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Saccharomyces boulardii produces a soluble anti-inflammatory factor that inhibits NF-κB-mediated IL-8 gene expression

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Abstract

Saccharomyces boulardii (*Sb*) is a non-pathogenic yeast that ameliorates intestinal injury and inflammation caused by a wide variety of enteric pathogens. We hypothesized that *Sb* may exert its probiotic effects by modulation of host cell signaling and pro-inflammatory gene expression. Human HT-29 colonocytes and THP-1 monocytes were stimulated with IL-1β, TNFα or LPS in the presence or absence of *Sb* culture supernatant (*Sb*S). IL-8 protein and mRNA levels were measured by ELISA and RT-PCR, respectively. The effect of *Sb*S on IκBα degradation was studied by Western blotting and on NF-κB-DNA binding by EMSA. NF-κB-regulated gene expression was evaluated by transient transfection of THP-1 cells with a NF-κB-responsive luciferase reporter gene. *Sb*S inhibited IL-8 protein production in IL-1β or TNFα stimulated HT-29 cells (by 75% and 85%, respectively; $P < 0.001$) and prevented IL-1β-induced up-regulation of IL-8 mRNA. *Sb*S also inhibited IL-8 production, prevented IκBα degradation, and reduced both NF-κB-DNA binding and NF-κB reporter gene up-regulation in IL-1β or LPS-stimulated THP-1 cells. Purification and characterization studies indicate that the *S. boulardii* anti-inflammatory factor (SAIF) is small (<1 kDa), heat stable, and water soluble. The probiotic yeast *Saccharomyces boulardii* exerts an anti-inflammatory effect by producing a low molecular weight soluble factor that blocks NF-κB activation and NF-κB-mediated IL-8 gene expression in intestinal epithelial cells and monocytes. SAIF may mediate, at least in part, the beneficial effects of *Saccharomyces boulardii* in infectious and non-infectious human intestinal disease.
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Saccharomyces boulardii (*Sb*) is a non-pathogenic yeast used for many years as a probiotic agent to prevent or treat a variety of human gastrointestinal disorders, including antibiotic associated diarrhea and recurrent *Clostridium difficile* disease [1,2]. A recent report also suggests that *Sb* may be useful in preventing clinical relapse in Crohn's disease [3]. Several studies indicate that *Sb* may exert its beneficial effects by multiple mechanisms including competition with pathogens for nutrients, inhibition of pathogen adhesion, strengthening of enterocyte tight junctions, neutralization of bacterial virulence factors and toxins, and enhancement of the mucosal immune response [4–6]. Since intestinal inflammation is a common characteristic in *C. difficile* colitis, enterocolitis caused by many other human enteric pathogens, and Crohn's disease, we hypothesized that *Sb* may interfere with the host signaling events that drive the intestinal inflammatory response.

Unpublished work from our laboratory indicated that *Sb* whole cell culture inhibited *C. difficile* toxin A or LPS-stimulated IL-8 production from THP-1 transformed human monocytic cells and from freshly isolated human peripheral monocytes. Moreover, a recent report suggests that the addition of *Sb* to T84 human enterocytes attenuates enterohemorrhagic *Escherichia coli*-induced NF-κB and MAP kinase activation through undefined mechanisms [15]. In an attempt to delineate the mechanism of action of *Sb* in NF-κB signaling, we tested the effectiveness of *Sb* culture supernatant on IL-8 production by both human

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monocytes and enterocytes after exposure to a variety of pro-inflammatory stimuli. Our results demonstrate that *Sb* produces a small molecular weight, water soluble factor that inhibits pro-inflammatory signaling in target cells by blocking activation of the transcription factor NF-κB.

Materials and methods

Cells and reagents

Human transformed monocytic (THP-1), colonic epithelial (HT-29), and gastric epithelial (AGS) cells were obtained from ATCC (American Type Culture Collection, Rockville, MD) and grown in RPMI 1640 media (Gibco-BRL). Recombinant human (rh) IL-1β and TNFα were purchased from R&D Systems (Minneapolis, MN). Purified LPS from *E. richia coli* 055:B5 was from Sigma (St. Louis, MO). Anti-human IκBα was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-NF-κB p65 was from Boehringer Mannheim (Mannheim, Germany). IL-8 protein levels in cell conditioned media were measured by an enzyme-linked immunosorbent assay, as previously described [16].

Preparation of Saccharomyces boulardii culture supernatant

Lyophilized *Sb* was provided by Biocodex Laboratories, Montrouge, France and was cultured in RPMI 1640 cell culture medium for 24 h in 37 °C. The suspension was then centrifuged at 7400 rpm for 15 min, the supernatant passed through a 0.22-mm filter (Fisher Scientific, Agawam, MA) and then fractionated through a 10 kDa filter (Millipore, Bedford, MA). The filtrate was designated as <10 kDa *Sb* supernatant (*Sb*S).

Analysis of NF-κB reporter gene activity in transiently transfected THP-1 cells

THP-1 cells were transfected with an NF-κB luciferase reporter plasmid carrying eight NF-κB binding sites [17] as previously described [18]. Following incubation with *Sb*S and/or purified LPS (100 ng/ml) for 5 h, THP-1 cells (8×10^6 cells per stimulus) were washed twice with ice-cold PBS, treated with 100 μl of reporter lysis buffer (Promega), and placed on a shaker for 15 min. Cells were then frozen at −70 °C for 15 h and cell lysates were thawed and centrifuged at 10,000 rpm at 4 °C for 8 min. Twenty microliters of lysate was combined with 100 μl of luciferase buffer (Promega) and quantified using a Turner TD-20e luminometer.

Western blotting

Ten milliliters of THP-1 cells was placed in 10-mm tissue culture dishes and stimulated with rhIL-1β (10 ng/ml) alone or in the presence of *Sb*S for 4 h. Whole cell lysates were prepared, separated in 10% SDS–PAGE, and transferred to Sequi-Blot PVDF membrane (Bio-Rad Laboratories, 2000 Hercules, CA). Western blots using anti-NF-κB p65 and anti-IκBα antibodies were performed as previously described [10,19].

