

1 **Full title: Cell-intrinsic genetic regulation of peripheral memory-phenotype T cell**
2 **frequencies**

3 **Short title:** Cell-intrinsic genetic regulation of T cell memory

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20

21 **Abstract**

22 Memory T and B lymphocyte numbers are thought to be regulated by recent and cumulative
23 microbial exposures. We report here that memory-phenotype lymphocyte frequencies in B, CD4
24 and CD8 T-cells in 3-monthly serial bleeds from healthy young adult humans were relatively
25 stable over a 1-year period, while recently activated -B and -CD4 T cell frequencies were not,
26 suggesting that recent environmental exposures affected steady state levels of recently activated
27 but not of memory lymphocyte subsets. Frequencies of memory B and CD4 T cells were not
28 correlated, suggesting that variation in them was unlikely to be determined by cumulative
29 antigenic exposures. Immunophenotyping of adult siblings showed high concordance in memory,
30 but not of recently activated lymphocyte subsets, suggesting genetic regulation of memory
31 lymphocyte frequencies. To explore this possibility further, we screened effector memory (EM)-
32 phenotype T cell frequencies in common independent inbred mice strains. Using two pairs from
33 these strains that differed predominantly in either CD4EM and/or CD8EM frequencies, we
34 constructed bi-parental bone marrow chimeras in F1 recipient mice, and found that memory T
35 cell frequencies in recipient mice were determined by donor genotypes. Together, these data
36 suggest cell-autonomous determination of memory T niche size, and suggest mechanisms
37 maintaining immune variability.

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42 **Introduction**

43

44 Gene-environment interplay in immune phenotypes has been extensively studied using steady-
45 state cellular immune profiles (1–3), functional immune responses (2,4–8), post-vaccination
46 responses (9,10) and V,D and J usage biases in naïve and memory T and B cell compartments
47 (11). Some of these studies have identified genomic correlates associated with specific steady-
48 state immune phenotypes (2) and vaccine responses (12). However, there are conflicting findings
49 regarding the relative importance of genetic versus environmental factors in regulation of
50 immune phenotype (1,2,13,14), warranting further investigations at the population-level in
51 humans and mechanistic studies in mice on regulation of individual immune phenotypes.

52

53 Memory subsets in T and B lymphocytes are immune populations that are generated in response
54 to past immunogen exposure. Immunological memory, providing long-term persistence of
55 antigen-experienced cells contributing to rapid and robust responses following re-exposure (15),
56 is likely to have evolved in an ecosystem where environmental challenges including repeated
57 infections would be the norm (16) and the persistence of long-lasting antigen-specific cells
58 generated during immune responses would confer survival advantage (17). However, it is
59 possible that larger memory lymphocyte pool sizes may carry costs such as restriction of space
60 for the more repertoire-diverse naïve T cell compartment (18), attrition of pre-existing memory
61 (19), and other bioenergetic costs (20). Such selection may well result in ‘optimum’ sizes of
62 memory lymphocyte pools (21), and these pool sizes could show population diversity, depending
63 on the diversity of pathogens in the ecosystem and their exposure rates (22). Diversity in pool
64 sizes of memory lymphocytes in a population could thus be determined by a combination of
65 genetic variability and diversity of environmental exposures.

66

67 A number of mechanisms can be envisaged regulating the pool size of the memory T cell
68 compartment, including cumulative life-time antigen-exposure and re-exposure, antigenic
69 persistence, degree of expansion, cell survival, attrition and niche-space availability (21,23–26).
70 Immune cells occupy a limited niche space in lymphoid organs (27) or in the periphery (28). This
71 niche size could be a function of size and/or structure of supporting lymphoid tissue architecture
72 (29), along with intrinsic properties of cells occupying the niche. Similar determinants could
73 affect steady state levels of transient cell populations such as immediate-effector T cells (30) and
74 plasmablasts, but their steady state levels would be expected to fluctuate more with short-term
75 environmental changes.

76

77 On this background, we have explored the possibility of gene-environment interplay affecting the
78 steady state pool sizes of lymphocytes post-activation. We have used peripheral blood leucocyte
79 immunophenotyping in serial bleeds from healthy young adult human volunteers to assess inter-
80 individual and temporal variability in lymphocyte memory and effector compartments, and report
81 that while both categories showed inter-individual variability, temporal variability was far less in
82 memory than in effector subsets. We have next used peripheral blood leucocyte
83 immunophenotyping of human siblings, and report that memory lymphocyte subset pool sizes
84 were far closer between siblings than between non-siblings. We then further confirmed a role for
85 genetic factors in determining memory lymphocyte pool sizes in independent inbred mouse
86 strains, in which bi-parental mixed bone marrow chimeras demonstrated a parental genotype-
87 driven inheritance of CD4 and CD8 T cell memory pool sizes. Together, our data from both
88 humans and mice provide novel insights in the determination of memory lymphocyte pool size.

