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Dorra Elhaj Mahmoud, Mariem Hanachi, Hajar Yaakoub, Simon Blanchard,
Pascale Pignon, Oussama Souiai, Yves Delneste, Jean-Philippe Bouchara, Nicolas
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► **To cite this version:**

Dorra Elhaj Mahmoud, Mariem Hanachi, Hajar Yaakoub, Simon Blanchard, Pascale Pignon, et al.. Functional insights into human macrophage response against *Scedosporium apiospermum* and *Scedosporium dehoogii*. *Cytokine*, 2023, 172, pp.156384. <10.1016/j.cyto.2023.156384>. <hal-04269253>

HAL Id: hal-04269253

<https://univ-angers.hal.science/hal-04269253v1>

Submitted on 11 Apr 2025

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1 **Functional insights into human macrophage response against *Scedosporium***
2 ***apiospermum* and *Scedosporium dehoogii***

3 Dorra Elhaj Mahmoud^a, Mariem Hanachi^b, Hajar Yaakoub^a, Simon Blanchard^c, Pascale
4 Pignon^c, Oussama Souiai^b, Yves Delneste^{c,d}, Jean-Philippe Bouchara^a, Nicolas Papon^a, Anaïs
5 Hérivaux^a

6 ^a Univ Angers, Univ Brest, IRF, SFR ICAT, 49000, Angers, France.

7 ^b Laboratory of Bioinformatics, Biomathematics and Biostatistics-LR16IPT09, Institut Pasteur de
8 Tunis, Tunis, Tunisia.

9 ^c Univ Angers, Nantes Université, Inserm, CNRS, CRCI²NA, SFR ICAT, F-49000 Angers, France.

10 ^d Immunology and Allergology laboratory, University Hospital of Angers, Angers, France.

11 **Corresponding author**

12 Dr. Anaïs Hérivaux (PharmD, PhD)

13 Fungal Respiratory Infections Unit

14 University of Angers, IRIS-IBS

15 4, rue Larrey – 49933 Angers

16 France

17 +33 (0) 244 688 363

18 anais.herivaux@univ-angers.fr

19

20 **Abstract**

21 Fungal infections caused by *Scedosporium* species are rising among immunocompromised
22 and immunocompetent patients. Within the immunocompetent group, patients with cystic
23 fibrosis (pwCF) are at high risk of developing a chronic airway colonization by these molds.
24 While *S. apiospermum* is one of the major species encountered in the lungs of pwCF, *S.*
25 *dehoogii* has rarely been reported. The innate immune response is believed to be critical for
26 host defense against fungal infections. However, its role has only recently been elucidated and
27 the immune mechanisms against *Scedosporium* species are currently unknown. In this
28 context, we undertook a comparative investigation of macrophage-mediated immune
29 responses toward *S. apiospermum* and *S. dehoogii* conidia. Our data showed that *S.*
30 *apiospermum* and *S. dehoogii* conidia strongly stimulated the expression of a set of pro-
31 inflammatory cytokines and chemokines such as IL-1 β , IL-8, IL-6 and TNF α . We
32 demonstrated that *S. dehoogii* was more potent in stimulating the early release of pro-
33 inflammatory cytokines and chemokines while *S. apiospermum* induced a late inflammatory
34 response at a higher level. Flow cytometry analysis showed that M1-like macrophages were
35 able to internalize both *S. apiospermum* and *S. dehoogii* conidia, with a similar intracellular
36 killing rate for both species. In conclusion, these results suggest that M1-like macrophages
37 can rapidly initiate a strong immune response against both *S. apiospermum* and *S. dehoogii*.
38 This response is characterized by a similar killing of internalized conidia, but a different time
39 course of cytokine production.

40 **Keywords:** *Scedosporium*, Macrophages, Inflammation, Immune response, Cytokines, Gene
41 expression

42

43 **1 Introduction**

44 *Scedosporium* species are environmental filamentous fungi that usually live as
45 saprophytes in highly polluted soils and contaminated water [1]. They are considered as
46 emerging fungal pathogens in both immunocompetent and immunocompromised individuals.
47 *Scedosporium* infections result in a wide range of clinical manifestations, from colonization
48 of the respiratory tract in patients with cystic fibrosis (pwCF) and subcutaneous mycetomas to
49 severe and often fatal disseminated infections in immunocompromised patients [2,3]. Within
50 the immunocompetent group, pwCF are among those at the highest risk of suffering from
51 these mycoses [4,5]. In fact, after *Aspergillus fumigatus*, *Scedosporium* species are the second
52 most frequent molds involved in the colonization of the airways of these patients with a
53 frequency ranging from 2 to 17.4% depending on the geographical area and the group of
54 pwCF studied [4,6,7]. Generally, the distribution of *Scedosporium* species in the CF lung
55 follows their respective frequency in the environment with the exception of *Scedosporium*
56 *dehoogii* [8]. Conversely to *Scedosporium apiospermum* and some other *Scedosporium*
57 species, *Scedosporium dehoogii* has only been isolated once from respiratory secretions,
58 whereas it is common in soil, thus questioning its ability to colonize the respiratory tract of
59 pwCF [9]. A comparative genomic analysis of the main *Scedosporium* species revealed a
60 number of candidate genes potentially involved in the adaptive mechanisms of pathogenic
61 *Scedosporium* species within the respiratory tract of pwCF. Interestingly, more than a hundred
62 genes exhibit point mutations that potentially lead to a loss of function in *S. dehoogii*,
63 including genes encoding some transcription factors, as well as some GPI-anchored proteins
64 and two thioredoxin-like proteins [10].

65 The airways are considered to be the primary route used by *Scedosporium* to colonize
66 or infect the host. In order to colonize the respiratory tract, fungi must evade the host immune
67 response. In the lungs, macrophages are one of the first lines of antifungal defense [11,12].
68 According to their activation state and function, macrophages can be broadly divided into
69 M1-type (pro-inflammatory phenotype) and M2-type (wound healing phenotype) [13,14]. M1
70 macrophages are characterized by CD80, CD86, CD40 and major histocompatibility complex
71 class II (MHC-II) surface molecules, whereas M2 macrophages express M2-specific markers
72 such as CD163 and CD206 [14]. It is now recognized that the M2 type comprises a diverse
73 group of macrophages rather than a single activation state. Accordingly, M2 macrophages can
74 be further-categorized into distinct subsets: M2a, M2b, M2c and M2d, based on their distinct
75 transcriptional profiles and biological functions [15]. Unlike M2 macrophages, M1

76 macrophages are typically fungicidal cells and their activation provides a host protection
77 against a myriad of fungal infections [16]. The common response of M1 macrophages to
78 fungal infections involves variety of pattern recognition receptors (PRRs) which may act
79 individually or in concert [13,17]. *Scedosporium* cell wall components, including
80 peptidorhamnomannans (PRMs), α -glucans, and glucosylceramides, are important activators
81 of immune cells following their recognition by Toll-like receptor 2 (TLR2), TLR4, and
82 Dectin-1 and through receptors that are yet unknown [18]. Following fungal recognition, M1
83 macrophages produce pro-inflammatory cytokines (dominated by interleukin (IL)-1 β , IL-6,
84 IL-12, IL-23 and Tumor Necrosis Factor (TNF) α) and chemokines that can recruit other
85 immune cells that contribute to fungal clearance [19,20]. Phagocytosis and the intracellular
86 killing of fungal elements constitute other key elements of the M1 macrophage-mediated
87 immune response [21,22].

88 Remarkably, very little research has focused on understanding the immune response
89 against *Scedosporium* species [23,24]. Among the unknowns that remain to be elucidated, the
90 most important is the complexity of the mechanisms developed by these fungi to evade the
91 host immune response and the differences in the inflammatory response induced by each
92 species. This may explain, at least in part, the inability of *S. dehoogii* to develop in the
93 respiratory tract and to cause a chronic colonization of the airways. In this context, we
94 undertook a comparative investigation of the M1-like macrophage-mediated immune
95 response upon conidia of *S. apiospermum* and *S. dehoogii*.

