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► **To cite this version:**

Jennifer Klunk, Tauras P Vilgalys, Christian E Demeure, Xiaoheng Cheng, Mari Shiratori, et al.. Evolution of immune genes is associated with the Black Death. *Nature*, 2022, 10.1038/s41586-022-05349-x . pasteur-03823087

HAL Id: pasteur-03823087

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Submitted on 25 Oct 2022

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Black Death shaped the evolution of immune genes

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46 Abstract

47 Infectious diseases have been among the strongest selective pressures during human
48 evolution. The single greatest mortality event in recorded history is the first outbreak of the
49 Second Pandemic of Plague, now commonly referred to as the Black Death (1346-1353 CE),
50 which was caused by the vector-borne pathogen *Yersinia pestis*¹. This pandemic devastated
51 Afro-Eurasia, killing up to 30-50% of the population². To identify targets of selection due to the
52 Black Death, we characterized genetic variation around immune-related genes from 321 ancient
53 DNA samples from two European populations before, during, and after the Black Death.
54 Immune loci are strongly enriched for highly differentiated sites, supporting the hypothesis of
55 genetic adaptation during the Black Death. In total, we identify 245 variants that are highly
56 differentiated within London, four of which were replicated in an independent cohort from
57 Denmark representing our strongest candidates for positive selection. The selected allele for one
58 of these variants is associated with a marked increase in the expression of *ERAP2* across a large
59 array of immune cells. This variant is also associated with increased ability to control
60 intracellular *Y. pestis* in monocyte-derived macrophages, functionally supporting an active role
61 of this variant in immune protection against *Y. pestis*. Finally, we show that several of our top
62 candidate variants overlap with alleles that are today associated with increased susceptibility to
63 autoimmune diseases, providing empirical evidence for the role played by past pandemics in
64 shaping present-day susceptibility to disease.

65

66 Main text

67 Infectious diseases have presented one of the strongest selective pressures in the
68 evolution of humans and other animals^{3,4,5}. Not surprisingly, many candidates for population-

69 specific positive selection in humans involve immune response genes, consistent with the
70 hypothesis that exposure to novel and or re-emerging pathogens has driven adaptation^{6,7}.
71 However, it is challenging to connect signatures of natural selection with their causative
72 pathogens unless the underlying loci are still associated with susceptibility to the same pathogen
73 in modern populations^{8,9}. Clarifying the dynamics that have shaped the human immune system is
74 key to understanding how historical diseases contributed to disease susceptibility today.

75

76 We sought to identify signatures of natural selection in Europeans imposed by *Yersinia*
77 *pestis*, the bacterium responsible for bubonic plague¹. The first plague pandemic began with the
78 Plague of Justinian in 541CE^{10,11}. Nearly 600 years later, the Black Death spread throughout
79 Europe, the Middle East, and Northern Africa, reducing the population by up to 30-50%^{2,12}. With
80 no recent exposure to plague, Europeans living through the Black Death represented
81 immunologically naïve populations with little to no adaptation to *Y. pestis*. Given the high
82 mortality rate, it seems reasonable to assume that genetic variants conferring protection against
83 *Y. pestis* infection be under strong selection at this time. Indeed, the nearly decadal plague
84 outbreaks over the subsequent four hundred years of the second pandemic in Europe often (but
85 not always) had reduced mortality^{12,13,14}, suggesting possible genetic adaptation to *Y. pestis*
86 although likely complicated by pathogen evolution and changing cultural practices.

87

88 Pre- and post-Black Death populations reveal candidate loci for plague-mediated
89 selection

90 Genomic targets of selection imposed by *Y. pestis* during the Black Death, if present,
91 have remained elusive^{15,16}. To better identify such loci, we characterized genetic variation from

92 ancient DNA extracts derived from individuals who died shortly before, during, or shortly after
93 the Black Death in London and across Denmark. This unique sampling design isolates, to the
94 greatest extent possible, signatures due to *Y. pestis* and not from other infectious diseases or
95 selective processes. From London, individuals stemmed from three cemeteries close to one
96 another, tightly dated by radiocarbon, stratigraphy and historical records to before, during and
97 after the Black Death (**Fig 1, Table S1, Supplementary Methods**). From Denmark, individuals
98 stemmed from five localities, geographically spread across the country, which were dated via
99 archaeological means (i.e. burial arm positions), stratigraphy and historical records. We grouped
100 all individuals into those that lived pre-Black Death (London: ~1000-1250 CE, Denmark: ~850
101 to ~1350 CE) and post-Black Death (London: 1350-1539 CE, Denmark: ~1350 to ~1800 CE).
102 Within London, we also included individuals buried in the plague cemetery, East Smithfield, all
103 of whom died during a two year window of the Black Death between 1348 and 1349²⁰. Analysis
104 of the mitogenomic diversity from these same individuals identifies solely European
105 mitochondrial haplotypes ruling out widespread population replacement from non-European
106 sources, which could confound the detection of natural selection²¹.

107

108 In total we screened 523 samples (n=325 from London; n=198 from across Denmark) for
109 the presence of human DNA using a modified PCR assay for the single copy nuclear cMYC
110 gene^{21,22} and identified 321 with sufficient endogenous DNA content for downstream enrichment
111 and sequencing of additional nuclear loci (Supplementary Methods). Since many of our samples
112 were poorly preserved and had low endogenous DNA content, we used hybridization capture to
113 enrich for and sequence 356 immune-related genes, 496 GWAS loci previously associated with
114 immune disorders, and 250 neutral regions (1.5kb each) based on their location >200kb from any

115 known gene (**Table S2**; Supplementary Methods). The targeted immune genes were manually
116 curated based on their role in immune related processes, and include innate immune receptors,
117 key immune transcriptions factors, cytokines and chemokines, and other effector molecules
118 (**Table S3**). To ensure that deamination and other forms of ancient DNA damage did not lead to
119 spurious genotype calls, we trimmed 4bp from the start and end of each sequencing read (**Fig S1**)
120 and excluded all singleton variants (n=106,757). Our final dataset contained 33,110 biallelic,
121 non-singleton variants within the targeted regions (2,669 near GWAS loci, 19,972 in immune
122 genes, and 10,469 in neutral regions), with a mean coverage of 4.6x reads per site per individual
123 (see **Table S1** for individual coverage). We further filtered our results by excluding samples with
124 missing genotype calls at more than 50% of those sites (retaining n=206 individuals) and
125 retaining variants with genotype calls for at least 10 individuals per time period and population.
126 Using genotype likelihoods, we then calculated the minor allele frequency (MAF) per population
127 at each time point, retaining only sites with a mean MAF (averaged across London and
128 Denmark) greater than 5% (n= 22,868 sites) since our power to detect selection for variants
129 below 5% is very low (**Fig S2**).

130

131 To detect alleles that may have conferred protection from, or susceptibility to, *Y. pestis*,
132 we searched within candidate regions (immune genes and GWAS loci) for variants exhibiting
133 unexpectedly large changes in allele frequency between pre- and post-Black Death. Specifically,
134 we identified alleles for which the degree of differentiation (F_{ST}) was larger than expected by
135 chance, when compared to the same neutral genetic regions in the same population. We used the
136 larger sample set from London as our discovery cohort. Burials in London were also more
137 precisely dated and better geographically controlled than those from Denmark, improving our

138 relative ability to detect selection in the cohort from London (Supplementary Methods). We
139 found an enrichment of highly differentiated variants, for all frequency bins with MAF > 10%,
140 relative to a null expectation established using our neutral loci (**Fig 2A**). Across these variants,
141 differentiation at immune loci exceeded the 99th percentile of neutral variants at 2.4x the rate
142 expected by chance (binomial test $p = 7.89 \times 10^{-12}$). For variants with a MAF > 30%, this
143 enrichment was even more pronounced (3.9x the rate expected by chance; binomial test $p =$
144 1.16×10^{-14}), likely due to increased power (**Fig S2**). Simulations show that differences in
145 recombination rate and background selection between neutral and candidate loci are insufficient
146 to explain the observed enrichments (**Fig S3**). To further validate the signatures of selection we
147 observed among immune loci from our London sample, we performed the same analyses using
148 the allele frequencies estimated from our Danish cohort. These samples were also enriched for
149 highly differentiated sites relative to the expectation from neutral loci (1.6x the rate expected by
150 chance, binomial test $p = 9.21 \times 10^{-4}$; **Fig 2B**), further supporting evidence for plague-induced
151 selection on immune genes.

152

153 To identify specific loci that represent the strongest candidates of selection, we applied a
154 series of stringent criteria that leveraged the time points and populations in this unique dataset.
155 First, we identified 245 common variants (MAF > 10%) that were highly differentiated (F_{ST}
156 > 95th percentile defined using neutral sites) when comparing pre- vs post-Black Death samples in
157 London alone (**Table S4**). Next, we reasoned that variants conferring increased susceptibility to,
158 or protection from, *Y. pestis* should show opposing frequency patterns before, during and after
159 the Black Death. Specifically, variants associated with susceptibility should increase in
160 frequency among people who died during the Black Death and should decrease in frequency

161 among individuals sampled post-Black Death (i.e., the survivors and/or descendants of the
162 survivors). Conversely, protective variants should show an inverse pattern. Using this reasoning,
163 we narrowed down our list of putatively selected loci from 245 to 35 (**Table S4**). Finally, we
164 asked if these loci were also highly differentiated before and after the Black Death in our Danish
165 replication cohort (i.e., among the top 10% most highly differentiated sites, and in the same
166 direction as seen in London). Four loci met our criteria, and therefore represented our strongest
167 candidates for selection (**Fig 2C-G**). We calculated the selection coefficient (s) for each of these
168 variants using a Hidden Markov Model (HMM) framework (based on ²⁴, Supplementary
169 Methods). Statistical support for non-neutral evolution ($s \neq 0$) among our four candidate loci is
170 strong when compared to that of neutral loci ($p < 0.001$ for each locus; **Table S5**). Despite the
171 large confidence intervals – an inherent limitation when trying to estimate s over a few
172 generations – the absolute values for the point estimate of s range from 0.26 to 0.4, which are
173 among the strongest selective coefficients yet reported in humans (**Fig S4, Table S5**).

174

175 Genes near candidate variants are differentially regulated in response to plague

176 None of our top candidate variants overlap (nor is in strong linkage disequilibrium with)

177 coding variants. Thus, it is likely that the selective advantage stems from their impact on gene

178 expression levels, particularly on immune cell types that participate in the host response to *Y.*

179 *pestis* infection. Macrophages in particular are recruited to sites of infection (i.e. flea bites)

180 where they interact with bacteria and have been shown to contribute to resistance to plague

181 ^{25,26,27}. Macrophages will phagocytize *Y. pestis*, but some bacteria survive and spread to the

182 lymph node where they replicate uncontrollably^{28,29}. To test whether our variants or nearby genes

183 are involved in the transcriptional response to *Y. pestis*, we incubated monocyte-derived

184 macrophages (MDMs) from 33 individuals with heat-killed *Y. pestis* and compared their
185 expression profiles to unstimulated control samples using RNA sequencing (Supplementary
186 Methods). As expected, macrophages responded robustly to *Y. pestis*, with principal component
187 (PC) 1 of the gene expression data explaining 56% of the total variance in gene expression levels
188 (**Fig S5**).

189

190 Seven genes within 100kb of our four candidate loci were expressed in this dataset: locus
191 1 (rs2549794): *ERAP1*, *ERAP2*, *LNPEP*; locus 3 (rs11571319): *CTLA4*, *ICOS*; and locus 4
192 (rs17473484): *TICAM2*, *TMED7*, *NFATC1*, the only gene nearby locus 2 (rs1052025), was not
193 expressed in this dataset. With the exception of *LNPEP*, all of these genes are differentially
194 expressed in response to *Y. pestis* stimulation (**Fig 3A**), supporting their putative role in the host
195 response. Macrophages from an additional panel of 8 individuals infected with live and fully
196 virulent *Y. pestis* showed similar directional changes in gene expression to those observed in
197 response to heat-killed bacteria; both genome-wide ($r = 0.88$, $p \approx 0$, **Fig S6A**) as well as at the
198 level of the genes nearby our candidate loci, with the exception of *ERAP1* that is up-regulated in
199 response to live bacteria but down-regulated in response to heat-killed bacteria (**Fig S6B**). Next,
200 we asked if changes in gene expression were specific to *Y. pestis* or shared with other infectious
201 agents. To do so, we analyzed gene expression data of macrophages infected with live *Listeria*
202 *monocytogenes* (a Gram-positive bacterium) and *Salmonella typhimurium* (a Gram-negative
203 bacterium)³⁰, as well as monocytes activated with bacterial and viral ligands targeting the Toll-
204 like receptor (TLR) pathways (TLR1/2, TLR4, and TLR7/8) and live influenza virus³¹. These
205 data show that all genes within our candidate loci (with the exception of *CTLA4*) respond to
206 other pathogenic agents but that the direction of change differs depending on the stimuli. For

207 example, *ERAP2* is downregulated in response to all live bacteria or bacterial stimuli, including
208 *Y. pestis*, but is up-regulated in response to viral agents (**Fig S7**).

209

210 Next, we asked if different genotypes at candidate loci had any impact upon the
211 expression levels of these genes (**Fig 3B-C**). We identified an association between rs17473484
212 genotypes and *TICAM2* expression where the protective allele was associated with higher
213 expression of the gene in the unstimulated condition (**Fig 3B**; $p = 2.5 \times 10^{-6}$), although this genetic
214 effect was smaller following *Y. pestis* stimulation ($p = 0.24$). This effect is intriguing as *TICAM2*
215 encodes an adaptor protein for Toll-Like Receptor 4 (*TLR4*). *In vivo*, *TLR4* detects *Y. pestis* via
216 the recognition of lipopolysaccharides (LPS) on the bacterial outer membrane³². *Y. pestis*
217 attempts to circumvent this detection by deacylating surface LPS, reducing the binding affinity
218 with *TLR4*^{33,34}. *TICAM2* ushers LPS-bound *TLR4* into endosomes and activates type I interferon
219 responses³⁵. It is therefore possible that increased *TICAM2* expression confers protection against
220 *Y. pestis* by increasing sensitivity to LPS and promoting an effective immune response.

221

222 The strongest association we identified was between rs2549794 and *ERAP2* expression,
223 where the protective allele (C) is associated with a 5-fold increase in expression of *ERAP2*
224 (relative to the susceptibility T allele, **Fig 3C**), in both unstimulated ($p = 4.4 \times 10^{-10}$) and *Y. pestis*
225 challenged ($p = 8.7 \times 10^{-7}$) cells. We observed similar strong associations from macrophages and
226 monocytes infected with other pathogens (*Salmonella*, *Listeria*, *influenza*) or stimulated with
227 TLR-activating ligands (all $p < 1 \times 10^{-10}$; **Fig S8**). Motivated by the strong regulatory impact of
228 rs2549794, we sought to systematically evaluate the relationship between rs2549794 genotypes
229 and the expression of *ERAP2* in a wider variety of immune cell types before and after infection

230 with live and fully virulent *Y. pestis*. We generated and sequenced expression data from
231 peripheral blood mononuclear cells from 10 individuals (5 homozygous for the protective
232 rs2549794 C allele and 5 homozygous for the susceptible T allele), using single-cell RNA
233 sequencing. Across all immune cell types profiled – B cells, CD4⁺ T cells, CD8⁺ T cells, NK
234 cells, and monocytes (**Fig 3D**) – we identified 5,570 genes where expression level is affected by
235 infection with *Y. pestis* (314 to 4,234 genes per cell type, 10% FDR; **Table S6**). Most genes near
236 our candidate loci are differentially expressed in response to *Y. pestis* infection, but both the
237 magnitude and direction of such effects is cell-type specific (**Fig S9**). For example, *ERAP2* is
238 upregulated upon stimulation in T and B cells, but downregulated in monocyte-derived
239 macrophages, suggesting that the regulation of *ERAP2* is likely to be controlled by a set of
240 distinct transcription factors and enhancers across cell types. As in macrophages and other
241 datasets, the protective rs2549794 genotype is associated with increased *ERAP2* expression
242 across cell types for both infected and non-infected cells (**Fig 3E-F**), but we found no association
243 between rs2549794 genotype and the magnitude of the fold change responses in *ERAP2* upon *Y.*
244 *pestis* infection. The much lower expression of *ERAP2* in individuals homozygous for the
245 susceptible T allele likely results from strong linkage ($R^2 > 0.8$; $D' = 1.0$) between the T allele and
246 a nearby alternative splicing variant which leads to a premature stop codon, nonsense mediated
247 decay, and a non-functional protein³⁶.

