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Effect of specific anti-*Brucella* antibodies and interferon-gamma on intracellular killing of *Brucella abortus* through oxidative burst in mice peritoneal macrophages

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Abstract
Brucella abortus is globally distributed facultative intracellular pathogen which cause significant economic losses in livestock and more often it is a neglected zoonotic threat for humans. Opsonophagocytosis mediated through host macrophages plays a pivotal role in destruction of threats inside host body, but its importance in recovery from infection with intracellular pathogens is still unclear. The main aim of this study was to access the role of *B. abortus* specific immune serum and interferon-gamma in post phagocytic clearance *B. abortus* through the flow cytometric measurement of reactive oxygen species produced by mice peritoneal macrophages. The study was conducted using mice peritoneal macrophages *in vitro*. Cultured macrophages were infected with opsonized and unopsonized *B. abortus* S19 (Vaccine strain) and S544 (virulent strain). Post-phagocytic clearance was measured in terms of extant of oxidative burst. Macrophages has undergone robust oxidative burst regardless of opsonized or unopsonized *Brucella*, measured by dihydrorhodamine 123. However, high rate of oxidative burst was observed in IFN-γ primed macrophages infected with opsonised strain.

Keywords: Macrophages, interferon-gamma, opsonophagocytosis, oxidative burst

1. Introduction
Brucella abortus is a Gram-negative, facultative intracellular bacterium that infects mainly cattle and imparts a zoonotic threat to humans across the globe. Also, it is classified as a category B bioterrorism agent because of its aerosol ease of transmission (Laine *et al*., 2022) [1].

It is reported that occurrence of more than 500,000 new human cases annually while the prevalence rate exceeding 10/100,000 in some developing countries (Rubach *et al*., 2013; Ahmed *et al*., 2016) [2, 3]. The organism is considered to be a pathogen wherever it is found in patient specimen. It causes abortion and infertility in cattle and undulant fever, endocarditis, arthritis and osteomyelitis in humans (Pappas *et al*., 2005) [4]. Since *B. abortus* is considered to be a facultative intracellular pathogen, cell-mediated immune (CMI) mechanisms have been overemphasized since long (Titball, 2008) [5], leading to a dogmatic preference for living vaccines. Though no classical virulence factors like cytolysins, exotoxins, secreted proteases, phage-encoded toxins, fimbriae, capsules, or plasmids have been identified in *B. abortus*, the sequence of events of the pathogenesis of the infection and the possible role of macrophages during *in vivo* dissemination of organism. All evidence till date suggests that *Brucella* do not persist within neutrophils (Barquero-Calvo *et al*., 2007) [6], but survive within macrophages. Therefore, macrophages are not only the primary target cells for replication of *B. abortus* but also considered as the warehouse for dissemination of infection within the host (Wang *et al*., 2017) [7]. Respiratory burst or oxidative burst is an anti-microbial activity that leads rapid release of reactive oxygen species (superoxide anion and hydrogen peroxide) from activated mammalian phagocytes for the optimal killing of a wide variety of bacteria and fungi. Respiratory burst is characterized by assembly of an enzyme that comprises NADPH oxidase on phagosome membrane. This leads to rapid release of reactive oxygen species (superoxide anion and hydrogen peroxide) from immune cells following phagocytosis and finally delivered into the phagosome. It is a crucial reaction to combat infection by degrading internalized particles and bacteria (Yang *et al*., 2015) [8].

The present study was therefore undertaken to delineate the interactive hidden roles of antibodies as opsonin and IFN-γ mediated mechanism involved with oxidative burst against *B. abortus* through flow cytometric measurement of reactive oxygen species.

2. Materials and Methods

2.1 Mice

All the experimental protocols carried out on mice were approved by the Animals Ethics Committee (AEC) of Indian Veterinary Research Institute (IVRI), Izatnagar-243122 (India). Mice were procured from the Laboratory Animal Resource Section of IVRI, kept in AEC approved facilities and received water and food *ad libtum*.

2.2 Bacterial strains

B. abortus S19 and S544 were obtained from *Brucella* Reference Repository Centre, Indian Veterinary Research Institute. Both the strains were maintained at 4 ºC on *Brucella* agar slants after confirming the purity and identity by appropriate methods. Prior to experimentation, suspension of each strain having the desired viable count was prepared and stored at 4 ºC.

