

Intestinal Candida albicans overgrowth in IgA deficiency

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1 Title: Intestinal Candida albicans overgrowth in IgA deficiency

2 Running title: **IgA impact on host-fungal mutualism**

3

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Symp: slgAd symptomatic; ASV: amplicon sequence variant; IC: intestinal cell

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41 Abstract

42 Background

43 Secretory IgA interacts with commensal bacteria, but its impact on human mycobiota ecology
44 has not been widely explored. In particular, it remains unknown whether human IgA-deficiency
45 is associated with gut fungal dysbiosis.

46 **Objectives**

47 Our goal was to study the impact of IgA on gut mycobiota ecology.

48 Methods

49 The Fungi-flow method was used to characterize fecal, systemic, and maternal IgA, IgM and 50 IgG responses against 14 representative fungal strains (yeast/spores or hyphae forms) in healthy 51 donors (HD, n= 34, 31 and 20, respectively), and also to compare gut mycobiota opsonization 52 by secretory antibodies in HD (n=28) and patients with selective IgA deficiency (SIgAd n=12). 53 Stool mycobiota composition was determined by ITS gene sequencing in HD (n= 23) and 54 SIgAd (n=17). Circulating CD4+ T cell cytokine secretion profiles were determined by 55 intracellular staining. Impact of secretory IgA, purified from breast milk (n=9), on candida growth and intestinal Caco-2 cell invasion was tested in vitro. 56

57 **Results**

Homeostatic IgA binds commensal fungi with a body fluid-selective pattern of recognition. In SIgAd patients, fungal gut ecology is preserved by compensatory IgM binding to commensal fungi. Gut *Candida albicans* overgrowth nevertheless occurs in this condition, but only in clinically symptomatic patients with decreased Th17/22 T cell responses. Indeed, secretory IgA can reduce *in vitro* budding and invasion of intestinal cells by *C. albicans*, and therefore exert control on this pathobiont.

64 **Conclusion**

65 IgA has a selective impact on *C. albicans* ecology to preserve fungal-host mutualism.

66 Clinical implication

67 Understanding of IgA-fungi interactions reveals the potential of secretory antibodies in68 modulating mycobiota for therapeutic purposes.

69

70 Capsule Summary

71 IgA show distinct commensal fungi-specific recognition patterns across body fluids and 72 contribute to the prevention of intestinal *Candida albicans* overgrowth that cannot be 73 compensated by IgM in symptomatic IgA-deficient patients.

- 74
- 75
- 76 Key words:
- 77 Mycobiota, homeostatic IgA, IgA deficiency, IgM, Candida albicans
- 78

79 Abbreviations

- 80 ASV : Amplicon sequence variants
- 81 HD : Healthy donors
- 82 IL : Interleukin
- 83 MFI : Median fluorescence intensity
- 84 PCA: Principal component analysis
- 85 sIgA : Secretory immunoglobulin A
- 86 SIgAd : Selective IgA deficiency

87 Introduction

Mounting evidence of gut mycobiota involvement in human health and disease has increased the interest in the immune mechanisms controlling host microbiota mutualism.¹ Commensal fungi induce innate responses, implicating CX3CR1⁺ macrophages via CARD-9 signaling pathway, as well as Th17 cell-mediated adaptive responses which are essential to maintain mycobiota homeostasis.² Th17 cells also exert innate immune mechanisms, notably via the production of interleukin (IL)-17A and IL-17F, both cytokines promoting local neutrophil recruitment and playing an active role in the maintenance of integrity of epithelial barrier sites.³

96 The role of antibodies in intestinal host-fungi symbiosis remains in contrast poorly 97 characterized. Secretory immunoglobulin A (sIgA) is of particular interest, because of its 98 mucosal localization and its reported involvement in mediating microbial commensalism.⁴ Although the human mycobiota contains a large number of fungal genera,⁵ sIgA-fungal 99 interactions have focused on Candida albicans, a human commensal and a major fungal 100 101 pathogen.⁶ Hence, in a mouse model of oropharyngeal candidiasis, opsonization of this 102 commensal by sIgA prevented fungal epithelial adhesion and invasion, contributing to control 103 commensal C. albicans carriage.⁷ Central to the pathogenic potential of C. albicans is its ability 104 to convert from round yeast cells to a hyphenated form able to invade mucosal epithelial cells, 105 leading to blood infection. Importantly, sIgA mainly recognizes the latter morphotype, 106 suggesting that sIgA opsonization constitutes a regulatory mechanism of intestinal fungal commensalism.^{8, 9} Indeed, in patients with Crohn's disease, reduced gut sIgA reactivity to 107 108 Candida hyphae-produced proteins was found to correlate with an increase in hyphal fungal 109 morphology.⁸

110

111 In addition to the regulatory role of intestinal sIgA, the involvement of systemic IgG in host-112 mycobiota interactions has recently been highlighted. Systemic IgG responses targeting 113 Saccharomyces cerevisiae were initially considered secondary to fungal translocations due to gut barrier defects in relation with intestinal bowel disease.¹⁰⁻¹³ We however recently reported 114 that systemic IgG responses against commensal fungi can also be detected in healthy subjects,¹⁴ 115 116 while CARD9-deficient patients with systemic candidiasis present decreased anti-fungal 117 commensal IgG levels.¹⁵ Finally, in a CARD9-deficiency murine model, intestinal C. albicans 118 interactions with CX3CR1⁺macrophages failed to elicit IgG class switch recombination and germinal center B cell expansion.⁸ 119

120

The above-mentioned recent studies support the view that, besides cellular responses, antifungal IgG responses are associated with a status of host-fungal mutualism. We postulated that it is IgA that plays a key role in the establishment of this equilibrium by preserving gut mycobiota diversity and intestinal barrier homeostasis. Here, we characterized fecal, systemic, and maternal antifungal humoral responses in a cohort of healthy donors and evaluated alterations of gut fungal diversity in a cohort of selective IgA deficient patients.