89

90 **Materials and methods**

91 Ethics approval and consent to participate

92 *Human:*

93 Informed written consent was obtained from all human volunteers who participated in the study.

94 The study was approved by ethics committees of National Institute of Immunology (approval

95 number IHEC/AKS/45/2013) and All India Institute of Medical Sciences (approval number

96 IEC/NP-471/2013). The methodologies used in the study were in accordance with approved

97 guidelines. All experimental protocols used in this study were approved by Institutional Ethics

98 Committee (Human Research) of National Institute of Immunology and All India Institute of

99 Medical Sciences.

100 *Animal:*

101 Mice were maintained and used according to the relevant rules and regulations of Government of

102 India and with the approval of Institutional Animal Ethics Committee, National Institute of

103 Immunology (approval number 381/15). All mice experimental protocols were in accordance

104 with approved guidelines and were approved by Institutional Animal Ethics Committee of

105 National Institute of Immunology.

106 Human subjects and blood collection:

107 Forty-five healthy adult volunteers were recruited into the study and were bled 3-monthly for 12

108 months (total of 4 bleeds). Individuals with acute or chronic illness, medication, recent

109 vaccination or pregnancy in women were excluded. Ten ml heparinized peripheral blood was

110 collected from healthy subjects after informed written consent. The study was approved by

111 institutional ethics committee of National Institute of Immunology (NII). All protocols were in

112 accordance with approved guidelines. For sibling study, 37 families were recruited, in which two

113 or more siblings were willing for participation. The family-based study was approved by

114 institutional ethics committees of NII and All India Institute of Medical Sciences (AIIMS).

115 Exclusion criteria were similar to that mentioned above. For looking at correlations between the
116 various memory and effector cell subsets, we utilized the published raw data from a larger (n=71)
117 cohort of adult healthy volunteers who participated in a previous study (31).

118 Sample processing and flow cytometry of human samples:

119 Blood samples were processed without delay (less than three hours). Total leukocyte counts and
120 differential counts were obtained from standard hematologic methods. Peripheral blood
121 mononuclear cells (PBMCs) were separated by density gradient centrifugation. PBMCs were
122 washed, counted and divided into aliquots of about 1 million cells per ml per vial and
123 cryopreserved in 10% DMSO in bovine serum until assays were performed. For flow cytometry,
124 PBMCs were thawed, washed and incubated with the following antibodies organized into two
125 cocktails: The T cell cocktails consisted of CD3 (UCHT1, eBioscience), CD4 (OKT4,
126 eBioscience), CD8 (SK1, eBioscience), CD45RO (UCHL1, eBioscience), CCR7 (150503, BD)
127 and the B cell cocktail consisted of CD19 (SJ25C1, BD), CD20 (2H7, eBioscience), CD38
128 (HIT2, eBioscience), CD43 (eBio84-3C1, eBioscience), CD27 (M-T271, eBioscience). A single
129 control sample was run along with all the samples to ensure that gating is comparable across
130 experiments. Samples were acquired in BD Verse and analysis was done using flowjo (Treestar).
131 CD4 and CD8 memory cells were defined as the CD45RO⁺ fractions (32) of CD8 and CD4 cell
132 compartments. Transient T effector-memory, RA⁺ve (TEMRA) cells were defined as CD45RO⁻
133 CCR7⁻ subsets (32) of CD8 cells and of CD4 cells. Memory B cells were defined as
134 CD27⁺CD43⁻ fraction of the CD19⁺CD20⁺ B cell subset (32). Plasmablasts were defined as the
135 CD38⁺CD20⁻ subset of CD19⁺ B cells (32).

136 Mice:

137 The following strains of mice were used for the study: C57Bl/6J, BALB/cJ, SJL/J, CBA/CaJ,
138 B6.SJL-Ptprca Pepcb/BoyJ (B6.SJL), CB10-H2b/LilMcdJ (BALB/b), FVB/NJ, C3H/HeOuj.
139 Mice strains used in the study were obtained as breeder stock from the Jackson Laboratory (Bar

140 Harbor, ME) and bred and maintained under specific-pathogen free conditions in the Small
141 Animal Facility of the National Institute of Immunology. All mice were maintained and used
142 according to the relevant rules and regulations of Government of India and with the approval of
143 Institutional Animal Ethics Committee, National Institute of Immunology. All experiments used
144 age and gender matched mice. Littermates were used as controls. Phenotyping of mouse spleen T
145 cells was done using the following antibodies (all from eBioscience): CD4 (RM4-5), CD8 (53-
146 6.7), CD44 (IM7), CD62L (MEL-14), CD25 (PC61.5), CD45.1 (A20) and CD45.2 (104). NK
147 cells were phenotyped using the following antibodies (all from eBioscience): CD90 (53-2.1),
148 B220 (RA3-6B2) and Ly49b (DX-5). Surface staining was done by incubating 1×10^6 cells in
149 staining buffer (PBS containing 2% BSA and 0.05% NaN₃) for 30 mins on ice. The cells were
150 washed thrice with cold staining buffer and samples were acquired on BD Verse and analysis was
151 done using flowjo (Treestar).

