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Saccharomyces boulardii inhibits lipopolysaccharide-induced activation of human dendritic cells and T cell proliferation

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Summary

Saccharomyces boulardii (*Sb*) is a probiotic yeast preparation that has demonstrated efficacy in inflammatory and infectious disorders of the gastrointestinal tract in controlled clinical trials. Although patients clearly benefit from treatment with *Sb*, little is known on how *Sb* unfolds its anti-inflammatory properties in humans. Dendritic cells (DC) balance tolerance and immunity and are involved critically in the control of T cell activation. Thus, they are believed to have a pivotal role in the initiation and perpetuation of chronic inflammatory disorders, not only in the gut. We therefore decided to investigate if *Sb* modulates DC function. Culture of primary (native, non-monocyte-derived) human myeloid $CD1c^+CD11c^+CD123^-$ DC (mDC) in the presence of *Sb* culture supernatant (active component molecular weight < 3 kDa, as evaluated by membrane partition chromatography) reduced significantly expression of the co-stimulatory molecules CD40 and CD80 ($P < 0{\cdot}01$) and the DC mobilization marker CC-chemokine receptor CCR7 (CD197) ($P < 0{\cdot}001$) induced by the prototypical microbial antigen lipopolysaccharide (LPS). Moreover, secretion of key proinflammatory cytokines such as tumour necrosis factor-α and interleukin (IL)-6 were notably reduced, while the secretion of anti-inflammatory IL-10 increased. Finally, *Sb* supernatant inhibited the proliferation of naive T cells in a mixed lymphocyte reaction with mDC. In summary, our data suggest that *Sb* may exhibit part of its anti-inflammatory potential through modulation of DC phenotype, function and migration by inhibition of their immune response to bacterial microbial surrogate antigens such as LPS.

Keywords: dendritic cells, inflammation, inflammatory bowel disease, *Saccharomyces boulardii*, yeast

Introduction

Lyophilized *Saccharomyces boulardii* (*Sb*) is a proprietary yeast preparation. It is currently the only yeast probiotic that has demonstrated efficacy in controlled clinical trials [1–6]. *Sb* belongs to the group of simple eukaryotic cells (such as fungi and algae) and thus differs from bacterial probiotics that are prokaryotes. Recent studies classified *Sb* genetically within the species of *S. cerevisiae* [7–9]. However, metabolically and physiologically it differs substantially from *S. cerevisiae* [10].

Preliminary studies have evaluated the effect of *Sb* in patients with inflammatory bowel disease (IBD). One study of patients suffering from Crohn's disease with moderate activity found that the addition of *Sb* to conventional treatment reduced stool frequency significantly [11]. A beneficial effect in the maintenance of remission in Crohn's disease has also been reported [12]. In another pilot study, *Sb* improved significantly the clinical activity index of patients with left-sided ulcerative colitis when added to their maintenance regimen with mesalamine [13]. Little is known about how *Sb* unfolds its anti-inflammatory properties in humans.

Dendritic cells (DC) control the critical balance between anergy and immunity because of their functional dichotomy of being either potent antigen presenters or effective inducers of (peripheral) tolerance [14,15].

Because of their expression of the entire spectrum of pattern recognition receptors such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) like receptors, they can sense virtually all

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microbe-associated molecular patterns, including yeasts [16,17]. This puts them in a pivotal position to co-ordinate innate and adaptive immune responses [16,18]. They are thought to be involved critically in inflammatory T cell polarization and activation observed in human IBD [19,20].

Here, we report novel anti-inflammatory effects of *Sb* on human myeloid DCs (mDCs) and T cells.

Materials and methods

Purification of mDCs

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers using a protocol published previously [21]. In brief, mDC were isolated by magnetic cell separation from PBMC using CD1c blood DC antigen 1 (BDCA-1) antibodies and MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) [22]. In blood the CD1c (BDCA-1) antigen is expressed specifically on DC, which are $CD11c^+$ $CD123^-$. Additionally, a subset of B cells also expresses this antigen. Thus, a depletion of B cells with CD19 MicroBeads was required prior to the enrichment of CD1c (BDCA-1) mDC. The purity of the isolated mDC population was checked with fluorescence activated cell sorting (FACS) and samples with fewer than 95% mDC were discarded.

Purification of naive T cells

Naive T cells were isolated by magnetic cell separation from PBMC using the CD4 MultiSort Kit and CD45RA MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) [22]. In brief, $CD4^+$ cells were selected positively with CD4 MultiSort MicroBeads. Afterwards magnetic particles were released enzymatically, which allows a second magnetic labelling and separation of the cells using CD45RA MicroBeads. $CD4^+CD45RA^+$ double-positive cells were selected positively from preselected $CD4^+$ cells. The purity of the isolated naive T cell population was checked with FACS and samples with fewer than 95% $CD4^+CD45RA^+$ double-positive T cells were discarded.