Electrophoretic mobility shift assay

THP-1 cells were stimulated with IL-1β (10 ng/ml), either alone or in the presence of *Sb*S, and nuclear extracts were then prepared as previously described [19]. The consensus NF-κB binding site was synthesized as a double stranded oligonucleotide by Operon (San Francisco, CA), and was end labeled with [^{32}P]dCTP by Klenow DNA Polymerase (New England Biolabs, Beverly, MA). The resulting probe was purified on a Quick-Sep Column (Isolab, Inc., Akron, OH) and percent binding was calculated. Electrophoretic mobility shift assay (EMSA) experiments were otherwise performed as previously described [10,20]. Certain reactions also contained 100-fold excess of the specific unlabeled consensus oligonucleotide in order to determine the specificity of the binding reaction.

RNA isolation, reverse transcription, and PCR amplification

HT-29 cells were seeded in 6-well plates and stimulated with IL-1β (10 ng/ml) in the presence or absence of *Sb*S. Cells were harvested at 30 min, 1, 2, and 4 h, and total RNA was extracted (Trizol, Invitrogen, Carlsbad, CA). One microgram of RNA was then reverse transcribed to yield cDNA that was subsequently subjected to PCR amplification for IL-8 and the housekeeping gene GAPDH, using appropriate primers, as we have previously described [21]. The PCR products were analyzed by electrophoresis through 1.2% agarose gels containing 100 ng/ml ethidium bromide. The DNA bands corresponding to IL-8 (199 bp) and GAPDH (600 bp) were visualized using an ultraviolet transilluminator (Bio-Rad), photographed, and their density quantified by Adobe Photoshop, Version 5.5. IL-8 mRNA level is presented as an expression index: the ratio of IL-8 signal to the corresponding signal from GAPDH, while the expression index for control cells cultured with medium alone was arbitrarily set to 1.0.

Cell viability assessment

The viability of HT-29 cells after exposure to *Sb*S was assessed by a modified MTT assay: the MTS (3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium) proliferation assay performed according to the manufacturer's instructions (Promega, Madison, WI) [22].

Purification and characterization studies

Heat stability. The <10 kDa *Sb*S was heated to 100 °C for 5 min and then tested for activity on IL-1β (10 ng/ml) treated HT-29 monolayers.

Lipid solubility. Lipids were extracted from the <10 kDa *Sb*S by liquid–liquid extraction using six volumes of chloroform–methanol (2:1, v/v) in a glass tube. After centrifugation at 800*g* for 3 min, the resulting organic (lower) phase was aspirated and transferred to a separate tube. The organic solvents were then evaporated in the presence of N_2 and the dried material was reconstituted in RPMI 1640 media by sonication. In some cases, the organic phase was subjected to a second cycle of the same procedure (double lipid extraction).

Density gradient ultra-centrifugation in cesium chloride. The <10 kDa *Sb*S was subjected to density gradient ultra-centrifugation in cesium chloride (CsCl), as we have previously described [23].

Measurement of neutral sugars. Neutral sugar content of the fractions was determined by the phenol–sulfuric acid method as originally described by Dubois et al. [24] and recently miniaturized for use with microsample plate reader [25].

Measurement of proteins. Protein contents of the fractions were determined using the bicinchoninic acid protein assay reagent (Pierce Laboratories, Rockford, Ill) used according to the manufacturer's instructions.

Dialysis–gel filtration. Fractions from density gradient ultra-centrifugation were dialyzed against water by using a 500 Da cut-off membrane mounted on a Harvard Apparatus dialyser (The Nest Group Inc, Southborough, MA). Removal of CsCl was monitored by measuring the conductivity of the dialysate and dialysis was continued until conductivity reached the same level as distilled water. After dialysis, all fractions were tested for their ability to inhibit IL-8 production by IL-1β stimulated HT29 monolayers. Finally, 500 μl of solution containing the active fraction was subjected to chromatography on a 1.5 × 12 cm Biogel P-2 column (Bio-Rad, Hercules, CA), as we have previously described [26]. Fifteen fractions of 1 ml were collected, and aliquots were assayed for neutral sugars and protein contents as well as for their ability to inhibit IL-8 secretion.

Statistical analyses

Results were expressed as means ± SEM. Data were analyzed using the SIGMA-STAT™ professional statistics software program (Jandel Scientific Software, San Rafael, CA). Analyses of variance (ANOVA) with protected *t* test were used for intergroup comparison.

Results

S. boulardii supernatant inhibits IL-8 secretion by cytokine-stimulated human colonic and gastric epithelial cell lines, the active component being less than 10 kDa in size

HT-29 cells were serum starved overnight and then stimulated with IL-1β (10 ng/ml), TNF-α (10 ng/ml) or LPS (100 ng/ml), in the presence or absence of *Sb*S (1:1, v/v). After 12 h incubation, the cell conditioned media were collected and IL-8 protein levels measured by ELISA. As illustrated in Fig. 1A resting HT-29 monolayers released little IL-8 protein while stimulation with IL-1β or TNFα markedly increased IL-8 protein production. The presence of *Sb*S significantly reduced IL-8 production in both IL-1β- and TNFα-stimulated HT29 cells.

As illustrated in Fig. 1B *Sb*S also markedly inhibited IL-8 protein production in AGS cells stimulated with IL-1β or TNFα. As expected, LPS resulted in minimal activation of IL-8 production by either HT29 or AGS cells [27]. A significant decrease in IL-8 production was also observed in both LPS- (100 ng/ml) and IL-1β- (10 ng/ml) stimulated THP-1 human monocytes incubated for 5 h in the presence of *Sb*S (data not shown).

Based on published evidence that *Sb* secretes large molecular weight proteins (>50 kDa) possessing biologic activity [28,29], we sought to determine whether the anti-inflammatory activity observed in this study arose from such molecules. We therefore examined the activity of *Sb*S after passage through a 10-kDa cut-off filter. As illustrated in Fig. 1C we found that the filtrate retained full activity indicating that the active component has a molecular weight of <10 kDa.