96 **2 Materials and Methods**

97 **2.1 *Scedosporium* strains and culture conditions**

98 The study was performed on two *Scedosporium* strains: *S. apiospermum* IHEM 14462,
99 originally isolated from a sputum sample from a CF patient and previously used for whole-
100 genome sequencing [25] and *S. dehoogii* UA 110354521 isolated from a soil sample from
101 Meknes (Morocco).

102 The strains were maintained at 37°C by weekly passages on potato dextrose agar (PDA)
103 (Condalab, containing in g/L: dextrose, 20; potato-infusion extract, 4; and bacteriological
104 agar, 15), supplemented with 0.5 g/L chloramphenicol. Conidia used in each experiment were
105 collected from 8-day-old cultures. Conidia were harvested from agar plates using sterile
106 water; then the obtained suspensions were filtered through sterilized Miracloth[®] (pore size

107 22–25 µm) to remove hyphae, and conidia in the filtrates were enumerated by
108 haematocytometer counts.

109 **2.2 M1-like macrophage generation and co-culture assays**

110 Peripheral blood mononuclear cells (PBMC) were obtained from healthy human
111 volunteers (Blood Collection Center, Angers, France; agreement CPDL-PLER-2021 038
112 approved by the Ethics Committee of the University Hospital of Angers) by standard density
113 gradient centrifugation on Lymphocyte Separation medium (Eurobio). To generate
114 macrophages, monocytes were purified from PBMC by positive selection using anti-CD14
115 mAb-coated magnetic beads (Miltenyi Biotec). Monocytes were cultured for 5 days in a
116 complete medium consisting of RPMI 1640 (Lonza) supplemented with 10% fetal calf serum
117 (Eurobio), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids,
118 10 mM HEPES, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Lonza) in the
119 presence of GM-CSF (50 ng/mL, R&D Systems) to generate M1-like macrophages. For
120 interaction studies, M1-like macrophages (5×10^5 /well in 24-well plates) were incubated with
121 *S. apiospermum* or *S. dehoogii* conidia at a 1:1 ratio at 37°C under 5% CO₂. Higher
122 macrophage-conidia ratios (e.g. 1:10) were not considered in this study since we observed
123 important cell death in M1-like macrophages 24 h after co-incubation.

124 **2.3 Analysis of immune-relevant gene expression by qPCR**

125 Total RNA was isolated from M1-like macrophages after 3 h and 24 h of co-culture
126 with *S. apiospermum* and *S. dehoogii* conidia using the PureLink RNA Mini Kit (Thermo
127 Scientific). The Qubit[®] 2.0 Fluorometer (Invitrogen) was used for RNA quantification. Total
128 RNA was reverse transcribed using the High-capacity cDNA Reverse Transcription Kit
129 (Applied Biosystems). After a 5-fold dilution, cDNA samples were used as templates for real-
130 time quantitative PCR (qPCR) using the StepOnePlus[®] thermocycler (Applied Biosystems).
131 Each qPCR reaction was performed in a final volume of 20 µL containing PowerUp SYBR
132 Green Master Mix (Applied Biosystems), 250 nM or 500 nM of each primer (primer
133 sequences are listed in Table 1), 6 µL of water, and 2 µL of diluted cDNA. PCR cycling
134 parameters were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for
135 3 s, and cooled to 60°C for 30 s (Fast cycling mode). Then, the PCR products were heated to
136 95°C for 15 s, cooled to 60°C for 1 min, and heated to 95°C to measure the dissociation
137 curves. Five-point serial dilutions of cDNA were used as templates to construct a standard
138 curve for each gene and determine the amplification efficiency (E). Thanks to its limited

139 variation between the different samples, glyceraldehyde-3-phosphate dehydrogenase
140 (GAPDH) was selected as an endogenous reference. The relative gene expression level (RQ)
141 was calculated using the $2^{-\Delta\Delta\text{Cycle.Threshold (CT)}}$ method according to the following formula.

142 $\Delta\text{CT experiment (stimulated macrophages)} = \text{CT target gene} - \text{CT GAPDH}$

143 $\Delta\text{CT control (untreated macrophages)} = \text{CT target gene} - \text{CT GAPDH}$

144 $\text{RQ} = 2^{-(\Delta\text{CT experiment} - \Delta\text{CT control})}$

145 **2.4 Multiplex cytokine/chemokine analysis**

146 Supernatants were collected after 3 h and 24 h of M1-like macrophage co-culture with
147 *S. apiospermum* and *S. dehoogii* conidia. They were centrifuged at 1,700 rpm at 4°C for 10
148 min, and stored at -80°C until use. Secreted cytokines and chemokines were measured in the
149 supernatants by multiplex fluorescent bead-based technology (Luminex 200), using a
150 commercial Luminex Screening Assay Kit from R&D Systems according to the
151 manufacturer's recommendations. Briefly, the samples were diluted 1:2 before incubation
152 with specific antibody-coated fluorescent beads. After washing, 50 beads were analyzed using
153 the Luminex 200 analyzer and Bio-Plex Manager software version 6 (Bio-Rad Laboratories),
154 and the analyte concentrations of the samples were estimated through the serial dilution of
155 cytokine standards: IL-1 β , sensitivity < 10 pg/mL; IL-6, sensitivity < 5 pg/mL; IL-8 (also
156 called C-X-C motif Ligand 8 (CXCL8)), sensitivity < 10 pg/mL; IL-18, sensitivity < 5
157 pg/mL; IL-23, sensitivity < 100 pg/mL; TNF α , sensitivity < 5 pg/mL; C-C motif chemokine
158 Ligand 2 (CCL2), sensitivity < 25 pg/mL; CCL3, sensitivity < 50 pg/mL; CCL5,
159 sensitivity < 10 pg/mL; and CXCL2, sensitivity < 70 pg/mL.

160 **2.5 Conidial killing experiments**

161 *S. apiospermum* and *S. dehoogii* conidia were stained with 40 $\mu\text{g/mL}$ of fluorescein
162 isothiocyanate (FITC; Sigma-Aldrich) in phosphate-buffered saline (PBS, 1X) for 30 min in
163 the dark with constant shaking (120 rpm), washed twice with PBS, and enumerated. FITC-
164 labelled conidia (5×10^5 cells) were co-cultured with adherent M1-like macrophages (cell
165 ratio of 1:1). The cells were then incubated for 2 h at 37°C under 5% CO₂ to allow the
166 internalization of the conidia. The medium containing the non-ingested conidia was removed,
167 wells were washed twice with pre-warmed PBS and a new medium was added. To measure
168 the conidiacidal capacity, M1-like macrophages were allowed to kill the ingested conidia for
169 3 h at 37°C under 5% CO₂. After the second incubation period, the medium was removed, and
170 the wells were washed twice with PBS. After microscopic examination to visualize adherence

171 and ingestion of the conidia, cells were lifted from the wells by scraping, transferred to
172 Eppendorf tubes, and centrifuged at 4,600 rpm for 5 min to remove PBS. The cell pellets were
173 resuspended with
174 500 μ L of water and incubated for 30 min to allow lysis of M1-like macrophages and release
175 of intracellular conidia. After microscopic control of macrophage lysis, the resulting
176 suspensions were centrifuged at 4,600 rpm for 5 min to pellet the conidia. Macrophage debris
177 was removed by treating the suspensions with DNase, RNase, and proteinase K (all from
178 Invitrogen). A negative control reflecting the live conidia (conidia not exposed to
179 macrophages) and a positive control referring to heat-killed conidia (85°C, 30 min) were
180 included in each assay. Conidia were then labelled with 25 μ g/mL propidium iodide (PI;
181 Sigma-Aldrich) for 30 min at room temperature in the dark prior to flow cytometry analysis.
182 Both controls were used to establish cut-offs for positivity of each marker. Conidia were
183 considered alive if they were only FITC positive and dead if they were double positive (FITC⁺
184 PI⁺). The percentage of killing was defined as the number of conidia killed by the
185 macrophages divided by the total number of conidia released by macrophages \times 100.

