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Fungal Chitin Induces Trained Immunity in Human Monocytes during Cross-talk of the Host with *Saccharomyces cerevisiae*

Lisa Rizzetto[1¶], Daniela C. Ifrim[2¶], Silvia Moretti[3], Noemi Tocci[1], Shih-Chin Cheng[2], Jessica Quintin[2#], Giorgia Renga[3], Vasilis Oikonomou[3], Carlotta De Filippo[1,4], Tobias Weil[1], Bastiaan A. Blok[2], Marcello S. Lenucci[5], Manuel A. S. Santos[6], Luigina Romani[3], Mihai G. Netea[2], and Duccio Cavalieri[1,4,7]

[1]Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige (TN), Italy; [2]Radboud University Medical Center, Department of Internal Medicine, Division of Experimental Internal Medicine, Nijmegen, The Netherlands; [3]Department of Experimental Medicine, University of Perugia, Polo Unico Sant'Andrea delle Fratte, Perugia, Italy; [4]Institute of Biometeorology (IBIMET), National Research Council (CNR), Florence, Italy; [5]Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali (Di.S.Te.B.A.), Università del Salento, Lecce, Italy; [6]Department of Biology and CESAM, University of Aveiro, Aveiro, Portugal; [7]Department of Biology, University of Florence, Sesto Fiorentino (FI), Italy.
[#]Current Address: Immunology of Fungal Infections group, Mycology Department, Institut Pasteur, 75724 Paris Cedex 15, France

Running Title: Chitin trains the immune system

To whom correspondence should be addressed: Duccio Cavalieri, Department of Biology, University of Florence, Via Madonna del Piano, 6 - 50019 Sesto Fiorentino (FI), Italy. Telephone: +39-055-2758322; Fax: +39-055-2756318; E-mail: duccio.cavalieri@unifi.it.

[¶]These authors equally contributed to the work

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ABSTRACT

The immune system is essential to maintain the mutualistic homeostatic interaction between the host and its micro- and mycobiota. Living as a commensal, *Saccharomyces cerevisiae* could potentially shape the immune response in a significant way. We observed that *S. cerevisiae* cells induce trained immunity in monocytes in a strain-dependent manner through enhanced TNFα and IL-6 production upon secondary stimulation with TLR ligands, as well as bacterial and fungal commensals. Differential chitin content accounts for the differences in training properties observed among strains, driving induction of trained immunity by increasing cytokine production and direct antimicrobial activity both *in vitro* and *in vivo*. These chitin-induced protective properties are intimately associated with its internalization, identifying a critical role of phagosome acidification to facilitate microbial digestion. This study reveals how commensal and passenger microorganisms could be important in promoting health and preventing mucosal diseases by modulating host defense towards pathogens and thus influencing the host microbiota-immune system interactions.

In several higher organisms, including plants, a central mechanism for efficient protection from infection is defense priming, the preconditioning of immunity induced by microbial colonization after germination or birth (1, 2), supporting the relevance of innate immune memory (3). This property of host defense is also referred as 'trained immunity', defined as an increased responsiveness of the innate immune system to secondary stimuli after an initial encounter with training microorganisms (4, 5).

Similarly to root symbioses, human microbiota functionally contributes to the nutrition and protection of the host, significantly shaping

mammalian immunity both at the mucosal surface of the host and systemically (6–9). Recent studies have shown that fungal microbiota, or mycobiota, is an important player in the host-microbes interaction, and that its effects are integrated with those of the dominant bacterial component (10–12). The complex interaction between bacterial microbiota, mycobiota and the immune system is critical for establishing a balance between immunity and tissue health. A recent publication (9) added another fundamental contribution to the role of skin microbiota in activating and educating host immunity, shedding new light on the interplay between immune systems and microbiota in shaping each other. It has been earlier demonstrated that *C. albicans* triggers differential immune signaling upon interaction with either inflammatory or tolerogenic dendritic cells (6) and, similarly to what occurred for *Aspergillus fumigatus* (8) and harmless fungi such as *Saccharomyces cerevisiae* (Di Paola et al, submitted, (13)), the type and intensity of immune reactivity is strain dependent (7). Moreover, fungi modulate responses towards other microorganisms such as intestinal and skin bacteria (14, 15).

Candida spp. are not the only fungal colonizer of humans. *Saccharomyces cerevisiae* has been earlier demonstrated to be a constant colonizer of skin (16) and human intestinal tract (17), and a recent study has shown that it may even surpass *Candida* species in some human populations (18). Even if it is a harmless fungus, *S. cerevisiae* shares with *Candida* many of the same cell wall structures, often leading to similar effects on host defense. In this work, we assessed the capacity of various *S. cerevisiae* strains to train human myeloid cells to react stronger towards other microorganisms such as bacteria or *Candida*. *S. cerevisiae* can efficiently induce trained immunity in human monocytes in a strain- and strain origin-dependent manner, leading to enhanced cytokine production *in vitro*. This effect was driven by chitin, explaining the major training properties observed upon challenge with strains presenting a chitin-rich cell wall, i.e. human isolates. The differences in the stimulatory capacities of *Saccharomyces* depending on the strain and the context from which they were isolated underlines the need to understand the interaction between mycobiota and the host, in order to identify the boundaries between friend and foe, and between health and disease.

EXPERIMENTAL PROCEDURES

Ethics statement - The *in vitro* study using human cells was designed in conformity with the international recommendation (Dir. EU 2001/20/EC) and its Italian counterpart (DM 15 Luglio 1997; D.Lvo 211/2003; D.L.vo 200/2007) for clinical trial and following the Declaration of Helsinki, to assure protection and care of subjects involved. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats provided by the Sanquin Bloodbank in Nijmegen or by Careggi Hospital in Florence. Before taking blood, informed written consent of each human subject was obtained. Dectin-1-deficient cells were isolated from patients homozygous for the stop mutation Y238X [40]. The study was approved by the review board of the Department of Medicine of the Radboud University Nijmegen Medical Centre and by Ethical Committee Nijmegen-Arnhem (approval nr. NL32357.091.10), by the Azienda Ospedaliera Careggi (Florence, approval nr. 87/10) and by Centro Trasfusionale Ospedale Santa Chiara (Trento, approval document nr. 54896583). All data analyzed were anonymized.

In mouse models, experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143-01 and Legislative decree 157/2008-B regarding the animal license obtained by the Italian Ministry of Health lasting for three years (2008–2011). Infections were performed under avertin anesthesia and all efforts were made to minimize suffering. The experimental protocol was designed in conformity with the recommendations of the European Economic Community (86/609/CEE) for the care and the use of laboratory animals, was in agreement with the Good Laboratory Practices and was approved by the animal care Committee of the University of Perugia (Perugia, Italy).

Reagents - Ficoll-Paque (GE Healthcare, Milan, Italy) was used to isolate PBMCs by differential centrifugation. RPMI 1640 Dutch Modification (RPMI, Sigma-Aldrich, Milan,

Italy), supplemented with 1% gentamicin, 1% L-glutamine, and 1% pyruvate (Life Technologies,Nieuwekerk, The Netherlands) was used as culture medium. Monocytes were isolates using magnetic CD14 positive selection (Miltenyi Biotec, Bologna, Italy). In a challenge experiment, IL-4 and GM-CSF (both from Gentaur, Kampenhout, Belgium) were added to induce monocyte differentiation into dendritic cells. Oregon green 488 and NucBlue Live Cell Stain Ready Probes reagent were from Molecular Probes, Life Technologies, Monza, Italy. Calcofluor white, PHK26 Red Fluorescent Cell Linker Kit, Cytochalasin D, the histone demethylase inhibitor Pargyline, the histone methyltransferase inhibitor (MTA, 5′-deoxy-5′(methylthio)adenosine) and standard for sugar's determination were purchased from Sigma-Aldrich, Milan, Italy. *C. albicans* and *S. cerevisiae* β-glucan and mannan were isolated and purified as described previously (19, 20). Pam_3Cys_4 and LPS (*Escherichia coli* serotype 055:B5) were purchased from Sigma-Aldrich, with an additional purification step for LPS (21). Only preparations with > 98% purity were used in the experiments.

