

# Immune strategies of phagocytic cells stimulated *in vitro* with live and heat-inactivated *Streptococcus pyogenes*

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**ABSTRACT:** *Streptococcus pyogenes* (group A *Streptococcus*) is frequently involved in a wide range of human diseases. Here we evaluated polymorphonuclear neutrophils and mononuclear cells from healthy subjects for their bactericidal function after stimulation with live and inactivated *Streptococcus pyogenes* (*Streptococcus* Group A). Mononuclear cells and Neutrophils were isolated from heparinized blood samples (n=18) using a Ficoll-Hypaque gradient and cultured in RPMI 1640 for 18 hours with a suspension of either live or inactivated *Streptococcus pyogenes*. Both the respiratory burst (flow cytometry) and nitrite, TNF and IL17 production (ELISA) were measured in the cell culture supernatants. An increased respiratory burst (expressed as R index) was induced by both live and inactivated bacteria. Also, increased nitrite, TNF and IL17 concentrations were found in cell culture supernatants in both cases. These findings may provide some explanation as to the roles played by neutrophils and mononuclear cells in *Streptococcus pyogenes* immunopathogenicity.

## Introduction

*Streptococcus pyogenes* (group A *Streptococcus*) is a frequent pathogenic bacterium in humans, which causes a wide range of diseases (Bisno, 1991; Cunningham, 2000) which span from localized infections, such as pharyngitis and pyoderma, to highly invasive diseases, such as necrotizing fasciitis, sepsis, and toxic shock-like syndrome. These infections can lead to the development of severe sequelae, such as rheumatic fever with carditis, acute poststreptococcal glomerulonephritis and serious inflammatory/autoimmune diseases (Guzmán et al., 1999; von Pawel-Rammigen, 2012).

Usually the immune system develops a successful response that is initiated by the recognition of this pathogen by immunocompetent cells of the innate immune system, which is followed by a variety of strategies resulting in a full protective response against the aggressor (Graztz et al., 2011). Among these responses, the phagocyte respiratory burst plays a crucial role to degrade internalized bacteria. Another important host defense mechanism is the endogenous production of nitric oxide (Moncada et al., 1991). An accurate regulation of the oxidase activity by cytokines such as TNF $\alpha$ , IL8, IL6, IL1 and IL17 produced during the immune and inflammatory responses to pathogens have been shown to play a critical role together with phagocytic cells' activities (Gougerot-Pocidallo et al., 1996). Some studies associate IL17 to the pathogenesis of inflammatory diseases, and several therapeutic programmes involve these cytokines in clinical developments (Pappu et al., 2011).

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Pathogenic bacteria utilize various mechanisms to invade their host, to obtain nutrients and to establish themselves in one or several microenvironments within the host. To colonize and cause disease, the bacteria must succeed over early defense mechanisms that normally should eliminate microorganisms from the mucosal surface (Lövkvist et al., 2008).

As *S. pyogenes* infections are frequently related to a strong inflammatory response, in this study we evaluated the activation process of peripheral mononuclear cells and polymorphonuclear neutrophils from normal subjects in presence of live and inactivated *S. pyogenes*, by measuring the respiratory burst, nitrites, TNF $\alpha$  and IL17 production in supernatant of cell cultures.

## Material and Methods

### *Study design and patients*

The purpose of this investigation was to evaluate neutrophils and mononuclear cells for their activation capacity when exposed to live and inactivated *S. pyogenes*. The sample population comprised 18 normal subjects (both sexes) with a mean age of  $20 \pm 8.5$  years (means  $\pm$  DS). Informed consent was obtained from each subject. The studies were approved by the Ethical and Biosecurity Committees of the Medical Sciences Faculty, National University of Rosario (on 30<sup>th</sup> May 2010). A heparinized blood sample (20 ml) was extracted from each subject.

### *S. pyogenes culture and heat inactivation*

Group A *Streptococcus* strain SP3832 is an M12 serotype that was originally isolated from a diagnosed patient with an infectious process. A swab was collected from a child who was suspected of having *S. pyogenes* pharyngitis. Each swab was used for culture. The material was seeded in sheep's blood agar (5 % final concentration), within 2 hours of collection. The plate was incubated for 24-48 hours and the agents were identified by established techniques. Then the bacteria were inactivated by incubating the tubes at 70 °C in a water bath for

30 minutes.

