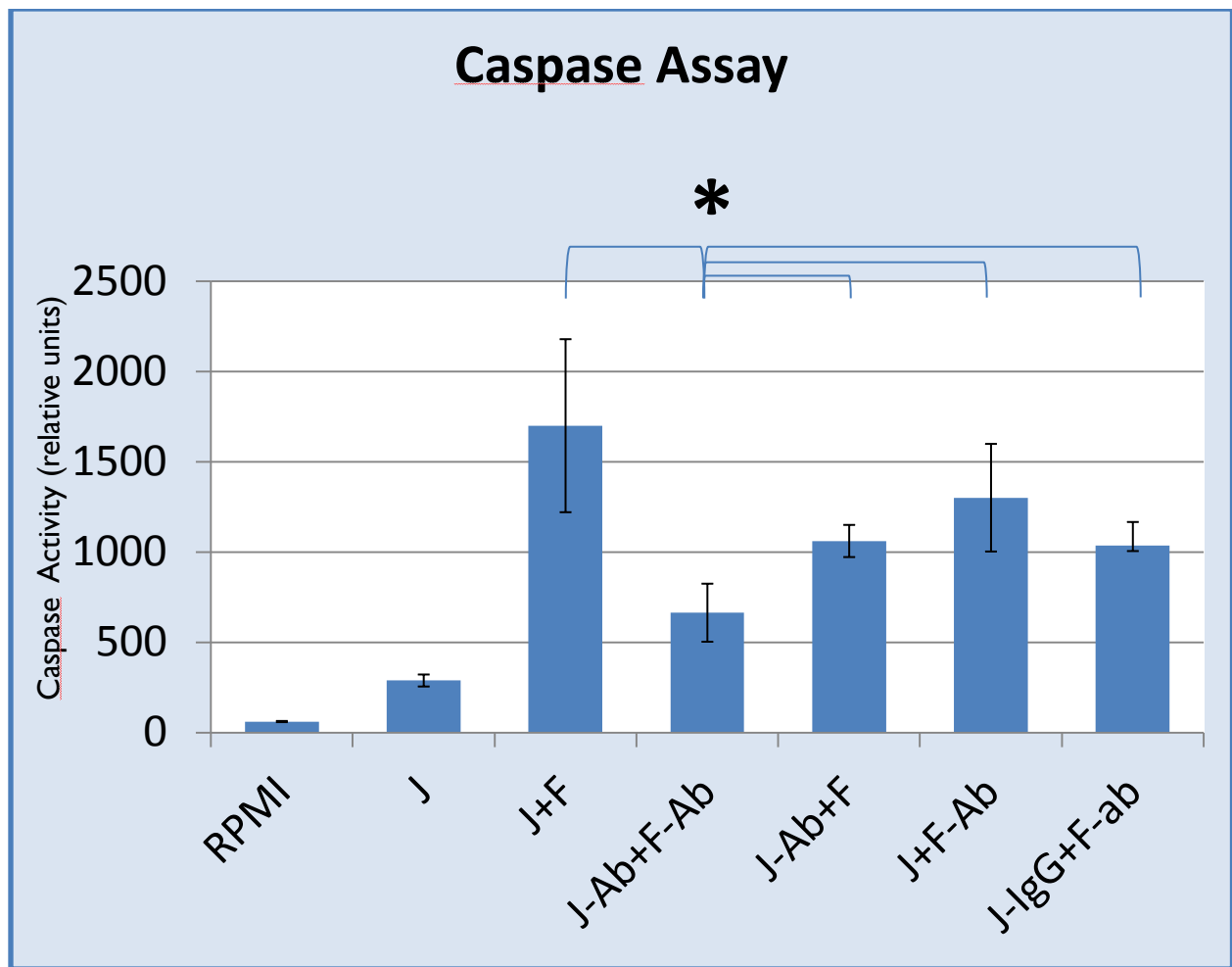
Figure 3: Caspase assay showed a significant difference between J-Anti-PGRP-S + F-Anti DnaK and all other groups (P<0.05) *.