2.3 Antiserum

Each animal of a group of 6 to 8 weeks old Swiss albino mice was immunized with *B. abortus* strain 19 (10^5 CFU/ml), subcutaneously. Blood samples were drawn after 5 weeks to collect immune sera (IS) which were pooled, filtered (22 µm MICRO-POR) and antibody titre of the pooled antiserum was determined by standard tube agglutination test (Alton *et al*., 1975) [9].

2.4 Isolation and purification of mouse peritoneal macrophages

The mouse peritoneal cavity provides an easily accessible site for harvesting moderate numbers of resident macrophages. Generally, macrophages isolated from the mouse peritoneal cavity will be mature quiescent macrophages (Fortier *et al*., 1982) [10]. The yields typically ~0.5–1 × 10^6 macrophages per mouse. This yield can be increased by injecting eliciting agents like proteose peptone intraperitoneally that induce inflammatory reaction & recruit immature macrophages into the peritoneum. In our study protocol the mice peritoneal macrophages were collected as per previously described method by Zhang *et al*., 2008 [11] with minor modifications. Briefly, 2 ml bacteriological peptone (10% solution) was injected into the peritoneum of 4–5 weeks old Swiss Albino mice. Following three days of injection, 5 ml of RPMI complete media (RCM) supplemented with 10% FBS (heat inactivated), 100U penicillin/ml, 100ug streptomycin/ml) and 0.5µg/ml fungizone was injected into the peritoneal cavity. After five minutes of gentle abdominal massage, the peritoneal fluid was aseptically aspirated. The cells from the fluid were recovered by centrifugation at 170 × g for 10 min at 4 °C, washed and resuspended in RCM. Viability count was performed by trypan blue dye exclusion method. Later, one hundred thousand (1 × 10^5) cells were seeded in each well of 24 well tissue culture plates & incubated at 37 °C with 5% CO_2 and 85% humidity. After 2 hr incubation, the non-adherent cells were removed and adherent cells were either left untreated or incubated with mouse INF-γ (100 ng/ml) for overnight in a 37 °C incubator with 5% CO_2 and 85% humidity. Fresh INF- γ were added to the macrophage cultures following the wash procedure. During the experimentation the cell morphology & viability was accessed by visualization through Olympus inverted microscope.

2.5 Opsonization of organisms

Both strains of *B. abortus* were treated with immune sera as per the method described by Martinez *et al*. (2008) [12]. Suspensions *Brucella abortus* S19 and S544 were incubated with 10% (V/V) anti-*B. abortus* murine immune serum and PBS control (as such bacterial suspension) for 45 min at 37 ºC with intermittent gentle rotation followed by brief vortexing. Following 45 min, both bacterial suspensions (immune serum opsonized and unopsonized) were centrifuged, washed thrice in sterile PBS and resuspended.

2.6 Oxidative burst study by flow cytometry

The oxidative burst in mice peritoneal macrophages was measured by ^{TM}BD FACS Calibur using dihydrorhodamine 123 (DHR 123, Sigma-Aldrich, USA) as per as per method described by Chang *et al*. (2001) [13], with our own modification. Briefly, DHR was added prior to infection of macrophages at a final concentration of 20 µM and incubated at 37 °C in the dark for 30 min. After 30 min incubation, the cells were infected with S544 and S19 with bacteria-macrophage ratio of 100:1 for 1 h. Relatively higher MOI was used to achieve a higher level of infection in most cells in the monolayer. Following infection, the cells were washed three times with PBS, scraped off from the well and resuspended in 0.5 ml PBS. To estimate ROI/RNI production, fluorescence intensities of 5,000 or 10,000 cells were recorded. The cells with and without 1 µg/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA) stimulus were used to set the positive and negative control, respectively.

2.7 Data analysis

Data are expressed as means ± standard errors of the mean (SEM) for each treatment group. Statistical comparisons were made using Student's t test, $p<0.05$ was considered significant. All the analyses were conducted using GraphPad Prism version 5 software.

3. Results

3.1 Confirmation of identity and maintenance of *B. abortus* strains

Brucella abortus S19 and S544 were used for the investigations. Both the strains were maintained on several slants at 4 °C after confirmation of their identity by morphological, cultural, biochemical, and serological tests. Both strains were found to be Gram-negative, non-spore-forming, non-motile, coccobacillary rods.