We next studied the time-course of the appearance of the inhibitory effect exerted by the <10 kDa *Sb*S. To do this, we stimulated HT29 monolayers with IL-1β at different time intervals up to 24 h in the presence or absence of the <10 kDa *Sb*S. Cell culture supernatants were then collected and tested for IL-8 levels by ELISA. As illustrated in Fig. 2A, we found that the <10 kDa *Sb*S significantly inhibited IL-8 production at all of the time points examined from 2 to 24 h.

We next stimulated HT29 monolayers with IL-1β alone or in the presence of serial twofold dilutions of the <10 kDa *Sb* supernatant in HT29 culture medium (v/v). After 12 h incubation, HT29 cell culture supernatants were collected and IL-8 was measured. As illustrated in Fig. 2B, we found that the <10 kDa *Sb*S reduced IL-8 production in a dose-dependent manner with significant inhibition at dilutions of 1:32 or less.

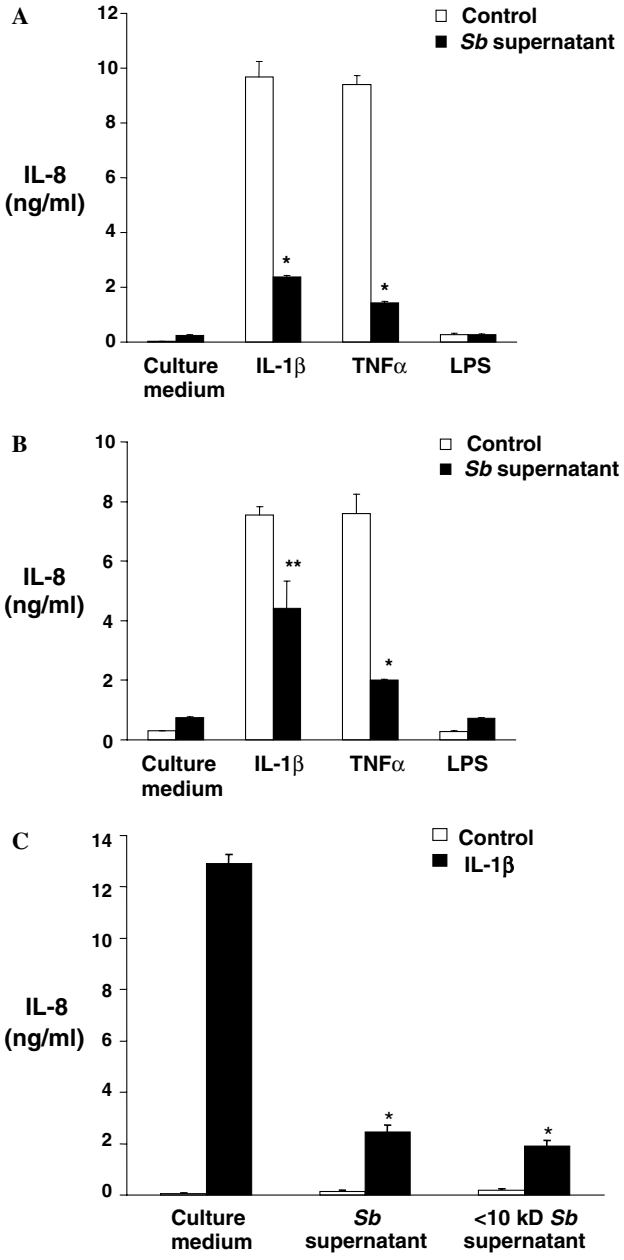

Fig. 1. Inhibition of IL-8 secretion by *Saccharomyces boulardii* supernatant. HT29 (A) and AGS (B) cell monolayers were stimulated with IL-1β (10 ng/ml) or TNFα (10 ng/ml) in the presence or absence of *Sb* supernatant (applied 1:1, v/v). IL-8 protein levels were measured in the culture media after 12 h. (C) Data from HT29 cell monolayers exposed to *Sb* supernatant that had been passaged through a <10 kDa molecular mass cut-off filter. Results are expressed as means ± SEM and correspond to representative experiments. All experiments were performed at least three times in quadruplicate. *, **$p < 0.001$ and $p = 0.01$, respectively, when compared to IL-1β or TNFα stimulation alone.

S. boulardii supernatant does not affect cell viability

To examine whether the inhibitory activity exerted by *Sb*S was due to a toxic effect, we tested whether the *Sb*S altered cell viability in HT29 colonocytes using the MTS

