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1 **Molecular characterisation of immunological memory following homologous or**
2 **heterologous challenges in the Schistosomiasis vector snail, *Biomphalaria***
3 ***glabrata*.**

4
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31 **Abstract**

32 Invertebrate immune response may be primed by a current infection in a sustained
33 manner, leading to the failure of a secondary infection with the same pathogen. The
34 present study focuses on the Schistosomiasis vector snail *Biomphalaria glabrata*, in
35 which a specific genotype-dependent immunological memory was demonstrated as a
36 shift from a cellular to a humoral immune response.

37 Herein, we investigate the complex molecular bases associated with this genotype-
38 dependent immunological memory response. We demonstrate that *Biomphalaria*
39 regulates a polymorphic set of immune recognition molecules and immune effector
40 repertoires to respond to different strains of *Schistosoma* parasites. These results
41 suggest a combinatorial usage of pathogen recognition receptors (PRRs) that
42 distinguish different strains of parasites during the acquisition of immunological
43 memory. Immunizations also show that snails become resistant after exposure to
44 parasite extracts. Hemolymph transfer and a label-free proteomic analysis proved
45 that circulating hemolymph compounds can be produced and released to more
46 efficiently kill the newly encountered parasite of the same genetic lineage.

47

48

49 **Keywords**

50 Immunological memory, specificity, Schistosoma, Biomphalaria, RNAseq, Proteomic,
51 Vaccination

52

53

54 **1. Introduction**

55 Living organisms have evolved complex immune systems to confront
56 pathogenicbiological-traits and diversity, from prokaryotes(Marraffini and Sontheimer,
57 2008) to eukaryotes,from plants (Calil and Fontes, 2016; Dangl et al., 2013) to
58 animals, and from invertebrates (Cirimotich et al., 2011; Haine et al., 2008; Moret and
59 Schmid-Hempel, 2000, 2001; Portela et al., 2013) to vertebrates (Cooper and Alder,
60 2006; Du Pasquier, 2004; Eason et al., 2004). “Benefiting from experience” or more
61 specifically “remembering pathogens” has been a pressure that has led many
62 immune systems to develop rapid and efficient protections against pathogens
63 encountered earlier in life. This is due to a chain of events referred to as
64 “immunological memory”. For innate immune systems, the memory processes are
65 derived from evolutionary convergences and could have very different features that
66 have led to different terminologies, i.e.trained immunity for vertebrates or immune
67 priming/innate immune memory for invertebrates (Gourbal et al., 2018; Milutinovic
68 and Kurtz, 2016; Netea et al., 2011; Pradeu and Du Pasquier, 2018).

69 In invertebrate species, despite the lack of a canonical adaptive immune system,
70 immunological memory and specificity of immunological memoryhave been
71 demonstrated using different strains of pathogens even though in most cases,a
72 short-term memory is difficult to distinguish from an ongoing response(Jorge
73 Contreras-Garduno et al., 2016; Kurtz and Franz, 2003; Lafont et al., 2017;
74 Milutinovic and Kurtz, 2016; Pham et al., 2007; Pinaud et al., 2016; Witteveldt et al.,
75 2004).

76 However, observations of memory specificity in invertebrate innate immune systems
77 are largely phenomenological; and the underlying molecular and/or cellular
78 mechanisms remain unknown. In vertebrates, trained immunity shows low or even no
79 specificity(Netea et al., 2011; Netea and van der Meer, 2017). Thus, it may not be
80 possible to explain the observations in invertebrate systems in terms of what we
81 know about non-self-recognition and trained immunity in vertebrates(Litman,
82 2006).Thus, if invertebrates do show specificity in their memory responses, what are
83 the molecular and cellular determinants of such a specific immunological memory?

84 In the present paper, we investigate this question in the Lophotrochozoan snail,
85 *Biomphalaria glabrata*, and its metazoan parasite, the trematode *Schistosoma*
86 *mansoni*. *Biomphalaria* snails are the vectorfor Schistosomiasis, which is the second

87 most widespread human parasitic disease after malaria, causing a substantial public-
88 health burden with about 200 million humans infected in 74 countries and more than
89 200,000 deaths annually (King et al., 2005).

90 We previously reported that the immunological memory response of *Biomphalaria* to
91 various strains of *Schistosoma* is specific (better protection observed in homologous
92 challenges compared to heterologous challenges), although cross-reaction was
93 certainly encountered (Portela et al., 2013). In the case of one Brazilian *Biomphalaria*
94 / sympatric *Schistosoma* interaction, a transcriptomic and comparative proteomic
95 approach revealed an apparent shift from a cellular immune response (where the
96 hemocyte encapsulates the parasite) following primo-infection, toward a humoral
97 immune response (soluble plasma factors will clear the parasite) following
98 challenge (Pinaud et al., 2016). Following primo-infection, 2/3 of the parasites were
99 encapsulated and 1/3 succeeded in infecting the snails that then harbour a viable,
100 developing and productive parasite infection (Portela et al., 2013). At the molecular
101 level, we identified a shift in transcript expression only associated with the
102 challenge (Pinaud et al., 2016).

103 In the present paper, we further explore the specificity of the immunological memory
104 response of the snail *Biomphalaria* particularly within the context of a specific
105 genotype-dependant immune response. Using experiments at different scales, as
106 well as transcriptomic responses, snail immunisation, *in-vitro* toxicity assays and a
107 comparative proteomic approach of plasma compartment, we compared host
108 response following homologous versus heterologous parasite challenges (figure 1)
109 and we bring new insights to help better understand the specificity of the
110 immunological memory response in *Biomphalaria glabrata* snails.

111

112 **2. Materials and methods**

113 **2.1. *Biomphalaria* snail and *Schistosoma* parasites**

114 One strain of *Biomphalaria glabrata* snail originating from Brazil (BgBRE) has been
115 used for all experiments. Its sympatric *Schistosoma mansoni* parasite strain from
116 Brazil (SmBRE) was used for homologous interactions while heterologous
117 interactions were conducted with two different geographic isolates, with the first
118 coming from the same species: *Schistosoma mansoni* from Venezuela (SmVEN);
119 and the second from a different species: *Schistosoma Rodhaini* from Burundi
120 (SROD). Infectivity phenotypes for all interactions were previously described as

121 highly compatible, with prevalence values from 80% to 100% (Portela et al., 2013;
122 Theron et al., 1997).

123

124 **2.2. Immunological memory experimental protocols**

125 Duration of the immune memory was tested by primo-infecting BgBRE snails (n=100)
126 with 10 SmBRE miracidia. Then, at 15, 25, 56, 72, and 140 days after primo-infection
127 (DPI), snails were challenged with 10 SmBRE miracidia and 15 days after challenge
128 snails were fixed in Raillet-Henri solution, and parasite prevalence was quantified as
129 previously described (Portela et al., 2013). For RNAseq, BgBRE snails were primed
130 with 10 SmBRE miracidia and then, 25 days later, challenged with 10 miracidia from
131 each strain (SmBRE, SmVEN, SROD). For vaccination, *in-vitro* toxicity and label-free
132 proteomic approaches from cell-free hemolymph, BgBRE snails (n=150) were primo-
133 infected or experimentally injected with parasite extracts and challenged with 10
134 SmBRE miracidia, 15 days later. Parasite prevalence was quantified
135 through dissection of Raillet-Henry fixed snails 15 days after the immune challenge as
136 previously described (Portela et al., 2013). For all of these experimental approaches,
137 uninfected naïve snails were used as negative controls.

138

139 **2.3. High throughput approach protocols**

140 For the RNAseq, pools of 20 BgBRE snails were recovered as previously described
141 (Pinaud et al., 2016) at 1, 4 and 15 days after the challenges and equimolar amounts
142 of each biological material collected at these time points were pooled together into 3
143 separate samples named BRE, VEN and ROD corresponding to the 3 strains. Two
144 pools of 20 naïve snails (naïve1 and naïve2) were sampled and used as controls.
145 Statistical analyses were performed as already described (Pinaud et al., 2016)
146 following the pipeline on our Galaxy Project server. Briefly, best quality reads were
147 filtered (phred >29) and then reads were aligned with Bowtie (v2.0.2, quality mapping
148 255) and PCR duplicates were removed thanks to SAM tools. DESeq2 software was
149 used to compare duplicate samples of uninfected snails (naïve1 and naïve2) with our
150 three challenge conditions (BRE, VEN and ROD) and all statistically different results
151 (P value <0.05) were annotated manually (Blast X and Interproscan against nr
152 database, Blast2GoPro for all the other available databases, and data from the
153 literature). A heatmap was constructed from a log₂ fold change for each of the
154 transcripts using Hierarchical Ascending Clustering (HAC) with Pearson correlation,

155 as applied by the Cluster 3.0 (Eisen et al., 1998) and JavaTreeView software
156 packages.

157

158 **2.4. Vaccination**

159 All developmental stages of *Schistosoma* parasites were recovered and whole
160 extracts were prepared. Miracidia and cercaria (free-living swimming stages) were
161 recovered in water, washed in PBS and pelleted by centrifugation. Primary
162 sporocysts (Sp1) were recovered by *in-vitro* transformation from miracidia as
163 previously described (Roger et al., 2008b) in CBSS for 24 hours. Secondary
164 sporocysts (Sp2) were recovered in the hepato-pancreas of 30-day infected snails
165 (Sp2 representing 70% of the hepato-pancreas at this time point). Hepato-pancreas
166 from uninfected naïve snails were also recovered and used as a control (named: Sp2
167 control). All of the samples were natively extracted in 150 µL TBS-Tween 0.05%
168 buffer. Three cycles of freeze-thaw (-80°C to 4°C) were followed by four pulses of
169 sonication (Vibra-cell 75185) at 20% max amplitude for 20 seconds at 4°C; then
170 proteins containing samples were titrated (2D Quant kit, GE Healthcare life sciences)
171 and parasite stage extracts were stored at -80°C. A pool combining an equimolar
172 amount of whole extracts from the 4 parasite stages (sample named “pool”) was
173 constituted and used as a test for antigen synergistic effect.

174 For the homologous vaccination, 500 uninfected BgBRE snails were anaesthetised
175 for 2h in 0.05% Pentobarbital sodium (200mg/ml Vetoquinol Dolethal) in 26°C drilling
176 water, then injected in the head-foot region with 1µg in 10µL TBS-Tween 0.05%
177 buffer of each SmBRE intra-molluscal stage protein extract (Miracidia, Sp1, Sp2,
178 Sp2Control, Cercaria or Pool). Then, individual snails were exposed to 10 SmBRE
179 miracidia 15 days after vaccination. An injection of TBS-Tween buffer alone was
180 used as a negative control. For heterologous vaccinations, 200 BgBRE naïve snails
181 were individually injected under the conditions previously described with Miracidia
182 and Sp1 extracts from SmVEN and SROD, then individually exposed to 10 SmBRE
183 miracidia 15 days after vaccination. Finally, fifteen days after infection, snails were
184 fixed in Raillet-Henry solution to quantify parasite prevalence as previously described
185 (Portela et al., 2013). Experiments were done in triplicate.