### *Mononuclear cells and neutrophils separation and culture*

For isolation of mononuclear cells and neutrophils, 20 mL heparinized blood obtained by venipuncture from normal donors was diluted 1:1 in phosphate buffer saline (PBS) in a polypropylene tube, layered over a Ficoll-Hypaque gradient (Sigma, density 1077-1078) and centrifuged at 400 g for 30 min at room temperature (19-22° C). The following three fractions were obtained after centrifugation: fraction 1: plasma; fraction 2, peripheral blood mononuclear cells, at the plasma-Ficoll interface; and fraction 3, granulocytes and red blood cells (pellet). Mononuclear cells were washed three times in PBS and centrifuged (30 g, 10 min). Neutrophils were also recovered from the red blood cells interface and washed three times in PBS by centrifuging at 300 g for 10 min. Lysis buffer (ClNH<sub>4</sub>) was added and the pellet was resuspended by gentle mixing. This tube was incubated for 10 min at room temperature and centrifuged for 10 min at 300 g. The supernatant was aspirated and washed twice with PBS. Next, mononuclear cells and neutrophils were resuspended in RPMI 1640 (Sigma) containing standard concentrations of L-glutamine. A cell count was performed and cells concentrations ( $5 \times 10^6$  cells/mL) were adjusted with RPMI 1640 and 250  $\mu$ L of the cell suspensions were distributed into polypropylene 6 mL tubes. Triplicate tubes were either unstimulated (basal), added with 5  $\mu$ L of RPMI 1640, or stimulated, to which 5  $\mu$ L of a suspension of live or heat inactivated *S. pyogenes* ( $1.6 \times 10^8$  /mL), in accordance to the McFarland Scale concentration) were added to the total volume. Cell cultures were incubated with such live and inactivated *S. pyogenes* concentration for 20 hs at 37 °C. Following incubation, culture supernatants were collected and stored at -70 °C for further assessment of TNF $\alpha$  and nitrites concentrations.

The cell pellets were washed 3 times with PBS and resuspended to their original volume in the same buffer. These cells were examined for respiratory burst by flow cytometry.

### Respiratory burst

For evaluation of the respiratory burst (Rothe and Valet, 1994), 100  $\mu\text{L}$  of either neutrophil or mononuclear cell suspensions (containing  $5 \times 10^6$  cells) were added to 0.9 mL of PBS in polypropylene tubes. Cells had been stimulated with either live or heat-inactivated *S. pyogenes*, and unstimulated cells were used as basal controls. Twenty five  $\mu\text{L}$  of dihydro-rhodamine-123 ( $50 \mu\text{g} \cdot \text{mL}^{-1}$ ) were added to each tube before incubation at  $37^\circ\text{C}$  for 15 min. Then, 10  $\mu\text{L}$  of phorbol myristate acetate (10  $\mu\text{L}$   $\mu\text{M}$ ) were added to each tube, and were incubated again for 15 min at  $37^\circ\text{C}$ . After incubation, the samples were centrifuged at 400 g for 5 min and the supernatants were discarded. Cells were finally resuspended in 500  $\mu\text{L}$  of FACS Flow BD, and  $10^4$  cells were acquired for fluorescence-activated-flow-cytometry (FACS) in a FACS-Calibur flow cytometer with computer-assisted evaluation of data (Becton-Dickinson/Cell Quest software). For each sample, 10,000 events were acquired with gates drawn around neutrophils or mononuclear cell preparations. Results were expressed as the percentage of fluorescent cells and as the oxidative index (R), which was calculated by dividing the mean fluorescence intensity of stimulated cells by that of unstimulated cells (mean fluorescence intensity is proportional to the amount of reactive oxygen intermediates generated).

### Measurement of TNF $\alpha$ and IL17 in culture supernatants of mononuclear cells and neutrophils

Levels of TNF $\alpha$  and IL17 in the supernatants of neutrophil and mononuclear cell cultures were determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits, as recommended by the manufacturer (R&D Systems, Minneapolis, MN, USA). Samples were assayed in duplicate and results expressed as the average of two readings in an ELISA reader at 450 nm. TNF $\alpha$  was quantified with reference to standard curves generated using a human recombinant cytokine. The sensitivity of the assay kit for TNF $\alpha$  was 4.4 pg/mL.

### Nitrite determination and evaluation

The method used involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrites formed by the spontaneous oxidation of NO under physiological conditions (Invitrogen, 2003). The detection limit for this method was 1.0  $\mu\text{M}$  nitrite.

