

# *Saccharomyces boulardii* Produces a Factor That Inhibits *Mycobacterium intracellulare* Burden in Human Macrophages

An Bai<sup>1,2</sup>, Michael Weaver<sup>2</sup>, Fukai Bao<sup>3</sup>, Edward D. Chan<sup>1,2,4</sup>, Xiyuan Bai<sup>1,2,4\*</sup>

<sup>1</sup>Department of Medicine, Denver Veterans Affairs Medical Center, Denver, CO, USA

<sup>2</sup>Departments of Medicine and Academic Affairs, National Jewish Health, Denver, CO, USA

<sup>3</sup>Yunnan Key Laboratory for Tropical Infectious Diseases, Department of Microbiology and Immunology, Kunming Medical University, Kunming, China

<sup>4</sup>Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, University of Colorado School of Medicine, Aurora, CO, USA

Email: \*Baix@njhealth.org

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## Abstract

Lung disease caused by Non-Tuberculosis Mycobacteria (NTM) is increasing in prevalence. NTM lung disease is notable for poor response to therapy. *Saccharomyces boulardii* is probiotic that can be effective in inflammatory gastrointestinal disease with diverse pathophysiology. The present study investigated the effects of the products of *S. boulardii*-B508 on burden of NTM-*Mycobacterium intracellulare* complex in human macrophage infection *in vitro*. It was found that the supernatant of *S. boulardii*-B508 inhibited the growth of *Mycobacterium intracellulare* in human macrophage infection and induced infected cell apoptosis. The data of RT-PCR showed that the products of *S. boulardii*-B508 inhibited IL-8 expression during *M. intracellulare* infection in human macrophages due to its effects on NF- $\kappa$ B activation. To the best of our knowledge, this is the first report of effective products of *S. boulardii* on NTM infection in human macrophage. *S. boulardii* possesses anti-NTM lung disease properties in human macrophage worthy of further evaluation in clinical studies.

## Keywords

*Saccharomyces boulardii*, Non-Tuberculosis Mycobacteria, Nuclear-Factor Kappa B

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## 1. Introduction

Mycobacteria are rod-shaped, non-motile and acid-fast bacteria that have high lipid content in their cell wall [1]. *Mycobacterium avium complex* (MAC) causes human

disease and is a group of pathogens, consisting of *Mycobacterium avium*, *Mycobacterium intracellulare*, principally found in soil, water, dust and other biofilms [2].

In the United States and most European countries, *M. avium* and *M. intracellulare* are the most common cause of pulmonary Non-Tuberculous Mycobacteria (NTM) disease that is increasingly reported [3]. *M. avium* and *M. intracellulare* were found in the majority of acquired immunodeficiency syndrome (AIDS) patients, and 10% of these patients have had symptom of diarrhea in the past [4]. Therefore, studies that could help us to potentially enhance the host immunomodulatory response and therapy against the mycobacteria is imminent.

*Saccharomyces boulardii* is a non-pathogenic yeast used for many years as a probiotic agent to prevent or treat variety of human gastrointestinal disorders, diarrhea and *Clostridium difficile* disease [5] [6]. Several studies have shown that there are beneficial effectors in *S. boulardii* by multiple mechanisms such as inhibition of pathogen adhesion, neutralization of bacterial virulence factors and toxins, and enhancement of the mucosal immune response [7] [8] [9]. Little is known about its effects on pulmonary Mycobacteria infection in human macrophages. In this study, we investigated the effect of *S. boulardii*-B508 supernatant on the growth of *M. intracellulare* in human macrophages. We tested the effectiveness of *S. boulardii*-B508 culture supernatant on IL-8 production by human macrophage after exposure in *M. intracellulare*. Our results demonstrate that *S. boulardii*-B508 potentially produces a molecule factor that inhibits the burden of *M. intracellulare* and pro-inflammatory signaling in target cells by blocking the activity of transcription factor NF- $\kappa$ B during *M. intracellulare* infection and induce host immune cell apoptosis due to enhancing infected cell apoptosis.