152 Ex vivo cell preparations

153 Spleen was dissected from mice euthanized by cervical dislocation and teased between a pair of
154 frosted slides to obtain single cell suspension. Red blood cells in the suspension were lysed by
155 osmotic shock using water, washed and then re-suspended in complete medium.

156 Mix bone marrow chimeras

157 F1 hybrids were irradiated at 800rads in gamma chamber (BARC, Mumbai) with Co⁶⁰ as a source
158 for gamma rays. Bone marrow cells from each parental strain were mixed in 1:2, 1:1 and 2:1
159 ratios and a total of 15 million cells transferred into irradiated F1 generation mice (n=24 for
160 B6.SJL - CBA/CaJ chimera and n=19 for SJL/J - BALB/cJ chimera) and reconstitution allowed
161 for two months. Based on the ratio of CD45.1 to CD45.2 in reconstituted chimera, biological
162 outliers showing extreme ratios more than 10- fold from the expected were removed from further
163 analysis (4 outliers in B6.SJL - CBA/CaJ chimera and 2 outliers in SJL/J - BALB/cJ chimera
164 were removed). Phenotyping was done by using above mentioned CD markers. Doublets were

165 excluded from the population using height and area parameters of forward and side scatter.

166 CD4 effector memory cells were defined as the CD4⁺ CD25⁻ CD44^{hi} CD62L^{lo} fractions and

167 CD8 effector memory cells were defined as the CD8⁺ CD44^{hi} CD62L^{lo} fractions.

168 Statistical analysis

169 For frequency and cell count comparisons, a non-parametric test (Mann Whitney) was used.

170 Correlations were estimated using Spearman's correlation coefficient. The Bonferroni method

171 was used to correct for multiple comparisons in testing the significance of elements of the

172 pairwise correlation coefficient matrix. Bootstrapping (resampling) methods were used for

173 comparison of intra-individual variances with between-individual variances and for comparison

174 of sibling pairs vs unrelated pairs. Comparison of multiple inbred mouse strains was done using

175 Analysis of Variance (ANOVA) with post-hoc testing for pair-wise comparison.

176 Gene expression analysis

177 Gene expression (microarray) data of sorted splenic 'naïve' CD4⁺ve CD62L⁻ve CD4 T cells of

178 mice published previously (33) was obtained from GEO database (accession: GSE60337) and

179 analysed using GEO2R tool. Genes with p-value of less than 0.05 after multiple testing

180 (Benjamini) was considered significantly differentially expressed. Gene Ontology enrichment

181 analysis was done using web-based Gene Ontology enrichment analysis and visualization tool

182 (34) (GORILLA) and Database for Annotation, Visualization and Integrated Discovery

183 (DAVID)(35). To reduce false positive results in enrichment, in all enrichment analyses, the

184 enrichment of candidate genes that were differentially expressed were analysed against a

185 background of all the genes that were used in array experiment.

186

187 **Results**

188

189 *Human peripheral T and B memory cell levels show temporal stability*

190

191 Firstly, we examined if variation in the memory T and B cell subsets in the population were
192 relatively stable over time, or were significantly affected by fluctuations possibly contributed by
193 short-term environmental exposures. To test this, we characterized the peripheral blood leucocyte
194 subset phenotype of a group of human volunteers [n=45, male: female ratio = 26:19, mean age =
195 27.16 years (SD = 6.75)] every 3 months for a period of 1 year. Gating strategy for the memory
196 subsets is indicated in supplementary figure 1 (Fig S1).

197 Representative data of frequencies of each subset over 4 time points are shown in Fig S2 and are
198 shown quantified in Figure 1. Variations of memory B, memory CD4 and memory CD8
199 frequencies were significantly lower within individuals than between individuals (Fig. 1A, 1B,
200 1C). On the other hand, intra-individual variances in plasmablasts (Fig. 1D) and CD4 TEMRA
201 cells (Fig. 1E) were no different from inter-individual variances. When absolute cell numbers per
202 μl blood were calculated, broadly similar trends were seen (Fig. 1G, 1H, 1I, 1J, 1K). Curiously,
203 CD8 TEMRA frequencies (Fig. 1F) and counts (Fig. 1L) tended to be similar to CD8 memory
204 (Fig. 1C, 1I) in that they showed lower intra-individual than inter-individual variation. These data
205 suggested that intra-individual short-term fluctuations in memory B and T subsets are unlikely to
206 be a major explanation for the variation that is seen in memory lymphocyte subsets in humans.

