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Yeast Modulation of Human Dendritic Cell Cytokine Secretion: An In Vitro Study

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Abstract

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. The concept of individual microorganisms influencing the makeup of T cell subsets via interactions with intestinal dendritic cells (DCs) appears to constitute the foundation for immunoregulatory effects of probiotics, and several studies have reported probiotic strains resulting in reduction of intestinal inflammation through modulation of DC function. Consequently, a focus on Saccharomyces boulardii as the fundamental probiotic yeast, very little is known about hundreds of non-Saccharomyces yeasts in terms of their interaction with the human gastrointestinal immune system. The aim of the present study was to evaluate 170 yeast strains representing 75 diverse species for modulation of inflammatory cytokine secretion by human DCs in vitro, as compared to cytokine responses induced by a S. boulardii reference strain with probiotic properties documented in clinical trials. Furthermore, we investigated whether cytokine inducing interactions between yeasts and human DCs are dependent upon yeast viability or rather a product of membrane interactions regardless of yeast metabolic function. We demonstrate high diversity in yeast induced cytokine profiles and employ multivariate data analysis to reveal distinct clustering of yeasts inducing similar cytokine profiles in DCs, highlighting clear species distinction within specific yeast genera. The observed differences in induced DC cytokine profiles add to the currently very limited knowledge of the cross-talk between yeasts and human immune cells and provide a foundation for selecting yeast strains for further characterization and development toward potentially novel yeast probiotics. Additionally, we present data to support a hypothesis that the interaction between yeasts and human DCs does not solely depend on yeast viability, a concept which may suggest a need for further classifications beyond the current definition of a probiotic.

Introduction

The mucosal-associated lymphoid tissues lining the human gastrointestinal tract contain a network of immune cells with the important task of distinguishing potentially dangerous antigens from harmless substances. Dendritic cells (DCs) govern the balance between immunity and tolerance by sampling of intestinal contents and initiating appropriate immune responses to luminal antigens through pattern recognition receptor signaling, cytokine secretion, and their ability to migrate and present antigen to naïve T cells in draining lymph nodes [1,2]. At homeostasis, DCs in the intestinal mucosa are conditioned by commensal microorganisms to promote proliferation of Foxp3+ regulatory T cells (Treg), strong producers of anti-inflammatory IL-10 contributing to intestinal tolerance [1,3,4]. During infection or active inflammation, pathogenic microorganisms bind to pattern recognition receptors expressed by DCs and activate signaling pathways involving MAP kinases and the nuclear transcription factor NFκB resulting in production and secretion of a wide range of chemokines and cytokines with distinct inflammatory effects. In this context, DC secretion of inflammatory cytokines such as TNFα and IL-1β is central for acute, innate inflammatory responses involving attraction of neutrophils and macrophages to the site of infection. In addition, DCs are central players in the regulation of adaptive immune responses. For example, DC secretion of IL-12 and IL-6 promotes the proliferation of Th1 and Th17 subpopulations, respectively, whereas DC modulation toward an IL-10 secreting phenotype contributes to induction of Treg responses promoting intestinal tolerance [5,6]. Furthermore, efficient antigen presentation relies upon DC maturation, a process involving upregulation of co-stimulatory surface molecules as well as modulation of chemokine receptor expression.

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [7]. Based on their role as key regulators of intestinal inflammation, DC involvement in probiotic functionality has been studied extensively [8–11]. The concept of individual commensal microorganisms influencing the makeup of intestinal T cell subsets via interactions with DCs appears to constitute the foundation for immunoregulatory effects of probiotics [4], and several studies have reported probiotic strains resulting in reduction of intestinal inflammation through modulation of DC function [8,9,12–14]. Consequently, modulation of DC cytokine secretion and maturation by various...
microorganisms has elucidated species and strain specific effects that have guided the selection of novel probiotic strains for further investigation [15–17].

Although the gut microbiota is dominated by bacteria [18], communities of eukaryotic microorganisms are part of the human microbiome [19–21]. In addition, eukaryotes such as food-related yeasts have been utilized for the production of fermented food and beverages and consumed by humans for centuries [22]. Thus, much like for prokaryotes, interactions between eukaryotic microorganisms and the intestinal immune system may influence human health in various ways. While the majority of probiotic microorganisms studied to date are lactic acid producing bacteria, research in yeasts with potentially beneficial influences on human health has mainly revolved around *Saccharomyces boulardii* [23,24], a yeast taxonomically acknowledged as belonging to the *S. cerevisiae* species [25,26] but in the following text referred to as *S. boulardii*. *S. boulardii* has been included in numerous randomized controlled trials and strong clinical evidence exists for the use of *S. boulardii* for the prevention of antibiotic associated diarrhea, Traveler’s diarrhea, and acute infectious diarrheas [27,28]. In addition, *S. boulardii* has shown a positive impact on disease outcome in clinical studies of inflammatory bowel diseases such as Crohn’s disease and ulcerative colitis [27], indicating an ability of *S. boulardii* to influence human immune responses underlying intestinal inflammation. The molecular basis for the beneficial effects of *S. boulardii* has been subject to extensive study, in vitro as well as in animal models, and *S. boulardii* has been found to impact inflammatory cytokine production by intestinal epithelial cells [29–34], peripheral blood mononuclear cells (PBMCs) [24], and DCs [23,35–37], reducing inflammatory scores in experimental colitis models in rodents [24,30,32,33,38–40].

Consequent to the intense research focus on *S. boulardii* as the fundamental probiotic yeast, very little is known about hundreds of non-*Saccharomyces* yeasts in terms of their interaction with the human gastrointestinal immune system. Other food-related yeast species typically associated with dairy products such as kefir and traditional cheeses include *Kluyveromyces lactis, K. marxianus, and Debaryomyces hansenii* [41]. While isolates of all three species have been evaluated for potential probiotic properties in *in vitro* experimental conditions assessing acid and bile survival, and adhesion to and modulation of cytokine secretion from intestinal epithelial cells [41–46], studies of the interactions between these yeasts and specialized immune cells have been far fewer [47,48].

The current definition of probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” places importance on the viability of probiotic microorganisms at the site of action, presumably the lower small intestines. This has led to numerous studies focusing on the ability of potentially probiotic microorganisms to survive the harsh conditions of the human gastrointestinal tract, i.e. the acidic environment in the gastric sac and the presence of bile salts in the proximal small intestines [41,42,47]. However, while probiotic effects caused by actively secreted molecules will depend on a probiotic microorganism being alive [23,30,34,35], other probiotic effects may depend solely on the interaction of microbial cell wall molecules and surface receptors expressed by host cells without the need for an active metabolic function of the probiotic. Indeed, several studies have found heat killed, UV irradiated, and live bacteria to display equal DC stimulatory patterns in *in vitro* [6,10,49–52]. Others have described the failure of nonviable *Saccharomyces* yeasts to prevent pathogen induced cytokine and chemokine expression in cultured epithelial cells [29,43], while a third study reported that *S. boulardii* maintained an inhibitory effect on *Salmonella* induced signaling pathways in epithelial cells even after being subjected to a membrane disrupting glass bead treatment, thus indicating the likely importance of yeast cell wall structures for the observed inhibition [32].