3.2 Mice peritoneal macrophages: Collection and characterization, maintenance of mice peritoneal macrophages

The cell yield was ~2-3× 10^6 macrophages per mouse. Viability of the cell populations was more than 95%. All macrophage monolayer remained intact and well adhered. The cells appeared healthy and viable throughout the 72h incubation period when observed under inverted microscope (Fig.1).

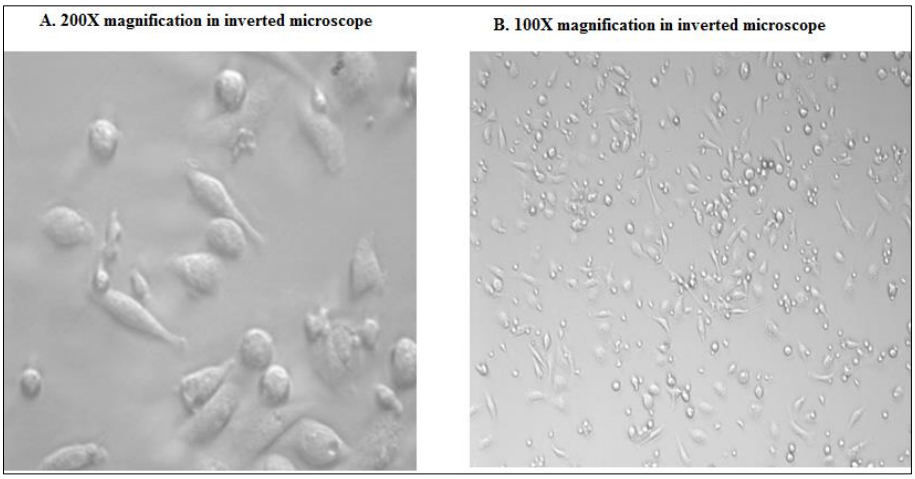

Fig 1: Mice peritoneal macrophages observed under inverted microscope 200X (A), 100X (B) magnification

3.3 Indirect determination by post-phagocytic oxidative burst activity of naïve unstimulated mice peritoneal macrophages: The key mediator of oxidative burst (ROI/RNI) act as a sharp weapon in host's antibacterial arsenal. These key components were analysed in naive unstimulated macrophages following internalization with opsonized or unopsonized *Brucella* strains through FACS. Both opsonized and unopsonized *Brucella* strains infected macrophages has undergone robust oxidative burst when compared to control (uninfected macrophages). The striking observation was non-significantly differed oxidative burst in opsonized and unopsonized *Brucella* strains. (Fig. 2).