Preparation of *Sb* supernatant

Lyophilized *Sb* was provided by Laboratories Biocodex (Montrouge, France). *Sb* supernatant (*Sb*S) was produced as described previously [23]. In brief, *Sb* was cultured in RPMI-1640 cell culture medium for 24 h at 37°C at an initial concentration of 100 mg/ml. To separate the yeast from the supernatant a 0·2 μm sterile filter (Fisher Scientific, Schwerte, Germany) was used. In contrast to the published procedure, initially we did not use the 10 kDa cut-off filter to exclude larger molecules, as we were interested in all potential biologically active components within the supernatant.

The *Sb*S fractionation by membrane partition chromatography

To characterize further the active component in the supernatant, serial fractionation by membrane partition chromatography was performed using Amicon Ultra-15 centrifugal filter units (Millipore, Carrigtwohill, Ireland) according to the manufacturer's guidelines based on a protocol published previously [24]. In brief, dissolved yeast supernatant was spun in an Amicon Ultra-15 centrifugal filter device with a 100 kDa nominal molecular weight limit at 4°C. The permeate (diffusate) was spun again in a filter device with a 50 kDa maximum weight limit following centrifugation steps with 30 kDa, 10 kDa and 3 kDa centrifugal filter devices.

Culture and stimulation of mDC

Highly purified mDC were cultured for 21 h in RPMI-1640 with 0·2% L-glutamine (Gibco, New York, NY, USA) + 10% human serum type AB (Cambrex, Charles City, IA, USA), +1% penicillin–streptomycin (Gibco). To mimic bacterial microbial activation, mDC were stimulated with lipopolysaccharide (LPS), a prototypical microbial antigen they may encounter in the gut [16], in the presence or absence of *Sb*S in different dilutions (1:2, 1:8, 1:32), and permeates (see *Sb*S fractionation above).

Ultra-pure LPS from *Escherichia coli* 055:B5 (Calbiochem, Darmstadt, Germany) was added at a concentration of 100 ng/ml [25]. Finally, mDC were harvested and stained with the appropriate antibodies to measure the expression of co-stimulatory molecules by FACS.

Mixed lymphocyte reaction of mDC and naive T cells

For mixed lymphocyte reaction (MLR) experiments 1×10^4 highly purified mDC were preincubated with LPS (12·5 ng/ml) in the presence or absence of *Sb*S in different dilutions (1:2, 1:8, 1:32) and permeates (see *Sb*S fractionation above) for 3 h at 37°C and 5% CO_2 in RPMI-1640 with 0·2% L-glutamine (Gibco) + 10% human serum type AB (Cambrex) + 1% penicillin–streptomycin (Gibco).

Thereafter, 1×10^5 naive, allogenic T cells from healthy volunteers were added and co-cultured for 5 days at 37°C and 5% CO_2. To rule out T cell unresponsiveness in MLR experiments staphylococcal enterotoxin B was added to some control experiments with naive T cells at a concentration of 6·7 μg/ml (Sigma, St Louis, MO, USA).

T cells were labelled with 5 μM carboxyfluorescein–diacetate–succinimidyl ester (CFSE) prior to co-culture experiments. This membrane-penetrating dye enters the cell and binds covalently to amino groups of cellular proteins. The cleavage of acetate groups by intracellular esterases yields in a highly fluorescent membrane-impermeable

product and results in a uniformly labelled cell population. During cell division the fluorescent dye is gradually lost and thereby distributed equally to the daughter cells. This mechanism allows the identification and enumeration of proliferating cells [26].

Antibodies

Monoclonal antibodies against CD4, CD11c, CD14, CD19, CD40, CD45RA, CD80, CD83, CD86 and CD197 (CCR7) were obtained from BD Biosciences (Heidelberg, Germany) against CD1c (BDCA-1) from Miltenyi Biotec as fluorochrome conjugates. To rule out unspecific binding mouse immunoglobulin G1 (IgG1) and IgG2b (BD Biosciences) and mouse IgG2a (Caltag, San Diego, CA, USA), isotype control antibodies were used.

The FACS analysis of mDC

Three- or four-colour flow cytometric analysis (FACS) was used to identify and enumerate blood mDC cells, as described previously [21]. Briefly, cells were stained with the appropriate amount of antibody and were incubated for 15 min on ice in the dark. After washing in FACS buffer [phosphate-buffered saline + 0·05% bovine serum albumin (Sigma, Steinheim, Germany) + 0·1% NaN_3 (Sigma, Steinheim, Germany)], cells were resuspended in FACS buffer and analysed immediately on a FACSCalibur (BD Biosciences) flow cytometer. Prior to running the samples, propidium iodide (PI) at a final concentration of 0·1 mg/ml (Sigma) was added to label dead cells. Data were analysed using Cell Quest™ (BD Biosciences) software. A major limitation in immunofluorescence measurements is the non-specific labelling of damaged and non-viable (dead) cells by some antibodies and the inability to distinguish non-specific from specific antibody labelling. Dead or damaged cells can represent a significant source of error in flow cytometric analysis of viable cells because of non-specific uptake of labelled antibody probes, increased cellular autofluorescence and altered cell-surface antigen expression. Light scatter has been used to discern non-viable from viable cells in homogeneous populations [27]. Another means of identifying damaged or non-viable cells has been the use of red-emitting fluorochromes which bind to nucleic acids by intercalation, i.e. PI [28]. When used at low concentrations, these highly sensitive dyes penetrate intact viable cells slowly, but enter damaged cells rapidly, where they bind to nucleic acids and fluoresce bright red. We combined both light forward-scatter and side-scatter (FSC/SCC) and PI to exclude unspecific staining and dead cells.