186

187 **2.5. Expression of relevant immunity genes following vaccination**

188 To determine if vaccination activates a functional immune response similar to the one
189 observed following natural primo-infection with *Schistosoma mansoni* miracidia, we
190 conducted a quantitative Real-time RT-PCR on relevant immune genes following
191 vaccination. The differential gene expressions from RT-PCR following vaccination
192 were compared to RNAseq Log2fold change data. Down- and up-regulated genes
193 were selected based on RNAseq data (see supplementary Table 1 for details on
194 primers and genes selected). BgBRE snails were injected with parasite extracts as
195 described above in the vaccination section, and then hemocytes were recovered and
196 gathered in 4 biological replicates of 3 individuals each. mRNAs were extracted using
197 TRIZOL® (Sigma Life Science, USA) and then reverse transcribed to first strand
198 cDNA as described by the manufacturer and treated with DNase (Maxima First
199 Strand cDNA Synthesis Kit with dsDNase, Thermo Scientific, USA). Real-time RT-
200 PCR analysis was performed in the LightCycler 480 System (Roche) with a volume of
201 10 µL comprised of 20ng cDNA diluted with MilliQ-H₂O in 2 µL, 5 µL of No Rox
202 SYBR Master Mix blue dTTP (Takyon) and 1.5 µL for each of the 2X primers. The
203 cycling program was: 95°C for 4 minutes, followed by 45 cycles of 95°C for 10 sec,
204 60°C for 20 sec and 72°C for 20 sec. Expression levels of targeted genes were
205 normalised relative to S19 gene thanks to the Pfaffl method (Pfaffl, 2001).

206

207 **2.6. Cell-free hemolymph transfer and protection phenotype**

208 Individual naïve BgBRE snails were primed with 10 SmBRE miracidia and
209 hemolymph was recovered from 30 snails 15 days later (sample was named 15DPI).
210 Next, the snails were challenged with 10 SmBRE miracidia, and hemolymph from
211 pools of 30 individuals, sampled at 1, 4 and 15 days after the challenge (samples
212 were named 1DC, 4DC and 15DC for “days post challenge”). Finally, a pool of
213 hemolymph from 30 naïve snails served as the control (termed naïve). All samples
214 were centrifuged (2,500 rpm for 15 min at 4°C) to pellet hemocytes and the
215 supernatant, referred to as “cell-free hemolymph”, was kept at -80°C until used.

216 Three hundred naïve snails (10-12 mm) were injected with 10 µL of cell-free
217 hemolymph of either naïve, 15DPI, 1DC, 4DC or 15DC samples or saline solution
218 (Chernin’s balanced salt solution, CBSS (used as negative control of injection)),
219 where 10 µl represented around 1/8 of the total hemolymph volume for the recipient
220 snail. Fifteen days after injection, all snails were individually exposed to 10 SmBRE
221 miracidia, and 15 days later, were fixed in Raillet-Henry solution to estimate parasite

222 prevalence. At the same time, 30 untreated snails were exposed to 10 SmBRE
223 miracidia to quantify control prevalence used for protection calculation. All
224 experimental approaches were conducted in triplicate.

225

226 **2.7. Comparative label-free proteomic of cell-free hemolymph**

227 Three hundred BgBRE snails were individually primo-infected with 10 SmBRE
228 miracidia; and 15 days later, 150 snails were exposed to a homologous challenged
229 with 10 SmBRE miracidia. For label-free proteomic sequencing, pools of hemolymph
230 were recovered from either naïve, 15 days after primo-infection (15DPI) or 1 day after
231 the challenge (1DC) snails. Twenty five µL of hemolymph was recovered from each
232 snail for a total of 3,500 µL for each of the three samples. Each sample was then
233 processed in the same way. Hemocytes were removed from hemolymph by
234 centrifugation at 2,000 g, 5 min at 26°C. Then, hemoglobin was depleted from cell-
235 free hemolymph by ultra-centrifugation at 40,000 g, 2.5 hours at 4°C. Cell-free
236 hemolymphs (hemoglobin depleted) were extracted in Laemmli buffer 4X (Biorad,
237 Hercules California, USA), boiled (95°C-100°C) for 5 min and stored at -80°C until
238 used for label-free sequencing (EDyP service facilities, CEA Grenoble, France).
239 Protein preparation and mass spectrometry-based proteomic analyses were
240 conducted as described in (Milbradt et al., 2014). Briefly, extracted proteins were
241 stacked in the top of an SDS-PAGE gel (NuPAGE 4 to 12%; Invitrogen) before in-gel
242 digestion was performed using trypsin (sequencing grade; Promega). Resulting
243 peptides were analysed in duplicate by online nanoscale liquid chromatography
244 tandem mass spectrometry (nanoLC-MS/MS) (UltiMate 3000 and LTQ-Orbitrap
245 Velos Pro; Thermo Scientific) using a 120-min gradient. Peptides and proteins were
246 identified using Mascot software (Matrix Science) and confronted against either,
247 Uniprot database and translated transcriptome of *Biomphalaria glabrata* snail
248 (available at: <http://ihpe.univ-perp.fr/acces-aux-donnees/>) or translated genome of
249 *Schistosoma mansoni* (available at: <http://schistodb.net/schisto/>). A differential
250 analysis was performed using extracted specific spectral counts (SSCs) where only
251 the qualitative differences between naïve, 15DPI and 1DC samples, were
252 considered.

253

254 **2.8. *In vitro* cell-free hemolymph toxicity assay: a validation of specificity**

255 Naïve BgBRE snails were primed either with 10 SmBRE miracidia or 10 SmVEN
256 miracidia, and then challenged by 10 homologous SmBRE or SmVEN miracidia. Cell-
257 free hemolymph was recovered from either thenaïve snails or 1 day after the
258 challenge (1DC) as described above and then kept at -80°C until used. *In vitro*
259 primary sporocysts (Sp1) of SmBRE, SmVEN and SROD were transformed *in-vitro*
260 from miracidia as previously described (Roger et al., 2008b). *In vitro* toxicity assays
261 were done by putting Sp1 in contact, at 26°C, with CBSS culture medium (control), or
262 with 500µL of cell-free hemolymph from naïve snails or from 1DC snails challenged
263 with SmBRE or SmVEN. Mortality was assessed after 4 hours of contact under a
264 light microscope, with the Sp1 considered “dead” when we failed to observe motility
265 and/or beating of the flame-cell flagella as previously described (Mone et al., 2011).
266 All experiments were done in triplicate with different preparations of sporocysts and
267 hemolymphs.

268

269 **3. Results**

270 **3.1. Long-lasting immunological memory in *Biomphalaria glabrata***

271 To test for duration of the innate immune memory process, BgBRE snails were
272 primo-infected with 10 SmBRE miracidia and challenged at 15, 25, 56, 72, and
273 140DPI, again with 10 SmBRE miracidia. For all-time points, primo-infected snails
274 were always resistant to challenge (Table 1). All snails died after 140 DPI due to
275 parasite pathogenesis (Table 1) as parasite from primo-infection developed to
276 patency in snail tissue. These results demonstrated that, (i) immunological memory
277 was acquired soon after infection (from 15 DPI) and (ii) was maintained during the
278 snail’s entire life span (Table 1).

279

280 **3.2. Transcriptomic results provide clues for specificity of immunological 281 memory**

282 A RNAseq approach was used to identify differentially expressed (DE) transcripts
283 following challenges with homologous (SmBRE) or heterologous (SmVEN or
284 SROD) parasites. Complete RNAseq results are available in supplementary Table 2.
285 Venn Diagram (Fig. 2A) shows that among 4,202 DE transcripts, 22% (representing
286 924 different transcripts) appeared as common regardless of the strain used for
287 immune challenges. Strain-specific transcripts were identified for the homologous
288 SmBRE challenge (45.8% representing 2,013 transcripts), for the heterologous

289 challenge with SmVEN (9.6% and 405 transcripts) and for the challenge with SROD
290 (5% and 210 transcripts). The number of DE transcripts is decreasing with an increase
291 in the genetic distance between the parasite used for the primo-infection and for the
292 one used in the challenge. The heat map of these DE transcripts provides more
293 precise information by highlighting 14 different transcript clusters (Fig. 2B). Clusters 1
294 to 7 corresponded to up-regulated transcripts and clusters 8 to 14 to down-
295 regulated ones. Both clusters 1 and 14 corresponded to transcripts shared between
296 all challenge conditions. With 3,339 DE transcripts, the homologous challenge with
297 SmBRE induced the largest response while the SmVEN strain induced the regulation
298 of 1,838 transcripts, and SROD only 1,525 transcripts (Fig. 2B). Interestingly, many
299 immunity-related transcripts can be identified in these clusters. Within them, three
300 main families can be defined, (i) the receptors that potentially bind pathogen factors,
301 (ii) effectors of immunity, capable of having a direct deleterious effect on the
302 pathogens or activation effect on host immune response and (iii) other transcripts
303 related to immunity triggering maturation, activation of immune response. In this way,
304 the putative immuno-receptors are widely distributed across clusters but with
305 differential distribution between the parasite strains. Immune effectors are
306 narrowly distributed on the heatmap. For example biomphalysin, a beta-Pore Forming
307 Toxin (β -PFT) secreted in snail plasma, capable of directly killing the parasite (Galinier
308 et al., 2013) is notably present in cluster 1 (Fig. 2B). Cluster 1 groups the up-
309 regulated transcripts differentially expressed regardless of the parasite strain. Thus,
310 biomphalysin could participate in the humoral memory phenotype observed following
311 the challenge in *B. glabrata* snails (Pinaud et al., 2016; Portela et al., 2013).
312 Biomphalysin DE transcripts suggest that numerous variants of biomphalysin family
313 members were involved in this immunological memory response (Supp-Table 2).
314 Variants of biomphalysin are specifically up-regulated following SmBRE, SmVEN
315 or SROD challenges while others are down-regulated following the SmVEN challenge
316 (Fig. 2B, and Supp-Table 2).

317 Anti-microbial peptides (AMPs) and the functionally related antimicrobial proteins
318 were also part of the immune effector family. Members of the *B. glabrata* *lbp/bpi*
319 (lipopolysaccharide binding protein/bactericidal permeability increasing protein)
320 family (22 transcript variants) are mainly down-regulated in response to the three
321 parasite strains (cluster 14). Two members were up-regulated in response to the
322 SmBRE strain (cluster 1) while one was up-regulated in the response shared by

323 SmBRE and SROD strains (cluster 4). One form of mytimacin, a cysteine-rich
324 antimicrobial peptide, previously described in the mussel *Mytilus* is up-regulated in the
325 SmBRE specific cluster 7, while a variant is up-regulated in the shared response
326 between SmBRE and SROD (Cluster 4).

327 For the third group of transcripts related to immunity, several immune regulators were
328 identified. Collagen, Hemagglutinin, Haemoglobin and SCO-spondin are components
329 of, or proteins associated with, the extra-cellular matrix (ECM), they showed a mosaic
330 of transcript expression depending on the parasite strain used for the challenge.
331 Collagen and hemagglutinin variants were associated with clusters 1 and 14.
332 Haemoglobin 11 variants was down-regulated following SmBRE challenge. These
333 results confirmed the complex restructuring of the ECM during the immune shift
334 from a cellular to a humoral immune response associated to immunological
335 memory. The serpin, a family of serine protease inhibitors, implicated mainly in
336 inflammatory processes, displayed a complex pattern of expression. A total of 26
337 variants were down-regulated in cluster 14 and 4 isoforms of serpin b6-b were up-
338 regulated following the homologous challenge (cluster 7). Cadherin and
339 protocadherin which belong to a superfamily of transmembrane proteins implicated in
340 cell-to-cell binding or adhesion, were also identified. Following the SmBRE immune
341 challenge, 40 different transcripts of this family were down-regulated including
342 protocadherin fats 1, 3, 4 and 20. Similar patterns of regulation were also observed
343 for 14 transcripts of hemocytin, a highly-glycosylated adhesive protein related to
344 haemostasis or the encapsulation of foreign substances for self-defence.

