ORIGINAL ARTICLE

Comparative Analysis of Inflammatory Markers Produced by Macrophages Inoculated with Invasive and Colonizing Strains of Streptococcus Agalactiae (Group B Streptococcus) and Evaluation of Patients' Clinical Data

Nassim Karimi Hosseini[1], Sharmili Vidyadaran[2] Shinsmon Jose[2], Narges Eskandarian[1], Zalina Ismail[3], Syafinaz Amin Nordin[1],*

1 Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
2 Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
3 Department of Medical Microbiology and Immunology, Faculty of Medicine, University Kebangsaan Malaysia, Cheras, Kuala Lumpur, Malaysia

ABSTRACT

Introduction: Group B Streptococcus (GBS), infection and recurrence in newborns and pregnant women can lead to chronic medical illness resulting in significant morbidity, and mortality. Pathogenesis of GBS may be due to reasons such as activation of the immune system, followed by the production of inflammatory markers and toxic components by immune cells including macrophages. **Methods:** The studies on invasive and colonizing GBS strains inoculated either with peripheral or brain macrophages, the expression of nitric oxide (NO), cell viability, and CD40 were also measured by Griess assay, methyl tetrazolium assay (MTT), and flow cytometry, respectively. Furthermore, the clinical manifestations of the selected patients were also assessed for this study. **Results:** Outcome of inflammatory markers studies, after GBS inoculation indicated that, invasive GBS strains induced higher inflammatory markers in comparison to colonizing GBS strains. Furthermore, patients' clinical data showed that patients with invasive GBS infections had severe condition unlike among patients with colonizing GBS strains. The fatality rate in patients with invasive GBS strain were 30.8% while there was no death among carriers. **Conclusion:** This study**,** aimed to understand the immune response to GBS, and strengthen the knowledge on GBS pathogenesis. It was concluded that invasive GBS strains not only showed higher expression of inflammatory markers on immune cells but also had higher pathogenesis effect in comparison to colonizing GBS strains.

Key words: Macrophages, *Streptococcus agalactiae***,** Streptococcal infection, Nitric oxide (NO), CD40, Pregnancy

Corresponding author
Assoc. Prof. Dr Syafinaz Amin Nordin,
Tel: +60-389472478
E-mail: syafinaz@upm.edu.my

INTRODUCTION

Streptococcus agalactiae, the group B streptococcus (GBS) is a gram-positive, encapsulated bacterium, displays beta-hemolytic activity on blood agar. It is a commensal flora in genital and lower gastrointestinal tract in 10-40% of every healthy adult (1). However, colonization of GBS in vagina during pregnancy can be clinically significant because of its association with neonatal meningitis and septicemia (2). In the last two decades, the percentage of GBS infection is found increasingly in adults, particularly those with other comorbidities, such as cirrhosis, renal failure and diabetes mellitus (3).

Pathogenesis of GBS infection involves several processes, such as inducing the production of various inflammatory markers and secretion of toxic components that are harmful to the human body. Macrophages plays a vital role in this response by production of these markers on their own as well as by stimulating other immune cells to do so (4, 5). The pro-inflammatory markers and toxic components such as reactive oxygen and nitrogen radicals produced by macrophages on exposure to GBS pathogens could cause cell and tissue injury in the human body (6, 7).

Understanding the pathogenesis in GBS infections is pivotal for the successful intervention at various course of the disease. Previous research has been done on invasive and colonizing GBS strains, however, on different classifications such as hemolytic and non-hemolytic GBS strains, or based on patients' symptoms. In the present study, we are taking an alternative approach by describing GBS strains which were collected from blood as invasive GBS strains, whereas GBS strains which were collected from high vaginal swab (HVS) as colonizing GBS strains. The purpose of this study is to add value to the previous works, by evaluating the differences in human immune inflammatory response elicitation, after inoculation of invasive and colonizing strains of GBS *in vitro* with macrophages. It can be hypothesized that there are differences between the colonizing and invasive GBS strains in their pathogenesis. Patients' clinical data were also collected and evaluated to see the correlation between the inflammatory markers and patients' symptoms.