Cytometric bead array analysis of cytokine secretion by mDC

Cytokine secretion in culture supernatants was measured by cytometric bead array (CBA) analysis (BD Biosciences), according to the manufacturer's guidelines. Briefly, bead populations with distinct fluorescence intensities coated with capture antibody proteins were first mixed with phycoerythrin-conjugated detection antibodies and recombinant standards or test samples, and then incubated to form sandwich complexes. With this assay the production of tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 was measured. After acquisition of sample data using FACS, the cytokine concentrations were calculated using proprietary flow cytometry analysis and processing (FCAP)™ (Softflow, New Brighton, MN, USA) analysis software [29].

Statistical analysis

For all studies, data are expressed as mean ± standard error of the mean. Comparisons are by two-tailed Mann–Whitney *U*-test with statistical significance accepted for $P < 0{\cdot}05$. For evaluation of cytokine secretion, one-way ANOVA with Tukey's multiple comparison test as post-test were used for intergroup comparison.

Ethical considerations

This study was approved by Charité's institutional review board.

Results

The *Sb*S decreases the number of CD40- and CD80-positive mDC after incubation with LPS

First, we studied the expression of CD40 and CD80, two co-stimulatory molecules which are known to be up-regulated on activated human mDC and critical for the activation of naive T cells[30–32]. After stimulation with LPS and incubation for 21 h in the presence or absence of *Sb*S in different dilutions, mDC were stained for CD40 and CD80 and analysed eventually by flow cytometry (Fig. 1).

While freshly isolated mDC express virtually no CD40 and CD80 (data not shown), stimulation with LPS induced a strong increase in the number of CD40 (80·5% ± 2·64) and CD80 (87·24% ± 2·41) positive cells respectively.

However, addition of *Sb*S to these cultures decreased significantly the number of CD40 and CD80 expressing mDC. Comparison between CD40 expressing cells in the presence or absence of *Sb*S showed significant differences ($P < 0{\cdot}01$) in the percentage of CD40-positive mDC for an *Sb*S dilution of 1:8 (39·91% ± 3·18). However, a dilution of 1:32 (72·45% ± 2·46) did not show any significant difference (Fig. 1a). We could observe the same trend for CD80, where the number of positive mDC was significantly ($P < 0{\cdot}01$) lower compared with culture approaches with LPS alone when *Sb*S was added to dilutions of 1:8 (55·34% ± 2·1) but not 1:32 (82·52% ± 2·72) (Fig. 1b).

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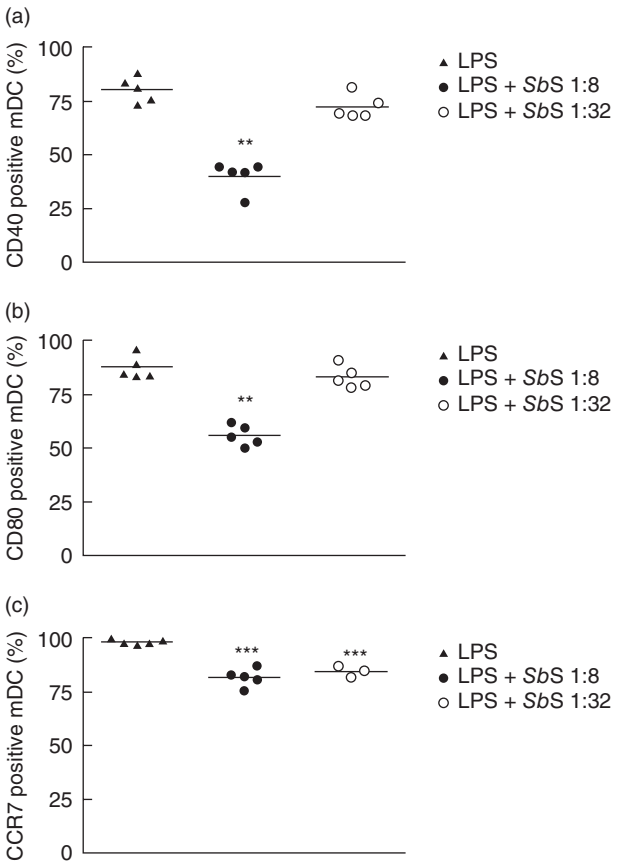

Fig. 1. *Saccharomyces boulardii* supernatant (*Sb*S) decreases the number of CD40 (a), CD80 (b) and CD197 (CCR7) (c) positive myeloid dendritic cells (mDC) dose-dependently when added to cultured mDC together with lipopolysaccharide (LPS) compared with LPS alone. The results represent data from five independent experiments. Asterisks denote statistical significance: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

The *Sb*S decreases the number of CD197 (CCR7)-positive mDC after incubation with LPS

Chemokine receptor-7 (CCR7) is not only another maturation marker for DCs, but is also involved in the steady state and inflammation-associated migration and mobilization of DCs and T cells [33].

