

1 **Decomposing variation in immune response in a wild rodent population**

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25 **Abstract**

26

27 Individuals vary in their immune response and, as a result, some are more susceptible to
28 infectious disease than others. Little is known about which components of immune pathways
29 are responsible for this variation, but understanding these underlying processes could allow
30 us to predict the outcome of infection for an individual, and to manage their health more
31 effectively. In this study, we describe transcriptome-wide variation in immune response (to a
32 standardised challenge) in a wild population of field voles (*Microtus agrestis*). We find that
33 this variation can be categorised into three main types. We also identify markers, across these
34 three categories, which display particularly strong individual variation in response. This work
35 shows how a simple standardised challenge performed on a natural population can reveal
36 complex patterns of natural variation in immune response.

37

38 **Introduction**

39

40 Individuals vary in their immune response. Within a population, some individuals may fail to
41 make protective immune responses following either natural infection or vaccination and so
42 are especially vulnerable to infectious disease¹⁻⁴. Defining the patterns of such variability will
43 enhance our ability to manage the health of individuals – especially those that are most
44 susceptible to infectious disease in human, livestock or wildlife populations.

45

46 Studies in laboratory mice are the cornerstone of immunology and have provided a detailed
47 understanding of the molecular and cellular pathways by which immune responses are
48 effected. This impressive mechanistic understanding, however, has only been achieved by
49 minimising genetic and environmental variation within a laboratory setting. Where laboratory

50 studies have examined the effects of variability – in genetics, microbiota or diet – both
51 qualitative and quantitative differences in immune responses have been observed, with
52 consequent effects on infection^{5–7}. Nevertheless, natural variability cannot be fully
53 reproduced in the laboratory, which has led to a recent effort to characterise the immune
54 response in wild populations of mice or other rodents. Recent work in mice from agricultural
55 and other anthropogenic settings is consistent with the expectation that exposure to complex
56 environments greatly alters immune function⁸. New populations of memory T cells, present
57 only in non-laboratory mice, have also been identified⁹.

58

59 One commonly used measure of an immune response is to assess the amount of one or more
60 markers (e.g. transcripts or proteins) produced by a population of cells following stimulation
61 by an immune agonist. From this *ex vivo* assay, one can gain insight into the types of immune
62 response that could be made to a pathogen *in vivo*. Such responses depend on the cell types,
63 the time points and the immune agonist used. Nevertheless, for any molecular marker with
64 such a response, individuals, in natural populations especially, could exhibit different marker
65 abundances prior to and/or following stimulation, leading to differences in their response to
66 stimulation (here defined as the difference between marker abundances prior to and following
67 stimulation). Furthermore, the most useful (and interesting) markers, in terms of
68 understanding why individuals vary in their ability to mount a successful immune response,
69 will be those for which response is most variable among individuals. In the laboratory, cell
70 populations are usually controlled, or at least well defined, so a difference in the abundance
71 of a particular marker can be attributed to differences in the activity of a particular cell type.
72 However, natural variability in the abundance of a marker, and by extension in the response
73 of individuals in the wild, could result from (i) differences in the composition of cell
74 populations, and/or (ii) differences in the activity levels of particular cell types. Both of these

75 components have the potential to shape the way an individual responds to immune challenge
76 in the wild. Our intention here is not to distinguish between the two, but rather to propose a
77 categorisation of responses, however generated.

78

79 We use a wild population of field voles (*Microtus agrestis*) to examine naturally occurring
80 patterns of individual variation in immune response, across the transcriptome, as a first step
81 towards furthering our understanding of the processes driving these patterns. The field
82 population we study, in Kielder Forest Northumberland, has been the subject of extensive
83 previous study on population ecology and pathogen dynamics¹⁰⁻¹³. Therefore, it allows us to
84 place our existing understanding of more established immunological mechanisms (largely
85 derived from the closely related laboratory mouse, *Mus musculus*) into a well-described, real-
86 world context.

87

88 We describe three main categories of immune response: (i) uncorrelated response, (ii)
89 constant response and (iii) baseline-dependent response (depicted in Fig. 1). We also identify
90 markers, across these categories, which show particularly high inter-individual variability in
91 response. We suggest that such categorisation is useful in organising natural immune
92 variation, since little is known about which components of immune pathways are responsible
93 for natural variability in immune response, or about the nature and possible causes of such
94 variability. Indeed, this categorisation is not limited to the components of conventional
95 immune pathways. The ability of an immune response to effect protection against infection,
96 for example, will be supported by a variety of non-immune functions, that will also be
97 activated following stimulation by an agonist, and vary to a greater or lesser extent among
98 individuals within a natural population. By identifying the components (whether
99 conventionally immunological or not) that are likely to be responsible for natural variability

100 in immune response, and by describing the nature of their variability, we are laying the
101 groundwork for exploring the processes, whether genetic or environmental, which drive inter-
102 individual variation in immune response.