186 **2.6 Protein–protein interaction (PPI) networks and biological process analysis**

187 Significantly differentially expressed genes and cytokines in response to conidial
188 challenge were used to construct PPI networks. The Search Tool for the Retrieval of
189 Interacting Genes/Proteins database (STRING v11.5) was used to construct and analyze all
190 PPI networks. PPIs were restricted to those with a confidence of more than 90% as calculated
191 by the String system. We also performed pathway enrichment analysis for each PPI network
192 to identify significantly enriched Gene Ontology (GO) biological processes in each set of
193 genes.

194 **2.7 Statistical analysis**

195 All experiments were performed in triplicate at least four times independently.
196 Statistical analysis and visualization were performed using the R 4.3.1 and GraphPad Prism9.
197 To assess the global transcriptomic response, principal component analysis was performed on
198 log-transformed fold-change ($\log_2(\text{FC})$) data obtained from qPCR. To illustrate gene
199 expression, heatmaps were generated based on $\log_2(\text{FC})$ and using the ComplexHeatmap R
200 package. Hierarchical clustering was performed based on complete linkage and Euclidean
201 distance measure. A significant difference in gene expression levels was defined as at least a
202 2- fold change up to or down, corresponding to $\log_2(\text{FC}) \geq 1$ or $\log_2(\text{FC}) \leq -1$ and a p -value

203 < 0.05 (treated M1-like macrophages vs. untreated cells). Differences between groups were
204 determined by a Mann-Whitney test (2 groups) or a one-way analysis of variance Kruskal-
205 Wallis test followed by Dunn's multiple comparison test (3 groups) using the R package
206 ggpurb. In all analyses, p -values < 0.05 were considered statistically significant.

207 **3 Results**

208 **3.1 *Scedosporium apiospermum* and *Scedosporium dehoogii* induce distinct** 209 **temporal transcriptomic profiles in M1-like macrophages**

210 To assess both the early (3 h) and late (24 h) macrophage response, we measured the
211 relative transcript levels of 19 receptors, cytokines, and chemokines from macrophages
212 challenged with conidia of both *S. apiospermum* and *S. dehoogii*. The overall expression
213 profile was subsequently explored by performing multivariate analysis based on principal
214 component analysis (PCA). PC1 accounted for 33.2% of the variability while PC2 explained
215 25.6% of variability (Fig. 1A).

216 Despite the overlapping confidence ellipses, the non-close centroids discriminated the early
217 and the late M1-like macrophage response against the two fungi (Fig. 1A, B and
218 Supplementary Table S1).

219 Furthermore, our global analysis highlighted a segregation in the early macrophage immune
220 response against *S. apiospermum* and *S. dehoogii* (Fig. 1A).

221 Taken together, these results suggest a distinct temporal M1-like macrophage transcriptional
222 profile after *Scedosporium* challenge.

223 **3.2 *Scedosporium dehoogii* is a more potent inducer of the early M1-like** 224 **macrophage transcriptomic response**

225 We further investigated the transcriptional profiles of M1-like macrophages after co-
226 culture with *S. apiospermum* or *S. dehoogii* by generating heatmaps of gene expression. We
227 then used the Mann-Whitney U test to determine the statistical differences between untreated
228 and infected macrophages, and between macrophages infected with *S. apiospermum* or *S.*
229 *dehoogii* conidia.

230 After 3 h of co-culture, 9 genes were upregulated in response to *S. apiospermum* conidia
231 compared with the untreated control (Fig. 2A, B and Supplementary Table S2). These genes

232 encode chemokines (IL-8, CXCL1, CXCL2, CCL3, and CCL5) and pro-inflammatory
233 cytokines (IL-1 α , IL-1 β , IL-6, and TNF α). With the exception of CXCL1, a similar profile
234 was observed for *S. dehoogii* (Fig. 2A and B). At the gene level, *S. dehoogii* conidia induced
235 significantly higher expression levels of genes encoding IL-8, IL-1 α , IL-1 β , CCL5, and TNF α
236 in M1-like macrophages than *S. apiospermum* conidia after 3 h of stimulation (Fig. 3A and
237 Supplementary Table S4). These differences were particularly observed for IL-1 α (572 fold
238 for *S. dehoogii* vs. 102 fold for *S. apiospermum*, p -value = 0.0079) and IL-8 expression (304
239 fold for *S. dehoogii* vs. 47 fold for *S. apiospermum*, p -value = 0.0079).

240 After 24 h of co-culture, 9 genes (encoding IL-1 α , IL-1 β , IL-6, IL-8, CXCL2, CCL3, CCL5,
241 TNF α , and IL-23A) were upregulated and 5 (encoding CCL2, IL-18, Dectin-1, HLA-DRA,
242 and TLR4) were downregulated in response to both fungal species compared to the untreated
243 control (Fig. 2C, D and Supplementary Table S3). Among these genes, IL-8 expression was
244 statistically more induced when M1-like macrophages were challenged with *S. dehoogii*
245 compared to *S. apiospermum* (1,700 fold for *S. dehoogii* vs. 154 fold for *S. apiospermum*, p -
246 value = 0.0285) (Fig. 3B and Supplementary Table S5). No significant changes were detected
247 in transcription of the genes encoding CXCL9, interferon gamma (IFN γ), IL-15, and TLR2,
248 regardless of the interactions and experimental time point.

249 Collectively, these data show that *S. apiospermum* and *S. dehoogii* conidia induce a common
250 early and late transcriptome signature (stimulated vs. untreated cells). However, the intensity
251 of the early induced response is species-specific.

252 **3.3 The cytokine release profile of M1-like macrophages confirms the strong** 253 **early pro-inflammatory potential of *Scedosporium dehoogii***

254 Since the transcriptomic analysis suggested that both *S. apiospermum* and *S. dehoogii*
255 stimulate the inflammatory response of macrophages, we aimed to verify these observations
256 at the protein level. In this line, we assessed the M1-like macrophage production of cytokines
257 and chemokines, which the corresponding gene expression level changed significantly, 3 h
258 and

259 24 h after challenge with conidia. The results are summarized in Figure 4. After exposure to
260 *S. dehoogii* conidia for 3 h, M1-like macrophages produced significantly higher levels of the
261 pro-inflammatory cytokines TNF α , IL-6, IL-23, and IL-1 β and the chemokines CCL3, CCL5,
262 and CXCL2 compared to unstimulated macrophages. Except for CCL3, CXCL2, and TNF α ,
263 *S. apiospermum* conidia did not induce higher levels of pro-inflammatory cytokines and

264 chemokines after 3 h of M1-like macrophages co-culture (Fig. 4A and Supplementary Table
265 S6). Furthermore, *S. dehoogii* conidia tended to induce a strong and early pro-inflammatory
266 response (but not statistically significant) compared to *S. apiospermum* (Fig. 4B). IL-8 and
267 CCL2 cytokine levels were too highly expressed after macrophages challenge with both fungi
268 to be evaluated precisely by the assay. At the 24 h time point, higher levels of IL-1 β , IL-6, IL-
269 18, IL-23, CXCL2, TNF α , CCL3 and CCL5 were produced by M1-like macrophages when
270 compared to the control (Fig. 4C and Supplementary Table S7). Interestingly, *S. apiospermum*
271 induced a higher production of the majority of the studied cytokines and chemokines than *S.*
272 *dehoogii* after 24 h of co-culture with M1-like macrophages (Fig. 4D).

273 Overall, these results are consistent with the transcriptomic profile of stimulated macrophages
274 and support that *S. dehoogii* exhibits a stronger early pro-inflammatory response compared to
275 *S. apiospermum*.

276 **3.4 *Scedosporium dehoogii* polysaccharides may orchestrate the specific early** 277 **M1-like macrophage response**

278 Given that *S. dehoogii* induced a strong early inflammatory response, we aimed to
279 investigate potentially relevant interactions between the induced mediators of inflammation.
280 The cytokines and chemokines, which were statistically upregulated (expression and/or
281 production *vs.* control) after macrophage co-culture with *S. dehoogii* conidia for 3 h, were
282 used to construct a protein–protein interaction (PPI) network. A high confidence threshold
283 (90%) was applied to counteract potential background. The network in Figure 5 represents 11
284 cytokines rapidly stimulated by *S. dehoogii* conidia (IL-1 α , IL-1 β , IL-6, IL-8, IL-18, IL-23,
285 CXCL2, CCL2, CCL3, CCL5 and TNF α).