Aim 2: To study the role of PGRP-S - DnaK complex in aggregation of immune cells by *F. nucleatum* :

Observation:

Epi-fluorescence observation with Eclipse TS100F illustrated decreased binding in the co-culture of Jurkat T cells with *F. nucleatum* upon the addition of PGRP-S and DnaK neutralizing monoclonal antibodies. Figure 4 illustrated an example of co-culture of Jurkat T cell and Fn under the high-intensity LED light (A1), under fluorescence light (A2) and with closed shutter (no light) when CellTracker Green fluoresce the green light in the binding sites between Jurkat T cells and *F. nucleatum* (A3). Figure 4 also showed an example of co-culture of Jurkat T cells with PGRP-S antibody and *F. nucleatum* with DnaK antibody under high-intensity LED light (B1), under fluorescence light (B2) and with closed shutter (no light) when CellTracker Green fluoresce the green light in the binding sites between Jurkat T cells and *F. nucleatum* (B3). Decreased level of aggregation between Jurkat T cells and F. nucleatum is noticed in presence of PGRP-S antibody and DnaK antibody when comparing B1 with A1. Also, decreased level of binding was observed between the two groups when comparing B3 with A3 based on the green light fluoresce from CellTracker Green.
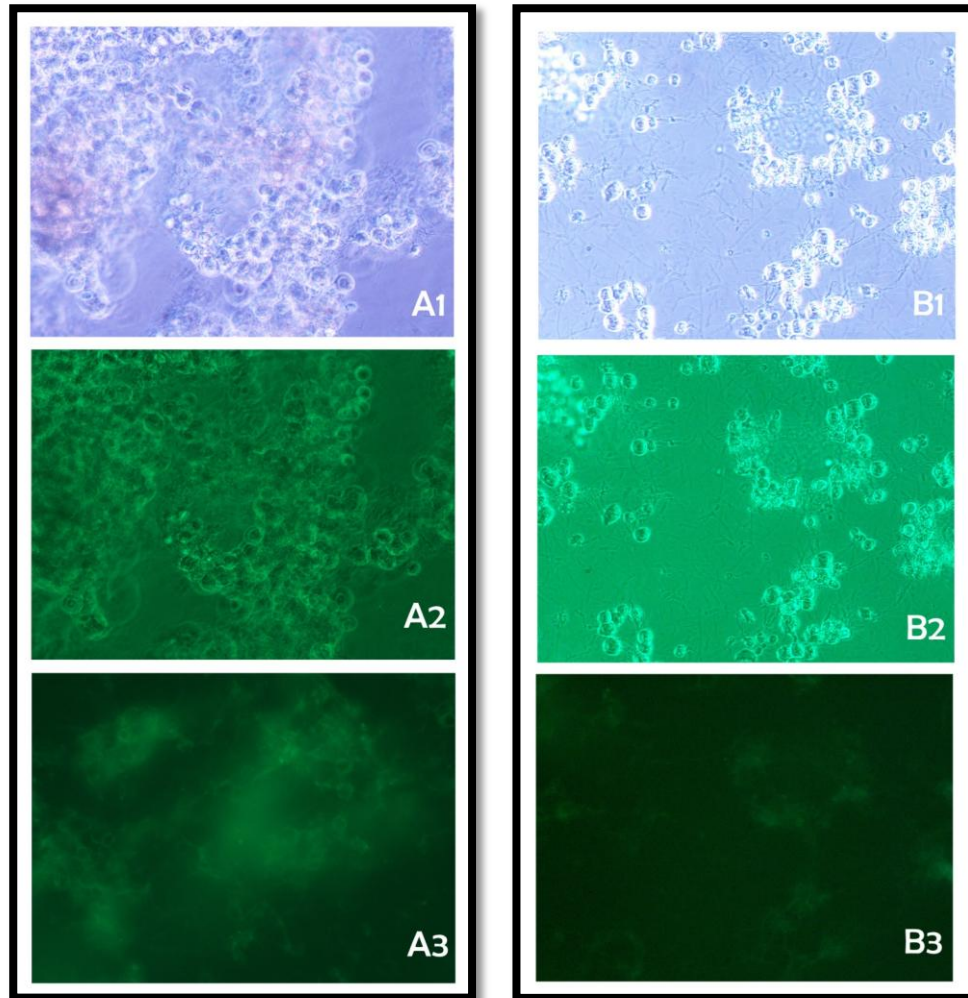
Figure 4: A1. Jurkat cells and *F. nucleatum* with CellTracker Green after 4hours of co-culture under the high-intensity LED light. A2. Under Epi-fluorescence light. A3. With no light when CellTracker Green in *F. nucleatum* fluoresce the green light in the binding sites. B1. Jurkat cells with PGRP-S Ab + *F. nucleatum* with DnaK Ab and CellTracker Green after 4hours of co-culture under the high-intensity LED light. B2. Under Epi-fluorescence light. B3. With no light when CellTracker Green in *F. nucleatum* fluoresce the green light in the binding site. Notice the decreased level of the green light in the binding sites compare to A3.