103

104 **Results**

105

106 **Stimulation with an immune agonist causes a widespread response**

107 Spleen cells from sixty-two field voles were split into two populations per individual vole.
108 One population was stimulated with anti-CD3 and anti-CD28 antibodies, while the other was
109 kept as an unstimulated control (hereafter referred to as the baseline). 1150 transcripts (5% of
110 all genes in the field vole genome and 85% of informative genes, those genes which were
111 more strongly expressed; see Methods) fell into one or more of the response categories set
112 out in Fig. 1. As expected, given that these antibodies are known to stimulate T-cell
113 proliferation¹⁴, they were enriched with transcripts (hereafter markers) associated with the T-
114 cell receptor (TCR) signalling pathway ($n = 27$; $p < 0.001$; Functional Enrichment Analysis
115 performed in DAVID; see Methods) and other T cell-related terms: positive regulation of T-
116 cell proliferation ($n = 12$; $p < 0.03$), TCR complex ($n = 7$; $p < 0.001$), positive thymic T-cell
117 selection ($n = 7$; $p < 0.01$), negative thymic T-cell selection ($n = 6$; $p = 0.03$) and alpha-beta
118 TCR complex ($n = 5$; $p < 0.001$). For the majority of these markers, a significant positive
119 linear relationship was found between baseline and stimulated abundance ($n = 844$). Only a
120 single marker, *Fam193b*, demonstrated a significant negative linear relationship between
121 baseline and stimulated abundance.

122

123

124

125 **There are three main categories of immune response**

126 Three main categories of immune response were identified based on the dependence of an
127 individual's response on its baseline abundance. Each of these categories demonstrates a
128 unique pattern (Fig. 1):

129

130 **Uncorrelated response:** markers for which individuals taken from the wild differ in their
131 baseline abundance, but the responses of different individuals are variable and independent of
132 their baseline, such that the slope of the relationship between baseline and stimulated
133 abundance is not significantly different from zero.

134

135 **Constant response:** markers for which individuals taken from the wild also differ in their
136 baseline abundance, but the responses of different individuals are (approximately) constant
137 and independent of their baseline, such that the slope of the relationship between baseline and
138 stimulated abundance is not significantly different from one and the intercept (indicating the
139 level of response) is significantly greater than zero.

140

141 **Baseline-dependent response:** markers for which individuals taken from the wild again
142 differ in their baseline abundance, but the responses of different individuals vary as a
143 function of their baseline level, either as a linear function of their baseline level (slope
144 significantly different from one), or as a quadratic function of their baseline level, where
145 stimulated levels either increase exponentially as a function of baseline levels or become
146 saturated at some upper limit.

147

148 We also identified markers, across these three categories, for which variability in baseline
149 and stimulated samples was significantly different, leading to high inter-individual variability
150 in response (see Methods). These can be divided into two categories (Fig. 1):

151

152 **Convergent response:** markers for which variability in baseline abundance is significantly
153 greater than variability in stimulated abundance.

154

155 **Divergent response:** markers for which variability in stimulated abundance is significantly
156 greater than variability in baseline abundance.

157

158 **The baseline-dependent response category is most common and is significantly enriched**
159 **in components of conventional immune pathways**

160 The baseline-dependent response category was the most common (Table 1), and included a
161 majority of markers for which stimulated levels were a linear function of baseline levels ($n =$
162 539), and a remainder for which they were a quadratic function ($n = 160$). The majority of
163 quadratic response markers showed evidence for saturation ($n = 138$), indicating some upper
164 limit on stimulated abundance. The general ontology term for immunity was enriched in the
165 linear response category of markers ($n = 20$; $p < 0.01$). The TCR signaling pathway was
166 enriched in the quadratic response category ($n = 7$; $p = 0.01$; Fig. 2).

167

168 **The uncorrelated response category is least common and lacks enrichment in**
169 **components of conventional immune pathways**

170 A number of markers showed no evidence for a relationship between baseline and stimulated
171 abundance ($n = 47$; Table 1). For the majority of these, mean abundance was significantly
172 greater for stimulated than for baseline samples ($n = 39$), suggesting that these markers were

173 (on average) responding to stimulation, but to an individually variable degree, independent of
174 baseline levels. These markers lacked any enrichment for immune-related terms (Fig. 2).

175

176 **A number of markers, including *Zap70*, show particularly high inter-individual
177 variability in response**

178 For a number of markers, variability in baseline and stimulated abundance was significantly
179 different, leading to high inter-individual variability in response ($n = 244$). The vast majority
180 of these markers showed a divergent ($n = 237$), rather than a convergent ($n = 7$) response
181 (Table 1). Within the (stimulated) TCR signalling pathway, the highest level of variability in
182 individual response, and the highest level of divergence, was demonstrated by *Zap70* (Fig. 3).

183 All convergent markers fell into one of the three main immune response categories. However,
184 over a third of divergent markers ($n = 98$), did not fall into any of these categories, appearing
185 instead as markers which (on average) did not respond to stimulation (Table 1). Mean
186 abundances for these markers were also not significantly different between stimulated and
187 baseline samples.

188

189 **Juveniles show more inter-individual variability in response than adults**

190 An age-specific analysis, run separately on samples from mature ($n = 43$) and juvenile ($n =$
191 19) field voles, showed that higher inter-individual variability in immune response (whether
192 divergent or convergent) was more common among juvenile voles (no. divergent markers =
193 108; no. convergent markers = 6) than mature voles (randomly sampled 1000 times as more
194 samples available; mean no. divergent markers = 50, empirical 95% interval = 0–338.2; mean
195 no. of convergent markers = 0.11, empirical 95% interval = 0–1).