286 GO analysis showed that the induced cytokines and chemokines were part of many biological
287 processes especially cellular response to lipopolysaccharide, neutrophil chemotaxis, and
288 myeloid leukocyte migration.

289 Taken together, these results suggest the involvement of *S. dehoogii* polysaccharides in the
290 induction of a strong early M1-like macrophage inflammatory response.

291 **3.5 M1-like macrophages can engulf and kill conidia of both *Scedosporium*** 292 **strains**

293 Microscopic analysis showed that *Scedosporium* conidia adhered to M1-like
294 macrophages. The macrophages were also able to internalize both *S. apiospermum* and *S.*

295 *dehoogii* conidia. Some macrophages appeared to interact with or engulf many fungal conidia
296 while others were not able to engulf any conidia (Fig. 6A and B).
297 No significant differences in killing activity were observed between the two strains, with
298 values of 16.26% for *S. apiospermum* vs. 18.96% for *S. dehoogii* (Fig.6C).
299 Collectively, these results show that M1-like macrophages interact with *S. apiospermum*/*S.*
300 *dehoogii* conidia and that the two strains are capable of surviving equally inside phagocytic
301 cells.

302 **4 Discussion**

303 Although *Scedosporium* species are the second most common filamentous fungi
304 colonizing the airways of CF patients, the immune mechanisms involved in host defense
305 against these fungi are largely unknown. The prevalence of these species is highly variable, *S.*
306 *apiospermum* being the most commonly recovered in the clinical setting [26,27]. To date, *S.*
307 *dehoogii* is considered as an environmental species and is not commonly associated with
308 human infections, despite the high virulence observed in murine models [26]. Myeloid cells,
309 including macrophages, express different PRRs that are important for *Scedosporium*
310 detection. Upon recognition of their respective ligands, these receptors induce cytokine and
311 chemokine production, which in turn regulate leukocyte trafficking and activation [17,28].
312 Our data showed that *S. apiospermum* and *S. dehoogii* conidia strongly stimulated the
313 expression of a number of pro-inflammatory cytokines and chemokines 3 h after incubation
314 with M1-like macrophages. The most significant gene expression changes were observed in
315 IL-1 α , IL-1 β , and IL-8, supporting their early involvement in the innate immune response
316 against *Scedosporium* fungi. Indeed, IL-8 attracts neutrophils to sites of infection and
317 activates them to kill the fungal cells [29]. While IL-1 α / β play an important role in mediating
318 immune responses, each cytokine possesses a distinct function. IL-1 α acts as an alarmin,
319 driving leukocyte recruitment and preventing fungal growth [30]. IL-1 β signaling enhances
320 phagocytosis and killing, increases reactive oxygen species/nitrogen oxide synthase
321 (ROS/NOS) production and promotes Th1 and Th17 immunity [31]. In our study, *S. dehoogii*
322 tended to be more potent in stimulating the early release of pro-inflammatory cytokines and
323 chemokines compared to *S. apiospermum*. This result may be related to the different
324 structural characteristics of the two species. Indeed, the presence of different pathogen-
325 associated molecular patterns (PAMPs) on the cell wall of the two *Scedosporium* conidia may

326 be related to different cytokine and chemokine signatures. In this context, a comparative
327 genomic analysis of the major *Scedosporium* species revealed several mutations in the *S.*
328 *dehoogii* genome that may lead to a loss of gene function. Some of these genes encode cell
329 wall components [10]. Given the importance of the cell wall in invasion of the conidia to the
330 immune response, the absence of certain molecules may rapidly expose *S. dehoogii* to
331 immune cells. Overall, it is tempting to speculate that the early abundant release of pro-
332 inflammatory cytokines and chemokines in response to *S. dehoogii* is associated with a lower
333 virulence. Despite the complexity of the immune mechanisms, it is currently assumed that, in
334 general, in the early immune response, the fewer pro-inflammatory cytokines are released by
335 an immune cell, the more virulent is the behaviour of the microorganism. *A. fumigatus*, the
336 most frequent filamentous fungus colonizing the airways of pwCF, and *Lomentospora*
337 *prolificans* have been shown to differ in their capacity to stimulate the early production of
338 pro-inflammatory cytokines (*L. prolificans* induces higher levels of TNF α and IL-1 β than *A.*
339 *fumigatus*) [21]. Interestingly, a recent study showed that *A. fumigatus* induces a lower
340 cytokine production in human lung adenocarcinoma cell line A549 compared to *S.*
341 *apiospermum* during the initial invasion period [32]. In term of cytokine levels, IL-21 levels
342 are extremely high with *S. apiospermum* co-culture whereas IL-1 α and IL-22 levels are
343 moderately elevated in the pneumocytic cells [32]. These observations may explain, at least in
344 part, *A. fumigatus* ranking the first among the filamentous fungi causing human respiratory
345 infections [21]. Similarly, Bruno *et al.* suggested that the early cytokine production induced
346 by yeasts of the *Candida auris* clade is inversely correlated with their virulence [33]. Indeed,
347 *C. auris* belonging to clade III, which is associated with invasive infections, shows a reduced
348 *in vitro* induction of cytokine production compared to other clades [33,34]. This is consistent
349 with the fact that the early production of pro-inflammatory cytokines and chemokines plays
350 an important role in protection against infection [12,24]. However, it is important to note that
351 a protective early response to fungal infection depends on several factors, including the host
352 immune status, site of infection, cell wall complexity, and fungal virulence traits [35]. A
353 previous study showed that *S. apiospermum* can efficiently destroy host proteins such as the
354 complement factors C3 and C1q, presumably by proteolytic degradation [36]. Furthermore,
355 some pathogenic *Scedosporium* species express surface glycoproteins with
356 immunomodulatory effects and produce some molecules masking PAMPs, which may make
357 recognition by the innate immune system difficult [37, 38].

358 By evaluating the late (24 h) *Scedosporium*-induced inflammatory response, we observed
359 substantial production of pro-inflammatory cytokines and chemokines. Considering that M1-
360 like macrophages did not inhibit the germinating process, this late response was probably
361 mediated by phagocytized conidia and *Scedosporium* hyphae. Indeed, *S. apiospermum* and *S.*
362 *dehoogii* conidia were able to germinate and to produce long hyphae during 24 h of contact
363 with M1-like macrophages. It should be pointed out that, in general, the morphology and
364 density of the monolayers of all M1-like macrophages used herein were preserved at the end
365 of the 24 h of interaction with *S. apiospermum* or *S. dehoogii*. Interestingly, our observations
366 indicated that, contrary to the early response, the late inflammatory pattern induced by *S.*
367 *apiospermum* was more intense than the one induced by *S. dehoogii*. As hyphae are the
368 invasive morphotype of *Scedosporium*, we can assume that the marked late pro-inflammatory
369 response by M1-like macrophages against *S. apiospermum* is aimed at enhancing the
370 recruitment of other immune cells, most of the innate defenses against hyphae relying on the
371 activity of other immune cells [17,39]. Although the design of our study did not allow us to
372 investigate the mechanisms by which M1-like macrophages recognize the two fungi, our PPI
373 network and pathway analysis suggested the involvement of *Scedosporium* polysaccharides in
374 the inflammatory response. Nevertheless, this predictive model may be subject to selection
375 bias due to the small number of genes investigated in this study. Further analysis based on
376 RNA sequencing would allow for a more comprehensive prediction of *Scedosporium*
377 structures involved in the pro-inflammatory response of M1-like macrophages.