Multiplexed immunoassays based on the principles of flow cytometry allow for simultaneous determination of numerous soluble proteins in very small sample volumes. The combination of high throughput and impressive accuracy, sensitivity, and reproducibility make these experimental techniques highly relevant for screening purposes where rapid quantification of multiple compounds is critical [33,54].

The aim of the present study was to evaluate a broad spectrum of yeasts (170 strains representing 75 diverse yeast species were included in the study) for modulation of inflammatory cytokine secretion by human DCs, as compared to cytokine responses induced by a *S. boulardii* (Ultra-Levure) reference strain with probiotic properties documented in clinical trials [27]. To our knowledge, this is the first large-scale study of highly diverse yeasts in terms of their modulation of DC function, incorporating secretion levels of several cytokines. Furthermore, we investigated whether cytokine inducing interactions between yeasts and human DCs are dependent upon yeast viability or rather a product of membrane interactions regardless of yeast metabolic function.

**Materials and Methods**

**Yeast strains and growth conditions**

Yeast strains included in this study were obtained from CBS (www.cbs.knaw.nl). 170 strains were selected based on a desire to include a broad range of yeast biodiversity (see complete list of included strains in Table 1). Strains were cultured in YPD media (0,5 % yeast extract, 1 % peptone, 1,1 % D-glucose) at 30°C under aerobic conditions. Early stationary growth phase yeast cultures were harvested by centrifugation, washed twice with DC media (RPMI 1640 supplemented with 10 mM HEPES (Sigma-Aldrich, Schnelldorf, Germany) and 50 μM 2-mercaptoethanol (Sigma-Aldrich, Schnelldorf, Germany)), OD adjusted in DC media containing 10 % glycerol, and cryopreserved at −80°C until time of DC stimulation. Viability of frozen yeast cultures was verified by staining with propidium iodide. For some experiments, yeast strains were UV irradiated (70,000 μW/cm² for 5 min) or heat treated (80°C at 650 rpm for 5 min) prior to cryopreservation at −80°C. Yeast cell viability after UV irradiation or heat treatment was assessed by propidium iodide staining (UV <40% intact cells; heat <20% intact cells), and the reproductive ability of UV irradiated and heat treated yeasts was determined by colony counts after 48 h incubation of YPD agar plates at 30°C.

**Monocyto-derived DC generation**

Immature monocyto-derived DCs were generated *in vitro* by a 6 day procedure as described [50]. Human buffy coats from healthy donors were supplied by Department of Clinical Immunology at Copenhagen University Hospital, Copenhagen, Denmark. Use of human samples with no identifying information was approved by The National Committee on Health Research and the Danish Society for Clinical Immunology, and all donors gave informed written consent upon donation. Briefly, human peripheral blood mononuclear cells were obtained from buffy coats by density gradient centrifugation using Ficol-Paque PLUS (GE Healthcare, Freiburg, Germany). Monocytes were isolated by positive selection for CD14 using magnetic-activated cell sorting with CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured at a density of 2x10⁶ cells/mL in complete DC media (RPMI 1640 supplemented with 10 mM HEPES (Sigma-Aldrich, Schnelldorf, Germany), 50 μM 2-mercaptoethanol (Sigma-Al-
Table 1. Yeast strains included in study.

| Genus                | Species                  | Strains                                      |
|----------------------|--------------------------|----------------------------------------------|
| Ambrosiozyma         | monospora                | CBS2554                                      |
| Barnettozyma         | pratensis                | CBS9053, CBS9055                            |
| Bensingtonia         | yamatoana                | CBS9336                                      |
| Botryozyma           | mucatilis                | CBS9042, CBS9043                            |
| Bretanomyces         | custersianus             | CBS4806, CBS5207, CBS5208                    |
|                      | naardenensis            | CBS7540                                      |
| Candida              | amphixiae                | CBS9877                                      |
|                      | anneliseae               | CBS9837                                      |
|                      | atakaporum               | CBS9833                                      |
|                      | athensensis              | CBS9840, CBS9841                            |
|                      | blattae                  | CBS9871                                      |
|                      | bohienisi                | CBS9897                                      |
|                      | boilotribri              | CBS9832                                      |
|                      | bombi                    | CBS9017                                      |
|                      | buenavistaensis          | CBS9895                                      |
|                      | chocktavorum             | CBS9831                                      |
|                      | chysomelidarum           | CBS9904                                      |
|                      | elateridarum             | CBS9842                                      |
|                      | ghanansis                | CBS8798                                      |
|                      | gigantensis              | CBS9896                                      |
|                      | litsaeae                 | CBS8799                                      |
|                      | michaelli                | CBS9878                                      |
|                      | palmirolephilis          | CBS8109                                      |
|                      | powellii                 | CBS8795                                      |
|                      | taliae                   | CBS9838                                      |
| Citeromyces          | siamensis                | CBS9152, CBS9153                            |
| Cryptococcus         | laurentii var. laurentii | CBS8796                                      |
|                      | podzolicus               | CBS9357, CBS9358                            |
| Cryptotrichosporon   | anacardii                | CBS9549, CBS9551                             |
| Debaryomyces         | fabryi                   | CBS10579                                     |
|                      | hansenii                 | CBS1116, CBS767, CBS773, CBS1101, CBS1119, CBS1123, CBS1129, CBS1519, CBS1791, CBS1795, CBS1962, CBS2331, CBS2343, CBS4890, CBS5139, CBS5514, CBS5609, CBS56574, CBS57032, CBS7848, CBS8339, CBS9682, CBS9685, CBS9696 |
|                      | subglobosus              | CBS792, CBS1128                              |
| Dekkera              | anomala                  | CBS777, CBS4212, CBS4711, CBS7250, CBS8138   |
|                      | bruxellensis             | CBS727, CBS755, CBS596, CBS2547, CBS4459, CBS4482, CBS4601, CBS4602, CBS6555 |
| Geotrichum           | curucidarum              | CBS9893                                      |
| Hanseniaspora        | lachancei                | CBS8818, CBS8819                             |
|                      | opuntiae                 | CBS8820, CBS9791                             |
| Kazachstania         | exigua                   | CBS9330                                      |
| Kluyveromyces        | lactis var. drosophilum  | CBS9056                                      |
|                      | lactis var. lactis       | CBS9057, CBS9058, CBS9059, CBS9060          |
|                      | manianus                 | CBS1553                                      |
| Kurtzmaniella        | cleridarium              | CBS8793                                      |
| Lachancea            | fermentati               | CBS797                                       |
|                      | kluvyeri                 | CBS6545, CBS6546, CBS6547                    |
|                      | thermotolerans           | CHCC5756                                     |
| Lodderomyces         | elongisporus             | CBS7803                                      |
| Metschnikowia        | arizonensis              | CBS9064                                      |
|                      | borealis                 | CBS8431, CBS8432                             |
Table 1. Cont.