196

197

198 **Response to stimulation is not limited to components of conventional immune pathways**

199 Non-immune related terms were enriched in the baseline-dependent response category,
200 including: insulin signalling pathway ($n = 9$; $p = 0.05$) and thyroid hormone signalling
201 pathway ($n = 8$; $p = 0.05$). The top convergent response marker, *Pdk1*, is also a component of
202 the insulin signalling pathway (Fig. 2).

203

204 **Discussion**

205

206 The need to better understand variation in immune response in natural populations is now
207 widely accepted^{15–18}. Our understanding of immune responses in laboratory settings comes
208 from animals that vary little either genetically or in prior experience. By contrast, animals in
209 natural populations vary (perhaps extensively) in both of these. In this study, we describe
210 natural variation in immune response in a wild population of rodents, and find that it can be
211 categorised into a limited number of types. We identify three main categories of immune
212 response: uncorrelated response, constant response and baseline-dependent response. We also
213 identify markers, across these categories, which show particularly high inter-individual
214 variability in response. Our work shows how a simple stimulatory assay performed on a
215 natural population can reveal underlying patterns of natural variation among individuals in
216 immune response.

217

218 The baseline-dependent response category is the largest. Markers in this category show a
219 relationship between baseline and stimulated abundance across individuals, and their
220 response to stimulation is (to a lesser or a greater extent) dependent on their baseline level. In
221 some cases, individuals already expressing the greatest abundance of a marker in their natural
222 setting went on to exhibit the greatest response to stimulation by an agonist. In others, the

223 opposite was true, and these individuals exhibited the smallest response to stimulation.

224 Similarly, previous work on humans has identified baseline (transcriptional) predictors of

225 influenza vaccination response^{19,20}. These differences in baseline level could be driven by

226 either genetic variation or individual differences in past experience. In humans, genetic

227 determinants of baseline immune cell population frequencies have been identified²¹. Even

228 though the stimulation we describe here was not antigen specific, previous challenge by a

229 parasite might also lead to changes in the baseline T-cell population within an individual's

230 spleen, affecting its response to any subsequent challenge. In fact, we find that voles infected

231 with *Babesia microti* (a blood parasite, common in our population²²) have larger spleens than

232 uninfected voles¹³. This prior experience may prime an individual, enabling a greater

233 response to subsequent challenge (e.g. slope greater than one; Fig. 1). However, individuals

234 may also have an upper limit on the number of immune cells they have available^{23,24}. An

235 individual that is already mounting an immune response to a parasite, and has a large number

236 of activated T cells, could therefore respond less to a similar challenge than an

237 'immunologically naïve' individual (slope less than one; Fig. 1). Membership of the baseline-

238 dependent response category recapitulates the known biology of the immune response (being

239 highly enriched for immune ontology terms). In doing so, it validates the approach we use

240 here, as a way of identifying markers of immune significance.

241

242 In some cases, individuals varied in their natural abundance of a marker but their response

243 was unrelated to this. They did nevertheless respond to stimulation, with the majority of these

244 markers occurring at a significantly higher mean abundance in stimulated samples than in

245 baseline samples. This uncorrelated response category, which contains a moderate number of

246 markers, also lacks any enrichment for immune-related ontology terms. This suggests that

247 markers in this category are not conventional immune markers but could be of immune

248 significance. We warn against omitting such markers from studies of immune response in the
249 laboratory. They could play an important part in our understanding of the immune response,
250 indicating for example, genetic variation in response among individuals, which is
251 independent of baseline level.

252

253 Cutting across this categorisation, a large number of markers displayed a pattern in which
254 variation between individuals was particularly strong. We describe two types of such
255 markers, both of which could be used in future studies as indicators of natural variability in
256 immune response. Markers in the less common, convergent, response category showed much
257 greater variation naturally than following stimulation. This pattern may be characteristic of
258 markers showing variable levels of prior activation, coupled with some maximum or
259 optimum abundance, and resulting in a stabilisation of the immune response across the
260 population following stimulation. We found that convergent patterns were more common
261 among juvenile voles. This could suggest that they are more constrained in the energy they
262 have available (as a result of the competing energetic demands of growth and development)
263 or the number of immune cells they have available (as a result of a developing immune
264 system). Either resource constraint could result in a maximum abundance, making them more
265 inclined to converge. Due to the costly nature of the immune response, individuals often
266 trade-off their investment in different arms of the immune system^{25,26}. Different types of
267 immune response are therefore likely to be associated with different optimum abundances (or
268 regions) and an individual already mounting an immune response, but to a different type of
269 challenge (associated with different cell types), may respond by down-regulating expression.

270

271 Divergent markers, which were more common, showed much greater variation following
272 stimulation than there was naturally. This pattern may be characteristic of (but not limited to)

273 markers showing genetic variation in response to the agonist, independent of baseline levels
274 e.g. subsets of animals that appear similar but respond more strongly to stimulation than
275 others. Our own recent work, where we found an association between polymorphism in a
276 single gene and a marker of a more tolerant immune response²⁷, is an example of such
277 genetic variation in immune response. Further supporting this hypothesis, here, we found
278 more divergent markers among juvenile voles than mature voles. Younger voles are expected
279 to have less variable exposure histories, as a result of their shorter life spans, making it easier
280 to detect genetic effects. Equally, though, divergent patterns could be the result of differences
281 in early life experiences. One would also expect these to be more easily detectable in
282 juveniles.