378 Cytokine induction is important after pathogen recognition, but the induction of phagocytosis
379 and killing is also critical [33]. Our data indicated that *S. apiospermum* and *S. dehoogii*
380 conidia were able to adhere and to be internalized by M1-like macrophages after only 2 h of
381 contact. A previous study conducted by Pinto *et al.* [40] reported that the surface PAMPs are
382 recognized by host cell PRRs, such as TLR4, characterizing it as a *S. apiospermum* adhesin.
383 Furthermore, different types of macrophages are capable of phagocytosing other
384 *Scedosporium* species despite the much larger size of their conidia [17,41]. However, the
385 intracellular outcome of the conidia is species-specific. Indeed, ingested conidia of *S.*
386 *aurantiacum* and *L. prolificans* are less likely to be killed than those of *S. minutisporum* [22].
387 This suggests specific mechanisms allowing *S. aurantiacum* and *L. prolificans* conidia to
388 survive inside macrophages [22]. In fact, several microbial systems including superoxide
389 dismutases (SODs) and components of the thioredoxin system can convert ROS into less toxic
390 products [39]. Interestingly, the expression of two thioredoxin-like proteins has been shown to

391 be affected in *S. dehoogii* [10]. Hence, one might have predicted an enhanced killing of *S.*
392 *dehoogii* conidia by M1-like macrophages. Surprisingly, our flow cytometry-based protocol
393 showed that the macrophage-mediated killing rates were similar for *S. dehoogii* and *S.*
394 *apiospermum* conidia. To the best of our knowledge, this study provides a first understanding
395 of the immune response against *S. dehoogii*.

396 There are several important limitations to the current work, including our exclusive focus on
397 the M1-like macrophage response to the two *Scedosporium* species. In fact, the immune
398 response to fungi involves many myeloid cells, such as dendritic cells and neutrophils, and
399 lymphoid cells, such as NK cells and lymphocytes. Furthermore, our study of the immune
400 response to *S. apiospermum* and *S. dehoogii* was limited to cells derived from healthy donors.
401 In fact, macrophages from pwCF differ significantly from healthy cells. They display
402 overexpression of several pro-inflammatory cytokines and have altered phagocytic capacity
403 and microbial killing [42]. We also selected only two well-characterized *S. apiospermum* and
404 *S. dehoogii* isolates for this study, which may have masked some intraspecific variations in
405 terms of virulence. Furthermore, the two strains used in our study were of different origin: *S.*
406 *apiospermum* was a clinical isolate, while *S. dehoogii* was an environmental one. Indeed, the
407 source of isolation (clinical or environmental) could affect the host-pathogen interaction and,
408 consequently, could also influence the induced immune response profile. Nevertheless, *S.*
409 *dehoogii* has been reported very rarely in humans, mainly as a cause of cutaneous or
410 subcutaneous infections due to traumatic inoculation of soil fungal elements, and only one
411 isolate has been reported as recovered from the respiratory tract [9]. In addition, a unique
412 study compared the susceptibility of clinical and environmental isolates of the main
413 pathogenic *Scedosporium* species to reactive oxidative species and no differences were seen
414 between isolates of the same species in their susceptibility to chemically induced oxidative
415 stresses or even a lower susceptibility of the environmental isolates [43]. Obviously,
416 replication of this work after incubation of different immune cells from pwCF with resting or
417 germinating conidia, and hyphae from clinical strains would allow a more comprehensive
418 perspective of the immune response against *Scedosporium* species.

419 In conclusion, our study showed that M1-like macrophages can rapidly initiate a host defense
420 response against both *S. apiospermum* and *S. dehoogii*. This response is characterized by a
421 similar killing efficiency of internalized conidia and a tendency to a species-specific cytokine
422 production. Our data pave the way for future research aiming at assessing the detailed

423 mechanisms of the macrophage inflammatory response against these underestimated
424 emerging pathogens.

425 **Declaration of interest**

426 The authors declare that they have no known competing financial interests or personal
427 relationships that could have appeared to influence the work reported in this paper.

428 **Acknowledgment**

429 We thank the members of the PACeM (Plateforme d'Analyse Cellulaire et Moléculaire) of the
430 University of Angers, Nathalie Clément, Charlotte Godon and Julia Razafimandimby for the
431 technical assistance. This work was supported by a grant from SFR ICAT (University of
432 Angers, Angers, France) and by institutional grants from Inserm and the University of
433 Angers. The funders had no role in the design and conduct of the study, nor the decision to
434 prepare and submit the manuscript for publication. This work was realized in the context of
435 the Laboratory of excellence Immunotherapy Graft-Oncology (LabEx IGO) program
436 supported by the National Research Agency (ANR) via the investments for the future
437 program ANR-11-LABEX-0016-01 and by the University of Angers - University Hospital of
438 Angers joint program (3I-Impact).

439 **Credit authorship contribution statement**

440 **Dorra Elhaj Mahmoud:** Conceptualization, Data curation, Formal analysis, Investigation,
441 Methodology, Writing-original draft, Writing-review & editing. **Meriem Hanachi:** Formal
442 analysis, Writing original draft. **Hajar Yaakoub:** Formal analysis, Writing-review & editing.
443 **Simon Blanchard:** Formal analysis, Writing-original draft. **Pascale Pignon:** Formal analysis,
444 Writing-review & editing. **Oussama Souiri:** Formal analysis. **Yves Delneste:** Funding
445 acquisition, Conceptualization, Writing-review & editing. **Jean-Philippe Bouchara:**
446 Conceptualization, Writing-original draft, Writing-review & editing. **Nicolas Papon:**
447 Supervision, Writing-original draft, Writing-review & editing. **Anaïs Hérivaux:** Funding
448 acquisition, Conceptualization, Project administration, Supervision, Validation, Writing-
449 original draft, Writing-review & editing.

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- 590

591 **Figure legends**

592 **Figure 1. Principal component analysis (PCA) of the M1-like macrophage response**
593 **during *S. apiospermum* and *S. dehoogii* co-culture.** The PCA was performed on scaled gene
594 expression counts ($\log_2(\text{FC})$) obtained from qPCR. (A) Individual plot: samples are depicted
595 as dots, the largest of which represents the group mean point. The dots are color coded
596 according to each experimental time point encountered in a given species. (B) Variables plot:
597 the principal components PC1 (or Dim1) and PC2 (or Dim2) of the most significant variables
598 are represented by vectors. The ellipses reflect a 95% confidence level.
599 CCL: (C-C motif) Ligand; CLEC: C-Type Lectin Domain Containing 7A; CXCL: (C-X-C
600 motif) Ligand; HLA-DRA: Major Histocompatibility Complex, Class II, DR Alpha; IFNG:
601 Interferon Gamma; IL: Interleukin; IL1A: Interleukin 1 Alpha; IL 1B: Interleukin1 Beta;
602 TLR: Toll-like receptor; TNFA: Tumor Necrosis Factor Alpha.

603 **Figure 2. Analysis of chemokine, chemokine and immune receptor mRNA expression by**
604 **M1-like macrophages after *Scedosporium* co-culture.** The expression heatmap was
605 generated using the $\log_2(\text{FC})$ of 19 genes encoding cytokines, chemokines and immune
606 receptors and boxed based on distinct expression profiles. Genes were ordered using full
607 hierarchical clustering. The color scale used for $\log_2(\text{FC})$ is green to red, where green
608 represents 1 and red represents -1. (A and C) Heatmaps of 19 genes expressed by M1-like
609 macrophages after *S. apiospermum* and *S. dehoogii* conidia challenge for 3 h and 24 h,
610 respectively. (B and D) Venn diagrams illustrating common and specific differentially
611 expressed genes between M1-like macrophages infected with *S. apiospermum* and M1-like
612 macrophages infected with *S. dehoogii* for 3 h and 24 h respectively. Upregulated genes:
613 $\log_2(\text{FC}) \geq 1$ and a p -value < 0.05 (treated M1-like macrophages vs. untreated cells) are
614 shown in green. Downregulated genes: $\log_2(\text{FC}) \leq -1$ and a p -value < 0.05 (treated M1-like
615 macrophages vs. untreated cells) are shown in red. Significance levels were calculated using
616 the Mann-Whitney U test (n=4-5 independent experiments).