| Genus                     | Species                   | Strains                                      |
|---------------------------|---------------------------|----------------------------------------------|
| drich, Schnelldorf, Germany), 2 mM L-glutamine (Life Technologies Ltd, Paisley, UK), 10 % heat-inactivated fetal bovine serum (Invitrogen, Paisley, UK), 100 U/mL penicillin (Biological Industries, Kibbutz Be’er-Haemek, Israel), and 100 μg/mL streptomycin (Biological Industries, Kibbutz Be’er-Haemek, Israel) containing 30 ng/mL human recombinant IL-4 and 20 ng/mL human recombinant GM-CSF (both from Sigma-Aldrich, Saint Louis, USA) at 37°C, 5 % CO2. Fresh complete DC media containing full doses of IL-4 and GM-CSF was added after three days of culture. At day 6, differentiation to immature DCs was verified by surface marker expression analysis (CD11c >90% expression; CD1a >75% expression).

DC stimulation

Immature DCs were resuspended in fresh complete DC media containing no antibiotics, seeded in 96-well plates at 1 x 105 cells/well, and allowed to acclimate at 37°C, 5 % CO2, for at least one hour before stimulation. DC stimulation using thawed yeast strains was performed at a yeast:DC ratio of 10:1, and stimulated DCs were incubated for 20 h at 37°C, 5 % CO2, as time-course experiments had shown a 20 h stimulation time to result in quantifiable levels of all cytokines of interest. After 20 h stimulation, DC supernatants were sterile filtered through a 0.2 μm AcroPrep Advance 96-well filter plate (Pall Corporation, Ann Arbor, MI, USA) and stored at −80°C until time of cytokine quantification.

DC staining for quantification of co-stimulatory molecules and chemokine receptors

Immediately following 20 h stimulation time, DCs were collected, centrifuged at 200x g for 5 min, and resuspended in cold PBS containing 2 % BSA. Staining was performed using the following monoclonal antibodies: FITC-conjugated anti-human CD80 (clone L307.4), FITC-conjugated anti-human CD86 (clone 2331), APC-conjugated anti-human CCR6 (clone 11A9), FITC-conjugated anti-human CCR7 (clone 150503), and appropriate isotype controls (all from BD Biosciences, Erembodegem, Belgium). DCs were incubated with mAb for 30 min on ice protected from light, followed by repeated wash steps using 1 mL cold PBS 2 % BSA. Finally, DCs were resuspended in PBS 2 % BSA and kept on ice until flow cytometric analysis. Samples were acquired on an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA) using FACSDiva software (BD Biosciences, San Jose, CA, USA).
Cytokine quantification

Secreted levels of IL-12, TNF, IL-10, IL-6, and IL-1β were quantified by the Human Inflammatory Cytokines cytometric bead array (CBA) kit (BD Biosciences, Erembodegem, Belgium) according to the manufacturer’s instructions. Briefly, fluorescent beads coated with monoclonal capture antibodies were mixed with PE conjugated detection antibodies and reconstituted standards or test samples and allowed to form sandwich complexes during 3 h incubation protected from light. After repeated wash steps, samples were acquired on an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA) and data analysis was performed using the FCAP Array 3 software (BD Biosciences, San Jose, CA, USA). Detection limits for individual cytokines were as follows: 1.9 pg/mL IL-12, 3.7 pg/mL TNF, 3.5 pg/mL IL-10, 2.5 pg/mL IL-6, and 7.2 pg/mL IL-1β.

Multivariate data analysis and statistical analysis

Multivariate data analysis was performed using SIMCA-P+ 13 (Umetrics, Umeå, Sweden). A PCA-class model was generated based on data for induced levels of IL-12, IL-10, IL-6, TNFα, and IL-1β by 12 biological replicates of the S. boulardii reference strain, thereby centering the plot point of origin on the cytokine profile induced by the S. boulardii reference strain. Induced cytokine data (IL-12, IL-10, IL-6, TNFα, and IL-1β) for the 170 yeast strains included in the screen constituted the prediction data set; i.e. the distance of a given yeast strain from the plot point of origin indicates how closely the induced cytokine profile resembles that of the S. boulardii reference strain.

Statistical analysis (one-way ANOVA with Bonferroni’s multiple comparison post test) was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, USA).

Results

Yeasts induce highly diverse cytokine profiles in human DCs

Given that modulation of DC cytokine secretion has been linked to probiotic functionality related to intestinal inflammation, we evaluated yeast modulation of DC secretion of five inflammation related cytokines in vitro. We exposed human DCs to each yeast strain in duplicate at a 10:1 yeast:DC ratio and quantified secreted levels of IL-12, IL-10, IL-6, TNFα, and IL-1β after 20 h stimulation. Stimulation time was selected based on time-course experiments indicating 20 h as ideal for obtaining quantifiable levels of cytokines produced at a slow rate (IL-12, IL-10, and IL-1β) without reaching saturation conditions for rapidly secreted cytokines (IL-6 and TNFα) (Fig 1). As a point of reference, we included a S. boulardii strain with probiotic effects documented in clinical trials [27]. As expected, S. boulardii engaged human immune cells, as evidenced by induction of a robust response across all five cytokines (Fig 1). In addition, S. boulardii induced high levels of the co-stimulatory molecules CD80 and CD86, indicative of strong activation of the immature DCs (Fig S2), and affected DC chemokine receptor expression, as observed by down-regulation of CCR6 and strong up-regulation of CCR7 (Fig S2), indicating that S. boulardii activates immature DCs to a mature phenotype primed for lymph node migration and efficient antigen presentation.