MATERIALS AND METHODS

Cell culture

BV-2 cells (transformed murine microglia cell line), and RAW 264.7 cells (transformed murine macrophage cell line) as representative for brain and peripheral macrophages respectively, were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, in 5% heat-inactivated Fetal Bovine Serum (FBS) for BV-2 cells and 10% FBS for RAW 264.7 cells, incubated at 37˚C with 5% CO_2. The cells were washed with phosphate buffered saline (PBS) twice and trypsinized with 0.25% Trypsin-EDTA solution. Cells with more than 95% viability (measured by trypan blue staining), were seeded into 24-well plate, 96- well plate, and 6-well plate for performing Griess assay, methyl tetrazolium (MTT) assay, and immunophenotyping, respectively, at the optimized cell seeding densities. In order to maximize the accuracy across different passage numbers, the passage number of used BV-2 and RAW 264.7 cells was restricted within five passage number difference in the experiments.

Bacterial culture, heat-killing, and inoculation

Sixteen different invasive GBS strains, and 15 different colonizing GBS strains, as well as *Streptococcus agalactiae* cultures (ATCC® 13813™), were grown on sheep blood agar followed by an overnight incubation, and the colonies were re-suspended in Brain Heart Infusion (27 g/mL of BHI powder in dH_2O ;OXOID; Cat No. CM 1135). The cultures were incubated at 37˚C overnight and diluted in BHI to acquire appropriate bacterial density based on 1.0 McFarland standard, and stored in 4˚C. Bacterial densities was optimized separately for each assay. GBS cultures were then heat killed by boiling for 30 minutes. The absence of any viable bacteria after the heat killing process was ensured by culturing on Columbia agar with 5% sheep blood at 37˚C incubator for three days. If there was no bacterial growth, GBS suspensions were used for cell treatment, however if bacterial growth was observed, the heat-killing procedure was repeated. The cells treated with lipopolysaccharide (with the optimized concentration for each assay) were used as positive control for all the assays.

Griess assay

Nitric oxide was measured by the Griess assay. Griess reagent was prepared by dissolving 1% sulfanilamide (Sigma, Batch No. 126K0001) and 0.1 % N-1-naphthyl-ethylenediamine hydrochloride (Sigma, Cat. No. 222488) in phosphoric acid. Two hundred microliter of supernatant from each well of the 24-well plate (bacterial treated cells, LPS treated cells and non-treated cells) was transferred into an eppendorf tube, and centrifuged at 24, 48, and 72 hours for 4 minutes with 14000 rpm and 50 µL of each sample was added in triplicate to a 96-well plates. Equal volume of prepared Griess reagent was added to the wells, and the OD of the samples was read by Dynex MRX II microplate reader at 530 nm wavelength. The NO levels were estimated by comparing the samples reading to the standard curve generated using serially diluted $NaNO_2$.

Methy tetrazolium (MTT) assay

Cell viability was measured by the MTT assay. MTT reagent was prepared by dissolving 5 mg/mL of MTT powder in dH_2O (Amresco, LOT No. 1247B021). MTT assay was performed at 72 hours by adding MTT reagent to the cells and the cells were incubated for 3 hours at 37˚C with 5% CO_2 in a humidified incubator. Afterwards, the culture supernatant was removed and DMSO was added to the wells and plates were incubated in the dark for 10 minutes and OD was read at 490 nm by Dynex MRX II microplate reader. Viability percentage was calculated with reference to a comparison between treated cells and untreated cells as control.