617 **Figure 3. Comparative analysis of M1-like macrophage gene expression after**
618 ***Scedosporium apiospermum* and *Scedosporium dehoogii* co-culture.** (A and B) Boxplots
619 comparing $\log_2(\text{FC})$ levels of the common up or downregulated genes after M1-like
620 macrophage challenge with *S. apiospermum* and *S. dehoogii* for 3 h (8 genes) and 24 h (14

621 genes). The boxes extend from the 25th to the 75th percentile, and the line in the middle is the
622 median. The statistical significance of the expression differences between the two species was
623 determined using the Mann-Whitney U test. (n=4-5 independent experiences). Levels of
624 significance were allocated as follow: ns, not significant, * p -value < 0.05, ** p -value <0.01.

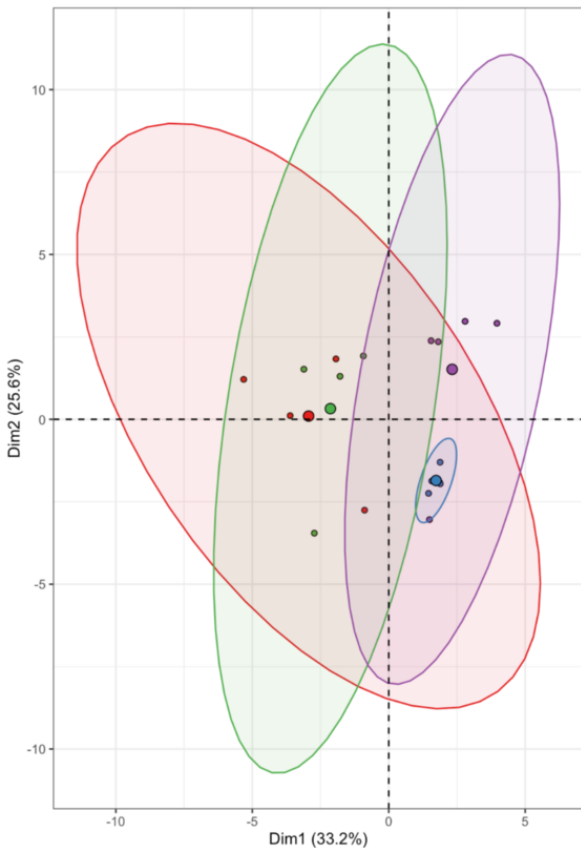
625 **Figure 4. Analysis of cytokine and chemokine release by M1-like macrophages after**
626 ***Scedosporium* co-culture.** (A and C) Boxplots showing cytokine concentrations in untreated
627 macrophages (CTRL), M1-like macrophages infected with *S. apiospermum* conidia and M1-
628 like macrophages infected with *S. dehoogii* conidia for 3 h and 24 h, respectively. The boxes
629 extend from the 25th to the 75th percentile, and the line in the middle is the median. (B and
630 D) Plot of relative cytokine abundance. The cytokine level in M1-like macrophages infected
631 with *S. apiospermum* for 3 h and 24 h, respectively (1) is represented by the vertical red line.
632 Bar graphs represent the mean of cytokine levels detected after *S. dehoogii* co-culture (3 h and
633 24 h) relative to the mean of cytokine levels released after *S. apiospermum* co-culture (3 h and
634 24 h). Significance levels were assigned as follows: * p < 0.05 and ** p < 0.01 by Kruskal-
635 Wallis test. (n=4-5 independent experiments).

636 **Figure 5. Protein–protein interaction (PPI) networks** of cytokines and chemokines up-
637 regulated (in expression and or in release) by *S. dehoogii* after 3 h of co-culture. The PPI
638 network was constrained to a 90% confidence level as calculated by the StringDB system.

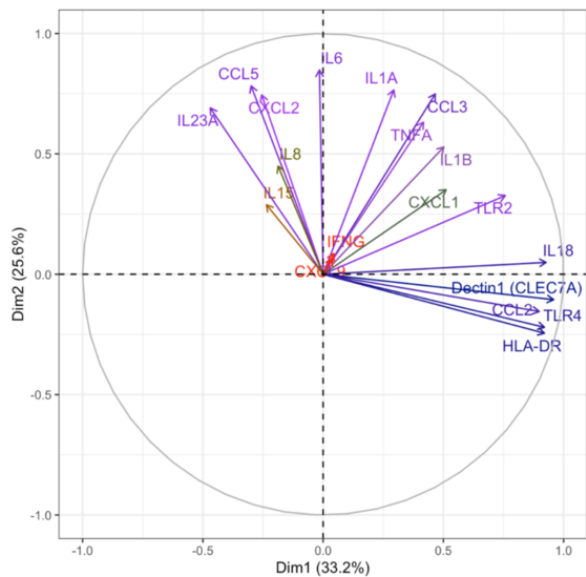
639 **Figure 6. Internalization and killing of *Scedosporium* conidia by M1-like macrophages.**
640 (A and B) Interaction between *Scedosporium* conidia and M1-like macrophages after 2 h of
641 co-culture. (C) Killing percentage was defined as the number of macrophage-killed conidia
642 defined by the total number of macrophage-released conidia \times 100; killing percentage data
643 were collected from four independent experiments. Levels of significance were allocated
644 using the Mann-Whitney U test.

A

Individuals - PCA

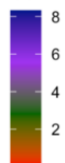
**B**

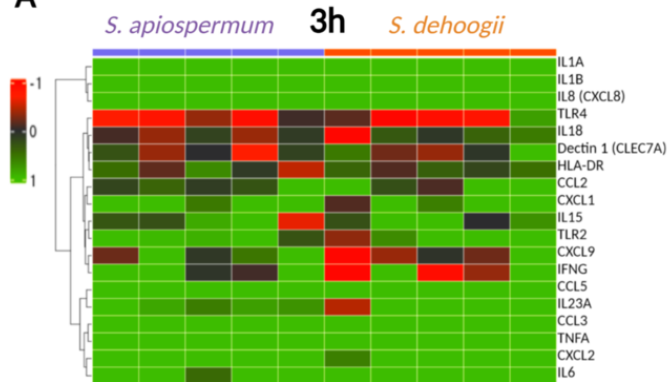
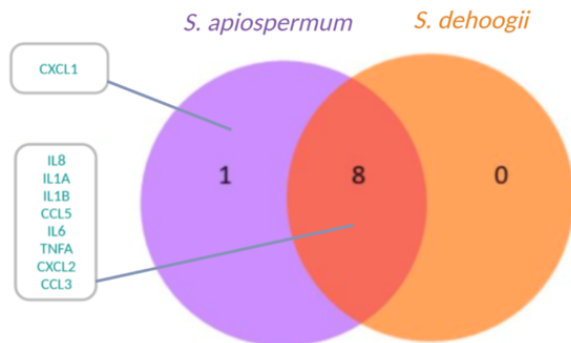
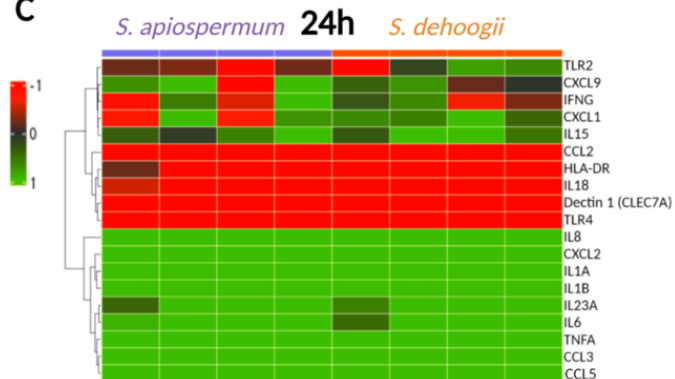
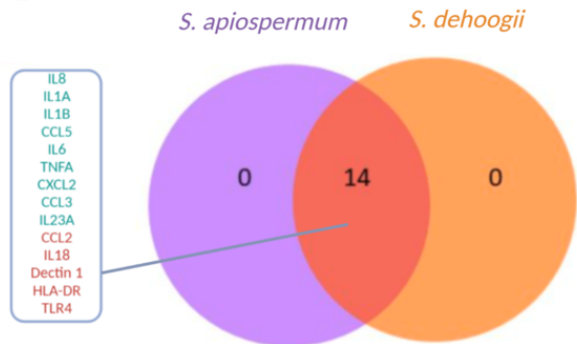
Variables - PCA