Nitrite accumulation, an indicator of NO synthesis, in the supernatant of 4-day cultured mononuclear cells was assayed by the Griess reaction. Briefly, equal amounts of the cell-free supernatant from each sample were added to an equal volume of freshly prepared Griess reagent. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 2.5%  $\text{H}_3\text{PO}_4$ , and 0.1% naphthylethylene diamine dihydrochloride in 2.5%  $\text{H}_3\text{PO}_4$ , and incubating for 10 min at room temperature. Absorbance was measured at 560 nm. Nitrite concentration was quantified using several  $\text{NaNO}_2$  concentrations in culture medium as the standard and data were expressed as  $\mu\text{M}$ .

### Statistical analysis

Comparisons were performed by means of non parametric tests: Kruskal Wallis analysis of variance, Mann-Whitney U test and Wilcoxon test.

## Results

### Respiratory burst

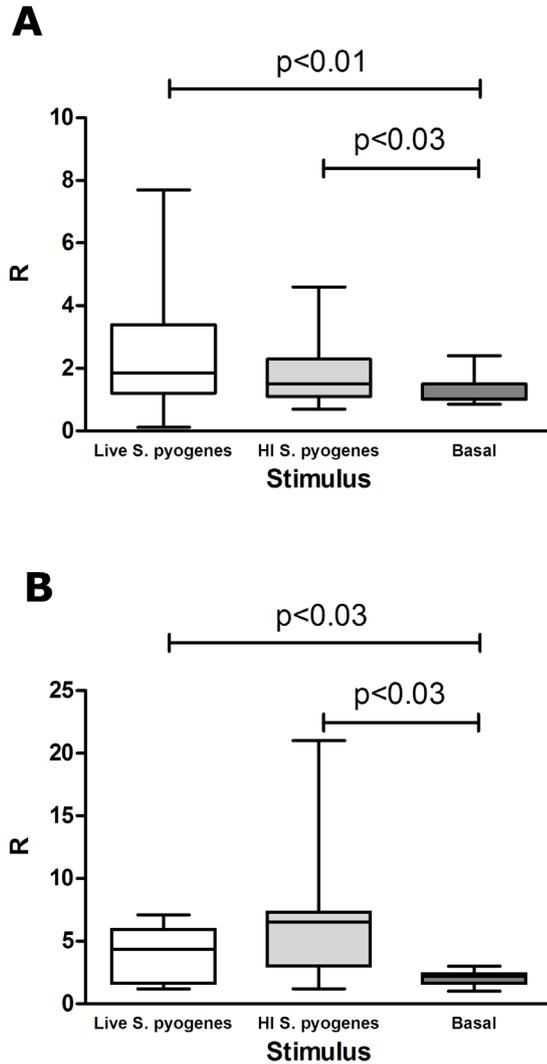
Differences in the respiratory burst (R index) are shown in Fig. 1. This index was significantly increased in both neutrophils and mononuclear cells, which were stimulated with either live or heat-inactivated *S. pyogenes* (as compared with basal unstimulated cells).

The percent of fluorescent cells (mean $\pm$ SE) was also increased by stimulation, as follows: Neutrophils, (a) basal,  $70.2 \pm 2.9$ ; (b) stimulated with live *S. pyogenes*,  $84.2 \pm 2.8$ , (c) stimulated with heat-inactivated *S. pyogenes*,  $80.6 \pm 2.5$ ;