Microorganisms - *S. cerevisiae* strains, previously isolated from different environmental sources, were cultured in complete medium (YPD, 2% yeast extract, 1% peptone, 2% glucose) for 18 hr, then collected. *C. albicans* ATCC MYA-3573 (UC820), was grown overnight to yeast cells in Sabouraud broth at 37°C. *Staphyloccus aureus* and *Escherichia coli* were grown overnight in Tryptic Soy Broth (TSB, Difco) at 37°C. Cells were harvested by centrifugation, washed twice with PBS, and resuspended in culture medium (RPMI; ICN Biomedicals, Aurora, OH). For trained immunity assays, *S. cerevisiae* yeasts and *Candida* were heat-killed for 30 min at 95°C and resuspended in culture medium to a cell inoculum size of 10^6 cells/ml.

Cell wall extraction - The sugar composition of cell walls was analyzed as described by Dallies et al. (22) with the following modifications. Briefly, about 20 mg cell dry mass of stationary phase cells were harvested and washed with deionized water. Cells were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.5) using glass beads (0.45-0.55 mm) and subsequently disrupted by three rounds of vortexing at maximum speed (30 s) and chilling on ice (1 min). Cell pellets were subjected to extraction with 100 µl of 72 % (w/w) H_2SO_4 for 3 hr at room temperature. The resulting slurry was diluted with MilliQ water, but without adding galactose, to a final volume of 1 ml and heated for four hr at 100 °C. The hydrolysate was then diluted to 9 ml with MilliQ water, neutralized with saturated $Ba(OH)_2$ and left overnight at 4 °C to allow the precipitation of sulfate ions. After centrifugation at 3800 g for 5 min, the supernatant was subjected to monosaccharide analysis with HPAEC-PAD.

Cell wall characterization and chitin purification - Cell wall characterization has been performed as described by Ferreira et al. (2006) (23) with modifications. Cells were cultured in YPD liquid medium at 28°C and collected at late exponential phase. The cells were washed once with distilled water and three times with 0.1 M Tris-HCl buffer (pH 8.5) containing 1 mM phenyl sulphonyl fluoride and disrupted in the same buffer with 0.5 mm-diameter glass beads by serial cycles of vortexing (30s at maximum speed) and ice cooling (20s). Cell walls were harvested by centrifugation for 30 min at 4200 g and dried in a speedvac concentrator. To extract chitin, cell wall dried biomass was subjected to alkaline extraction, followed by acid extraction according to the protocol described by Ferreira et al. (2006) (23). Chitin was finally obtained by dialysis of the extract and lyophilized to determine the dry weight. Only preparation with > 98% purity were used in the experiments.

Quantification of sugars by HPAEC - All samples were filtered through a 0.2mm Spartan 13 filter (Schleicher & Schuell Microscience, Dassel, Germany) prior to analysis on a Dionex HPAEC equipped with a CarboPac PA10 (4 x 50 mm) guard column and a CarboPac PA10 (4 x 250 mm) analytical column. Separation was performed according to Dallies et al (22). Sugars were quantified with a pulsed amperometric detector (PAD) with gold electrode. Glucosammine, galactose, glucose and mannose (for chitin, glucan and mannan content determination, respectively) were identified by comparison with reference compounds and quantified according to calibration curves obtained for each sugar.

Human cell preparation - The PBMC fraction was obtained by density centrifugation of diluted blood (1:1 ratio between blood and pyrogen-free saline) over Ficoll-Paque. PBMCs were washed twice in saline and resuspended in culture medium. Monocytes were isolated from low density PBMCs by magnetic enrichment with anti-CD14 beads. In a challenge experiment, monocytes were cultured in the presence of GM-CSF (800 U/ml) and recombinant IL-4 (1000 U/ml) for 6 days to allow DC differentiation (24). DC activation was induced by *S. cerevisiae* strains. A serial dilution of live yeast preparations was added to the moDCs at different stimuli-to-DC ratios. For confirmation experiments, PBMCs from the same healthy subjects were used; stimulation was performed as in DC challenge experiments. Depending on the experiment, supernatants were collected after 24 hr or 5 d, and stored at -20°C until the assays.

In-vitro *induction of trained immunity* - Monocytes (10^6 cells/ml) were added in a volume of 100 µl/well in flat-bottom 96-well plates (Greiner, Nurnberg, Germany). Cells were incubated with one of the first stimuli for 24 hr, rested for 5 days and re-incubated for another 24 hr with one of the second stimuli. As priming stimuli we used 1 X 10^4 cells/ml of the different *S. cerevisiae* strains and *S. cerevisiae*-derived chitin (10 µg/ml). After 24 hr, cells were washed to remove all stimuli, and they were allowed to rest for 5 days before being exposed for 24 h to a second stimulation with various stimuli. Cytokines were measured after the second stimulation. For specific experiments to inhibit phagocytosis, Cytochalasin D has been dissolved at a concentration of 5 mg/ml in DMSO and used in a concentration of 1 µg/ml 60 min before chitin exposure. Inhibition of phagosomal acidification was done by preloading the cells with 50 nM Bafilomycin A (Calbiochem, San Diego, CA) 60 min before the stimulation. Cells pretreated with the same volume of vehicle (DMSO) were used as a negative control for both inhibitors. For inhibiting the methylation processes, before training with *S. cerevisiae* or with chitin, monocytes were preincubated for 1 hr with Pargyline (3 µM), or MTA (1 mM).

In-vivo *induction of trained immunity* - C57BL/6 mice were intraperitoneally injected with 1 mg of chitin particles from *Saccharomyces cerevisiae* or sterile PBS on days –7 and –4 prior to tail vein inoculation with $5x10^5$ *C. albicans* yeasts (Marakalala, Williams et al. 2013). Mice were then monitored at 3 and 10 days post infection (dpi) for survival, fungal burden in kidney, liver and brain, kidney histopathology and cytokine production in kidney homogenates.

Cytokine assay - TNFα and IL-6 levels on monocytes were determined by enzyme-linked immunosorbent assays on 24 hr monocyte cell culture supernatants according to manufacturer's instructions (R&D Systems). For the other experiments, at the indicated times, supernatants from human cell cultures were collected and cytokine detection was performed using the Milliplex® MAP human cytokine/chemokine kit (Merck-Millipore), according to the manufacturer's instructions.

Microorganism survival following uptake by primed monocytes - Human monocytes (10^6 cells/ml) were added in a volume of 100 µl/well in flat-bottom 96-well plates (Greiner, Nurnberg, Germany). Cells were incubated with one of the first stimuli for 24 hr, rested for 5 days and re-incubated for 6 hr with one of the second stimuli. As a priming stimulus, we used 10^4 cells/ml of YP4 *S. cerevisiae* strain and YP4 derived chitin (10 µg/ml). The second stimuli were *E. coli* (10^7 cells/ml), *S. aureus* (10^7 cells/ml), or *C. albicans* (10^6 cells/ml) in a final total volume of 200 µl. After that, monocytes were collected, washed three times with PBS, treated with zymolyase (2 mg/ml) or lysozyme (10 mg/ml), and washed twice; cells lysated with a hypotonic solution (0.05% KCl) to release intracellular microorganisms were plated on solid complete medium (YPD for yeast cells, TSB for bacterial cells). Survival of microorganisms after uptake are reported as the percentage of CFUs after 1 day (bacteria) or 3 days (fungus) relative to the total number of cells growing in the absence of monocytes exposure. Control experiments were carried out to verify that the hypotonic solution was not toxic to bacteria and yeasts.