Quantitative evaluation:

The fluoresce FITC green light levels (binding marker) read by LSR II flow cytometer (BD, Franklin Lakes, NJ) using FACSDiva software in different groups were presented in Table 2.

The results showed that addition of PGRP-S antibody to Jurkat cells and DnaK antibody to *F. nucleatum* significantly reduced binding florescent of the co-culture of Jurkat cells + *F. nucleatum* ($p < 0.05$) while addition of Mouse IgG1 Isotype control (Positive control) did not affect the binding compared to pure co-culture(Table 2 and Figure 5).

Figure 6 and 7 illustrate the examples of the flow cytometry results of JF-dye and JFAb- dye group to show the shift and decrease in green light fluorescence after blocking both PGRP-S and DnaK .

Table 2 : The fluoresce FITC green light levels (binding marker) in Q4 read by LSR II flow cytometer using FACSDiva software in different groups.

groups	Caspase levels (mean ± SD)
Jurkat cells	0 ± 0 [a*]
F. nucleatum	0 ± 0 [a]
Jurkat cells + *F. nucleatum*	0 ± 0 [a]
Jurkat cells + dye	64.3 ± 1.5 [b]
F. nucleatum **+ dye**	18.7 ± 3.1 [c]
Jurkat cells + *F. nucleatum* **+ dye**	58.9 ± 5.8 [b]

