

1 **Differential modulation of mouse heart gene expression by** 2 **infection with two *Trypanosoma cruzi* strains: a transcriptome** 3 **analysis**

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10 The protozoan *Trypanosoma cruzi* (*T. cruzi*) is a well-adapted parasite to mammalian hosts and
11 the pathogen of Chagas disease in humans. As both host and *T. cruzi* are highly genetically diverse,
12 many variables come into play during infection, making disease outcomes difficult to predict. One
13 important challenge in the field of Chagas disease research is determining the main factors leading
14 to parasite establishment in the chronic stage in some organs, mainly the heart and/or digestive
15 system. Our group previously showed that distinct strains of *T. cruzi* (JG and Col1.7G2) acquired
16 differential tissue distribution in the chronic stage in dually-infected BALB/c mice. To investigate
17 changes in the host triggered by the two distinct *T. cruzi* strains, we assessed the gene expression
18 profile of BALB/c mouse hearts infected with either JG, Col1.7G2 or an equivalent mixture of
19 both parasites during the initial phase of infection. This study demonstrates a clear distinction in
20 host gene expression modulation by both parasites. Col1.7G2 strongly activated Th1-polarized
21 immune signature genes, whereas JG showed only minor activation of the host immune response.
22 Moreover, JG strongly reduced the expression of genes for ribosomal proteins and mitochondrial
23 proteins related to the electron transport chain. Interestingly, evaluation of gene expression in mice
24 inoculated with the mixture of parasites showed expression profiles for both up- and down-
25 regulated genes, indicating the coexistence of both parasite strains in the heart during the acute
26 phase. This study suggests that different strains of *T. cruzi* may be distinguished by their efficiency

- 27 in activating the immune system, modulating host energy and reactive oxygen species production
28 and decreasing protein synthesis during early infection, which may be crucial in defining parasite
29 persistence in specific organs.

31 **Author Summary**

32 The causative agent of Chagas disease, *Trypanosoma cruzi*, retains high genetic diversity, and its
33 populations vary greatly across geographic locations. The *T. cruzi* mammalian hosts, including
34 humans, also have high genetic variation, making it difficult to predict the disease outcome.
35 Accordingly, this variability must be taken into account in several studies aiming to interrogate
36 the effect of polyparasitism in drug trials, vaccines, diagnosis or basic research. Therefore, there
37 is a growing need to consider the interaction between the pathogen and the host immune system
38 in mixed infections. In the present work, we present an in-depth analysis of the gene expression of
39 hearts from BALB/c mice infected with Col1.7G2 and JG alone or a mixture of both strains.
40 Col1.7G2 induced a higher Th1 inflammatory response, while JG exhibited a weaker activation of
41 immune response genes. Furthermore, JG-infected mice showed a notable reduction in the
42 expression of genes responsible for mitochondrial oxidative phosphorylation and protein
43 synthesis. Interestingly, the mixture-infected group displayed changes in gene expression as
44 caused by both strains. Overall, we provided new insights into the host-pathogen interaction in the
45 context of single and dual infection, showing remarkable differences in host gene expression
46 modulation by two *T. cruzi* strains.

47 **Introduction**

48 Chagas disease (CD) is a parasitic illness caused by the kinetoplastid protozoan *Trypanosoma*
49 *cruzi*. Six to seven million people are estimated to be compromised by this disease, which affects
50 mostly poor communities in rural areas of Latin America. [1]. Despite being the prototype of a
51 neglected tropical disease, CD has recently gained attention in nonendemic areas due to increasing
52 emigration of affected people from endemic to nonendemic countries, with new cases occurring

53 mainly by infected blood transfusion, organ transplantation and congenital transmission [2-4].
54 Currently, there are six discrete typing units (DTUs I-VI) described for *T. cruzi*, according to a
55 series of genetic markers, such as rDNA 24S α , miniexon and mitochondrial polymorphisms [5-8],
56 and a seventh has been postulated (Tcbat) [9]. This broad genetic diversity makes *T. cruzi* a highly
57 complex organism and also plays an essential role in the differential tropism in host tissues, which
58 culminates in diverse clinical manifestations observed in chronic patients and experimental models
59 of CD [10-13]. Notably, the term tropism has been used with different meanings by many authors
60 in the scientific literature [14-16], and here, we refer to tissue tropism as the ability of a particular
61 pathogen to infect and persist within an organ or set of organs [17].

62 Elucidating the molecular mechanisms dictating the interaction between the pathogen and its host
63 is crucial for understanding the disease progression and the development of new treatments.
64 Previously, Andrade *et al.* showed that after inoculating BALB/c mice with a mixture of different
65 strains of *T. cruzi*, namely, JG (*T. cruzi* I) and Col1.7G2 (*T. cruzi* II), the two parasite strains did
66 not evenly distribute among different tissues in the chronic phase of the disease. JG was primarily
67 found in the heart, while Col1.7G2 was encountered in the rectum of the animals [11]. Curiously,
68 mice infected with only one strain did not exhibit this pronounced tissue tropism. In addition,
69 different mouse lineages such as C57BL/6J and SWISS were unable to reproduce the
70 aforementioned tissue tropism, indicating the role of the host in the different behaviors of *T. cruzi*
71 strains [12]. In parallel, such tropism was also detected in human patients during the chronic phase
72 of CD, as different organs were the subject of distinct *T. cruzi* DTUs establishment, leading to the
73 proposition of the 'clonal-histotropic model' hypothesis [10, 11, 18].

74 Recently, extensive research has helped to better understand the immunological and molecular
75 interaction between *T. cruzi* and its mammalian host. An efficient and non-exaggerated immune

76 response is crucial to pathogen clearance, without much damage to the host tissue [19]. The innate
77 immune system provides the first line of defense to initiate an effective response against a parasite
78 via pattern recognition receptors (PRRs), of which the Toll-like receptors (TLRs) are the best
79 known [20]. Among the most well-studied TLRs in the context of CD are *Tlr-2* and *Tlr-9* [21].
80 The Glycosylphosphatidylinositol (GPI)-anchored mucin-like glycoproteins (tGPI-mucin), widely
81 present on the parasite surface, and unmethylated CpG DNA sequences are the primary
82 immunostimulatory ligands of these TLRs, respectively. They have been recognized to trigger the
83 release of *Il-12* and *TNF* by dendritic cells and macrophages, which are pivotal for host resistance
84 at the beginning of the acute phase of CD [22, 23]. Moreover, the establishment of a Th1 response,
85 which is dependent on the release of *Ifn-γ*, has been extensively studied and is essential for parasite
86 control and host survival [21, 24]. However, there is a lack of comparative studies showing the
87 peculiarities of the host response against distinct strains of *T. cruzi* and their role in differential
88 parasite tissue preferences. Experiments in mice and rats comparing JG (*T. cruzi* II) and CL-
89 Brenner (*T. cruzi* VI) have shown remarkable differences in the systemic production of
90 inflammatory cytokines and inflammatory cells [25, 26]. Even though much has been achieved to
91 understand the complexity of tissue preferences in the parasite, real changes in host gene
92 expression during infection with distinct *T. cruzi* strains and mixtures have not yet been
93 investigated.

94 In the present work, we report a thorough gene expression analysis of BALB/c hearts during the
95 acute stage of infection by different strains of *T. cruzi* and their mixture. Our data reveals that JG-
96 infected mice display less pronounced induction of both innate and adaptive immune response
97 genes in contrast to Col1.7G2-infected mice. Moreover, biological processes such as translation
98 and mitochondrial oxidative phosphorylation are intensely downregulated in JG, indicating a

99 cellular metabolism modulation that might benefit JG parasites. Remarkably, the mixture-infected
100 animals showed both profiles simultaneously. Our data demonstrate the complexity of host-
101 pathogen interactions in the context of experimental CD and how distinct *T. cruzi* strains
102 differently affect gene expression in mouse hearts.

103 **Results and Discussion**

104 Differential performance of infection by JG, Col1.7G2 and their mixture in BALB/c
105 mice

106 One critical aspect of distinct *T. cruzi* strains is their high genetic variability and differential
107 parasitism when infecting a mammal host. Our research group has intensively studied the
108 Col1.7G2 (*T. cruzi I*) and JG (*T. cruzi II*) strains regarding characteristics of their infection in
109 different mouse lineages and tissue tropism [12, 13, 27]. For example, the JG strain rarely causes
110 death in BALB/c mice, while Col1.7G2 presents high virulence, killing these animals during early
111 moments of infection (Fig 1A). On the other hand, this virulence does not appear to be caused by
112 parasite burden, since JG display higher parasitemia than Col1.7G2 in the acute phase of our
113 experiments (Fig 1B). Interestingly, animals infected by an equivalent mixture of both strains
114 present high parasitemia, and a high mortality rate (Fig 1A-B). Such phenomenon is also noted by
115 Campos *et al.*, in which BALB/c mice infected by two *T. cruzi* I strains (AQ1-7 and MUTUM)
116 present undetectable parasitemia, but when coinfect with JG, a synergic effect in parasitemia is
117 observed [28]. In general, highly multiplicative intracellular pathogens exacerbate the immune
118 response and promote early activation of CD8+ T cells [29]. This effect does not appear to be the
119 case of JG, since experiments confirmed the presence of lower levels of inflammatory cytokines
120 in infected mice and rats during the acute phase in comparative studies [25, 26]. A possible
121 explanation for this diverse mortality and parasitemia levels may be the exacerbation of the

122 inflammatory response by virulent strains, and the remarkable ability to evade the innate
123 mechanism presented by nonvirulent strains.

124 Currently, the reason for strain preferences for particular tissues is not completely understood. In
125 humans, it has been suggested that distinct *T. cruzi* strains can prevail in different organs and cause
126 variable damage to its hosts, culminating in distinct CD outcomes in the chronic phase [10, 11].
127 Thus, different forms of CD would be dependent on the parasite strain and its tropism for specific
128 niches and on the host genetic variability, a concept from which the ‘clonal-histotropic model’ for
129 the pathogenesis of the disease was constructed [30]. Severe chronic manifestations of CD result
130 from persistence of the intracellular stage of the parasite (amastigotes) in heart and muscle cells,
131 enteric ganglia, and adipose tissue [31, 32]. It is still a matter of controversy whether *T. cruzi*
132 parasites exhibit true tissue tropism for heart cells due to tissue-specific protein-protein
133 interactions in the cell surface, or if differential proliferation and selection by immune system/drug
134 resistance affect survival and persistence, leading to the outcome observed in chronic stages [33].
135 At the acute stage of infection, *T. cruzi* parasites can infect virtually any nuclear cell or organ,
136 bringing the real meaning of the tissue tropism concept to question. We cannot discard the
137 hypothesis that the differential strain ‘preference’ may represent the consequence of competition
138 between two or more distinct *T. cruzi* strains for a specific niche, as determined by strain resistance
139 and adaptation to the immune system. Experiments with Holtzman rats that attested to the
140 efficiency of the JG strain to persist in the heart, compared to the CL-Brener strain (*T. cruzi* VI),
141 at the end of the acute phase as well as the chronic phase [26]. It has been suggested that *T. cruzi*
142 persists preferentially in cells with high rates of fatty acid metabolism, and therefore, those cells
143 can provide adequate nutrients for the parasites [34]. However, it is interesting to note that cells

144 where *T. cruzi* parasites are predominant in the chronic phase, such as adipocytes, cardiomyocytes,
145 skeletal fibers, and neurons, are the cells with minor turnover rates in mammals [35] and may
146 represent tissues that can harbor latent parasites for long periods.

147 Heart transcriptome acquisition, quality assessment, and gene clustering

148 To help us to understand the modulation of heart gene expression by parasite infection, we infected
149 BALB/c mice with the JG, Col1.7G2 and an equal mixture of both strains (Fig 2A). mRNA from
150 independent biological replicates of each group was sequenced to generate up to 120 million
151 paired-end reads in total (S1 Table), followed by processing through our RNA-Seq analysis
152 pipeline (S1 Fig). The tendency within our data was evaluated by executing principal component
153 analysis (PCA) using the regularized-log (log2) transformed data matrix from normalized read
154 counts. The dimensionality reduction through PCA evidenced the separation between control and
155 experimental groups by the first principal component. The second principal component separated
156 Col1.7G2 and JG-infected groups, while the mixed infection were distributed between both
157 profiles (Fig 2B). The relationship among samples was revealed using a heatmap of the Euclidean
158 distances between samples. As seen before, experimental and control groups clustered separately,
159 and Col1.7G2 and JG groups are shown to be distinctive. Interestingly, the mixture group shows
160 moderate similarity among samples from each infected mouse, but the control group does not (Fig
161 2C).

162 Revealing the Differentially Expressed Genes (DEGs)

163 We found approximately 16,400 genes expressed in mouse hearts in all experimental groups (S2
164 Table). 2,583 (S3 Table) genes presented a minimum 2-fold change (1 log2 Fold Change) with a
165 1% false discovery rate (FDR) when comparing infected groups to the control non-infected groups

166 and were considered differentially expressed genes DEGs. From these, 2,396 genes are protein
167 coding, 47 are long intervening noncoding RNA (lincRNA), and 140 belong to other biotypes (S4
168 Table). The distribution of DEGs in each group is represented by volcano plots (Fig 3A-C). It is
169 worth noting that there are remarkable differences in fold changes of DEGs among groups.
170 Col1.7G2-infected animals exhibit more upregulated (999 in Col1.7G2 and 729 in JG) and less
171 downregulated DEGs (145 in Col1.7G2 and 673 in JG) relative to the control group in comparison
172 to JG infected-animals. Notably, mouse hearts from the mixed infection presented a higher number
173 of downregulated DEGs (775) and a higher number of upregulated DEGs (1278) relative to the
174 control group, compared to both single-infected groups.