## 2. Materials and Methods

### 2.1. Materials

The human monocytic cell line THP-1 (TIB-202) and *Mycobacterium intracellulare* were obtained from the American Type Culture Collection (Manassas, VA). Cell culture medium (RPMI 1640) was obtained from Cambrex (East Rutherford, NJ), reagents for Middlebrook 7H10 solid agar medium from Difco (Detroit, MI), and phorbolmyristate acetate (PMA) from Sigma (St. Louis, MO). TRIzol reagent from Invitrogen Inc, ISCRIP cDNA Synthesis Kit and the PCR Kit were purchased from Bio-Rad Inc (Bio-Rad, Hercules CA, USA). ELISA kit for detecting IL-8 was purchased from R & D System Inc (Minneapolis, MN), The TransAM NF- $\kappa$ B p65 Transcription Factor Assay Kit was obtained from Active Motif (Carlsbad, CA, USA).

### 2.2. Culture and Differentiation of THP-1 Cell Infected with/without *M. intracellulare*

THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mM glutamine. Prior to experimentation, THP-1 cells were stimulated with 15 ng/mL PMA (phorbolmyristate acetate) for 24 h to allow differentiation into macrophages. The PMA-containing medium was removed and replaced with fresh medium just prior

to exposing the cells to infection. Differentiated macrophages were plated with *M. intracellulare* (MAI) at a multiplicity of infection (MOI) of 10 bacilli to 1 macrophage as previously described [10]. For culture of cell-associated MAI, briefly, after one-hour infection, the cells were washed with a 1:1 solution of RPMI:1 × PBS. Adherent cells were lysed with 250 µl of 0.25% SDS solution per well, followed by addition of 250 µL of 7H9 plating broth. Serial dilution of cell lysates was prepared and then 5 µl of each dilution was plated on Middlebrook 7H10 agar. For 48 h and 96 h, the cells were washed after the initial 1 h of infection and replaced with fresh culture medium or the supernatant of *S. boulardii*-B508 as previously described [11].

### 2.3. RNA Isolation, Reverse Transcription, and PCR Amplification

THP-1 cells were plated into each well of a six-well tissue culture plates ( $1 \times 10^6$  cells/well), differentiated with PMA overnight for experiments. Total RNA was extracted using the TRIzol reagent, and cDNA was prepared using reverse transcriptase, following the manufacturer's instructions (Invitrogen Life Technologies) and as previously described [12]. Briefly single-stranded cDNA products were used as templates in a 30 µL PCR reaction according to the instruction of the ISCRIP cDNA Synthesis Kit and the PCR Kit (Bio-Rad, Hercules CA, USA). The following primers were used: IL-8 sense primer: 5'-CAT GAC TTC CAA GCT GGC CGTG-3' and IL-8 antisense primer: 5'-TCA CTG ATT CTT GGA TAC CAC AGAG-3'; GAPDH sense primer: 5'-TCC ATG ACA ACT TTG GTA TCG TG -3' and GAPDH antisense primer: 5'-TGT CGC TGT TGA AGT CAG AGGA-3'. PCR amplification parameters were as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles of amplification (94°C for 35 s, 55°C for 50 s, 72°C for 1 min), and finally terminated at 72°C for 4 min and kept 4°C until use. PCR products were analyzed by electrophoresis through 1.5% agarose gels containing ethidium bromide. The DNA bands corresponding to IL-8 (489 bp) and GAPDH (501 bp) were visualized using an ultraviolet dock. Images were captured and then quantified using Adobe Photoshop.

### 2.4. Preparation of *S. boulardii* Culture Supernatant

*S. boulardii*-B508 was cultured in RPMI 1640 cell culture medium for 48 h in 28°C. The supernatant was then centrifuged at 6500 rpm for 20 min, the supernatant was prepared to use.

### 2.5. TUNEL Assay

The THP-1 cells were plated in each well of a four-chamber culture slide, followed by differentiation with PMA of THP-1 cells and infection with MAI. Before of infection, the cells were washed and added with or without supernatant of *S. boulardii*-B508. For each condition, triplicate slides were prepared. To assay for apoptosis at single cell based on labeling of DNA strand breaks was used the *in situ* cell death detection kit from Roche Diagnostics GmbH and followed according to manufacturer's instructions and enumeration of apoptotic cells as previously described [10].

## 2.6. NF- $\kappa$ B p65 Activation Assay

Activation of the p65 subunit of NF- $\kappa$ B was quantified using the TransAMNF- $\kappa$ B p65 Transcription Factor Assay Kit according to manufacturer's instructions. For each condition, 10  $\mu$ g of nuclear protein extracts from treated cells were incubated in 96-well plates coated with an oligonucleotide containing the NF- $\kappa$ B consensus binding site (5' GGGACTTCC-3' oligonucleotide) or a mutated oligonucleotide for one hour at room temperature. After washing three times, NF- $\kappa$ B p65 antibody was added for one hour followed by HRP-conjugated secondary antibody. Binding of activated p65 NF- $\kappa$ B was determined colorimetrically.