248

249 *ERAP1* and *ERAP2* are aminopeptidases that work synergistically to trim peptides for
250 presentation to CD8⁺ T cells by MHC class I molecules^{37,38}. Given their central role in antigen
251 presentation, it is not surprising that polymorphisms in these genes, including rs2549794, have
252 been associated with susceptibility to a variety of infectious agents^{39,40}. Interestingly, *ERAP2*

253 deficiency leads to a significant remodeling of the repertoire of antigens that are presented by
254 MHC to CD8⁺ T cells^{41,42,43}, including MHC ligands that have high homology to peptide
255 sequences derived from *Yersinia* species⁴³. It is therefore likely that having a functional version
256 of *ERAP2* would help promote the presentation of a more diverse array of *Yersinia*-derived
257 antigens to CD8⁺ T cells, which play an important role in protection against *Yersinia*
258 infections^{44,45}. Indeed, mice depleted of CD8⁺ T cells all die within one-week post-infection with
259 a milder *Yersinia* spp., *Y. pseudotuberculosis*, whereas all wild-type mice survive⁴⁴.
260 Complementing our findings, a recent study reported positive selection on several MHC class I
261 alleles⁴⁶, further strengthening the link between CD8⁺ T cell responses and selection by the
262 plague.

263

264 In addition to its canonical role in antigen presentation and CD8⁺ T cell activation,
265 *ERAP2* is also involved in viral clearance and cytokine responses⁴⁷. We sought to test whether
266 *ERAP2* genotype is associated with variation in the cytokine response to *Y. pestis* infection. To
267 do so, we infected an additional set of MDMs from 25 individuals (9 homozygous for the
268 protective *ERAP2* haplotype, 9 heterozygous, and 7 homozygous for the deleterious haplotype)
269 with live and virulent *Y. pestis* and measured the protein levels of 10 cytokines involved in
270 various aspects of the immune response, at baseline and at 24 hours post-infection. No
271 differences in cytokine levels existed at baseline (all $p > 0.05$; **Table S7**), but four cytokines
272 showed a significant association with *ERAP2* genotype upon stimulation. Specifically, the levels
273 of G-CSF ($p = 0.0155$), IL1 β ($p = 0.00262$), and IL10 ($p = 0.00248$) significantly decreased with
274 the number of protective C alleles, while we observed the opposite pattern for the chemokine
275 CCL3 levels ($p = 0.0216$), which is involved in the recruitment of neutrophils upon infection

276 (Fig 4A-D; Table S7)^{48,49}. We also assayed the ability of macrophages to control internalized *Y.*
277 *pestis* replication as a function of genotype at the *ERAP2* locus. Individuals homozygous for the
278 protective *ERAP2* allele were better able to limit *Y. pestis* replication ($p = 0.02$; Fig 4E), further
279 supporting a protective role for this variant against infection.

280

281 Discussion

282 Our results provide strong empirical evidence that the Black Death was an important
283 selective force that shaped genetic diversity around some immune loci. Because our unique
284 sampling design included individuals who died before, during, and after the Black Death, we
285 minimized the degree to which other historical events (i.e. tuberculosis⁹ or famine^{50,51,52}) could
286 affect the inference of selection. To support our ancient genomic data, we confirmed that the
287 strongest candidates for positive selection have a direct impact on the immune response to *Y.*
288 *pestis* using functional data.

289

290 We identified four loci which were strongly differentiated before and after the Black
291 Death in London and replicated in an independent sample cohort from Denmark as the strongest
292 candidates of selection. However, given our small sample sizes and the low sequencing coverage
293 inherent to ancient DNA studies, our replication power is limited and some of the other 245
294 highly differentiated loci in London were also likely targets of selection, but did not survive our
295 conservative, multi-step filtering criteria. Increased sample sizes coupled with additional
296 functional data will be required to further dissect the evolutionary role played by these variants
297 in immune protection against *Y. pestis*.

298

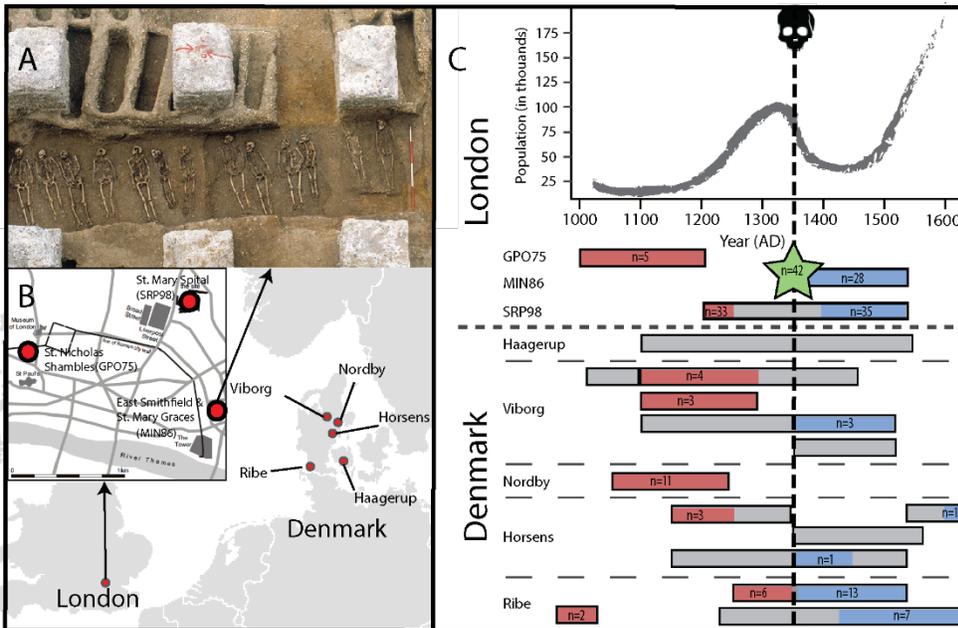
299 *ERAP2* showed the most compelling evidence for selection, both from a genetic and
300 functional perspective, with an estimated selection coefficient of 0.4 (95% CI 0.19,0.62),
301 suggesting that individuals homozygous for the protective allele were approximately 40% more
302 likely to survive the Black Death than those homozygous for the deleterious variant. The selected
303 allele is associated with increased expression of *ERAP2*, an effect that we expect to be
304 exacerbated at the protein level because the deleterious haplotype harbors a splicing variant that
305 leads to a premature stop codon (ref). Thus, only individuals with the protective allele have
306 *ERAP2* aminopeptidase activity. We hypothesize that having *ERAP2* activity increases the ability
307 of antigen presenting cells to present *Yersinia*-derived antigens to CD8+ T cells. Furthermore,
308 we show that the protective *ERAP2* haplotype is strongly associated with the secretion of several
309 cytokines in macrophages infected with *Y. pestis* and the ability of macrophages to limit *Y. pestis*
310 replication *in vitro*. In general, individuals with more copies of the protective haplotype
311 displayed a weaker cytokine response to infection but a better ability to limit bacterial growth.
312 For example, levels of IL1 β , a key proinflammatory cytokine often associated with pyroptotic
313 cell death⁵³, were 3-fold lower in individuals homozygous for the protective *ERAP2* genotype
314 when compared to individuals homozygous for the susceptible one. Therefore, subjects with the
315 protective haplotype are both more efficient at controlling internalized bacteria and at resisting
316 *Y. pestis*-induced cell death than subjects with the deleterious haplotype, abilities which may
317 help reduce bystander tissue damage during infection. On the other hand, the increased secretion
318 of CCL3 in individuals with the protective haplotype suggests that CCL3-induced neutrophil
319 recruitment may be beneficial^{48,49}. However, since these experiments have been done *in vitro* and
320 rodent models only possess a single *ERAP* aminopeptidase that is homologous to *ERAP1*, we
321 were unable to directly evaluate the impact of *ERAP2* genotype on tissue damage, immune cell

322 recruitment, and survival.

323

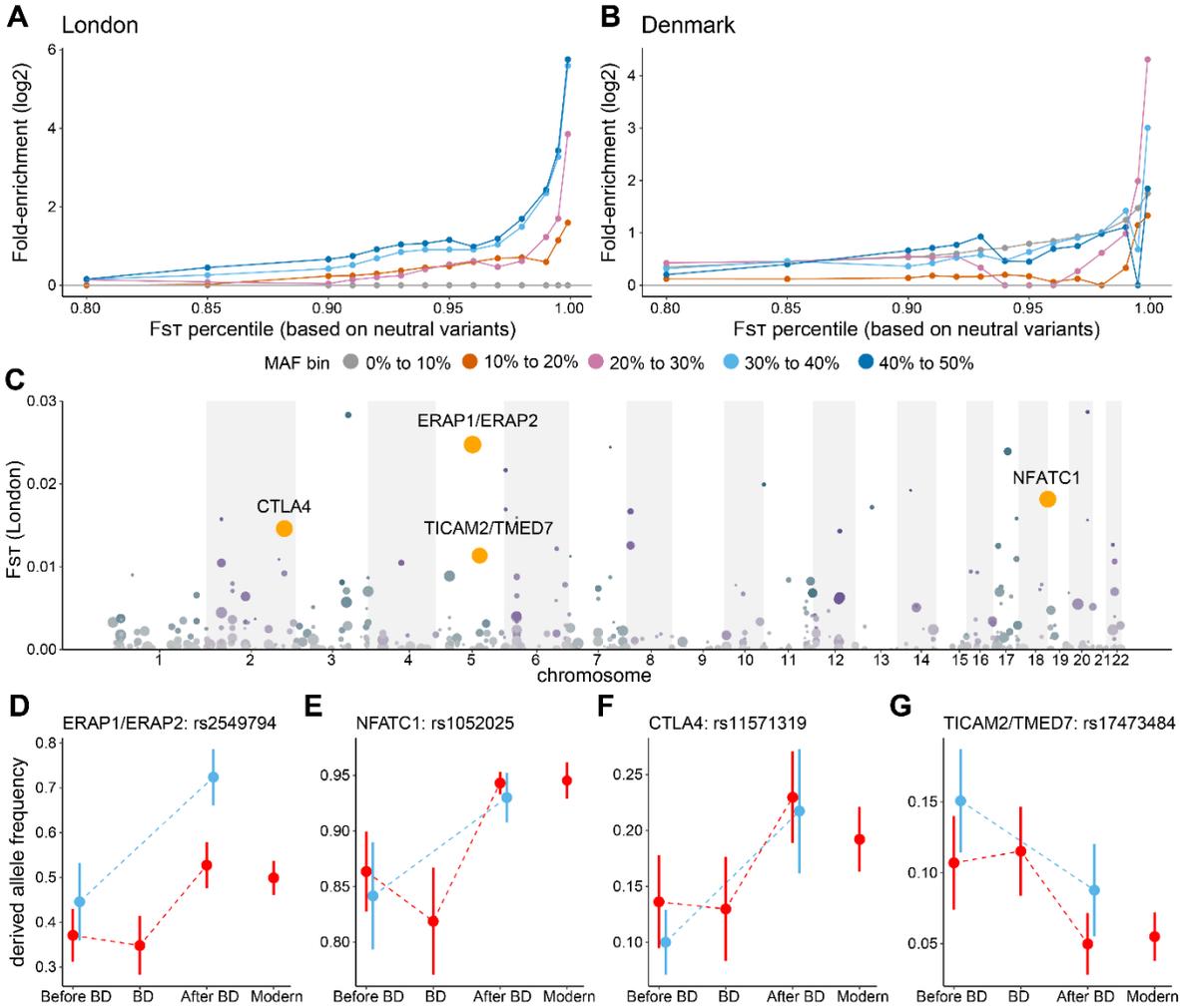
324 More broadly, our results highlight the contribution of natural selection to present-day
325 susceptibility towards chronic inflammatory and autoimmune disease. We show that *ERAP2* is
326 transcriptionally responsive to stimulation with a large array of pathogens, supporting its key role
327 in the regulation of immune responses. Therefore, selection imposed by *Y. pestis* on *ERAP2*
328 likely affected the immune response to other pathogens or disease traits. Consistent with this
329 hypothesis, the protective *ERAP2* variant is a known risk factor for Crohn's disease⁵⁴, and
330 *ERAP2* variation has also been associated with other infectious diseases^{39,40}. Thus, fluctuating
331 selection based on the presence of various pathogens (including *Y. pestis*) weighed against the
332 costs of immune disorders likely explains why this locus appears to be evolving under long-term
333 balancing selection³⁶. Likewise, another one of our top candidate loci (rs11571319 near *CTLA4*)
334 is associated with an increased risk of rheumatoid arthritis⁵⁵ and systemic lupus erythematosus⁵⁶,
335 with the protective allele during the Black Death conferring increased risk for autoimmune
336 disease. To date, most of our evidence of an association between autoimmune risk alleles and
337 adaptation to past infectious disease remains indirect, primarily because the etiological agents
338 driving selection remain hidden. Our ancient genomic and functional analyses suggest that *Y.*
339 *pestis* has been one such agent, representing, to our knowledge, the first empirical evidence
340 connecting the selective force of past pandemics to present-day susceptibility to disease.

341 Figures
 342



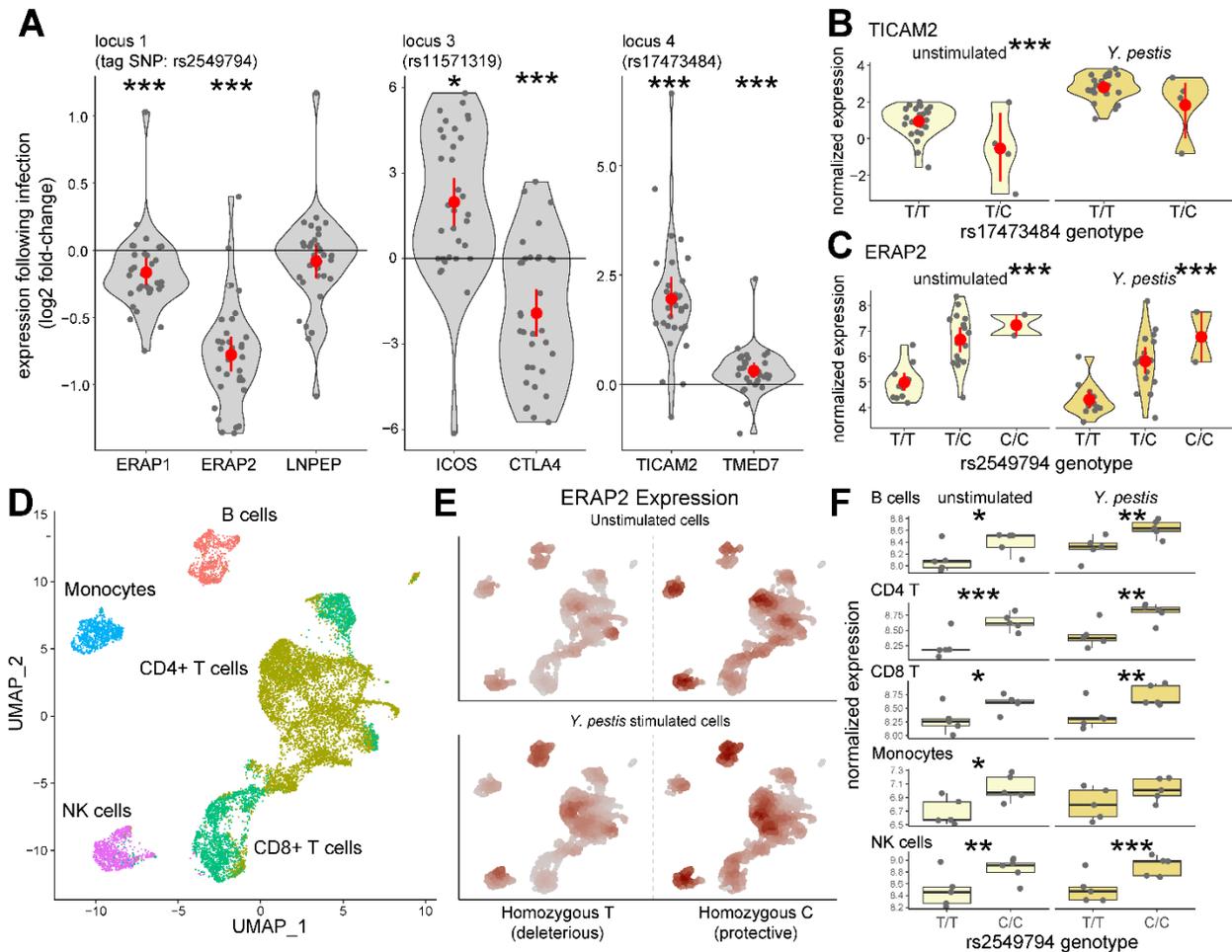
343
 344 **Figure 1. East Smithfield mass burial, sample locations with their date ranges along with**
 345 **final sample numbers used for the present study (A-C).** (A) East Smithfield Black Death
 346 mass burial site from 1348-1349 (courtesy of the Museum of London); (B) Site locations for
 347 samples from London (top left, after ¹⁷ © Museum of London Archaeology) and from across
 348 Denmark (bottom right); (C) Top: populations size estimates for London for ~6 centuries (data
 349 taken from ^{15,18,19}; **Table S1**); Bottom: Site locations with associated date ranges, colored boxes
 350 indicate date range for samples, numbers in boxes indicate samples meeting all criteria for
 351 inclusion in final analyses (see main text and SI). Numbers in green star stem from East
 352 Smithfield.