175 At the acute stage of the mixed infection, it is improbable that many host cells are infected by both
176 strains, since we inoculated mice with a very low load of parasites in both single and mixed
177 infections. Therefore, based on the observation that in the acute phase of mixed infection, hearts
178 present both JG and Col1.7G2 expression patterns, but in the chronic phase, Col1.7G2 seems to
179 be eliminated from this organ, as seen by Andrade *et al.*, 1999, we suggest that JG proliferates at
180 a higher rate than Col1.7G2, predominating in the heart in the chronic phase. This hypothesis
181 corroborates previous reports showing that both JG and Col1.7G2 were detected in the hearts of
182 dually-infected BALB/c mice by low-stringency PCR in the acute phase of infection, but at the
183 chronic stage (3 to 6 months after infection), there was a predominance of JG in the hearts of these
184 animals [12, 13]. Additionally, recent findings by Dias *et al.* demonstrated that Col1.7G2 is less
185 efficient than JG in proliferating in the cardiomyocytes of neonate BALB/c mice in culture, which
186 reinforces our hypothesis [36].

187 We asked if the transcriptome profile of infected mice would differ between *T. cruzi* strains. Thus,
188 we investigated whether DEGs are shared or exclusively expressed in a specific experimental

189 group, as shown in a Venn diagram (Fig 3D). A total of 501 (25%) DEGs are shared among all
190 groups and can represent genes intrinsically influenced by the *T. cruzi* infection (S5 Table).
191 Interestingly, for exclusive DEGs, the mixture-infected animals show an equivalent number of up-
192 and downregulated genes (271 up and 275 down), while JG-infected animals present more
193 downregulated genes (132 up and 206 down), and Col1.7G2- present more upregulated (131 up
194 and 55 down) DEGs. Most DEGs from the Mixture-infected group (1507 or 74%) correspond to
195 DEGs also found in JG- and Col1.7G2- single infected animals. We decided to analyze the
196 exclusive DEGs from the mixture-infected group (~21%), since it remains an open question
197 whether a mixed infection leads to exacerbation of the pathological effect of single strains
198 (synergistic effect) or if it is merely the sum of the impact of both strains [25, 37, 38]. We
199 concluded that the mixed infection is much more similar to the sum of every infection than
200 previously understood. We examined the FDR distribution of exclusive DEGs from Col1.7G2, JG
201 and Mixture-infected groups that were not considered as DEGs in the other groups (Fig 3E).
202 Notably, an accumulation of mixture-exclusive DEGs close to the threshold to also be considered
203 a DEG (FDR < 0.01) in the JG or Col1.7G2 groups, but this tendency was not observed for
204 exclusive JG DEGs present in the Col1.7G2 group and vice-versa, demonstrating that these two
205 groups are distinct. Thus, it is plausible that these two parasite strains induce different gene
206 expression responses in the host, and that mixture-infected animals combine cells infected by both
207 strains. The effect of gene expression modulation in the heart of mixture-infected animals will
208 depend on how many cells are infected by JG or Col1.7G2, leading to profiles that are more similar
209 to Col1.7G2 single infection or to JG single infection.
210 Different *T. cruzi* strains stimulate distinct gene expression response in mouse hearts

211 We next asked which biological processes are mainly represented by DEGs, and whether
212 differences could be detected in the more representative categories when comparing the different
213 groups. To achieve this, we separately analyzed the genes upregulated and downregulated upon
214 JG, Col1.7G2, or mixture infection versus uninfected control in mouse hearts for functionally
215 enriched biological processes (GO - Gene Ontology analysis) (Fig. 4 and S6 Table). For
216 upregulated genes, we found that all groups present significantly enriched biological processes
217 related to the innate, adaptive immune, and inflammatory responses of the host against the
218 pathogen (among the top 30 most represented GO categories in these groups – Table S6). We also
219 found significantly enriched categories of cellular response to interferon-beta and gamma, and
220 defense response to virus, bacteria, and protozoa. This result highlights the intersection of
221 intracellular pathogen defense mechanisms. However, the number of DEGs was not similar in each
222 category. Col1.7G2-infected and mixture-infected groups displayed the highest number of DEGs
223 in all upregulated biological processes (Figs 4A and 4C, respectively) and the JG-infected group
224 presented the lowest number of DEGs in the upregulated categories (Fig 4B). Notably, both the
225 mixture and Col1.7G2-infected groups presented enriched biological processes with statistical
226 significance (-log10 pvalue) that were higher than those encountered in the JG-infected group.
227 This observation correlates with previous studies showing that JG leads to lower cytokine
228 production and immune system activation during acute infection [25]. Every DEG encountered in
229 each GO is available in the supplementary material (Table S7), as well as all non-DEGs from the
230 background (Table S8).

231 Remarkably, the enriched biological processes observed in the mixture-infected group are
232 consistent with those seen in the individual infected groups (Fig 4C). For instance, a high number

233 of upregulated genes were related to immune response (Col1.7G2 profile), and a high number of
234 downregulated genes were related to translation, ATP synthesis and respiration (JG profile).
235 Hence, the coexistence of both profiles in the mixture is substantial evidence of the presence of
236 both *T. cruzi* strains in the mice during the acute phase of infection. Both JG- and the mixture-
237 infected groups displayed categories of protein translation, ATP synthesis coupled respiratory
238 electron chain and a significantly enriched tricarboxylic acid cycle.

239 To visualize the interactions between the enriched pathways and the genes within our list of DEGs,
240 we constructed a functional network of each infected group compared to the control using the
241 Cytoscape plugin, ClueGO. Here, we can appreciate the Col1.7G2 activated infection genes (Fig
242 5A). Protein phosphorylation mediated by MAPKinase follows the transcription factor NfkB
243 activation with subsequent immune-mediated pathway stimulation, such as cytokin production that
244 leads to the establishment of a strong immune response [39]. Interestingly, JG infected samples
245 exhibit less immune-mediated pathways when compared to Col1.7G2. However, it is evident that
246 mitochondrial processes and electron transport chain genes are downregulated due to JG infection,
247 particularly during the early phase (Fig 5B). It is worth noting that the Mixture-infected group
248 displays a rich immunological network and also mitochondrial related gene downregulation,
249 evidencing the concurrently presence of Col1.7G2 and JG-infected cells (Fig 5C).

250 To visualize the effect of JG and Mixture-infected downmodulation of genes that play specific
251 roles within the mitochondria, we generated a heatmap from the KEGG database. The complete
252 expression data of all genes and experimental groups are presented in the supplementary material
253 (S2 and S3 Table). Notably, from the 13 protein-coding genes of the mitochondrial genome, 7

254 genes (Nd1, Nd2, Nd4, Nd5, Nd6, Co1 and Cytb) were downregulated in all experimental groups.
255 In the JG- and Mixture-infected animals, they were almost four-fold downregulated and were
256 approximately two-fold downregulated in the Col1.7G2-infected group (S3 Table). In addition,
257 large and small ribosome subunits were significantly downregulated in the JG-infected but not in
258 the Col1.7G2-infected group (Fig 6A). Interestingly, metabolic pathways, such as Oxidative
259 phosphorylation (Fig 6B) and Citric acid cycle were strongly downregulated in JG and Mixture-
260 infected animals when compared to Col1.7G2. As previously noted, these findings suggests that
261 JG promotes a drastic reduction in oxidative metabolism and protein synthesis in the infected
262 cardiac cells. Previous microarray studies showed the same oxidative phosphorylation
263 downregulation pattern in cardiomyocytes infected by other *T. cruzi* strains [40, 41]. The
264 downregulation of the electron transport chain and oxidative phosphorylation gene expression
265 could either increase or decrease reactive oxygen species (ROS) levels, depending on the proton
266 motive force, NADH/NAD⁺ and CoQH₂/CoQ ratios and O₂ concentration [42]. However,
267 previous studies have shown that ROS is a double-edged sword for *T. cruzi* parasites, as it acts as
268 a signaling molecule for *T. cruzi* replication in macrophages at low concentrations [43, 44], but it
269 can be harmful at higher concentrations [45]. Interestingly, treatment with catalase reduces the
270 multiplication of JG in cardiomyocytes, but not Col1.7G2, suggesting that H₂O₂ acts as a signaling
271 molecule for JG growth in these cells [36]. Notably, *T. cruzi* lacks the catalase gene, which may
272 be important for the parasite to sense and overcome oxidative stress. Furthermore, *T. cruzi*
273 parasites transfected with catalase showed increased resistance to H₂O₂ and impaired signals for
274 cell differentiation compared with wild-type parasites [46]. Excessive ROS level causes oxidative
275 DNA damage, such as 8-oxo-7,8-dihydroguanine (OG) accumulation, which slows pathogen
276 growth. Remarkably, Campos et al. have shown that JG and Esmeraldo (both *T. cruzi* II) are more

277 sensitive to oxidative DNA damage caused by H₂O₂ treatment when compared to Col1.7G2 and
278 Silvio (both *T. cruzi* I) [47]. They also showed that even before treatment with 200 μM H₂O₂, JG
279 displayed more oxidative lesions in DNA than Col1.7G2, as determined by the amount of OG
280 present in the nucleus. The role of OG in *T. cruzi* replication signaling is still poorly understood,
281 but recent studies have shown that parasites overexpressing MutT, which are responsible for
282 removing OG from DNA, increased intracellular parasite replication (CL Brener strain) in
283 fibroblasts compared to WT [45]. Interestingly, the 8-oxo-dGMP MutT product may act as a stress
284 signal carrier, and OG DNA lesions might act as an epigenetic modification, which serves as a
285 sensor for oxidative stress, increasing gene expression [48]. Thus, the available evidence suggests
286 that the differential multiplication rate between different *T. cruzi* strains caused by sensing and
287 responding distinctively to ROS, may be crucial to determine the strain colonization in the heart
288 of mice, and possibly other mammals such as humans.

289 Next, we sought to visualize DEGs involved in the immune response detected as enriched
290 biological processes to capture differences between *T. cruzi* strains during the acute stage of
291 infection. To visualize DEGs involved with CD we accessed KEGG pathway 05142 which
292 evidenced a clear distinction between the JG- and Col1.7G2-infected animals (Fig 6C). As noticed
293 earlier, we observed that the JG-infected group exhibited weaker activation of several immune
294 response genes compared to Col1.7G2. For instance, in Col1.7G2-infected animals, *Tlr9* and *Tlr2*
295 had a positive fold-change of approximately 5, relative to control animals, while the JG-infected
296 group showed a positive fold-change of 2.7 (Fig 6C, Table S3). The high expression profile of
297 cytokines such as *Ifn-γ*, *Il-6*, *Tnf* and *IL-12* observed in Col1.7 G2-infected animals corroborates
298 the previously described Th1 host response induced by *T. cruzi* [24]. However, JG-infected

299 animals exhibit lower expression of these same genes. Similar to the CXC and CC chemokines
300 involved in neutrophil and macrophage recruitment, transcriptional factors such as the Class II
301 Major Histocompatibility Complex Transactivator (Ciita) and Tbx21/Tbet, a well-known Th1 cell-
302 specific transcription factors, also displays lower expression levels in the JG group compared to
303 the Col1.7G2 group (Fig 7C). Notably, animals inoculated with a mixture of both parasites
304 generally exhibit the same pattern of immune response DEGs as the Col1.7G2-infected group.
305 This expression pattern suggests that the Col1.7G2 strain is more likely to be detected early by the
306 immune system and initiate a stronger immune response than the JG strain.

307 The evasion capability of each strain can further explain why there are differences in the expression
308 of genes involved in the immune response when animals are infected with different parasite strains.
309 From earlier studies, parasite-specific CD8+ T cells were not seen in the bloodstream of BALB/c
310 mice until day 9 of infection by the Y strain (a nonvirulent *T. cruzi* II strain similar to JG). To
311 prove whether this effect was due to immune evasion or immunosuppression, the authors
312 administered agonists of *Tlr*-2 and *Tlr*-9 to emulate immune system recognition prior to infection.
313 They showed that treated mice developed parasite-specific CD8+ T cells earlier than nontreated
314 groups [49-51]. Therefore, the lower expression of immune response genes by JG-infected animals
315 can be the result of an effective immune-system evasion of this strain, which could also be
316 responsible for the persistence of this parasite in the heart of BALB/c mice in the chronic phase of
317 the disease. One possible explanation for this evasion ability of *T. cruzi* is the transfer of sialic acid
318 from host proteins to parasite surface proteins, such as mucins. Indeed, some of the most well-
319 studied surface proteins of *T. cruzi*, namely, mucins, mucin-associated surface proteins (MASPs)
320 and trans-sialidases (TSs) are strongly related to immune system evasion, cellular membrane

321 adhesion and cellular invasion [52-56]. Nevertheless, *T. cruzi* I strains have fewer genes coding
322 for the mucin, MASP and TS families compared to *T. cruzi* II [57].