283

284 The divergent category (predominantly) included markers for which individuals made (on
285 average) the same response to stimulation and markers that did not respond (on average) to
286 stimulation. Standard differential expression analysis would miss the individual variation
287 present in the former group, and would fail to pick up the latter group of markers altogether.
288 Both warn against looking at average (population-level) response, and point instead, to the
289 value of looking at individual-level differences in immune response. This is particularly
290 important because divergent markers may act as critical regulators of pathways. For example,
291 *Zap70*, which demonstrates particularly high levels of variability in individual response and
292 is centrally located in the TCR signalling pathway, interacts with many other markers (Fig.
293 3). We suggest that *Zap70* expression could be used as a marker of response in larger studies.
294 Indeed, it is already linked to major seasonal immune variation in wild fish²⁸ and is being
295 used as a prognostic marker for B-cell chronic lymphocytic leukemia in humans, with
296 potential implications for determining a patient's treatment path (recently reviewed in Liu *et*
297 *al.*²⁹). Other potential prognostic (or diagnostic) factors which may have been missed using

298 standard differential expression analyses may be present in this category and warrant further
299 investigation.

300

301 The immune response categories we describe here are based on spleen cells stimulated with
302 anti-CD28 and anti-CD3 antibodies and sampled at 24 hours. However, the relative
303 frequency of the response categories reported here may vary depending on the choice of
304 agonist and/or time point. For example, markers are known to follow different response
305 trajectories, with some immediately responding and reaching peak activation, and others
306 taking longer to reach this point³⁰. Sampling at a later time point, then, when the ‘slower’
307 markers have reached peak activation, may lead to more convergence than reported here. In
308 order to fully account for this temporal variation, multiple time points need to be averaged
309 across. We argue that both time-specific and averaged responses are of functional
310 significance, but hope others will extend our work. We use RNASeq here in order to give a
311 broad view of the immune response. Single-cell RNASeq could be used to quantify
312 differences in individual response resulting solely from differences in cell-specific activity.
313 Previous work has shown that transcript levels generally correlate with protein levels across
314 genes³¹. However, more work is needed to confirm these response categories at the functional
315 level³². In future, Q-PCR or protein-level data could be used in order to include weakly
316 expressed markers, which were excluded here as a result of the heteroscedasticity inherent in
317 RNASeq data.

318

319 Markers that responded to stimulation were not limited to immune pathways as
320 conventionally defined. They included, for example, markers involved in the insulin
321 signalling pathway. This is in line with previous studies, which suggest that insulin plays a
322 key role in coordinating an organism’s response to infection, influencing, in particular, the

323 allocation of resources^{33,34}. One of these markers, *Pdk1*, was also among the top convergent
324 markers. This could be representative of the high levels of variability in the (baseline)
325 nutritional status of individuals in a natural population, coupled with an upper limit on the
326 processes involved in glucose metabolism.

327

328 The immune categories we presented here, therefore, highlight markers not traditionally
329 associated with immune functions, and offer a promising avenue for identifying potential
330 prognostic (or diagnostic) factors for disease, like *Zap70*. They also point to both genetics
331 and prior experience as drivers of natural variation in immune response. Our future work will
332 further decompose this natural variation into that driven by these two components.

333

334 **Methods**

335

336 **Field methods**

337 Sixty-two field voles were collected between July and October 2015 to assay expression by
338 RNASeq. These voles came from four sites in Kielder Forest, Northumberland (55°130N,
339 2°330W). Each site contained a trapping grid of regularly spaced traps (at approx. 5 m
340 intervals) and was also used for other components of a larger field study (for more details see
341 Wanelik *et al.*²⁷).

342

343 **Ethics statement**

344 All animal procedures, carried out as part of this field study, were performed with approval
345 from the University of Liverpool Welfare Committee and under the authority of the UK
346 Home Office (Animals (Scientific Procedures) Act 1986) project licence number PPL

347 70/8210 to SP. Voles were killed by a rising concentration of CO₂ followed by
348 exsanguination.

349

350 **Cell culture methods**

351 Splenocyte cultures from each vole were split into two populations, one of which was
352 stimulated with anti-CD3 antibodies (Hamster Anti-Mouse CD3e, Clone 500A2 from BD
353 Pharmingen) and anti-CD28 antibodies (Hamster Anti-Mouse CD28, Clone 37.51 from BD
354 Tombo Biosciences) at concentrations of 2 µg/ml and of 1 µg/ml respectively for 24hr, and
355 the other was left as an unstimulated control to act as a reference level. We refer to this
356 reference level as the baseline, and control samples as baseline samples. However, it is
357 important to note that this level will vary for an individual, not only on a day to day basis, but
358 throughout its life. Culture conditions were otherwise equivalent to those used in Jackson *et*
359 *al.* (2011)³⁵. Costimulation with anti-CD3 and anti-CD28 antibodies was used to selectively
360 promote the proliferation of T cells¹⁴, our assumption being that this would reflect the
361 potential response of T-cell populations *in vivo*. Cell populations within splenocyte cultures
362 were variable but left undefined.