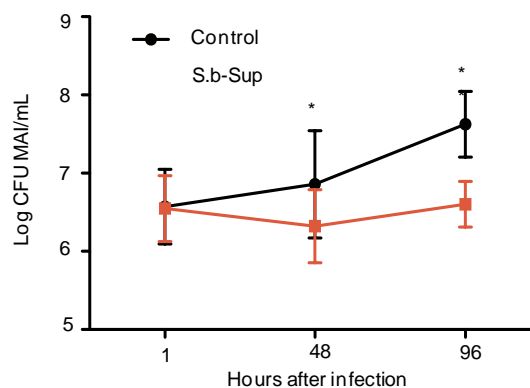
## 2.7. Statistical Analysis

Replicated experiments were independent, and where appropriate, summary results are presented as means  $\pm$  SEM. Differences were considered significant for  $P < 0.05$ , and all reported p-values used a two-sided test. For most experiments, group means were compared by ANOVA using Fisher's least significance difference procedure.

## 3. Results

### 3.1. Secreted Products of *S. boulardii*-B508 Inhibit the Growth of *M. intracellulare* in Human Macrophages

To study the effects of *S. boulardii*-B508 on the growth of *M. intracellulare* in human macrophages, we cultured differentiated THP-1 cells with and without the supernatant of *S. boulardii*-B508, and then infected the cells with *M. intracellulare* for one hour, 48 or 96 hours. After the indicated times of infection, the cells were washed, lysed and *M. intracellulare* quantified. The number of *M. intracellulare* was significantly decreased after two days or four days of infection in cells cultured with *S. boulardii*-B508 supernatant compared with the control THP-1 cells (Figure 1). We conclude from these



**Figure 1.** The products of *S. boulardii*-B508 inhibit the growth of *M. intracellulare* in human macrophages. THP-1 cell differentiated macrophages were pretreated with the supernatant of *S. boulardii*-B508 for one hour, followed by infection with *M. intracellulare*. One hour, 48 and 96 hours after infection, the cells were lysed and cultured for *M. intracellulare*. Data shown as mean  $\pm$  SEM,  $n = 3$ , \* $p < 0.05$ , \*\* $p < 0.01$ .

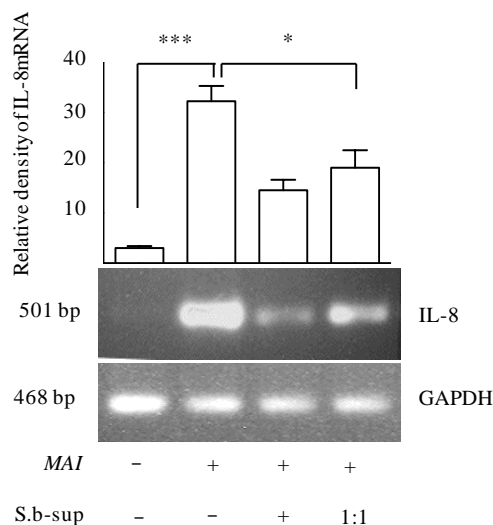
findings that the secreted products of *S. boulardii*-B508 directly inhibit the proliferation of *M. intracellulare* enhances macrophage killing of *M. intracellulare* or both.

### 3.2. The Supernatant of *S. boulardii*-B508 Inhibits IL-8 mRNA Expression after Infection of *M. intracellulare* in Human Macrophages

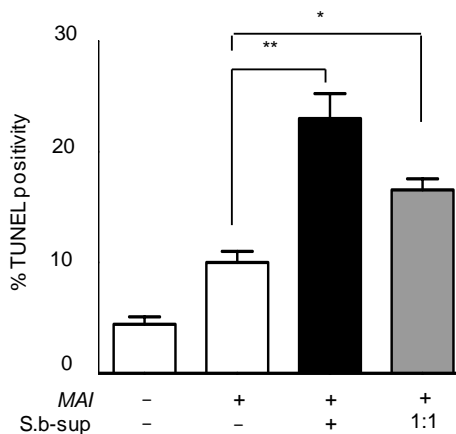
It is well established that IL-8 production is regulated at the level of gene transcription [13]. To examine whether the supernatant of *S. boulardii*-B508 affects IL-8 mRNA expression, we stimulated THP-1-derived differentiated macrophages seeded in 6 well plates with or without *M. intracellulare* infection in the presence or absence of the supernatant treatment of *S. boulardii*-B508 for 18 hours. Total RNA was isolated and RT-PCR was performed with sense and anti-sense primers for IL-8. *M. intracellulare* infection induced the production of IL-8 mRNA, with the stronger induction occurring after infection. Our data indicated that the total supernatant or 50% supernatant of *S. boulardii*-B508 inhibits significantly IL-8 mRNA expression (Figure 2).