323 To confirm the expression data obtained from the RNA-Seq, we performed qPCR for validation
324 (Fig 7). Quantitative PCR data shows that the fold change of most tested genes corroborates the
325 reported RNA-Seq expression tendency. Highly upregulated genes in RNA-Seq analysis, such as
326 Cxcl9 and Igtp, are also seen to be upregulated by qPCR when compared to the Gapdh, and genes
327 coding for proteins acting in the mitochondria are seen to be downregulated by both methods. Data
328 represents one sample from each group with three technical replicates.

329 In the present work, we depicted the differential ability of two *T. cruzi* strains (JG and Col1.7G2)
330 to modulate gene expression in hearts of BALB/c mice at the acute phase of infection. We also
331 showed how the mixture of these *T. cruzi* strains affects host gene expression. We described two
332 major distinct behaviors: hearts of mice inoculated with JG or with the mixture of both strains
333 exhibit many genes from oxidative metabolism and translation downregulated compared to the
334 uninfected controls. However, hearts of mice inoculated with Col1.7G2 and with the mixture of
335 both strains showed a strong activation of genes from the innate and adaptive immune response
336 compared to noninfected hearts. Corroborating previous findings, we propose here that the
337 remarkable differences between the two *T. cruzi* strains in their ability to persist in BALB/c hearts
338 in the chronic stage of CD could be explained by differences in the higher intracellular proliferation
339 rate of JG and its ability to slowly activate the immune response of the host at the acute phase of
340 infection, in contrast to Col1.7G2, which strongly activates the host immune response and has a
341 slower proliferation rate in cardiomyocytes. JG parasites would also benefit from the robust
342 reduction of the overall energetic status of the cell and augmentation of ROS production to better
343 establish in the tissue (Fig. 8). We suggest here that the different features of each *T. cruzi* strain,

344 such as ROS signaling, proliferation and immune system evasion, could determine the survival of
345 one strain over others and prevail in host tissues. Altogether, our research highlights the need for
346 a better understanding of the effect of *T. cruzi* polyparasitism, as well as the uniqueness of each *T.*
347 *cruzi* strain and its interaction with the host.

348 **Materials and Methods**

349 **Ethics Statement**

350 All procedures complied with the standards stated in the Guide for the Care and Use of Laboratory
351 Animals and were conducted under conditions approved by the local animal ethics committee [58].
352 The Institutional Committee for Animal Ethics of UFMG (CEUA-UFMG, license 64/12) approved
353 all experimental procedures used in this study. Both noninfected and infected animals were kept
354 in plastic boxes with food and water *ad libitum* with the same appropriate conditions of technical
355 management in cages that were properly identified and sealed in a 12-h light dark cycle
356 environment. All animal procedures were performed under anesthesia using a mixture of ketamine
357 (50 mg/kg) and xylazine (10 mg/kg).

358 *T. cruzi* populations used in this study, Col1.7G2 and JG, belong to lineages TCI and TCII,
359 respectively. JG strain was originally isolated in 1995 by Professor Eliane Lages-Silva
360 (Universidade Federal do Triângulo Mineiro, Brazil) from a chronic patient with megaesophagus.
361 Col1.7G2 strain is a clone from Colombian strain, which was originally isolated by Federici in
362 1964 from a chronic patient with cardiac disorder [59]. Both JG and Col1.7G2 were formerly
363 characterized as monoclonal population, through the analysis of the eight microsatellite loci
364 according to previously described methodology [60]. Both strains belong to the *T. cruzi* collection
365 from Laboratory of Chagas Disease (UFMG), coordinated by Professor Egler Chiari. Infective
366 trypomastigote forms were thawed from liquid nitrogen preservation and parasites were injected
367 into SWISS mice for population expansion and diluted to 50 parasites/100 µl of sterile PBS for
368 further use in BALB/c infections.

369 **Experimental Infection**

370 Inbred 6-8-week-old male BALB/c mice were obtained from the Centro de Bioterismo
371 (CEBIO/ICB, Belo Horizonte, Brazil) and housed in our local animal facility under the same
372 conditions. Mice were randomly divided into four groups of 5 individuals each. The noninfected
373 controls were injected intraperitoneally (i.p.) with phosphate-buffered saline (PBS-vehicle), while
374 the three experimental groups were injected (i.p.) with 50 trypomastigotes of the JG (*T. cruzi* II),
375 50 of Col1.7G2 (*T. cruzi* I) and an artificial mixture of both strains (50 + 50 parasites). After
376 infection, mice were caged according to group. Blood parasitemia was verified by counting the
377 parasites present in 5 µL of blood using an optical microscope at the 14th day post infection. On
378 the 15th day, mice were anesthetized, and their hearts were collected, rapidly washed in sterile PBS
379 and immediately frozen in liquid nitrogen.

380 **RNA extraction, quality assessment, and sequencing**

381 Frozen hearts were kept in liquid nitrogen and thoroughly pulverized into a powder using a sterile
382 porcelain crucible. The total RNA for sequencing was extracted using the Trizol reagent (Life
383 Technologies) following the manufacturer's protocol and precipitated with isopropanol. The
384 quality and integrity of the samples were verified by electrophoresis on a Bioanalyser 2100
385 (Agilent Technologies), and only samples with an RNA integrity number (RIN) greater than seven
386 were selected for library construction (S9 Table).

387 The cDNA libraries were prepared and sequenced at the Beijing Genomics Institute (Shenzhen,
388 China). In short, polyadenylated RNA was purified from total RNA, converted to cDNA using
389 random hexamer primers, sheared, and size-selected for fragments ~200 bp in length using the
390 Illumina TruSeq RNA Sample Preparation Kit v2. Sequencing was performed on the Illumina
391 Hiseq 2000 (Illumina, CA) platform and generated approximately 12 million paired-end reads,
392 which were 90 nucleotides in length for each sample. Each group was sequenced in triplicate.

393 **Processing of raw sequencing reads**

394 Raw reads were first checked for quality using FastQC (Babraham Bioinformatics, Cambridge,
395 UK) [61]. Since all samples displayed high-quality scores, no sequence trimming was performed.
396 STAR aligner (v2.5) was used with default parameters to map read locations in the mm10
397 (GRCM38) mouse reference genome [62]. Only uniquely mapping reads were retained for use in
398 the downstream analysis (S1 Fig).

399 **Differential Expression, and graphic visualization**

400 Information on transcript abundances was obtained by raw read counts using HTseq-count [63],
401 and differential expression analysis was performed using DESeq2 [64] in R version 3.2.4. For this
402 study, we considered as differentially expressed genes presenting a 2-fold change in relation to the
403 control group, with a false discovery rate (FDR) of 1%.

404 Principal component analysis (PCA) was performed using the regularized-logarithm
405 transformation (rlog) offered by the DESeq2, and the heatmaps were constructed using the
406 heatmap.2 function of gplots. A Venn diagram was created with an area-proportional web
407 application [65].

408 All figures were mounted and treated using vector graphics quality within Adobe Illustrator 2017.

409 **Enrichment analysis**

410 We conducted the enrichment analysis using an R package with mouse genome-wide annotations
411 [66]. Functional enrichment for biological processes (based on implemented Fisher's exact test)
412 was calculated with the Bioconductor package topGO [67]. Of note, topGO makes a nonbiased
413 background adjustment of data for enrichment analysis, since it considers as background only non-

414 DE genes with similar expression to DE genes as controls for comparison. We model the
415 background by considering only genes expressed in the samples analyzed. In addition, we analyzed
416 the upregulated and downregulated genes separately, as described previously [68].

417 **Functional network analysis**

418 The networks of enriched functional terms were constructed with the ClueGO plug-in using
419 Cytoscape software [69, 70]. Our lists of DEGs was analyzed using the biological process EBI-
420 QuickGO mouse annotation. Pathways with p-values less than 0.001 were considered statistically
421 significant after Bonferroni step-down correction.

422 **Quantitative Real-Time PCR (qPCR)**

423 Total RNA of mouse hearts was extracted using TRIzol (Life Technologies, CA, US) and treated
424 with TURBO™ DNase (Thermo Fisher Scientific, MA, US). cDNA was then produced with
425 Superscript II kit first strand synthesis (Invitrogen, CA, US). PCR experiments were performed in
426 a 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, US) using the PowerUp SYBR
427 Green Master Mix (Thermo Fisher Scientific, MA, US). Tested genes and primer sequences are
428 provided in the Supplementary Table 10.

429 **Data availability**

430 All raw and processed sequencing data are available in the GEO (Gene Expression Omnibus)
431 under the accession code: GSEXXXXXX.

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451

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637

638 **Figure legends**

639 **Figure 1. Differences between two *T. cruzi* strains.** (A) Survival curve of mice with *T. cruzi*
640 infection for 60 days. (B) Mouse parasitemia 14 days after infection. Data represents mean \pm SEM,
641 n=5 mice/group * p<0.05 comparing Col1.7G2 group to JG and mixture.

642 **Figure 2. Experimental Design.** (A) BALB/c mice were divided into one control and three
643 experimental groups, infected with Col1.7G2, JG, or mixed strains of equivalent quantities of both
644 strains. After 15 days, during the acute phase, mice were euthanized, and hearts were removed for
645 total RNA extraction and sequencing with Illumina HiSeq2000. (B) To capture overall expression
646 profile of each infected mouse sample, controls (blank dots), Col1.7G2-infected (green dots), JG-
647 infected (yellow dots) and Mixture-infected (blue dots), were compared using principal component
648 analysis (PCA). The first two principal components (PC1 and PC2) are plotted according to rlog-
649 transformed data of read counts for each gene. (C) Hierarchical clustering of rlog-transformed data
650 matrix exhibited as heatmap of Euclidian distances between samples.

651 **Figure 3. Comparative analysis of mouse gene expression after exposure to single and mixed**
652 ***T. cruzi* infections.** Volcano plot of (A) Col1.7G2-, (B) JG- and (C) mixture-infected groups
653 showing log2 of fold changes of each detected gene on the x-axis, along with -log10 of adjusted
654 p-values (FDR) after comparison with noninfected controls. Differentially expressed gene are in
655 blue (log2fold-change < -1) for downregulated genes and red (log2fold-change > +1) for
656 upregulated genes. Grey dots represent genes that were not considered differentially expressed.
657 Genes with -log10 FDR equal or smaller than 50 are shown as diamond shapes. (D) The scaled
658 Venn diagram shows the proportion of shared and exclusive genes in each group. Up- and

659 downregulated genes are shown in red and blue, respectively. (E) Distribution of FDRs of
660 exclusive genes from mixture in the Col1.7G2-infected (MiC) or JG-infected (MiJ) groups and
661 exclusive genes from Col1.7G2 in JG-infected group (CiJ) or vice versa (JiC) dataset are shown.

662 **Figure 4. Analysis of enriched Gene Ontology categories present in differentially expressed**
663 **genes.** All genes from (A) Col1.7G2, (B) JG and (C) mixture groups were analyzed in accordance
664 with their Gene Ontology terms using the topGO package for R. Encountered GO terms derived
665 from up and downregulated genes are shown in red and blue, respectively. Bars represent -log10
666 of Fisher elim p-value ($p < 0.01$). Histogram values represent the number of differentially
667 expressed genes by the number of expressed genes in the background.

668 **Figure 5. Functional networks of enriched biological processes.** Network of biological
669 processes of (A) Col1.7G2- (B) JG- and (C) Mixture-infected groups. Functional groups with more
670 than 90% of upregulated or downregulated genes are shown in red or blue, respectively. Data
671 represents only gene clusters with p-values less than 0.001 after Bonferroni step-down correction.

672 **Figure 6. Heatmap of DEGs** included in (A) Ribosomes subunits, (B) Oxidative phosphorylation
673 pathway, (C) Chagas disease related genes and (D) Chemokine signaling. Data represent z-score
674 of normalized read counts (log2 scale) for each gene. Base mean track shows average gene
675 expression between samples.

676 **Figure 7. Quantitative real-time RT-PCR validation of differentially expressed genes.**
677 Expression levels of tested genes were normalized to Gapdh (housekeeping gene) and fold-change
678 values were calculated with the $2^{-\Delta\Delta CT}$ method. All PCR reactions were performed with three
679 technical replicates.

680 **Figure 8. Proposed model for preferential prevalence of JG over Col1.7G2 in BALB/c mouse**

681 **hearts.** Throughout the acute stage of *T. cruzi* infection, Col1.7G2 parasites induce a strong Th1-
682 polarized immune response in mouse hearts. Although a potent Th1 response helps developing
683 against parasites, amastigote nests are capable to survive later at chronic stages. Mice infected with
684 the JG strain exhibit lower Th1 response and higher survival rates compared to Col1.7G2. In
685 parallel, JG parasites, stimulated by higher sensitivity to ROS generated by mitochondrial
686 metabolism down-modulation, display rapid increase in clonal burden. During a mixed infection
687 with both strains, JG parasites can overcome Col1.7G2 population due to an enhanced
688 proliferation.