* Results with different superscript letter indicates that the results are statistically significantly different from the other group (Student T test, $P<0.05$)
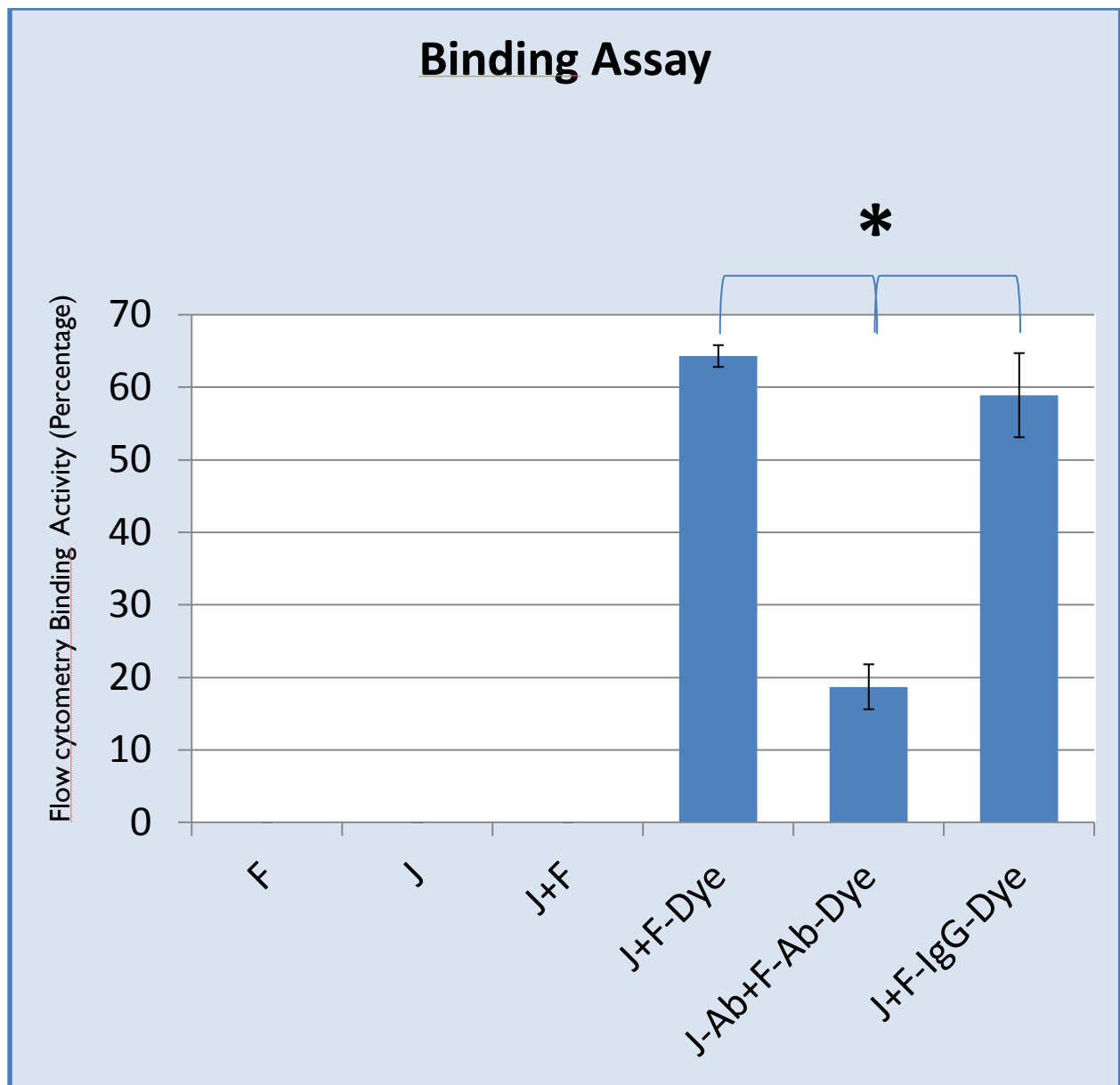
Figure 5: Flow cytometry demonstrated a significant decrease