127

128 Material and methods

129 Human samples

130 Samples from patients with a selective IgA deficiency (SIgAd) and healthy donors (HD) used 131 in the present cross-sectional study are part of a previously published collection.¹⁶ The main 132 criterium for the inclusion of SIgAd patients were undetectable serum IgA (<0.07 mg/mL), but 133 normal IgG levels. Exclusion criteria were antibiotic therapy and laxative drug use during the 134 last 3 months before stool collection. Clinical and biological data were collected at inclusion 135 time. Fresh stool, fecal waters, purified microbiota, DNA from fresh stools, serum samples and 136 data from immunological status of HD and SIgAd patients were generated, as described previously.¹⁶ Oral and written consent was obtained from patients before inclusion in the study. 137 Fresh breast milk of healthy lactating women was obtained from the Lactarium Regional d'Ile 138 139 de France (Hopital Necker Enfants Malades, Pôle Mère-Enfant). Breast milk sample use for 140 research approved by the ethical committee (Avis CENEM was 2020-VR 141 Biocol lactothèque v1.0 20190408). IgA was purified from breast milk and IgM from sera of 142 SIgAd patients using peptide M (InvivoGen).

143

144 Fungal strains and culture conditions

145 A fungal biobank, including 14 fungal strains, was generated from different genera commonly described in human skin, lung, and intestinal mycobiota.¹⁴ Fungal reference strains were 146 obtained for C. albicans (ATCC90028) and Aspergillus fumigatus (ATCC204305), whereas 147 148 other fungal strains, isolated for clinical purpose, were obtained from *in vitro* culture of human 149 stool, skin and bronchoalveolar lavage samples. Fungi were cultured in Sabouraud agar medium 150 (Bio-Rad) between 30 and 35 °C, depending on the fungus studied. To generate budding forms, yeast or spores from filamentous fungi were kept at 4 °C in RPMI-1640 medium (Gibco) for 2 151 h and then cultured at 30 °C under stirring for 9 h. At the end of the incubation period, cultures 152

were centrifuged at 21,000 g for 20 min. Supernatant was removed and fungi were resuspendedin cryopreservation medium.

155

156 Quantification of humoral responses

157 Systemic, as well as fecal water- and breast milk-derived, secretory IgA responses were determined using the Fungi-flow method,¹⁴ as follows. Twenty-five microliters of fixed (2% 158 PFA/PBS) samples containing 2.5 10⁵ fungi were distributed in a 96 well conic bottom plate. 159 160 Twenty-five microliters of normalized samples were then distributed in each well and incubated 161 for 20 min at 4 °C. Seventy microliters of PBS were added and samples were centrifuged at 162 3200g during 10 min. After removal of supernatant, fungi were incubated with a 25 µL mix (10 163 µg/mL) of goat anti-human IgG-Alexa Fluor 647 (Jackson Immunoresearch) or goat anti-164 human IgA-FITC (Jackson Immunoresearch) or donkey anti-human IgM-PE (Jackson 165 Immunoresearch) for 20 min at 4 °C. After one washing step with PBS, fungi were resuspended 166 in 150 µL of PBS and analyzed on a Cytoflex flow cytometer (Beckman coulter). Unstained 167 spores or budding forms were used as negative control to determine background fluorescence. 168 Flow cytometry data were analyzed using the FlowJo program (version 10; Becton Dickinson) 169 and expressed as median fluorescence intensity (MFI).

170

171 Quantification of *in vivo* antibody-binding levels to commensal fungi

172 Whole microbiota was extracted by gradient purification in anaerobic conditions (Freter 173 chamber) as previously described.¹⁷ Microbiota samples were stored at -80°C and thawed 174 before use. Three microliters (10⁷ microorganisms/ μ L) were fixed in 500 μ L of 2% (w/v) PFA 175 in PBS, supplemented with 4 % of calcofluor white dye (Sigma) for 20 min at 4°C. After 176 washing with 1 mL PBS (9000 g for 10min at 4°C), bacteria and fungi were resuspended in 177 PBS at a concentration of 3x10⁷/mL with the appropriate secondary staining antibody, washed and analyzed as above. An IgA isotype control antibody (FITC ChromPure Goat Serum, Jackson Immunoresearch) was used to correct for unspecific binding. In order to better define mycobiota flow cytometry events among fecal microbiota, we determined the SSC/FSC distribution of a fungal mock population containing most prevalent fungi found in human mycobiota,⁵ to determine flow cytometry gates (Fig E1, A).

183

184 DNA sequencing

185 Sequencing libraries were constructed by amplifying the 18S rRNA gene ITS2 region using the PCR amplification protocol previously described.14 DNA samples were subjected to PCR 186 187 amplification using the following ITS2 FwTag primers: 188 (5'CTTTCCCTACACGACGCTCTTCCGATCTGTGARTCATCGAATCT TT-3') and ITS2 189 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTGATATGCTTAAGTTCAGCGG RvTag 190 GT3'). Amplification reaction was performed with DNA MolTaq (Molzym, Bremen, Germany) 191 in a total volume of 50 µL containing 1 µM of each primer and 1 µL genomic DNA from 192 extracted stool samples. Cycling conditions were initial denaturation at 94 °C for 60 s, 35 cycles 193 of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 45 s, 194 followed by a final elongation step at 72 °C for 7 min. PCR amplicon libraries were sequenced 195 on a MiSeq Illumina instrument (Genotoul, Toulouse, France) producing 2 X 300 bp paired-196 end reads.