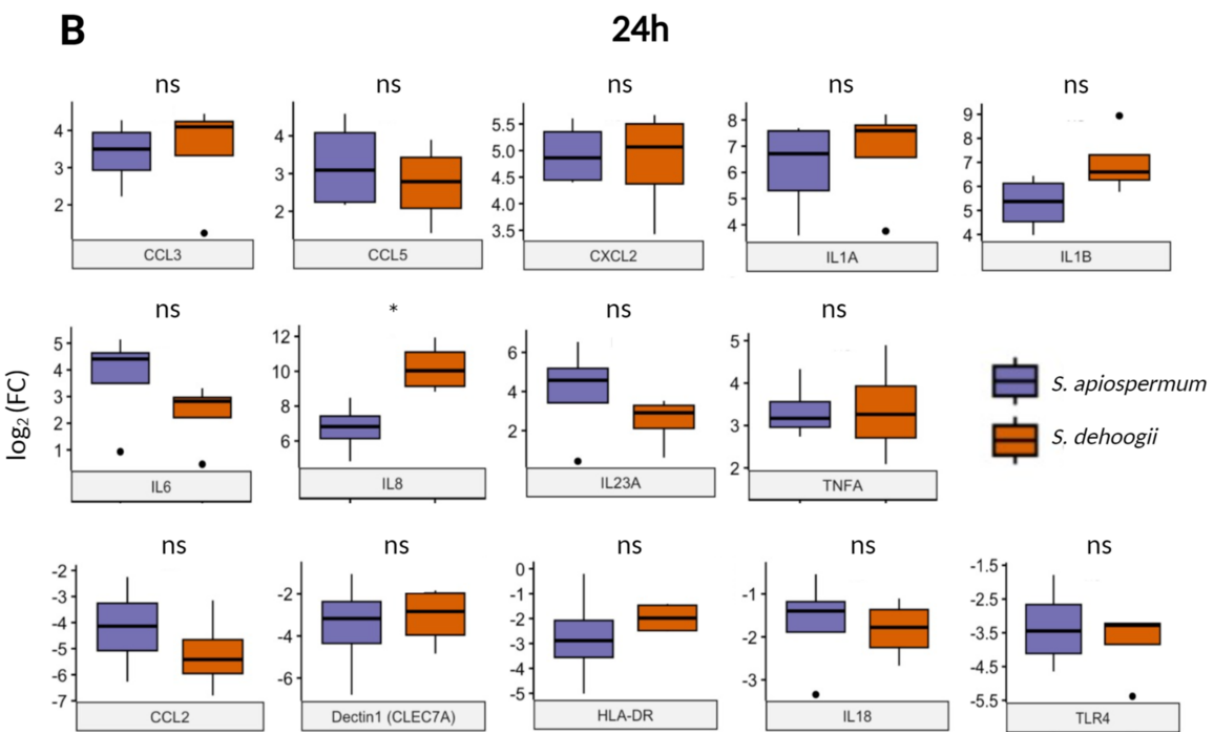
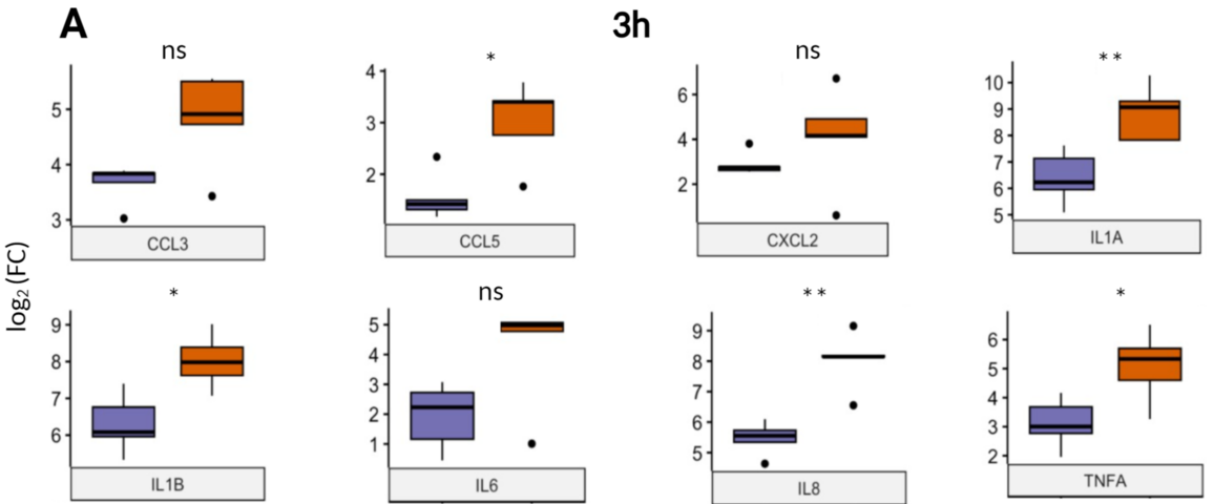


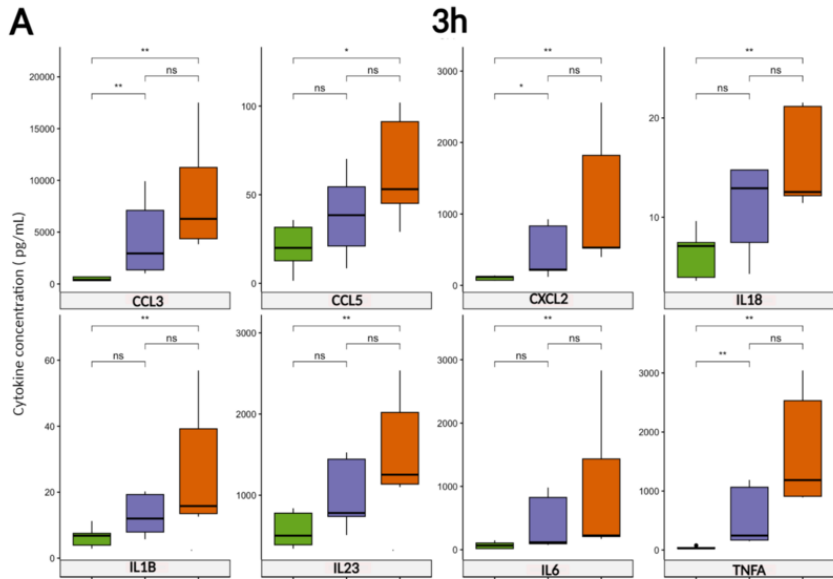
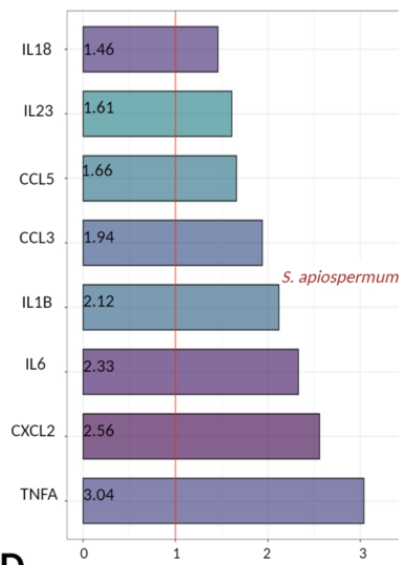
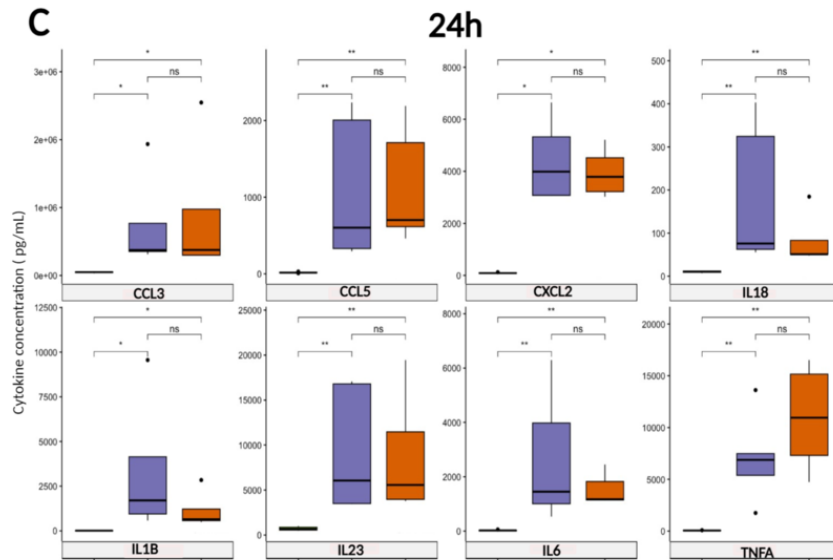
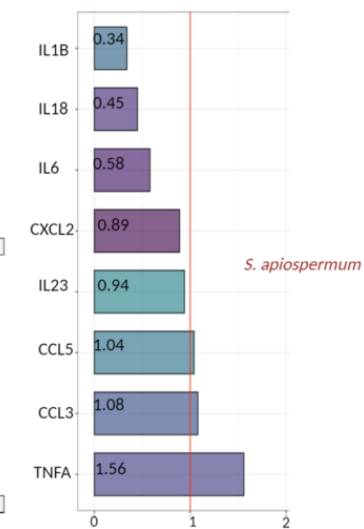
- *S. apiopermum* 24h
- *S. apiospermum* 3h
- *S. dehoogii* 24h
- *S. dehoogii* 3h