In vitro *phagocytosis assay* - Human primed monocytes (as above) will be seeded into poly-L-lysine slides and incubated for 30 min at 37 °C and 5% CO_2 and then infected with *E. coli*

or *C. albicans*. For *C. albicans* internalization evaluation, after PBS washing and medium replacement, cells were infected with Oregon green 488 (Molecular Probes, Life Technologies) prelabelled *Candida* cells (10^6 cells/ml in complete RPMI) and further incubated for 1.5 hr. Thereafter, calcofluor white was added (20 µl/well) 15 min before each end point. Cells were washed with PBS to remove nonadherent yeasts and fixed with cold ethanol for 30min at -20°C and labeled in red with PHK26. Calcofluor white staining of fungi allows one to discriminate between adherent (Calcofluor white accessible) and phagocytized (i.e. internalized, Calcofluor white) yeast cells. When visualized by epifluorescence microscopy, all the yeast cells appeared as green (independently of the localization), while the not-internalized yeast cells were blue stained. Finally, merging of Oregon green 488 and Calcofluor white images allowed one to definitively exclude the extracellular yeast cells. By contrast, *E. coli* were labeled using Oregon green 488 only without distinguishing between internalized or not internalized bacteria. Cells where stained NucBlue Live Cell Stain Ready Probes reagent. A minimum of 200 monocytes per group were scored and any cell containing one or more particles was counted as phagocytic.

Phagolysosome acidification assay - To visualize the acid yeast-containing vesicles, chamber slides were prepared as in Phagocytosis assay; then, primed monocytes were exposed to the acidotropic dye LysoTracker DND-99 (75 nM) and infected as described above. Afterwards the samples were fixed with cold ethanol and immediately examined by fluorescence microscopy. Acidification control groups consisted of uninfected cells. A minimum of 200 yeast/bacteria-containing phagosomes was scored; the percent of acid phagolysosomes was evaluated as the ratio between the number of LysoTracker-labelled phagosomes and the total number of yeast-containing phagosomes.

Statistical analysis - The paired Wilcoxon test was used to compare differences between the effect of a particular stimulus and the RPMI medium control. The level of significance was set at $p<0.05$.

RESULTS

S. cerevisiae *isolates train differently the cytokine responses towards TLR ligands and fungi* - As *C. albicans* enhanced the cytokines response towards TLR ligands and colonizing bacteria (14), we tested whether *S. cerevisiae* was also able to induce trained immunity in monocytes. To investigate this effect, monocytes from healthy donors were first exposed to low amounts of *S. cerevisiae* (Fig. 1*A*) and *C. albicans* for 24 hr (Fig. 1*B*). After that, the initial stimulus was removed and the cells were washed. After an additional 5-days resting period, cells were restimulated with pure components of the bacterial cell wall or with *C. albicans* (Figs. 1*A*, *B*). Exposure of monocytes to a laboratory strain of *S. cerevisiae* yeast potentiated the production of TNFα induced by a second bacterial stimulus such as LPS, the lipopeptide analog Pam_3Cys_4, or by the fungal pathogen *C. albicans* (Fig. 1*A*) to the same extent as *C. albicans* (Fig. 1*B*).

Considering the variability in stimulating pro- and anti-inflammatory cytokines of fungal strains either pathogenic (7, 8) or harmless (13) species, we probed the ability of a set of *S. cerevisiae* strains, having different inflammatory properties [data not shown, Di Paola et al. submitted, (13)], to induce trained immunity towards different microbial ligands such as *E. coli* LPS (Fig. 2*A*) and Pam_3Cys_4 (Fig. 2*B*). This set comprises: 2 laboratory strains [SK1 (25) and BY4741 (26) used as reference strains in previous studies (27)], 8 clinical isolates (Di Paola et al., submitted, (13)) [7 from CD patients, one from healthy subject], 4 wild strains from Barriada wine region of Portugal, and 4 grape isolates from Tuscan vineyards (28) (Table 1). The results showed that all the *S. cerevisiae* strains train TNFα production in response to both TLR ligands, in a similar or even higher extent than *C. albicans* (14). In contrast to the clinical and Tuscan grape isolates, laboratory and Barriada wine (wild) strains showed little ability to train the monocytes response to Pam_3Cys_4 (Figs. 2*A*, *B*). All strains were able to modulate TNFα and IL-6 production upon challenge with LPS, albeit to different extents. The clinical YP4 isolate

showed the greatest potential to train cytokine production (Figs. 2*A*, *B*).

Since one of human commensal microorganisms is *C. albicans* itself, we tested whether *S. cerevisiae* isolates from different environmental niches train cytokine production in response to *C. albicans* stimulation. As shown in Fig. 2*C*, clinical and wild isolates enhanced monocyte response to *C. albicans* with different extents via modulation of TNFα and IL-6 production.

Fungal chitin drives the immune training lead by S. cerevisiae - As in the case of *C. albicans*, the outer layer of the cell wall of *S. cerevisiae* is enriched with mannoproteins, whereas the inner layer is composed of chitin and β-glucan. Both mannosylated proteins, phospholipomannan and β-glucan are known inducers of proinflammatory cytokines through C-type lectin receptors.

We speculate that differences in the cell wall composition of the isolates tested influenced their ability to induce and train cytokine production, and could be the result of the different environmental selective forces.
Thus, we investigated the cell wall composition of 13 strains with different ecological niches tested for their training properties (Fig. 1): one laboratory strain, 3 wild strains, 6 clinical isolates and 3 grape isolates (Table 2). While β-glucan content did not differ among the isolates (no statistical differences), chitin content was 3- to 10 - fold higher in clinical strains with respect to the others (Fig. 3*A* and Table 2). We isolated chitin from those strains and assessed the immune training properties of this fungal cell wall component towards either *C. albicans* or extracellular TLR secondary challenges. Chitin was able to induce a potent trained immune response in terms of IL-6 and TNFα induction (Fig. 3*B*). Similarly, chitin was able to enhance cytokine production upon signaling through endosomal TLR ligands, such as TLR9 ligand CpG, TLR3 ligand PolyI:C and TLR8 ligand risiquimod (R848) (Fig. 3*C*). To corroborate the result showing chitin as the driving training force in *S. cerevisiae*, we exposed monocytes to a strain having an high chitin content, one with a low chitin content, and we added chitin to the latter to a concentration similar to the concentration that would have been obtained by using a chitin rich strain. As shown in Fig. 3*D*, the presence of chitin increases the training properties of an isolate normally bearing a low chitin content on its cell wall. The training level herein restored was similar to the one exerted by a high chitin strain (Fig. 3*D*) and chitin itself (Figs. 3*B*,*C*).

We thereafter corroborated our *in vitro* results by *in vivo* mouse experiment, as indicated in the Material and Methods section. Chitin training before fungal intraperitoneal infection mediates resistance against *C. albicans* systemic infection (Fig. 4). In fact, in contrast to the high mortality in the control group (infected), treatment with chitin markedly enhanced survival of infected mice, both in terms of percentage of survivors and MST (Fig. 4*A*), and significantly reduced the fungal burden in the kidney, liver and brain at 3 dpi (Fig. 4*B*). *Candida* hyphae were markedly reduced in chitin-trained mice (Fig. 4C) confirming the reduced fungal burden observed at 3 dpi in this group. In addition, the inflammatory lesions of the kidney in control mice, in which a prominent cell infiltration around renal papilla was observed (Fig. 4*C*), were ameliorated upon treatment. It has been demonstrated that priming with β-glucan induces the production of TNFα and IL-6 *in vivo* (15). Consistently, chitin increased levels of IL-6, TNFα and IL-1β in kidney homogenates along with heightened anti-inflammatory IL-10 (Fig. 4*D*).

Chitin training potentiate monocytes intracellular killing of fungi and bacteria - We thereafter compared the ability of whole *S. cerevisiae* cell and chitin to induce trained immunity for killing both fungi and bacteria. In this set of experiments, human monocytes trained as previously described were exposed for 6 hr to live fungus *C. albicans*, to the Gram-positive bacteria *S. aureus* or to the Gram-negative bacteria *E. coli*. Trained monocytes showed enhanced killing ability with respect to non-trained monocytes (Fig. 5*A*). Particularly, chitin-trained monocytes showed a potent antimicrobial killing. This result demonstrates an enhancement of direct antimicrobial killing capacity of monocytes after induction of trained immunity by *S. cerevisiae*, and this effect is driven by chitin.