197

198 ITS amplicon profiling

We applied DADA2 (version 1.20.0) for further processing of demultiplexed sequence reads¹⁸ in R (version 4.1.0). The full amplicon DADA2 workflow: filtering, dereplication, sample inference, chimaera identification, and merging of paired-end reads was employed by using a default setting. We used standard filtering parameters: maxN=0, truncQ=2, rm.phix=TRUE and

maxEE=2. The maxEE parameter established the maximum number of "expected errors" 203 204 allowed in a read to 2, and cut off 210 bp for forward and 200 bp for reverse reads. Amplicon 205 sequence variants (ASV) generated by DADA2 analysis of the quality-filtered sequence data 206 classified against UNITE were taxonomically database (sh general release dynamic 10.05.2021 dev.fasta (release May 11th, 2021)).¹⁹ All ASVs 207 208 not matching taxa in the UNITE database were given the designation "NA" (unassigned). ASVs 209 without identified phylum were excluded from the analysis. To incorporate the ASVs that 210 included all taxonomically unassigned sequences, multiple sequencing comparisons were performed using Blast.²⁰ The ASV sequences with > 97% similarity were identified as the same 211 212 species, > 95 % using as the same genus.

213

Mycobiome alpha and beta diversity were studied with the Phyloseq (version 1.36.0) and vegan R package (2.5-7). Rarefaction was used to simulate an even number of reads per sample. The rarefaction depth chosen was 90% of the minimum sample depth in the dataset (in this case 11725 reads per sample). DESeq2²¹ was used to produce a matrix containing differential abundance values (e.g. log2 fold changes) corresponding significance measures for species and genus abundance (*p* and adjusted *p* values). An adjusted *p*-value < 0.01 was considered statistically significant.

221

222 Peripheral blood CD4+ T cell profiling

Cytokines secreting T cells from frozen PBMC samples were detected after a 6-hour Phorbol
12-Myristate 13-Acetate (PMA) – Ionomycin calcium salt (Sigma Aldrich) stimulation and
Golgi blocking (Golgi stop, BD Biosciences) and stained with: CD4-APC-Cy7 (BD
Biosciences), CD3-PerCP (BD Biosciences), Aqua (LIVE/DEAD® Fixable Dead Cell Stain
Kit, Invitrogen), IL-22-A647 (e-Bioscience), IFN-γ-FITC (BD Biosciences), IL-17A-PE

(eBioscience) and analyzed on a FACSCanto II flow cytometer (BD Biosciences) and FACS
Diva (BD) software (BD Biosciences).

230 Intestine enterocyte cell (IEC) line and growth conditions

The cell line Caco-2, derived from a human colon adenocarcinoma, was purchased from the 231 232 American Type Culture Collection (ATCC n°HTB-37TM). Cells were grown in Dulbecco's modified Eagle's minimum essential medium (Gibco, Life Technologies) supplemented with 233 234 10% FBS (PAN Biotech) and 0.1mM non-essential amino acids (Dominique Dutscher), without 235 antibiotics or antifungal agents. Three hundred thousand cells were seeded onto 14 mm 236 diameter glass coverslips previously placed in 24-well plates. Cells were maintained in a humidified incubator at 37°C with 5% CO₂ and were used for experiments 15 to 21 days after 237 238 seeding (Passages 10 to 20) after full differentiation.

239

240 *C. albicans* filamentation adhesion and invasion assays

For filamentation, adhesion and invasion assays, C. albicans (SC5314) were grown in liquid 241 242 YPD medium (yeast extract 1%, bacto-peptone 2%, and dextrose 2%, Fischer Bioreagent) 243 overnight at 37°C, in a shaking incubator. Fungal cells were then diluted in fresh liquid YPD 244 medium to a OD_{600nm} of 0.28 to 0.32 and then grown to log phase for another 2 hours in the same conditions. A pellet of 5.10^4 yeasts was resuspended with 100 µg/mL purified sIgA or 245 246 IgM. After an incubation for 20 min at 4°C, the yeast concentration was adjusted to 5.10⁴ 247 yeasts/mL in cell culture media for further investigation. For filamentous assays, C. albicans, either pre-treated or not with sIgA or IgM in RPMI-1640 medium were incubated into a 6 248 249 plastic dishes for 3 hours at 37°C, 5% CO₂. Fungal cells were then washed with PBS, scrapped 250 from the dishes and observed with a BX51 microscope using a X40 magnification (Olympus). 251 Adhesion and invasion assays were performed using differentiated Caco-2 cells. Briefly,

252	intestinal cells cultured on coverslips were infected with C. albicans pre-treated or not with
253	sIgA or IgM for 30 min or 2 hours at 37°C, respectively, as described elsewhere. ²²

255 Statistical analysis

- 256 Statistical analysis was performed using GraphPad Prism (version 9) and JMP Pro (version 17).
- 257 Mann-Whitney test, Wilcoxon/Kruskal-Wallis test and Chi-square test were used whenever
- 258 necessary. Principal component Analysis (PCA) was performed using RStudio v1.3.1093
- 259 with FactoMineR PCA and Factoextra fviz_pca_biplot functions on MFI values.