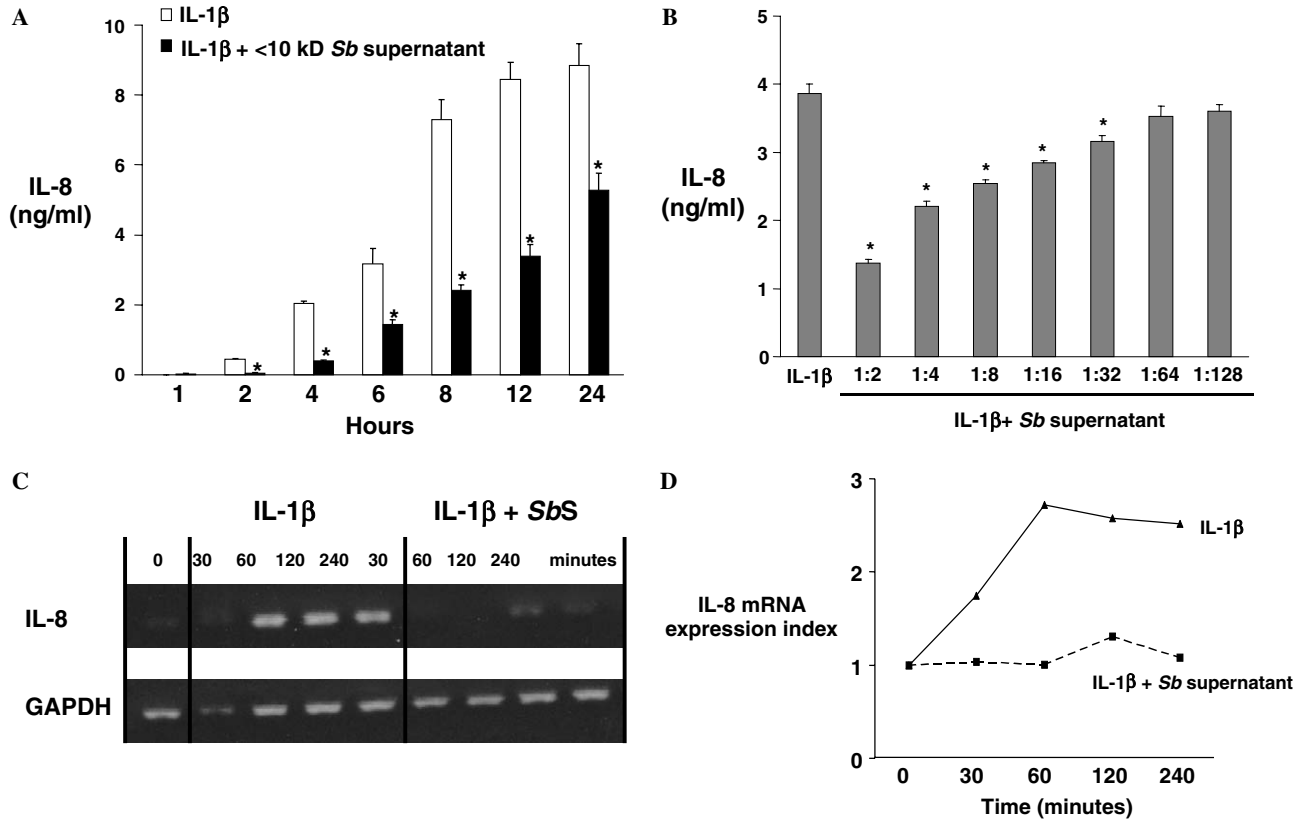

Fig. 2. Inhibitory effect of <10 kDa *Sb* supernatant on IL8 protein and mRNA up-regulation. HT29 cell monolayers were stimulated with IL-1β (10 ng/ml) in the presence or absence of the <10 kDa *Sb* supernatant and IL-8 protein was measured in the conditioned media by ELISA after varying periods of time (A, $*p < 0.01$ compared to IL-1β stimulation alone at each respective time point). (B) IL-8 protein levels in HT29 cell conditioned media collected 12 h after IL-1β stimulation in the presence of varying dilutions of the <10 kDa *Sb* supernatant (means ± SEM shown; $p < 0.001$ by ANOVA; $*p < 0.05$ when compared to IL-1β stimulation alone). (C) RT-PCR results for both IL-8 and GAPDH from HT-29 cells stimulated with IL-1β (10 ng/ml) in the presence or absence of the <10 kDa *Sb* supernatant. (D) HT29 cell RT-PCR data after densitometry and calculation of the IL-8 mRNA index (IL-8 signal/GAPDH signal).

assay. The 490 nm absorbance recorded from untreated cells and cells exposed to *Sb*S for 12 h did not differ significantly (0.845 ± 0.09 versus 0.785 ± 0.03, respectively; OD_{490}, mean ± SD; $p = 0.32$). These data indicate that *Sb*S does not have an adverse effect on cell viability within the tested time window during which IL-8 production is inhibited.

We also studied the time-course of the appearance and disappearance of the inhibitory effect exerted by the *Sb*S. To do this, we pretreated HT29 monolayers with *Sb*S for either 2 or 4 h. We then removed the *Sb*S and stimulated the cells with IL-1β. The conditioned media were collected and tested for IL-8 levels by ELISA after time periods of up to 8 h. We found that pretreatment of cells with *Sb*S for either 2 or 4 h was followed by a significant inhibition of IL-8 production. This inhibition lasted for up to 4 h (41.7% inhibition following the 2 h pretreatment and 59.9% following the 4 h pretreatment). However, after 8 h the inhibitory effect had been reversed and IL-8 production returned to control levels. These findings indicate that the inhibitory effect of *Sb*S is reversible and not associated with loss of cell viability or protein production.

S. boulardii supernatant prevents IL-1β-mediated increases in IL-8 mRNA levels in HT29 colonic epithelial cells

It is well established that IL-8 production is regulated at the level of gene transcription [14,30]. To examine whether *Sb*S affects IL-8 mRNA expression, we stimulated HT29 colonocytes seeded in 6-well plates with IL-1β (10 ng/ml) in the presence or absence of *Sb*S. Our results illustrated in Figs. 2C and D showed that IL-1β induced IL-8 mRNA within 30 min, with maximum induction at 1 h. The addition of *Sb*S markedly reduced IL-8 mRNA levels, at all time points studied. Taken together with the results shown in Fig. 1, our data indicated that *Sb* releases a soluble factor that inhibits IL-8 gene transcription.

S. boulardii supernatant prevents NF-κB reporter gene transactivation and IκBα degradation

Given that NF-κB is the prime regulator of IL-8 gene transcription in both epithelial cells and monocytes [14,30], we sought to determine the effect of *Sb*S on NF-κB activation. These experiments were performed in