363

364 **RNASeq preparation and mapping**

365 RNA was extracted using Invitrogen PureLink kits. Following extraction, cDNA libraries
366 were prepared using Illumina RiboZero kits and libraries were constructed with NEBNext
367 Ultra directional RNA library prep kit according to the manufacturers protocols. Samples
368 were sequenced to produce 2 x 75 bp paired-end reads on an Illumina HiSeq4000 platform.
369 Adaptor sequences were removed with CUTADAPT version 1.2. and further trimmed with
370 SICKLE version 1.200 (minimum window quality score of 20). This resulted in a mean
371 library size of 18 million (range = 5–50 million) paired-end reads.

372

373 High-quality reads were mapped against a draft genome for *M. agrestis* (GenBank Accession
374 no. LIQJ00000000) using TOPHAT version 2.1.0, and a set of predicted gene models was
375 generated using BRAKER. Mapped reads were counted using FEATURECOUNTS. Further
376 analysis was performed on counts of mapped reads for each gene in R version 3.4.2³⁶. These
377 count data were initially filtered to remove unexpressed genes (those genes with fewer than
378 three counts per million across all samples; $n = 13$). Following filtering, library sizes were
379 recalculated and data were normalised to represent counts per million (cpm). These data were
380 found to be correlated with quantitative PCR (Q-PCR) data (see Supplementary Fig. 1). No
381 correction for gene length was necessary as all analyses were based on comparisons across
382 (rather than within) samples. Transcript abundance for a particular gene here represents a
383 single, functional measure of its activity across some, undefined, cell population. In order to
384 maximise the power of our analysis to identify biologically relevant patterns, we focussed on
385 those genes which were expressed at an informative level in the spleen prior to and/or
386 following stimulation ($n = 1350$ or 6%). Genes expressed at a mean level greater than 200
387 cpm were considered informative. As weakly expressed genes were removed (minimising
388 heteroscedasticity), log-transformation of data was unnecessary (Supplementary Fig. 2).

389

390 **Statistical analysis**

391 Genes for which a response to stimulation was observed across individuals were identified,
392 and, as elaborated in the Results, categorised on the basis of (i) the dependence of an
393 individual's response on its baseline level, and (ii) the degree of inter-individual variability in
394 response across individuals.

395

396 **Baseline-dependence of response.** The dependence of an individual's response on its
397 baseline level was quantified by testing the relationship between that individual's baseline
398 abundance (cpm_{base}) and its stimulated abundance (cpm_{stim}) using a linear regression, taking
399 the form

400

401
$$\text{cpm}_{\text{stim}} \sim \text{cpm}_{\text{base}}$$

402

403 as well as a quadratic regression, taking the form

404

$$\text{cpm}_{\text{stim}} \sim \text{cpm}_{\text{base}} + \text{cpm}_{\text{base}}^2$$

405

406 For approximately one third of genes ($n = 466$), the residuals from both of these regressions
407 deviated significantly from the assumptions of normality and/or homoscedasticity, and a non-
408 parametric Kendall–Theil linear regression was fitted instead. Regression fits varied from
409 gene to gene (R^2 ranging from <0.001 to 0.85).

410

411 **Inter-individual variability in response.** Inter-individual variability in response was
412 quantified by comparing the coefficient of variation (CV) for baseline abundances across
413 individuals (CV_{base}) and the CV for stimulated abundances across individuals (CV_{stim}). As
414 response is defined as the difference between baseline and stimulated abundance, a large
415 difference in their CVs, either

416

$$\text{CV}_{\text{base}} > \text{CV}_{\text{stim}}$$

417

418 or

$$CV_{\text{stim}} > CV_{\text{base}}$$

419

420 indicates a high level of variability in response. A relationship between gene-wise mean
421 expression levels and CV is typically found in RNASeq data, with low mean transcript
422 abundance being strongly associated with high variability³⁷. As we restricted our analysis to
423 informative genes only, excluding those genes with low mean abundance, it was not
424 necessary to account for this relationship (Supplementary Fig. 2). Asymptotic tests for the
425 equality of CVs were run using the cvequality package. All *p*-values were corrected for
426 multiple testing using the Benjamini-Hochberg method³⁸.

427

428 **Functional annotation.** Functional enrichment analyses were run using The Database for
429 Annotation, Visualization and Integrated Discovery (DAVID) version 6.8^{39,40}. Benjamini-
430 Hochberg corrected *p*-values and gene counts are reported alongside ontology terms,
431 including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to indicate their
432 level of enrichment⁴¹⁻⁴³.

433

434 **Age-specific analysis.** In order to begin to investigate the relative importance of genetic
435 variation versus prior stimulation for shaping patterns of variation in immune response, the
436 same analysis was performed separately on juvenile and mature voles. As we had more
437 samples from mature voles (*n* = 43) than juvenile voles (*n* = 19), we randomly sampled the
438 mature population (with replacement) 1000 times and averaged across these samples. The
439 number (juveniles) or mean number (matures) of genes in each of these age classes is
440 presented in the text.

441

442

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444

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544

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550

551 **Author contributions**

552 M.B., J.E.B., J.A.J. and S.P. designed the study. E.A. undertook the stimulatory assays.

553 K.M.W. analysed the data. All authors wrote the manuscript.

554

555 **Competing interest statement**

556 The authors declare no competing financial interests.

557

558 **Figure captions**

559

560 **Fig. 1 Different categories of immune response.** These are based on two overlapping sets
561 of criteria, baseline-dependence of response (blue) and inter-individual variability in response
562 (yellow background). Arrows represent individual immune responses. No response (for
563 reference): markers for which individuals (on average) show no response to stimulation
564 (intercept not significantly different from zero; slope not significantly different from one).