As the internalization ability of chitin-trained immune cells did not differ from one of non-trained cells (Figs. 5*B*,*C*), to investigate the events following internalization by monocytes, we evaluated the fate of the yeast-containing phagosomes in terms of acidification. As shown in Figs. 5*D*,*E*, the percent of *E. coli*-containing as well as *C. albicans* containing-acid phagolysosomes was increased in chitin-trained cells with respect to not-trained monocytes, thus accounting for the more efficient ability to kill the pathogens. If chitin trains by increasing the phagocytic activity of human monocytes, we hypothesized that blocking of its internalization by using Cytochalasin D (an inhibitor of phagocytosis and phagolysosome fusion), will restrict the intracellular survival of the pathogen and the induced cytokine responses (Fig. 5*F*, *G*). Indeed, inhibition of phagocytosis increased the survival of microbes (Fig. 5*F*) and blocked the production of TNFα and IL-6 (Fig. 5*G*) affecting the training induced by chitin. To determine whether formation of phagosome maturation was required for chitin training effects, we perturbed phagosome acidification prior to secondary infection using Bafilomycin A, a specific inhibitor of v-ATPase. As already shown (29), Bafilomycin A decreased both TNFα and IL-6 secretion after *S. aureus* stimulation but had no effect on *E. coli* recognition (Fig. 5*H*), while when Bafilomycin A is added prior secondary stimulation after chitin training, the effect of the training is almost abolished (Fig. 5*H*). Together, these data indicate that chitin must be internalized and delivered into an acidic phagolysosome to trigger its training properties.

Involvement of histone methylation for the chitin-induced training in monocytes – The previous observation of the training effect of β-glucan (15) prompted us to investigate whether a functional reprogramming of chitin-trained monocytes occurred via epigenetic changes such as histone methylation. Inhibition of histone demethylases by a specific inhibitor had no effect on the training of monocytes (Figs. 6*A*, *B*). In contrast, inhibition of histone methyltransferases using MTA inhibited monocyte training by *S. cerevisiae* or chitin (Figs. 6*C*, *D*), supporting the hypothesis that histone methylation is involved in the training of monocytes.

DISCUSSION

Although an anti-inflammatory potential of microbial/yeast strains was occasionally suggested *in vitro* (30, 31) or in experimental models (30, 31), few reliable observations were reported in terms of strain variability and of specific mechanisms involved (32, 33). To our knowledge, no studies addressed *S. cerevisiae* immune reactivity and its ability to modulate the cytokines response of human primary cells to bacteria and fungi. All the different *S. cerevisiae* isolates tested induce trained immunity in monocytes, resulting in an enhanced pro-inflammatory cytokine production when they are exposed to a secondary bacterial or fungal stimulus. The induction of trained immunity is dependent on the carbohydrate components of the cell wall of *S. cerevisiae*.

Cell wall characterization in terms of β-glucan and chitin content indicated how isolates from different ecological niches showed a remarkable different cell wall composition, with the clinical isolates being highly enriched in chitin. Under normal growth condition, chitin is a minor component of *C. albicans* cell wall, comprising only the 2-3% of its dry weight (34): however, chitin content of clinical *S. cerevisiae* isolates represented up to 8% of the dry weight of the yeast. Our results show that clinical strains, specifically isolated from human fecal samples from healthy and Crohn disease's patients are able to strongly modulate cytokines response to bacterial antigens and fungi, and this effect is largely driven on chitin. Moreover, the preliminary *in vivo* results show that chitin increases resistance of mice to subsequent systemic infection with *C. albicans* via a fine modulation of inflammatory/anti-inflammatory cytokines. Our data suggested that the training immunity driven by chitin is intimately associated with its internalization and identified a critical role of phagosome acidification to facilitate microbial digestion.

In addition, the enhanced cytokine production capacity induced by chitin and *S. cerevisiae* has been reduced by inhibition of histone methyl-transferases. This finding is

similar to the trained immunity induced by □–glucan (15), and suggests the involvement of epigenetic mechanisms (e.g. histone methylation such as H3K4me3), for effects of chitin and *S. cereviesiae.* A detailed assessment of the epiegentic landscape induced by these stimuli is thus warranted, and is the subject of future studies.

Even though chitin is an essential structural polysaccharide of fungal pathogens and parasites, some controversy persists on its role in human immune response (35–38). Earlier studies have shown that *C. albicans*-derived chitin reduces direct stimulation of *in vivo* LPS-induced inflammation and contributes to dampening the immune response by NOD2 and TLR9-mediated secretion of IL-10 (38). In a previous finding we showed how *S. cerevisiae* spores, which wall is largely composed by chitin, elicit inflammatory IL-17 responses, while cells of the same strain induce tolerance (27). Thus, by increasing the chitin content in their cell wall, fungi may influence the immune system in two ways: 1. favoring their persistence by influencing immune homeostasis and 2. training the innate host response against commensals.

The results presented here give a novel perspective on the role of non-pathogenic microorganisms for immune homeostasis. The chitin-induced training suggests how chitin levels could importantly help breaking potential dysbiosis, priming organismal protection against pathogens. This training effect does not require colonization but can be elicited simply via exposure to chitin rich *S. cerevisiae* yeast cells or spores and possibly, crustacean shell. Thus yeasts would play a fundamental role in shaping host microbiota simply when passengers, rather than necessarily persisting as continuous colonizers. This finding is in agreement with recent evidence that probiotic microorganisms are potentially effective through immune-mediated selection of the gut microbiota, rather than thanks to their ability to directly compete with other microorganisms present in the gut (39).

We can speculate that the recent elimination of *S. cerevisiae*, as well as of other immune regulators, from fermented foods, and overall the reduction of early exposure to beneficial microbiomes, is not only causing a reduction in anti-inflammatory signals, but is more importantly causing a decreased ability of "training" our immune system to handle pathogenic microorganisms, resulting in potentially uncontrolled immune reactions. The finding that the best "trainers" are strains isolated from Crohn's patients leads us to hypothesize an evolutionary advantage of chitin production in surviving and colonizing the human gut. It remains unclear if, in addition to these potential beneficial effects, induction of trained immunity may also have unintended deleterious effects, since fungi have been reported to increase the severity of intestinal inflammation in murine models of colitis (40, 41).

Several directions of research are opened by this study: on the one hand future investigations should deeply assess the molecular pathways that are involved in the induction of trained immunity by *S. cerevisiae* and chitin, as recently shown to involve genetic determinants (42) for epigenetic (15, 43) and metabolic (44) reprogramming for β-glucan. Monocytes metabolism changes during *S. cerevisia*e-cells interaction could control and enhance the immune cell function as recently described for T cells, DCs and macrophages (43–47). This could occur also through metabolites produced by the fungus itself during challenge, with *S. cerevisiae* strains behaving differently according to the different adaptation to the environment from which they were isolated. It was previously demonstrated that *C. albicans* can actively shift the balance of tryptophan metabolism in the host through soluble factors (48) and a recent study revealed the glucosamine modulatory properties on T cells (49). Indeed, fungus metabolites could somehow allow for the epigenetic changes that then form the basis for the training event. In this light, changing in the metabolic pathways induced by chitin might enable the cells to respond more efficiently to the invading pathogens, e.g. by enhancing the phagocytic activity.

On the other hand translational studies should assess the clinical potential of these effects. Further studies on the ability to induce trained immunity of yeasts in Crohn's mouse model will

reveal whether trained immunity responses to fungi are potentially associated with onset of remission of Crohn's disease. Discovering the factors that drive microbial colonization will help us develop new antimicrobial therapeutics based on their potential to induce trained immunity. Selected strains of the 'Generally Recognized as Safe' *S. cerevisiae* described in this study could be specially suited for this task and for developing novel therapeutic approaches to IBD, or in general, to disease associated to inflammatory responses towards an unbalanced microbiota.

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FOOTNOTES

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The abbreviations used are: DC, dendritic cells; IL, interleukin; Baf A, Bafilomycin A; Cyt D, Cytochalasin D; MTA, 5′-deoxy-5′(methylthio)adenosine.