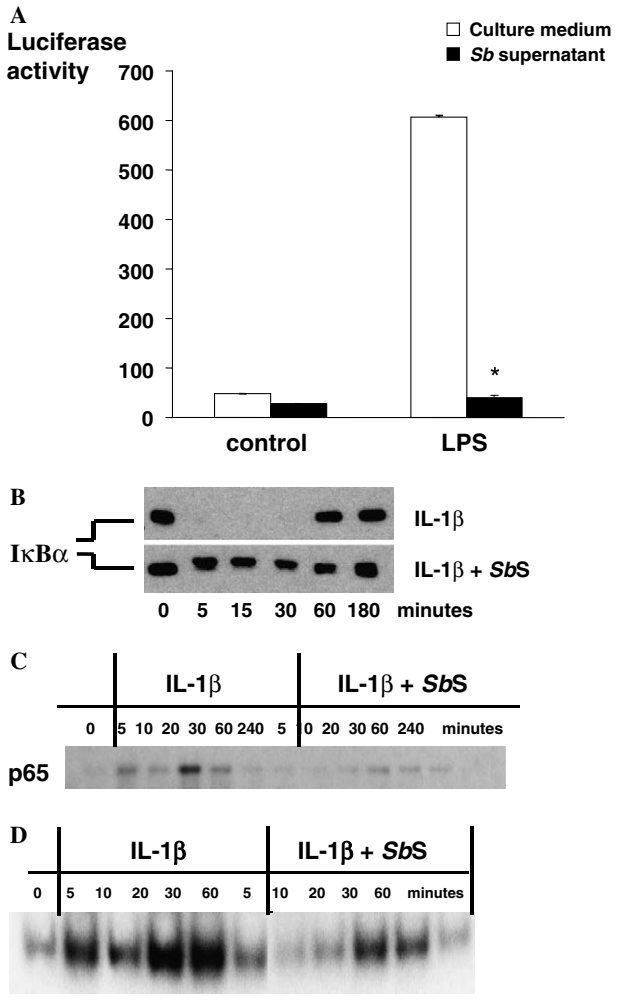

Fig. 3. Inhibition of IκBα degradation and NF-κB transactivation by *Sb* supernatant. (A) THP-1 cells were transiently transfected with 5 μg of an NF-κB-luciferase reporter plasmid, as described in the Materials and methods, and stimulated with LPS (100 ng/ml) in the presence or absence of *Sb* supernatant. (B) Cytoplasmic extracts were prepared from THP-1 cells at the indicated time points, resolved by SDS–PAGE, and analyzed by Western blotting with an anti-IκBα antibody. (C) THP-1 cells were stimulated with IL-1β (10 ng/ml), nuclear extracts were prepared at the indicated time points and subjected to Western blotting to measure nuclear levels of p65 by using an anti-p65 antibody. (D) NF-κB-DNA binding activity was examined by EMSA with a ^{32}P-labeled probe corresponding to the consensus NF-κB binding site.

THP-1 monocytic cells since it has been reported that HT29 colonocytes exhibit altered regulation of IκBα proteolysis [7,31,32]. As shown in Fig. 3A, LPS stimulation for 5 h caused a 12-fold increase in NF-κB luciferase reporter activity compared to unstimulated cells and this effect was dramatically inhibited after exposure of THP-1 cells to *Sb*S. These results indicate that *Sb*S reduces IL-8 protein levels by a mechanism involving NF-κB inhibition. To further explore this NF-κB response, we examined the effect of *Sb*S on a key intracellular step in NF-κB activation, IκBα degradation [10]. THP-1 cells were stimulated with IL-1β (10 ng/ml) in the presence or absence of *Sb*S for 3 h and IκBα protein levels in cytoplasmic extracts were examined by Western blotting. As shown in Fig. 3B, IL-1β stimulation resulted in complete IκBα degradation evident as early as 5 min and persisting for 30 min post-stimulation. In contrast, addition of *Sb*S prevented IκBα degradation during the same period. These findings further support the notion that the inhibitory effect of *Sb*S on IL-8 production is mediated via NF-κB inhibition. It is noteworthy that the non-degraded IκBα band in the *Sb* supernatant-treated cells has higher molecular weight than expected, suggesting that IκBα may be phosphorylated and/or ubiquitinated but not degraded.

S. boulardii supernatant reduces p65 nuclear translocation and NF-κB-DNA binding

Since *Sb*S prevents IκBα degradation, it is to be expected that NF-κB is retained in the cytoplasm and does not translocate to the nucleus to function. To test this hypothesis, we determined p65 protein levels in nuclear extracts of THP-1 cells stimulated for 4 h in the presence or absence of *Sb*S. As shown in Fig. 3C (left panel), IL-1β stimulation results in rapid increase of p65 in the nucleus, starting at 5 min with a peak at 20 min. In contrast, in cells co-treated with *Sb*S the amount of p65 protein in the nucleus was less at all time points studied (Fig. 3C, right panel). These findings, together with the IκBα degradation results (Fig. 3B), indicate that the p65 NF-κB subunit is retained in the cytoplasm in *Sb* supernatant-treated THP-1 cells.

We next determined whether the observed reduction in the amount of p65 in the nucleus results in attenuated NF-κB-DNA binding activity. After THP-1 cells were stimulated with IL-1β (10 ng/ml) for 1 h, in the presence or absence of *Sb*S, NF-κB-DNA binding activity in nuclear extracts was determined by EMSA. As shown in Fig. 3D (left panel), NF-κB-DNA binding is rapidly induced (5 min) following IL-1β stimulation; the activation peaks at 20 min and declines by 60 min. Co-treatment with *Sb*S results in marked reduction of NF-κB-DNA binding at all studied time points.

The active substance in the S. boulardii supernatant is a heat stable, water soluble molecule with a mass of less than 1 kDa

We next sought to characterize the active component(s) in *Sb*S that suppresses NF-κB activation and thereby reduces IL-8 production. We first determined the heat stability of the active component and observed that boiling the <10 kDa *Sb*S for 5 min did not diminish its activity (data not shown). We extracted lipids from the <10 kDa *Sb*S as described in Materials and methods, and tested the lipid fractions for IL-8 inhibitory activity in IL-1β stimulated HT-29 colonocytes. As shown in Fig. 4A, the lipid containing fractions (after single or double extraction) did not exhibit any inhibitory activity on IL-8 production. These results suggest that the active factor in *Sb*S is neither a pure protein nor a lipid.