### 3.3. The Supernatant of *S. boulardii*-B508 Induces Apoptosis with Infection of *M. intracellulare* in Human Macrophages

Our previous studies suggested that induced apoptosis is known to inhibits the growth of intracellular mycobacteria [11] [12]. In order to determine whether the supernatant of *S. boulardii*-B508 induces the apoptosis of the macrophages THP-1 cells infected with *M. intracellulare*. TUNEL staining were performed, there was a dose-dependent increase in apoptosis in infected cell incubated with supernatant of *S. boulardii*-B508 (Figure 3). *M. intracellulare* infection of THP-1 cells also induced a modest increase in



**Figure 2.** The supernatant of *S. boulardii*-B508 inhibits IL-8 mRNA expression by infection of *MAI* in human macrophages THP-1. The top panels shown that relative density of IL-8 mRNA expression were semi quantified for RT-PCR for IL-8. Data shown represent the mean  $\pm$  SEM of three independent experiments \* $p < 0.05$ , \*\*\* $p < 0.001$ .



**Figure 3.** The supernatant of *S. boulardii*-B508 induces apoptosis by infection of MAI in human macrophages THP-1 cells. The amount of apoptosis was measured after 18 hours of incubation by quantifying percent TUNEL positivity. Data shown represent the mean  $\pm$  SEM of three independent experiments \* $p < 0.05$ , \*\* $p < 0.01$ .

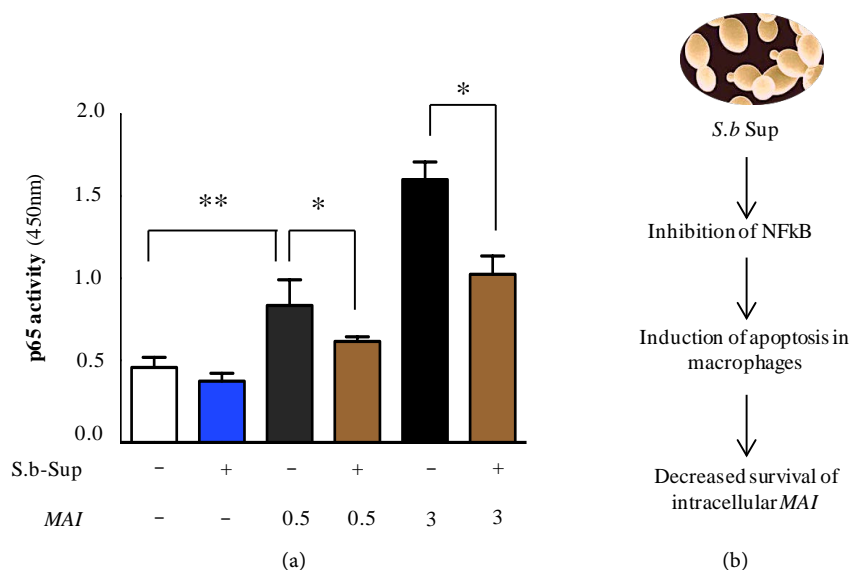
apoptosis, but in the presence of supernatant of *S. boulardii*-B508, there was a significant increase in the percentage of TUNEL-positive cells, although the supernatant of *S. boulardii*-B508 were 1:1 dilution (Figure 3).

### 3.4. The Supernatant of *S. Boulardii*-B508 Inhibits NF- $\kappa$ B Activity after Infection of *M. intracellulare* in Human Macrophages THP-1

Since *M. avium* organisms have been shown to activate I $\kappa$ B $\alpha$  kinase- NF- $\kappa$ B and the mitogen-activated protein kinase (MAPK) signaling pathway [14] [15]. Given that NF- $\kappa$ B is the prime regulator of IL-8 gene transcription in both epithelial cells and monocytes [13] [16], we sought to determine the effects of *S. boulardii* on NF- $\kappa$ B activation by *M. intracellulare* infection. In our previous studies showed that NF- $\kappa$ B activation inhibits both apoptosis and autophagy in *M. tuberculosis*-infected human Macrophages, it was impairing their control of the infection [11]. THP-1-derived macrophages were infected with *M. intracellulare* for 30 minutes and three hours with or without adding the supernatant of *S. boulardii*-B508. p65NF- $\kappa$ B binding to its consensus oligonucleotide was then quantified. As shown in Figure 4(a), MAI induced NF- $\kappa$ B p65 binding at both time points and the supernatant of *S. boulardii*-B508 significantly inhibited that activation.