260 **Results**

261 Intestinal, systemic and maternal IgA differentially target commensal fungi

262 Characterization of homeostatic IgA binding to 14 fungal strains from different genera of the 263 two main divisions Ascomycota and Basidiomycota was carried out by flow cytometry in fecal 264 waters, serum, and breast milk from a cohort of HD (Fig 1, A). Free sIgA fecal water binding 265 was observed against all fungal genera studied and showed high inter-individual variability (Fig 1, B). sIgA responses against members of the Ascomycota phylum, and Saccharomycetales 266 267 order, such as Saccharomyces, Debaryomyces, Candida, Cyberlindnera and Yarrowia were lower than those of the Hypocreales order, Fusarium or Acremonium, or those of the 268 269 Basidiomycota phylum, Rhodotorula, Malassezia and Trichosporon. Analysis of Ig-opsonized 270 fungi in human purified gut microbiota (Fig 1, C and Fig E1, A) showed a preponderance of sIgA⁺ as compared to sIgM⁺ fungal populations (38.2 \pm 23 % vs 5.7 \pm 10 %; p<0.0001). 271 272 Systemic IgA responses exhibited also an inter-taxonomic variability, even against closely 273 related taxons of the Saccharomycetales order (Fig 1, D). The more intense responses were 274 observed against the Candida genus but also against Aspergillus, Fusarium, Acremonium and Penicillium. Finally, as the maternal milk IgA repertoire largely mirrored the intestinal IgA 275 repertoire,²³ we characterized anti-fungal IgA activity in breast milk from healthy lactating 276 277 women (Fig 1, E). sIgA responses were mainly observed against *Debaryomyces*, *Candida* and 278 Malassezia. We also assessed IgA responses against the filamentous forms of Candida, 279 Aspergillus, and Fusarium. Interestingly, the more intense responses were observed against 280 Candida hyphae in serum and so less in breast milk as well, as compared to Fusarium and Aspergillus (Fig 1, B-E). Principal component analysis (PCA) highlighted three distinct 281 282 patterns of IgA anti-mycobiota combined reactivity profiles (Fig 1, F) and further underline the 283 focalization of serum IgA responses against Candida hyphae as compared to Aspergillus and 284 Fusarium in healthy individuals (Fig 1, G). Together, these results show that homeostatic IgA

responses preferentially target specific members of the fungal community and suggest that intestinal, systemic and maternal IgA repertoires are focused on different combinations of genera, as underlined by combined PCA.

288

289 *C. albicans* overgrowth in SIgAd patients

290 To decipher the impact of IgA on homeostasis of gut mycobiota and its diversity, we 291 characterized the mycobiota composition in a cohort of IgA deficient patients (Fig 2, A and Fig 292 E1, B). Using the strategy described above, we first analyzed the percentage of fungal cells in 293 purified microbiota samples from HD and SIgAd patients (Fig 2, B). The results show that the 294 proportion of the fungal population in mycobiota was similar in HD and SIgAd patients (1.1% 295 \pm 1,15 and 0.71% \pm 0.64, respectively). We then analyzed the percentage of IgA-opsonized 296 fungi. As expected, $38.2\% \pm 23.32$ of the gut fungal population was opsonized by intestinal 297 IgA in the HD population, whereas the IgA-opsonized population was absent in all but two 298 SIgAd patients.

299

300 To determine how the lack of IgA could alter the gut mycobiota ecology, we performed ITS-301 gene sequencing of DNA obtained fecal samples (Fig 2, C-F). We observed a trend toward 302 increased diversity in SIgAd subjects for multiple alpha diversity metrics (observed ASVs, 303 Shannon Diversity Index and Chao1), although none of these differences reached statistical 304 significance (p < 0.05). We then analyzed whether IgA deficiency might affect the composition 305 of fungal populations from the phylum to the species level in terms of ASVs, relative abundancy 306 and prevalence. Interestingly, at the phylum level, significant differences were observed for the 307 Ascomycota phylum between the gut mycobiota of SIgAd subjects and that of HD (p=0.003) 308 (Fig 2, D). We detected 72 fungal genera, with Saccharomyces being the more prevalent one, followed by Candida, Debaryomyces, Penicillium, Malassezia and Yaworria genera (Fig E2). 309

Thirty-three out of 72 genera described were common to both populations whereas the other 39 genera, were either present in HD or SIgAd donors and belonged to the mycobiota rare biosphere. Genera only present in the SIgAd population were yeasts such as *Nakaseomyces* or *Metschnikowia* or filamentous fungi associated with diet or environment like *Aspergillus*, *Podosphaera* or *Claviceps*.

315

316 Analysis at the class, order, family, genus and species level revealed important differences 317 between both populations with a significant increase in C. albicans overgrowth in SIgAd 318 mycobiota (p=0.0046) (Fig 2, E). We furthermore observed a loss of species diversity among 319 the Candida genus (Fig 2, F). Whereas five Candida species, in particular C. sake, were present 320 in HD, only three *Candida* species were identified in SIgAd patients with *C. albicans* being the 321 most abundant. This phenomenon was also observed for Penicillium species. In contrast, we 322 observed an increase in the species diversity of the Kazarstania and Yaworria genera in fecal samples of SIgAd patients. Therefore, although IgA deficiency does not induce a global 323 324 dysbiosis, it is associated with a promotion of *C. albicans* development.