Contrib

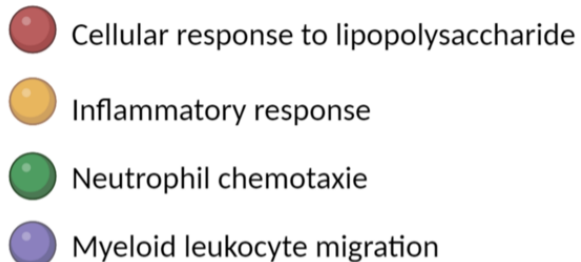
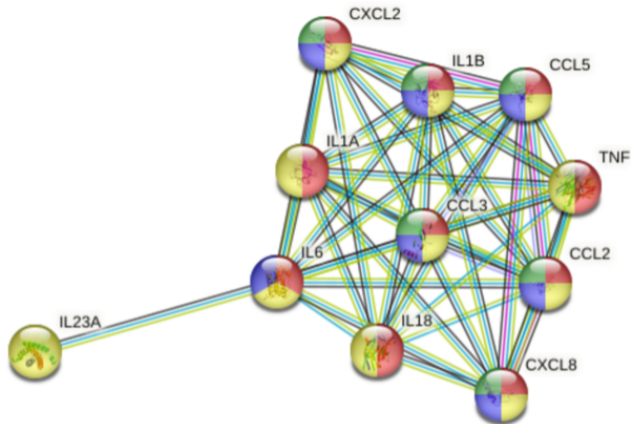


A**B****C****D**



A**B****C****D**

CTRL
S. apiospermum
S. dehoogii



PPI Legend

Known interactions

- from curated databases
- experimentally determined

Predicted interactions

- gene neighborhood
- gene fusions
- gene co-occurrence

Others

- textmining
- co-expression
- protein homology

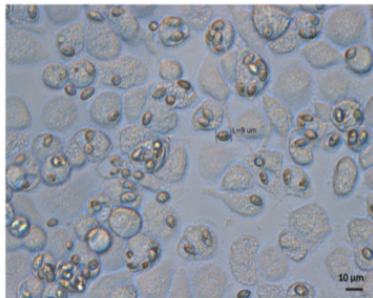
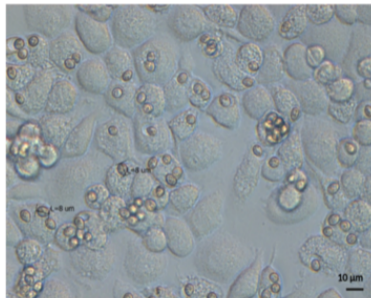
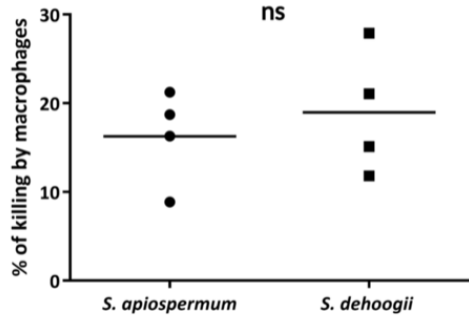
A*S. apiospermum***B***S. dehoogii***C**

Table 1: Primers used in qPCR

Gene	Forward primer	Reverse primer	Product size (bp)
CCL2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT	190
CCL3	TTCCGTCACCTGCTCAGAATC	GTGATGCAGAGAACTGGTTGC	94
CCL5	CCTGCTGCTTTGCCTACATTGC	ACACACTTGGCGGTTCTTTCCGG	125
CXCL1	CTGGCTTAGAACAAAGGGGCT	TAAAGGTAGCCCTTGTTTCCCC	112
CXCL2	TTCACAGTGTGTGGTCAACAT	TCTCTGCTCTAACACAGAGGGA	150
CXCL9	TGAGAAAGGGTCGCTGTTCC	GGGCTTGGGGCAAATTGTTT	91
Dectin1 (CLEC7A)	ACAATGCTGGCAACTGGGCTCT	AGAGCCATGGTACCTCAGTCTG	143
GAPDH	ACTTCAACAGCGACACCCACTC	TACCAGGAAATGAGCTTGACAAAG	88
HLA-DRA	CCGATCACCAATGTACCTCCA	CGAAGCCACGTGACATTGAC	128
IFNγ	ACTGTCGCCAGCAGCTAAAA	TATTGCAGGCAGGACAACCA	96
IL-1α	TGTATGTGACTGCCCAAGATGAAG	AGAGGAGGTTGGTCTCACTACC	96
IL-1β	AAATACCTGTGGCCTTGGGC	TTGGGATCTACACTCTCCAGCT	101
IL-6	GTAGCCGCCCCACACAGA	CATGTCTCCTTCTCAGGGCTG	101
IL-8	ATAAAGACATACTCCAAACCTTTCCAC	AAGCTTTACAATAATTTCTGTGTTGGC	102
IL-15	AACAGAAGCCAAGTGGGTGAATG	CTCCAAGAGAAAGCACTTCATTGC	148
IL-18	TGCAGTCTACACAGCTTCGG	ACTGGTTCAGCAGCCATCTT	129
IL-23A	GAGCCTTCTCTGCTCCCTGATA	GACTGAGGCTTGGAAATCTGCTG	121
TLR2	CTTCACTCAGGAGCAGCAAGCA	ACACCAGTGCTGTCTGTGACA	146
TLR4	CCCTGAGGCATTTAGGCAGCTA	AGGTAGAGAGGTGGCTTAGGCT	126
TNFα	CACAGTGAAGTGCTGGCAAC	GATCAAAGCTGTAGGCCCA	75

Abbreviations: bp: basepair; CCL: (C-C motif) Ligand; CLEC7A: C-Type Lectin Domain Containing 7A; CXCL: (C-X-C motif) Ligand; Abbreviations: bp: basepair; CCL: (C-C motif) Ligand; CLEC: C-Type Lectin Domain Containing 7A; CXCL: (C-X-C motif) Ligand ; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HLA-DRA: Major Histocompatibility Complex, Class II, DR Alpha; IFN γ : Interferon Gamma; IL: Interleukin; TLR: Toll-like receptor; TNF α : Tumor Necrosis Factor Alpha.