207

208 **Figure 1: Comparison of intra-individual and inter-individual variance for immune subsets**
209 **in Humans.**

210 Box plots indicate median and interquartile ranges of variances of cell frequencies and counts in
211 Human volunteers. Upper whisker extends till the highest value that is within 1.5 times the

212 interquartile range from 3rd quartile. Lower whisker extends till the lowest value that is within
213 1.5 times the interquartile range from 1st quartile. Outliers are shown as dots. Intra-individual
214 variances indicate variance of subset frequency or count over 4 time points in each individual
215 (n=45). Inter-individual variances indicate variance of subset frequency or count in randomly
216 chosen set of different individuals (n=45). P-values obtained by bootstrapping are as indicated in
217 the panels.

218

219

220 *Correlations between CD4, CD8 and B cell memory*

221

222 Since memory cells can be long-lived and could have been generated by antigenic exposure in the
223 relatively remote past, it remained possible that cumulative antigen exposures contribute to
224 determining steady-state memory T and B cell levels. Microbial exposure commonly leads to
225 generation of CD4 T-dependent B cell responses, since CD4 T cells are necessary for B cell
226 germinal centre responses and hence vital to memory B cell generation. Thus, it would be
227 expected that there would be coordinated accumulation of memory cells in the CD4 T and B cell
228 compartments leading to a positive correlation between CD4 T memory and B memory cell
229 frequencies, if cumulative antigenic exposures were to contribute substantially to determining
230 memory T and B cell frequencies. However, there was no correlation between CD4 T cell
231 memory frequencies and B cell memory frequencies in a given individual (Fig. 2A). Thus,
232 cumulative antigen exposure may not be a major determinant of memory CD4 and B cell levels.
233 Interestingly, CD4 and CD8 memory T cell subsets showed a strong correlation (correlation
234 coefficient = 0.61, $p < 0.001$) (Fig. 2B), suggesting shared determinants of memory cell subsets
235 within the T cell lineage. On the other hand, frequencies of CD4 TEMRA or CD8 TEMRA cells

236 or plasmablasts did not show any correlations with memory T or B cell frequencies or with each
 237 other (Table 1).

238

Pairs of variables	Correlation coefficient (Spearman)	Adjusted p-value (Bonferroni)
Plasmablast – B memory	0.17	1
CD4 memory - B memory	0.0009	1
CD4 TEMRA - B memory	-0.006	1
CD8 memory - B memory	-0.09	1
CD8 TEMRA - B memory	0.038	1
CD4 memory - Plasmablast	-0.04	1
CD4 TEMRA - Plasmablast	0.09	1
CD8 memory – Plasmablast	-0.0017	1
CD8 TEMRA - Plasmablast	0.16	1
CD4 TEMRA - CD4 memory	-0.26	0.335
CD8 memory - CD4 memory	0.6	8.90E-08
CD8 TEMRA - CD4 memory	-0.064	1
CD8 memory - CD4 TEMRA	-0.16	1
CD8 TEMRA - CD4 TEMRA	0.32	0.065
CD8 TEMRA - CD8 memory	0.2	1

239

240 **Table 1: Correlation coefficient (Spearman) of correlation between cell subsets in adult**
 241 **volunteers**

242

243

244

245 **Figure 2: Correlation between memory B and memory CD4 T cell frequencies (A) and**
246 **between memory CD4 and memory CD8 T cell frequencies (B).** Memory CD4, memory CD8
247 and memory B cell frequencies are expressed as percentage of total CD4, CD8 and B cells
248 respectively. Each dot represents data from one individual donor (n=71). Correlation coefficient
249 (Spearman) and p-value are as indicated.

250

251

252 *Siblings show concordance in T and B cell memory levels*

253

254 Since these descriptive and correlative data from humans showed associations that suggested that
255 memory phenotypes might be genetically regulated, we explored this further by a family based
256 study where peripheral blood leucocytes from 80 full siblings from 37 families were
257 immunophenotyped [male: female ratio = 38:42, mean age = 31.8 years (SD=12.7)]. We
258 compared the differences in cell frequencies between siblings with the differences between
259 random pairs of non-siblings for memory CD4, memory CD8 and memory B cells, and for
260 plasmablasts, CD4 TEMRA or CD8 TEMRA cell frequencies. The analysis showed that
261 differences in cell frequencies between siblings were significantly less than the differences
262 between random pairs of non-siblings for memory CD4, memory CD8 and memory B cells, but
263 not for plasmablasts, CD4 TEMRA or CD8 TEMRA cell frequencies (Table 2), suggesting that
264 there was indeed likely to be a genetic contribution to the variations in memory cell frequencies,
265 although possible contributions from early life co-habitation could not of course be ruled out.