345 In order to investigate the specificity of immunological memory towards the complex
346 mixture of antigenic epitopes presented by *Schistosoma*, we focussed on putative
347 immuno-receptors and we tried to decipher how a specific repertoire is
348 activated following homologous or heterologous parasite challenges (Fig.
349 3). Cumulative log₂ fold-change of each DE immuno-receptor transcript was used to
350 highlight quantitative differences while the number of transcripts was used to highlight
351 qualitative differences (Fig. 3). Homologous systems led to +70.59 and -70.70 log₂
352 fold-changes for 82 DE transcripts (Fig. 3A-B). The heterologous SmVEN and SROD
353 challenges led to the modulation of 62 and 48 DE transcripts, respectively (Fig. 3A-
354 B). The level of expression stands between +22.77 and -80.05 cumulative log₂ fold-
355 change for SmVEN and +35.52 and -41.37 log₂ fold-change for SROD (Fig. 3A).
356 Expression patterns of these transcripts differed strongly between parasites of

357 different genetic backgrounds (Fig. 3B). Only one fourth of the transcripts shared a
358 similar modulation irrespective of the parasite strain used for the challenge (N=29,
359 24.4%) (Fig. 3B). SmBRE-challenged snails expressed the most specific pattern of
360 DE transcripts (N=42, 35.3%) while snails expressed less and less transcripts
361 when challenged with SmVEN (N=19, 16.0%) or SROD (N=14, 11.8%) (Fig. 3B).
362 Annotations provide important information concerning the families of immune
363 recognition receptors selected by the host to cope with the diversity of the pathogens
364 used for the challenges. In cluster 1 (shared up-regulated transcripts), selectin, c-type
365 lectin, FREP 3.3, tenascin, CD205 protein, lymphocyte antigen 75-like and
366 macrophage mannose receptor 1-like were identified (Fig. 3C). In cluster 12 (shared
367 down-regulated transcripts), we identified: 3 transcripts of Pathogenesis Related
368 Proteins (PathoRP), 2 variants of C1q-like 4 (C1q complement-like), 3 transcripts of
369 c-type lectin (calcium-dependant lectin), 1 transcript of collectin-10, 2 transcripts of c-
370 type mannose receptor-2, one CD209, a dermatopontin-3 and one transcript of
371 Thioester-containing protein (TEP) (Fig. 3C). For the strain specific response,
372 homologous challenge led to up-regulation of macrophage mannose receptor 1-like, 3
373 transcripts of selectin, 5 Peptidoglycan recognition proteins (PGRP), 8 Fibrinogen
374 related proteins (FREPs), one galectin, 4 transcripts of c-type lectin, 2 of alpha-2-
375 macroglobulin and 2 of dermatopontin (cluster 6) (Fig. 3C). For down-regulation, one
376 transcript of macrophage mannose receptor-1 like, BgFREP1, a Toll-like receptor, a
377 mucin-5AC, a Galectin-3 and one transcript of TEP were identified (cluster 7). For the
378 SmVEN heterologous response, most of the transcripts were down-regulated
379 (clusters 5-8) including 3 transcripts of C1q-like, 4 transcripts of c-type lectin, and
380 5 transcripts of macrophage-mannose receptor. For the SROD heterologous
381 challenge, 2 transcripts of FREPs were down-regulated together with a Toll-like
382 receptor and one C-type lectin (cluster 9) while 2 transcripts of macrophage mannose
383 receptor 1-like were up-regulated with 8 transcripts of peptidoglycan binding domain-
384 containing protein (cluster 4).

385

386 **3.3. Vaccination with parasite stage extracts**

387 The parasite developed into 3 successive stages within snail tissue (Fig. 4A). Parasite
388 stages were thus recovered independently and used for vaccination. Vaccination with
389 miracidia (prevalence 82.6% (N=86)), Sp2 (66% (N=50)), snail tissues (9% (N=83)),
390 cercaria (81.4% (N=43)) and pool extracts (72.4% (N=98)) did not result in significant

391 protection(Fisher exact test, $p>0.01$)compared to TBS-Tween injections (80.3%
392 (N=61)) (Fig. 4B). However, Sp1 extract induceda significant protection (Fisher exact
393 test, $p=0.000145$) with a prevalence of 47.9%(N=73 individuals) (Fig. 4B). Thus,
394 snails were partially protected solely by Sp1 extract immunization, as other parasite
395 stage extracts appearednot enoughimmunogenic to activate a significant protection.

396

397 **3.4. Vaccination:a tool to validate immunological memory specificity**

398 We analysed immune specificity using vaccination with Sp1 parasite extracts.
399 Vaccinations with Sp1 protein extract from SmBRE, SmVEN or SROD were
400 compared to TBS-Tween injection (Fig. 5). Only homologous vaccination provided
401 significant protection from parasite infection (prevalence 47.9% (N=73), (Fisher exact
402 test, $p=0.000145$)) (Fig. 5). Heterologous vaccinations did not differ from the TBS-
403 Tween control with a prevalence of84.8% (N=33) for SmVEN, and 83.8% (N=37) for
404 SROD (Fig. 5),respectively.

405

406 **3.5. Immune relevant gene-expression following vaccination**

407 To demonstrate that vaccination mimics natural infection and results in the activation
408 of snail immune response, qRT-PCR on relevant immune genes wasperformed on
409 hemocytes following vaccination and then compared to natural infections
410 (Supplementary Fig. 1). Six candidate genes were selected for their putative role in
411 immunological memoryin *Biomphalaria glabrata* based on a whole body RNAseq
412 experiment (Supplementary Table 2). These candidates were selected based on their
413 differential regulation following challenge and for their presence in the shared
414 clusters 1 and 14 (same pattern of expression irrespective of the parasite strain).
415 Vaccination induced the same pattern of response for these immune recognition
416 receptors asthe known response generated by natural infection (Supplementary Fig.
417 1). The transcripts of FREP 3.3, C-type lectin and Selectin are down-regulated
418 following primo-infection in the qRT-PCR approach,similar to the regulation observed
419 in the RNAseq experiment(Supplementary Fig. 1). Glutamate synthase was up-
420 regulated in both natural infection and vaccination(Supplementary Fig. 1). However,
421 some differences exist for TEP and biomphalysin, which displayed opposite
422 expression patternscomparingvaccination and natural infections(Supplementary Fig.
423 1). Altogether, these results suggest that vaccination can partially mimica natural

424 primary infection and can prepare the snail immune system to respond more
425 efficiently to a subsequent parasite infection.

426

427 **3.6. Cell-free hemolymph transfer and acquisition of protection**

428 RNAseq approach reveals that most of the molecules identified as differentially
429 represented between homologous and heterologous infections, corresponded to
430 circulating immune recognition and/or effector factors. We thus investigated how the
431 transfer of cell-free hemolymph from donor snails to naïve recipient snails could
432 transfer protection against infection. Naïve, 15DPI, 4DC and 15DC hemolymph did
433 not significantly transfer resistance to naïve snails, compared to saline injection
434 (Fig.6). Parasite prevalences were as follows: 76% (N=29) for saline injection, 76.5%
435 (N=34) for naïve, 64.4% (N=59) for 15DPI, 59.4% (N=35) for 4DC and 81.8% (N=33)
436 for 15DC (Fig. 6). However, transfer of cell-free hemolymph recovered one-day after
437 the challenge (1DC) led to the acquisition of a significant protection against SmbRE
438 infection (Fisher exact test, $p=0.000145$), with a prevalence of 42.9% (N=35) (Fig. 6).
439 These results demonstrate that 1 day after the challenge, relevant immune
440 recognition molecules, immune mediators or cytotoxic/cytolytic effectors were present
441 in the snail hemolymph and, when injected, were able to activate and maintain an
442 efficient immune protection even 15 days after cell-free hemolymph transfer to naïve
443 recipient snails.

444

445 **3.7. Comparative label-free proteomic of cell-free hemolymph**

446 Cell-free hemolymph recovered 1 day after the challenge (1DC) was able to confer a
447 significant protection when transferred to naïve snails. Thus the characterisation of
448 cell-free hemolymph composition was crucial for the understanding of immune
449 protection following the challenge. Using a comparative label-free proteomic
450 sequencing approach, we characterised cell-free hemolymph circulating protein from
451 naïve, 15 DPI and 1DC snails (Table 2). Here, biomphalysin was identified as the
452 main protein in the 1DC cell-free hemolymph compounds (Table 2). This confirms, at
453 the protein level, the RNAseq results showing that biomphalysin was up-regulated
454 following the challenge (Fig. 2, cluster 1). For all cell-free hemolymph samples, the
455 other identified proteins belong to lectins, recognition proteins, immune cell response
456 mediators, glycan hydrolase, or extracellular matrix components (Table 2). These
457 molecules support the immune shift phenotype observed in *Biomphalaria* snails that

458 turns from a cellular immune response following the primo infection, towards a
459 humoral immune response following the challenge. Moreover, the same functional
460 categories of molecules were observed in the RNAseq results (Fig.2), resulting in a
461 reciprocal confirmation of transcriptomic and proteomic data.

462

463 **3.8. Specificity validation by *in-vitro* cell-free hemolymph toxicity assay**

464 The transcriptomic reprogramming is associated with a higher difference in the set of
465 recognition proteins and effector molecules depending on the interaction with
466 homologous or heterologous parasites. Moreover, the massive proteomic-sequencing
467 approach demonstrated that most of these molecules were released into the
468 hemolymph (Table 2). To confirm that cell-free hemolymph contains factors that
469 support a specific activity against *in-vitro* cultured Sp1 of SmBRE, SmVEN or SROD, a
470 cell-free hemolymph toxicity assay was conducted. SROD sporocysts were difficult to
471 culture *in-vitro* (*i.e.*, 89% of mortality for SROD cultured in buffer saline, Table 3) thus
472 the effects of naïve, SmBRE/SmBRE or SmVEN/SmVEN hemolymphs on SROD
473 Sp1 survival have not been tested due to this high mortality rate (Table 3).