in binding between Jurkat T cells and *F. nucleatum* before and after neutralizing the DnaK/PGRP-S complex formation (P<0.05) *.
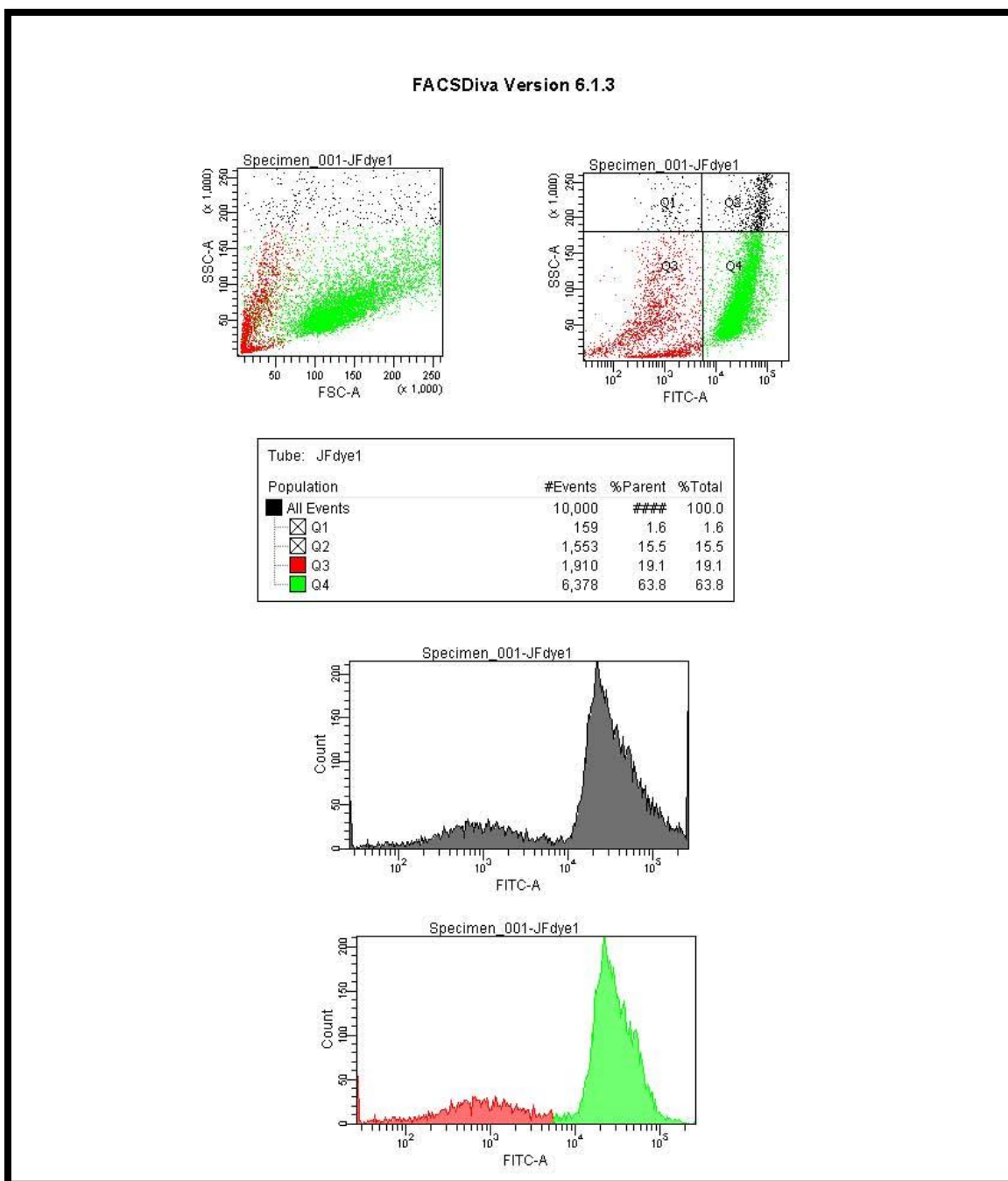
Figure 6: Q4: FITC green light level (binding marker) read by LSR II flow cytometer using