FIGURE LEGENDS

FIGURE 1. *In vitro* training of monocytes with *S. cerevisiae*. Monocytes were trained with *S. cerevisiae* (*A*) or *C. albicans* (*B*) (priming stimulus) and after 24 hr washed to remove the stimulus. TNFα and IL-6 were measured in supernatants after bacterial cell wall pure components or *C. albicans* second stimulation (n = 8; four independent experiments). Bars indicate mean + SD (n = 10, four independent experiments). *p<0.05 trained cells versus RPMI–stimulated, Wilcoxon nonparametric test for two related samples. P3C, Pam_3Cys_4.

FIGURE 2. *S. cerevisiae* strains enhance differently the cytokine responses of cells restimulated with TLR ligands. *In vitro* training of monocytes with different strains of *S. cerevisiae* towards LPS (*A*), Pam_3Cys_4 (*B*) or *C. albicans* (*C*) stimulation. TNFα and IL-6 have been measured on culture supernatants 24 hr adding of stimulator inducer. White bars: laboratory strains; dark grey: clinical strains; light grey: Tuscan wine isolates; dashed: Barriada wild isolates. Bars indicate mean + SD (N=6). *p<0.05 trained cells versus RPMI–stimulated, Wilcoxon nonparametric test for two related samples. P3C, Pam_3Cys_4.

FIGURE 3. Chitin drives training immune properties of *S. cerevisiae* and enhanced intracellular killing of primed monocytes. (*A*) Chitin content of strains isolated from different environments. Chitin is expressed as percentage of the total cell wall. Data are presented as mean ± SD. (N=3), *p<0.05, clinical versus other isolates, Wilcoxon nonparametric test for two related samples. (*B, C*) Monocytes were exposed to RPMI or *S. cerevisiae* derived chitin (10 µg/ml), isolated from YP4 strain, for 24 hr, after which the cells were washed to remove the stimulus. After 5 days of resting, monocytes were restimulated with pure ligands or *C. albicans*. TNFα and IL-6 were measured in supernatants. Data are presented as fold increase with respect to not-trained (RPMI-stimulated) cells. Bars indicate mean ± SD (N = 4). *p<0.05, **p<0.01 trained cells versus RPMI–stimulated cells, Wilcoxon nonparametric test for two related samples. (*D*) Monocytes were exposed to RPMI, *S. cerevisiae* YP4 whole cell (high) (-), *S. cerevisiae* SK1 whole cell (-) and *S. cerevisiae* whole cell in presence of chitin (1 µg/ml) (+) for 24 hr, after which the cells were washed to remove the stimulus. After 5 days of resting, monocytes were restimulated with pure ligands or *C. albicans*. TNFα and IL-6 were measured in supernatants. Data are presented as fold increase with respect to not-trained (RPMI-stimulated) cells. N=3, mean ± SD,. *p<0.05, **p<0.01, whole cell + chitin (+) vs whole cell training (-),§p<0.05, §§p<0.01, whole cell high chitin (high) vs whole cell training (-)Wilcoxon nonparametric test for two related samples. LPS, lypopolisaccharyde; P3C, Pam_3Cys_4.

FIGURE 4. Chitin mediates resistance against *C. albicans* systemic infection. C57BL/6 mice were intraperitoneally trained at day -7 and -4 with 1 mg chitin from *S. cerevisiae* YP4 strain prior systemic infection with $5x10^5$ *C. albicans* yeasts. Mice were monitored for (*A*) survival, (*B*) fungal load, (*C*) kidney histopathology at4x and 60x magnification, white arrows indicate *C. albicans* hyphae, (*D*) levels of cytokine in kidney homogenates.. Data are expressed as mean ± SD. *p< 0.05, **p< 0.01, ***p<0.001, ****p<0.0001, chitin-treated vs untreated C57BL/6 mice (n = 10 mice/group from one -3 dpi- or two -10 dpi one experiments). Naïve, uninfected mice. Infected, infected and untreated mice. Treated, chitin-trained and infected mice. dpi: days post infection.

FIGURE 5. Chitin does not change internalization properties but promotes killing by increasing the acidification of microbial containing phagolysosomes. (*A*) Survival of *C. albicans* and $Gram^+$ (*S. aureus*) and $Gram^-$ (*E. coli*) bacteria after killing by trained monocytes. Data are expressed as percentage of CFU of microorganisms growing after 24 hr in solid growth medium at the appropriate microbial temperature. (*B,C*) Monocytes were exposed to RPMI or *S. cerevisiae* derived chitin (10 µg/ml) for 24 hr, after which

the cells were washed to remove the stimulus. After 5 days of resting, monocytes were restimulated for 1.5 hr to green-labeled *E. coli* (*B*) or *C. albicans* (*C*). After appropriate staining, cells were visualized by fluorescence microscopy. Representative images of 3 independent experiments. (*D*) Oregon green 488 prelabelled microorganims were exposed to Lyso-Traker labeled monocytes (E : T=1 : 5); samples were then fixed and visualized by fluorescence microscopy. The results, expressed as percent of acid phagolysosomes, were calculated by evaluating the number of red-stained vacuoles among 200 yeast-containing vacuoles. (*E*) Representative images of *C. albicans* containing- acid phagolysosomes. Bars indicate mean + SD (N=3). *p<0.05 trained cells versus RPMI–stimulated, Wilcoxon nonparametric test for two related samples. (*F*) Survival of *C. albicans* and Gram$^+$ (*S. aureus*) and Gram$^-$ (*E. coli*) bacteria after killing by trained monocytes. Before chitin training, cells have been treated or not for 1h with 1 µg/ml Cytochalasin D (Cyto D). Data are expressed as percentage of CFU of microorganisms growing after 24 h in solid growth medium at the appropriate microbial temperature. Bars indicate mean + SD (N=3). *p<0.05 Cyto D trained cells versus trained cells, Wilcoxon nonparametric test for two related samples. (*G*) *In vitro* training of monocytes with chitin towards *C. albicans*, *S. aureus* or *E. coli* stimulation. Before chitin training, cells have been treated or not for 1 hr with 1 µg/ml Cytochalasin D. TNFα and IL-6 have been measured on culture supernatants 24 hr adding of stimulator inducer. Bars indicate mean + SD (N=3). *p<0.05 Cyto D trained cells versus trained cells, for each stimulator inducer respectively, Wilcoxon nonparametric test for two related samples. (*H*) *In vitro* training of monocytes with chitin towards *C. albicans*, *S. aureus* or *E. coli* stimulation. Before addition of the stimulatory inducer, cells have been treated or not for 1 hr with 50 nM Bafylomicin A (BafA). TNFα and IL-6 have been measured on culture supernatants 24 hr adding of stimulator inducer. Bars indicate mean + SD (N=3). *p<0.05 BafA trained cells versus trained cells, §p<0.05 BafA versus RPMI for each stimulator inducer respectively, Wilcoxon nonparametric test for two related samples.

FIGURE 6. Hystone methylation is involved in chitin-training effects. (A-D) Cytokine production in supernatants of adherent monocytes primed 24 hr with either cell culture medium or *S. cerevisiae* (A, C) or chitin (B, D) in the absence or presence of the histone demethylase inhibitor pargyline (A and B) and the histone methyltransferase inhibitor 5′-deoxy-5′(methylthio)adenosine (MTA) (C and D) and restimulated with LPS or *C. albicans*. Data are presented as mean ± SD (N=3). *p<0.05, **p<0.01, treated vs not-treated cells.