474 However, two conditions were tested, including hemolymph that was either recovered
475 from BgBRE snails primed and challenged by SmBRE miracidia, or hemolymph that
476 was primed and challenged by SmVEN miracidia. A significant difference in
477 toxicity was observed when comparing mortalities induced by challenged snail
478 hemolymph vs naïve snail hemolymph or CBSS culture medium. Hemolymph
479 recovered from challenged snails increased the mortality of sporocysts compared to
480 controls (Fisher exact tests: control vs SmBRE/SmBRE hemolymph for SmBRE
481 sporocyst $p = 0.0262$ and controls vs SmVEN/SmVEN hemolymph for SmBRE
482 sporocyst $p = 0.00001$ or for SmVEN sporocysts $p = 0.00001$). However, one
483 exception to this finding occurred for the controls vs SmBRE/SmBRE hemolymph for
484 SmVEN sporocysts (Fisher exact test $p = 0.8633$) (Table 3). Interestingly, the
485 hemolymph recovered from challenged snails always induced greater mortality for
486 homologous sporocysts compared to heterologous ones (Fisher exact tests:
487 SmBRE/SmBRE hemolymph on SmBRE (47.48%) vs SmVEN (23.07%) sporocysts:
488 $p = 0.0000252$ and SmVEN/SmVEN hemolymph on SmBRE (84.19%) vs SmVEN
489 (95.20%) sporocysts: $p = 0.000258$) (Table 3). These results confirmed the specificity
490 in the humoral factors that are released in the hemolymph after the challenge that can
491 participate in the binding, recognition and killing of primary sporocysts.

492

493 **4. DISCUSSION**

494 An acquired resistance following a primary antigenic stimulation or encounter with a
495 pathogen has been demonstrated for numerous animal models in various
496 invertebrate phyla. Arthropods have been the most investigated group (Brown and
497 Rodriguez-Lanetty, 2015; Kurtz and Franz, 2003; Rodrigues et al., 2010; Roth and
498 Kurtz, 2009) but Cephalochordata, Echinodermata, Nematoda, Cnidaria, Ctenophora
499 and Mollusca have also been investigated (Jorge Contreras-Garduno et al., 2016;
500 Milutinovic and Kurtz, 2016). Some of these studies demonstrated the existence of a
501 specific immunological memory against viruses, yeasts, and bacteria. However, these
502 pathogens express a limited set of potential antigens or pathogen associated
503 molecular patterns (PAMPs) like peptidoglycans, beta-glucans or Lipo-poly-
504 saccharides (LPS), which can be easily recognised by the host immune
505 system (Jorge Contreras-Garduno et al., 2016).

506 In the *Macrocyclops albidus*/*Schistocephalus solidus* model (Kurtz and Franz,
507 2003) and *Biomphalaria glabrata*/*Schistosoma mansoni* model (Pinaud et al., 2016;
508 Portela et al., 2013), both partners are phylogenetically related as they belong to the
509 metazoa, bilateria lineage. This relative phylogenetic proximity is particularly
510 interesting when studying the mechanisms involved in the specificity of immunological
511 response. Here, both partners would be expected to share several antigenic
512 determinants (Yoshino et al., 2012), and fine-scale host recognition processes would
513 thus be expected to recognise specific pathogen determinants and to avoid auto-
514 immunity. A genotype-dependant immunological memory has been demonstrated for
515 *Biomphalaria glabrata* suggesting that snail immune response can distinguish
516 between different parasite species and strains (Portela et al., 2013). Herein, we go
517 further in the characterisation of the molecular bases for this specific immunological
518 memory response.

519 An atypical transcriptomic profile was described following a natural primo-infection
520 and challenge in *Biomphalaria glabrata* snails and was termed an 'immune
521 shift' (Coustau et al., 2016; Pinaud et al., 2016). This response is characterised by an
522 incomplete or absence of immune response following the primary encounter with
523 *Schistosoma* worms (Portet et al., 2018b). This first interaction is considered compatible
524 and thus the parasite can develop to patency in snail tissue. At the transcriptomic
525 level, no strong immune response was observed in response to infection. However,

526 following the secondary encounter with the same pathogen, the parasite was unable
527 to develop into snail tissue as the snails became totally resistant to infection. Only
528 parasites from the challenge were killed, when parasites from the primo-infection
529 persisted in snail tissue producing Sp2 and cercariae that lead to snail death 140
530 days after infection (Table 1).

531 Interestingly an analogous situation was demonstrated in experimental
532 schistosomiasis infections in mice and monkeys (Smithers and Terry, 1967). The
533 term 'concomitant immunity' has been used to describe the ability of the vertebrate
534 host to mount an effective immune memory response against larval stages of the
535 parasite whilst being unable to clear the persistent adult worms. The adult
536 schistosomes are capable of evading the immune response which they
537 provoke (Smithers and Terry, 1969). As the worms developed, a gradual acquisition
538 of host antigens were detected and provide a possible explanation of concomitant
539 immunity, and demonstrate the ability of adult worms to evade the immune response
540 which acts against the unprotected schistosomula in a challenge infection (Clegg et
541 al., 1971). These host antigens may be molecules of host origin incorporated by the
542 parasite into its surface membrane or molecules synthesised by the parasite that
543 mimic a host antigenic determinant. This refers to Raymond Damian's concept of
544 'molecular mimicry' or 'antigen sharing' between host and parasite which proposed
545 that one of the mechanisms by which parasites could avoid the host immune
546 response was by mimicking host molecules (Damian, 1997). Carbohydrate epitopes
547 or protein glycosylation were more likely to be mimicked by the parasite and
548 appeared as the best candidate to explain concomitant immunity (Damian, 1987).

549 In snail/Schistosome interactions, similar processes have been demonstrated and
550 host snail antigens shared with miracidia and sporocysts have been described on
551 parasite larval surfaces and constitute another example of molecular
552 mimicry (Yoshino and Bayne, 1983). In this context, our recent work on miracidium
553 and sporocyst glycoproteins, the *Schistosoma mansoni* polymorphic mucins
554 (SmPoMucs) (Galinier et al., 2017; Mone et al., 2010; Roger et al., 2008a), were
555 identified as relevant carbohydrate or glycan candidates that share potential
556 antigenic epitopes with snail antigens and support the "molecular mimicry" or "smoke
557 screen" strategies developed by the parasite to avoid recognition (Mitta et al., 2012;
558 Mitta et al., 2017; Portet et al., 2017). Thus concomitant immunity may exist solely if

559 efficient immune memory response exists, and this is exactly what we have observed
560 in the present paper.

561 Here, a shift in the immune response was observed, following the first encounter with
562 the parasite. First, a cellular immune response, clears most of the parasites through
563 hemocyte encapsulation. Then, during the secondary encounter with the same strain
564 of parasite, circulating/plasma factors were involved in the elimination of the parasite.
565 This shift in phenotype is supported by numerous differentially regulated immune
566 transcripts identified in the RNAseq approach only following the challenge (Pinaud et
567 al., 2016) (Fig. 2). This pattern of specific massive transcriptomic response appeared
568 as a characteristic of immunological memory in invertebrates (Greenwood et al.,
569 2017).

570 This pattern was also confirmed in the present study, irrespective of the strain of
571 parasite used for the secondary challenge. However, if homologous SmBRE/SmBRE
572 infection and challenge resulted in a total immune protection (100% of snail
573 resistant and 2,013 DE transcripts), the heterologous SmBRE/SmVEN system solely
574 induced a partial protection of 71% with 405 DE transcripts and the SmBRE/SROD
575 system led to 58% protection with only 210 DE transcripts (Fig. 2). However, it would
576 be difficult to assume that the number of DE transcripts alone could explain both the
577 improvement in protection observed and the specificity, even if they appeared to be
578 correlated. The correlation between qualitative/quantitative transcript expression and
579 protection in homologous or heterologous challenges has to be considered in
580 association with their potential involvement in pathogen recognition, pathogen killing,
581 specificity and memory (Figs. 2 and 3).

582

583 **4.1. Immunological memory against *Schistosoma sp.***

584 Transcripts in the cluster 1 are up-regulated and correspond to the basal
585 challenge response observed for all the parasite strains, symmetrically, those in
586 cluster 14 correspond to down-regulated transcripts (Fig. 2). Within these clusters, we
587 identified numerous factors already implicated in immune response pathways. Within
588 immune recognition molecules or effectors, the lectin families are considered
589 recognition factors in *Biomphalaria glabrata* snails. Lectins are defined as molecules
590 that contain a lectin domain, previously described as carbohydrate-binding
591 proteins (Dheilly et al., 2015; Portet et al., 2017; Tetreau et al., 2017; Wu et al., 2017).
592 The presence of macrophage mannose receptor family members, FREP3.3, C-type

593 lectin and selectin in cluster 1, highlights the importance of pathogen sensing
594 in response to the secondary challenge. Lectin families have been shown to be highly
595 diversified and polymorphic to cover an important range of pathogen diversity (Dheilly
596 et al., 2015). This diversity should be enough to face the potential parasite
597 strains/species antigenic diversity and to support the specific immune response
598 observed herein. Numerous immune response-associated genes are frequently found
599 in deep-sequencing analyses but their role within the context of immunity was seldom
600 discussed. For example, snail mucins and collagens are often described as
601 components of the extra-cellular matrix, but they must also be involved in cell motility
602 or migration (Hunt et al., 1996). An up-regulation of these variants within these families
603 may induce several pathways implicated in the recruitment of competent cells in order
604 to block incompetent cells or pathogens as previously described for *Streptococcus*
605 infection of macrophages (Dhar et al., 2017).

606 The biomphalysin appeared as a particularly relevant candidate. As a beta-pore
607 forming toxin (Galinier et al., 2013), it remains the sole candidate able to directly kill
608 schistosomes (Galinier et al., 2013). This highly toxic molecule has already been
609 described in the immune memory protection observed after challenge in
610 *Biomphalaria* snails (Pinaud et al., 2016). In the present work, its presence and its
611 expression increase against all homologous or heterologous tested strains and
612 species of parasites (Figs. 2 and 3) and is identified in cell-free hemolymph
613 immediately following the challenge (Table 2). This circulating toxin, may be
614 potentially released by competent immune cells that have been trained following the
615 primary infection, and could be considered as the primary factor supporting immune
616 shift in *Biomphalaria* immunological memory. Different biomphalysin transcripts are
617 identified in massive transcriptomic approaches and these transcripts are difficult to
618 align with reference biomphalysin genes, leaving the possibility that several isoforms of
619 this toxin could exist in *Biomphalaria* snails (Fig. 2B and Supp-Table 2). Label free
620 proteomic (Table 3) and comparative interactome experiments between living
621 pathogens and snail hemolymph demonstrate that it is often difficult to assign the
622 identified peptides to a specific biomphalysin gene (Galinier et al., 2013; Tetreau et
623 al., 2017). Altogether, these results provide clues for the potential role of this toxin in
624 the humoral immunological memory response observed in *Biomphalaria glabrata*
625 snails. Other immune effectors like lbp/bpi or mytimacin, described as antimicrobial
626 peptides (AMPs) or proteins (Adema et al., 2017; Baron et al., 2016; Baron et al.,

627 2013), can also participate in the regulation of the immune response (Hu et al.,
628 2017), displaying cytokine functions, attracting haemocytes towards sites of injuries
629 (Destoumieux-Garzon et al., 2016; Li and Song, 2010).

630 Finally, thioester-containing proteins (TEP) are complement-like proteins that
631 act as opsonin, promoting phagocytosis, or lysis and sometimes even melanisation,
632 in invertebrates (Le Clec'h et al., 2016), and arthropods (Blandin and Levashina,
633 2004; Buresova et al., 2011; Volohonsky et al., 2017) and appear to also participate
634 in primary cellular immune response and encapsulation in *Biomphalaria glabrata*
635 snails (Mitta et al., 2012; Mone et al., 2010; Portet et al., 2018a). Following
636 immunological memory acquisition in *Biomphalaria*, the cellular immune
637 encapsulation response shifts to a humoral response (Pinaud et al., 2016) and is
638 associated with the down-regulation of TEP in the homologous challenge RNAseq
639 data 15 days after the primo-infection (Figs. 2 and 3) as well as the absence of TEP
640 in 1DC proteome (Table 2).