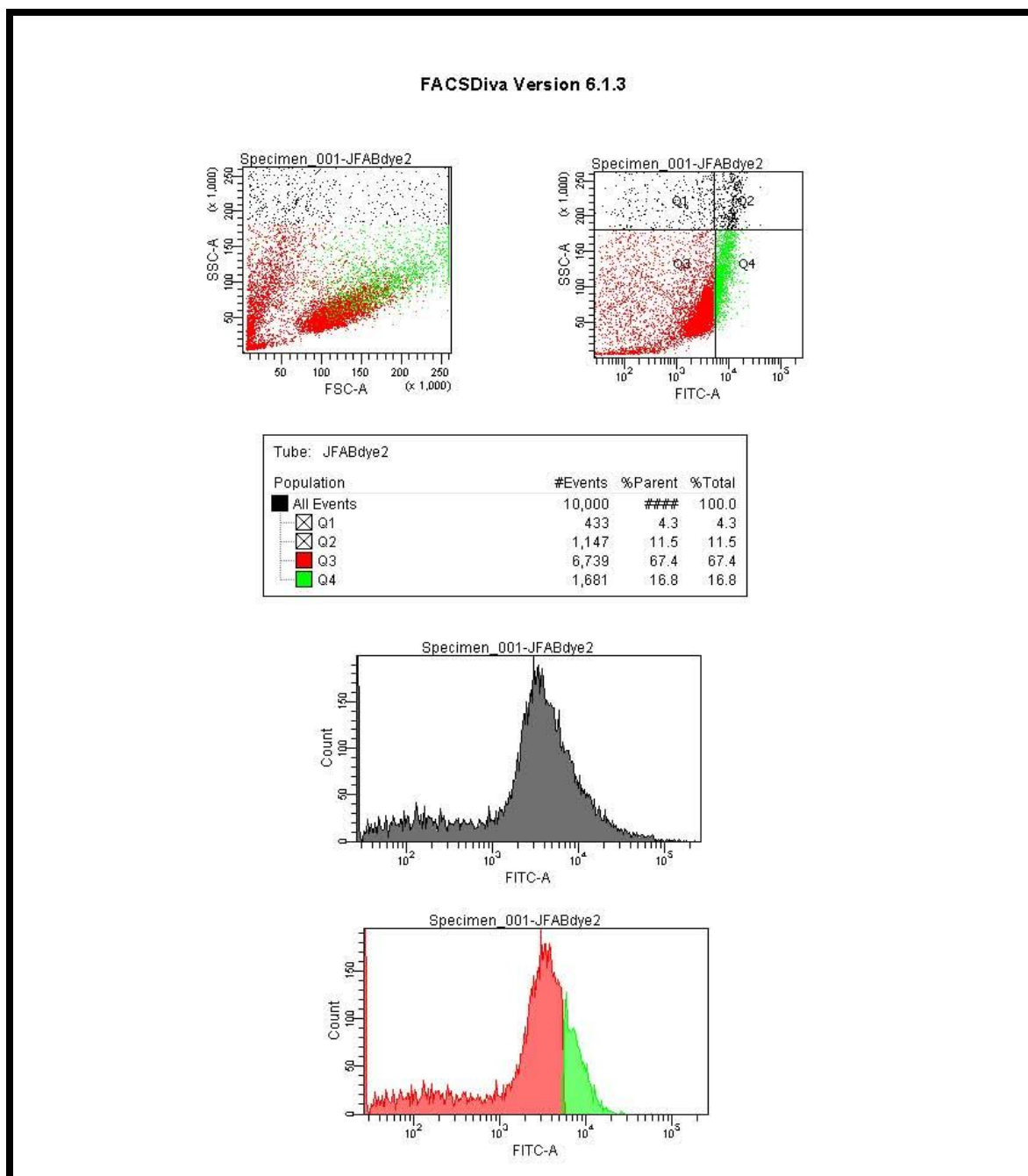FACSDiva software in Jurkat cells + *F. nucleatum* with CellTracker Green (JF-dye) group.