325

326 Mycobiota dysbiosis in symptomatic SIgAd patients

327 Although many IgA-deficient individuals are asymptomatic, some are affected by recurrent 328 infections, have metabolic and gastrointestinal diseases and are at greater risk for developing colorectal cancer.²⁴ To explore whether mycobiota composition could be related to clinical 329 330 phenotypes in the SIgAd population, we compared the intestinal mycobiota of HD and 331 asymptomatic SIgAd patients with that of symptomatic patients suffering from auto-immunity, 332 infections and/or intestinal disorders. The results show that alpha diversity, in particular the 333 Shannon, Simpson and invSimpson index, are significantly higher in symptomatic patients when compared with HD and SIgAd patients (Fig 3, A). No significant differences were 334

335 observed in alpha diversity metrics between mycobiota of HD and asymptomatic SIgAd 336 patients. Comparison of relative abundance at class, order, genus and species level pointed out 337 that *C. albicans* and *S. cerevisiae* were overrepresented in symptomatic patients or 338 asymptomatic patients, respectively (Fig 3, B). Analysis of the observed ASV in both groups 339 illustrated a shift of mycobiota composition related to clinical phenotype, with a significant 340 increase of *C. albicans* and a decrease of *S. cerevisiae* in symptomatic patients (Fig 3, C).

341

342 Symptomatic IgA-deficient individuals present with decreased Th17/22 counts

343 As C. albicans is known to have an inflammatory effect,⁶ we then compared the frequency of 344 cytokine-secreting circulating CD4⁺ T cells in asymptomatic and symptomatic IgA-deficient 345 individuals (Fig 3, D-E). Proportions of interferon- γ (IFN- γ)⁺CD4⁺ T cells differed between HD and asymptomatic but no differences were observed in SIgAd patients (18.8 \pm 14 % vs 26.1 \pm 346 6.4 %, p=0.516). IL-17⁺- and IL-22⁺-secreting CD4⁺ T cells were increased in both populations 347 348 of SIgAd patients however, these cell populations were significantly lower in symptomatic patients $(1.14 \pm 0.7 \% vs \ 2.5 \pm 0.9 \%, p=0.014 \text{ and } 0.75 \pm 0.5 \% vs \ 2.0 \pm 1.3 \%, p=0.019$ 349 350 respectively). Double-positive IFNy⁺IL-17⁺ CD4⁺ T cells were also decreased in symptomatic patients as compared with asymptomatic patients (0.18 \pm 0.1 % vs 0.4 \pm 0.2 , p=0.034 351 respectively). Despite the limited number of individuals studied, our results underscore a 352 353 significant decrease of systemic Th17-type lymphocyte responses in symptomatic IgA-354 deficient individuals.

355

356 Secretory IgA controls *C. albicans* development and intestinal cell invasion

357 The increase in *C. albicans* prevalence in the mycobiota of SIgAd donors prompted us to study

358 the impact of IgA responses in *C. albicans* yeast-to-hyphal transition, intestinal cell adhesion

and invasion. To this aim, we used an *in vitro* model of *C. albicans*-Caco-2 cells and IgA

360 purified from breast milk. The impact of purified systemic IgM from six SIgAd patients was 361 also evaluated. SIgA from nine different donors were found to significantly reduce C. albicans 362 hyphae development with reduction rates ranging from 10.27% to 23.8% (p=0.002; IgA versus no-IgA conditions) (Fig 4, A). Breast-milk-derived sIgA also reduced yeast adhesion to Caco-363 364 2 cells (reduction rates: 27.5% to 60%), although these differences did not reach statistical significance (Fig 4B). Interestingly, yeast intestinal cell invasion was significantly reduced by 365 366 sIgA from eight of nine donors tested with a mean reduction rate of $33.68 \pm 4.3\%$ (p=0.0012; 367 IgA versus no IgA conditions) (Fig 4, C). In comparison, purified IgM did not significantly impact hyphae development, nor intestinal cell adhesion or invasion. Thus, it appears that in 368 369 vitro antibody-mediated repression of C. albicans budding, intestinal cell adhesion and invasion 370 is mainly IgA-mediated.

371

372 Compensatory secretory IgM binding to commensal fungi in SIgAd patients

373 Although we did not *in vitro* measure a significant impact of IgM on C. albicans budding and 374 cell invasion, previous studies have nevertheless suggested that IgM might play in vivo a compensatory role which might explain why IgA deficiency is not associated with massive 375 perturbations of gut microbial ecology.^{16, 25, 26} To explore whether sIgM could exert a similar 376 effect resulting in the preservation of gut mycobiota homeostasis in SIgAd patients, we 377 378 analyzed the sIgM-opsonized fungal community in purified microbiota. Indeed, sIgM was 379 detected at mycobiota surface in all SIgAd patients tested (53.3 $\% \pm 16.2$) (Fig 5, A). We also 380 characterized sIgM responses in fecal waters (Fig 5, B). We observed a profile similar to that 381 observed for sIgA in HD population with a higher binding for those fungi that are less 382 represented in the gut, such as Aspergillus, Malassezia or Thrichosporon as compared with that observed for fungi from the Saccharomycetales order. This finding might suggest that 383

- antibodies specific for abundant targets, such as fungi from the *Saccharomycetales* order, are
 adsorbed at the surface of the latter and, are thus, less represented in fecal waters.
- 386

387 One classically described function of secretory immunoglobulins is immune exclusion of its 388 targets, in which antibody binding retains antigen in the intestinal lumen, thereby precluding the priming of other immune responses, and lethal dissemination of commensal bacteria.²⁷ To 389 390 indirectly determine whether sIgM could play a role in the immune exclusion of fungal targets, 391 we compared antifungal systemic IgG responses in SIgAd and HD subjects. The results show 392 that IgG responses in both groups were similar with only significant differences in IgG 393 responses against two fungal genera (Fusarium, p=0.0188; Trichosporon, p=0.0006) (Fig 5, C). 394 We then compared systemic anti-fungal IgM responses in both populations (Fig E3). Systemic 395 IgM bound a large range of commensal fungi in HD as well as in SIgAd patients, with a 396 comparable inter-individual variability. Finally, the profile of systemic IgA responses in HD 397 was compared with that observed for IgM in SIgAd population in order to unveil a potential 398 compensatory role for IgM. Our results show that the IgA and IgM recognition patterns of the 399 sporulated forms did not differ between both groups (Fig 5, D). In contrast, comparison of the 400 patterns of filamentous form recognition showed that systemic IgA had a more selective 401 reactivity profile than IgM, with a preferential targeting of the C. albicans filament. Altogether, 402 these results confirm a partial compensatory impact of IgM on gut mycobiota in the absence of 403 IgA that does not fully extend to *C. albicans*.