Analysis of CD40 expression

CD40 expression was analyzed on RAW 264.7 cells 72 hours post exposure to the GBS strains. Cells were harvested using trypsin and aliquoted into FACS tubes (0.5×106 cells/tube) and rinsed with 0.2% BSA (Amresco, LOT No. 1621C094) in PBS at 2000 rpm for 5 minutes. The cells were resuspended in 0.2% BSA/PBS, and mouse serum (AbDseroTec; Datasheet C11SCZ) was added to the cells, to block the unspecific bindings. The cells were incubated at room temperature for 15 minutes, and stained with CD40-FITC Ab (BD Pharminogen FITC Hamster Anti-Mouse; LOT No. 18883). The cells were incubated at 4˚ C in the dark for 20 minutes, and resuspended in 0.2% BSA/PBS after being centrifuged at 2000 rpm for 5 minutes. CD40 expression was acquired by recording 10000 events per sample using BD FACS Calibur and analyzed with CellQuest Pro software (BD) at 24 hours of treatment.

Ethical clearance

Ethical clearance was obtained from ethic committees of Universiti Putra Malaysia (UPM/FPSK/PADS/T7-MJKEtikaPer/F01(LECT(JMPP)_OKT(10)01) and Universiti Kebangsaan Malaysia Medical Centre (FF-196-2011).

Clinical data evaluation

In order to enhance the accuracy of our results in terms of finding any correlation between the inflammatory markers and patient's symptoms, patient's clinical data was collected along with the samples from Universiti Kebangsaan Malaysia Medical Centre. It was aimed to assess whether the invasive GBS strains isolated from patients with severe symptoms, induced higher inflammatory markers expression, in comparison to colonizing GBS strains which were isolated from asymptomatic patients.

Statistical analysis

Experimental data are expressed as mean ± SD. The statistical analysis was performed by using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Inflammatory markers analysis

Bacterial density was optimized before inoculation with BV-2 and RAW 264.7 cells. Both invasive and colonizing GBS strains induced NO production in BV-2 and RAW