While freshly isolated mDC express virtually no CCR7, stimulation with LPS induced expression of CCR7 by virtually all mDC (97·49% ± 0·5)-positive cells respectively (Fig. 1c).

However, addition of *Sb*S to these cultures decreased significantly the number of CCR7 expressing mDC. Comparison between CCR7-expressing cells in the presence or absence of *Sb*S showed significant differences ($P < 0{\cdot}001$) in the percentage of CCR7-positive mDC for *Sb*S dilutions of 1:8 (80·89% ± 1·92) and 1:32 (83·77% ± 1·54) (Fig. 1c).

There were no significant differences between the numbers of CD83 and CD86 expressing mDC (data not shown).

The *Sb*S reduces secretion of TNF-α and IL-6 and increases secretion of IL-10 by mDC

To investigate further the cytokine secretion by LPS-stimulated mDC in the presence or absence of *Sb*S we analysed the culture supernatants from the previous experiment. Therefore, the key proinflammatory cytokines TNF-α and IL-6 as well as the anti-inflammatory cytokine IL-10 were measured by CBA (Fig. 2).

Because cytokine secretion of mDC after stimulation with LPS varied within a wide range (228·2–933·4 pg/ml for TNF-α, 661·6–5365·0 pg/ml for IL-6 and 150·8–2030·0 pg/ml for IL-10), we decided to normalize these controls to 100% and investigate further the increase or decrease of cytokine secretion depending on the presence of *Sb*S.

The secretion of TNF-α by LPS-stimulated mDC decreased significantly ($P < 0{\cdot}001$) when *Sb*S was added to the culture at dilutions of 1:8 to 48·42% ± 10·43 and 1:32 to 38·91% ± 7·73 (Fig. 2a). We then studied the production of IL-6, which echoed the results seen with TNF-α. LPS-stimulated mDC secreted significantly ($P < 0{\cdot}01$) more IL-6 than samples with supplemental *Sb*S (56·01% ± 11·32 for *Sb*S 1:8 and 57·54% ± 6·95 for *Sb*S 1:32) (Fig. 2b).

As DC are known to have a dual role in inflammation and may also help to control inflammation independent of regulatory T cells, we decided to also look at IL-10 secretion [14,34]. LPS-stimulated mDC secreted significantly ($P < 0{\cdot}01$) more IL-10 when *Sb*S was added at a dilution of 1:8 (161·90% ± 16·96). This effect was dose-dependent, as a further significant ($P < 0{\cdot}001$) increase in the production of IL-10 could be observed when *Sb*S was used in a dilution of 1:32 (204·40% ± 10·98) (Fig. 2c).

The *Sb*S inhibits T cell proliferation in allogenic MLR

As the first set of experiments suggested an effect of *Sb*S on co-stimulatory molecules and DC maturation markers highly relevant for T cell activation, we decided to investigate its direct effect on T cell proliferation. We studied the effect in an allogenic MLR of naive T cells and mDC (Fig. 3).

Naive T cells showed almost no autoproliferation after stimulation with LPS alone (2·47% ± 1·21) (Fig. 3a and e). However, the allogenic MLR with LPS-stimulated mDC induced a robust proliferation (36·11% ± 2·46) (Fig. 3b and e). Addition of *Sb*S at a dilution of 1:32 revealed no significant effect (35·01% ± 2·28) (Fig. 3d and e), whereas dilution of 1:8 reduced proliferation significantly to 16·40% ± 2·75 (Fig. 3c and e).

The active component in *Sb*S has a molecular weight of < 3 kDa

Because we had decided not to use the 10 kDa cut-off filter to avoid exclusion of potentially biologically active larger