689 **Supporting information**

690 **S1 Table. Total number of reads and mapping statistics.** Number of sequences from each
691 sample and proportion of reads mapping at single positions using GRCm38 mouse genome.

692 **S2 Table. Dataset of all detected genes.** Full dataset of gene expression analysis after comparing
693 experimental groups with controls, including Ensembl gene ID, external gene name, gene biotype,
694 chromosome coordinates, expression mean, fold change statistics and adjusted p-values (FDR).

695 **S3 Table. Filtered dataset from genes considered differentially expressed.** Complete list of
696 differentially expressed genes (DEG) from all experimental groups after filtering the full dataset
697 of detected genes following a 2-fold change and 1% FDR.

698 **S4 Table. Gene biotype statistics.** Statistics of all differentially expressed genes with their biotype
699 classification.

700 **S5 Table. Venn diagram dataset.** List of all shared genes in each experimental group and their
701 expression levels which were used to generate the Venn diagram.

702 **S6 Table. Complete Gene Ontology analysis dataset.** Table with a complete list of terms
703 containing up- or downregulated genes. Each functional category is shown with the number of
704 detected (annotated) and differentially expressed (significant) genes.

705 **S7 Table. Differentially expressed genes per GO category.** Table with a complete list of all
706 DEGs encountered in each enriched GO category.

707 **S8 Table. Genes per GO category.** Table with a complete list of all expressed gene encountered
708 in dataset per enriched GO category.

709 **S9 Table. Sample description and RNA quality statistics.** RNA quality attributes of samples
710 used in library construction.

711 **S1 Fig. Overall workflow.** All major bioinformatics steps performed in this work.

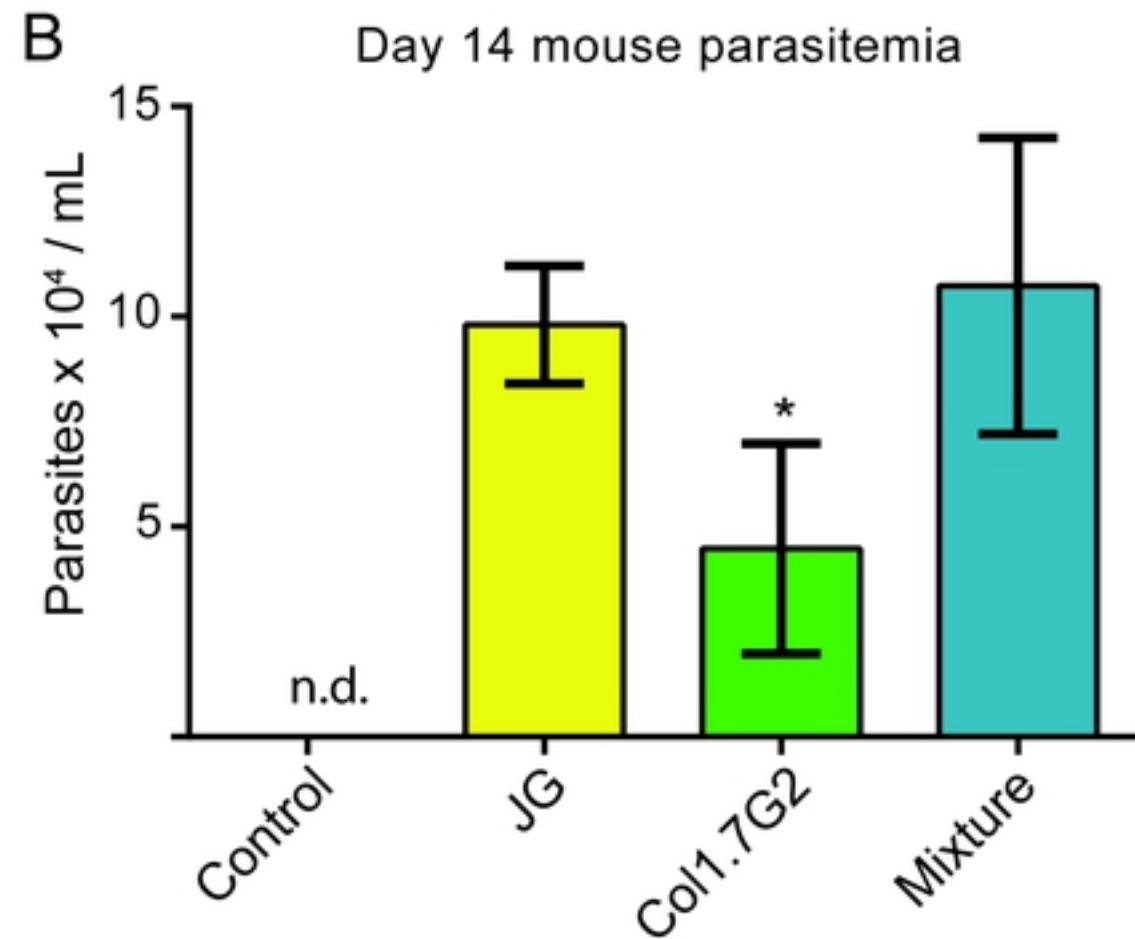
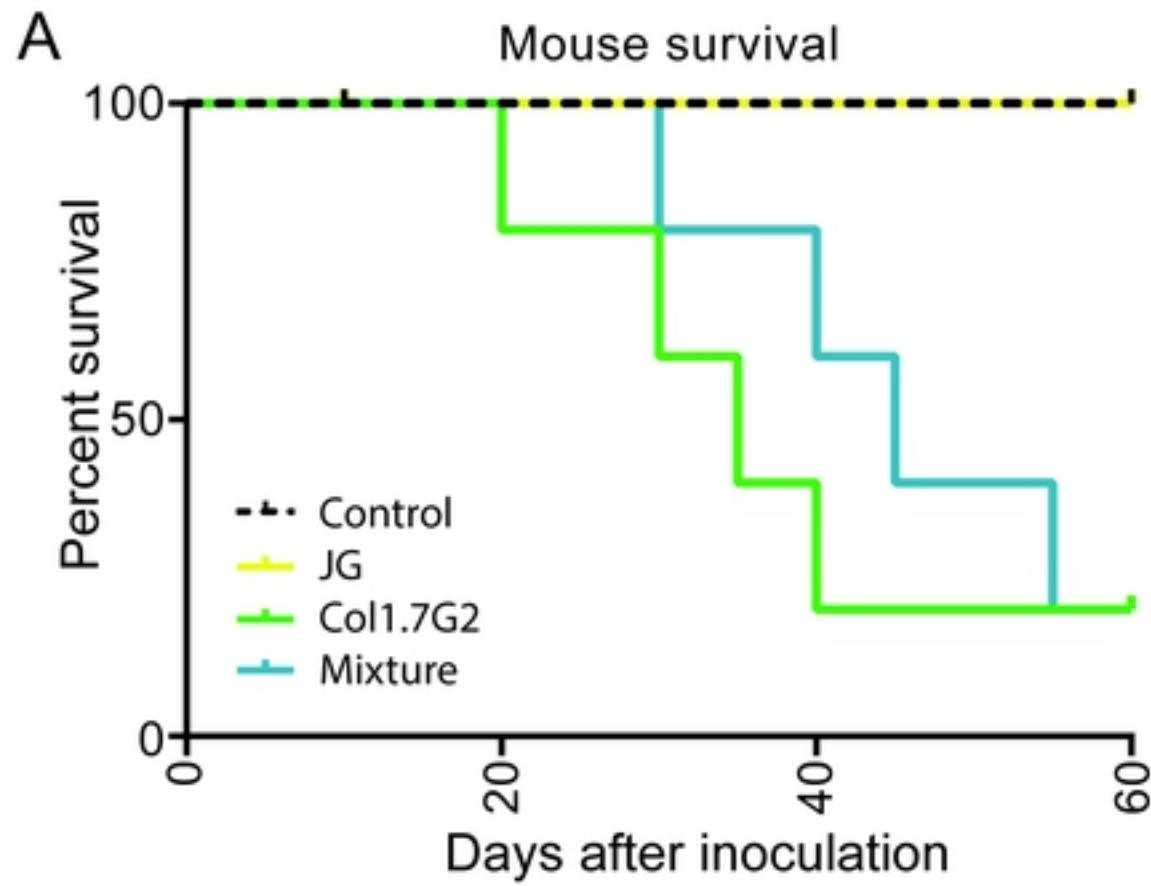


Figure 1

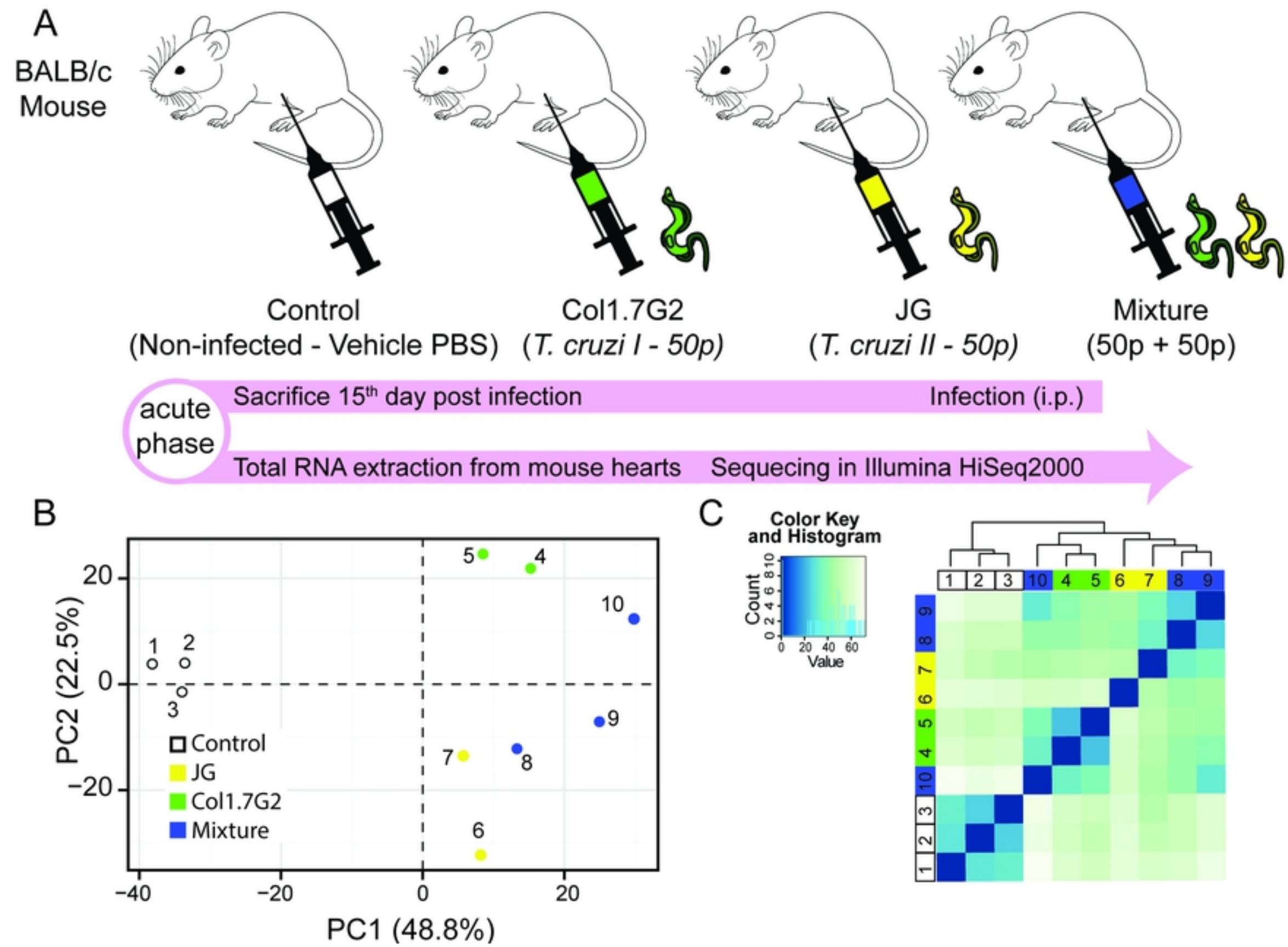
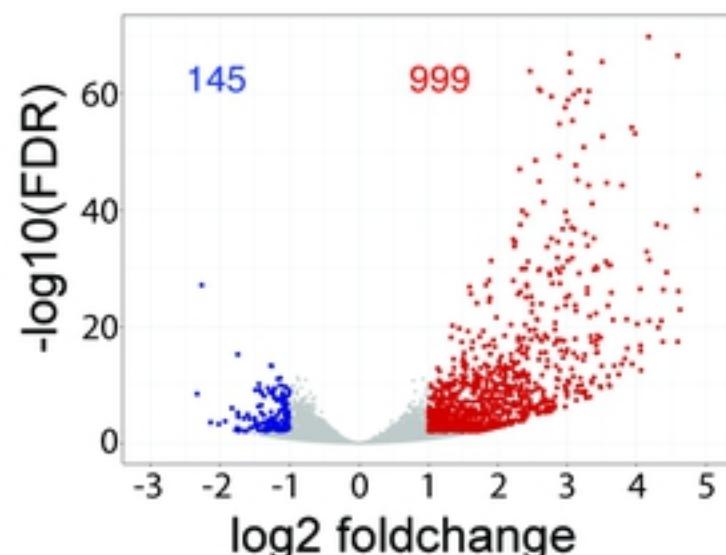