## 4. Discussion

MAC lung disease is the most common lung disease caused by NTM in the United States and its incidence and prevalence are increasing worldwide in the last decades [17] [18] [19] [20]. MAC was originally composed of two species, *M. avium* and *M. intracellulare* [21]. *M. intracellulare* is the major cause of MAC lung disease in many countries [18]. The long-term success rate in the treatment of pulmonary MAC remains relatively low [22]. Since an increasing number of potential health benefits are being attributed to



**Figure 4.** The supernatant of *S. boulardii-B508* inhibits NFκB Activity by infection of *MAI* in human THP-1 macrophages. (a) THP-1 cells were incubated in medium alone, supernatant alone or with/without *MAI* infection for half hour, three hours, nuclear protein extracted and then NFκB (p65) binding to its consensus oligonucleotide was quantified. Data shown are the mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ . (b) Diagram of the anti-*MAI* mechanisms of supernatant of *S. boulardii-B508* in macrophages based on the findings from this study.

probiotic treatments [23], a greater understanding of the role that probiotics play with regard to MAC organisms may lead to new treatment approaches that enhance protective immunity.

*S. boulardii* as a probiotic is used in the treatment of *Clostridium difficile* diarrhea, colitis and protects *C. difficile*-induced inflammatory diarrhea in human due, in part, to proteolytic digestion of toxin A and B molecules by a secreted protease [24]. This effect is mediated by the release of 54 kDa protease from *S. boulardii* cultures that digests both toxin A and its receptor binding sites [8]. *S. boulardii* has been tested for clinical efficacy in several types of chronic diseases including Crohn's disease, ulcerative colitis, irritable bowel syndrome, parasitic infections and human immunodeficiency virus (HIV)-related diarrhea [25].

We report here that the yeast *S. boulardii-B508*, a probiotic, inhibits the growth of *M. intracellulare* in human macrophages, potentially produces a small soluble molecule called Saccharomyces anti-inflammatory factor (SAIF) [26] that may inhibit the activation of NF-κB and a transcription factor that plays a central role in human inflammatory response. Our results demonstrate that the supernatant contained SAIF of *S. boulardii-B508* potentially induces apoptosis and inhibits NF-κB-dependent IL-8 production for *M. intracellulare* infection in human macrophages.

Dahan *et al.* previously showed that whole yeast cells of *S. boulardii* inhibited NF-κB-DNA binding activity, phosphorylation and degradation of IκBα and activation of the three members of the MAP kinases in infected T84 human colonocytes [27]. Sougioult-

zis *et al.* reported that *S. boulardii* produced a soluble anti-inflammatory factor that inhibited the activation of NF- $\kappa$ B by inhibiting I $\kappa$ B $\alpha$  degradation [26]. We have previously shown that pharmacologic inhibition of NF- $\kappa$ B activation resulted in proved macrophages control of *M. tuberculosis* infection [11]. Inhibition of NF- $\kappa$ B activation is an attractive therapeutic target in a lot of human diseases such as arthritis, asthma, and inflammatory bowel disease [28]. The factors of the supernatant in *S. boulardii* B-508 induce infected cell apoptosis through inhibiting NF- $\kappa$ B activity to decrease *M. intracellulare* survival (Figure 4(b)).

In conclusion, we provide evidences that the probiotic *S. boulardii*-B508 produces a factor that induces apoptosis and inhibits NF- $\kappa$ B activation and mediates pro-inflammatory signaling in host cell macrophages by *M. intracellulare* infection. Future studies on the molecular basis for *S. boulardii*-B508 regulation of human macrophages and immune cell function will enable us to better understand the mechanisms of therapeutic and prophylactic role of this probiotic yeast in MAC disease. It may shed light on new potential applications for *S. boulardii*-B508 in MAC lung diseases.

### Conflict of Interests

The authors declare that there is no competing financial interests regarding this study and without support of any research funding.

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