For comparison of the yeast induced DC cytokine profiles, multivariate data analysis was applied as a valuable tool for visualizing and grouping yeast strains based on quantified levels of all five cytokines. A PCA-class model was generated based on data for induced levels of IL-12, IL-10, IL-6, TNFα, and IL-1β by 12 biological replicates of the S. boulardii reference strain, thereby centering the PCA plot point of origin on the cytokine profile induced by the reference strain (Fig 2). Induced cytokine data (IL-12, IL-10, IL-6, TNFα, and IL-1β) for the 170 yeast strains included in the study constituted the prediction data set; i.e. the distance of a yeast strain from the plot point of origin indicates how closely the induced cytokine profile resembles that of the S. boulardii reference strain. Visualizing the obtained cytokine profiles in this way revealed the interesting fact that induction of all five cytokines was positively correlated in our study, as shown by the loadings of individual cytokines in the Figure 2 insert.

As displayed in Figure 2, the yeasts included in this study induced highly diverse cytokine profiles in human DCs. Not surprisingly, the majority of Saccharomyces yeasts induced cytokine profiles very similar to the S. boulardii reference strain, as indicated by their location very close to the plot point of origin in Figure 2. In addition, this overview plot shows Saccharomyces yeasts as strong cytokine inducers, with very few non-Saccharomyces yeasts inducing cytokine levels higher than the S. boulardii reference strain (i.e. not many yeast strains present in the upper right quadrant of the plot). For non-Saccharomyces yeasts, we observe a broad range of cytokine inducing properties. For instance, a third of the included Debaryomyces strains, several Dekkera strains, and all included Zygosaccharomyces isolates dominate a distinct cluster of very low cytokine inducing yeasts present at the bottom left corner of the plot (Fig 2).

Distinct differences observed in cytokine inducing properties of individual yeast genera

Next, we focused on the induced DC cytokine profiles of individual yeast genera. Six Kluyveromyces strains representing the species K. marxianus, K. lactis var. lactis, and K. lactis var. drosophilaeum were included in our study, and multivariate data analysis of the induced DC cytokine profiles revealed clear species distinctions in immune stimulating capacities (Fig 1A). K. marxianus (CBS1553) induced DC cytokine levels statistically indistinguishable (P>0.05) from the profile induced by the S. boulardii reference strain for every one of the quantified cytokines (Fig 1B-F). In contrast, the four K. lactis var. lactis strains (CBS9057, CBS9058, CBS9059, CBS9060) induced much lower levels of cytokines; in particular, induced levels of IL-12, IL-10, and IL-1β were near or below the detection limit of the assay. Strikingly, no significant differences were observed between the DC cytokine profiles induced by the four K. lactis var. lactis strains (Fig 1B-F, P>0.05 for all quantified cytokines).

For Debaryomyces yeasts, 25 of the 28 strains included in our study represented the species D. hansenii, and the induced DC cytokine profiles revealed a remarkable diversity in immune stimulating properties (Fig 3A). The strain CBS1121 induced DC cytokine levels similar to the S. boulardii reference strain (Fig 3B-F), as indicated by secreted levels of IL-1β, IL-6, and TNFα being statistically indistinguishable from S. boulardii induced levels. The D. hansenii type strain (CBS767) displayed much poorer cytokine induction capabilities, as seen by significantly lower induction of the pro-inflammatory cytokines IL-12, IL-6, and TNFα. Finally, the D. hansenii strain CBS7348 induced a DC cytokine profile characterized by levels of IL-1β, IL-10, IL-6, and TNFα significantly higher than the S. boulardii reference strain, yet failed to induce detectable levels of IL-12.

The yeast genus Metschnikowia represents a large family of yeasts which, to the best of our knowledge, has not been explored for properties relating to human health. The PCA plot in Figure 4A displays the DC cytokine profiles induced by the 16 isolates representing seven Metschnikowia species included in our study. The plot reveals striking species distinctions separating species with
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A

B

C

D

E

F
highly diverse cytokine inducing properties. While isolates of *M. reukaufii* induced DC cytokine profiles very similar to the *S. boulardii* reference strain across all five cytokines, *M. gruessii* isolates induced robust levels of IL-1β, IL-10, IL-6, and TNFα, yet undetectable levels of IL-12 (Fig 4B-F). In contrast, *M. borealis* displayed poor cytokine inducing properties in general, as seen by an induced DC cytokine profile characterized by significantly lower cytokine levels compared to the *S. boulardii* reference strain.

Yeasts are capable of DC stimulation independently of viability

Next, we investigated whether the observed interactions between yeasts and DCs were dependent upon yeast viability. We hypothesized that yeasts would be able to induce DC activation regardless of metabolic activity and conducted experiments to compare DC stimulation with live, UV irradiated, and heat treated yeast. UV irradiation conditions were designed to generate relatively intact yeast cells unable to reproduce, whereas heat treatment was intended to cause severe yeast cell membrane disruption. Propidium iodide staining confirmed a reduction in the proportion of intact yeast cells to levels below 40% after UV

Figure 1. DC cytokine secretion induced by *Kluyveromyces* yeasts. A. Principal component analysis (PCA) scatter plot of DC cytokine profiles induced by *Kluyveromyces* yeast strains and the *S. boulardii* reference strain. Each dot represents induced cytokine data (IL-12, IL-10, IL-6, TNFα, and IL-1β) for one yeast strain, entered as the mean value of two biological replicates, and colored according to yeast species. The six red dots representing biological replicates of the *S. boulardii* reference strain show the deviation in the model. The insert represents loadings of individual cytokines included in the PCA model; for example, high IL-12 inducing yeasts are placed high along the plot Y axis, whereas strong IL-10 inducing yeasts are located to the right along the plot X axis. Levels of B, IL-1β, C, IL-12, D, IL-10, E, IL-6, and F, TNFα secreted by human monocyte-derived DCs following 20 h stimulation with DC media containing 10% glycerol (unstimulated) or *S. boulardii* (Ultra-Levure), *K. marxianus* (CBS1553), or *K. lactis* var. lactis (CBS9057, CBS9058, CBS9059, CBS9060) at a yeast:DC ratio of 10:1. Data are representative of two independent experiments, error bars represent SEM. One-way ANOVA, Bonferroni’s multiple comparison post test, indicating significant differences from cytokine levels induced by *S. boulardii*. ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.

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Figure 2. Yeast induced cytokine profiles in human DCs. Principal component analysis (PCA) scatter plot of induced cytokine data for 170 yeast strains included in screen. Each dot represents induced cytokine data (IL-12, IL-10, IL-6, TNFα, and IL-1β) for one yeast strain, entered as the mean value of two biological replicates, and colored according to yeast genera. The plot point of origin is centered on the cytokine profile induced by the *S. boulardii* reference strain, and thus the distance of a given yeast strain from the plot point of origin indicates how closely the induced cytokine profile resembles that of the *S. boulardii* reference strain. The insert represents loadings of individual cytokines included in the PCA model; for example, high IL-12 inducing yeasts are placed high along the plot Y axis, whereas strong IL-10 inducing yeasts are located to the right along the plot X axis.