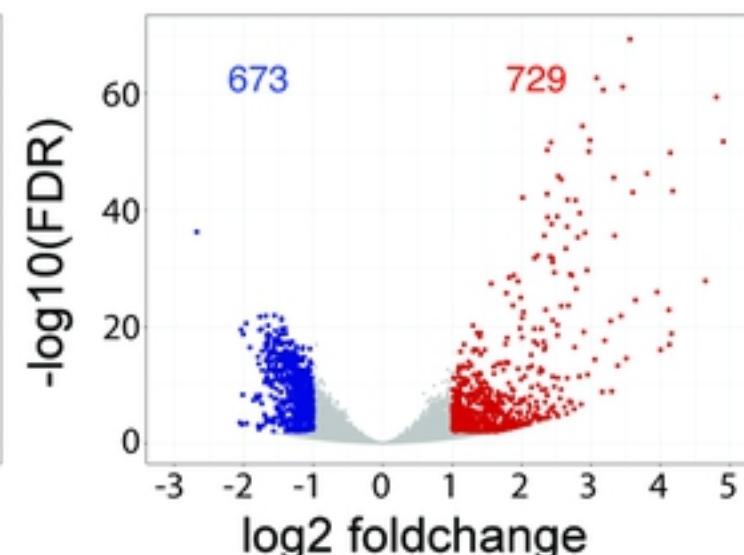
Figure 2

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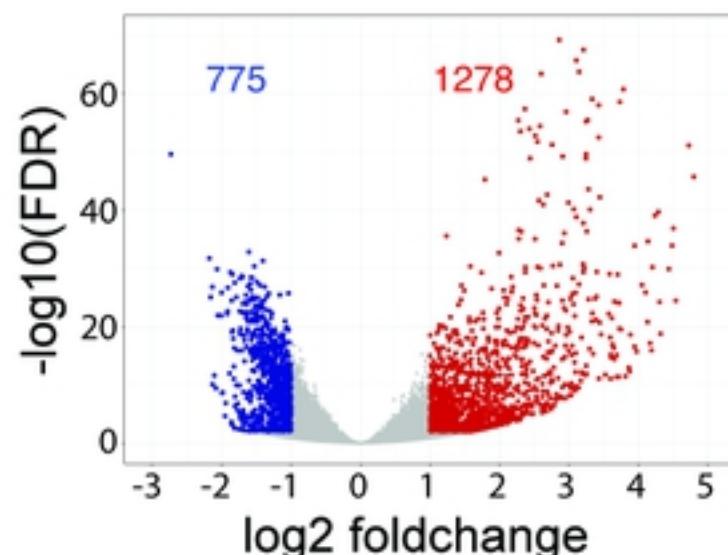
Control vs Col1.7G2

**B**

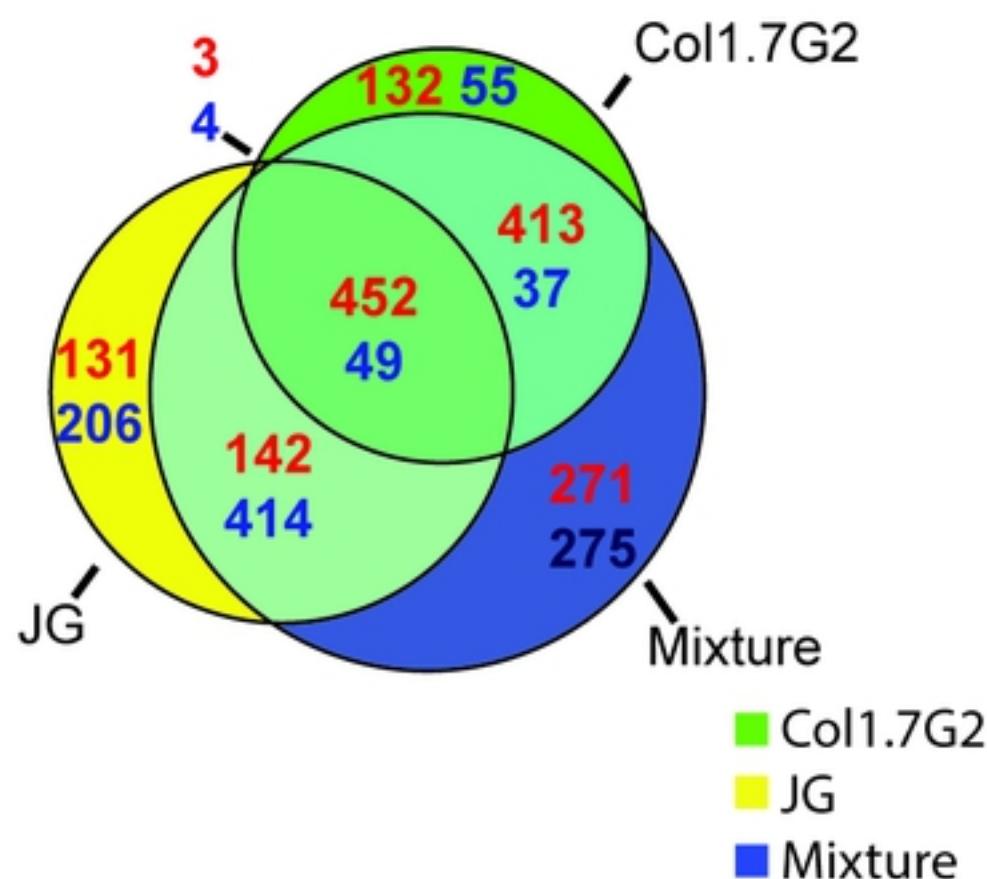
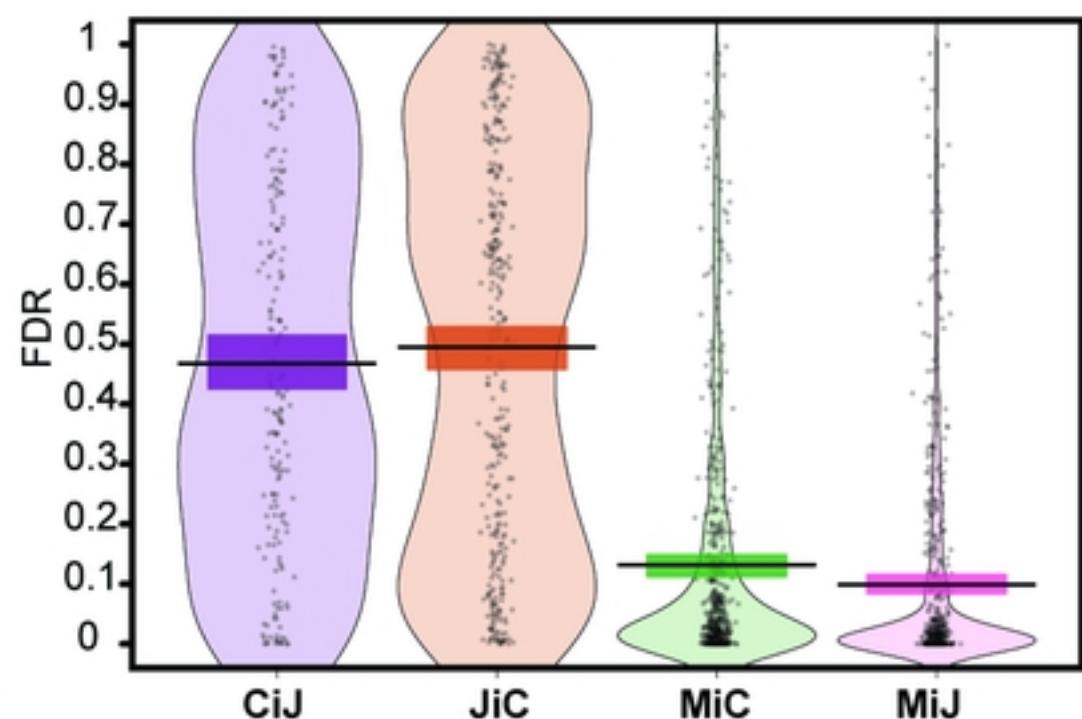
Control vs JG

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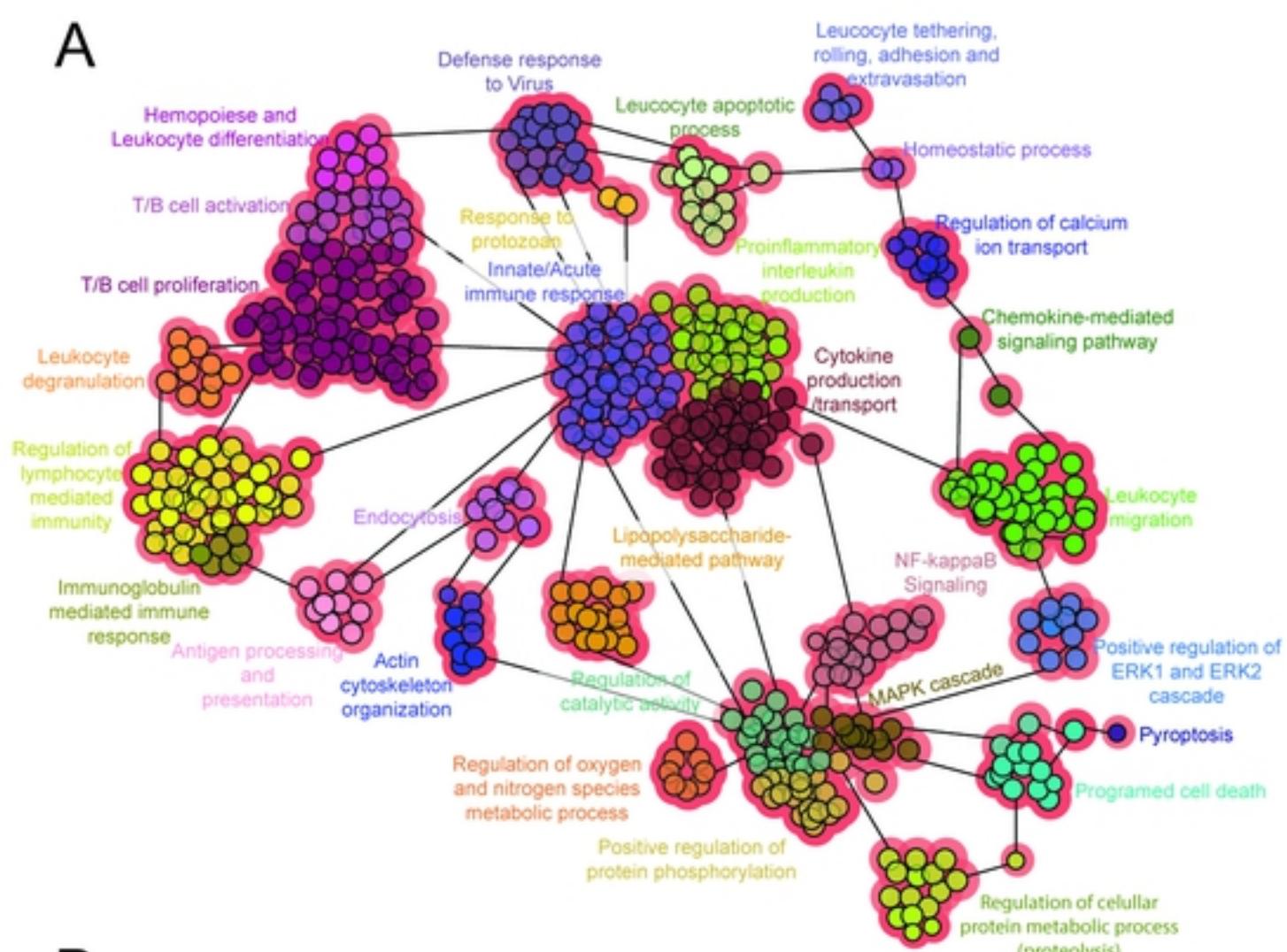
Control vs Mixture