We next subjected the <10 kDa *Sb*S to density gradient ultra-centrifugation in cesium chloride and recovered nine

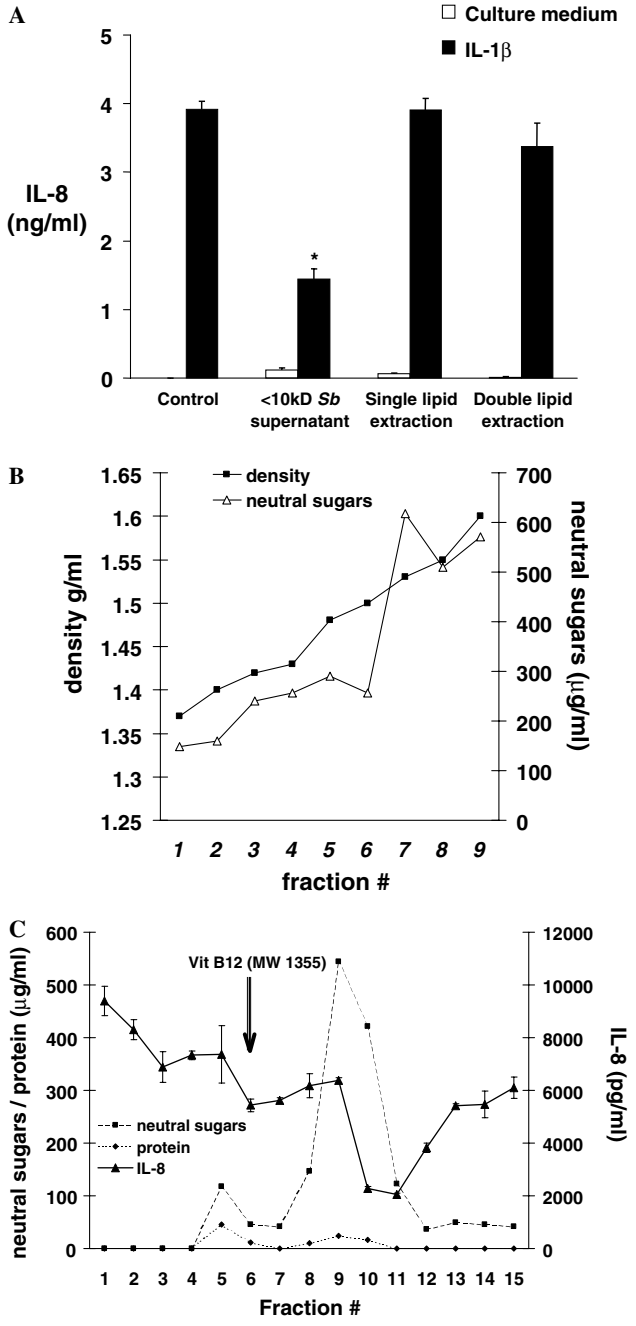

Fig. 4. Initial characterization of SAIF by lipid extraction, density gradient separation, and gel filtration. (A) HT29 cell monolayers were treated with IL-1β (10 ng/ml) alone or together with the <10 kDa *Sb* supernatant or with lipid extracts from the <10 kDa *Sb* supernatant (see Materials and methods). (B) Proteins were separated from glycoproteins by subjecting the <10 kDa supernatant to cesium chloride ultra-centrifugation. Fractions 7–9, corresponding to high density fractions, also contain high neutral sugar levels (e.g., glucose, galactose) and were found to be potent inhibitors of IL-8 production by IL-1β stimulated HT29 colonocytes. (C) Fractions 7–9 from the cesium chloride ultra-centrifugation experiments illustrated in (B) were pooled and 500 μl of the mixture was subjected to gel filtration on a Biogel P-2 column. The active fractions (10 and 11) had measurable levels of neutral sugars and protein by the phenol–sulfuric acid and BCA method, respectively. Vitamin B12, used as a molecular weight marker, was eluted under the same conditions at fraction 6, indicating that the active substance is <1 kDa.

1 ml fractions ranging in density from 1.37 to 1.60 g/ml, the former expected to contain pure proteins and the latter glycoproteins/proteoglycans. When tested for inhibitory activity, we observed that fractions 7–9 (high density, high neutral sugar content) were the most active (data not shown). Fractions 7–9 were then pooled, loaded onto a Biogel P-2 column, eluted with water, and 15 fractions collected. As shown in Fig. 4C, only fractions 10 and 11 significantly inhibited IL-8 production, compared to control (fraction 1) (ANOVA, $p < 0.001$. Bonferroni test for fractions #10, 11: $p < 0.05$ for each, compared to control; for all other fractions $p > 0.05$). Both of these fractions contained measurable neutral sugars and protein by the phenol–sulfuric acid and BCA method, respectively. The fractionation range of the Biogel P-2 column is 100–1800 Da. To gain a more accurate estimation of the molecular weight of the active factor, a colored solution of vitamin B12 (molecular weight 1355) was loaded on the Biogel P-2 column under the same conditions. B12 was eluted with a maximum peak at fraction #6 (Fig. 4C), suggesting that the active substance, eluting at fractions #10, 11, has a molecular weight less than 1 kDa.

Discussion

We report here that the yeast *Saccharomyces boulardii*, a probiotic widely used to treat or prevent a variety of human diarrheal diseases, produces SAIF (*Saccharomyces* anti-inflammatory factor) a small molecular weight, water soluble molecule that inhibits the activation of NF-κB, a transcription factor that plays a central role in human inflammatory responses. The mechanism of NF-κB inhibition exerted by SAIF involves inhibition of IκBα degradation, thereby resulting in sequestration of NF-κB in the cytoplasm. Our results also demonstrate that SAIF potently inhibits NF-κB-dependent IL-8 production from human transformed intestinal epithelial cells, gastric epithelial cells, and monocytes stimulated by a variety of pro-inflammatory stimuli.

Previous studies have shown that whole *Sb* yeast cells inhibit NF-κB-DNA binding activity, phosphorylation and degradation of IκBα, and activation of the three members of the MAP kinases (extracellular signal-regulated protein kinases 1 and 2, p38, and c-jun N-terminal kinase) in enteropathogenic *E. coli* (EPEC) infected T84 human colonocytes [15,33]. Preincubation of the yeast with the colonocytes as well as its continuous presence during EPEC infection were required for *Sb* to exert its effects. In those studies *Sb* did not seem to modify the number of adherent bacteria and the mediator(s) of its inhibitory effects on the above signaling pathways were not identified.