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272

273 **Table 2: P-values of difference between within-sibling pairs and between-sibling pairs**

274

Compartment	Sibling (Mean ± SD)	Unrelated Individuals (Mean ± SD)	p-value
B cell memory	6.88 ± 4.45	8.84 ± 1.08	0.03
Plasmablasts	2.05 ± 3.08	2.32 ± 0.33	0.207
CD4 memory	11.17 ± 8.24	15.41 ± 1.91	0.01
CD4 TEMRA	1.56 ± 1.39	0.92 ± 0.14	0.99
CD8 memory	12.66 ± 10.67	17.64 ± 2.19	0.008
CD8 TEMRA	10.04 ± 8.76	10.2 ± 1.27	0.46

275

276

277

278 *Mouse strains differ in their memory T cell frequencies and show cell-autonomous regulation of*
279 *memory T cell phenotype*

280

281 These data from humans indicated the possibility of a genetic contribution to the determination of
282 memory T and B cell pool sizes. To examine this possibility more rigorously, we examined
283 independent inbred strains of mice. Since specific markers for B cell memory are uncertain (36)
284 and since this compartment in mice may be smaller than in humans (37), we restricted these
285 studies to T cell memory subsets alone.

286

287 The gating strategy used for defining effector memory (EM) CD4 (Fig 3A to 3D) and CD8 T
288 cells (Fig 3E to 3H) in a group of representative mice available in our laboratory are shown in
289 Figure 3. EM populations in CD4 and CD8 subsets were distinguished by unambiguous contours,
290 whereas in some strains (eg. BALB/cJ) the central memory (CM) populations were difficult to
291 gate out from the CD44 negative naïve pool using the conventional markers of memory (CD44
292 and CD62L). CM phenotype CD4 T cells in the BALB/c strain have been previously shown to
293 contain recent thymic emigrants with a CD44-high phenotype (38). CM phenotype CD8 T cells
294 have also been reported to contain antigen-inexperienced, non-memory (“virtual memory”) cell
295 types (39) as well as regulatory CD8 T cells (40), further confounding our analysis of memory
296 subsets. Hence, we have avoided CM phenotype T cells and focused on the unambiguously
297 defined EM populations for interpreting differences in memory between the genetically different
298 strains.

299

300 **Figure 3: Gating strategy for memory subsets in mouse splenic CD4 T cells (A to D) and**
301 **CD8 T cells (E to H).** Each plot shows representative gates from a single mouse strain as
302 indicated. CD4 memory subsets are gated on conventional CD4⁺ CD25⁻ gate. CD8 memory
303 subsets are gated on CD8⁺ gate.

304

305

306 Our preliminary data showed that CBA/CaJ and C57BL/6J showed differences in both EMCD4
307 and EMCD8 compartments with C57BL/6J showing higher EMCD4 and EMCD8 frequencies
308 and counts than CBA/CaJ (Fig 4A - 4D). On the other hand, BALB/cJ and SJL/J differed in
309 EMCD4 but not EMCD8 compartment (Fig 4E - 4H), with SJL/J showing significantly higher
310 EMCD4 frequencies and counts than BALB/cJ (Fig 4E, 4F).

311

312 **Figure 4: EMCD4 and EMCD8 frequencies and counts (in millions) in spleen in C57BL/6J**
313 **versus CBA/CaJ (A to D) and BALB/cJ versus SJL/J (E to H).** Each dot represents data from
314 one individual mouse ($n > 25$ per group). EMCD4 and EMCD8 frequencies indicate frequencies
315 of EM compartment out of total conventional CD4 (CD4+CD25-) and CD8 T cells respectively.
316 Cell counts are expressed in millions. Box plots indicate median and interquartile ranges. Upper
317 whisker extends till the highest value that is within 1.5 times the interquartile range from 3rd
318 quartile. Lower whisker extends till the lowest value that is within 1.5 times the interquartile
319 range from 1st quartile. The p-values are as indicated in the panels.