■ Downregulated genes ■ Upregulated genes

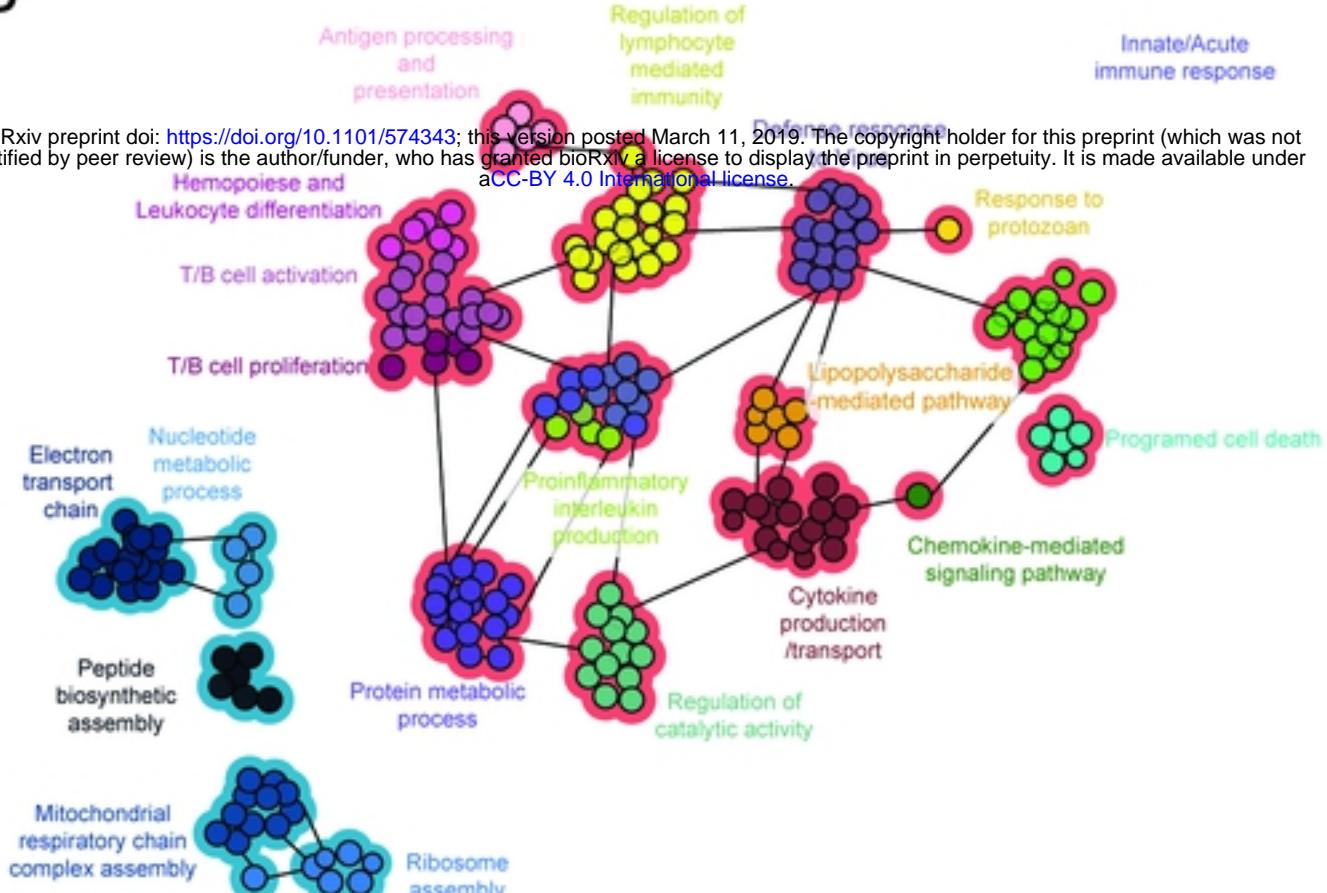
D**E****Figure 3**

A



B

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C

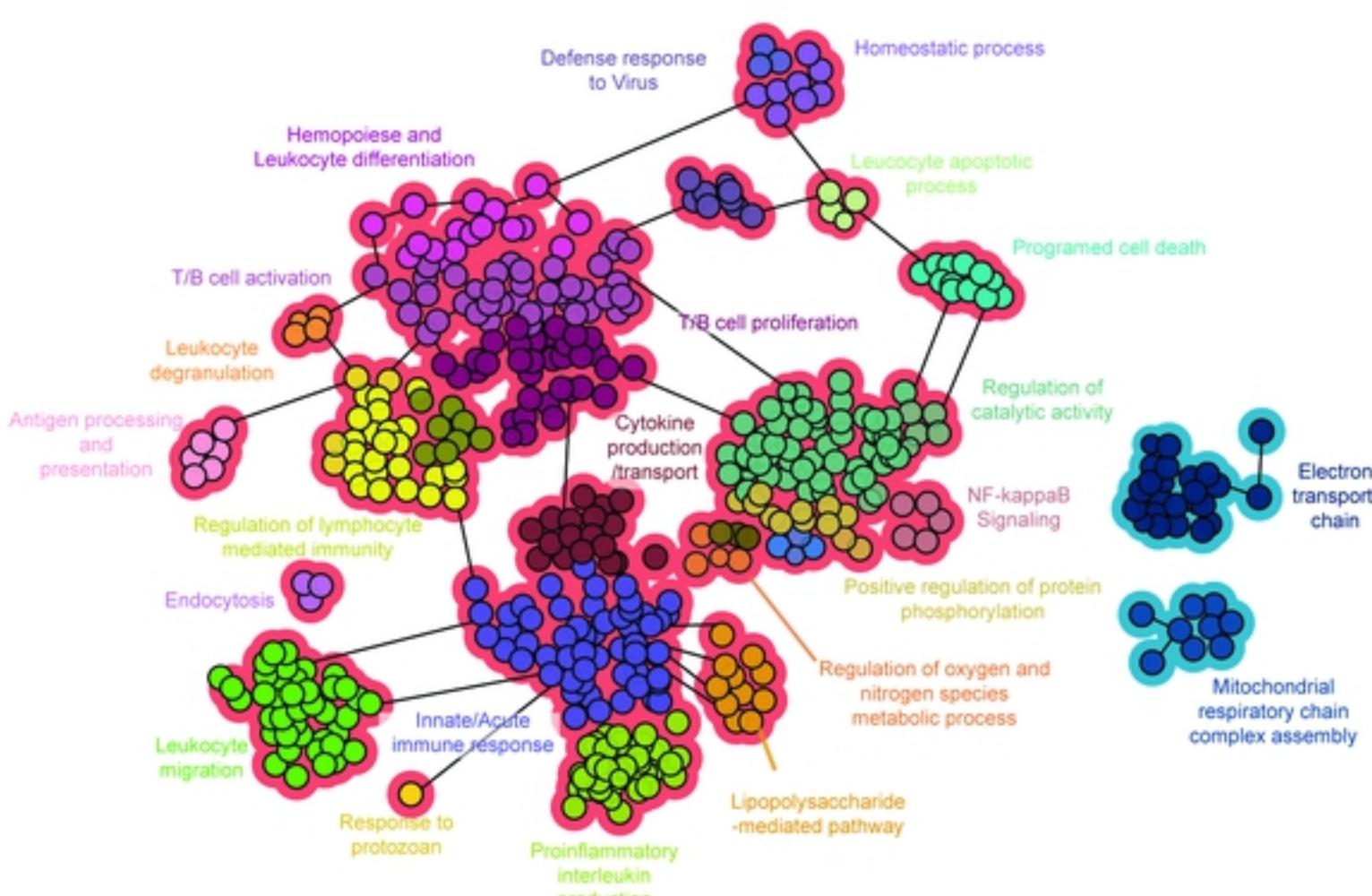
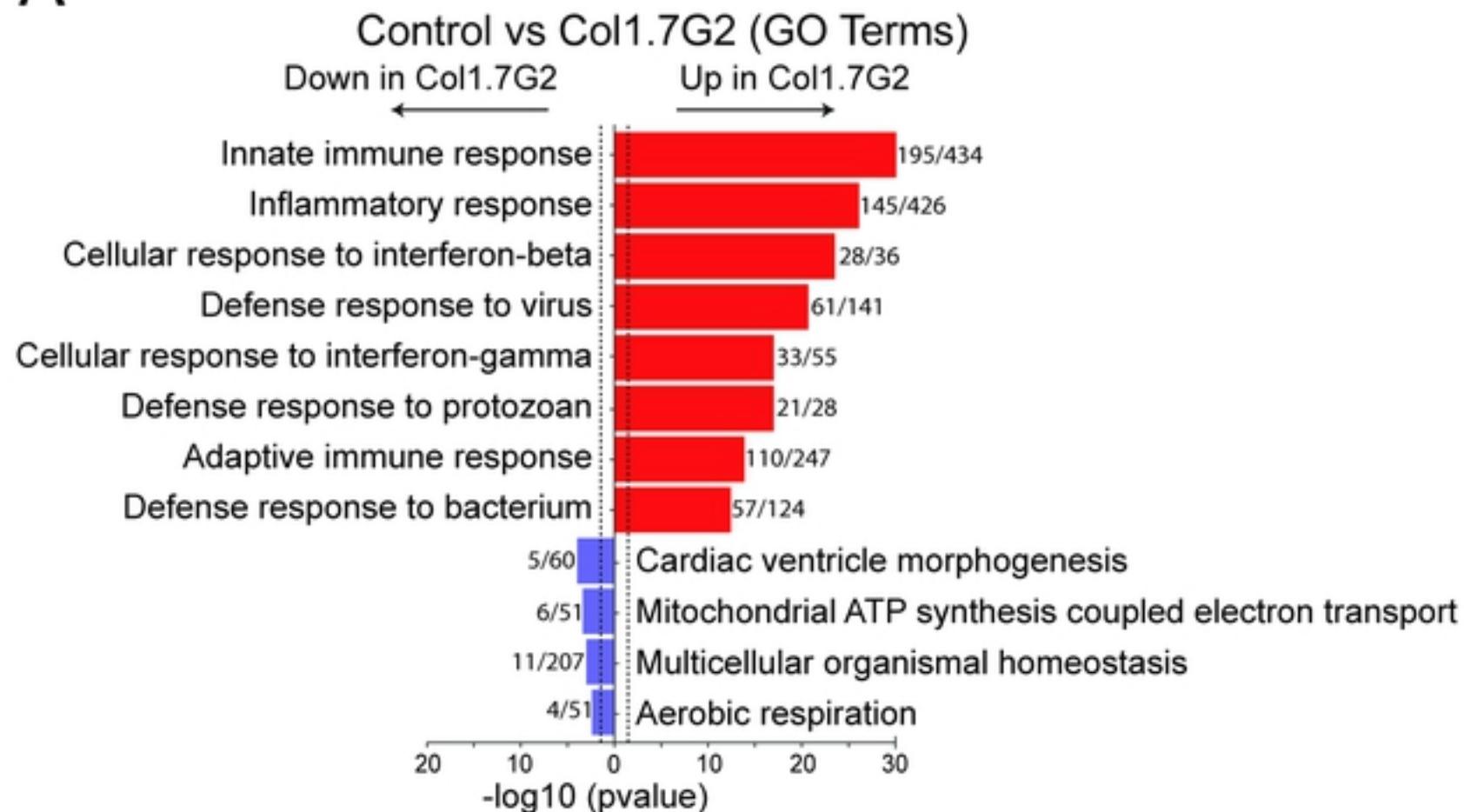
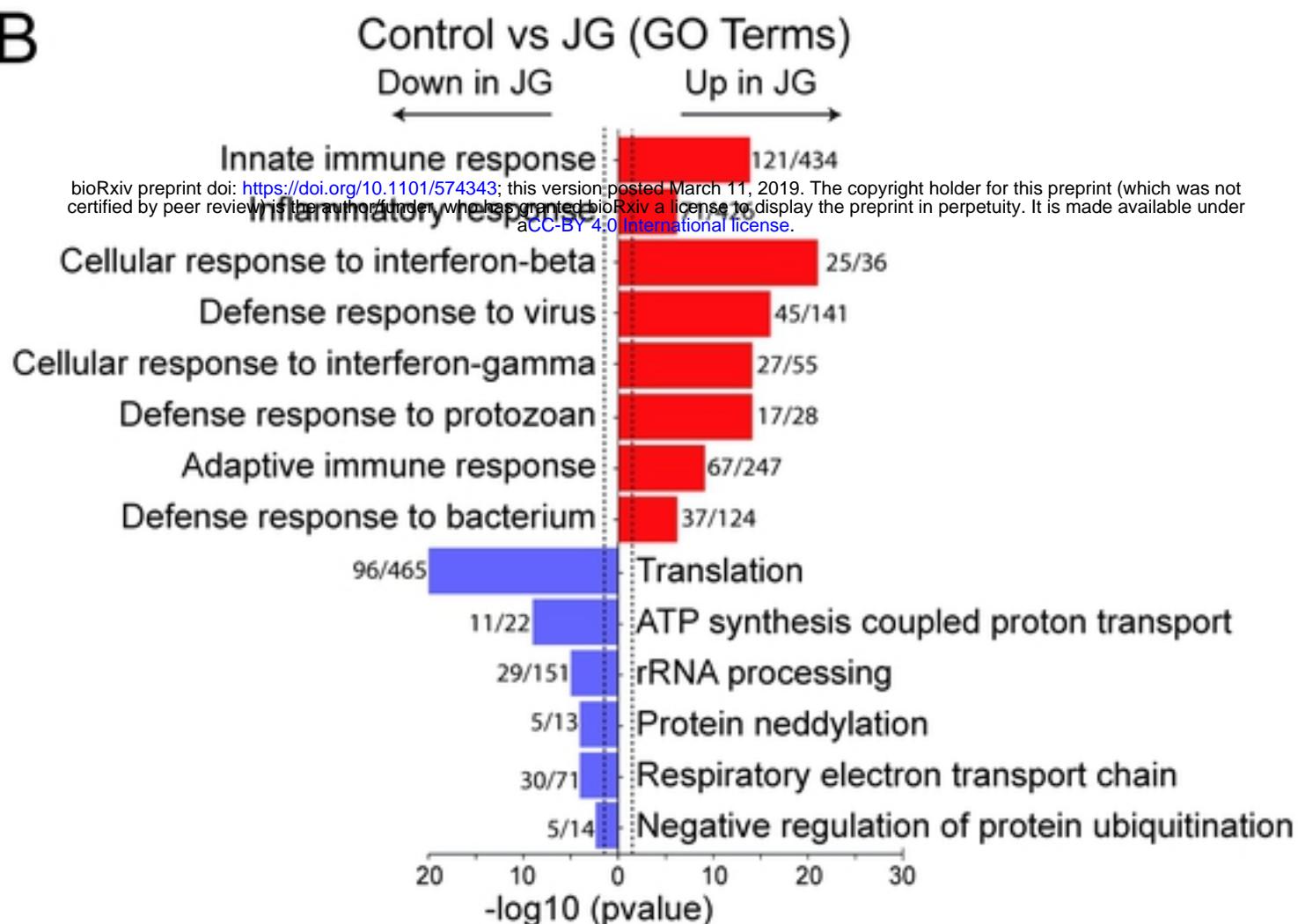