641

642 **4.2. Total protection in homologous challenge**

643 Following the homologous challenge, we observed a total protection. Immune
644 recognition molecules and effectors were particularly up-regulated with an increase in
645 both, the expression and diversity of different lectins (Fibrinogen-related proteins
646 (FREPs), c-type lectin, and galectin-4) (Fig. 2 and 3, Supp Fig. 2).

647 In this context, FREPs were of particular interest. These molecules have been
648 highlighted in several different contexts in the study of *Biomphalaria* snails, (i)
649 pathogen recognition (Mone et al., 2010; Roger et al., 2008b), (ii) resistance against
650 parasites (Hanington et al., 2010; Hanington et al., 2012) and (iii) innate immune
651 memory (Pinaud et al., 2016). RNAseq results showed that following a homologous
652 challenge, the diversity and expression of FREP transcripts increase (Fig. 2, 3, and
653 Supp Fig. 2) compared to heterologous challenges, leading to a potential increase in
654 the ability of the host to recognise/bind to the encountered parasite following the
655 challenge. Certain BgFREPs have been shown to form multimers, viewed using
656 native PAGE or SDS-PAGE gels as noncovalent or covalent complexes (Adema et
657 al., 1997; Zhang et al., 2008). For example, BgFREP4 is commonly found as a
658 tetramer in snail plasma (Gordy et al., 2015; Zhang et al., 2008). Moreover, even if
659 not demonstrated for *Biomphalaria* snails, other lectins like the galectin are known to
660 form multimers and their association leads to the activation of bactericidal activity in

661 mammals and molluscs(Blazevits et al., 2016; Kawsar et al., 2011). Thus, we can
662 hypothesize a putative dual-role of theselectin families, with both single-member
663 pathogen recognition/binding and association with different members in
664 dimer/multimer, as well as homo or hetero-multimers,providing additional functions to
665 proteins that participate in an immunological response. Multimerization could be an
666 additional process to increase the mollusc pathogen recognition repertoire, by
667 increasing binding avidity or enhancing pathogen recognition specificityand making
668 the invertebrate immune response more efficient(Schulenburg et al., 2007).

669 In the present study, we observe a complex expression pattern of diverse immune
670 recognition molecules and/or effectors that drives a total immune protection following
671 a homologous challenge. This complexity of immune recognitioneffectorsresults from
672 the complexity of the antigenic landscape offered by *Schistosoma* parasite and may
673 explained why previous RNAi studies targeting solely the FREPs or the BgTLR,
674 resulted only in the partial invalidation of snail resistance or immunological
675 memory(Hanington et al., 2010; Hanington et al., 2012; Pila et al., 2016; Pinaud et
676 al., 2016).

677

678 **4.3. Vaccination as proof of immune memory**

679 Vaccination is the process whereby an individual is made immune to an infectious
680 agent. This immunization with pathogen extracts, stimulates the immune system and
681 yields a state of protection (i.e., immune response is stored and recalled) against
682 subsequent infection. Thus, immunization is a tool that has the capacity to
683 demonstrate the existence of an enhanced immune activity or immune memory in
684 invertebrates. Here, vaccinations were developedusing native parasite extracts that
685 correspondto a complex molecular extract containing proteins, sugars, salts, ions,
686 hormones, nucleic acids, or other potential viral or bacterial associated organisms
687 fromthe parasite. Thus it can be difficult to conclude which specific compounds
688 (proteins, sugars, nucleic acids)were responsible for immunization and for the
689 acquisition of resistance in immunized snails.However inflammatory response
690 associated with needle injection of saline buffer (used as control of injection) was
691 notenoughto activate protection(Fig.4). An absence of protection was also observed
692 following immunization with hepato-pancreas extracts isolated from healthy
693 individuals (used as Sp2 control injection) (Fig. 4). A tissue extract fromindividual
694 snailwasnot enough to immunize recipient snails against*Schistosoma*infection.

695 However , vaccination with primary sporocyst extract (Sp1) was able to induce a
696 significant protection (47.9% of snails not infected following exposure), vaccination
697 with Sp2 extract seemed to have a slight effect (not significant) and vaccination with
698 cercariae extract did not protect snail at all (Fig. 4). While the snail immune system
699 seemed able to recognise foreign pathogenic antigens and could activate an adapted
700 and efficient immune response against them, it seems that it was unable to recognise
701 antigens belonging to snails with same genetic background.

702 Efficient recognition of Sp1 suggests that evolutionary pressure targeted recognition
703 to the parasite's first larval stage, one could thus expect that the Sp1 was the most
704 immunogenic in comparison with those that developed later (Sp2 and cercariae). This
705 observation fits well with the "molecular mimicry" hypothesis (Damian, 1987, 1997)
706 and the gradual acquisition of host antigens by parasites during their development
707 within host tissues.

708 Finally, snails are better protected when vaccinated with homologous parasite
709 extracts than with heterologous ones. These results revealed specificity of
710 immunological memory in *Biomphalaria glabrata*. However, vaccination with Sp1
711 extracts from SmBRE, SmVEN or SROD neither activated nor induced a total
712 protection against reinfection, as it can be observed following natural infection.
713 Vaccination with whole extracts of SmBRE Sp1 induced a partial protection against
714 the homologous SmBRE challenge while heterologous Sp1 vaccination (SmVEN,
715 SROD) did not result in any protection (fig. 5). Even immunizations with sympatric
716 Sp1 or pools of whole parasite extracts cannot induce a total protection (Fig. 4B).
717 This suggests that other immune pathways or mechanisms have to be activated to
718 reach a 100% of protection. In this context, the parasite's development within snail
719 tissues may be the source of a permanent snail immune stimulation through the
720 release of proteins, nucleic acids, or excreted/secreted products by the parasite
721 (Guillou et al., 2007; Lodes and Yoshino, 1990; Nowacki et al., 2015; Yoshino et al.,
722 2014).

723 So, if vaccination reveals such a high degree of efficiency and potential
724 specificity in the reaction of the *Biomphalaria glabrata* immune system, it did not
725 succeed in inducing a complete protection for all individuals tested within a
726 heterogeneous population. The RT-q-PCR experiment that was based on a few
727 candidate genes selected because they were differentially regulated in the massive
728 transcriptomic approach, confirms that vaccination did not perfectly mimic natural

729 infection (Supp Fig. 1). Together, vaccination experiments demonstrate unequivocally
730 the acquisition of immunological memory in *Biomphalaria* snails. Vaccination in
731 invertebrates with economic importance remains mainly focused on viral disease
732 where successful vaccinations have been demonstrated (Chang et al., 2017; Green et
733 al., 2016; Lafont et al., 2017; Syed Musthaq and Kwang, 2014; Taengchaiyaphum et
734 al., 2017). However, our results on vaccination in the schistosomiasis vector snail
735 *Biomphalaria glabrata* presents the first anti-metazoan vaccination in an invertebrate
736 but highlighted also the necessity to conduct additional investigations on the process
737 of vaccination in molluscs and more generally in invertebrates.

738

739 **4.4. Cell-free hemolymph transfer**

740 When hemolymph is recovered 1 day after the immune challenge and injected into
741 naïve snails, a high protection against infection (51% of snails protected following
742 exposure) is observed (Fig. 5). Other plasma samples tested did not transfer any
743 significant protection (Fig. 5). This result suggests that secretion of relevant immune
744 factors into the plasma occurs early after the challenge and participates immediately
745 in the defence of the organism against newly infecting parasites, while those from the
746 primo-infection remained unharmed. These active molecules may decrease in the
747 hemolymph as they are consumed in the anti-parasitic response. This may explain
748 why the 4DC and 15DC cell-free hemolymph no longer provide protection when
749 transferred to naïve snails (Fig. 5). These factors seem important in support of the
750 immune humoral response observed following secondary challenge infection and in
751 the killing of the parasite. The label-free proteomic approach (Table 2) helped to
752 identify some of these factors and in particular, the biomphalysin, which might act as
753 the main humoral factor responsible for this immunological memory response.

754 Moreover, while natural infection induces a 100% protection to secondary infection,
755 vaccination and cell-free hemolymph transfer only reach around 50% of protection.
756 This difference would be resolved by the sequence composition of plasma and
757 parasite protein extract, but this must be studied independently. Immunization means
758 that the parasite -exogenous- molecule can activate the host's immune system and
759 can lead to an acquired immune resistance while the plasma transfer experiments
760 confirm that circulating host molecules from infected individuals can activate the
761 immune system of naïve individuals to reach a comparable immune protection.

762

763 **4.5. *In-vitro* toxicity and specificity of primed plasma**

764 Toxicity assays show for the first time, the implication of humoral factors in the
765 specificity of parasite clearance in an invertebrate. Homologous system displays a
766 higher *in-vitro* toxicity despite the origin of the strain used (Fig. 6 and Table
767 3). Immune factors are produced and secreted in the hemolymph compartment to
768 specifically bind and kill the homologous parasite strains more efficiently than the
769 heterologous ones (Table 3). These results suggest that the genotype-dependent
770 specific memory response in *Biomphalaria* is supported by complex repertoires of
771 circulating recognition molecules or effectors secreted in the hemolymph early after
772 the challenge.

773 Cytotoxic activities may be due to some toxic molecules like the
774 biomphalysin (Galinier et al., 2013; Pinaud et al., 2016) (Table 2). This beta-pore
775 forming toxin may be transferred during vaccination and may remain toxic 15 days
776 after injection to protect more than 50% of the tested snails (Fig. 6). Interestingly,
777 biomphalysin is the main protein released in the hemolymph 1 day after the challenge
778 (Table 2, Fig. 2 and 3) that can participate in parasite killing after challenge (Galinier
779 et al., 2013; Pinaud et al., 2016). Moreover, a recent interactome approach revealed
780 that different biomphalysin variants were found to interact with different pathogen
781 species (bacteria, yeast, trematodes) (Tetreau et al., 2017) (Supp-Table 2), arguing
782 for the potential role of biomphalysin in both immunological recognition/binding and
783 pathogen clearance, leading to a higher level of specificity of immunological memory
784 response in *Biomphalaria* snails.