320

321

322 To test the source of the differences observed in EMCD4 frequencies between the strains, we
323 prepared bi-parental mixed bone marrow chimeras in which bone marrow cells from both
324 parents, in various ratios, were transferred into irradiated F1 mice. We used heavy irradiation that
325 is reported to successfully engraft parental donor cells in F1 recipients (41–44) and ensured
326 endogenous NK cell depletion post-irradiation (Fig S3 A and B). We also confirmed that
327 chimerism is successful by comparing injected donor cell ratios with ratios of CD45.1 to CD45.2
328 lymphocytes in reconstituted F1 mice (Fig S3 C and D) and found no statistically significant
329 differences, ruling out the possibility of unequal reconstitution by donor strains. The mean
330 frequency of endogenous cells was 10.1 % in B6.SJL – CBA/CaJ chimera (SD-6.5) and 25.9 %
331 in BALB/cJ - SJL/J chimera (SD-12.3). After waiting two months for generation of memory cells
332 from the donor genotypes, these mice were phenotyped for CD4 and CD8 EM compartments.
333 Memory CD4 and CD8 frequencies of the donor-derived cell population (Fig 5A to 5D) in these
334 chimeras showed similar trends as that in parental strains (Fig 4). These results suggest that
335 genetic factors contribute in cell intrinsic fashion to determining EM T cell frequencies.

336

337 **Figure 5: Frequencies of EMCD4 and EMCD8 in the spleen from donor partner in mixed**
338 **bone marrow chimera of B6.SJL-CBA/CaJ pair (A, B) and BALB/cJ-SJL/J pair (C, D).**

339 Each dot represents data from one individual mouse ($n > 9$ per group) and individual mice data
340 are connected by lines. The p-values are as indicated in the panels.

341

342

343 *CD4 gene expression in strains that differ in CD4 effector memory phenotype*

344

345 Since bone marrow chimera data suggested cell-autonomous regulation of memory phenotype,
346 we hypothesized that intrinsic gene expression differences in naïve CD4 T cells between the
347 mouse strains could contribute to differences in EMCD4 frequencies that are observed across the
348 strains. We tested this using gene expression data (microarray) of splenic CD4 T cells from
349 multiple mouse strains available from the immunological genome project (33). Immgen gene
350 expression data comes from sorted CD4⁺ CD62L⁺ spleen cells ('non-EM' CD4 T cells), which
351 could potentially include CD44-high CMCD4 population in addition to genuine naïve CD4 T
352 cells. Hence, we selected 2 mouse strains (CBA/CaJ and SJL/J) that showed maximum
353 differences in EMCD4 frequencies and counts, without showing differences in CMCD4 levels
354 (Fig S4 A to E, p-values shown in supplementary table S5). CMCD4 frequencies were very low
355 in these 2 strains (Figure 3B and 3D), making comparison of CD4⁺ CD62L⁺ gene expression
356 between these strains interpretable. A comparison of gene expression profile of CD4⁺ CD62L⁺ve
357 splenocytes (obtained from GEO accession: GSE60337) of CBA/CaJ and SJL/J identified 290
358 genes that were significantly different (p values < 0.05 after multiple correction) (supplementary
359 table S6). Gene ontology enrichment analysis identified significantly enriched GO processes
360 associated with the 290-gene list (supplementary table S6). As expected, MHC molecule related

361 transcripts were differentially expressed and came up as significantly enriched, reassuring that the
362 analysis we performed could actually pick up biologically relevant differences (table S6). In
363 addition, there was significant enrichment for lipid biosynthesis (11 genes, p value: 1.78E-05,
364 adjusted p value: 0.004) and lipid metabolism (16 genes, p value: 1.13E-04, adjusted p value:
365 0.02) (supplementary file S6), implying that these pathways could be of biological relevance to
366 regulation of CD4 T cell memory phenotype. When data from multiple strains were pooled
367 together, there was a positive correlation between EMCD4 and EMCD8 frequencies (Fig S4F),
368 similar to what is observed for humans (Fig 2B), suggesting similar mechanisms in regulation of
369 CD4 and CD8 effector memory or dependence of one subset on the other.

370

371

372

373 **Discussion**

374

375 There are a number of possible explanations for the observation of a substantial extent of
376 variation in the relative frequencies of memory-phenotype B cells, CD4 T cells and CD8 T cells.
377 Such variations in biological parameters are commonly interpreted as errors in technical or
378 experimental factors, although in real populations, biological variance may well have meaningful
379 interpretative value (31). At the population level, we have used descriptive characterization of the
380 human peripheral blood leucocyte phenotype to examine the possible significance of population
381 level variation in these leucocyte subsets.