404 **Discussion**

405 Here we show that homeostatic IgA binds to distinct strains of commensal fungi with a body 406 fluid-selective pattern of recognition in a cohort of healthy subjects. The profile of fungal 407 recognition by free sIgA in fecal waters was characterized by a reduced binding to the more 408 abundant genera, Saccharomyces, Debaryomyces and Candida and was related with a high rate 409 of opsonization of mycobiota by intestinal IgA. In contrast, systemic IgA was found to 410 preferentially target genera mainly implicated in human allergic responses or invasive fungal 411 infections, including *Penicillium*, *Candida*, *Fusarium* and *Aspergillus*,^{6, 28-30}. Finally, breast 412 milk-derived IgA responses were directed against Candida, Debaryomyces and Malassezia, previously reported to colonize the mycobiota of healthy lactating mothers and their infants³¹ 413 414 and, in case of fungal overgrowth, to be associated with the occurrence of inflammatory bowel disease.32-34 415

416

417 To get further insight into the impact of IgA on host-mycobiota mutualism, we then compared 418 the gut mycobiota of IgA-deficient individuals and healthy subjects. Molecular characterization 419 of fungal commensal communities in both populations showed that IgA deficiency is not 420 associated with a significant perturbation of gut microbial ecology, given that, in SIgAd 421 patients, only an increase in intestinal diversity of fungal species belonging to the rare 422 mycobiota biosphere was observed. However, results from differential ASV analysis between 423 the mycobiota of HD and SIgAd patients assigned at order, class, family, genus and species 424 level showed an association of IgA deficiency with a significant proliferation of C. albicans. This proliferation was associated with a reduction of other Candida species in SIgAd patients, 425 426 as compared with HD, suggesting an elective role of sIgA on species regulation inside the 427 Candida genus or revealing the preferential fitness of C. albicans over other species. Intestinal 428 mycobiota analysis within the population of the SIgAd patients also revealed a significantly

higher diversity with an overrepresentation of C. albicans in those presenting clinical 429 430 complications that contrasted with the dominant presence of S. cerevisiae in asymptomatic SIgAd patients. The known anti-inflammatory and pro-inflammatory effects of S. cerevisiae³⁵ 431 432 and C. albicans⁶ respectively, might contribute to the clinical phenotype observed in the SIgAd 433 population. While gut proliferation of C. albicans is associated with an increased CD4⁺Th17 systemic response,³⁶ C. albicans SIgAd-colonized patients showed lower IL-17⁺CD4⁺, IL-434 $22^{+}CD4^{+}$ and IFN- $\gamma^{+}IL17^{+}$ responses. These results corroborate and extend previous findings 435 showing that systematic SIgAd is associated with immune response defects³⁷ that may account 436 437 for their mycobiota altered ecological status and clinical manifestations observed in some 438 SIgAd patients. This interpretation is nevertheless limited by the fact that we did not have access to gut biopsies in order to confirm that a global Th17 decrease is observed in 439 440 symptomatic SIgAd patients.

441

442 C. albicans is a commensal fungus of the human gut, but also causes life-threatening invasive infections.³⁸ As reported previously in an experimental mouse model^{9, 15} and corroborated by 443 the present study, the pathogenic hyphal morphotype of C. albicans, once established, is 444 445 targeted by intestinal IgA. However, the *in vivo* study of IgA C. albicans-interaction on human fungal-host mutualism is challenging given that C. albicans is not a mouse commensal. The 446 447 demonstration in the present study that sIgA significantly reduces hyphae development 448 corroborates a previous report showing that the lack of sIgA, directed against the C. albicans-449 specific hypha antigen Sap6, in Crohn disease patients was associated with an increase in hyphal fungal morphology.¹⁵ Moreover, sIgA was found to significantly reduce C. albicans 450 451 intestinal cell invasion, thereby implying that C. albicans opsonization by sIgA controls fungal 452 fitness and could reduce pathogenic fungal migration through the intestinal barrier.

453 It has been previously reported that IgA uniquely uncouples fungi colonization from pathogenesis in humans.⁹ The clear in vivo and in vitro effects of IgA on C. albicans 454 455 development notwithstanding, it is important to stress that increased C. albicans fitness leading 456 to invasive or mucocutaneus candidiasis is never observed in human IgA deficiency.³⁹ 457 Reminiscent to the compensatory effect of IgM specific for bacterial microbiota in human IgA 458 deficiency, we show that, in SIgAd patients, gut mycobiota is opsonized by IgM and that free 459 sIgM is able to recognize a diversity of fungal genera. The lack of gut fungal dysbiosis in these 460 patients therefore points to a compensatory role of IgM-fungal opsonization in order to maintain 461 mycobiota diversity. Our results also suggest that IgM might play an important role in 462 maintaining intestinal barrier integrity and controlling fungal antigen transfer because systemic 463 IgG responses against both sporulated and filamentous forms of commensal fungi are not increased. Moreover, profiles of systemic IgA responses in HD against sporulated forms are 464 465 reminiscent of IgM responses in SIgAd patients, except for the hyphae forms of C. albicans, 466 for which IgA shows a higher selectivity. The later result is corroborated by the fact we did not 467 observe a significant impact of IgM on C. albicans in vitro budding and cell invasion and could 468 consequently explain the C. albicans overgrowth observed in SIgAd patients. IgM has nevertheless a 469 broad fungal reactivity that could be related to low affinity interactions of this isotype for the β -(1,3)-glucan and chitin antigens which are conserved major fungal cell wall components.⁴⁰ 470