565 Uncorrelated response: markers for which responses of different individuals are variable and

566 independent of their baseline level (slope not significantly different from zero). Constant

567 response: markers for which the responses of different individuals response are
568 (approximately) constant and independent of their baseline (intercept significantly greater
569 than zero; slope not significantly different from one). Baseline-dependent response: markers
570 for which responses of different individuals vary as a function of their baseline level, either
571 as a linear function of their baseline (slope significantly different from one; slope greater than
572 one is depicted but could equally be less than one), or as a quadratic function of their baseline
573 (a saturating function is depicted but could equally be exponential). Convergent response:
574 markers for which the coefficient of variation (CV) for baseline abundances is significantly
575 greater than the CV for stimulated abundances across individuals ($CV_{base} > CV_{stim}$).
576 Divergent response: markers for which CV for stimulated abundances is significantly greater
577 than CV for baseline abundances across individuals ($CV_{stim} > CV_{base}$). Both convergent and
578 divergent markers depicted as, but not limited to, markers for which response is uncorrelated.
579

580 **Fig. 2 Top 10 markers and enriched ontology terms in each immune response category.**
581 Each box represents a category of immune response (as in Fig. 1). For each category,
582 top 10 annotated markers for which we had the most confidence in their categorisation
583 (markers were ranked on R^2 and p -values) are listed, one or two of these are represented in
584 plots showing stimulated versus baseline abundances across individuals (solid line indicates
585 significant relationship between baseline and stimulated abundance; dashed line indicates
586 slope equal to one for reference). In the case of the convergent category, which only included
587 a total of six annotated markers, all markers are listed. Ontology terms of interest, from an
588 enrichment analysis preformed on all markers within a category (where possible), are also
589 included (immune-related terms in black).
590

591 **Fig. 3 Map of the T-cell receptor signalling KEGG pathway for *Mus musculus*, with the**
592 **colour of nodes representing level of inter-individual variability in response to**
593 **stimulation with anti-CD3 and anti-CD28 antibodies in *Microtus agrestis*.** Namely the *p*-
594 value from an asymptotic test for the equality of variance in gene expression levels for
595 baseline and stimulated samples (range = < 0.001–0.97). Dark blue indicates high inter-
596 individual variability in response, whereas light blue or white indicates low inter-individual
597 variability in response. Grey nodes represent genes for which no information is available,
598 either because they are unannotated in the *M. agrestis* genome, or because they are weakly
599 expressed in the spleen.

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616 **Tables**

617

618 **Table 1 Table summarising the number of markers found in each of the three main**
619 **categories of immune response. For each of these categories, the number of convergent**
620 **and divergent markers is shown.**

621

Immune response category	Total no. markers	No. convergent	No. divergent
Uncorrelated	47	2	1
Constant	306	0	91
Baseline-dependent	699	5	47

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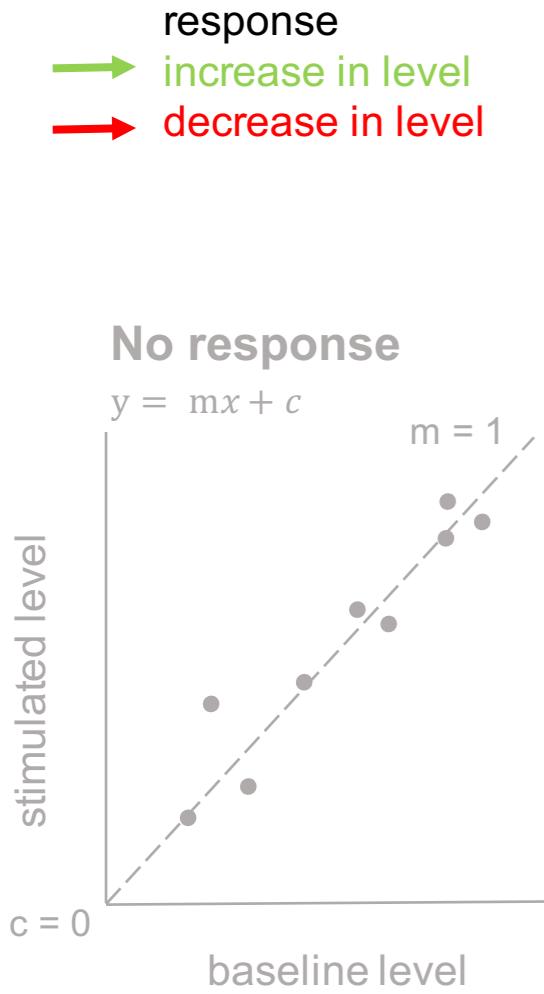
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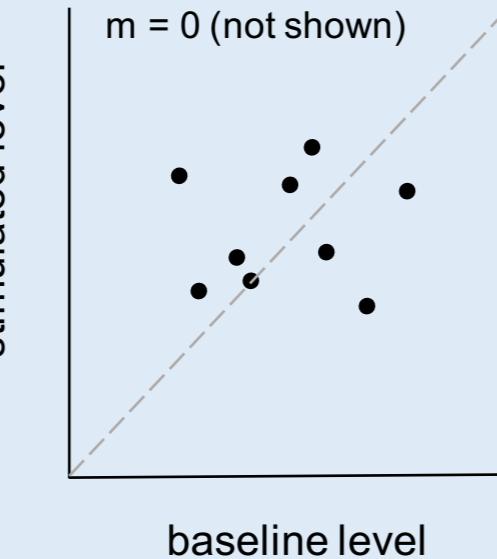
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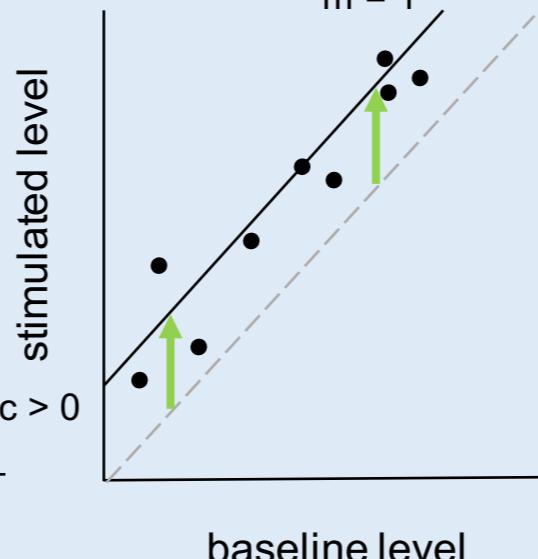
Baseline-dependence