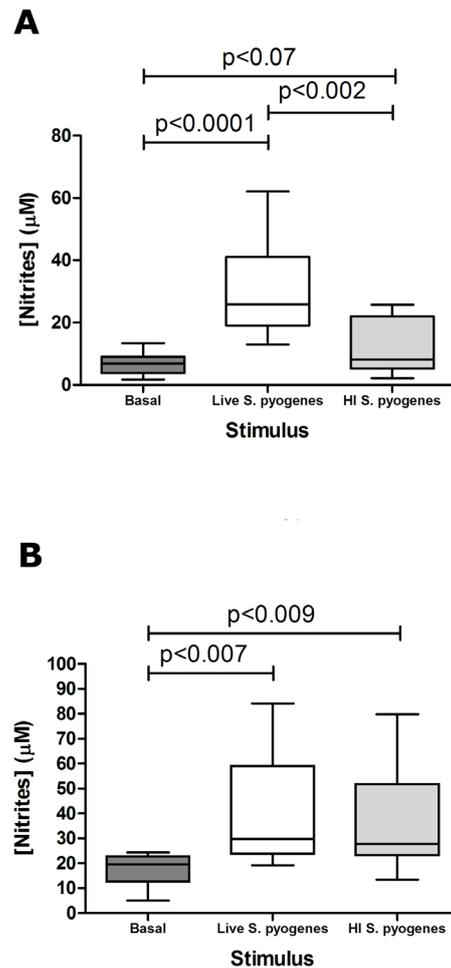
Mononuclear cells, (a) basal,  $19.9 \pm 3.5$ ; (b) stimulated with live *S. pyogenes*,  $36.3 \pm 4.5$ , (c) stimulated with heat-inactivated *S. pyogenes*,  $32.4 \pm 3.6$ . Differences between basal and stimulated cells were statistically significant ( $P < 0.03$  or better).

*NO* concentration

Fig. 2 shows that nitrite concentrations were increased in the supernatant of neutrophil and mononuclear cell cultures ( $N=18$ ) when the cells had been stimulated with either live or heat-inactivated *S. pyogenes* (as compared with basal unstimulated cells). Differences between basal and stimulated cells were statistically significant (Fig. 2).



**Figure 1.** Respiratory burst (R index) by mononuclear cells (panel A) and polymorphonuclear neutrophils (panel B). Cells were previously stimulated or not with either live or heat-inactivated (HI) *Streptococcus pyogenes*. Data are expressed as median and range.



**Figure 2.** Nitrite concentration ( $\mu\text{M}$ ) in the supernatant of cultured mononuclear cells (panel A) and neutrophils (panel B). Cells were previously stimulated or not with either live or heat-inactivated (HI) *Streptococcus pyogenes*. Data are expressed as median and range.

### *TNF $\alpha$ and IL17 production*

TNF $\alpha$  concentrations (pg/ml, mean $\pm$ SE) were increased in supernatants of neutrophil and mononuclear cell cultures (N=18) when the cells had been stimulated with either live or heat-inactivated *S. pyogenes* (as compared with basal unstimulated cells, as follows: Neutrophils: (a) basal, 55.0 $\pm$ 9.1; (b) stimulated with live *S. pyogenes*, 98.3 $\pm$ 8.2, (c) stimulated with heat-inactivated *S. pyogenes*, 128.9 $\pm$ 14.2; Mononuclear cells, (a) basal, 100.8 $\pm$ 3.2; (b) stimulated with live *S. pyogenes*, 223.5 $\pm$ 21.5, (c) stimulated with heat-inactivated *S. pyogenes*, 202.8 $\pm$ 37.4. Differences between basal and stimulated cells were statistically significant (P<0.02 or better). IL17 concentrations (pg/ml, mean $\pm$ SE) were also increased in supernatants of neutrophil and mononuclear cell cultures (N=18) when the cells had been stimulated with either live or heat-inactivated *S. pyogenes* (as compared with basal unstimulated cells, as follows: Neutrophils: (a) basal, 1.33 $\pm$ 0.2; (b) stimulated with live *S. pyogenes*, 3.27 $\pm$ 0.37, (c) stimulated with heat-inactivated *S. pyogenes*, 3.27 $\pm$ 0.37; Mononuclear cells, (a) basal, 1.15 $\pm$ 0.09; (b) stimulated with live *S. pyogenes*, 3.10 $\pm$ 0.24; (c) stimulated with heat-inactivated *S. pyogenes*, 3.86 $\pm$ 0.50;. Differences between basal and stimulated cells were statistically significant (P<0.02 or better).

## **Discussion**

### *Respiratory burst*

*S. pyogenes* stimulation *in vitro* results in a significantly increased respiratory burst by both phagocytic cells which may be relevant for controlling infection progression. Effective phagocytosis and the subsequent production of ROIs are essential for the killing of *S. pyogenes* at the site of infection, and hence, defects of these functions may favor disease progression.