785

786 **4.6. Combinatorial repertoire of host recognition molecules and effectors to** 787 **face the very large number of parasite epitopes**

788 The peptidoglycan recognition protein family, FREPs, C-type lectins, macrophage
789 mannose receptors, TLRs, selectin and galectin are all members of different putative
790 immune recognition superfamilies (Adema et al., 2017; Dheilly et al., 2015; Ferrandon
791 et al., 2007) (Figs. 2, 3). They contain domains capable of directly binding to pathogens
792 as carbohydrate recognition domain (CRD), fibrinogen (FBG) or immunoglobulin (Ig)
793 for the FREPs, the leucine-rich repeats (LRR) for TLR or carbohydrate-binding
794 protein domain for C-type lectins (Ferrandon et al., 2004; Pasare and Medzhitov,
795 2004). FREPs or C-type lectin, for example, were previously described in the
796 interaction between *Biomphalaria* and *Schistosoma* in immune protein

797 complexes(Mone et al., 2010; Tetreau et al., 2017; Wu et al., 2017) as well as in
798 transcriptomic response(Hanington et al., 2012; Pinaud et al., 2016). Herein, we
799 reveal for the first time, the complex repertoire of recognition and effectorfactors
800 requiredto express an efficientimmunological memory response (Fig. 3 and Supp Fig.
801 2).These putative recognition molecules oreffectorsneed to be produced as
802 extracellular or humoral factorsin order to cooperate altogether(Hargreaves and
803 Medzhitov, 2005; Schulenburg et al., 2007). Such moleculesmayby successively
804 expressed orassociated with each other to work in coherent multimers to increase
805 recognition capabilities or to enhance substrate affinity(Brewer et al., 2002; Choe et
806 al., 2005; De Marzi et al., 2015; Hargreaves and Medzhitov, 2005; Kang et al., 1998;
807 Lee and Lee, 2000; Sharma et al., 2011; Wang et al., 2008).Together, the complex
808 regulation and/or selection of different types of recognition molecules and effectors
809 from the innate immune system certainly reflects the great antigenic complexity of
810 immunogens facing the host. This complex response involving a multitude of often
811 polymorphic genotype-dependent elements that, independently of each other or
812 perhaps in combination with each other, contributes to provide the *Biomphalaria* snail
813 with an efficient and specific immunological memory response. Recently we
814 hypothesised that a molecular or epigenetic reprogramming of hemocyte sub-
815 populationsmightallow for the expression of a higher quantity and diversity of immune
816 recognition molecules and/or effectors, and support theimmunological
817 memoryspecificity in*Biomphalaria*snails (Gourbal et al., 2018).We are convinced that
818 investigating themolecular mechanisms supporting hemocyte reprogrammingwould
819 now be mandatory to better understand the innate immunity, specificity and memory
820 in invertebrates, and to decipher the overall complexity, originand evolution of
821 metazoan innate and adaptive immune systems.

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823

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833 *Author contributions*

834 SP, AP, JFA, RG, DD, BG design and performed the experiments and analysis. CSB,
835 NA participated in animal breeding and parasite experimental infections. LB
836 conducted the Label free proteomic experiments. SP, LdP, BG drafted the
837 manuscript. JFA, DD, RG, LdP participated in the reviewing process. All authors read
838 and approved the final version of the manuscript.

839 All data generated or analysed during this study are included in this published article
840 [and its supplementary information files]. Transcriptome of *Biomphalaria* snail is
841 available at: <http://ihpe.univ-perp.fr/acces-aux-donnees/>.

842

843 *Conflict of interest*

844 The authors declare that no conflict of interest exists.

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1123 **Figure 1. Overview of experimental protocols and results.**

1124

1125 **Figure 2. Transcriptomic response exploration of innate immune memory**
1126 **specificity.**

1127 (A) Venn diagram of differentially expressed (DE) transcripts following homologous or
1128 heterologous challenges. Snails were primo-infected by SmBRE and then challenged
1129 by SmBRE, SmVEN or SROD parasites. A correlation is observed between the level
1130 of protection following challenge and the number of DE transcripts. The homologous
1131 interaction SmBRE/SmBRE: 100% of protection and 2,013 specific DE
1132 transcripts; heterologous SmBRE/SmVEN: 71% of protection and 405 specific DE
1133 transcripts; heterologous SmBRE/SROD: 58% of protection and 210 specific DE
1134 transcripts. (B) Heat map of differentially expressed transcripts following homologous
1135 and heterologous challenges. Each line represents a DE transcript (padj value
1136 <0.05), each row represents the strain of parasite used for the challenge. Colours
1137 represent the level of expression, green for down-regulated transcripts and blue for
1138 up-regulated transcripts. Last column of the heat map highlighted the immune related
1139 transcripts identified based on Blast2Go annotation, domain search and known
1140 immune genes of the literature. Names in bold correspond to transcripts that have
1141 been yet described as involved in *Biomphalaria* / *Schistosoma* interaction in the
1142 literature.

1143

1144 **Figure 3. Immune recognition-related transcripts following homologous or**
1145 **heterologous challenge.**

1146 (A) Graph showing cumulative Log₂FC of DE immune recognition-related transcripts
1147 identified in *BgBRE* snail primo-infected by SmBRE and challenged with homologous
1148 (SmBRE) or heterologous (SmVEN or SROD) parasites. Each colour in the graph
1149 correspond to a different DE transcript. (B) Venn diagram that indicates a relationship
1150 between the number of DE transcripts related to immune recognition and the
1151 homologous/heterologous associated protection (C) Heatmap of DE transcripts
1152 related to immune recognition. Each line is a DE transcript (padj value <0.05), each
1153 row the strain of parasite used and the colours depend on the level of expression,
1154 green for down-regulated and blue for up-regulated transcripts. Last column of the
1155 heat map indicates the immune receptor or recognition transcripts identified in each
1156 cluster. Transcript annotations were defined using Blast2Go annotation, Blastp
1157 against opened protein databases, domain searches and data from the literature.

1158

1159 **Figure 4. Vaccination with *Schistosoma* parasite extracts.**

1160 (A) Representation of *Schistosoma* parasite stages development into snail tissues
1161 (B) Prevalence observed following *SmBRE* infection in *BgBRE* snails previously
1162 vaccinated with different parasite native extracts (Miracidia, Sp1: Primary sporocyst,
1163 Sp2: Secondary sporocyst, Cercaria and an equimolar pool of Miracidia, Sp1, Sp2
1164 and Cercaria.)

1165

1166 **Figure 5. Homologous versus heterologous vaccination with sporocyst**
1167 **extracts**

1168 Prevalence observed following *SmBRE* infection in *BgBRE* previously vaccinated
1169 with sporocyst (Sp1) native extracts from homologous (*SmBRE*) or heterologous
1170 (*SmVEN*, *SROD*) parasites.

1171

1172 **Figure 6. Protection acquired following hemolymph transfer**

1173 Protection against *SmBRE* infection observed in naïve *BgBRE* snails following
1174 different hemolymph transfer treatments. Naïve *BgBRE* snails were subjected to
1175 injection with, (i) saline solution (CBSS); (ii) cell-free hemolymph (plasma) of naïve
1176 snails, (iii) plasma from *BgBRE* snails primed with *SmBRE*; or (iv) plasma from
1177 *BgBRE* snails challenged with *SmBRE* and recovered at 1 day, 4 days or 15 days
1178 after challenge. Protection has been estimated using the following formula: (control
1179 prevalence-experimental sample prevalence)/(control prevalence). Control
1180 prevalence correspond to prevalence calculated for untreated snail following infection
1181 with 10 *SmBRE* miracidia.

1182

1183 **Supplementary figure 1. Q-RT-PCR expression of innate immune memory**
1184 **candidate genes following vaccination.**

1185 *BgBRE* snails were either vaccinated or primo-infected with *SmBRE* Sp1 and gene
1186 expression was quantified 15 days after treatment. Q-RT-PCR was conducted after
1187 vaccination and gene expression was compared to RNAseq data following primo-
1188 infection. See supplementary table 1 for gene details.

1189

1190 **Supplementary Figure 2. RNAseq-based FREP expressions following**
1191 **homologous or heterologous challenges.**

1192 Histogram displays cumulative RNAseq Log2FC of differentially expressed FREP-
1193 related transcripts identified in *BgBRE* primo-infected by *SmBRE* and challenged with
1194 homologous (*SmBRE*) or heterologous (*SmVEN* and *SROD*) parasites.

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1200 Table 1. Long-lasting immunological memory in *Biomphalaria glabrata* snails.

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Prevalence primo-infection		
BgBRE/SmBRE		
BgBRE	N=15	100%
Prevalence challenge		
BgBRE/SmBRE/SmBRE		
15 DPI BgBRE	N= 15	0%
25 DPI BgBRE	N=15	0%
56 DPI BgBRE	N= 15	0%
72 DPI BgBRE	N= 15	6%
140 DPI BgBRE	N= 15	0%
162 DPI BgBRE	N= 15	NA

BgBRE/SmBRE: BgBRE snails primo-infected by SmBRE.

BgBRE/SmBRE/SmBRE: BgBRE snails primo-infected by SmBRE and Challenged by SmBRE.

DPI: days post-primo-infection.

NA: not available.

1225 **TABLE 2: Label free proteomic of cell-free hemolymph**

1226

15DPI		1DC		Accession # (Biomphalaria transcriptome)	
Pep.	SC	SSC	WSC	Pep.	WSC
	7	10	2	647	89/Locus_580_Transcript_6/13_Confidence_0.012_Len
	3	3	3	5.8	88/98/Locus_10707_Transcript_2/13_Confidence_0.4
	3	3	3		cdh1est_out_L4502
	3	3	3		86/Locus_1894_Transcript_1/1_Confidence_0.000_L4
	3	3	3		86/Locus_1894_Transcript_2/1_Confidence_0.000_L4
	3	3	3		86/Locus_1894_Transcript_3/1_Confidence_0.000_L4
	3	3	3		86/Locus_1894_Transcript_4/1_Confidence_0.000_L4
	2	2	2	495	6
	2	2	2	4	7
	2	2	2	3	5
	2	2	2	4	4
	2	2	2	4	4
	1	1	1	2	3
	1	1	1	2	3
	1	1	1	2	3
	3	3	3		88/Locus_1648_Transcript_4/7_Confidence_0.182_Len
	2	2	2		Locus_900_Transcript_L_50/213_Confidence_0.000_Leng
	2	2	2		88/Locus_1448_Transcript_3/5_Confidence_0.300_Len
	2	2	2		88/Locus_1448_Transcript_4/5_Confidence_0.300_Len
	2	2	2		88/Locus_3280_Transcript_1/2_Confidence_0.667_Len
	3	3	3		88/Locus_7652_Transcript_1/1_Confidence_0.000_Len
	3	3	3		88/Locus_1838_Transcript_20/75_Confidence_0.348_Lu
	2	2	2		Locus_651_Transcript_L_39/39_Confidence_0.000_Lengh
	2	2	2		88/98/Locus_1461_Transcript_1/1_Confidence_0.181
	2	2	2		88/Locus_1461_Transcript_2/1_Confidence_0.181
	2	2	2		88/Locus_2653_Transcript_2/7_Confidence_0.000_Len
	2	2	2		88/98/Locus_7968_Transcript_1/1_Confidence_0.000
	2	2	2		88/Locus_5998_Transcript_1/1_Confidence_0.000_Len
	3	3	3		88/98/Locus_230_Transcript_32/53_Confidence_0.040
	2	2	2		88/Locus_11898_Transcript_1/1_Confidence_0.000_Lu
	2	2	2		Locus_8595_Transcript_42/49_Confidence_0.100_Km

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1228

1229 Pep: Number of identified peptides

1230 SC: Spectral Counts : mass spectra allow the identification of peptides belonging to the protein

1231 SSC: Specific Spectral Counts : mass spectra allow the identification of peptides belonging specifically to the protein

1232 WSC: Weighted Spectral Counts : SSC + the weight of unspecific SC (in the case of peptides shared between several proteins)