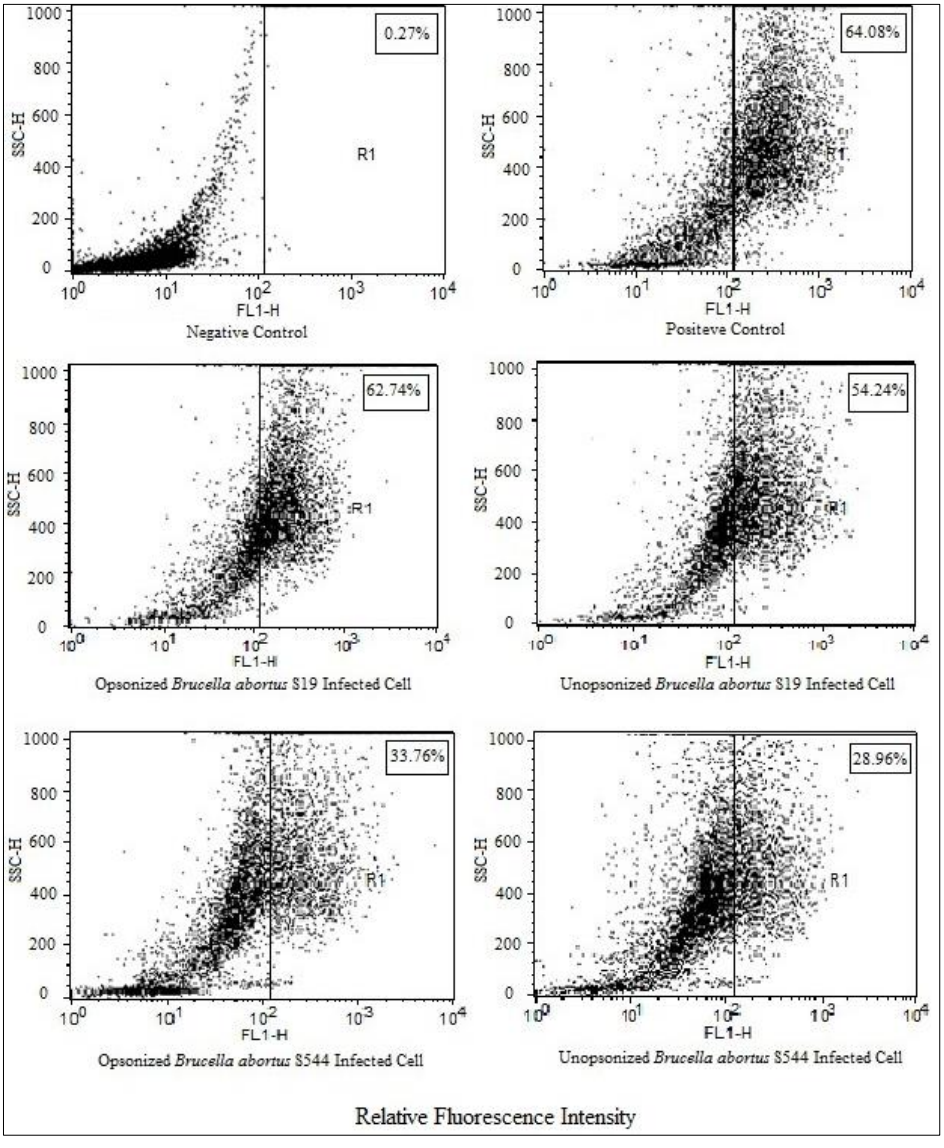

Fig 2: Representative flow cytometry plots of *B. abortus* S19 and S544 infected mice peritoneal macrophages

Table 1: Comparative DHR oxidation in IFN-γ stimulated and unstimulated mice peritoneal macrophages following infection with opsonized and unopsonized *B. abortus* S19 and S544

B. abortus	Percent DHR positive cells (Mean ± SD)	
	Unstimulated	IFN-γ stimulated
Opsonized S19	55 ± 10.8	65.7 ± 0.53
Unopsonized S19	47.3 ± 9.7	59.6 ± 2.05
Opsonized S544	49.3 ± 12.4	60.1 ± 10.6
Unopsonized S544	41.5 ± 5.8	54.2 ± 9.6

3.4 Indirect determination by post-phagocytic oxidative burst activity of IFN-γ stimulated mice peritoneal macrophages

Though the IFN-γ stimulated macrophages induced significantly (p<0.01) more oxidative burst than uninfected control as in case of naïve macrophages, there was non-significant difference between the values obtained with opsonized and unopsonized strains (Table 1 and Fig 3).