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A

B

C

D

E

F

IL-1β

IL-12

IL-10

IL-6

TNFα

Concentration (pg/mL)

Concentration (pg/mL)

Concentration (pg/mL)

Concentration (pg/mL)

Concentration (pg/mL)

Concentration (pg/mL)

Unstimulated

S. boulardii (Ultra-Levure)

D. hansenii CBS1121

D. hansenii CBS767

D. hansenii CBS748

Unstimulated

S. boulardii (Ultra-Levure)

D. hansenii CBS1121

D. hansenii CBS767

D. hansenii CBS748

Unstimulated

S. boulardii (Ultra-Levure)

D. hansenii CBS1121

D. hansenii CBS767

D. hansenii CBS748

Unstimulated

S. boulardii (Ultra-Levure)

D. hansenii CBS1121

D. hansenii CBS767

D. hansenii CBS748

Unstimulated

S. boulardii (Ultra-Levure)

D. hansenii CBS1121

D. hansenii CBS767

D. hansenii CBS748

Unstimulated

S. boulardii (Ultra-Levure)

D. hansenii CBS1121

D. hansenii CBS767

D. hansenii CBS748
irradiation and below 20% after heat treatment, and the reproductive inability of UV irradiated and heat treated yeasts was verified by colony counts (data not shown). As presented in Figure 5, the obtained data show no significant differences in cytokine inducing properties between live, UV irradiated, and heat treated S. boulardii. Additionally, UV irradiated and heat treated S. boulardii were as effective as live yeast in inducing DC co-stimulatory functions and altering chemokine receptor expression levels (Fig S2). Regardless of UV irradiation or heat treatment, S. boulardii induced high levels of the co-stimulatory molecules CD80 and CD86, and modulated DC chemokine receptor expression, as observed by down-regulation of CCR6 and strong up-regulation of CCR7 (Fig S2).

For non-Saccharomyces yeasts, we observed a remarkably similar pattern. K. marxianus induced DC secretion of IL-12, IL-10, IL-6, TNFα, and IL-1β was unaffected by UV irradiation or heat treatment of the yeast (Fig 5). In contrast, the ability of K. lactis var. lactis (CBS9057) to induce IL-10 appeared slightly stronger after heat treatment, whereas induced levels of IL-12, IL-6, TNFα, and IL-1β remained unchanged. D. hansenii (CBS1121) induced cytokine secretion was unaffected by UV irradiation as well as heat treatment (Fig 5). For Metschnikowia yeasts, we observed differential modification of cytokine inducing properties between the three species examined. Whereas M. gratissi and M. borealis were unaffected by UV irradiation as well as heat treatment, the IL-10 inducing properties of M. rouxii were slightly potentiated by the heat treatment (Fig 5).

Discussion

The complex interplay between the gut microbiota and the intestinal immune system as this relates to human health and disease has received increasing attention recently [3,4,19]. Novel understandings of how the composition of resident populations of intestinal microorganisms can significantly impact human health in areas as diverse as obesity, allergy, and inflammatory disorders, is making this an area of intense research [19]. Maintenance of intestinal homeostasis requires a carefully regulated network of immune cells responsible for controlling the delicate balance between populations of potentially pro-inflammatory effector cells such as Th1 and Th17 cells and anti-inflammatory Treg cells [4], and intestinal DCs play a central role in orchestrating both innate and adaptive immune responses to commensal microorganisms as well as invasive pathogens [1,10].

As research in yeasts with a potentially beneficial impact on human health has focused almost exclusively on S. boulardii, the aim of the present study was to evaluate immune modulation by a large number of highly diverse yeasts in order to build a data set to serve as a foundation for selecting strains for further mechanistic studies. Choosing an experimental system involving human monocyte-derived DCs resulted in the necessary throughput and by employing a multiplexed immunoassay and multivariate data analysis to incorporate data for yeast induced DC secretion of five cytokines with distinct inflammatory effects we were able to assess the modulation of DC cytokine secretion by 170 yeast strains representing a broad spectrum of biodiversity. In contrast to studies focusing on modulation of a challenge induced DC cytokine response [23,36], our experimental setup evaluated the ability of yeasts to engage DCs per se.

The ability of S. boulardii to engage human immune cells and influence cytokine secretion in vitro is well established and has been linked to improved inflammatory scores in rodent colitis models. Likewise, commensal bacterial strains have been found to induce strong DC maturation and inflammatory cytokine secretion, yet display tolerogenic properties promoting T cell hyporesponsiveness [10], and strains with known probiotic properties have been shown to induce transient pro-inflammatory host responses in rodents upon initial colonization [55]. Accordingly, we observed robust induction of DC cytokines when human DCs were cultured in the presence of a S. boulardii reference strain with probiotic properties documented in clinical trials [27], indicating the expected ability of S. boulardii to engage human DCs and impact their secretion of cytokines with distinct inflammatory effects. In addition, we found the S. boulardii reference strain capable of inducing co-stimulatory functions and modulating chemokine receptor expression towards an activated DC phenotype primed for lymph node migration and efficient antigen presentation. These findings are supported by reports of S. boulardii modulating LPS induced CD80 and CCR7 expression [23,35], and echo reports of commensal bacterial strains inducing DC maturation, as indicated by increased surface expression of CD80, CD86, and CCR7 [8,10]. Our observation that yeasts belonging to the Saccharomyces genus are among the strongest cytokine inducing yeasts included in the present study, and that all included Saccharomyces yeasts induce DC cytokine profiles very similar to that induced by the S. boulardii reference strain, parallels the finding of a recent study where six live yeast strains representing the species S. bayanus, S. cerevisiae, and S. pastorianus induced non-discriminatory cytokine profiles in human PBMCs [24].

The remarkable diversity in cytokine inducing properties observed for non-Saccharomyces yeasts reflects the high diversity among the included yeast isolates. As the selection criteria for inclusion in the present study were based on a desire to include a broad representation of yeast biodiversity and thus had no apparent link to properties of immune modulation, it is not surprising that while some non-Saccharomyces yeasts exhibit strong cytokine inducing properties, others present as far more immunologically inert.

It is generally accepted that probiotic properties of bacteria are not only species but also strain dependent [56–58]. Interestingly, whereas our data agree with this notion for a number of included yeast species, other yeast genera display distinct species clustering in DC cytokine inducing properties.