1235 Biomphalaria transcriptome and proteome were availables at: <http://ihpe.univ-perp.fr/acces-aux-donnees/>

1237 15DPI: 15 days post primo-infection

1238 1DC: 1 day post challenge

1239 Table 3. Cell-free hemolymph toxicity

1240

		% of mortality					
		Sporocysts SmBRE		Sporocysts SmVEN	Sporocysts SROD		
CBSS culture medium		N= 71	23.94%	N= 89	21.34%	N= 72	89.47%
	naive hemolymph	N= 60	21.05%	N= 118	17.41%		NA
CBSS supplemented with	SmBRE/SmBRE hemolymph	N= 106	47.48% ^{a,b}	N= 143	23.07% ^b		NA
	SmVEN/SmVEN hemolymph	N= 203	84.19% ^{a,c}	N= 162	95.20% ^{a,c}		NA

1241

1242

1243 a, Fisher exact test < 0.05 compared to controls

1244 b, Fisher exact test < 0.05 comparing SmBRE or SmVEN sporocysts with SmBRE/SmBRE

1245 hemolymph

1246 c, Fisher exact test < 0.05 comparing SmBRE or SmVEN sporocysts with SmVEN/SmVEN

1247 hemolymph

1248

1249 NA: not available

1250

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1256 Supplementary Table 1. Primers of Q-RT-PCR

1257

1258

Transcript names	Annotations	Primer names and sequences [§]	
BBVBR_Locus_52081_Transcript_1/1_Confidence_0.000_Length_987	fibrinogen-related protein 3.3 [Biomphalaria glabrata]	Frep3.3	Forward: CCTCTGTCGAAGCGGAACCTTATT Reverse: AACGTCGGCCTTTCAACAGTTATT
BB_Locus_14654_Transcript_1/1_Confidence_0.333_Length_433	PREDICTED: c-type lectin	C-type Lectin	Forward: TAATCGGAGCTGTACACACAA Reverse: AGCGTCAGCCGTTACAATACA
BBVBR_Locus_2588_Transcript_1/2_Confidence_0.667_Length_595	uncharacterized protein [Aplysia californica]_&_selectin partial	Selectin	Forward: GGGCGTTTGAATGGCGATGAAT Reverse: TGCATTCGTCGAGGAGGTGT
BB_Locus_44392_Transcript_1/3_Confidence_0.667_Length_1083	putative glutamate synthase [NADPH]-like isoform X1 [Aplysia californica]	Glutamate synthase	Forward: TGCAGGTCCACTGCCAATGATA Reverse: CTCCTTGTGAGGGAGCATGTGT
cdhittest_out_225206	thioester-containing protein [Euphaedusa tau]	TEP	Forward: GGGGTGGAATCAATCTGGTCTA Reverse: TGGAAGTGAGTCTGATGGTGTT
BB_Locus_4989_Transcript_4/4_Confidence_0.000_Length_1252	biomphalysin [Biomphalaria glabrata]	Biomphalysin	Forward: CGTGGAGGAATGTTTGGGTAGT Reverse: TCAACGACAAGCAGCTGGAAGA

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1261 § Synthesized by ©Eurogentec, Belgium Sepop Desalt

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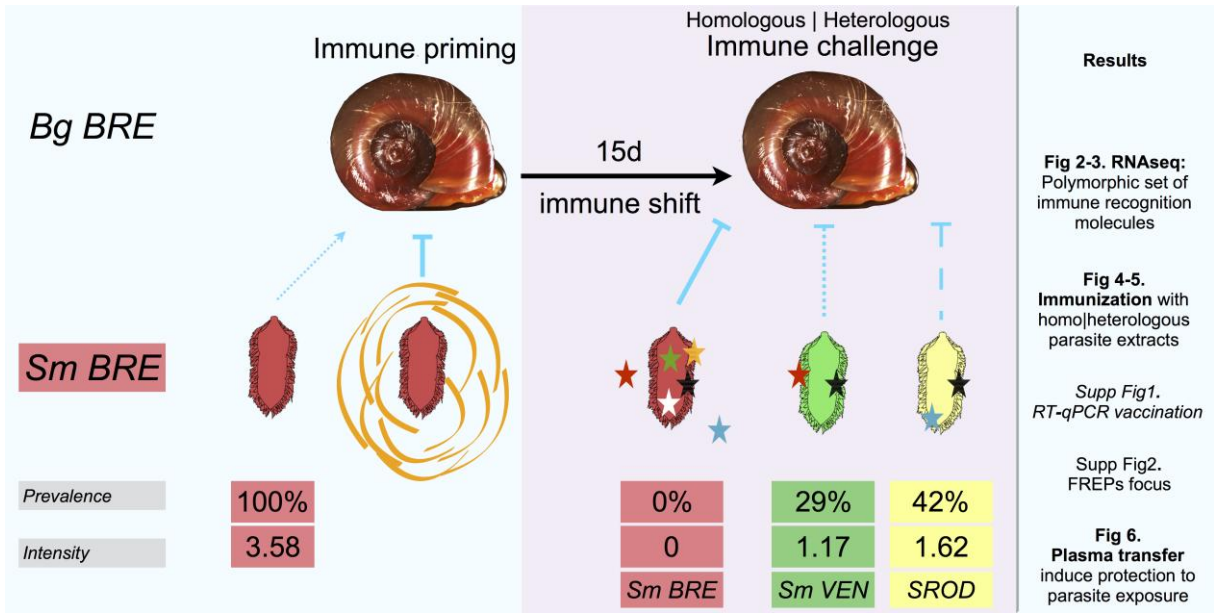
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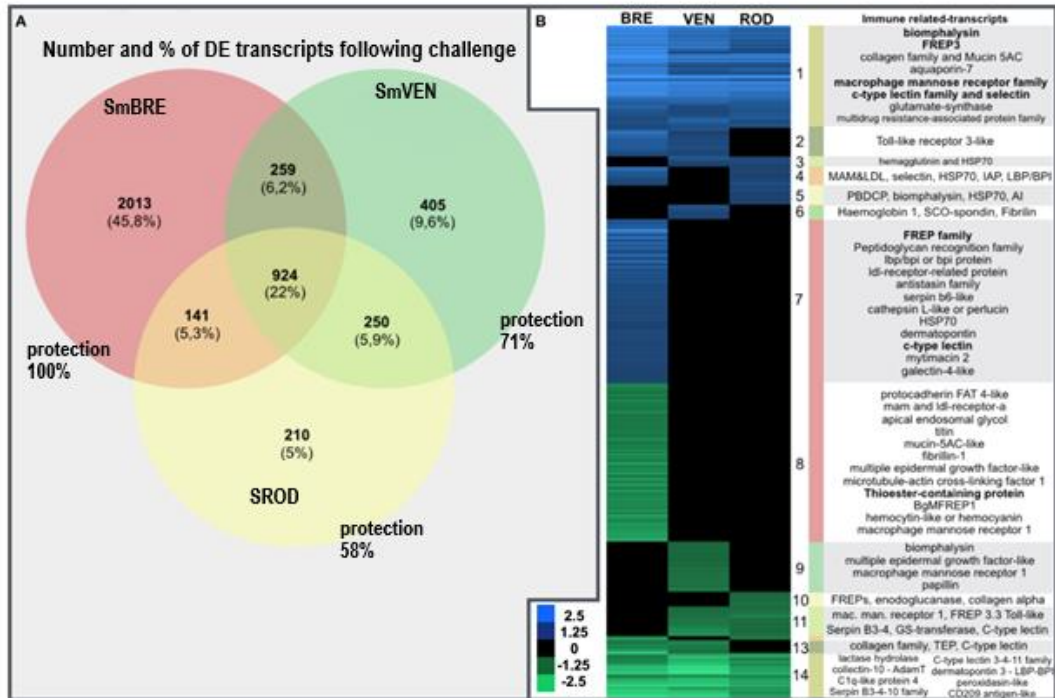
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1282 Figure 1
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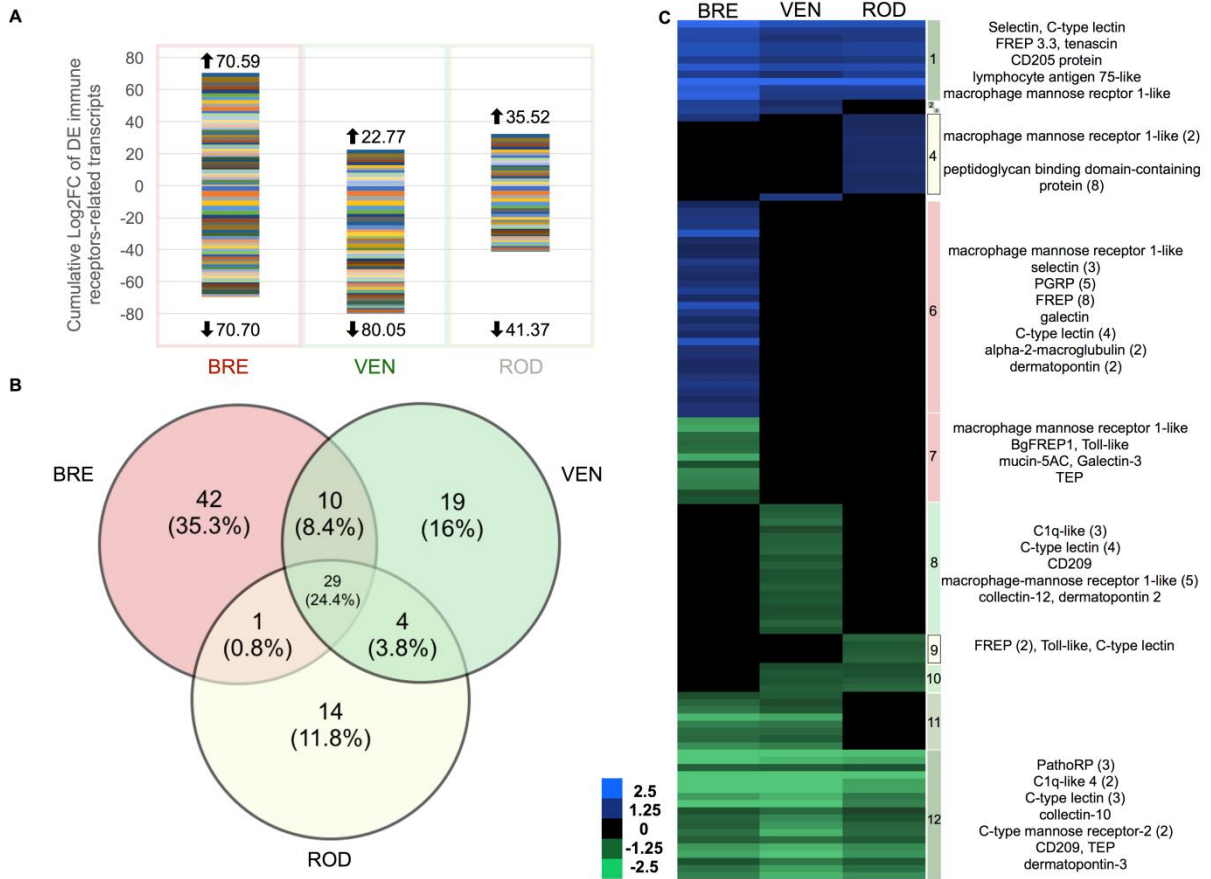