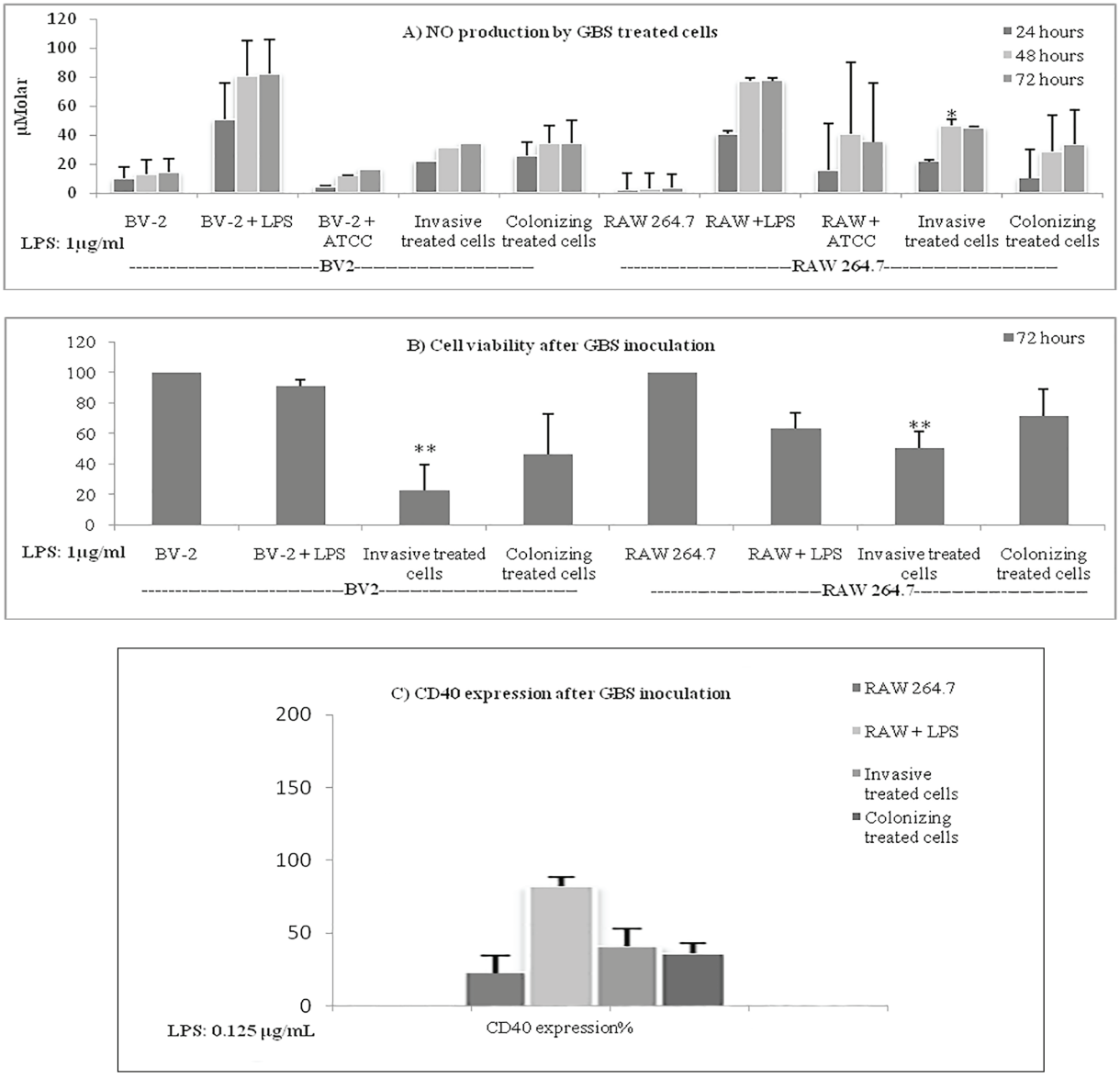

Fig1. Inflammatory marker evaluation, produced by macrophages, after inoculation of GBS. (A)NO production: The cell density of BV-2 and RAW 264.7 cells were 25000 cells/well and 50000 cells/well respectively. (B) Viability measurement: The cell density of BV-2 and RAW 264.7 cells were 6250 cells/well and 12500 cells/well respectively. (C) Evaluation of CD40 expression: The cell density of RAW 264.7 was 0.8×10^6 cells/well.

264.7 cells at all time points tested. At 48 hours, NO levels in RAW 264.7 cells induced by invasive GBS strains were higher than those induced by colonizing GBS strains (46.35 ± 25.11 μM NO compared to 28.3 ± 20.67 μM NO respectively; *P* value <0.05) (Figure 1A). Moreover, results of viability measurement showed that there was a significant decrease in BV-2 and RAW 264.7 cell viability after inoculation of invasive GBS heat killed strains (to 22.7 ± 16.9% and 50.25 ± 11.16% respectively), in comparison to the cell viability after inoculation of colonizing GBS heat killed strains (to 46.01 ± 26.54% and 71.21 ± 17.49% respectively) by 72 hours (*P* < 0.01) (Figure 1B). RAW 264.7 cells also increased expression of CD40 following LPS stimulation, indicating microglia activation. Similar to NO expression, invasive GBS strains appeared to induce higher CD40 expression (40.95 ± 11.98%; Figure 1C) compared to colonizing GBS heat killed strains (35.72 ± 7.44%) although the difference was not statistically significant.

Clinical data evaluation

Among 17 patients with invasive GBS infections, no data were available for four patients. The clinical presentations are listed in Table 1 and presented as follows: septicemia (n = 6, 46.2%), pneumonia (n = 3, 23.1%), peritonitis (n = 3, 23.1%), meningitis (n = 1, 7.7%) and prematurity (n=1, 7.7%). There were only one case of meningitis and prematurity. The most common clinical syndrome among all the patients was septicemia. Of the three cases of pneumonia, one was community-acquired and two were hospital-acquired. The underlying medical conditions of all patients are shown in Table 2 and all patients had more than two underlying conditions. All patients survived except four patients (30.9%) with the cause of brain abscess, peritonitis, septicemia in three adults and prematurity in one neonate. On the other hand, among 14 carriers, no data was available for one patient. Out of the 13 cases, eleven (84.6%) were in the postpartum period with the history of GBS positive in previous pregnancy