TABLES

TABLE 1. List of *S. cerevisiae* isolates used in the study

strain	*origin*		*study*
SK1	rotten fig	laboratory	(50)
BY4741		laboratory	(51)
BT2440	Barriada, Portugal	vineyards	This study
BB1533	Barriada, Portugal	vineyards	This study
BB2148	Barriada, Portugal	vineyards	This study
BR2435	Barriada, Portugal	vineyards	This study
YA5	clinical, Crohn disease	human feces	Di Paola et al., submitted, (13)
YB7	clinical, Crohn disease	human feces	Di Paola et al., submitted, (13)
YP4	clinical, Crohn disease	human feces	Di Paola et al., submitted, (13)
YD1	clinical, Crohn disease	human feces	Di Paola et al., submitted, (13)
YH1	clinical, Crohn disease	human feces	Di Paola et al., submitted, (13)
YUC22	clinical, Crohn disease	human feces	Di Paola et al., submitted, (13)
YN19	clinical, healthy	human feces	Di Paola et al., submitted, (13)
Y13EU	clinical, healthy	human feces	Di Paola et al., submitted, (13)
Sgu421	Tuscany, Italy	grape	(52)
Sgu165	Tuscany, Italy	grape	(52)
M28-1A	Tuscany, Italy	grape	(52)
M28-1B	Tuscany, Italy	grape	(52)

TABLE 2. Cell wall sugar composition of *S. cerevisiae* isolates. Sugars composition is expressed in percentage of the total cell wall (mean ± SD, n=3)

origin	*strain*	*chitin*	*glucan*	*mannan*
lab	SK1	2.24 ± 0,87	43.38 ± 2,24	54.37 ± 2.00
wild	BT2440	2.90 ± 0,54	40.32 ± 3.68	56.77 ± 5,43
	BB1533	3.00 ± 0.14	44.01 ± 3.48	52.97 ± 3.24
	BB2148	3.86 ± 1.36	45.66 ± 4.28	50.46 ± 3.26
clinical	YA5	8.63 ± 2.35	57.68 ± 2.98	33.67 ± 7.76
	YB7	8.65 ± 1.20	55.76 ± 3.52	35.58 ± 6.11
	YP4	15.84 ± 3.77	56.04 ± 5.17	28.11 ± 0.43
	YD1	16.42 ± 2.69	54.632 ± 5.14	28.9 ± 1.44
	YH1	12.31 ± 1.68	51.06 ± 1.79	36.62 ± 8.01
	Y13EU	13.5 ± 3.87	56.51 ± 7.02	29.97 ± 2.11
grape	SGU421	3.33 ± 1.18	45.03 ± 4.99	51.63 ± 4.40
	M28-1A	0.7 ± 0.42	68.94 ± 1.49	30.36 ± 1.08
	M28-1B	1.42 ± 0,86	56.11 ± 10.74	42.45 ± 14.15