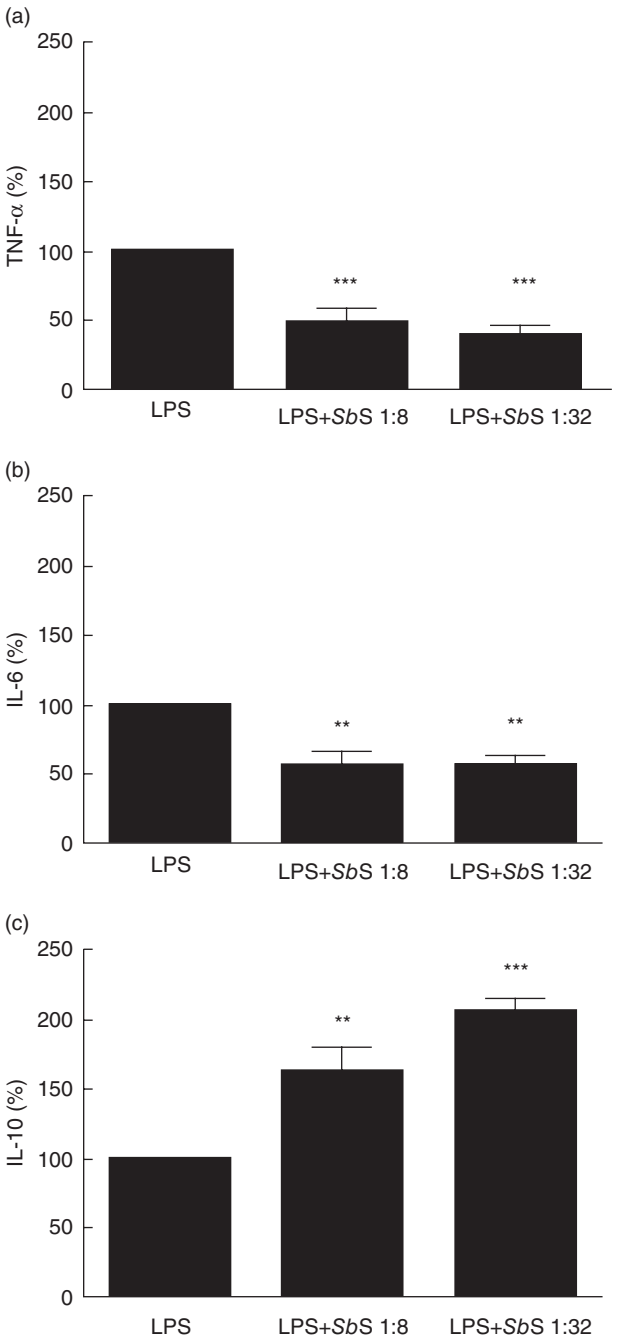

Fig. 2. *Saccharomyces boulardii* supernatant (*Sb*S) reduces the secretion of tumour necrosis factor (TNF)-α (a), interleukin (IL)-6 (b) and shows an increase in the secretion of anti-inflammatory IL-10 (c) by lipopolysaccharide (LPS)-stimulated myeloid dendritic cells (mDC). Graphs show means ± standard error of the mean from five independent experiments. Controls (i.e. mDC cultured with LPS alone) were normalized to 100%. Asterisks denote statistical significance: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

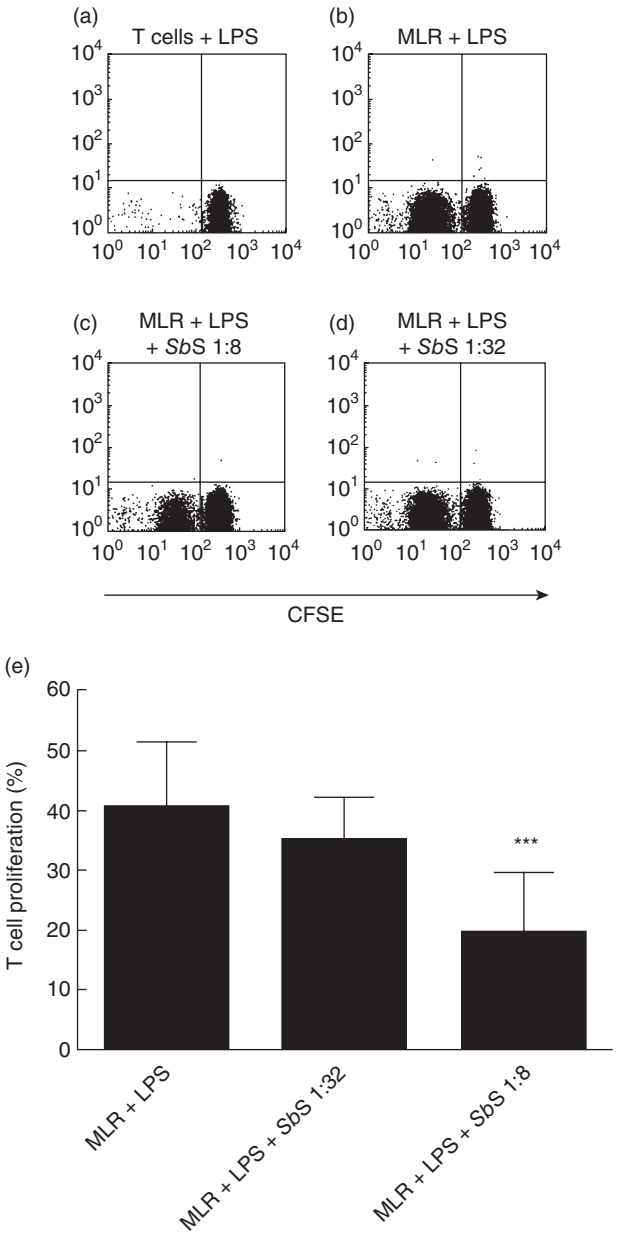

Fig. 3. *Saccharomyces boulardii* supernatant (*Sb*S) inhibits T cell activation and proliferation in an allogenic mixed lymphocyte reaction (MLR) with mDC ($n = 10$). This effect is dose-dependent and reproducible. Fluorescence activated cell sorter plots from representative experiments proliferation assessed by carboxyfluorescein–diacetate–succinimidyl ester staining. Naive T cells cultured without myeloid dendritic cells (mDC) in the presence of lipopolysaccharide (LPS) alone served as controls (a). MLRs of T cells and mDC preincubated with LPS show a robust proliferation in the absence of *Sb*S (b). Adding *Sb*S to the MLR results in a significant decline of T cell proliferation for a *Sb*S dilution of 1 : 8 (c). Further dilution of *Sb*S to 1 : 32 could not maintain a significant inhibitory effect on T cell proliferation (d). Data from these experiments are summarized in bar graphs (e). Bars represent means ± standard error of the mean. Asterisks denote statistical significance: $^{***}P < 0{\cdot}001$.