Our findings strengthen the notion that the beneficial effects of this probiotic in enteric diseases may be mediated through attenuation of host mucosal pro-inflammatory responses, thereby maintaining the intestinal hyporesponsiveness to pathogen determinants and other noxious stimuli within the enteric lumen [34]. Consistent with this notion

is a recent study by Neish et al. [35], who demonstrated that infection of T84 cells with non-pathogenic *Salmonella* strains prevents NF-κB activation induced by TNFα or pathogenic *Salmonella* by a mechanism involving blockade of IκBα degradation. IκBα was found phosphorylated but not polyubiquitinated, suggesting that non-pathogenic *Salmonella* strains inhibit the β-TrCP IκB-specific ubiquitin ligase [35]. Our results indicate that *Sb* produces a soluble factor that acts also by inhibiting IκBα degradation. It is also interesting that the non-degraded IκBα obtained after treatment with the *Sb* supernatant appears to migrate higher than native IκBα (Fig. 3B), suggesting that IκBα may be phosphorylated, and/or ubiquitinated. Recently, Petrof et al. [36] reported that the probiotic VSL#3 (composed of *Streptococcus thermophilus* and several species of *Lactobacillus* and *Bifidobacteria*) inhibits NF-κB through proteosome inhibition. Studies are under way in our laboratory to find whether inhibition of IκBα degradation is due to blockade of IκBα phosphorylation, ubiquitination, proteasome inhibition or another mechanism [37].

Inhibition of NF-κB activation is an attractive therapeutic target in a wide range of human diseases such as arthritis, asthma, and inflammatory bowel disease [38]. It is well known that the mechanism of action of many widely used anti-inflammatory compounds including salicylates and corticosteroids, drugs with proven therapeutic value in inflammatory bowel disease, involves NF-κB inhibition [39,40].

Our initial characterization of SAIF employed density gradient ultra-centrifugation in cesium chloride which has been successfully used for the separation of highly glycosylated epithelial glycoproteins (mucins) from lipids and proteins in respiratory and gastrointestinal mucus ([23]). This method is based on the difference in buoyant density between proteins (∼1.3 g/ml) and carbohydrates (∼1.6 g/ml). Heavily glycosylated mucins (80% carbohydrate) have a buoyant density of ∼1.5 g/ml. This method has the advantage that after the separation there is almost 100% recovery of material unlike in conventional chromatography methods and this was an important consideration in recovering the biologically active factor. The fact that SAIF is recovered in the high density fraction suggests that it is glycosylated ([23]). This was further confirmed by our gel filtration studies where the biologically active fractions #10 and #11 (Fig. 4C) contain substantial amounts of neutral sugars. Moreover, based on the biologic activity of the fractions derived from gel filtration, we conclude that SAIF has a molecular weight of <1 kDa. Taken together, these data suggest that the active factor is a glycan. However, since we have not performed in-depth structural analyses yet, the possibility that the biologic activity is mediated by another organic substance that simply co-migrates with sugars cannot be excluded definitely.

In conclusion, we provide evidence that the probiotic *S. myces boulardii* produces a soluble anti-inflammatory factor, SAIF, that inhibits NF-κB activation. Attenuation of pro-inflammatory signaling in host cells is a putative mechanism that may mediate, at least in part, the beneficial effects of *S. boulardii* in infectious and non-infectious human intestinal disease. Initial characterization of SAIF indicates that it may be a useful pharmacologic agent in that it is small, water soluble, and stable.

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Competing interests statement. Drs. Sougioultzis, Bhaskar, Pothoulakis, and Kelly are named co-inventors on a patent application on *Saccharomyces* anti-inflammatory factor (SAIF) filed by Beth Israel Deaconess Medical Center, Boston, MA.

Dr. Pothoulakis has acted as a consultant or scientific advisor to companies interested in developing therapeutic interventions for intestinal infectious and inflammatory disorders; those companies are: ActivBiotics Inc., Bicodex Laboratories, Salix Pharmaceuticals Inc.

Dr. Kelly has acted as a consultant or scientific advisor to companies interested in developing therapeutic interventions for intestinal infectious and inflammatory disorders; those companies are: ActivBiotics Inc., Bicodex Laboratories, Genzyme Inc., Salix Pharmaceuticals, Inc., Oscient Pharmaceuticals Inc.

References

[1] G.W. Elmer, C.M. Surawicz, L.V. McFarland, Biotherapeutic agents. A neglected modality for the treatment and prevention of selected intestinal and vaginal infections, JAMA 275 (11) (1996) 870–876.

[2] A. Sullivan, C.E. Nord, The place of probiotics in human intestinal infections, Int. J. Antimicrob. Agents 20 (5) (2002) 313–319.

[3] M. Guslandi, G. Mezzi, M. Sorghi, P.A. Testoni, *Saccharomyces boulardii* in maintenance treatment of Crohn's disease, Dig. Dis. Sci. 45 (7) (2000) 1462–1464.

[4] C. Pothoulakis, C.P. Kelly, M.A. Joshi, N. Gao, C.J. O'Keane, et al., *Saccharomyces boulardii* inhibits *Clostridium* difficile toxin A binding and enterotoxicity in rat ileum, Gastroenterology 104 (4) (1993) 1108–1115.

[5] I. Castagliuolo, M.F. Riegler, L. Valenick, J.T. LaMont, C. Pothoulakis, *Saccharomyces boulardii* protease inhibits the effects of *Clostridium* difficile toxins A and B in human colonic mucosa, Infect. Immun. 67 (1) (1999) 302–307.

[6] D. Czerucka, P. Rampal, Experimental effects of *Saccharomyces boulardii* on diarrheal pathogens, Microbes Infect. 4 (7) (2002) 733–739.

[7] C. Jobin, R.B. Sartor, The I kappa B/NF-kappa B system: a key determinant of mucosal inflammation and protection, Am. J. Physiol. Cell Physiol. 278 (3) (2000) C451–C462.

[10] S. Simeonidis, D. Stauber, G. Chen, W.A. Hendrickson, D. Thanos, Mechanisms by which IkappaB proteins control NF-kappaB activity, Proc. Natl. Acad. Sci. USA 96 (1) (1999) 49–54.