Figure 1

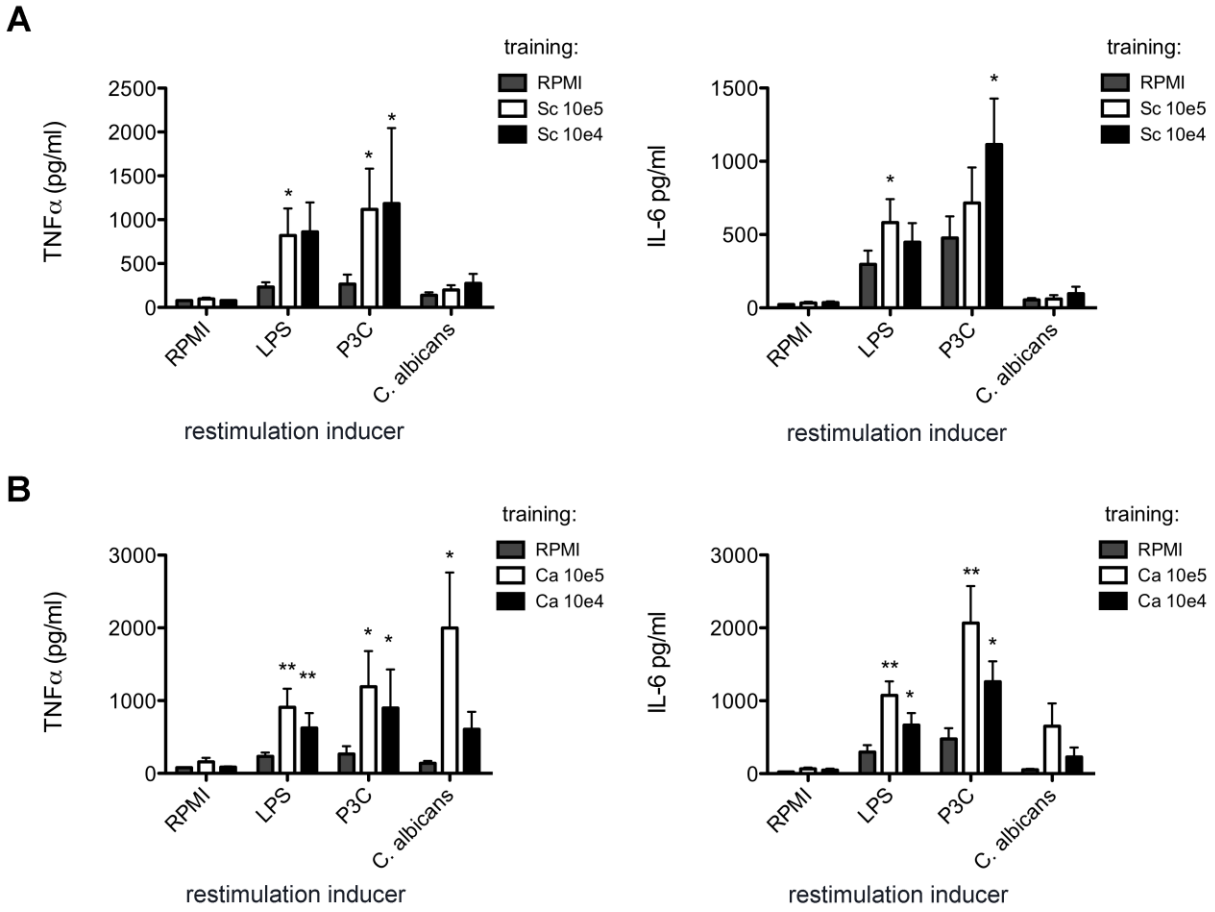


Figure 2

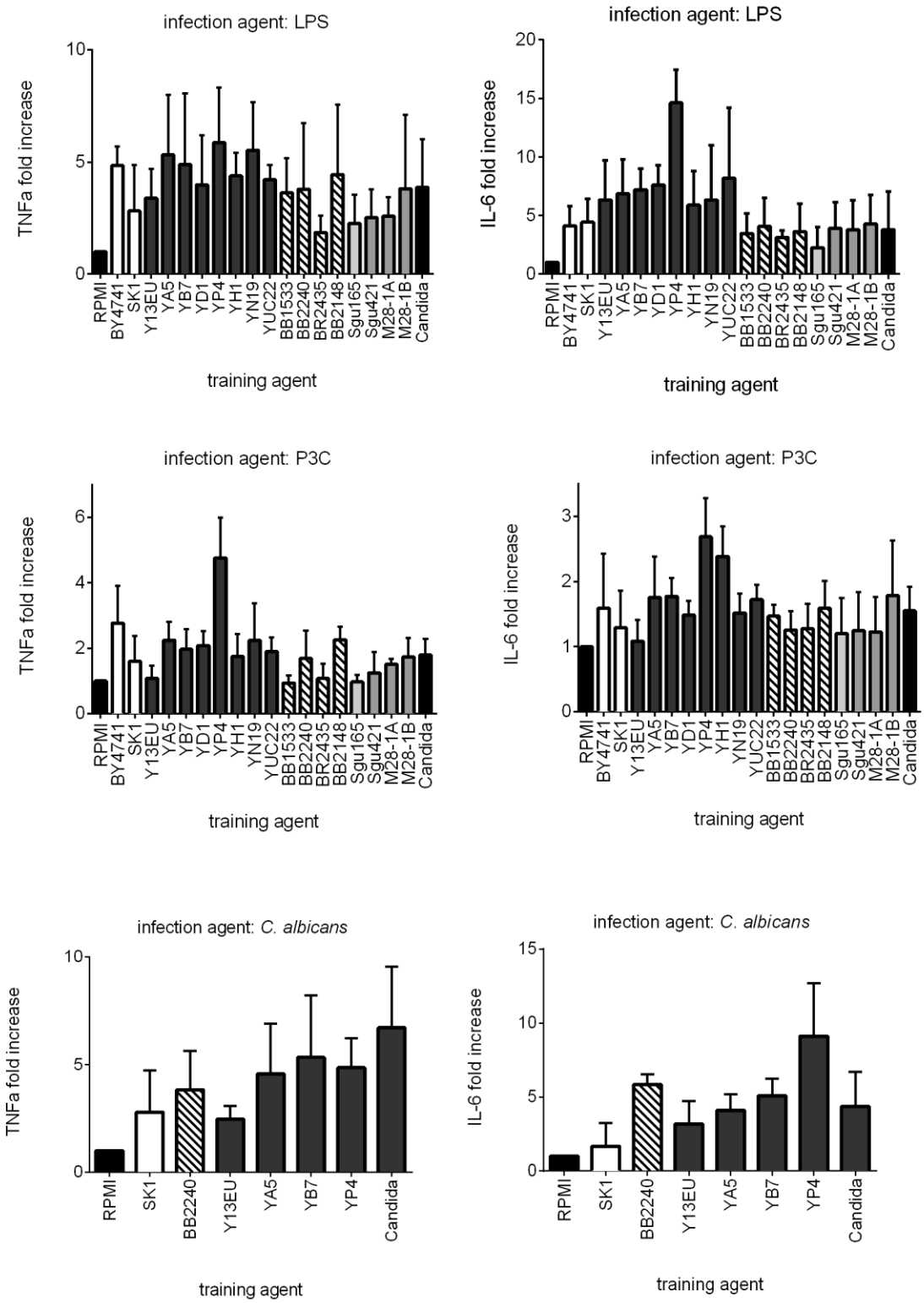


Figure 3

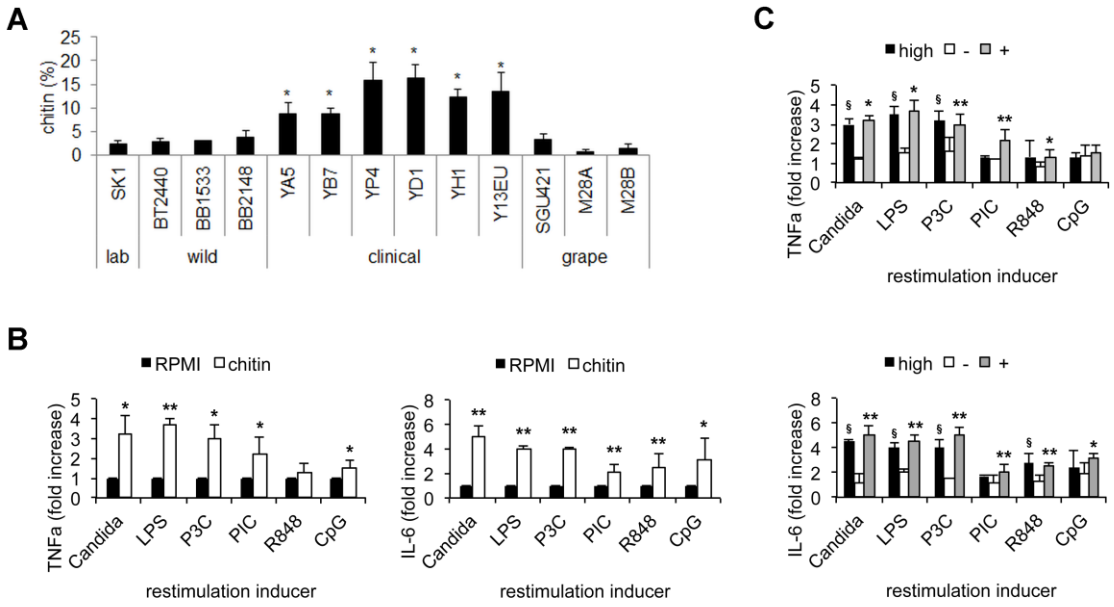


Figure 4

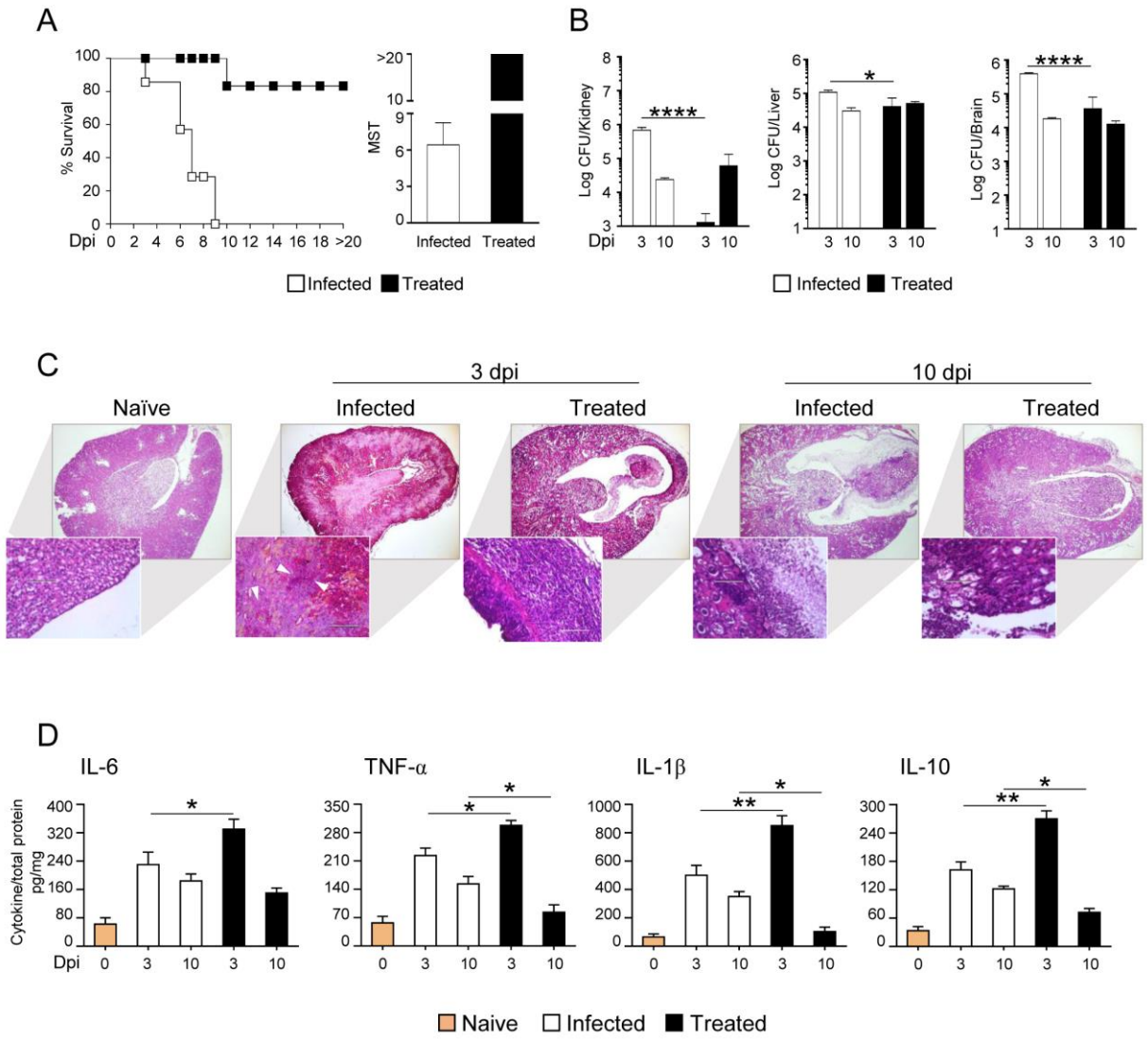


Figure 5

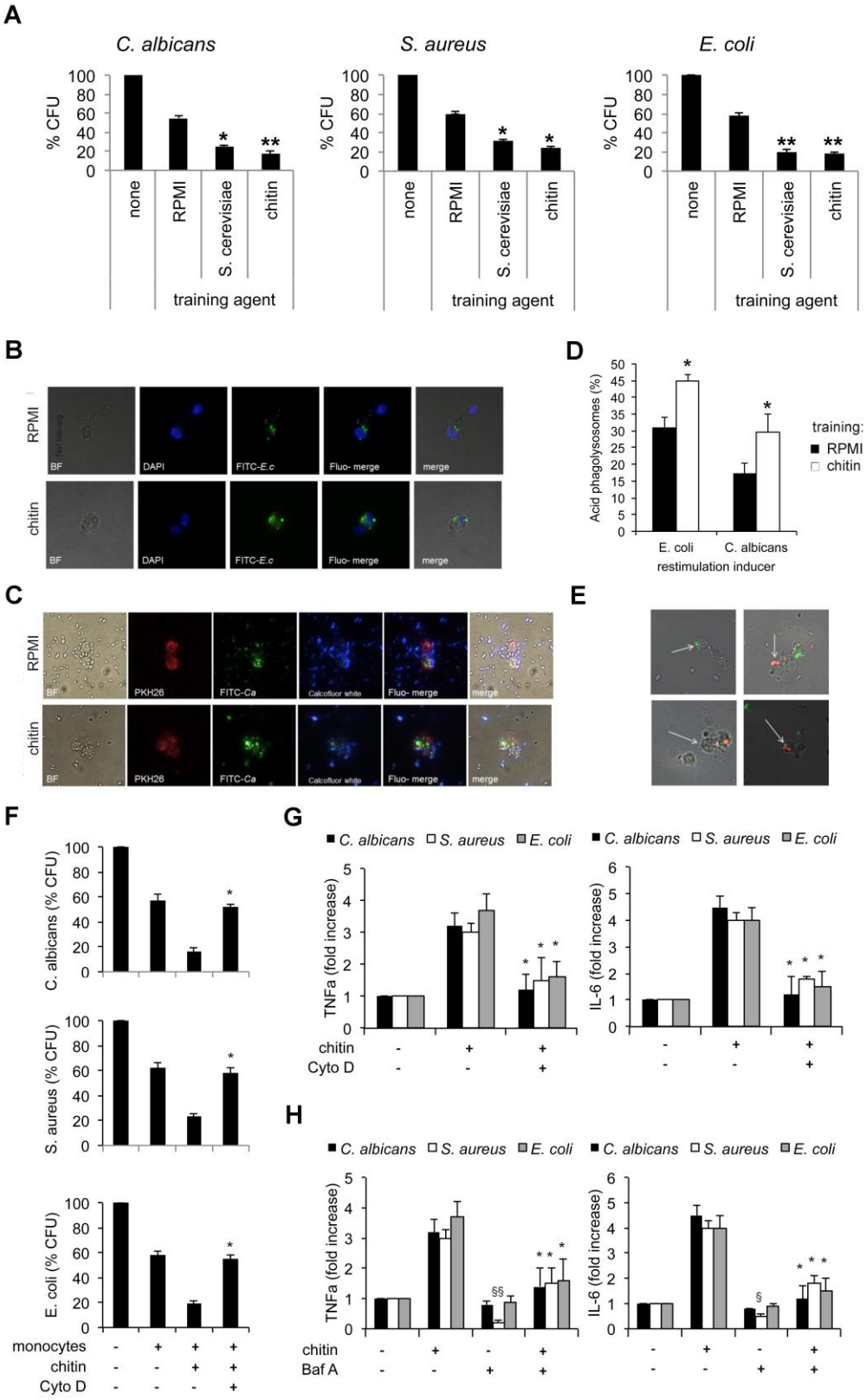
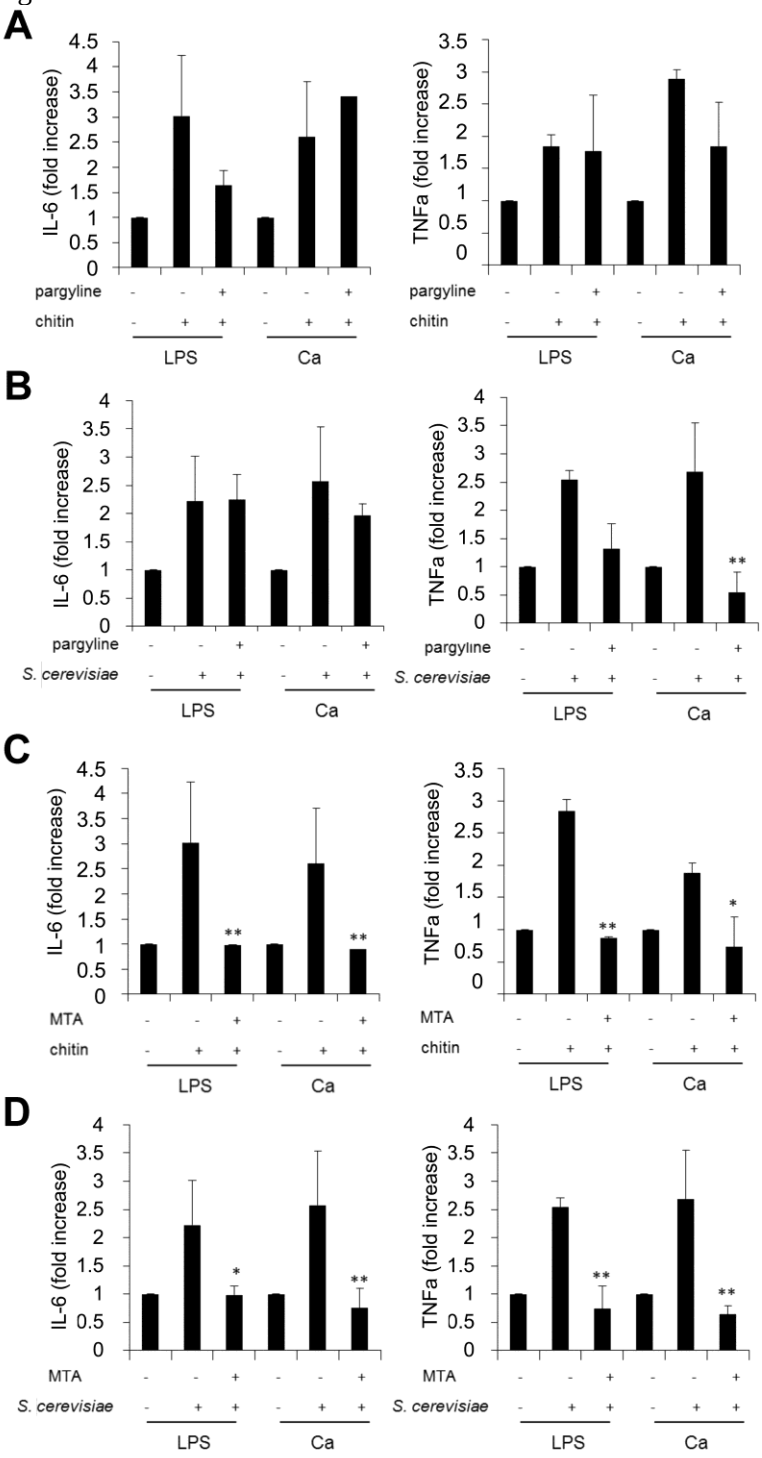


Figure 6



Fungal chitin induces trained immunity in human monocytes during cross-talk of the host with Saccharomyces cerevisiae

Lisa Rizzetto, Daniela C. Ifrim, Silvia Moretti, Noemi Tocci, Shih-Chin Cheng, Jessica Quintin, Giorgia Renga, Vasilis Oikonomou, Carlotta De Filippo, Tobias Weil, Bastiaan A. Blok, Marcello S. Lenucci, Manuel A. S. Santos, Luigina Romani, Mihai G. Netea and Duccio Cavalieri

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