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molecules, we now aimed to determine the approximate molecular weight of the active *Sb*S component by repeating the phenotypic and cytokine secretion studies with *Sb*S permeates (diffusates) ranging from < 100 kDa down to < 3 kDa (see above) (Figs 4 and 5).

Compared with LPS-stimulated mDC alone, the presence of 1:8 diluted *Sb*S and all tested permeates in these cultures decreased significantly the number of CD40, CD80 and CD197 (CCR7) expressing mDC ($P < 0{\cdot}001$) (Fig. 4).

*Sb*S permeates reduced significantly the percentage of CD40 expressing mDC at < 100 kDa (data not shown), < 50 kDa (74·25% ± 1·71), < 30 kDa (data not shown), 10 kDa (data not shown) and < 3 kDa (77·12% ± 2·4) (Fig. 4a).

The same trend was observed for CD80, where *Sb*S permeates reduced significantly the percentage of expressing mDC at < 100 kDa, < 50 kDa (81·46% ± 3·4), < 30 kDa, 10 kDa and < 3 kDa (80·09% ± 3·8) (Fig. 4b).

Finally, *Sb*S permeates reduced significantly the percentage of CD197 expressing mDC at < 100 kDa (data not shown), < 50 kDa (85·25% ± 3·8), < 30 kDa, 10 kDa and < 3 kDa (83·08% ± 2·5) (Fig. 4c).

Compared with LPS-stimulated mDC alone, the presence of 1:8 diluted *Sb*S and all tested permeates in these cultures decreased significantly the secretion of TNF-α and IL-6 and increased the secretion of IL-10 (Fig. 5).

The *Sb*S permeates reduced significantly the secretion of TNF-α by mDC at < 100 kDa, < 50 kDa (56·69% ± 6·7), < 30 kDa, 10 kDa and < 3 kDa (43·99% ± 8·5) (Fig. 5a).

The same trend was observed for IL-6, where *Sb*S permeates reduced significantly the production of mDC at < 100 kDa, < 50 kDa (64·54% ± 10·04), < 30 kDa, 10 kDa and < 3 kDa (57·45% ± 7·3) (Fig. 5b).

In contrast, *Sb*S permeates increased the production of IL-10 by mDC at < 100 kDa, < 50 kDa (122·5% ± 12·4), < 30 kDa, 10 kDa and < 3 kDa (133·5% ± 21·25) (Fig. 5c). However, unlike unfractionated *Sb*S, the permeate comparisons did not reach statistical significance.

To corroborate these data further, we used the retentates of the membrane partition chromatography process in identical experiments and found the opposite results (data not shown). In other words, the retentates did not induce the phenotypic changes and cytokine secretion pattern seen with the *Sb*S permeates. This suggests that the active component is included in the permeate.

In summary, as all permeates down to < 3 kDa resulted in a similar phenotype and cytokine secretion compared with non-ultrafiltrated *Sb*S, the active component in *Sb*S appears to have a molecular weight smaller than < 3 kDa.