353
 354
 355



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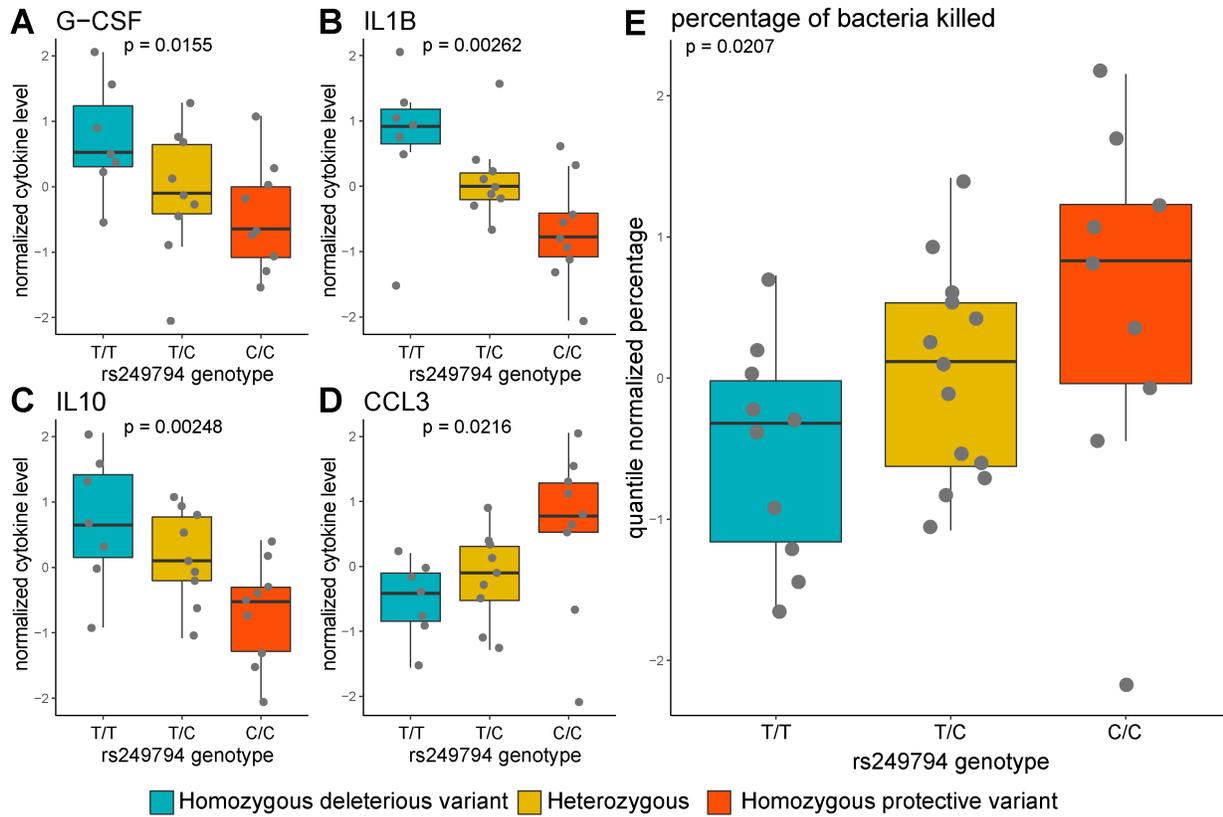
357 **Figure 2: Positive selection at immune loci.** (A-B) Enrichment of highly differentiated sites in
 358 functional regions relative to neutral regions when comparing the pre-Black Death (BD)
 359 population to the post-BD population in London (A) and Denmark (B). (C) F_{ST} between London
 360 before and after the Black Death, limited to the 535 sites which show qualitative patterns
 361 consistent with natural selection (namely allele frequency changes in the same direction in both
 362 London and Denmark after the Black Death, and the opposite direction for individuals who died
 363 during the Black Death, **Table S4**). Manhattan plot showing loci with patterns indicative of
 364 positive selection. Point size and color intensity (which alternates by chromosome) represents the
 365 $-\log_{10}$ p-value comparing populations in London before and after the plague, points colored in
 366 orange represent the 4 sites which are highly differentiated in Denmark as well. (D-G) Patterns
 367 of allelic change over time for the four strongest candidates for positive selection. Error bars
 368 represent the standard deviation based on bootstrapping individuals from that population and
 369 each time point 10,000 times. Allele frequencies for London are shown in red and for Denmark
 370 are shown in blue. Modern allele frequencies are derived from 1000 Genomes data for Great
 371 Britain in London²³.



372

373 **Figure 3. Positively selected loci are associated with changes in gene regulation upon *Y.***
 374 ***pestis* stimulation. (A)** Normalized log₂ fold-change response to incubation of primary
 375 macrophages for 4 hours with heat killed *Y. pestis* of genes within 100kb of candidate variants
 376 that are expressed in macrophages. Gray dot corresponds to the fold-change observed for each of
 377 the 33 individuals tested; red dots and bars represent the mean ± standard deviation. With the
 378 exception of *LNPEP*, all associations are significant (10% FDR). **(B-C)** Effect of rs1747384
 379 genotype upon *TICAM2* expression **(B)** and rs2549794 genotype upon *ERAP2* expression **(C)**,
 380 split by macrophages stimulated for 4 hours with heat killed *Y. pestis* and non-stimulated
 381 macrophages. Red dots and bars represent the mean ± standard deviation. **(D)** UMAP projection
 382 of single-cell RNA sequencing data of non-infected cells and cells infected with live *Y. pestis* for
 383 5 hours (strain CO92, at an MOI of 1:10), after integrating samples and colored by the major
 384 immune cell types. **(E)** Comparison of *ERAP2* expression among *Y. pestis*-infected and non-
 385 infected scRNA-sequencing data in individuals with homozygous rs2549794 genotypes. Color
 386 intensity reflects the level of *ERAP2* expression, standardized for unstimulated or infected cells.
 387 **(F)** Effects of rs2549794 genotype upon *ERAP2* expression, split by infected and non-infected
 388 conditions, for each cell type. *** p < 0.001; ** p < 0.01; * p < 0.05.

389



390

391 **Figure 4. *ERAP2* genotype is associated with cytokine response to *Y. pestis* stimulation. (A-**
 392 **D) Effect of genotype upon cytokine levels for granulocyte colony-stimulating factor (G-CSF),**
 393 **interleukin 1 β (IL1B), interleukin 10 (IL10), and C-C motif chemokine ligand 3 (CCL3).**
 394 **Remaining cytokines showed no significant effects and are included in Table S7. (E) Boxplots**
 395 **showing the quantile normalized values of the percentage of bacteria killed (y-axis) by**
 396 **macrophages infected for 24 hours macrophages as a function of *ERAP2* genotype (x-axis). The**
 397 **percentage of bacteria killed was calculated as the $CFU_{2h} - CFU_{24h} / CFU_{2h}$. The *p* value results**
 398 **from a linear model examining the association between *ERAP2* SNP genotypes (coded as the**
 399 **number of protective rs2549794 alleles) and the percentage of bacteria killed coded as the**
 400 **number of protective alleles found in each individual; 0, 1 or 2) and the percentage of bacteria**
 401 **killed. We reach similar conclusions to quantile normalization with either ranked CFU levels**
 402 **(i.e., the percentage of *Y. pestis* killed 24 hours post infection; $p = 0.0155$, $r = 0.424$) or using the**
 403 **Kendall-Theil Sen Siegel nonparametric linear regression ($p < 10^{-5}$) on the raw values.**

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533
 534 **Data Availability**

535 Hybridization capture data from the ancient individuals have been deposited in the NCBI
 536 Sequence Read Archive (SRA) under BioProject PRJNA798381. Expression data have been
 537 deposited into the NCBI Gene Expression Omnibus (GEO) under project GSE194118. Cytokine
 538 data is available in **Table S8**.

539
 540 **Code Availability**

541 Scripts for all data analyses are available at github.com/TaurVil/VilgalysKlunk_yersinia_pestis/.

542

543 **Acknowledgements**

544 We thank all members of the Barreiro lab and the Poinar lab for their constructive comments and
545 feedback. Computational resources were provided by the University of Chicago Research
546 Computing Center. Sequencing was performed at the Farncombe Sequencing Facility McMaster
547 University. We thank the Cytometry and Biomarkers platform at the Institut Pasteur for support
548 in conducting this study with a special thanks to Caroline Petidmange for help running the
549 Luminex assay. Finally, we thank Xinjun Zhang for assistance in simulating allele frequency
550 changes under neutral evolution. This work was supported by grant R01-GM134376 to LBB,
551 HP, and JP-C, a grant from the Wenner-Gren Foundation to JB (8702), and the UChicago
552 DDRCC, Center for Interdisciplinary Study of Inflammatory Intestinal Disorders (C-IID)
553 (NIDDK P30 DK042086). HNP was supported by an Insight Grant #20008499 from the Social
554 Sciences and Humanities Research Council of Canada (SSHRC) and The Canadian Institute for
555 Advanced Research under the Humans and the Microbiome program. TPV was supported by
556 NIH F32GM140568. We also thank the University of Chicago Genomics Facility
557 (RRID:SCR_019196), especially P Faber, for their assistance with RNA-sequencing. HP thanks
558 Debi Poinar for continued support and manuscript suggestions and editing.

559

560 **Author contributions**

561 LBB and HP directed the study. JK designed the enrichment assays and generated ancient
562 genomic data. TPV led all data and computational analyses, with contributions from J-CG. XC
563 performed all analyses to estimate selection coefficients under the supervision of MSt. JFB, RS. ,
564 and VY performed challenge experiments with macrophages and heat-killed *Y. Pestis*. MS and
565 ADu performed the infections experiments on PBMCs and generated the single cell RNA-
566 sequencing data, with assistance from DE and DM. CED and JP-C performed and designed the
567 infection experiments with live *Y. pestis* on macrophages and generated both cytokine and CFU
568 data, with assistance from JM and RB. RR, SND, JG. JLB provided access to samples,
569 archeological information, including dating, and other relevant information. AC and NV
570 provided insights into historical context. KE and GBG provided additional sampling and
571 bioinformatic processing and cluster maintenance. ADe and JMR provided insight on targeted
572 enrichment and modified versions of baits used for immune enrichment. GAR provided genomic
573 input on loci and contributed financially to the sequencing of targets. TPV, JK, HP, and LBB
574 wrote the manuscript, with input from all authors.

575

576 **Competing interest declaration**

577 JK, ADe, and J-MR declare financial interest in Daicel Arbor Biosciences, who provided the
578 myBaits hybridization capture kits for this work.

579

580 **Additional information**

581 Supplementary Information is available for this paper. Correspondence and requests for materials
582 should be addressed to lbarreiro@uchicago.edu and poinarh@mcmaster.ca.

583

SUPPLEMENTARY INFORMATION

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Black Death shaped the evolution of immune genes

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715 1. Supplementary Methods

716 a. Sampling locations and dates

717 The details of all remains used in this study, including burial locations and associated dates can
 718 be found in ²¹ as well as in the **Table S1**. For the purposes of our study, we split the samples
 719 from London into three time points: early or Pre-Black Death (~1000-1250), Black Death (1348-
 720 1350) and late or Post-Black Death (1350-1500) (**Table S1**). The London cemeteries are dated
 721 based on a combination of primary sources, Bayesian radiocarbon dating, and archaeological
 722 remains (primarily coins). We did the same for our Danish samples, as best we could from
 723 associated dates, which are less precise due to the lack of records associated with many of the
 724 cemeteries and the imprecision of the dating method based on arm position at burial used to date
 725 medieval burials in this region. We therefore group Danish samples into either an Early (~850-
 726 1350) or Late (1350-1800) period corresponding to before or after the Black Death period.

728 b. Ancient DNA sample processing, bait design, enrichment, sequencing and data 729 processing

730 i. Sample processing

731 All sample processing was performed in the specialized cleanroom facilities of the McMaster
 732 Ancient DNA Centre (McMaster University, Hamilton, Ontario). Individuals from three
 733 cemeteries in London, England (n=325), and five localities from Denmark (n=198) were
 734 included in this study (**Fig 1**). Both reagent blanks and carrier blanks (bacteriophage lambda
 735 DNA sheared to an average of 150 bp) were processed alongside the samples at 1:20 reagent
 736 blank and 3:20 carrier blank ratio. Subsamples of 10-300 mg of long bone, tooth (root or pulp
 737 cavity), or petrous portion of the temporal bone were pulverized manually. The resulting bone or
 738 tooth fragments and powder were demineralized and digested using a previously described
 739 protocol^{57,21}. The DNA was purified using either a modified phenol:chloroform:isoamyl alcohol
 740 (PCI) method or a large-volume guanidinium hydrochloride method designed to retain short
 741 DNA fragments^{57,58,59}. Samples were screened for the presence of single copy nuclear DNA
 742 using an 81bp quantitative PCR assay targeting a portion of the nuclear cMYC gene²² or a
 743 shortened 52 bp version of the assay²¹. Extracts of samples that returned positive results were
 744 prepared into double-stranded, double indexed Illumina-style sequencing libraries^{60,61} for
 745 downstream enrichment.

747 ii. Bait design

748 Three sets of baits were designed and used for the enrichment of i) neutral, ii) GWAS, and iii)
 749 exon targets (**Tables S2 & S3**).

- 750 1. **Neutral.** 250 independent (non-linked) and putative neutral loci were randomly
 751 selected from a larger set of “neutral loci” defined by Gronau and colleagues⁶².
 752 Briefly, these are regions outside any known protein-coding and conserved noncoding
 753 region of the genome, and with low intralocus recombination but sufficient between
 754 regions that the genealogies are largely uncorrelated. All bait regions were initially
 755 designed to target 1,500 bp, but some of the targets required shortening to avoid non-
 756 specific hybridization. The final neutral bait set included 16,062 probes, each 80bp in
 757 length tiled every 20 bp.