Figure 7: Q4: FITC green light level (binding marker) read by LSR II flow cytometer using FACSDiva software in Jurkat cells with PGRP-S antibody + *F. nucleatum* with DnaK antibody and CellTracker Green (JFAb- dye) group which showed a significant decrease in binding level (Green light) compare to JF-dye group .

Aim 3: To examine the role of PGRP-S - DnaK complex in the modulation of FasL expression upon the co-culture of F. nucleatum with Jurkat T cells:

Observation:

Epi-fluorescence observation with Eclipse TS100F illustrated decreased up-regulation of FasL expression in the co-culture of Jurkats with *F. nucleatum* upon the addition of PGRP-S and DnaK neutralizing monoclonal antibodies. Figure 8 illustrated an example of co-culture of Jurkat T cell and Fn covered with anti-Fas (human, activating) and Mouse Anti-Human Fas Ligand-FITC secondary antibody under the high-intensity LED light (A1), under fluorescence light (A2) and with closed shutter (no light) when Anti-Human Fas Ligand-FITC secondary antibody fluoresce the green light in the binding sites between Jurkat T cells and *F. nucleatum* (A3). Figure 8 also showed an example of co-culture of Jurkat T cells with PGRP-S antibody and *F. nucleatum* with DnaK antibody covered with anti-Fas (human, activating) and Mouse Anti-Human Fas Ligand-FITC secondary antibody under high-intensity LED light (B1), under fluorescence light (B2) and with closed shutter (no light) when Anti-Human Fas Ligand-FITC secondary antibody fluoresce the green light in the binding sites between Jurkat T cells and *F. nucleatum* (B3). Decreased level of aggregation between Jurkat T cells and F. nucleatum is noticed in presence of PGRP-S antibody and DnaK antibody when comparing B1 with A1. Also, decreased level of FasL expression was observed between the two groups when comparing B3 with A3 based on the green light fluoresce from Mouse Anti-Human Fas Ligand-FITC secondary antibody.
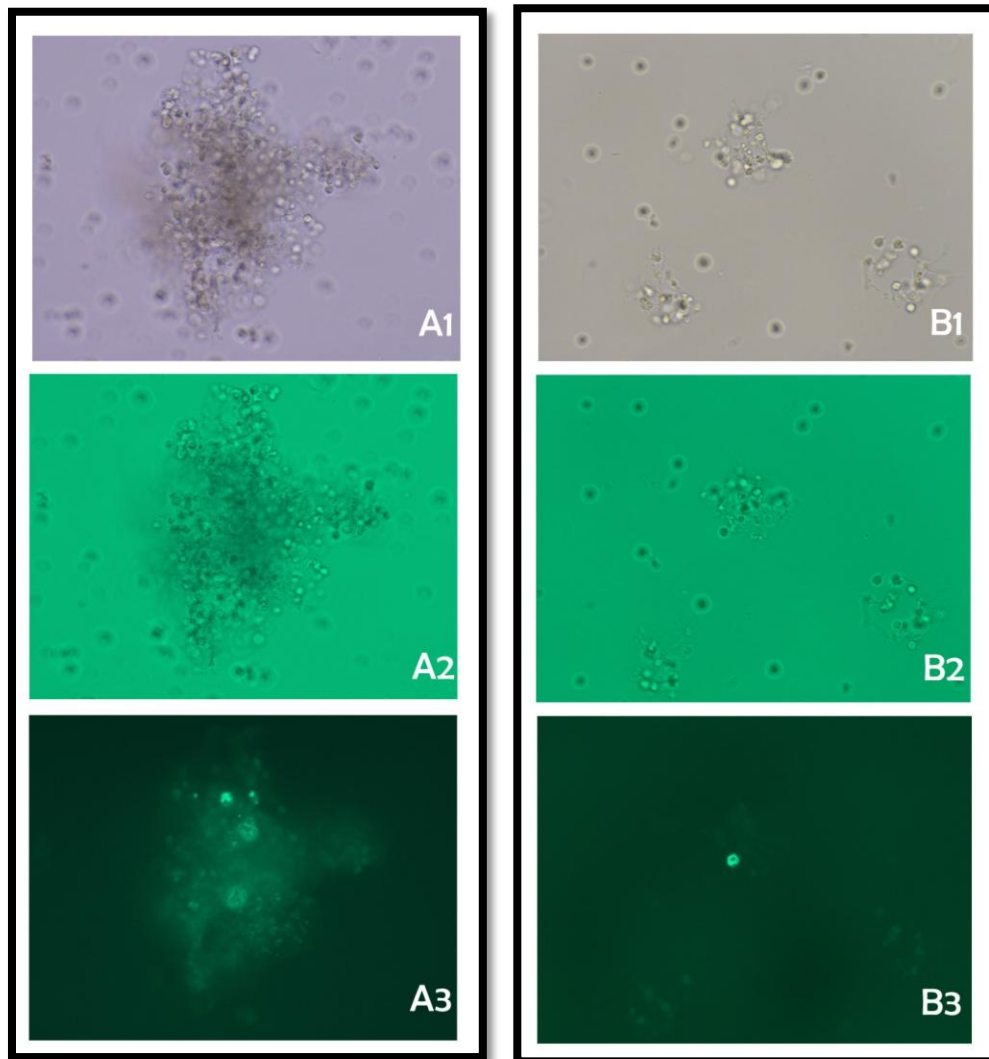
Figure 8: A1. Jurkat cells and *F. nucleatum* covered with anti-Fas (human, activating) and Mouse Anti-Human Fas Ligand-FITC secondary antibody after 4hours of co-culture under LED light. A2. Under Epi-fluorescence light. A3. With no light: FasL fluoresce the green light in binding sites. B1. Jurkat cells with PGRP-S Ab + *F. nucleatum* with DnaK Ab covered with anti-Fas (human, activating) and Mouse Anti-Human Fas Ligand-FITC secondary Ab after 4hours of co-culture under LED light. B2. Under Epi-fluorescence light. B3. With no light: FasL fluoresce decreased level of the green light in binding sites compare to A3.

DISCUSSION

Fusobacterium nucleatum is one of the most frequently identified endodontic pathogens isolated from root canals, and its pathogenicity has been shown to be synergistic with other oral bacteria (6, 11, 13, 15). There is increasing evidence that bacteria can modulate the apoptotic signaling cascade of host cells (18). These results indicate that apoptosis of the host cell may represent as a defense mechanism by pathogens. Certain bacteria have developed these complex mechanisms to modulate the fate of the host cell, which include induction or blockage of apoptosis (19). Two main pathways for the induction of the apoptosis have been identified: the intrinsic and extrinsic pathway (receptor mediated). In the extrinsic pathway, typical death receptors include: Fas (CD 95), TNF receptor and Trail (20). Bacterial induced cell death in human lymphocytes is a key virulence factor which allows bacteria to fight the host immune system and establish pathogenesis (21). Our results support the extrinsic pathway with the modulation of FasL. However, further investigation is necessary to quantify this effect.