[14] K.A. Roebuck, Regulation of interleukin-8 gene expression, J. Interferon Cytokine Res. 19 (5) (1999) 429–438.

[15] S. Dahan, G. Dalmasso, V. Imbert, J.F. Peyron, P. Rampal, D. Czerucka, *Saccharomyces boulardii* interferes with enterohemorrhagic

Escherichia coli-induced signaling pathways in T84 cells, Infect. Immun. 71 (2) (2003) 766–773.

[16] S. Keates, S. Sougioultzis, A.C. Keates, D. Zhao, R.M. Peek Jr., L.M. Shaw, et al., cag+ *Helicobacter pylori* induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells, J. Biol. Chem. 276 (51) (2001) 48127–48134.

[17] A.T. Ting, F.X. Pimentel-Muinos, B. Seed, RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis, EMBO J. 15 (22) (1996) 6189–6196.

[18] M. Warny, A.C. Keates, S. Keates, I. Castagliuolo, J.K. Zacks, S. Aboudola, et al., p38 MAP kinase activation by *Clostridium* difficile toxin A mediates monocyte necrosis, IL-8 production, and enteritis, J. Clin. Invest. 105 (8) (2000) 1147–1156.

[19] S. Simeonidis, I. Castagliuolo, A. Pan, J. Liu, C.C. Wang, A. Mykoniatis, et al., Regulation of the NK-1 receptor gene expression in human macrophage cells via an NF-kappa B site on its promoter, Proc. Natl. Acad. Sci. USA 100 (5) (2003) 2957–2962.

[20] M. Merika, A.J. Williams, G. Chen, T. Collins, D. Thanos, Recruitment of CBP/p300 by the IFN beta enhanceosome is required for synergistic activation of transcription, Mol. Cell 1 (2) (1998) 277–287.

[21] S. Keates, A.C. Keates, E. Mizoguchi, A. Bhan, C.P. Kelly, Enterocytes are the primary source of the chemokine ENA-78 in normal colon and ulcerative colitis, Am. J. Physiol. 273 (1, Pt. 1) (1997) G75–G82.

[22] A.H. Cory, T.C. Owen, J.A. Barltrop, J.G. Cory, Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture, Cancer Commun. 3 (7) (1991) 207–212.

[23] K.R. Bhaskar, L. Reid, Application of density gradient methods for the study of mucus glycoprotein and other macromolecular components of the sol and gel phases of asthmatic sputa, J. Biol. Chem. 256 (14) (1981) 7583–7589.

[24] M. Dubois, K. Gilles, J. Hamilton, P. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (3) (1956) 350–356.

[25] J.D. Fox, J.F. Robyt, Miniaturization of three carbohydrate analyses using a microsample plate reader, Anal. Biochem. 195 (1) (1991) 93–96.

[26] K.R. Bhaskar, B.S. Turner, S.A. Grubman, D.M. Jefferson, J.T. LaMont, Dysregulation of proteoglycan production by intrahepatic biliary epithelial cells bearing defective (delta-f508) cystic fibrosis transmembrane conductance regulator, Hepatology 27 (1) (1998) 7–14.

[27] M.T. Abreu, P. Vora, E. Faure, L.S. Thomas, E.T. Arnold, M. Arditi, Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide, J. Immunol. 167 (3) (2001) 1609–1616.

[28] I. Castagliuolo, J.T. Lamont, S.T. Nikulasson, C. Pothoulakis, *Saccharomyces boulardii* protease inhibits *Clostridium* difficile toxin A effects in the rat ileum, Infect. Immun. 64 (12) (1996) 5225–5232.

[29] D. Czerucka, I. Roux, P. Rampal, *Saccharomyces boulardii* inhibits secretagogue-mediated adenosine 3′,5′-cyclic monophosphate induction in intestinal cells, Gastroenterology 106 (1) (1994) 65–72.

[30] A. Ben Baruch, D.F. Michiel, J.J. Oppenheim, Signals and receptors involved in recruitment of inflammatory cells, J. Biol. Chem. 270 (20) (1995) 11703–11706.

[31] D. Elewaut, J.A. DiDonato, J.M. Kim, F. Truong, L. Eckmann, M.F. Kagnoff, NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria, J. Immunol. 163 (3) (1999) 1457–1466.

[32] C. Jobin, S. Haskill, L. Mayer, A. Panja, R.B. Sartor, Evidence for altered regulation of I kappa B alpha degradation in human colonic epithelial cells, J. Immunol. 158 (1) (1997) 226–234.

[33] D. Czerucka, S. Dahan, B. Mograbi, B. Rossi, P. Rampal, *Saccharomyces boulardii* preserves the barrier function and modulates the signal transduction pathway induced in enteropathogenic *Escherichia coli*-infected T84 cells, Infect. Immun. 68 (10) (2000) 5998–6004.

[34] R.J. Xavier, D.K. Podolsky, Microbiology. How to get along–friendly microbes in a hostile world, Science 289 (5484) (2000) 1483–1484.

[35] A.S. Neish, A.T. Gewirtz, H. Zeng, A.N. Young, M.E. Hobert, V. Karmali, et al., Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination, Science 289 (5484) (2000) 1560–1563.

[36] E.O. Petrof, K. Kojima, M.J. Ropeleski, M.W. Musch, Y. Tao, C. De Simone, et al., Probiotics inhibit nuclear factor-kappaB and induce heat shock proteins in colonic epithelial cells through proteasome inhibition, Gastroenterology 127 (5) (2004) 1474–1487.

[37] M. Karin, Y. Ben Neriah, Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity, Annu. Rev. Immunol. 18 (2000) 621–663.

[38] Y. Yamamoto, R.B. Gaynor, Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer, J. Clin. Invest. 107 (2) (2001) 135–142.

[39] E. Kopp, S. Ghosh, Inhibition of NF-kappa B by sodium salicylate and aspirin, Science 265 (5174) (1994) 956–959.

[40] N. Auphan, J.A. DiDonato, C. Rosette, A. Helmberg, M. Karin, Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis, Science 270 (5234) (1995) 286–290.