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Altogether, we show here that secretory and systemic IgA is able to recognize a large range of fungal members of the human mycobiota and that it participates in fungal-host mutualism. At the species level, intestinal *C. albicans* overgrowth in human IgA deficiency underscores the pivotal role IgA in the intestinal fitness of this pathobiont fungus. Finally, the importance of IgA-mediated fungal recognition in healthy subjects and the paradoxical lack of fungal dysbiosis in human IgA deficiency could be explained by a partial compensatory role of IgM.

478 Author contributions

479 A. Moreno-Sabater, D. Sterlin, C. Fieschi, M. Malphettes, J. Fadlallah collected and prepared 480 the specimens from healthy donors and selective IgA deficient patients. V. Rigourd provided 481 breast milk samples. A. Moreno-Sabater, A-C. Normand, C. Bachmeyer, C. Hennequin 482 provided fungal strains. A. Moreno-Sabater, D. Sterlin, L. Imamovic, F. Bon, A-C. Normand, 483 C. Gonin, M. Bensalah, M. Gazzano, K. Dorgan, E. Ben Salah, A. Acherar, C. Parizot, F. Dalle, 484 J. Fadlallah designed and performed experiments. A. Moreno-Sabater, D. Sterlin, Lejla 485 Imamovic, A-C Normand, A. Acherar performed data analysis. A. Moreno-Sabater, D. Sterlin, L. Imamovic, A-C. Normand and prepared figures. A. Moreno-Sabater, D. Sterlin, L. 486 487 Imamovic, Hans Yssel, J. Fadlallah, G. Gorochov designed the study and wrote the manuscript. 488

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597 Figures Legends

598 Figure 1. Selective homeostatic targeting of fungal community members by IgA shows body 599 fluid-specific features. (A) Flow cytometry protocol used to probe IgA binding to fungal strains 600 (yeast/spores or hyphae forms) following incubation with fecal water, serum or breast milk and 601 secondary fluorophore-conjugated anti-IgA. (B) Analysis of human fecal free IgA binding 602 (n=34) to a panel of cultivated strains covering 14 fungal genera. (C) Protocol used to probe 603 immunoglobulins (Ig)-opsonized fungal populations of the human gut microbiota using flow 604 cytometry. Calcofluor white (CFW) staining. Percentage of the gut mycobiota opsonized by 605 secretory (s) IgA or IgM in healthy donors (n=28). (D-E) Analysis of systemic (n=31) and 606 breast milk (n=20) IgA binding. (F-G) Principal component analysis of data from B, D, E 607 figures and factorial analysis (variable relative contributions indicated by length and direction 608 of respective arrow). Ellipses represent the 95% CI of patient distribution in each group. (B, D, 609 E) Results are displayed as median fluorescence intensity (MFI) values. Bar plots show 610 individual data with mean + s.d. Sac: Saccharomyces; Deb: Debaryomyces; Can: Candida; 611 Cyb: Cyberlindnera; Yar: Yarrowia; Klu: Kluyveromyces; Asp: Aspergillus; Pen: Penicillium; 612 Fus: Fusarium; Acr: Acremonium; Rho: Rhodotorula; Mal: Malassezia; Tri: Trichosporon; 613 Cry: Cryptococcus. Images were created with BioRender.com.

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Figure 2. Fungal gut ecology perturbation in selective IgA deficient (SIgAd) patients. (A) Strategy used to assess gut immunoglobulins (Ig) opsonized fungal population by flow cytometry and to characterize mycobiota composition by internal transcribed spacer (ITS) gene sequencing in healthy donors (HD) and SIgAd patients. (B) Proportion of CFW^{bright} population and double IgA⁺CFW^{bright} population among total gut microbiota in HD (n=28) and SIgAd (n=12). (C) Alpha diversity index in HD (n=23) and SIgAd (n=17). ASV: Amplicon sequence variant. (D) Observed ASV inside the Ascomycota and Basidiomycota phylum of the studied

622 populations. (E) Differential ASV between HD and SIgAd assigned at order, class, family, 623 genus and species level, ranked by statistical difference. Blue histograms represent fungal 624 population overrepresented in HD and red histograms in SIgAd patients. Significant differences 625 determined by Wilcoxon/Kruskal-Wallis test (*p<0.05; **p<0.01). (F) Changes in the relative 626 abundance at species level in the Candida, Penicillium, Kazachtania and Yarrowia genera. Patients with residual secretory IgA are colour-coded (black symbols). Bar plots show 627 628 individual data with mean + s.d. (B-D). Indicated p values were calculated using a Mann-629 Whitney test. Images were created with BioRender.com