- 758 2. **GWAS.** A total of 496 GWAS variants associated with immune-related disorders
 759 (**Table S2**). Initially, the targets included 50 bp on either side of the variant of interest
 760 but were redesigned to include 80 bp on either side after experiments showed better
 761 capture success. The redesigned baits outperformed the old baits in target coverage
 762 breadth and depth by a factor of 3, at approximately 4.8x lower sequencing depth.
 763 Probes for both variant alleles were designed. The final GWAS bait set included
 764 5,800 probes, 80 bp in length tiled every 20 bp.
- 765 3. **Exons.** A total of 3,512 sequences, including all exons from 356 genes associated
 766 with immune function were included in the exon bait set. The genes were manually
 767 curated and chosen based on their known role in immune processes. 69% of all 399
 768 genes are associated with one or more of the following GO terms (“immune
 769 function”, “immune system process”, “Innate Immunity”, “adaptive immunity”,
 770 “cytokine”, “NF-kappaB”, and “interleukin”). The total number of genes targeted was
 771 constrained by the library volume remaining from these precious remains. Using the
 772 maximum size of existing bait sets as our threshold we fitted the 3,512 sequences,
 773 representing a total of 2,506,004 bp into a bait set of 93,180 probes. The probes were
 774 designed to be 60 bp in length and tiled every 20 bp.

775

776 **iii. Enrichment**

777 Samples were enriched using baits targeting the neutral and GWAS loci (75ng of each probe set,
 778 resulting in a final mass of 150ng of baits per reaction) according to the myBaits v3 protocol
 779 (Daicel Arbor Biosciences), with minor modifications to result in the recommended final
 780 concentrations at a final volume of 26 μ L instead of 30 μ L, allowing a maximum of 10.75 μ L
 781 template input instead of 7 μ L template input., and using 20 μ L of beads (pre- and post- wash)
 782 instead of 30 μ L pre-wash and 70 μ L post-wash. A volume of 5 μ L template input was used.
 783 During the removal of the supernatant post-incubation with the magnetic beads (2B.5 Bead
 784 Washing, step 1), the supernatant was saved, labeled as the non-enriched fraction (NEF), and
 785 frozen at -20°C. 25 μ L of the NEF was used as input to the new enrichment for the exon probes,
 786 with 150 ng baits, with incubations at 55°C. Samples were reamplified with the following
 787 conditions: Real-time PCR was performed in 10 μ L reactions consisting of: 1X KAPA SYBR®
 788 FAST qPCR Master Mix (2X), 150 nM of each primer (IS5_long_amp.P5:
 789 AATGATACGGCGACCACCGA and IS6_long_amp.P7: CAAGCAGAAGACGGCATAACGA),
 790 and 18.8 μ L template. The cycling conditions were: 5 minutes of initialization at 95°C, followed
 791 by 12 cycles of a 30 second denaturation at 95°C (30 sec) and a 45 second annealing/extension at
 792 60°C, followed by a final extension for 3 minutes at 60°C, and ending with a 1 minute hold at
 793 4°C. A second round of enrichment was performed with the modifications above. For libraries
 794 requiring a second exon enrichment, a new aliquot of library was indexed and 5 μ L was used as
 795 the input for capture using the protocol described above.

796

797 **iv. Pooling, size selection, and sequencing**

798 Sample concentration was quantified via qPCR in 0.001x dilutions against the 425bp- 525bp
 799 PhiX Control (Illumina) standard serially diluted from 1 nM to 62.5 fM. Real-time PCR was
 800 performed in 10 μ L reactions consisting of: 1X KAPA SYBR® FAST qPCR Master Mix (2X),

801 200 nM of each primer (IS5_long_amp.P5 and IS6_long_amp.P7, as above), 4 μ L template, and
 802 nuclease-free water. The cycling conditions were: 5 minutes of initialization at 95°C, followed
 803 by 35 cycles of a 30 second denaturation at 95°C (30 sec) and a 45 second annealing/extension at
 804 60°C, ending with a 30 second hold at 8°C.

805
 806 Samples were pooled at equimolar ratios. If the pool volume was greater than 12 μ L, the pool
 807 was concentrated over a Minelute PCR Purification column, with above modifications. Size
 808 selection was performed on a 3% NuSieve GTG Agarose Gel with a GeneRuler 50 bp ladder
 809 (Fermentas), run for 35 minutes at 100V, and then bands 150-500 bp were excised. Gel slices
 810 were purified using a Minelute Gel Extraction kit, with the above modifications. Pools were
 811 eluted in 20 μ L Qiagen Buffer EB. Pools were sequenced at the Farncombe Metagenomics
 812 Facility on the Illumina HiSeq 1500 platform, using V2 Rapid chemistry, with 2x90 paired end
 813 reads. Each sample received an average of 732,209 clusters (range: 3,549- 20,052,880).

814

815 **v. Data processing**

816 Adapters were trimmed and overlapping reads were merged using *leeHom* with aDNA specific
 817 settings⁶³. Reads were mapped to each target set separately using a modified version of *BWA*
 818 which allows for bam-to-bam mapping⁶⁴ (<https://github.com/udo-stenzel/network-aware-bwa>).
 819 PCR duplicates were removed with biohazard software ([https://github.com/udo-](https://github.com/udo-stenzel/biohazard)
 820 [stenzel/biohazard](https://github.com/udo-stenzel/biohazard)) and reads with quality 30 and length below 24bp were removed with
 821 *samtools*⁶⁵. Unique indexed libraries stemming from the same individual were merged using
 822 *samtools* and RG tags were standardized with a custom perl script.

823

824 Reads mapped strictly the targeted region of the genome produced excess heterozygosity,
 825 presumably because of slightly divergent sequences which result in off-target enrichment and are
 826 falsely interpreted as alternative alleles⁶⁶. To correct this, we remapped reads to the entire human
 827 genome (hg19) and only retained uniquely mapped reads. Resulting bam files were sorted and
 828 indexed using *samtools*⁶⁵. We then merged the exon, immune loci, and neutral loci enriched
 829 reads together, reasoning that off-target reads which overlap other regions of interest remain
 830 valuable and that sequencing from the same sample should share similar patterns of ancient DNA
 831 damage. Quality scores in each bam file were adjusted for DNA degradation using *mapDamage*
 832 *2.0*⁶⁷, and then trimmed of the first and last 4 bases using bamUtil (trimBam -L 4 -R 4)⁶⁸, where
 833 ancient DNA damage is typically concentrated.

834

835 Analysis of damage patterns was performed with *mapDamage 2.0* and plotted in *R* using the
 836 *ggplot2* package^{67,69}. No blanks yielded enough mappable sequences to be included in any
 837 downstream analyses. Our final dataset contained 33,110 biallelic, non-singleton variants within
 838 the targeted regions (2,669 near GWAS loci, 19,972 in immune genes, and 10,469 in neutral
 839 regions), with a mean coverage of 4.6x reads per site per individual (see **Table S1** for individual
 840 coverage). **While low relative to sequencing of modern genomes, 4.6x coverage is quite high**
 841 **compared published ancient genomes (for example, a large dataset of ancient genomes compiled**
 842 **across multiple studies has a median autosomal coverage of 2.2x with 164 of 804 individuals**
 843 **have greater than 4x coverage⁷⁰). Furthermore, to account for the uncertainty in genotype calls**
 844 **associated with low coverage data all our analyses used genotype likelihood (see point C.**
 845 **below), and we required our top candidates for selection to be replicated in both the London and**
 846 **Denmark ancient DNA cohorts.**

847

848 **c. Genotype calling**

849 Genotypes were called for each individual using GATK's Haplotype Caller in gVCF mode
 850 (version 4.1.4.1) with default setting and a minimum base quality of 20⁷¹. Genotype calls across
 851 individuals were then combined using the CombineGVCFs and GenotypeGVCFs functions, and
 852 filtered for biallelic sites with minQ ≥ 30 in regions of the genome which were targeted for
 853 enrichment (n=139,867 sites). We next excluded singleton variants (n=106,757) as they are more
 854 prone to reflect DNA damage or sequencing errors, resulting in a dataset of 33,110 biallelic, non-
 855 singleton variants within the targeted regions (2,669 near GWAS loci, 19,972 in immune exons,
 856 and 10,469 in neutral regions). We then excluded samples with missing genotype calls at more
 857 than 50% of those sites (retaining n=206 individuals) and filtered for sites with at least 10
 858 individuals per population and any given time point. Because of the low coverage intrinsic to
 859 ancient genomic studies, many genotype calls were based on a few reads. We therefore capture
 860 uncertainty in genotype calls by extracting the genotype likelihoods for each individual at each
 861 site using `filtbaboon1b` from *LCLAE*⁷². From these estimates, we calculated the expected number
 862 of alternate alleles as the likelihood the individual is heterozygous plus 2x the likelihood the
 863 individual is homozygous alternate. Based on these genotype likelihoods, we then calculated the
 864 minor allele frequency per population and time point, retaining only sites with a mean minor
 865 allele frequency between London and Denmark greater than 0.05 (n= 22,868 sites), as our power
 866 to detect selection for variants below 5% frequency is very low (**Fig S2**).

867

868 **d. Genetic differentiation following the Black Death**

869 We calculated F_{ST} for each variant as the loss in expected heterozygosity between time points
 870 relative to the expected heterozygosity of a panmictic population⁷³. Specifically, $F_{ST} =$
 871 $\frac{E_{het_{panmictic}} - \text{mean}(E_{het_{populations}})}{E_{het_{panmictic}}}$, where E_{het} (the expected heterozygosity) is calculated for each
 872 population-time point and a hypothetical panmictic population from the allele frequency
 873 assuming Hardy-Weinberg equilibria. Because high variance in estimating F_{ST} at low allele
 874 frequencies can lead to high false positive rates⁷⁴, we also filtered out variants with minor allele
 875 frequencies less than 10% (calculated as the mean across populations). After this, we compared
 876 3,293 sites in functional regions to 760 putatively neutral loci; functional loci showed an
 877 enrichment for high levels of differentiation compared to the neutral loci (**Fig 2A-B**).

878

879 We then asked whether sites in functional regions were more differentiated than expected based
 880 upon neutral loci. Specifically, we compared the F_{ST} values in functional regions to neutral loci,
 881 calculating a p-value for each site as the proportion of neutral loci with a more extreme F_{ST} . As
 882 these proportions were generally stable when controlling for minor allele frequency, we
 883 maximized the number of neutral sites to compare against by not further restricting the set of
 884 neutral loci based on difference in minor allele frequency from the tested variant. Enrichment in
 885 the Denmark cohort is smaller than in London, although still considerably higher than expected
 886 based on forward simulations (**section 1.e**). There are several reasons why the population in
 887 London is better suited to detect positive selection relative to those in Denmark: the dataset from
 888 London is ~50% larger (which directly impacts our ability to accurately estimate allele
 889 frequencies; hence, power), comes from a smaller geographic area, and from samples which
 890 were more accurately dated and occurred over a shorter period of time. Therefore, putatively
 891 neutral sites in Denmark show larger F_{ST} values before vs after the Black Death than those in

892 London (78% larger on average, paired t-test $p = 3.4 \times 10^{-10}$), reflecting increased population
 893 heterogeneity and/or our reduced ability to accurately estimate allele frequencies in Denmark
 894 given the small sample sizes. Thus, the reduced signal in Denmark (which nonetheless remains
 895 quite large: 1.6x the number of variants expected by chance exceed the 99th percentile of neutral
 896 loci) can likely reflect contributions of these technical and demographic differences.

897
 898 The strongest evidence of selection would come from candidate loci with concordant evidence of
 899 high differentiation across populations and time points. In particular, we required three
 900 conditions be met for a loci to be of interest: (i) individuals who survived the Black Death in
 901 both London and Denmark, have the same direction of allele-frequency change, (ii) within
 902 London, allele frequencies of Black Death survivors are shifted in the opposite direction of
 903 individuals who died during the plague (e.g. those buried in East Smithfield), and (iii) variants of
 904 interest are more differentiated than expected based on variation at neutral loci. To address point
 905 iii, we required sites be more differentiated than 95% of neutral loci in London before vs after
 906 the plague and be more differentiated than 90% of neutral loci in Denmark before vs after the
 907 plague.

908
 909 To ensure that widespread gene flow did not drive these signatures of selection, we proceeded
 910 with two additional analyses. We note that a population replacement would affect the totality of
 911 the genome and therefore the fact that our enrichments of highly-differentiated variation among
 912 immune loci is relative to putative neutral regions sequenced in the same exact samples should
 913 alleviate that concern. That said, we cannot exclude the possibility that the highly differentiated
 914 sites that we identified correspond to sites that were already unusually differentiated between the
 915 local population in and the one replacing it. Therefore, we first reasoned that if our highly
 916 differentiated loci in London when comparing pre- and post-Black Death samples were the result
 917 of a significant population replacement, those same loci should be amongst the most
 918 differentiated amongst contemporary European populations. We found no evidence that the 235
 919 variants we identify as outliers in London before vs after the plague are enriched for large F_{ST}
 920 values among European populations, relative to variants that were not highly differentiated. Out
 921 of all pairwise comparisons of the five European 1000 Genomes populations, the only evidence
 922 for elevated genetic differentiation at candidate loci was observed when contrasting between
 923 Iberian (IBS) and Tuscany (TSI) (t-test $p=0.0051$), which is an implausible populations pair to
 924 explain populations replacements in London in the 14th and 15th century (especially given that
 925 Great Britain is not even included in this pair, despite the fact that among our strongest candidate
 926 variants, post-plague allele frequencies in London are most similar to modern 1000 Genomes
 927 frequencies from Great Britains in London: mean difference of 1.8% vs 5.5-6.7% for other
 928 European populations).

929

930 e. Forward simulations of neutral evolution

931 Our results indicate an excess of highly diverged alleles near immune-related genes and GWAS
 932 variants relative to neutral regions of the genome. However, the genomic context of candidate
 933 sites differs from neutral regions in ways that may affect the variance in F_{ST} ^{75,76}. We therefore
 934 contrasted the recombination rate (derived from the DeCODE project⁷⁷) and the level of
 935 background selection (as measured by the B statistic⁷⁸) between the neutral regions and our
 936 targeted immune loci. Recombination rates were significantly different between neutral regions
 937 and candidate loci, with an overall trend for higher recombination rates around our candidate loci

938 ($p = 4.03 \times 10^{-9}$; **Fig S3A**). Levels of background selection were also significantly higher around
 939 immune loci as compared to putative neutral regions ($p \approx 0$; **Fig S3B**). Importantly, neither
 940 recombination rate nor background selection was enriched for candidates of positive selection
 941 relative to other tested loci in exonic regions or near GWAS hits ($p > 0.5$, **Fig S3A-B**).

942
 943 Nevertheless, since recombination rate and background selection can affect the variance in F_{ST}
 944 (albeit in opposing directions^{79,80}), we used forward simulations to confirm that the differences in
 945 genetic differentiation observed between neutral and candidate regions are unlikely under neutral
 946 evolution. Specifically, we used SLiM^{75,76} to simulate the neutral evolution of full genomes in a
 947 population of 10,000 individuals while controlling for recombination rates (estimated by the
 948 DeCODE project⁷⁷) and negative selection against mutations in coding regions (defined using the
 949 UCSC table of canonical genes⁸¹). Deleterious mutations were introduced at a rate of 7.4×10^{-8}
 950 mutations per base pair per generation, and an exponential distribution of fitness effects with a
 951 mean deleterious effect size of 2.5×10^{-3} (inferred by⁷⁸). We simulated 1,000 generations under
 952 this model, allowing for neutral and deleterious variation to accumulate and stabilize, before we
 953 simulated our data collection surrounding the Black Death. At 1,000 generations, we extracted
 954 “pre-BD” allele frequencies for variants within 5kb of genomic regions targeted in this study.
 955 Assuming a generation time of approximately 30 years, we simulated an additional 8 generations
 956 before the BD event (equivalent to the mean number of generations of our pre-plague London
 957 samples), the BD which led to 50% mortality in the population, and a further 7 generations after
 958 the BD event (equivalent to the mean age for our post-plague London samples), at which point
 959 we sampled “post-BD” allele frequencies.