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 1286 Figure 2
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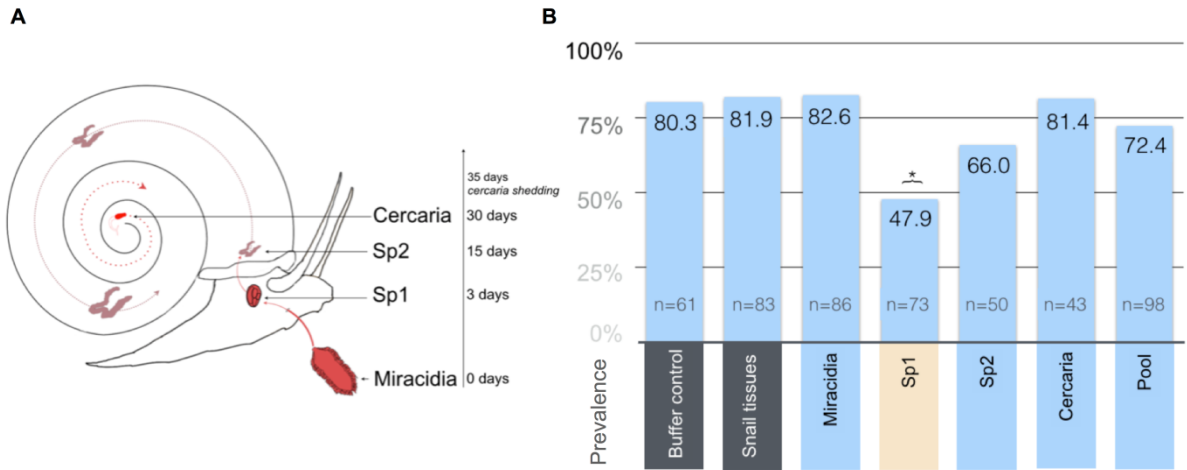
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1290 Figure 3



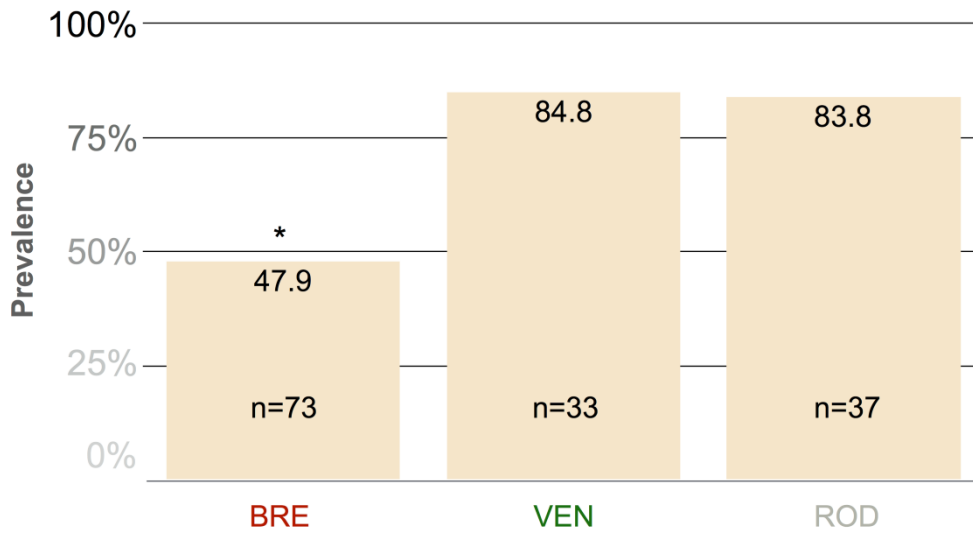
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Figure 4

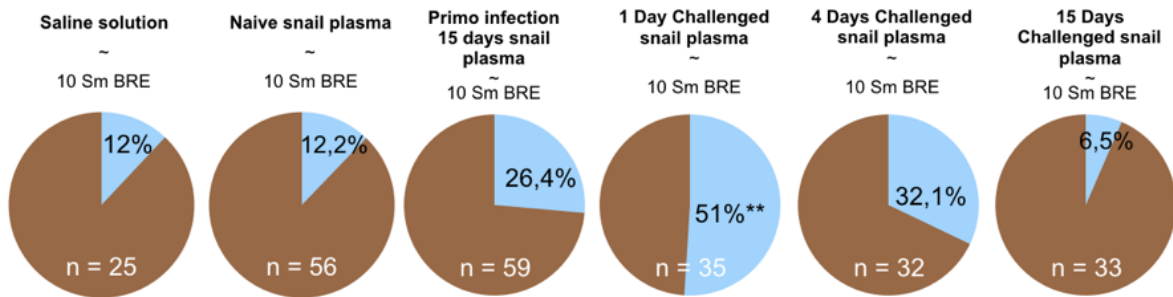


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1303 Figure 5

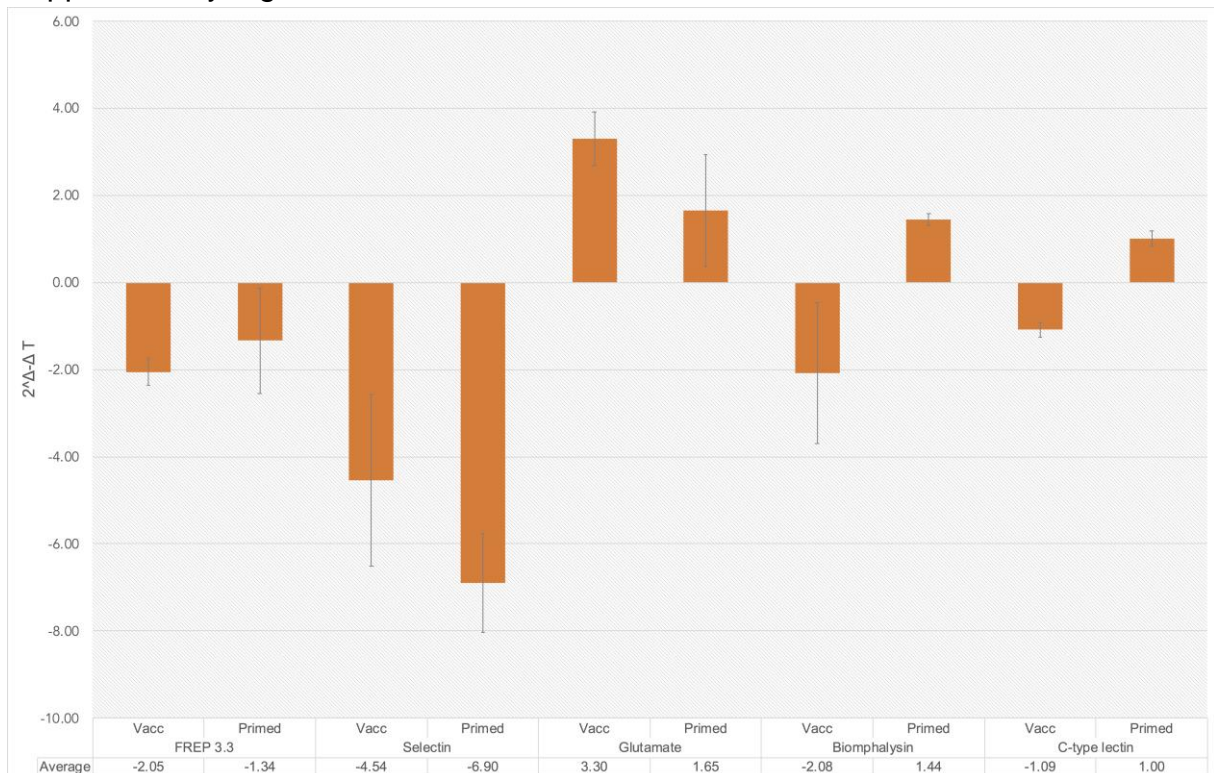


1304 Figure 6
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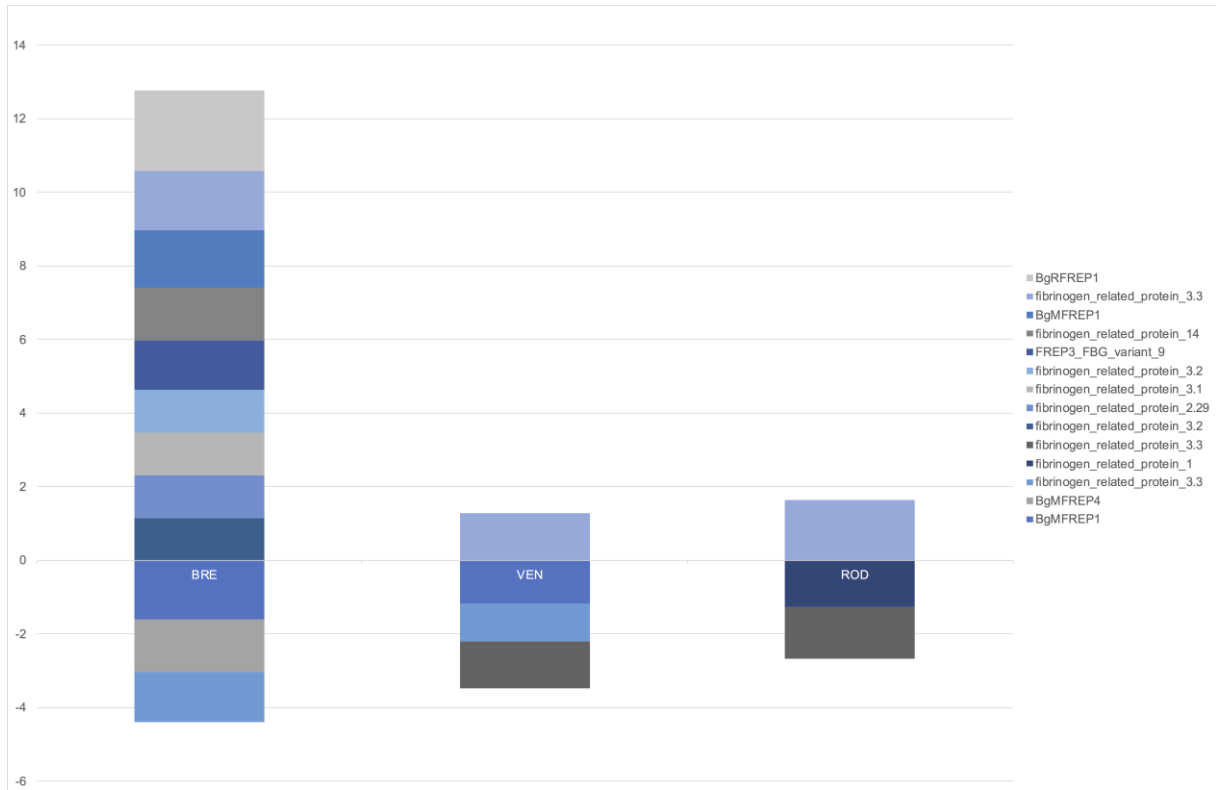
Supplementray Figure 1



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1312 Supplementray Figure 2



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