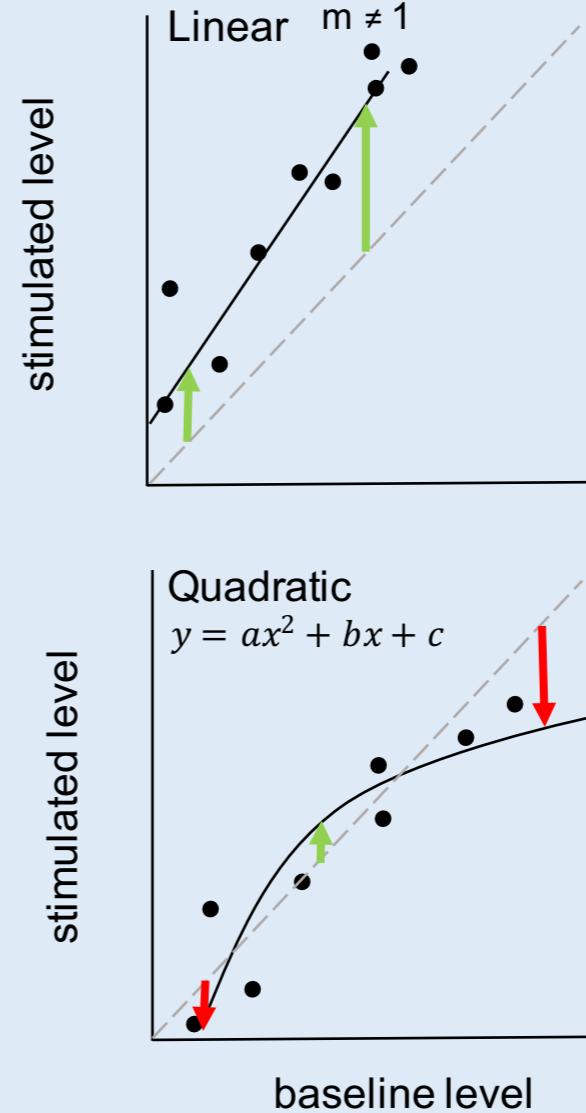
Uncorrelated response



Constant response



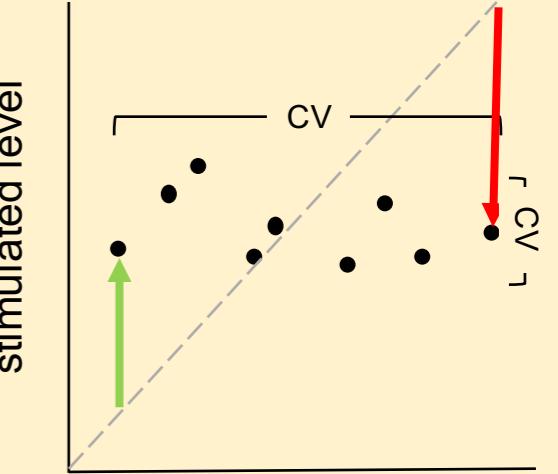
Baseline-dependent response



Variability

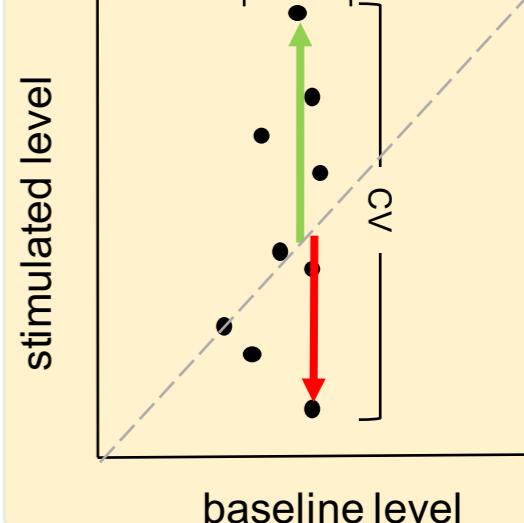
Convergent response

$$CV_{\text{base}} > CV_{\text{stim}}$$



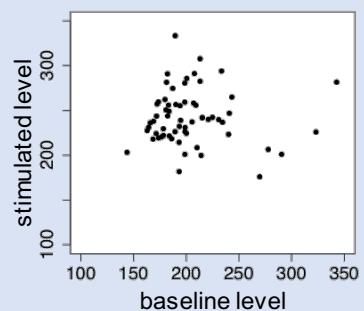
Divergent response

$$CV_{\text{stim}} > CV_{\text{base}}$$



Baseline-dependence

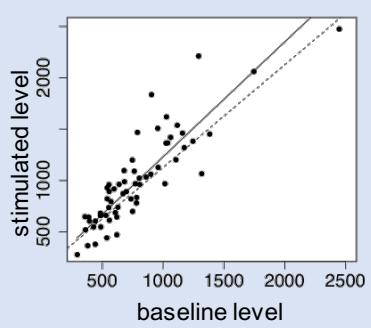
Uncorrelated response



Mon2 *Smarcc2*
Glyr1 *Rbm5*
Setx *Ap3d1*
Baz2b *Zfp280d*
Clasp1 *Zfp445*

mRNA splicing (4)

Constant response

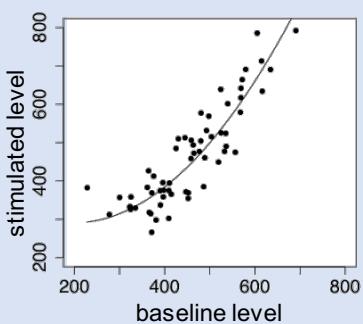
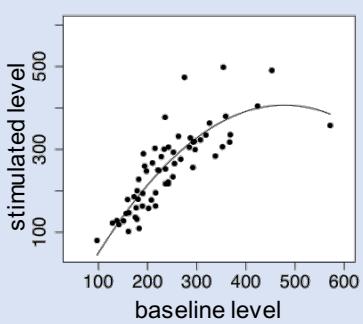


Lyst *Rapgef1*
Mgat4a *Swap70*
Rgs9 *Ckap5*
Fyn *Sik3*
Ddr1 *Hfe*

TCR pathway (9)
Natural killer cell mediated cytotoxicity (8)
Fc epsilon RI signaling pathway (7)
Fc gamma R-mediated phagocytosis (7)
Insulin signaling pathway (9)
thyroid hormone signaling pathway (8)

Baseline-dependent response

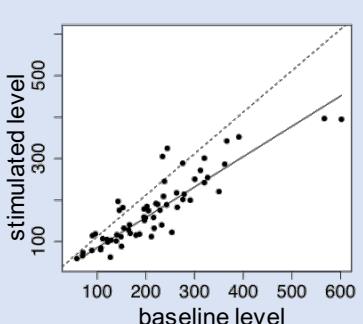