960
 961 As in our main analyses, we then asked whether sites near candidate regions were more strongly
 962 differentiated before vs after the plague than those of neutral regions. We detected a slight
 963 enrichment of highly differentiated sites that was much smaller than those observed for our
 964 empirical data (**Fig S3C-D**), suggesting background selection and recombination are insufficient
 965 to explain the enrichment of highly differentiated sites we observe. Importantly, these results are
 966 consistent across a range of parameter values including population sizes up to 50,000 (the
 967 approximate census population in London at the beginning of the Black Death) and mortality
 968 rates from 30 to 50%.

969

970 f. Gene regulatory response to *Y. pestis*

971 **i. Isolation of monocytes, differentiation of macrophages, and challenge with heat-killed *Y.***
 972 ***pestis***

973 This study has been approved by the Institutional Review Board at the University of Chicago
 974 (protocol #: IRB19-0432). All Buffy coats from 33 healthy donors were obtained from the
 975 Indiana Blood Center (Indianapolis, IN, USA). All individuals recruited in this study were males,
 976 self-identified as African-American (AA) (n = 17) or European-American (EA) (n = 16) between
 977 the age of 18 and 55 years old. Only individuals self-reported as currently healthy and not under
 978 medication were included in the study. In addition, each donor’s blood was tested for Hepatitis
 979 B, Hepatitis C, Human Immunodeficiency Virus (HIV), and West Nile Virus, and only samples
 980 negative for all of the tested pathogens were used.

981

982 Blood mononuclear cells were isolated by density gradient centrifugation (Ficoll-Paque
 983 Premium, Sigma Aldrich, St. Louis, MI, USA). Monocytes were purified from peripheral blood

984 mononuclear cells by positive selection with magnetic CD14 MicroBeads (Miltenyi Biotech,
 985 Bergisch Gladbach, Germany) using the autoMACS Pro Separator. The purity of the isolated
 986 monocytes was verified using an antibody against CD14 (BD Biosciences) and only samples
 987 showing > 90% purity were used to differentiate into macrophages. Monocytes were then
 988 cultured for 7 days in RPMI-1640 (Fisher) supplemented with 10% heat-inactivated FBS (FBS
 989 premium, US origin, Wisent), L-glutamine (Fisher) and M-CSF (20ng/mL; R&D systems). Cell
 990 cultures were fed every 2 days with complete medium supplemented with the cytokines
 991 previously mentioned. Before inoculation with *Y. pestis*, we checked the
 992 differentiation/activation status of the monocyte-derived macrophages by flow cytometry, using
 993 antibodies against CD1a, CD14, CD83, and HLA-DR (BD Biosciences). All samples presented
 994 the expected phenotype for non-activated macrophages (CD1a+, CD14+, CD83, and HLA-
 995 DRlow). Macrophages were stimulated for 4 hours with lysate of heat-killed *Y. pestis* (strain
 996 CO92) at a value of 10 colony forming units (CFUs) per cell, or with endotoxin-free water
 997 (negative control).
 998

999 **ii. RNA Extraction, Library Preparation, and Sequencing**

1000 Total RNA was extracted from the non-stimulated and stimulated macrophages using the
 1001 miRNeasy kit (QIAGEN). RNA quantity was evaluated spectrophotometrically, and the quality
 1002 was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with no
 1003 evidence for RNA degradation (RNA integrity number > 8) were kept for further experiments.
 1004 RNA-sequencing libraries were prepared using the Illumina TruSeq protocol. Once prepared,
 1005 indexed cDNA libraries were pooled (6 or 7 libraries per pool) in equimolar amounts and were
 1006 sequenced with single-end 100bp reads on an Illumina HiSeq2500.
 1007

1008 Adaptor sequences and low quality score bases (Phred score < 20) were first trimmed using Trim
 1009 Galore (version 0.2.7). The resulting reads were then mapped to the human genome reference
 1010 sequence (Ensembl GRCh37 release 75) using STAR (2.4.1d)⁸² with a hg19 transcript annotation
 1011 GTF file downloaded from Ensembl. Gene-level expression estimates were calculated using
 1012 RSEM (version 1.2.21)⁸³ with default parameters.
 1013

1014 After removing lowly expressed genes (mean counts per million < 1 in both stimulated and null
 1015 conditions), we normalized expression data using voom and analyzed 5,874 genes using the R
 1016 package *limma* (v3.48.3). We first tested for an effect of stimulation with *Y. pestis* while
 1017 controlling for individual identity for an effect of stimulation on expression level, while
 1018 controlling for individual identity. We then calculated a permutation-based FDR, comparing our
 1019 observed results to permuted results observed from randomizing which sample was stimulated
 1020 within each individual (repeated 25 times). We extracted expressed genes within 100kb of our
 1021 candidate loci for positive selection, retaining *ERAP1*, *ERAP2*, *LNPEP*, *TICAM2*, *TMED7*,
 1022 *CTLA4*, and *ICOS*. We then used previously published genotype data for these individuals³⁰ to
 1023 test whether genotype at our candidate variants was associated with expression level using a
 1024 linear model. Finally, we tested for an interaction effect between stimulation and genotype.
 1025 Despite several trends which appear to resemble interaction effects, our sample was
 1026 underpowered to identify any significant interactions. We applied an identical computational
 1027 pipeline to test for effects of stimulation and genotype in previously published datasets using
 1028 different bacterial and viral pathogens and ligands^{30,31}.
 1029

1030 **iii. Comparison with Quach et al. and Nedelec et al. data**

1031 To evaluate if the differences in gene expression identified among our candidate loci were
 1032 specific to flu or shared in response to other pathogens we re-analyzed existing datasets: Nedelec
 1033 and colleagues³⁰ measured the gene expression response of monocyte-derived macrophages to
 1034 infection with two live intracellular bacteria: *Listeria monocytogenes* (a Gram-positive
 1035 bacterium) and *Salmonella typhimurium* (a Gram-negative bacterium), using the same
 1036 experimental design and time point as the current study. Quach and colleagues³¹ characterized
 1037 the transcriptional response of primary monocytes to bacterial and viral ligands activating Toll-
 1038 like receptor pathways (TLR1/2, TLR4, and TLR7/8) and live influenza virus 6 hours after
 1039 stimulation. Processing of the RNAseq data from these datasets and analysis was done using the
 1040 same methods described above in section ii.

1041

1042 **iv. Infection of macrophages with live *Y. pestis*: cytokine and gentamycin protection assays**

1043 In addition to the stimulation of macrophages with heat-killed *Y. pestis* (section 1.f.i above), we
 1044 also performed a separate set of infections with live *Y. pestis* of a set monocyte-derived
 1045 macrophages derived from 33 healthy anonymous donors (Etablissement Français du Sang). This
 1046 experiment was specifically designed to (i) evaluate if the differences in gene expression
 1047 observed in response to heat-killed *Y. pestis* were similar to those observed in response to live *Y.*
 1048 *pestis* (this was done on a subset of 8 individuals); and (ii) evaluate if the *ERAP2* protective
 1049 allele impacted cytokine secretion levels or intracellular bacteria levels. DNA from 200µl of
 1050 blood was extracted using PureLink Genomic DNA kits (Invitrogen, Waltham, MA, USA) and
 1051 allelic discrimination at the rs2248374 SNP was determined by TaqMan qPCR with Applied
 1052 Biosystems Human *ERAP2* probes. CD14+ monocytes were isolated and were differentiated into
 1053 macrophages as described above. Fully virulent *Y. pestis* (strain CO92) was grown on LB Agar
 1054 supplemented with 0.0002% porcine hemin and cells (25×10^4 /well) were infected with live
 1055 bacteria at a multiplicity of infection of 10 bacteria/cell. Supernatants (for cytokine profiles) or
 1056 cells (for RNA-sequencing) were collected after 5 hours of incubation without washing cells, or
 1057 cells were washed with PBS after 1h and incubated in fresh medium for a further 23 hours in the
 1058 presence of gentamycin (40µg/ml). Samples were sterilized by centrifugation over 0.22µm Spin-
 1059 X filters (Costar) before deep-freezing. Levels of ten cytokines (IL-1β, IL-1RA, TNFα, IL-6, IL-
 1060 10, G-CSF, GM-CSF, CCL2/MCP-1, CCL3/MIP-1α, CXCL8/IL-8) were determined using
 1061 Magnetic Luminex performance assays from R&DSYSTEMS with Curiox DropArray plates and
 1062 LT washing station for 25 individuals. Data were acquired on a Luminex LX-200 reader. For 8
 1063 individuals, we performed RNA-sequencing on infected and non-infected cells. Sequencing data
 1064 were mapped and analyzed for an effect of *Y. pestis* stimulation as described above in **section**

1065 **1.f.ii.**

1066

1067 For all 33 individuals, macrophages (6 biological replicates per condition) were infected with *Y.*
 1068 *pestis* (MOI of 10 bacteria/cell) with gentle centrifugation (300xG, 5 min) to enhance contacts.
 1069 After one hour, samples were washed twice with PBS, and new medium containing Gentamycin
 1070 (40 µg/ml) was added to kill extracellular bacteria. Then, another hour or 24h, macrophages were
 1071 washed twice with PBS, lysed with sterile water and scratched from plastic. The lysate was
 1072 serially diluted, and samples immediately cultured at 28°C on LB agar + 0.002% Hemin to count
 1073 the number of colony forming units (CFU) of bacteria. Intracellular bacterial killing after 24h
 1074 was calculated as $100 \times (\text{CFU at 2h} - \text{CFU at 24h}) / (\text{CFU at 2h})$. This strategy is the most used
 1075 strategy to analyze intracellular survival or killing of bacteria inside macrophages⁸⁴. For both

1076 cytokine and CFU data, we quantile-normalized the data across individuals to minimize the risk
 1077 of spurious association due to outliers, **and to ensure that the data was normally distributed**. We
 1078 then examined the association between SNP genotypes (coded as the number of protective “C”
 1079 alleles at rs2549794: 0, 1, or 2) and cytokine or CFU levels by using a linear regression model in
 1080 which phenotype was regressed against genotype. This model returns a single p value that
 1081 reflects the relationship between genotype (i.e., the number of protective “C” alleles) and
 1082 cytokine/CFU levels.

1083

1084 **v. Single-cell RNA-sequencing of PBMCs before and after infection with *Y. pestis*.**

1085 *Experimental design*

1086 To consider the response to *Y. pestis* infection across a broader array of immune cell types, we
 1087 also conducted single-cell RNA-sequencing of peripheral blood mononuclear cells (PBMCs) for
 1088 10 individuals of European ancestry, five of whom were homozygous for the reference allele at
 1089 rs2549754 and five of whom were homozygous for the alternate allele. These individuals were
 1090 previously used to study the response to viral infections and the full details of sample providence
 1091 can be found in previous work⁸⁵. Briefly, samples were obtained from BioIVT along with a
 1092 signed, written consent from healthy participants at a collection site in Miami, Florida (United
 1093 States) utilizing a standard protocol with a sodium heparin anticoagulant. PBMCs were extracted
 1094 from whole blood using a density gradient, washed with HBSS, reconstituted in CryoStor CS10
 1095 to a concentration of 10 million cells/mL, and cryopreserved in sets of 5-10 million cells a vial.

1096

1097 PBMCs were thawed approximately 14 hours prior to infection and cultured overnight in RPMI
 1098 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 μ g/mL
 1099 gentamycin. Infection experiments were balanced for individual genotypes. The morning of the
 1100 experiment 10 individuals were collected, counted, plated at 1 million cells per ml per well, and
 1101 infected with *Y. pestis* (strain CO92), at an MOI of ten. Following 5 hours of infection, cells
 1102 were collected, washed, and prepared for single-cell capture using the 10X workflow.
 1103 Multiplexed cell pools of 6 samples were used as input for the single cell captures, and for each
 1104 pool 10,000 cells were targeted using the Chromium Single Cell 3' Reagent (v3 chemistry) kit
 1105 (10X Genomics #CG000183 Rev A). Post Gel Bead-in-Emulsion (GEM) generation, the reverse
 1106 transcription (RT) reaction was performed in a thermal cycler as described (53°C for 45 min,
 1107 85°C 537 for 5 min), and post-RT products were temporarily stored at -20°C. After all captures
 1108 were complete, post-RT reaction cleanup, cDNA amplification, and sequencing library
 1109 preparation were performed as described in the Single Cell 3' Reagents Kits v3 User Guide (10X
 1110 Genomics #CG000183 Rev A). Briefly, cDNA was cleaned with DynaBeads MyOne SILANE
 1111 beads (ThermoFisher Scientific) and amplified in a thermal cycler using the following program:
 1112 98°C for 3 min, 11 cycles x 98°C for 15 s, 63°C for 20 s, 72°C for 1 min, and 72°C 1 min. After
 1113 cleanup with the SPRIselect reagent kit (Beckman Coulter), the libraries were constructed by
 1114 performing the following steps: fragmentation, end-repair, A-tailing, SPRIselect cleanup, adaptor
 1115 ligation, SPRIselect cleanup, sample index PCR (98°C for 45 s, 13 or 14 cycles x 98°C for 20 s,
 1116 54°C for 30 s, 72°C for 20 s, and 72°C 1 min), and SPRIselect size selection. Libraries were
 1117 sequenced 100 base pair paired-end (R1: 30 cycles, I1: 10 cycles, R2: 85 cycles) on an Illumina
 1118 NovaSeq. Cell pools contained infected or uninfected samples, to limit the possibility for cross-
 1119 contamination.

1120

1121 *Mapping, demultiplexing, cell filtering, and UMAP analysis*

1122 FASTQ files were mapped to a custom reference containing GRCh38 and the *Y. pestis* reference
 1123 genome (downloaded from NCBI, created using cellranger mkref) using the cellranger (v3.0.2)
 1124 (10X Genomics) count function. Souporecell (v2.0, Singularity v3.4.0)⁸⁶ in --skip_remap mode
 1125 was used to demultiplex cells based on genotypes from a common variants file (1000GP samples
 1126 filtered to SNPs with $\geq 2\%$ allele frequency in the population, downloaded from
 1127 <https://github.com/wheaton5/souporcell>). Briefly, souporcell clusters cells based on cell allele
 1128 counts in common variants, assigning all cells with similar allele counts to a single cluster
 1129 corresponding to one individual, while also estimating singlet/doublet/negative status for that
 1130 cell. Hierarchical clustering of the true genotypes known for each individual (obtained from low-
 1131 pass whole-genome-sequencing) and the cluster genotypes estimated from souporcell was used
 1132 to assign individual IDs to souporcell cell clusters. All 10 individuals were successfully assigned
 1133 to a single cluster.

1134

1135 After demultiplexing cells into samples, Seurat (v4.0.4, R v4.1.1)⁸⁷ was used to perform quality
 1136 control filtering of cells. Cells were considered “high quality” and retained for downstream
 1137 analysis if they had: 1) a “singlet” status called by souporcell, 2) between 200 – 2500 genes
 1138 detected (nFeature_RNA), and 3) a mitochondrial reads percentage $< 20\%$. In total, we captured
 1139 14,174 high quality cells (range of cells per individual: 780 to 2030).

1140

1141 We split the cells by infection status (uninfected or *Y. pestis*) and individual, then ran
 1142 SCTransform (Seurat v4.0.4) to normalize and scale the UMI counts within condition. In this
 1143 step, we simultaneously regressed out variables corresponding to experiment batch and percent
 1144 mitochondrial reads per cell. Cells were assigned to cell types using Seurat’s Multimodal
 1145 Reference Mapping workflow. Briefly, we applied SCTransform, FindTransferAnchors, and
 1146 MaqQuery to assign each cell a predicted cell type based on published data from PBMCs⁸⁸. Data
 1147 were then integrated on infection status and individual using reciprocal PCA on SCTransformed
 1148 data (https://satijalab.org/seurat/articles/integration_rpca.html) prior to dimensionality reduction
 1149 (RunUMAP in Seurat, with the first 20 dimensions). Reassuringly, predicted cell types reflected
 1150 major structure in the UMAP plot.