382

383 By comparison of intra-individual variance of memory and effector subsets of T and B cells, we
384 find that immune subsets that respond to day-to-day fluctuations, such as the plasmablasts and
385 CD4 TEMRA cells, show similar intra-individual and inter-individual variance in the population,
386 while B cell memory, CD4 memory and CD8 memory cell frequencies are very much stable over
387 a one-year period within individuals. This suggests that memory subsets are not determined by
388 short-term environmental fluctuations, unlike CD4 TEMRA and plasmablasts. Within-individual
389 variation can thus possibly be an explanatory factor in the population-level variation for effector
390 T cells and plasmablast subsets, but not for memory T and B cell subsets. A recent study (45) that
391 quantified technical and biological variation in human immune phenotype also found high intra-
392 individual variation in CD4 TEMRA cells in comparison to other immune lineages. Our data
393 show that CD8 TEMRA cells did not behave similar to CD4 TEMRA cells in that they do not
394 show considerable intra-individual fluctuation. This could be related to indications that CD8
395 TEMRA cells may be functionally different from CD4 TEMRA cells, despite being
396 phenotypically similar (46).

397

398 Our findings of a lack of correlation between B cell memory and CD4 memory also argue against
399 the possibility of cumulative exposures determining both the memory subsets, although it
400 remains possible that cumulative exposures regulate one but not the other subset. The positive
401 correlation we find between CD4 memory and CD8 memory suggest that T cell memory levels
402 could be regulated by similar mechanisms, distinct from how memory B cell levels are regulated.
403
404 We attempted to quantify the degree of similarity in immune phenotype between siblings to see if
405 there was any indication that these memory phenotypes are heritable. Although our sibling study
406 was not as powerful as previously published twin studies (1,2,4,14) for understanding heritability,
407 our sibling data strongly support and extend the interpretations from our serial bleed data. Thus,
408 siblings did not show concordance in effector T and B subsets, suggesting that variation in those
409 subsets is environmentally driven. On the other hand, siblings were far similar to each other in
410 memory subset frequencies than non-siblings were, consistent with a genetic component
411 regulating memory lymphocyte frequencies. However, it must be acknowledged that any
412 similarity between siblings could be attributed to early life influences, since it is well recognized
413 that both intra-uterine (47,48) and neonatal (49) stress impacts immune system development, and
414 a recent study (13) has shown a prominent role for co-habitation in determining immune
415 phenotypes. Our findings are consistent with two previous studies (1,14) which report high
416 degree of heritability for majority of immune subsets. Another study (2), too, reported high
417 degree of heritability for CD4 memory subsets, although it found non-heritable factors to be
418 important for most other immune phenotypes. Since our interpretations are predominantly based
419 on associations, we complemented the study with experimental data from mice.
420
421 Although laboratory animals show restricted genetic diversity compared to human populations,
422 we exploited the fact that independent inbred strains are genetically distinct and are grown in a

423 homogeneous environment in a controlled animal facility. Hence, it is worth examining if
424 differences in leucocyte subset levels between strains are genetically driven. We
425 immunophenotyped some common strains of mice and chose two pairs showing substantial
426 differences, SJL/J and BALB/cJ for further experiments on CD4 memory and C57BL/6J and
427 CBA/CaJ for CD8 and CD4 T cell memory phenotype. It is notable that not only did splenic CD4
428 and CD8 memory frequencies show differences, but that total numbers of these cells per organ
429 also showed the same differences, indicating that subset frequencies are reasonable surrogates for
430 the pool size of these subsets. We also have noted that animals maintained in our facility had
431 relatively high frequencies of EM phenotype T cells even at steady state. Even though mice were
432 harbored in a specific-pathogen-free facility, it remains possible that there might be variations in
433 microbial antigenic burden or gut microbiome composition between laboratories, although these
434 factors are difficult to quantify. Long years of reproductive isolation because of inbreeding could
435 also have contributed to these differences.

436

437 Our mixed bone marrow chimera experiments allowed us to examine whether the CD4 and/or
438 CD8 memory levels were genetically determined and whether these genetic influences were T
439 cell-intrinsic in nature. Our data suggest that donor genotype-specific cell-intrinsic factors
440 strongly influence both CD4 and CD8 memory T cell pool size.

441

442 Using gene expression data of mouse splenic CD4+ve CD62L+ve T cells available in the public
443 domain (33) we attempted to characterize genetic differences that could explain differences in
444 CD4 memory phenotype. There are a number of limitations in our approach. Firstly, we do not
445 evaluate gene expression differences in the same mice (or even mice from the same small animal
446 facility) that we experimentally find phenotypic differences for, and differences in phenotype that
447 we observe could be influenced by additional facility-specific environmental factors as well.

448 Secondly, the array based gene expression data available in the public domain that we used (33)
449 contain only 2 replicates for each strain (except for C57BL/6J), limiting statistical power and
450 increasing the chances of picking up false positive differences. Thirdly, the "naive" population as
451 defined by Immgen is based on CD4+ve CD62L+ve gate, and does not exclude CD44+ cells,
452 potentially including central memory cells into the population. Our analysis attempts to overcome
453 this limitation by comparing those strains which differ only in EMCD4, and not CMCD4 subsets.
454 In spite of these caveats, our analysis is still useful as a preliminary exploration that can be
455 extended in further experiments using multiple replicates and strains. Remarkably, lipid/fatty acid
456 metabolism pathways that came up as significant in as limited an analysis as this, was recently
457 suggested to play crucial roles in T cell activation (50). This exploratory analysis is thus likely to
458 provide further clues to direct future mechanistic studies.