The genus Kluyveromyces includes food-related yeasts typically isolated from fermented dairy products, and the health-promoting effects associated with the consumption of these products have led
Yeast Modulation of Human DC Cytokine Secretion

A

B

IL-1β

Concentration (pg/mL)

Unstimulated
S. boulardii (Ultra-Levure)
M. reukaufii CBS8018
M. reukaufii CBS8019
M. reukaufii CBS8020
M. reukaufii CBS8021
M. reukaufii CBS8022
M. reukaufii CBS8029
M. reukaufii CBS8030
M. gruvelii CBS8031
M. borellii CBS8032
***
ns
ns
ns
ns

C

D

IL-10

Concentration (pg/mL)

Unstimulated
S. boulardii (Ultra-Levure)
M. reukaufii CBS8018
M. reukaufii CBS8019
M. reukaufii CBS8020
M. reukaufii CBS8021
M. reukaufii CBS8022
M. reukaufii CBS8029
M. reukaufii CBS8030
M. gruvelii CBS8031
M. borellii CBS8032
***
ns
ns
ns
ns

E

F

IL-6

Concentration (pg/mL)

Unstimulated
S. boulardii (Ultra-Levure)
M. reukaufii CBS8018
M. reukaufii CBS8019
M. reukaufii CBS8020
M. reukaufii CBS8021
M. reukaufii CBS8022
M. reukaufii CBS8029
M. reukaufii CBS8030
M. gruvelii CBS8031
M. borellii CBS8032
ns
ns
ns
ns

TNFα

Concentration (pg/mL)

Unstimulated
S. boulardii (Ultra-Levure)
M. reukaufii CBS8018
M. reukaufii CBS8019
M. reukaufii CBS8020
M. reukaufii CBS8021
M. reukaufii CBS8022
M. reukaufii CBS8029
M. reukaufii CBS8030
M. gruvelii CBS8031
M. borellii CBS8032
***
to several studies investigating individual isolates from properties related to human health. A recent study evaluating the immune modulatory properties of a K. marxianus strain in vitro found a significant impact on the cytokine secretion of IL-6, TNFα, and IL-1β by human PBMCs [47]. While the K. marxianus isolate included in the present study is not identical, this does support our observation that K. marxianus induces a cytokine profile statistically indistinguishable from that of the S. bouardi reference strain, clearly indicating the ability of K. marxianus to engage specialized immune cells and impact the secretion of cytokines with distinct effects on adaptive immune responses. We observed distinct species differences clearly separating K. marxianus and K. lactis var. lactis in terms of their ability to induce DC cytokine secretion, with all four K. lactis var. lactis strains displaying an apparent inability to induce significant levels of cytokines. This is supported by a study finding K. lactis unable to induce detectable levels of the cytokines IL-6 and TNFα in a system evaluating interactions between yeast and cultured intestinal epithelial cells [44]. As the same study found K. lactis yeasts able to stimulate IL-8 secretion, our data may reflect differences between yeasts mainly interacting with highly abundant epithelial cells triggering innate immune mechanisms such as neutrophil and monocyte recruitment and yeasts capable of engaging specialized immune cells playing an active role in adaptive immune responses.

As discussed above, the species distinction in DC cytokine inducing properties is unmistakable for Kluyveromyces yeasts included in the present study. In contrast, the included isolates representing another genus comprising many food-related yeasts, namely Debaryomyces, display highly diverse and strain dependent DC inducing properties. Despite being highly abundant in nature and well known as food-related yeasts typically isolated from dairy, meat, and fermented soy products [59], all of which suggest likely human contact with these microorganisms, Debaryomyces yeasts have not been thoroughly studied for properties relating to human health.

The aim of the present study was to provide a first step in a search for novel yeasts with the ability to impact human health through interactions with the intestinal immune system, and naturally, safety constitutes an important factor when considering novel microorganisms with the potential for development of products aimed at human consumption. Consequently, focusing further studies solely on yeast species approved by the European Food Safety Authority for holding qualified presumption of safety (QPS) becomes an attractive proposition. This would suggest an emphasis on K. marxianus, K. lactis, and D. hansenii, all of which hold QPS status [60] and, in the present study, displayed DC cytokine induction properties worthy of further investigation. However, limiting future efforts to already well-known food-related yeasts would present the inherent risk of missing less-studied yeasts with potentially superior properties. The yeast genus Metschnikowia, comprising a large family of yeasts primarily isolated from flowers and bees across the European continent, provides an example. To the best of our knowledge, Metschnikowia yeasts have not been subject to studies relating to human health, yet the numerous isolates included in our study exhibited a remarkable species distinction in cytokine inducing properties warranting further examination.

While the experimental conditions in this study were intended to simulate the in vivo situation where microorganisms encounter mucosal DCs during passage through the human gut; naturally, some limitations need to be kept in mind when interpreting the data. Notably, single strain stimulation of DCs may be a necessity for data analysis when evaluating numerous strains but it fails to account for interactions between different members of the intestinal flora. Given the complexity of the human microbiota, the likely interactions between yeasts and other microorganisms present in the intestinal tract may result in different DC cytokine profiles. This highlights the importance of extending the current knowledge of select strains through additional studies incorporating a more complex mix of microorganisms resembling that of the human gastrointestinal tract.

From an industrial point of view, it is of importance to know whether dead yeast cells can be used in the same manner as viable cells. Additionally, yeast cells present in the human gastrointestinal tract may be either viable or dead, which makes it relevant to investigate whether the immunological responses are the same. Further, as yeast cell wall composition and structure are known to vary depending on yeast growth phase and any treatment, the prospect that cell wall structures of nonviable yeast cells may differ from those of viable yeast cells and in turn potentially trigger a different immunological response, led us to explore whether DC cytokine induction was affected by yeast viability. We observed no significant differences between the DC cytokine inducing properties of live, UV irradiated, and heat treated S. bouardi, and in addition, DC co-stimulatory functions were upregulated to an equal extent by live and reproducibly unable yeast.

The remarkable consistency in DC cytokine inducing properties observed across very diverse yeast species and genera regardless of viability appears to support our hypothesis that interactions between yeasts and immune cells are likely to rely upon contact between (possibly conserved) yeast cell wall structures and DC surface receptors. Further, our findings suggest that this phenomenon holds true for a number of diverse yeast species beyond the widely studied Saccharomyces genus. In this context, it is interesting to note that a recent study have found yeast cell wall fractions, and particularly β-glucan fractions, prepared from either S. cerevisiae or C. albicans, able to modulate intestinal inflammation in vivo [40], potentially through interactions with the C-type lectin receptor Dectin-1 expressed by DCs [61,62]. Thus, while our findings for nonviable yeasts beyond S. cerevisiae and C. albicans appear to extend their observation that yeast cell wall molecules are capable of interacting with immune cells, their findings support our hypothesis that yeast modulation of intestinal inflammation can take place without the need for live yeast cells.