### *Nitrite production*

Nitric oxide (NO) production by inducible NO synthase (iNOS) during inflammatory response is an important factor of antimicrobial immunity but can also contribute to host-induced tissue damage. Results in this study showed that *in vitro* stimulation with *S. pyogenes* increases nitrite by phagocytic cells. And that this may be an efficient protector mechanism against *S. pyogenes*. It has been shown that cell wall components present in culture supernatants of Gram-positive bacteria, such as lipoteichoic acid, can lead to NO production in tissue cultures (Lonchampt et al., 1992; Sriskandan et al., 1997). It is likely that such components induce NO through their ability to produce proinflammatory cytokines, in particular TNF $\alpha$  (Sriskandan et al., 1997; Heumann et al., 1994; Timmerman et al., 1993; Wilkinson et al., 1995).

### *IL17 and TNF $\alpha$ production*

It has been shown here that stimulation of neutrophils and mononuclear cells with *S. pyogenes* induced an increased production of IL17 and TNF $\alpha$ . Recognition of *S. pyogenes* by phagocytic cells leads to cell activation and production of cytokines, which by themselves induce further activation and cytokine production in a complex process of regulation and cross-regulation (Kuby, 2007). This cytokine network plays a crucial role in the inflammatory response against *S. pyogenes* infections. Interleukin 17 is a cytokine that operates by increasing chemokine production in delayed-type reactions in various tissues to recruit mononuclear cells and neutrophils to the place of inflammation, similar to IFN $\alpha$ . The IL17 production by T-helper cells is induced by IL-23 and as consequence, a destructive tissue damage in delayed-type reactions happens. IL 17 acts as a proinflammatory cytokine in response to the attack by extracellular pathogens and induces the pathogen's destruction (Kuby, 2007).

IL17 works synergistically with TNF $\alpha$  and may induce the production of other cytokines as IL6, G-CSF, GM-CSF, IL1 $\beta$ , TGF $\beta$ , TNF $\alpha$ , chemokines including IL8, GRO- $\alpha$ , and MCP-1, and also prostaglandins as PGE2 (Aggarwal and Gurney, 2002; Chiricozzi et al., 2011; Miossec et al., 2009).

The recruited neutrophils, display relevant immunomodulatory activities when activated, which result in the release of proinflammatory cytokines and chemokines, a significant ability that intensify the initial chemotactic role of resident macrophages and dendritic cells. Among the cytokines secreted by activated neutrophils are the proinflammatory IL1 $\beta$  and TNF $\alpha$  (Kasama et al., 2005), which also increase the production by several cells of chemokines that primarily attract neutrophils or monocytes (Silva, 2010). TNF $\alpha$  is a pleiotropic cytokine playing a key role in inflammatory process, macrophage and PMN activation, respiratory burst activity and regulation of anti- *S. pyogenes* activity. Thus, TNF $\alpha$  occupies a central role in the regulation of the inflammatory cascade (Blake et al., 2002). As such, the increased production of this proinflammatory cytokine, reported here, may be accounting for the inflammatory process observed in the *S. pyogenes* infected patients. Severe infections with *S. pyogenes*, are associated with massive inflammatory reactions in the human host. Pählman et al., (2006) reported that streptococcal M protein interacts with TLR2 on human peripheral blood mononuclear cells, and as a consequence, monocytes express the cytokines IL6, IL1 $\beta$ , and TNF $\alpha$ . Neutrophils act mainly by phagocytosing pathogens and exposing them to two destructive mechanisms as reactive oxygen species generated by the enzyme called the phagocyte NADPH oxidase 2 and hydrolytic granule proteins (Roos et al., 2003). The antimicrobial action of phagocytes is decisive in the host innate defense against infection and successful pathogens should avoid internalization and subsequent killing. Mononuclear cells and neutrophils are the most efficient phagocytes, which play an effective and specific role in response to pathogens (Silva, 2010). Both types of phagocytic cells, share several functions, as strong phagocytosis, and possess similar resources for controlling external aggression under inflammatory/infectious conditions, and also exhibit antimicrobial and immunomodulatory

characteristics (Silva, 2010).

The present report shows that stimulation by either live or heat inactivated *S. pyogenes* enhances the participation of phagocytic cells (polymorphonuclear neutrophils and mononuclear cells) in the defense mechanisms against this important pathogen.

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## Ethical Considerations

In the reported studies the procedures followed on the human subjects were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

The corresponding author states that a written informed consent was obtained from all participants.

This article has obtained the approval from the Ethics Committee and the Biosecurity Committee from the Secretariat for Science and Technology of Medicine Faculty, Rosario National University (Argentina). These studies have also the Rosario National University accreditation (Resolution C.S. N° 713/2012).

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