631 Figure 3. Clinical status of healthy donors (HD) and selective IgA deficient (SIgAd) patients 632 in relation with mycobiota composition and circulating CD4⁺ T cells. (A) Alpha diversity in 633 twenty-three HD and five asymptomatic (Asym) and eleven symptomatic (Sym) SIgAd 634 patients. Sym patients suffer from infections and/or autoimmune and/or intestinal 635 complications. ASV: amplicon sequence variants. (B) Differential ASV between asym and sym 636 patients assigned at order, class, family, genus and species level, ranked by statistical 637 difference. Red and black histograms represent fungal population overrepresented in asym and 638 in sym patients, respectively. F.i.s: fam incertae sedis; c.n.a.: class not assigned. Significant 639 differences determined by Wilcoxon/Kruskal-Wallis test (*p<0.05; **p<0.01). (C) Number of 640 observed ASV for C. albicans and S. cerevisiae in gut mycobiota of HD, asym and sym SIgAd patients. (D) Systemic inflammatory markers. Graphs show percentages of CD4⁺ T cells 641 642 expressing indicated cytokines determined by intracellular staining. (E) CD4⁺IL-22⁺, IL17⁺, IFNg⁺IL17⁺ data from sym, and asym patients were grouped by hierarchical cluster analysis 643 644 according to Ward's method and plotted as a heat map. For all panels, bar plots show individual 645 data with mean \pm s.d, and p values were calculated with a Mann-Whitney test.

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Figure 4. Impact of secretory IgA and IgM on C. albicans host mutualism. (A) C. albicans 647 648 (yeast form) was incubated in medium alone or in the presence of purified secretory IgA (sIgA) 649 or systemic IgM (B-C) in absence or presence of human intestinal Caco-2 cell monolayer. For 650 hyphae assay, C. albicans (yeast form) was incubated alone or in the presence of sIgA or IgM 651 for 3 hours at 37°C and observed with a BX51 microscope using X40 magnification (Olympus). 652 For adherence and invasiveness assays, C. albicans (yeast form) pre-incubated with or without 653 purified antibodies for 30 min or 2 hours, respectively, at 37°C. C. albicans assay concentration 654 were 5.10⁴ cfu/mL. Purified sIgA from nine healthy lactating women and purified IgM from 655 six SIgAd patients were used at a final concentration of 100 µg/mL. Bar plots show the ratio 656 between IgA or IgM conditions and control conditions of three independent assays, P values 657 were calculated using a Mann-Whitney test. Images were created with BioRender.com.

659 Figure 5. Compensatory role of IgM in selective IgA deficiency (SIgAd). (A) Percentage of 660 the gut mycobiota fraction opsonized by secretory IgM in healthy donors (HD, n=28) and 661 SIgAd patients (n=12) determined by flow cytometry. (B) SIgAd fecal water IgM binding (n=21) to yeast and hyphae forms of 12 commensal fungi. Plots show individual data with mean 662 663 \pm s.d. (C) Characterization of IgG systemic response in HD (n=31) and SIgAd (n= 21). Box 664 limits: upper and lower quartiles. MFI: Mean fluorescence intensity. (D) Radar plot comparison 665 of systemic IgM responses in SIgAd patients versus IgA responses in HD against spores/yeast or hyphae forms of cultivated commensal fungi. Sac: Saccharomyces; Deb: Debaryomyces; 666 667 Can: Candida; Cyb: Cyberlindnera; Yar: Yarrowia; Klu: Kluyveromyces; Asp: Aspergillus; 668 Pen: Penicillium; Fus: Fusarium; Acr: Acremonium; Rho: Rhodotorula; Mal: Malassezia; Tri: 669 Trichosporon; Cry: Cryptococcus.

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671 Figure E1. (A) Gatting strategy to analyze intestinal IgA-opsonized mycobiota. A minimal 672 mock mycobiota corresponding to the most abundant fungal genera from the human gut 673 mycobiota (Saccharomyces, Candida, and Debaryomyces) was used to gate on fungal events 674 among the complex purified microbiota from a representative healthy donor (HD). Calcofluor 675 white (CFW) fungal-selective staining was used to probe fungal populations among gut 676 microbiota. The percentage of IgA-opsonized mycobiota among CFW^{bright} population is 677 indicated. (B-C) Systemic and fecal water immunoglobulin levels in HD (n=31) and selective 678 IgA-deficient (SIgAd) patients (n=21). Bar plots show individual data with mean + s.d. 679 Indicated *p* values were calculated using a Mann-Whitney test.

681 Figure E2. Comparative analysis of gut mycobiota diversity performed at genus level 682 highlights quantitative dominance of few genera from the Ascomycota phylum in both healthy 683 donors (HD) and in selective IgA deficient (SIgAd) patients. Analysis of fungal genera, relative 684 abundance and prevalence does not show significant differences between HD (n=23) and SIgAd 685 patients (n=17) suggesting that compensatory mechanisms occur in SIgAd to preserve gut 686 fungal diversity (Mann-Whitney and Chi-square tests). Bar plots show relative abundance 687 (RA), calculated as log 10 (1/RA). In circles are indicated the prevalence of fungal genera in 688 HD (blue) as compared with SIgAd (red). The phylogenetic tree was constructed using Mega 689 X program and iTOL web application (https://itol.embl.de/ last accessed: 11/2021).

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Figure E3. Systemic IgM response against commensal fungi. Characterization by flow cytometry of IgM systemic responses in healthy donors (HD, n=31) and selective IgA deficient patients (SIgAd, n=21). Box limits: upper and lower quartiles. MFI, Mean fluorescence intensity. Sac: *Saccharomyces*; Deb: *Debaryomyces*; Can: *Candida*; Cyb: *Cyberlindnera*; Yar:

- 695 Yarrowia; Klu: Kluyveromyces; Asp: Aspergillus; Pen: Penicillium; Fus: Fusarium; Acr:
- 696 Acremonium; Rho: Rhodotorula; Mal: Malassezia; Tri: Trichosporon; Cry: Cryptococcus.

Figure 1

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Figure 3



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Figure 4









Figure E2

Relative abundance