In conclusion, the present study provides the first large-scale study of immune modulation by highly diverse yeasts described in

Figure 4. DC cytokine secretion induced by Metschnikowia yeasts. A. Principal component analysis (PCA) scatter plot of DC cytokine profiles induced by Metschnikowia yeast strains and the S. bouardi reference strain. Each dot represents induced cytokine data (IL-12, IL-10, IL-6, TNFα, and IL-1β) for one yeast strain, entered as the mean value of two biological replicates, and colored according to yeast species. The six red dots representing biological replicates of the S. bouardi reference strain show the deviation in the model. The insert represents loadings of individual cytokines included in the PCA model; for example, high IL-12 inducing yeasts are placed high along the plot Y axis, whereas strong IL-10 inducing yeasts are located to the right along the plot X axis. Levels of B, IL-1β, C, IL-12, D, IL-10, E, IL-6, and F, TNFα secreted by human monocyte-derived DCs following 20 h stimulation with DC media containing 10 % glycerol (unstimulated) or S. bouardi (Ultra-Levure), M. reukaufii (CBS9018, CBS9019, CBS9020, CBS9021, CBS9022), M. gruessii (CBS9029, CBS9030), or M. borealis (CBS8431, CBS8432) at a yeast:DC ratio of 10:1. Data are representative of two independent experiments, error bars represent SEM. One-way ANOVA, Bonferroni’s multiple comparison post test, indicating significant differences from cytokine levels induced by S. bouardi. ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001. doi:10.1371/journal.pone.0096595.g004
the scientific literature. Our data clearly demonstrate high
diversity in yeast induced cytokine secretion across a broad
spectrum of yeasts, and by employing multivariable data analysis we
reveal distinct clustering of yeasts inducing similar cytokine profiles
in DCs, highlighting clear species distinction within specific yeast
genera. The observed differences in induced DC cytokine profiles
may indicate distinct modes of interaction between yeasts and
human immune cells, and will aid in the selection of strains for
further characterization and development toward potentially novel
yeast probiotics. Additionally, we present data to support a
hypothesis that the interaction between yeasts and human DCs
does not solely depend on yeast viability, a concept which may
suggest a need for further classifications beyond the current
definition of a probiotic.

Supporting Information

Figure S1 Time-course of S. boulardii induced DC
cytokine secretion supports a 20 h stimulation time.
Levels of IL-12, IL-10, IL-1β, IL-6, and TNFα secreted by human
monocyte-derived DCs following incubation with S. boulardii
(Ultra-Levure) at a yeast:DC ratio of 10:1. Data are expressed as
mean±SEM (n=2). (TIF)

References

1. Cosmesos JL, Povers F (2008) Dendritic cells in intestinal immune regulation.
Rev Imunol 8: 433–446.
2. Rescigno M, Urbano M, Valzasina B, Franchin M, Rotta G, et al. (2001)
Dendritic cells express tight junction proteins and penetrate gut epithelial
monolayers to sample bacteria. Nat Imunol 2: 361–367.
3. Maynard CL, Elson CO, Hatton RD, Weaver CT (2012) Reciprocal
interactions of the intestinal microbiota and immune system. Nature 489:
231–241.
4. Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the
microbiota and the immune system. Science 336: 1268–1273.
5. Manicasamy S, Ravindran R, Deng J, Olouch H, Denning TL, et al. (2009)
Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in
dendritic cells promotes T regulatory responses and inhibits autoimmunity.
Nat Med 15: 401–409.
6. Donkor ON, Ravikumar M, Proudfoot O, Day SL, Apostolopoulos V, et al.
(2012) Cytokine profile and induction of T helper type 17 and regulatory T cells
by human peripheral mononuclear cells after microbial exposure. Clin Exp
Immunol 167: 282–295.
7. Joint FAO/WHO Working Group (2002) Guidelines for the evaluation of
probiotics in food.
8. Foligne B, Zoumpoulou G, Devault J, Ben Younes A, Chareyre F, et al. (2007)
A key role of dendritic cells in probiotic functionality. PLoS One 2: e313.
9. Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, et al. (2010) Generation of
regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration
suppresses immune disorders. Proc Natl Acad Sci U S A 107: 2159–2164.
10. Baba N, Samson S, Bourdet-Sicard R, Rubio M, Sarfati M (2008) Commensal
bifidobacterium induces IL-10-producing Tr1 cells in the colon. PLoS Pathog 4:
648–476.
11. Konieczna P, Groeger D, Ziegler M, Frei R, Fersl R, et al. (2012)
Bifidobacterium infantis 35624 administration induces Foxp3+ regulatory T cells
in human peripheral blood. Potential role for myeloid and plasmacytid
dendritic cells. Gut 61: 354–366.
12. Jeon SG, Kayama H, Ueda Y, Takahashi T, Arahata T, et al. (2012) Probiotic
bifidobacterium breve induces IL-10-producing Tr1 cells in the colon. PLoS
Pathog 8: e1002714.
13. Di Giacinto C, Marinaro M, Sanchez M, Strober W, Boivin A (2005)
Probiotics ameliorate recurrent Th1-mediated mucosal infl ammation by inducing
IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol 174:
3237–3246.

Figure 5. Yeast induced DC cytokine secretion occurs independently of yeast metabolic function. Levels of A. IL-12, B. IL-10, C. IL-6, D. TNFα, and E. IL-1β secreted by human monocyte-derived DCs following 20 h stimulation with DC media containing 10 % glycerol (unstimulated) or S. boulardii (Ultra-Levure), K. marxianus (CBS1553), K. lactis var. lactis (CBS59057), D. hansenii (CBS59021), M. gruessii (CBS9029), or M. borealis (CBS8431). For each yeast strain, DC stimulation was performed with live yeast, UV irradiated yeast, and heat treated yeast at a yeast:DC ratio of 10:1. Data are expressed as mean±SEM (n=4). One-way ANOVA, Bonferroni’s multiple comparison post test, indicating significant differences between cytokine levels induced by live, UV irradiated, and heat treated yeast for each strain. ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.
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Figure S2 Modulation of DC co-stimulatory functions and chemokine receptor expression occurs independently of yeast metabolic function. DC surface expression of CD80, CD86, CCR6, and CCR7 following 20 h stimulation with DC media containing 10% glycerol (unstimulated) or either live, UV irradiated, or heat killed S. boulardii (Ultra-Levure) at a yeast:DC ratio of 10:1. Data are expressed as mean±SEM (n=4). One-way ANOVA, Bonferroni’s multiple comparison post test, indicating significant differences between cytokine levels induced by live, UV treated, and heat killed yeast. ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.