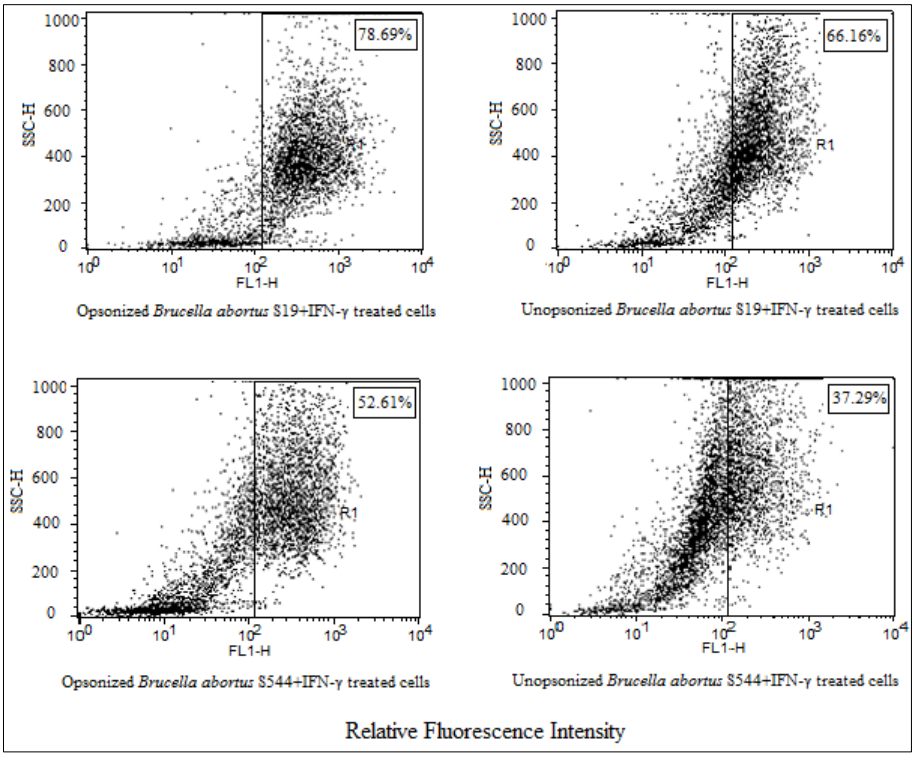

Fig 3: Representative flow cytometry plots of IFN-γ stimulated macrophages infected with *B. abortus* S19 and S544

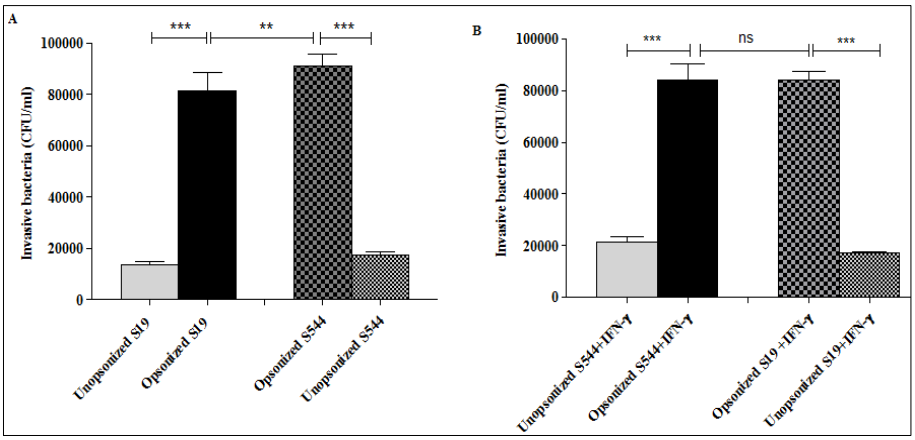

Fig 4: Graph showing initiation of oxidative burst in terms of percent dihydrorhodamine (DHR) oxidation positive cells following infection with *B. abortus* S19 and S544 in naïve unstimulated (A) and IFN- γ stimulated (B) mice peritoneal macrophages

4. Discussion

The extent of oxidative burst directly correlated with reduction in percent survival considering it as innate immune component. The key mediator of oxidative burst (ROI/RNI) act as a sharp weapon in host's antibacterial arsenal. These key components were analysed in naïve (Fig.2) and IFN-γ pre-treated (Fig.3) macrophages following internalization with opsonized or unopsonized *Brucella* strains through FACS. Opsonized and unopsonized S19 induced maximum burst in both macrophage groups, though the IFN-γ pre-treated group produced a higher quantity of oxidative burst intensity as compared to naive macrophages. Similar pattern was also observed with S544 though the quantity of oxidative burst intensity produced was comparatively less than that produced by S19 infected macrophages. The bactericidal environment created by IFN-γ is mediated through up-regulation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) by the process of

respiratory burst (Nathan *et al*., 1983) [14]. The observations on oxidative bursts of various macrophage groups indicated that intracellular killing is related to stimulation of respiratory burst activity. The increment of oxidative burst in IFN-γ pre-treated macrophages is complete agreement with previous study where IFN-γ primed neutrophil has undergone high rate of oxidative burst (Filiz *et al*., 2015) [15]. Hence, opsonophagocytosis and IFN-γ acts as a milestone to eliminate the notorious pathogen *B. abortus* which adopts stealthy strategy to evade the host immne response and potentially develop an intracellular niche in macrophages that leads chronic infection.

5. Conclusion
In conclusion, our *in vitro* experiment provides valuable information on the indirect way of post-phagocytic clearance involving oxidative burst. Intracellular killing of *B. abortus* was not found solely and directly related to stimulation of oxidative burst activity.

6. Conflicts of interest
We have no conflict of interest to declare.

7. Acknowledgements
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