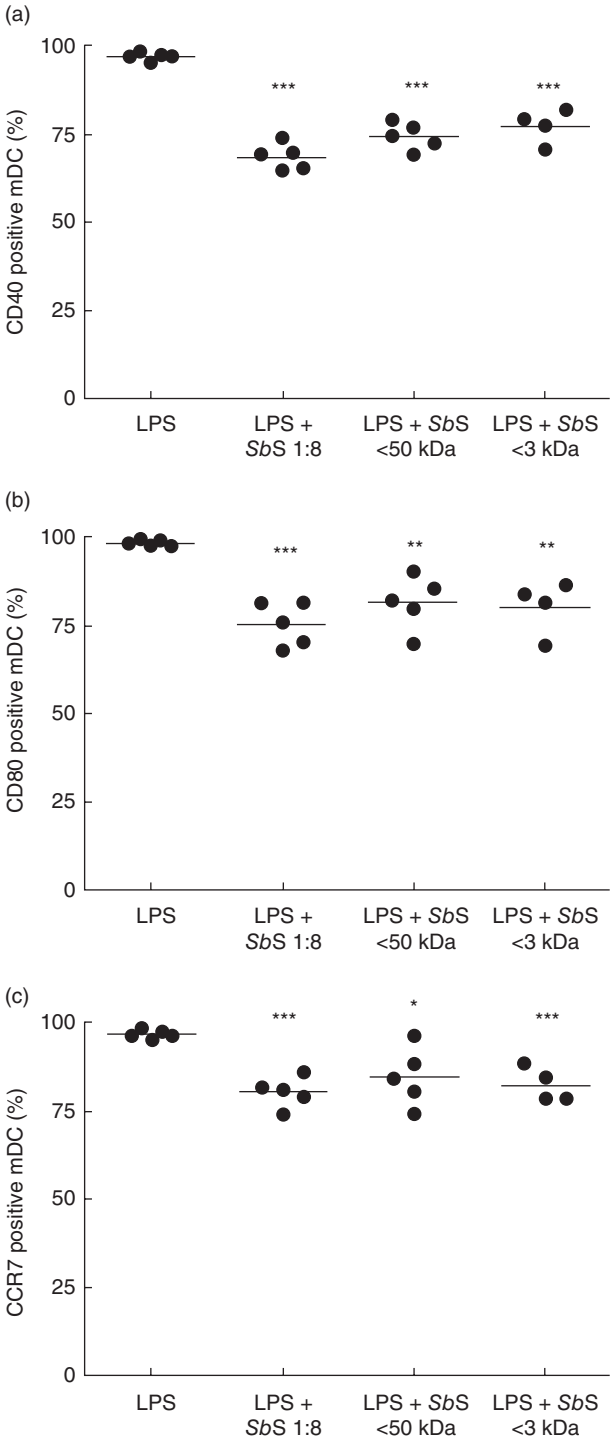

Fig. 4. Compared with lipopolysaccharide-stimulated myeloid dendritic cells (mDC) alone, the presence of 1:8 diluted *Saccharomyces boulardii* supernatant (*Sb*S) and *Sb*S permeates ranging from < 100 kDa to < 3 kDa (not all data shown) in these cultures decreased significantly the number of CD40, CD80 and CD197 (CCR7) expressing mDC. The results represent data from five independent experiments. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Discussion

The mechanisms of *Sb* therapeutic efficacy in diarrhoeal illnesses are still understood incompletely [35]. Most experimental research from *in vitro* studies with organoid or cell

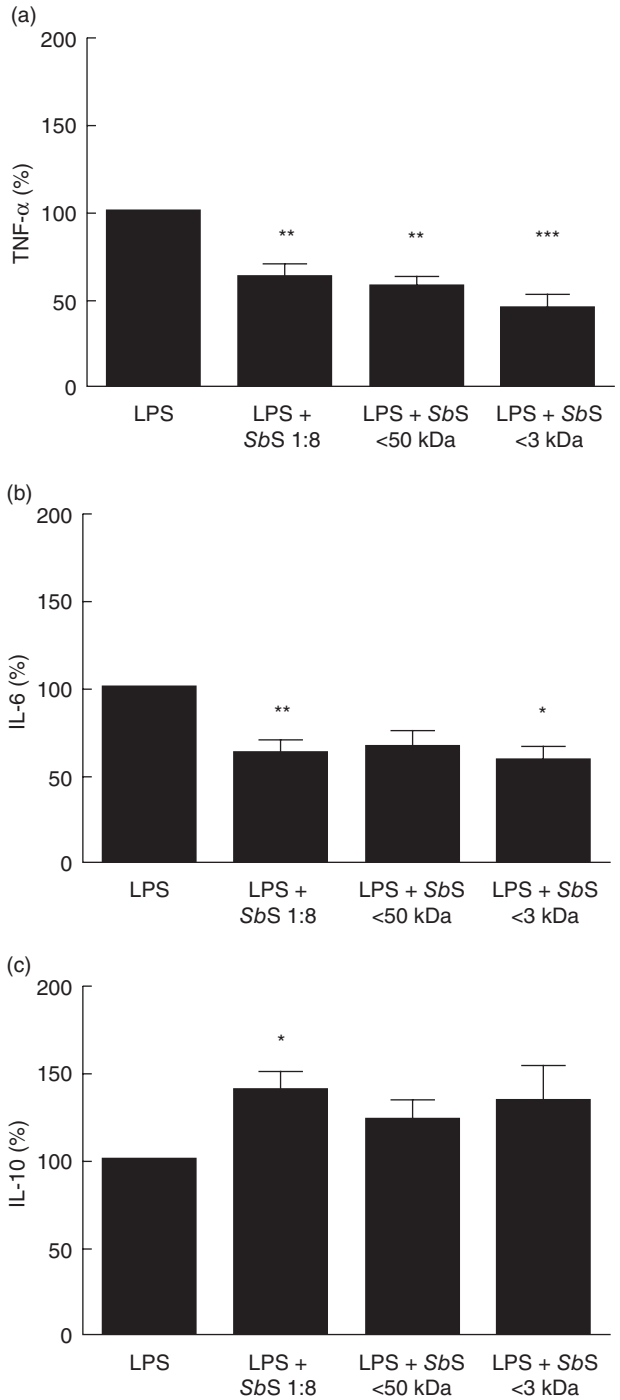

Fig. 5. Compared with lipopolysaccharide (LPS)-stimulated myeloid dendritic cells (mDC) alone, the presence of 1:8 diluted *Saccharomyces boulardii* supernatant (*Sb*S) and *Sb*S permeates ranging from < 100 kDa to < 3 kDa (not all data shown) in these cultures decreased significantly the secretion of tumour necrosis factor (TNF)-α (a), interleukin (IL)-6 (b), and increased in the secretion of anti-inflammatory IL-10 (C). Graphs show means ± standard error of the mean from five independent experiments. Controls (i.e. mDCs cultured with LPS alone) were normalized to 100%. Asterisks denote statistical significance: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

cultures and animal models has been focused upon the prevention of microbial pathogen adherence [36,37], translocation of the commensal microbial flora [38,39], investigation of neutralization of bacterial toxins (i.e. *Clostridium difficile* toxin A [23,40,41] or *Cholera* toxin [42]), toxin-related signalling [42–44], maintenance of normal intestinal permeability and barrier function [41,45], as well as control of epithelial electrolyte transport and luminal secretion [23,41,46,47].