1151

1152 *Differential expression analysis*

1153 To model expression data for each cell type, we converted raw counts per cell into pseudobulk
 1154 mean counts for each cell type and sample (as in ⁸⁵). Within each cell type for each sample, raw
 1155 counts were summed across all cells assigned to that cell type and sample for each gene using the
 1156 sparse_Sums function in textTinyR (v1.1.3)

1157 (<https://cran.rproject.org/web/packages/textTinyR/textTinyR.pdf>), yielding an $n \times m$ expression
 1158 matrix, where n is the number of samples ($n = 20$) and m is the number of genes detected. For
 1159 each cell type, we removed lowly expressed genes ($\text{cpm} < 1$) and then voom-normalized the
 1160 count data to control for library. Using lmFit (R *limma* package v3.48.3), we modeled effects of
 1161 stimulation and an effect of rs2549754 genotype within each condition, while controlling for the
 1162 number of cells. False discovery rates were estimated using the q-value approach⁸⁹ relative to an
 1163 empirical null distribution constructed by randomly assigning which sample condition within
 1164 each individual and by randomizing genotypes between individuals ($n=10$ iterations). Results for
 1165 stimulation of each gene and each cell type are available on in **Table S6**. **Finally, we find no**
 1166 **evidence for widespread *trans*-effects of *ERAP2* haplotype on the gene expression levels of other**

1167 genes across the genome after correction for multiple testing. However, we urge caution in
 1168 interpreting this results as this particular data set is severely underpowered to identify interaction
 1169 effects of this type. Moreover, we are only able to capture a snapshot of the immune response *in*
 1170 *vitro* (i.e., measures of gene expression 5 hours post-infection). It is possible that differential
 1171 gene regulation between the protective and deleterious haplotypes may occur at later time points,
 1172 or in an *in vivo* setting where many different cell types interact with each other.

1173
 1174 g. Hidden-Markov Model-based inference of selection coefficients
 1175 We analyzed the four SNPs identified in the main text (rs2549794, rs17473484, rs1052025, and
 1176 rs11571319) further to quantify the evolutionary advantage that the respective alleles conferred.
 1177 To this end, we aimed to infer a selection coefficient from the temporal genetic variation at each
 1178 of these SNPs. Assuming the allelic effects are additive, we define s (the selection coefficient) as
 1179 the expected number of offspring of an individual with genotype aa , Aa , or AA is 1 , $1 + s/2$, or 1
 1180 $+ s$, respectively (where a is the ancestral allele and A is the derived variant).

1181
 1182 To infer this selection coefficient s from the temporal data, we used a common Hidden Markov
 1183 Model (HMM) framework²⁴. Briefly, in this framework, the underlying population allele
 1184 frequency is treated as the hidden state, and the observed genetic variation at the respective times
 1185 present the emissions, which are binomially distributed, given the underlying allele frequency.
 1186 To compute the likelihood of the data, the framework integrates over all possible hidden allele
 1187 frequency trajectories under the Wright-Fisher model. Since the selection coefficient s affects the
 1188 likelihood of these trajectories (larger s makes an increase in frequency more likely), this allows
 1189 us to infer s using a maximum likelihood approach. Here, we use an implementation of this
 1190 HMM framework available at https://github.com/steinrue/diplo_locus which allows efficient
 1191 computation of the likelihood by discretizing the allele frequency space and using a Gaussian
 1192 approximation to compute the transition probabilities of the Wright-Fisher model⁹⁰.

1193
 1194 With this implementation, we computed joint likelihoods of observing samples in both
 1195 populations across time, which yields maximum-likelihood point estimates of s . In addition, we
 1196 applied this implementation to simulated data to provide confidence intervals and assess the
 1197 statistical power of our inference scheme. Because the sampling times across both populations
 1198 span no more than 20 generations, we set the mutation rate to zero for convenience. All scripts to
 1199 perform the analyses are available at
 1200 [https://github.com/TaurVil/VilgalysKlunk_yersinia_pestis/tree/main/part1_aDNA/infer_selection](https://github.com/TaurVil/VilgalysKlunk_yersinia_pestis/tree/main/part1_aDNA/infer_selection_coefficients)
 1201 [coefficients](https://github.com/TaurVil/VilgalysKlunk_yersinia_pestis/tree/main/part1_aDNA/infer_selection_coefficients).

1203 **i. Maximum likelihood estimation of selection coefficients from data**

1204 We follow the analysis in the main text and group the samples into before, during, and after the
 1205 Black Death pandemic. We use the expected derived allele frequency in each group for each
 1206 candidate SNP, computed as $\mathcal{P}_{Aa} + 2\mathcal{P}_{AA}$ for each individual and the mean across individuals in
 1207 each group, where \mathcal{P} is the genotype likelihood per individual inferred in section 1.c.

1208
 1209 For samples from London (blue lines and boxes in **Fig S10**), we assume an effective population
 1210 size of 5,000 and that the individuals before, during, and after the pandemic are sampled in
 1211 generation 1, 9, and 16, respectively. Considering that multiple generations of people have been
 1212 impacted by the circulating disease, we assume selection acted during generation 6, 7, and 8, the

1213 three generations preceding the mid-pandemic sample. Because the mid-pandemic samples in
 1214 London are known to be from individuals who died from the Black Death, we assume that the
 1215 lineages leading to mid-pandemic samples experience opposite selective pressure from those
 1216 who survived the pandemic (the post-pandemic samples).

1217
 1218 For samples from Denmark (brown lines and orange boxes in **Fig S10**), we only consider
 1219 samples taken before and after the pandemic. We assume an effective population size of 5,000,
 1220 that the samples were taken at generation 0 and 17, and that selection occurred during generation
 1221 6, 7, and 8 (same as in London). The generation times were chosen to represent approximate
 1222 mid-points of the pre- and post-Black Death samples. For each candidate SNP, we computed log-
 1223 likelihoods on a grid of s values in the interval $[-0.9, 0.9]$, with a higher density of grid-points
 1224 around zero, for three different scenarios (see **Fig S10**, straight lines in the lower half): before-to-
 1225 during in London, before-to-after in London, and before-to-after in Denmark. To reduce the
 1226 number of free parameters, we assumed the selection coefficient s in all three scenarios has the
 1227 same absolute value, but the coefficient for before-to-during in London has inverted sign. The
 1228 rationale for this is that the samples before-to-after represent the survivors, whereas before-to-
 1229 during samples in London represent the ones who did not survive. The final composite log-
 1230 likelihood for each locus is then obtained by summing the log-likelihoods from the three
 1231 scenarios.

1232
 1233 To estimate \hat{s}_{MLE} , we interpolate the composite log-likelihood at each locus as a function of s
 1234 using cubic splines (with the `scipy.interpolate` module in Python3), and infer the maximum in the
 1235 $[-0.9, 0.9]$ interval (with the `scipy.optimize` module) from this interpolated function. The log-
 1236 likelihood surfaces and the resulting maximum-likelihood estimates (MLEs), \hat{s}_{MLE} , are depicted
 1237 in **Fig S11** for the four SNPs. We also performed the analysis with an effective population size of
 1238 10,000 in both populations, but the results did not change substantially.

1239

1240 **ii. Simulation study to assess confidence in selection coefficients**

1241 To assess the statistical properties of our estimation procedure, we simulated allele frequency
 1242 trajectories under the Wright-Fisher model, and simulated samples at the respective times given
 1243 the underlying allele frequency. In the simulations of the London population, the three sampling
 1244 times are set to be generation 1, 9, and 16. In simulations for Denmark, sampling times are set as
 1245 generations 0 and 17. As sample sizes, we used the sample sizes in the empirical data at the
 1246 respective SNPs. The effective population sizes for London and Denmark populations are set to
 1247 be 5,000, and the mutation rate is set to zero. As in the likelihood-computations, selection was
 1248 assumed to act in generations 6, 7, and 8.

1249

1250 To reflect that the mid-pandemic samples from London were taken from people who died of the
 1251 disease, we simulate this set of samples by assuming a parallel population where the allele
 1252 frequency experiences reverse changes during the pandemic. In particular, for each replicate, we
 1253 track the allele frequency throughout the period of selection (pandemic), take the logit-
 1254 transformed difference between the start and end, and deduct it from the logit-transformed pre-
 1255 pandemic frequency. The use of logit here ensures that all values are bounded within $(0, 1)$. This
 1256 then gives the frequency in the parallel population who died of Black Death, from which mid-
 1257 pandemic samples are generated. To reflect uncertainty of the initial frequency in the empirical
 1258 data, at each of the four target SNPs, we re-sample each pool of pre-pandemic samples with

1259 replacement and use the resulting allele frequencies as the initial frequencies for the simulated
 1260 replicates.

1261
 1262 We simulate 5,000 replicates for a grid of s -values ranging from -0.35 to 0.5 with increments of
 1263 0.05, and then applied the same procedure as described in the previous section to each replicate
 1264 to obtain \hat{s}_{MLE} . **Fig S12** shows the empirical distribution of these estimates, highlighting the
 1265 mean, median, and 2.5-th and 97.5-th percentiles. We observed a slight bias, albeit very small, in
 1266 the estimates. Thus, we performed an empirical bootstrap bias-correction of the estimates as
 1267 follows: We interpolate the values of the median of the empirical \hat{s}_{MLE} distributions as functions
 1268 of true s value simulated. With these interpolated functions, we use the \hat{s}_{MLE} directly computed
 1269 from the likelihood for the four SNPs to identify which underlying value of s would result in such
 1270 an estimate. We denote the latter bootstrap corrected MLE by \tilde{s}_{MLE} .

1271
 1272 To provide confidence intervals, for each of the four SNPs, we simulated 5,000 replicates using
 1273 the bootstrap-corrected \tilde{s}_{MLE} as the underlying true selection coefficient and re-sampled initial
 1274 frequencies, as before. We then adjust the MLEs from all replicates by the same amount that
 1275 \hat{s}_{MLE} was adjusted to obtain \tilde{s}_{MLE} , for each SNP. After this adjustment, we report the 2.5-th and
 1276 97.5-th percentiles of the empirical distribution of the MLEs as confidence intervals for our
 1277 estimates in **Fig S4A** and **Table S5**. We also simulated sets of 5,000 replicates with the same
 1278 initial conditions but under $s = 0$, applied the same inference procedure to assess the power, and
 1279 report Receiver Operating Characteristics (ROC) curves and Precision-Recall curves in **Fig S4B-**
 1280 **C**. Note that the power is affected by the selection coefficient and the initial frequencies, and
 1281 thus varies between the SNPs.

1282
 1283 To provide further evidence for selection acting at the four focal SNPs, we applied the same
 1284 inference procedure that was used to obtain \hat{s}_{MLE} for the focal SNPs to all SNPs in the neutral
 1285 regions (described in **section 1.b**), since these reflect the empirical neutral distribution. For each
 1286 SNP, we then computed the log-likelihood-ratio by subtracting the log-likelihood at $s = 0$ from
 1287 value of the log-likelihood at \hat{s}_{MLE} . We report the percentile of the log-likelihood-ratio for the
 1288 four focal SNPs in this empirical neutral distribution in **Table S5**, with the rationale that low
 1289 percentiles indicate strong evidence that non-neutral processes were driving the allele frequency
 1290 change at the respective SNP. We note for neutral SNPs where the ancestral allele is unknown,
 1291 we polarized for the reference allele. Since this only changes the sign of the estimated s , the
 1292 polarization does not affect the value of the log-likelihood-ratio.

1293
 1294 Last, we note that $1 + s$ yields the expected number of viable offspring of an individual that is
 1295 homozygous for the derived allele at the respective locus. Thus, we can loosely interpret $1 + s$
 1296 as reflecting the chance of an individual to survive the Black Death pandemic. With this
 1297 interpretation, we can quantify the increase in chance of survival of individuals homozygous for
 1298 the derived allele over individuals homozygous for to ancestral allele by $p_{surv} := (1 + s)/1$.

1300 h. Supplementary References

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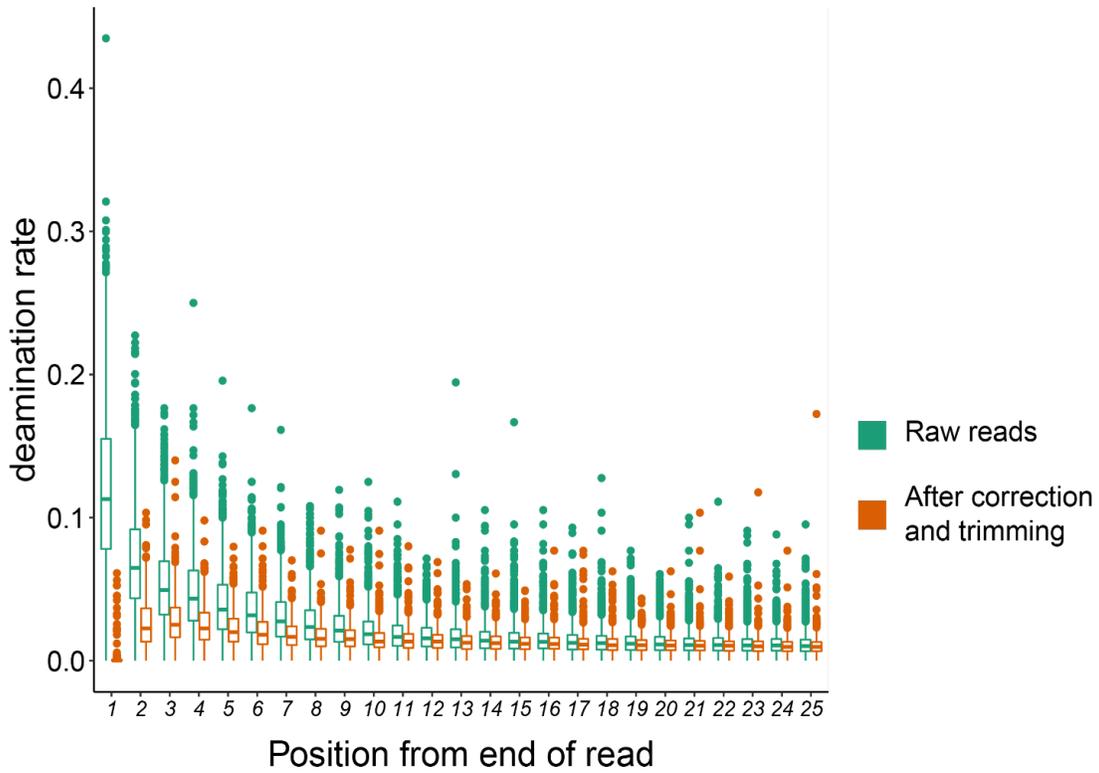
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1376 2. Supplementary Figures