The co-incubations of *F. nucleatum* with immune cells results in aggregation of the immune cells within minutes of the addition of the bacteria (24, 25, 26). Also, the ability to induce cell death in immune cells appears to be mediated through the immune cells being aggregated, which might have important implications for *F. nucleatum* pathogenesis (25). In addition, up-regulation of Fas death ligands and receptors play a significant role in immune cell apoptosis and periapical bone resorption (27). Increased aggregation of cells by *F. nucleatum* can aid in signaling for cell death at several levels. It can provide a bacterial scaffold that could bring the cells to close proximity to each other for cross receptor

signaling from one cell to another. Our data is consistence with the aforementioned studies in the role of aggregation in the pathogenicity of *F. nucleatum*.

In the present study, we aimed to investigate the role of PGRP-S - DnaK complex in aggregation of immune cells and apoptosis by *F. nucleatum*. Also we evaluated the possible role of FasL in these interactions. In our *in-vitro* experiments, we used Jurkat T cells as a representative of immune cells. Jurkats is an immortalized T cell line that has been previously utilized by other groups to study apoptotic pathways (36).

In our *in-vitro* model, it appears that in conditions where the PGRP-S and DnaK were neutralized individually by their respective inhibitory monoclonal antibodies to prevent the formation of PGRP-S - DnaK complex, induction of apoptosis by *F. nucleatum* was significantly reduced compare to all other experimental groups in our test (P<0.05). Blocking PGRP-S alone decreased the cell death induction which confirmed the result that was previously presented by Evans (AADR 2011) and the same was true about naturalizing the DnaK. However, blocking both of them and preventing the formation of PGRP-S - DnaK complex, has a synergistic effect in cell death reduction. On the other hand, when compared to the background levels of apoptotic activity, there was not a complete reduction of the caspase activity, suggesting that other mechanisms may be involved in the apoptotic process.

Epi-fluorescence observation revealed decrease binding in the co-culture of Jurkats with *F. nucleatum* upon the addition of PGRP-S and DnaK neutralizing monoclonal antibodies. Quantitative evaluation by Flow cytometry confirmed these significant decreases in the binding in the co-culture of Jurkats with *F. nucleatum* by preventing the formation of PGRP-

S - DnaK complex ($P<0.05$). Our results support previous findings of Huynh (2010) that aggregation may play a significant role in immune cell death induction by *F. nucleatum* (26)*.*

Epi-fluorescence observation illustrated decreased up-regulation of FasL in the co-culture of Jurkats with *F. nucleatum* upon the addition of PGRP-S and DnaK neutralizing monoclonal antibodies. This could describe the possible mechanism for PGRP-S - DnaK complex action. Future quantitative evaluation is necessary to confirm this observation.

It is imperative to acknowledge that the findings of this study may add a new player, PGRP-S - DnaK complex, to the apoptotic pathway to help us have a better understanding of the interaction between *F. nucleatum* and immune cells. Further immunohistologic studies are recommended to investigate these interactions.

CONCLUSIONS

Fusobacterium nucleatum is one of the most prevalent microbes found in endodontic infections. *F. nucleatum* has been shown to have immunosuppressive properties. Our group previously has been shown that *F. nucleatum* is able to induce apoptotic cell death in immune cells. Aggregation of immune cells and the induction of the death ligand FASL appear to be the mechanism of cell death induction. However, the manner in which binding and aggregation occurs remains unknown.

The findings of our *in-vitro* study were the first to suggest that PGRP-S/DnaK Complex may play a significant role in promoting apoptosis in immune cells after aggregation caused by *Fusobacterium nucleatum.* Our results demonstrate that blocking the formation of PGRP-S/DnaK Complex significantly decreased aggregation and apoptosis of Jurkat T cell induced by *Fusobacterium nucleatum.*

Future studies should be conducted to support these *in vitro* findings including:

1) Quantitative evaluation of role of PGRP-S - DnaK complex in the modulation of FasL expression

2) Perform PGRP-S and DnaK knockdown studies in Jurkat T cells and Fusobacterium nucleatum respectively to validate our neutralizing antibody findings

3) Evaluate *F. nucleatum's* ability to induce endodontic abscess in PGRP-S knockout mice compared to wildtype mice

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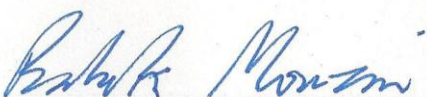
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