However, a positive outcome in clinical pilot studies in Crohn's disease and ulcerative colitis [11–13] and beneficial effects of *Sb* in immunological (adoptive transfer model in SCID mice) and chemical [dextran sulphate sodium (DSS) colitis] [48,49] animal models of IBD imply additional, directly anti-inflammatory properties of *Sb* beyond those identified for and studied in infectious diarrhoea.

To the best of our knowledge, this is the first study to investigate the effects of *Sb*S on human DCs and their control of T cell activation. An important prerequisite for T cell activation is the expression of co-stimulatory molecules on antigen-presenting cells [50]. Their up-regulation and the additional expression of activation molecules on the DC surface mark not only a phenotypic, but dramatic functional change from being inducers of peripheral tolerance to potent activators of immune effector cells, such as T cells [14,51].

In the first set of our experiments we investigated this process by assessing the expression of CD40, CD80 and CD197 (CCR7) on LPS-stimulated mDC with or without the presence of different dilutions of *Sb*S. Therefore, we decided to use the microbial antigen surrogate motif and model TLR ligand LPS to stimulate mDC, as this mimics the situation these cells may encounter in the gut. Consistent with the literature, freshly isolated, i.e. immature and inactive, mDC expressed virtually no detectable numbers of the two activation markers (data not shown) [52]. The addition of LPS induced the expected maturation of DC as indicated by the increased expression of these molecules. However, when cultured in the presence of LPS and *Sb*S, substantially fewer mDC express CD40, CD80 and CD197 (CCR7).

CD197 (CCR7)-mediated signals control the migration of immune cells to secondary lymphoid organs and subsequently their positioning within defined functional compartments [33]. The *Sb*S-dependent reduced expression of CD197 in mDC observed by us may prevent their migration from the peripheral circulation to the sites of inflammation known to occur in chronic inflammatory conditions, such as IBD [21,53].

As well as the ability of *Sb*S to reduce the number of activated mDC (i.e. mDCs expressing the co-stimulatory surface markers CD40, CD80 and the migration marker CD197 (CCR7), *Sb*S moreover modulates mDC to secrete less TNF-α and IL-6 upon contact with LPS compared with LPS-only stimulated mDC. These data underscore the observed phenotypic differences discussed above. An increased secretion of these cytokines upon contact with

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microbial antigens encountered in the gut, such as LPS, attracts other leucocytes and may thereby contribute to the perpetuation of inflammation known to occur in IBD[19,20]. However, DC are also known for their dual role in the polarization of immune responses. Interestingly, *Sb*S was not only able to reduce the secretion of proinflammatory cytokines, but also able to increase the secretion of anti-inflammatory IL-10 to boost the natural regulatory potential of mDC [14].

While more research is required to describe the molecular structure of the active component in *Sb*S, our fractionation series demonstrates that it appears to have a molecular weight smaller than 3 kDa.

The effects of *Sb*S on mDC were not restricted to phenotypic changes but paralleled by functional consequences, as the second set of experiments shows. Here we were able to demonstrate that *Sb*S was able to suppress mDC-mediated T cell activation in an allogenic MLR. This effect was reproducible and dose-dependent, as we were able to establish in the third set of experiments.

We cannot rule out that *Sb*S induced a down-regulation of co-stimulatory molecules on LPS-stimulated mDC is mediated, in part, through direct effects of *Sb*S on LPS. One animal study identified, in *Sb*, a protein phosphatase that had a greater ability to dephosphorylate LPS of *E. coli*, which when injected in rats produced substantially less TNF-α and no organic lesions compared with the non-*Sb* exposed LPS [54]. However, this observation would not explain the further up-regulation of IL-10, which is also increased by LPS alone (data not shown). Alternative explanations and additional evidence for a modulation of the immune response by *Sb*, which may also help to explain that its clinical benefit in inflammatory and infectious conditions, comes from *in vitro* and animal studies. Orally administered *Sb* was shown to increase the production of secretory IgA (sIgA) and the secretory component of Ig in growing rats and monoassociated germ-free mice, thereby augmenting the host's first line of defence of the innate immune system in the gut [55,56]. Furthermore, it has been shown that *Sb* blocks nuclear factor (NF)-κB activation, IL-8 gene expression, IL-8 production, TNF-α gene expression and secretion by lymphoid and non-lymphoid cells [45,57–59].

In summary, our data suggest that *Sb* may exhibit its anti-inflammatory potential through modulation of DCs phenotype, function and migration by inhibition of their immune response to bacterial microbial surrogate antigens such as LPS.

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