Figure 5

A



B



C

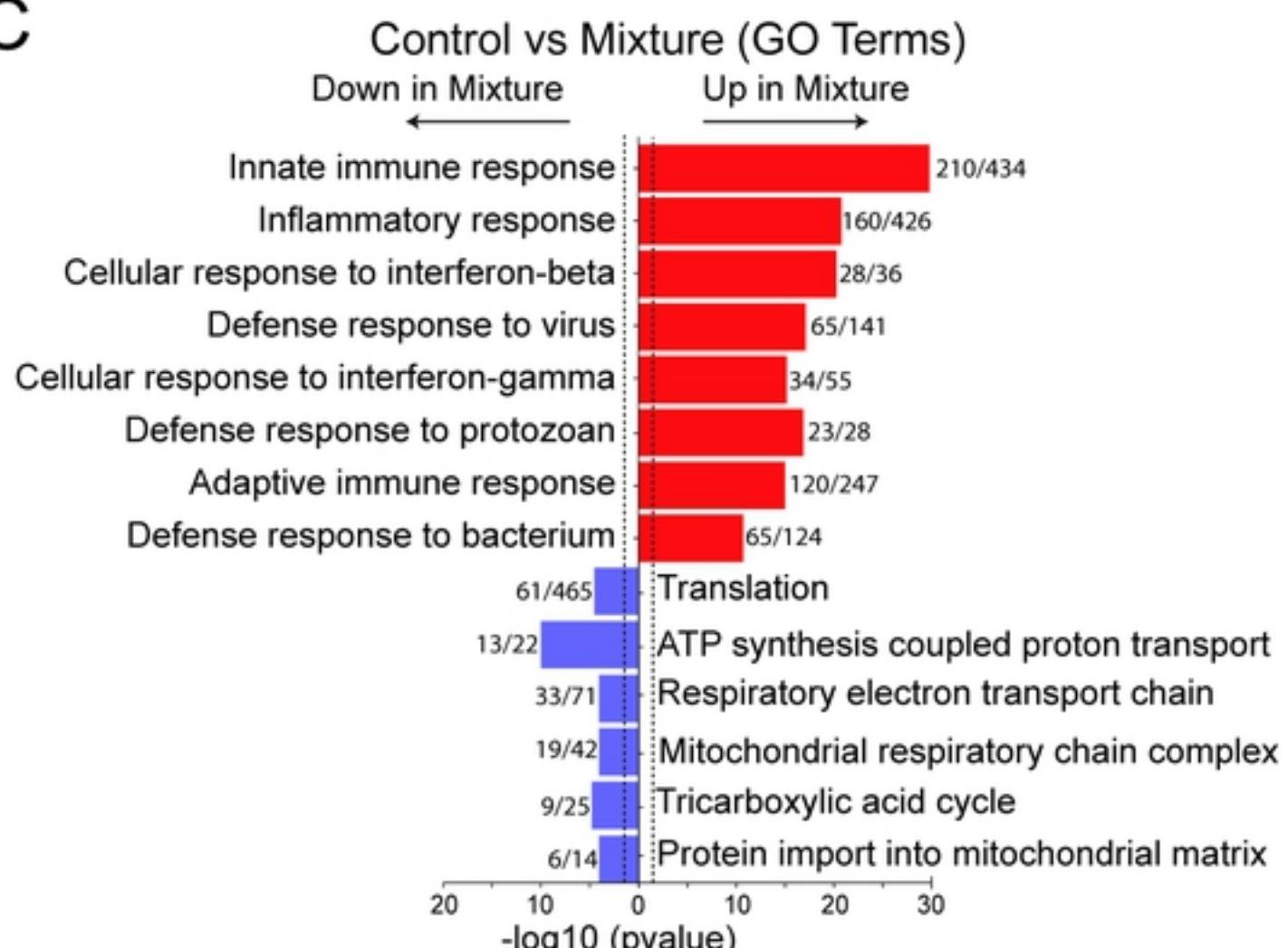
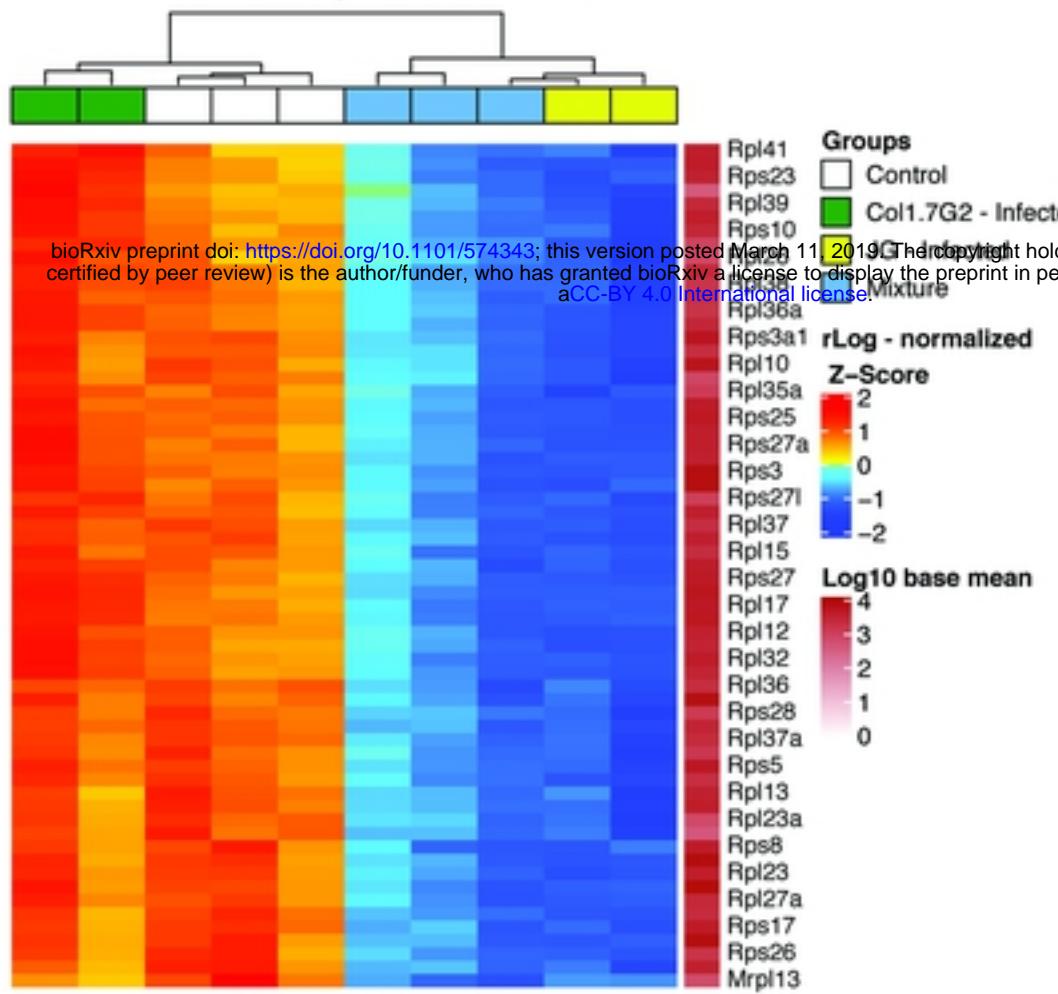