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Author Contributions

Conceived and designed the experiments: IMS, JEC NA IJ. Performed the experiments: IMS, JEC. Analyzed the data: IMS, JEC. Contributed reagents/materials/analysis tools: IMS, JEC. Wrote the paper: IMS.
20. Dinleyci EC, Ezen M, Ozen M, Yargic ZA, Vandenplas Y (2012) Effectiveness and safety of saccharomyces boulardii for acute infectious diarrhea. Expert Opin Biol Ther 12: 395–410.

21. Zanello G, Berri M, Dupont J, Sizaret PY, D’Inca R, et al. (2011) Saccharomyces cerevisiae modulates immune gene expressions and inhibits ETEC-mediated ERK1/2 and p38 signaling pathways in intestinal epithelial cells. PLoS One 6: e18573.

22. Chen X, Kokkotou EG, Mustafa N, Bhaskar KR, Sougioultzis S, et al. (2006) Saccharomyces boulardii inhibits ERK1/2 mitogen-activated protein kinase activation both in vitro and in vivo and protects against colistininduced peritonitis. J Biol Chem 281: 24449–24454.

23. Daham S, Dalmaso G, Inbert V, Peyron JF, Rampal P, et al. (2003) Saccharomyces boulardii interferes with enterohemorrhagic escherichia coli-induced signaling pathways in T84 cells. Infect Immun 71: 766–773.

24. Martins FS, Dalmaso G, Arantes RM, Doye A, Lemichez E, et al. (2010) Interaction of saccharomyces boulardii with salmonella enterica serovar typhimurium protects mice and modifies T84 cell response to the infection. PLoS One 5: e9895.

25. Lee SK, Kim YW, Chi SG, Joo YS, Kim HJ (2009) The effect of saccharomyces boulardii on human colon cells and inflammation in rats with trimethobenzene sulfoxide acid-induced colitis. Dig Dis Sci 54: 255–263.

26. Sougioultzis S, Simopoulos S, Bhaskar KR, Chen X, Anton PM, et al. (2006) Saccharomyces boulardii produces a soluble anti-inflammatory factor that inhibits NF-kappaB-mediated IL-6 gene expression. Biochem Biophys Res Commun 345: 69–76.

27. Thomas S, Metzke D, Schmitz J, Dorffel Y, Baumgart DC (2011) Anti-inflammatory effects of saccharomyces boulardii mediated by myeloid dendritic cells from patients with crohn’s disease and ulcerative colitis. Am J Physiol Gastrointest Liver Physiol.

28. Gad M, Ravn P, Soborg DA, Lund-Jensen K, Ousehald AC, et al. (2011) Regulation of the IL-10/IL-12 axis in human dendritic cells with probiotic bacteria. FEMS Immunol Med Microbiol 63: 93–107.

29. Pothoulakis C (2009) Review article: Anti-inflammatory mechanisms of action of saccharomyces boulardii. Aliment Pharmacol Ther 30: 826–833.

30. Martins FS, Nardi RM, Arantes RM, Rosa CA, Neves MJ, et al. (2005) Screening of yeasts as probiotic based on capacities to colonize the gastrointestinal tract and to protect against enteropathogen challenge in mice. J Gen Appl Microbiol 51: 85–92.

31. Dalmaso G, Cottrez F, Inbert V, Lagarde P, Peyron JF, et al. (2006) Saccharomyces boulardii inhibits inflammatory bowel disease by trapping T cells in mesenteric lymph nodes. Gastroenterology 131: 1812–1825.

32. Jawhara S, Habib K, Maggiotto F, Pignede G, Vandekerckove P, et al. (2012) Down-regulation of intestinal epithelial innate response by probiotic Kluyveromyces marxianus B0399 modulates the immune response in caco-2 cells and peripheral blood mononuclear cells and impacts the human gut microbiota in an in vitro colonic model system. Appl Environ Microbiol 78: 956–964.

33. Kouréni A, Kotzanamidou C, Litopoulos-Tzanetaki E, Papaconstantinou J, Tzanetakis N, et al. (2010) Immunomodulatory activity of potential probiotic yeast strains in the doral air pouch system and the gut mucosa. J Appl Microbiol 109: 260–271.

34. Weiss G, Rasmussen S, Nielsens F, Hjerpe A, Nielsens B, et al. (2010) Immunomodulatory activity of gut-derived lactobacillus acidophilus in murine dendritic cells. PLoS One 5: e11065.

35. Zeuthen LH, Fink LN, Frohleker H (2008) Toll-like receptor 2 and nucleotide-binding oligomerization domain-2 play divergent roles in the recognition of gut-derived lactobacillus and bifidobacteria in dendritic cells. Immunology 124: 499–502.

36. Baba N, Samson S, Bourdet-Sicard R, Rubio M, Sarfati M (2009) Selected commensal-related bacteria and toll-like receptor 2 agonist combinatorial codes synergistically induce interleukin-12 production by dendritic cells to trigger a T helper type 1 polarizing programme. Immunology 128: e223–31.

37. Zeuthen LH, Fink LN, Frohleker H (2008) Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta. Immunology 123: 197–208.

38. Carson RT, Vignali DA (1999) Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. J Immunol Methods 227: 41–52.

39. Vignali DA (2000) Multiplexed particle-based flow cytometric assays. J Immunol Methods 243: 243–255.

40. Ruiz PA, Hoffmann M, Szczesny S, Blaut M, Haller D (2005) Innate mechanisms for bifidobacterium lactis to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats. Immunology 115: 441–450.

41. Wall R, Marques TM, O’Sullivan O, Ross RP, Shanahan F, et al. (2012) Contrastive effects of bifidobacterium breve NCIMB 702256 and bifidobacterium breve DPC 6330 on the composition of murine brain fatty acids and gut microbiota. Am J Clin Nutr 95: 526–537.

42. Smelt MJ, de Haan BJ, Bron PA, van Swam I, Meijerink M, et al. (2012) L. plantarum, L. salivarius, and L. lactis attenuate Th2 responses and increase Treg frequencies in healthy mice in a strain dependent manner. PLoS One 7: e47244.

43. Wall R, Marques TM, O’Sullivan O, Ross RP, Shanahan F, et al. (2012) Contrastive effects of bifidobacterium breve NCIMB 702256 and bifidobacterium breve DPC 6330 on the composition of murine brain fatty acids and gut microbiota. Am J Clin Nutr 95: 526–537.

44. Yeast Modulation of Human DC Cytokine Secretion

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