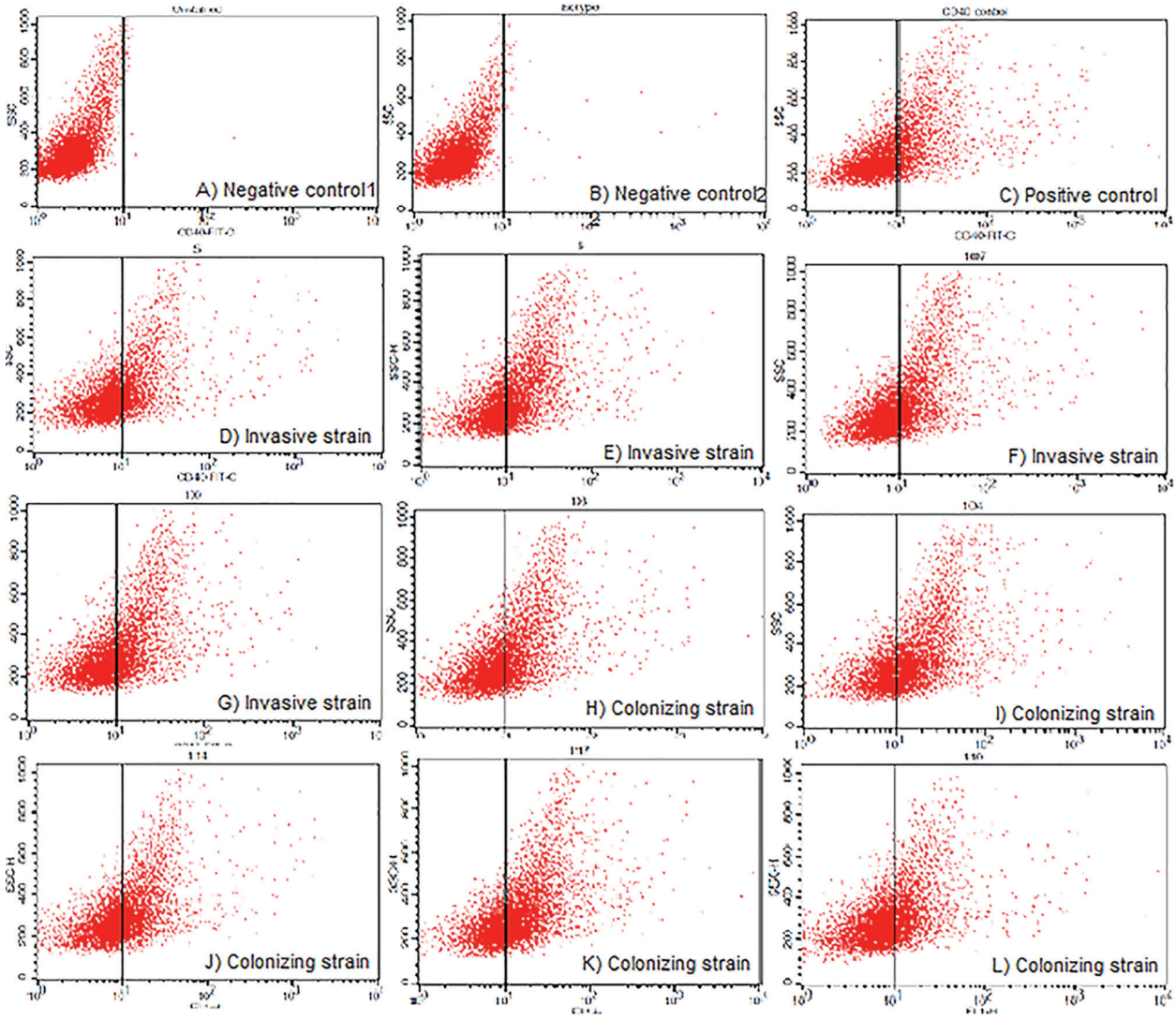

Fig 2. Immunophenotyping assay to measure CD40 expression in RAW 264.7 cells, after GBS inoculation**.** A) Negative control 1. B) Negative control 2. C) Positive control. D), and E) Invasive GBS strains-treated cells. F), G), H), I), J), K), and L) Colonizing GBS strains-treated cells. The recorded events were 10000.