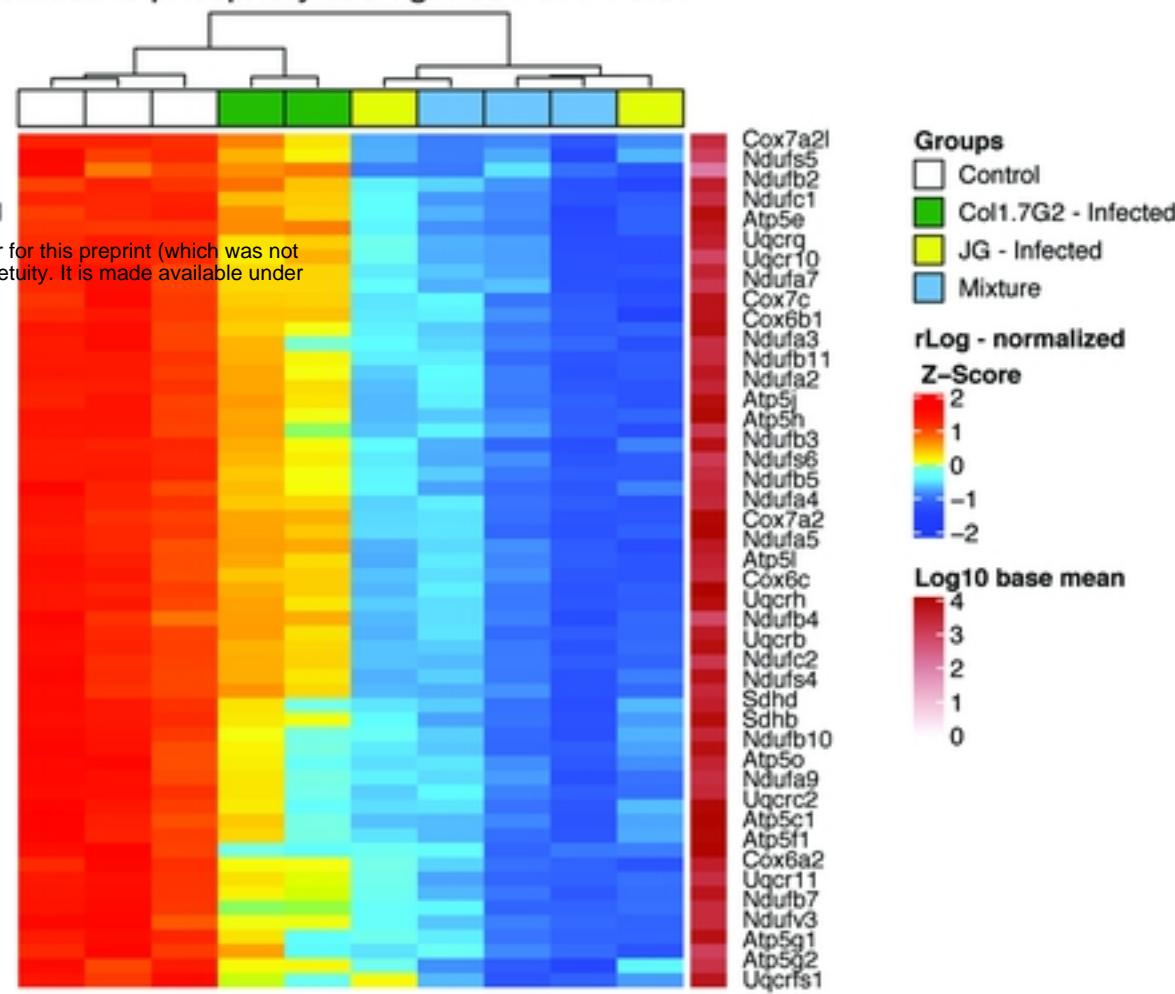
Figure 4

A

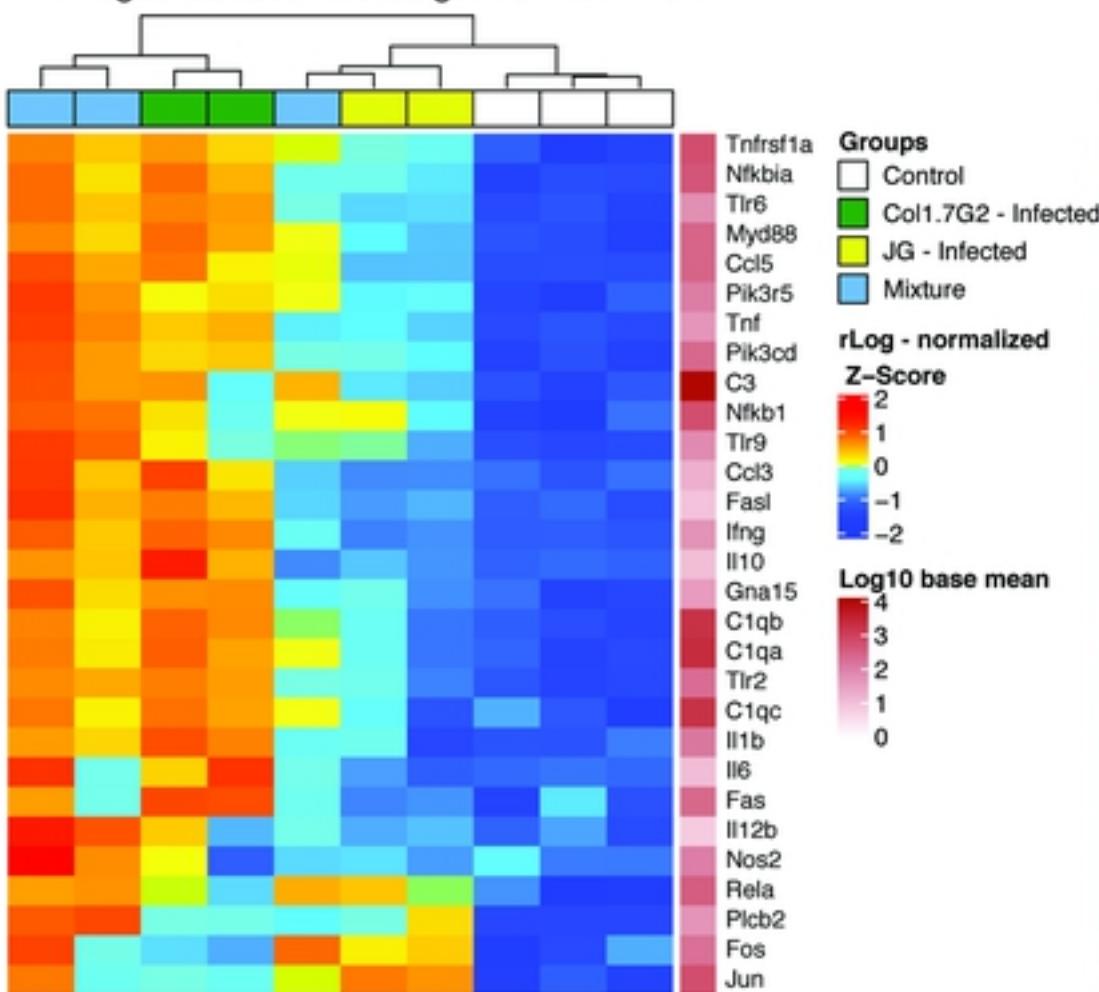
Ribosome Subunit genes FDR < 0.01

**B**

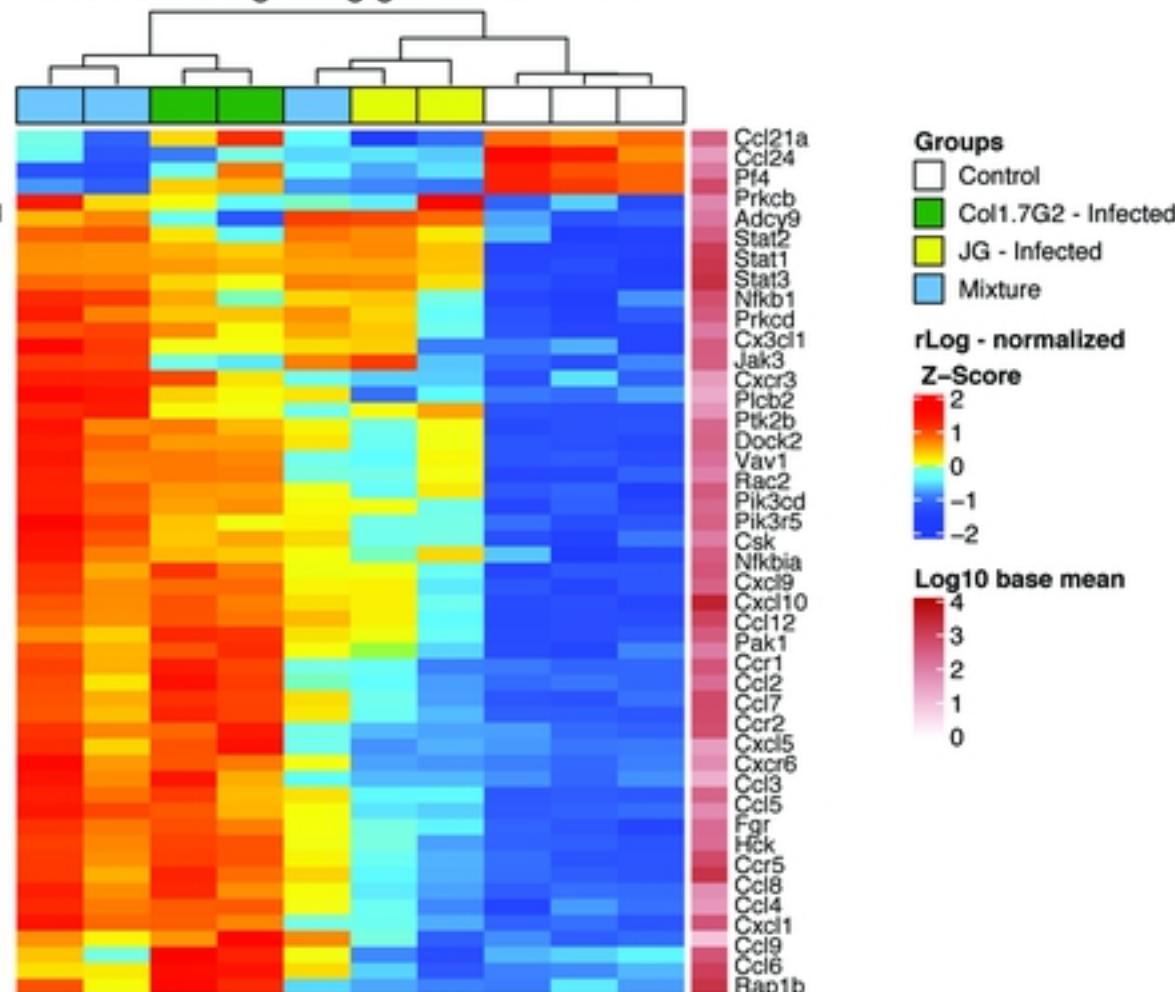
Oxidative phosphorylation genes FDR < 0.01

**C**

Chagas disease related genes FDR < 0.01

**D**

Chemokine signaling genes FDR < 0.01

**Figure 6**

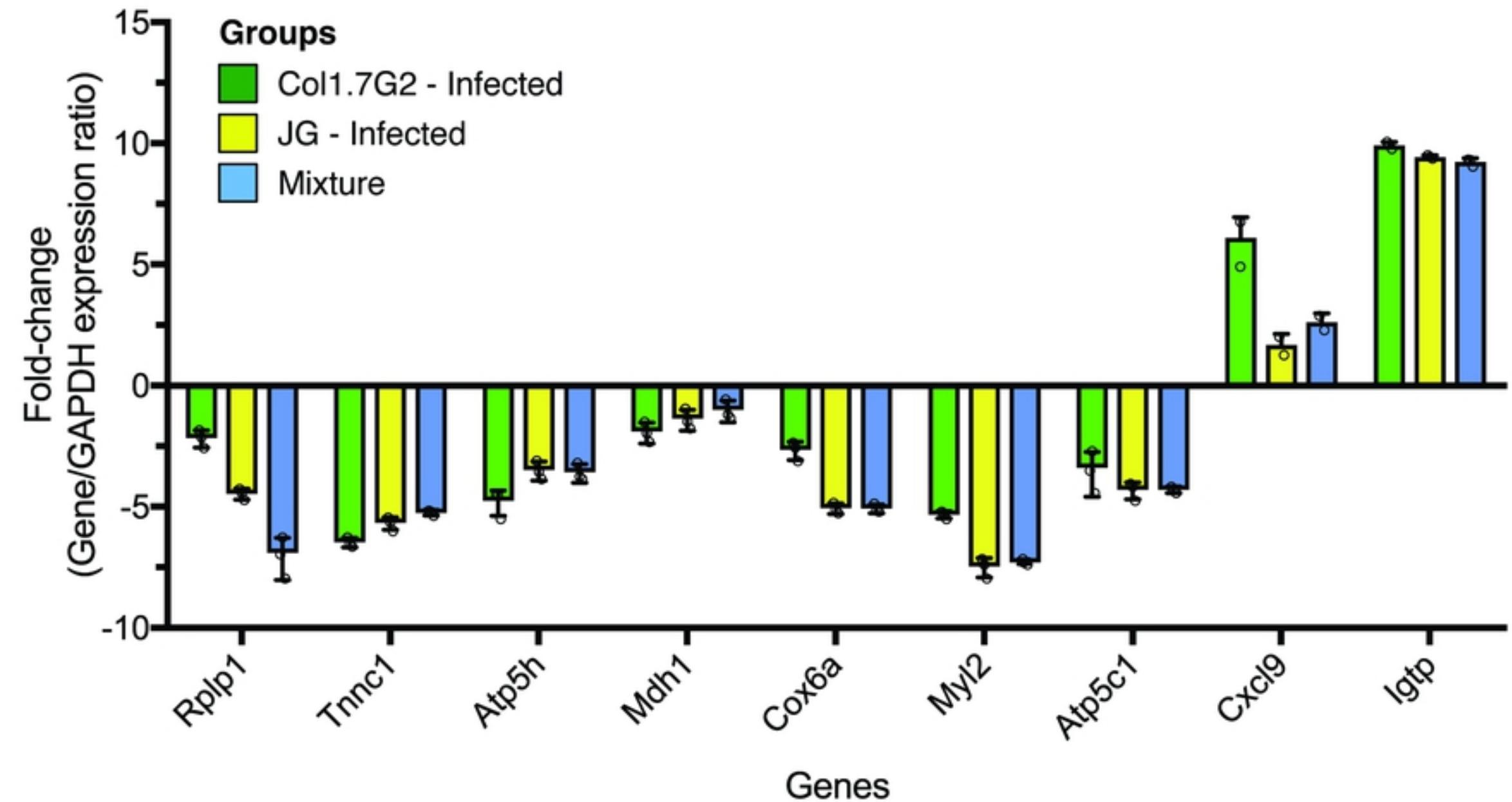


Figure 7

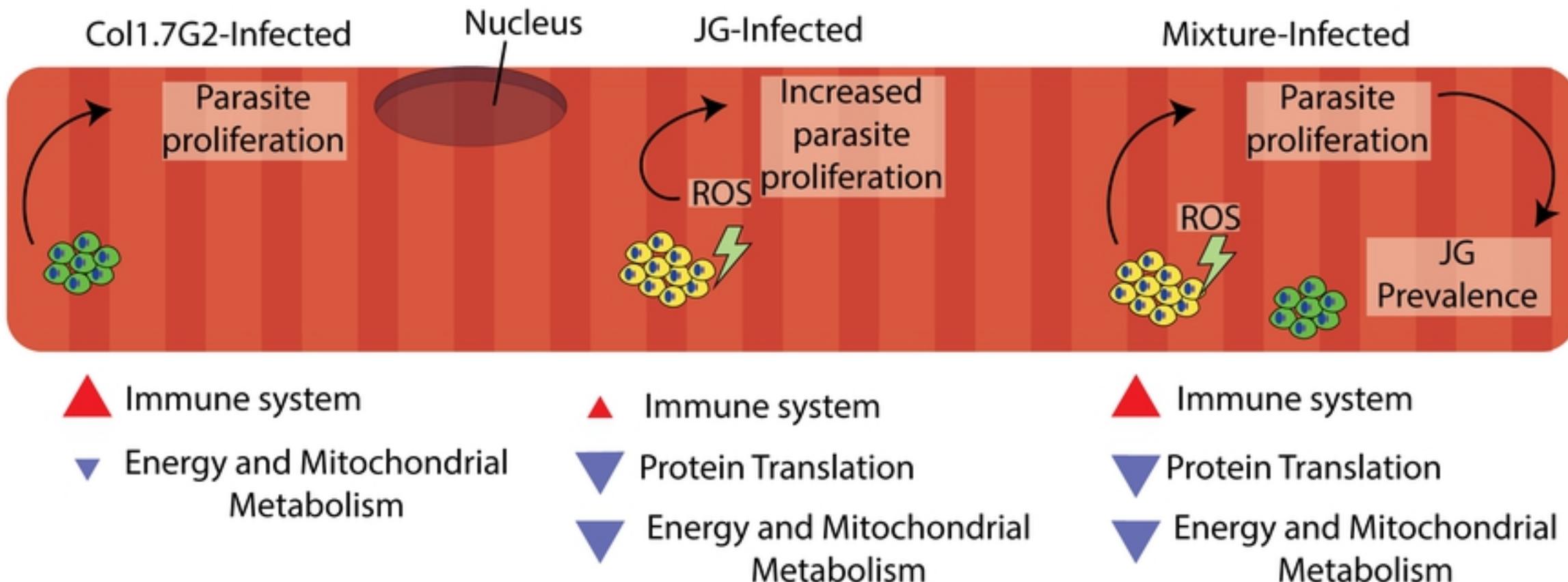


Figure 8