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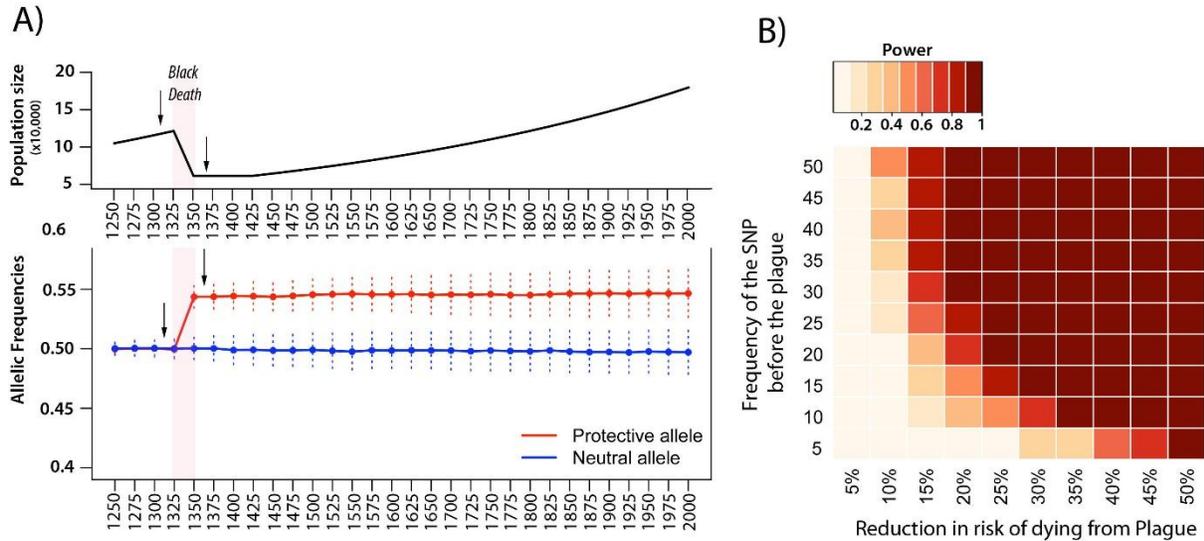


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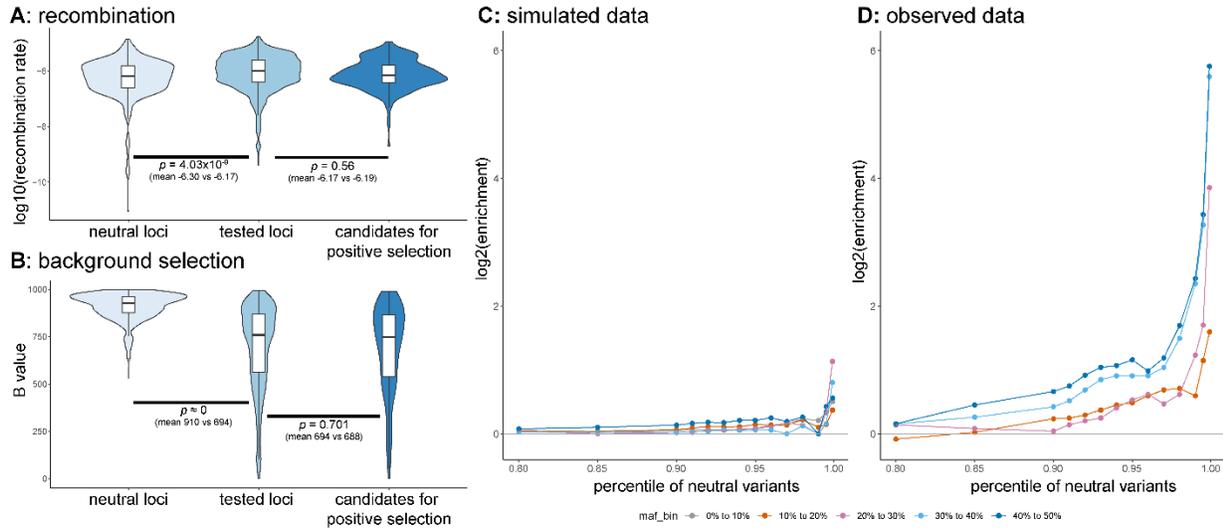
1381 **Figure S1: Correcting for ancient genomic damage.** Damage and degradation in ancient DNA samples
1382 result in an increased deamination rate near the end of reads (green). We correct for this source of DNA
1383 damage by using *mapDamage*⁵⁹ and trimming the terminal 4 bases of each read, which eliminates the bias due
1384 to DNA damage (orange). After correcting and trimming reads, error rates are lower for the first base pair
1385 because *mapDamage* is more liberal in masking possible DNA damage near the ends of reads.

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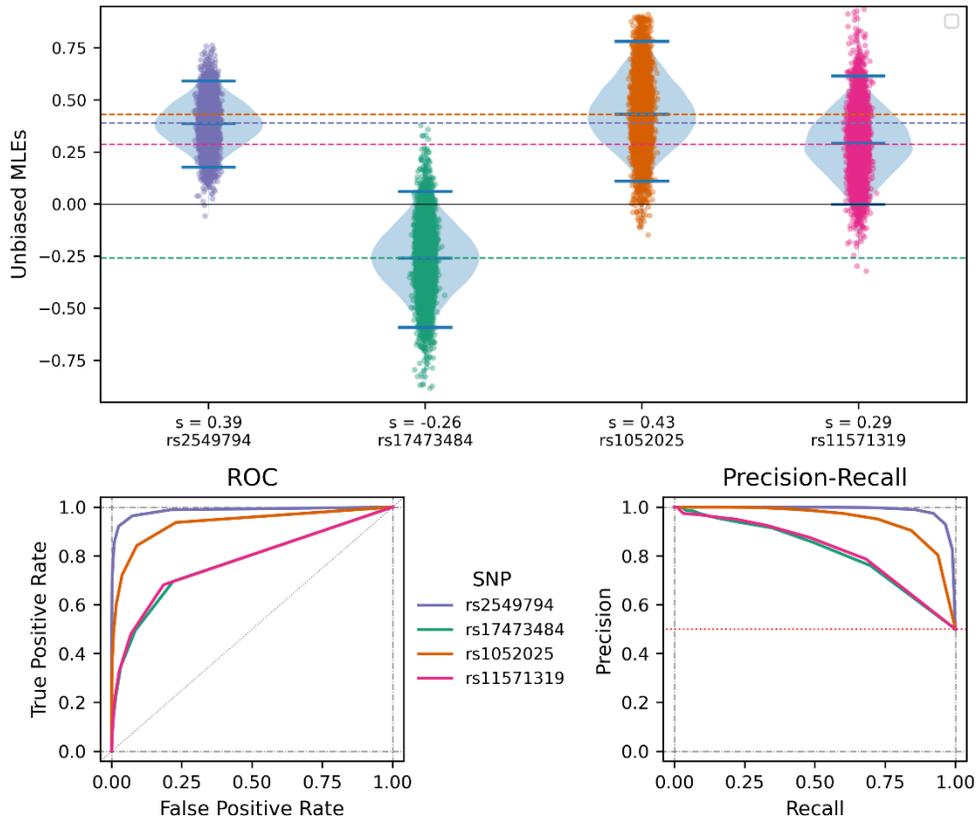
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Figure S2: Power to identify protective alleles against Black Death. (A) Simulations of the expected changes in allele frequencies across generations for a protective allele (red) that decreases the risk of mortality from plague by 30% in homozygous individuals and 15% in heterozygous individuals (i.e., additive effect) as compared to a neutral allele (blue), with a starting allele frequency of the protective or neutral allele of 50%. Dashed lines - standard deviation for 100 simulated alleles. Under the simulated scenario the protective allele increases by ~5% in a single generation (in red), which is significantly more than what would be expected by a neutral allele (in blue). (B) Power to detect protective alleles as a function of their frequency prior to *Black Death* and their level of protection against the disease (the figure shows the level of protection for homozygous individuals) by calculating *Fst* values between pre- and post-*Black Death* populations. Power was calculated by comparing *Fst* values for a simulated set of 100 protective alleles between the pre- and post-*Black Death* populations with respect to *Fst* values observed among 1,000 simulated neutral alleles sampled from the same populations.



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Figure S3: Differences in recombination rate and background selection are insufficient to explain the marked enrichment of high F_{ST} values among immune loci. Comparison of recombination rate (A) and background selection levels (B) between neutral loci and our candidate regions. Candidate regions were stratified into those which were tested and those which were candidates for positive selection based on high differentiation in London pre- vs post-BD. (C) Forward simulations matched for the rates of recombination and background selection of the regions targeted in our study show a slight enrichment of highly differentiated sites in candidate regions, but far from the level of enrichment observed in the real data (D), replicated from Fig 2A for comparison. For example, whereas in the real data differentiation at immune loci exceeded the 99th percentile of neutral variants at 2.4x the rate expected by chance (among variants with a MAF > 10%), the same enrichment is less than 1.2x in the simulated data.



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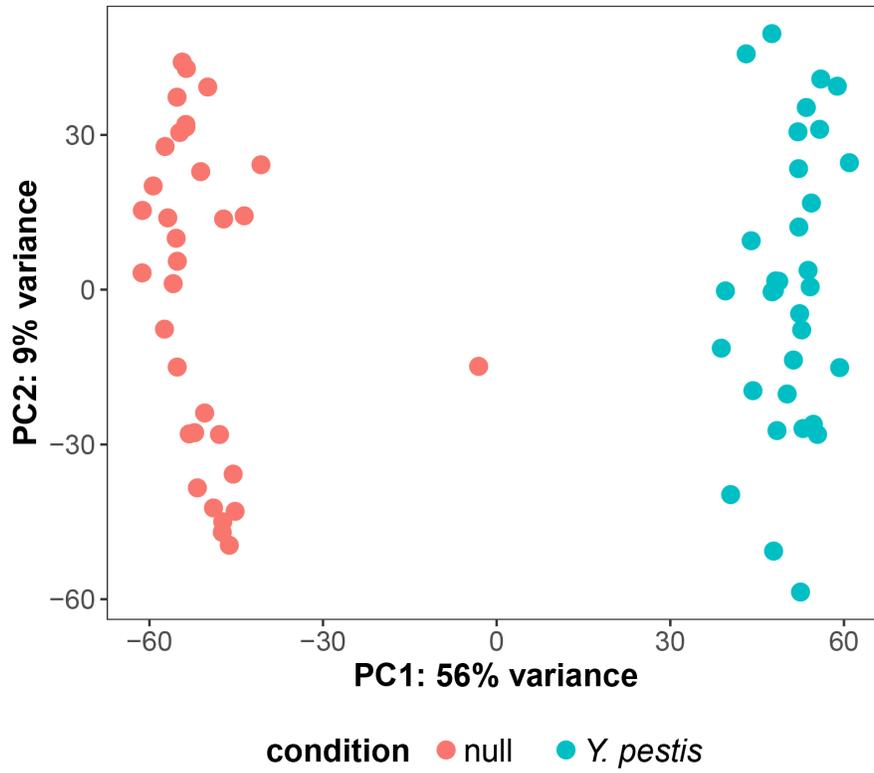
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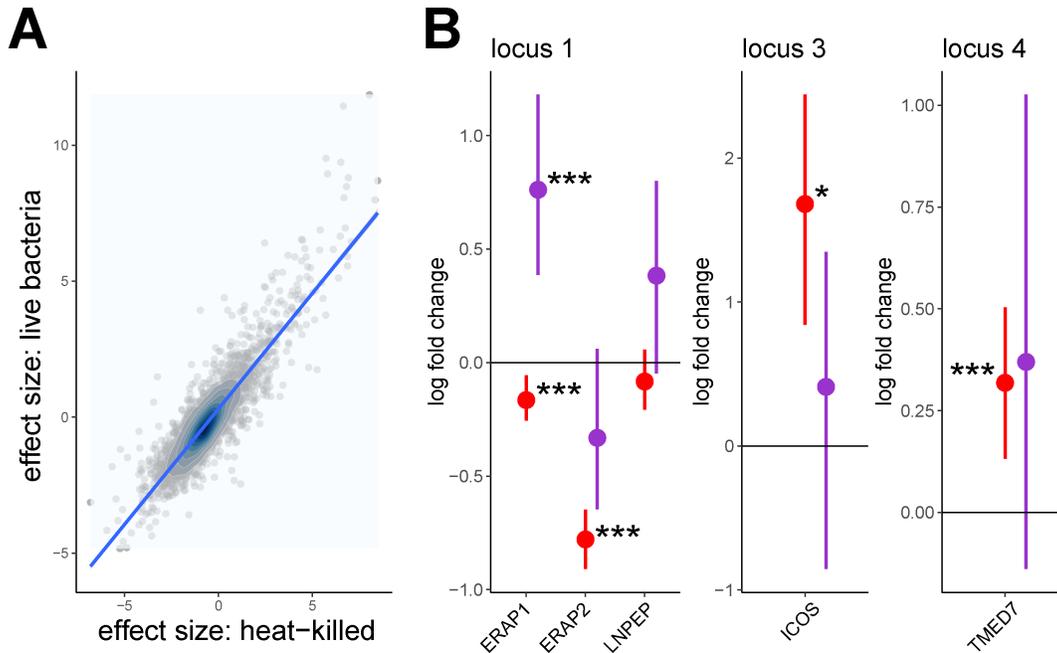
Figure S4: Estimates of the selection coefficients for the four SNPs of interest and power of the inference procedure. (A) Distributions of \hat{s}_{MLE} for the four SNPs when replicates are simulated with the corresponding bootstrapped allele frequency distributions as initial conditions and bootstrap-corrected estimates \tilde{s}_{MLE} . Whiskers on the violin plots label the 2.5-, 50-, and 97.5-percentiles of their respective distributions. **(B)** ROC and **(C)** Precision-Recall curves for the estimation procedure to distinguish replicates under selection from those under neutrality.



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Figure S5: Principal components of gene expression for macrophages stimulated with heat-killed *Y. pestis*. The first principal component clearly separates stimulated samples from matched controls.

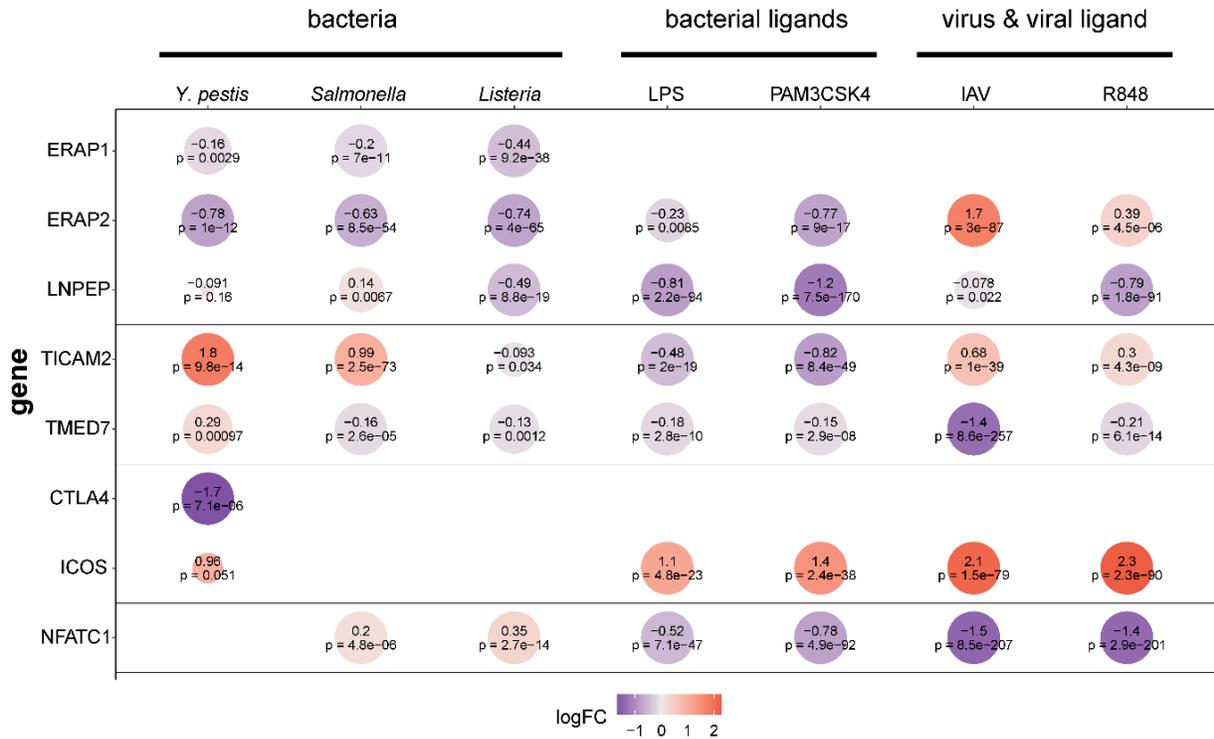
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Figure S6. Response to *Y. pestis* is similar between macrophages stimulated with heat-killed and live *Y. pestis*. Effect size of *Y. pestis* stimulation compared between heat-killed *Y. pestis* (x-axis, n=33 individuals) and live *Y. pestis* (y-axis, n=8 individuals). **(A)** shows all genes, with a blue line representing the best fit line ($r = 0.88$). **(B)** compares effect sizes at genes near candidates for positive selection profiled in both expression datasets (red: heat-killed; purple: live bacteria). Error bars represent the standard error in estimating the effect size. The direction of effect is consistent except for *LNPEP* (which is not significant in either analysis) and *ERAP1*. *CTLA4* and *TICAM2* are now shown because there were not expressed at sufficiently highly levels in the macrophages from the 8 individuals infected with live *Y. pestis*. Asterisks placed near the point estimate of each value represent the significance: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

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Figure S7. Transcriptional changes of genes nearby candidate loci in response to bacterial and viral stimuli. Data are derived from Nedelec *et al.*³⁰ and Quach *et al.* Nedelec *et al.*³¹ measured the gene expression response of monocyte-derived macrophages to infection with two live intracellular bacteria: *Listeria monocytogenes* (a Gram-positive bacterium) and *Salmonella typhimurium* (a Gram-negative bacterium). Quach *et al.* characterized the transcriptional response at 6 hours of primary monocytes to bacterial and viral stimuli ligands activating Toll-like receptor pathways (TLR1/2, TLR4, and TLR7/8) and live influenza virus. **The data for *Y. pestis* are the fold change responses observed in response to heat-killed bacteria.** A negative estimate in plot (purple) indicates that the gene is downregulated and a positive value (red) indicates that the gene is up-regulated. The statistical support for the reported changes is given by the associated *p* values. Larger circle sizes represent smaller *p* values and empty circles refer to cases where that the gene was not expressed in that dataset.