in two (15.4%) cases. In two (15.4%) pregnant women, the baby was delivered via EMLSCS (one due to fetal distress and one due to leaking liquor) and in one (7.7%) pregnant women, there was the presence of PROM < 18 hours. There was one case of placenta praevia and two cases of STD (one had aspirate pneumonia) in women with postpartum condition. In the case of non-pregnant women (n=2, 15.4%) with colonizing GBS strain, one presented with uterine fibroid and one had UTI (Table 2). There was no death and all patients survived.

DISCUSSION

The foremost research on oxidative burst, parallelly with phagocytosis on monocytes, induced by GBS, was done by McCloskey *et al.* in 2000 to measure reactive oxygen species (8). Oxidative burst has not been clearly investigated in monocytes/macrophages inoculated by GBS in previous research. However, it has been done on other bacteria such as *Salmonella typhimurium, Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus* (8). In the previous studies, it has been shown that NO induced by GBS, plays a crucial role in the brain injury (9). Moreover, in a study by Ulett *et al.* in 2005, was shown that GBS-induced macrophages, had a significant increase in NO production at 72 hrs (10). In addition, all the methods which were used for measuring NO were listed by Mur and colleagues in 2011, although NO electrodes and Griess assay are preferred (11). Ring *et al.* in 2000 reported that NO production by RAW 264.7 cells was significantly higher after inoculation of invasive (hemolytic) GBS strains (even in absence of stimuli), in comparison to non-invasive (non-hemolytic) GBS strains (even after adding stimuli) (12). In the current study, GBS strains, were classified based on where they were isolated from, invasive GBS strains, induced higher NO production, in comparison to colonizing GBS strains.

MTT assay was optimized by Gerlier *et al.* in 1986 on various cells (13). In addition, the dyes used for cell viability measurement were optimized by Skehan *et al.* in 1990. In previous studies, microglia have been exposed to heat-killed GBS strains, and it was shown that the number of the viable cells, were significantly lower in bacterial-treated cells, than in control wells (14), with adverse effect of time and bacterial dose (7, 9). Moreover, it was shown by them that live GBS strains induced significantly higher and faster cell death, in comparison to heat-killed strains. In the current study, bacterial dose-dependence of MTT assay followed the previous studies, and the results showed that viability of invasive GBS treated macrophages, were significantly lower than colonizing GBS treated macrophages.

CD40 - CD40L engagement is crucial for microglial activation and neurotoxin production, which leads to the pathology of neuro inflammatory diseases, following chronic inflammation (15). In this study, it was indicated that invasive GBS strains, induced higher CD40 expression on macrophages, in compare to colonizing GBS strains.

CONCLUSIONS

It was proven in this study that all selected markers as representative for immune response, have shown that invasive GBS strains had more effects on two selected immune cells, in comparison to colonizing GBS strains. Although some of the results were not statistically significant. Moreover, clinical data showed that patients, from whom invasive GBS samples were isolated from, had severe clinical conditions and the high rate of fatality, in comparison to the patients that colonizing GBS samples were isolated from.

There were no sufficient data on the similar classification in previous studies, and GBS strains were classified into invasive and non-invasive groups, according to different criteria such as being hemolytic or non-hemolytic, the frequency of the serotypes isolated from neonates with sepsis (serotype III showed the most frequency), and the patients' symptoms that the samples were isolated from (16, 17). However, in the current study GBS strains were classified based on the isolations whereby the samples which were isolated from blood were considered as invasive GBS strains, and the samples which were isolated from HVS as colonizing strains. Hence the results of this study were supported by the most relevant studies, as it has been discussed above.

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