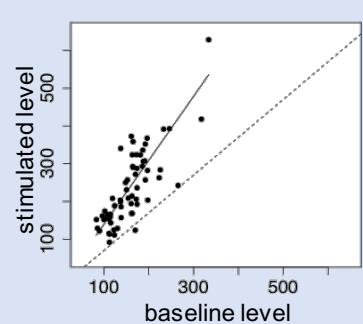
Quadratic



Cd247 *Fam135a*
Snrk *Card11*
Cpd *Traf1*
Itga6 *Sik2*
Cd3d *Ralgapa2*

TCR pathway (7)
Biological rhythms (6)
RNA-mediated gene silencing (4)

Linear

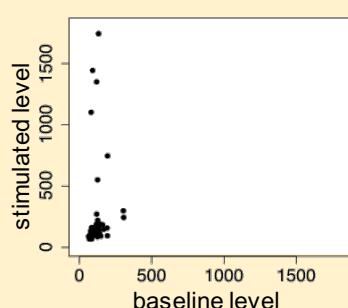


Jchain *Cybb*
Klri1 *Ncf2*
Ikzf2 *Cd8a*
Gbp2/2b *Tcf4*
Man2a2 *Txndc5*

Immunity (20)
Leukocyte transendothelial migration (14)
T Cytotoxic Cell Surface Molecules (7)
HTLV-I infection (20)
Leukocyte cell-cell adhesion (7)
Immunological synapse (6)
Antigen processing and presentation of
endogenous peptide antigen via MHC class I (4)

Highly variable response

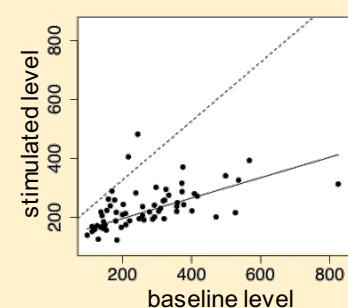
Divergent



Mdn1 *Jun*
Xpo1 *Ddx21*
Akap13 *Usp28*
Serbp1 *Hspf1*
Ptbp1 *Sik2*

Immunological synapse (5)
Viral carcinogenesis (12)
Epstein-Barr virus infection (11)
Heat shock protein 70 family (4)
Transcription factor Jun (3)
Toxin transport (6)
Positive regulation of telomere maintenance via
telomerase (5)

Convergent



Pdk1
Ero1l
Pja2
Lonp1
Mon2
Tcigr1