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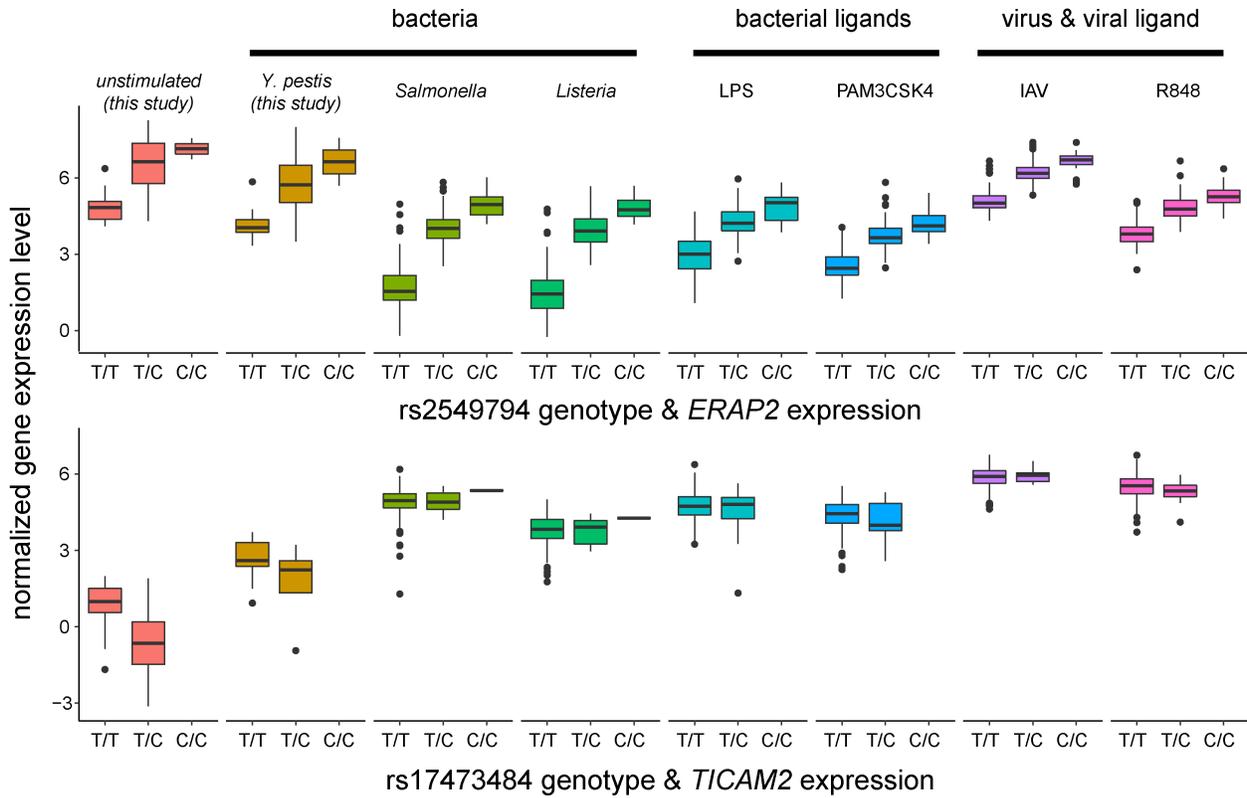
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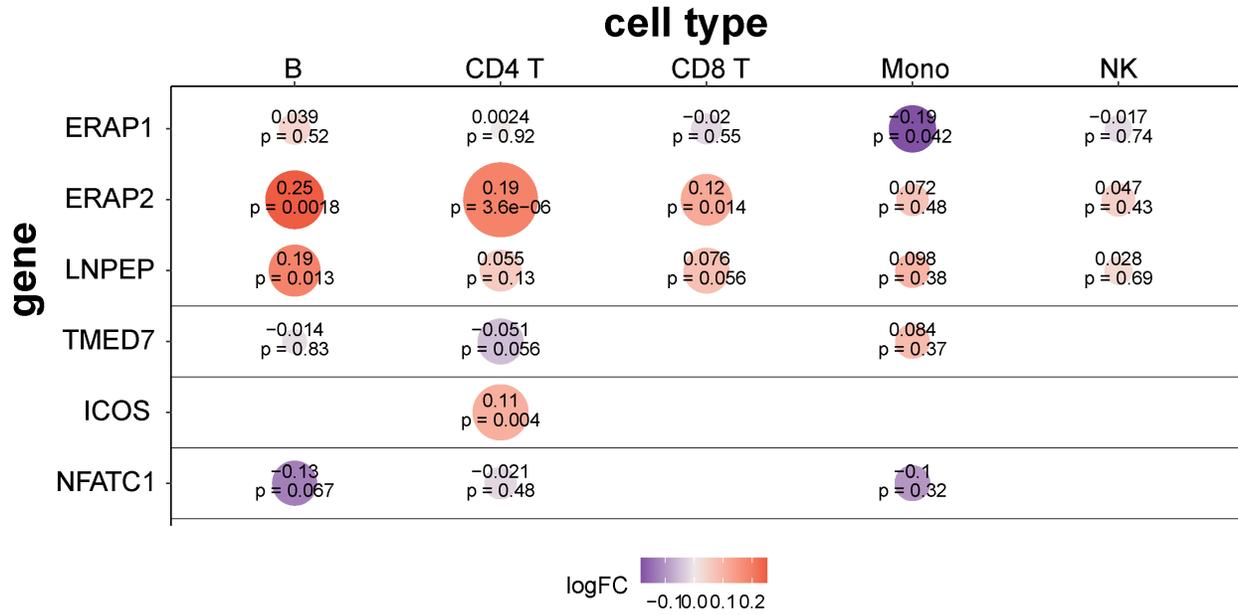
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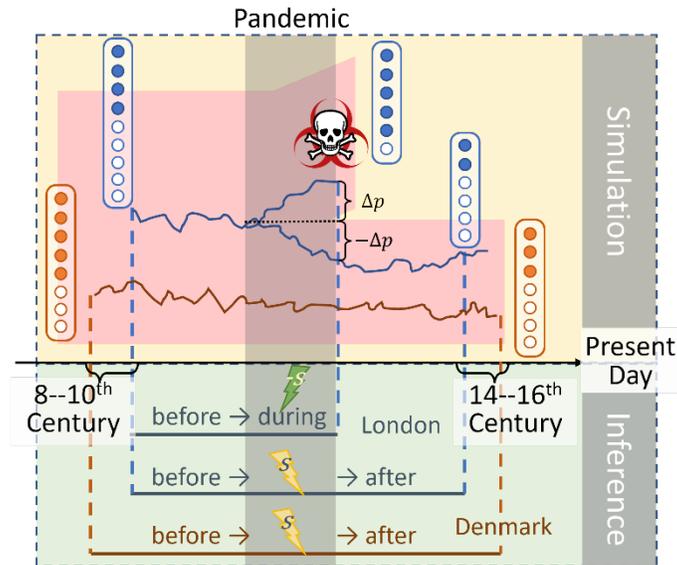
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Figure S8. Genotype effects on transcription at candidate loci. Effect of genotype at nearby loci on the expression of *ERAP2* (top) and *TICAM2* (bottom), across experimental conditions in this study and previously published^{30,31}. For *ERAP2*, the protective “T” haplotype increases expression in all conditions ($p < 0.001$). For *TICAM2*, the protective reference haplotype decreases expression only in the unstimulated condition ($p = 2.5 \times 10^{-6}$; $p > 0.05$ in all other conditions).



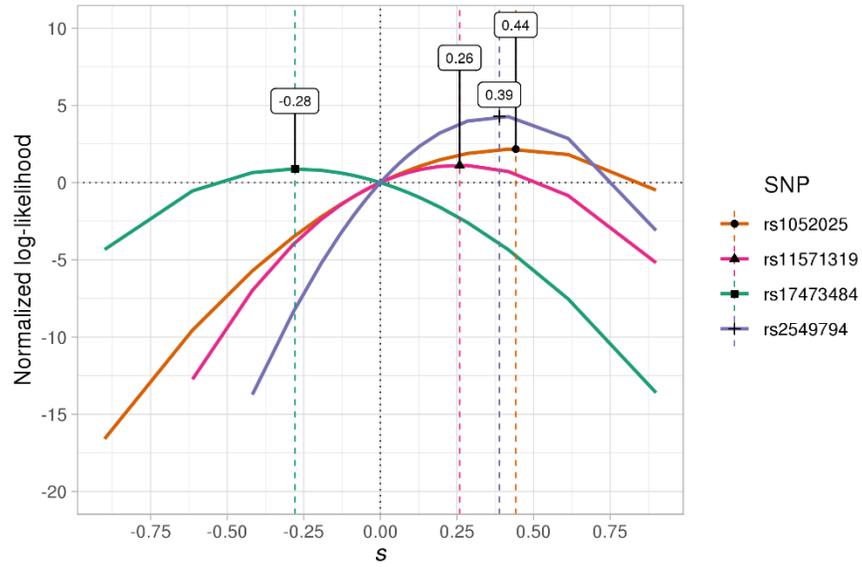
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Figure S9. Transcriptional changes of genes nearby candidate loci in response to *Y. pestis* infection across cell types. For each cell type profiled using single-cell RNA sequencing, we show the effect of *Y. pestis* infection upon gene expression. A negative estimate (purple) indicates that the gene is downregulated and a positive value (red) indicates that the gene is up-regulated. The statistical support for the reported changes is given by the associated *p* values. Larger circle sizes represent smaller *p* values and empty circles refer to cases where that the gene was not expressed in that cell type.



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Figure S10: Schematics for estimating selection coefficients. The time axis serves as an approximate reference of the relative sampling times for the empirical samples. Dashed vertical lines indicate the relative sampling time for each group of samples considered in the analysis, and the floating boxes represent pools of samples from a bi-allelic locus. Above and below the time axis are sketches that respectively correspond to the simulation scheme and the likelihood computations. The shaded red horizontal tree represents the population-continuity along approximate time (x-axis), with the Black Death pandemic occurring in the dark shaded period. The shortened branch with a skull at the end represents people who died of the disease. In each simulated replicate, Δp and $-\Delta p$ mark the respective changes of allele frequency during the pandemic in the mid-pandemic and post-pandemic sample pools. In the inference schematics, each horizontal straight line represents a sampling scheme from which a likelihood was computed. Lightning bolts labeled with s or $-s$ represent the selection coefficients.



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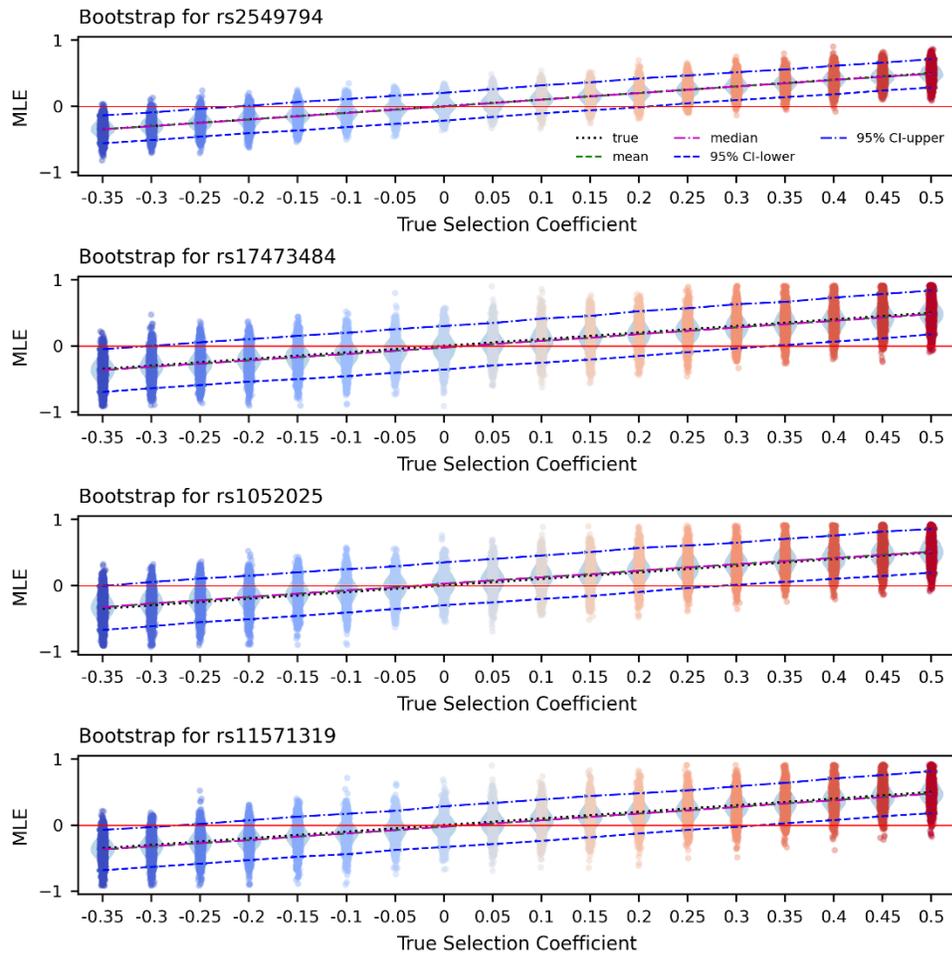
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Figure S11: Log-likelihood as a function of the selection coefficient s for each target SNP. Labeled numbers show the approximate values of respective interpolated \hat{s}_{MLE} . The log-likelihood is centered to 0 for $s = 0$.

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1496 **Figure S12: Distributions of \hat{s}_{MLE} for simulated data starting with the pre-pandemic frequencies of the**
 1497 **four target SNPs.** X-axes label the values of the true s used in the simulations, and y-axes show \hat{s}_{MLE} . The
 1498 black dotted lines are the $y = x$ reference lines. The green dashed lines and red dash-dotted lines indicate the
 1499 means and medians of the \hat{s}_{MLE} . The blue dashed and dot-dashed lines represent the 2.5-th and 97.5-th
 1500 percentiles of the \hat{s}_{MLE} distributions.
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