459

460 Thus, our data suggest genetic and cell-intrinsic factors as major determinants of the memory
461 pool size of T cells as well as, probably, B cells. However, in a natural ecosystem where animals
462 are exposed to substantial and continual antigenic exposures, the balance between cell-intrinsic
463 genetic effects on memory and the effects of environmental factors is likely to be nuanced and
464 quantitative. Further studies using varying antigenic burdens and experimental immunizations
465 will be necessary to address these issues. Mechanistically, it will be interesting to explore
466 whether genetic cell-intrinsic factors affect memory generation during immune response or
467 memory attrition after the peak of immune response. Our data provide interesting insights and
468 directions for future work in understanding the genesis and consequences of the regulation of
469 niche size of lymphocyte memory.

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483

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485 **Key words:** memory, T-cell, B-cell

486 **List of abbreviations:**

487 EM: Effector memory

488 TEMRA: T, effector memory, RA positive

489 CD: Cluster of differentiation

490

491

492 **References**

- 493 1. Orrù V, Steri M, Sole G, Sidore C, Viridis F, Dei M, et al. Genetic variants regulating
494 immune cell levels in health and disease. *Cell*. 2013 Sep 26;155(1):242–56.
- 495 2. Brodin P, Jovic V, Gao T, Bhattacharya S, Angel CJL, Furman D, et al. Variation in the
496 human immune system is largely driven by non-heritable influences. *Cell*. 2015 Jan
497 15;160(1–2):37–47.
- 498 3. Lu Y, Biancotto A, Cheung F, Remmers E, Shah N, McCoy JP, et al. Systematic Analysis
499 of Cell-to-Cell Expression Variation of T Lymphocytes in a Human Cohort Identifies Aging
500 and Genetic Associations. *Immunity*. 2016 Nov 15;45(5):1162–75.
- 501 4. de Craen AJM, Posthuma D, Remarque EJ, van den Biggelaar AHJ, Westendorp RGJ,
502 Boomsma DI. Heritability estimates of innate immunity: an extended twin study. *Genes
503 Immun*. 2005 Mar;6(2):167–70.
- 504 5. Li Y, Oosting M, Deelen P, Ricaño-Ponce I, Smeekens S, Jaeger M, et al. Inter-individual
505 variability and genetic influences on cytokine responses to bacteria and fungi. *Nat Med*.
506 2016 Aug;22(8):952–60.
- 507 6. Ye CJ, Feng T, Kwon H-K, Raj T, Wilson MT, Asinovski N, et al. Intersection of
508 population variation and autoimmunity genetics in human T cell activation. *Science*. 2014
509 Sep 12;345(6202):1254665.
- 510 7. Li Y, Oosting M, Smeekens SP, Jaeger M, Aguirre-Gamboa R, Le KTT, et al. A Functional
511 Genomics Approach to Understand Variation in Cytokine Production in Humans. *Cell*. 2016
512 Nov 3;167(4):1099-1110.e14.
- 513 8. Horst R ter, Jaeger M, Smeekens SP, Oosting M, Swertz MA, Li Y, et al. Host and
514 Environmental Factors Influencing Individual Human Cytokine Responses. *Cell*. 2016 Nov
515 3;167(4):1111-1124.e13.
- 516 9. Lee YC, Newport MJ, Goetghebuer T, Siegrist CA, Weiss HA, Pollard AJ, et al. Influence
517 of genetic and environmental factors on the immunogenicity of Hib vaccine in Gambian
518 twins. *Vaccine*. 2006 Jun 19;24(25):5335–40.
- 519 10. Yan K, Cai W, Cao F, Sun H, Chen S, Xu R, et al. Genetic effects have a dominant role on
520 poor responses to infant vaccination to hepatitis B virus. *J Hum Genet*. 2013
521 May;58(5):293–7.
- 522 11. Rubelt F, Bolen CR, McGuire HM, Vander Heiden JA, Gadala-Maria D, Levin M, et al.
523 Individual heritable differences result in unique cell lymphocyte receptor repertoires of
524 naïve and antigen-experienced cells. *Nat Commun*. 2016;7:11112.
- 525 12. Majumder PP, Sarkar-Roy N, Staats H, Ramamurthy T, Maiti S, Chowdhury G, et al.
526 Genomic correlates of variability in immune response to an oral cholera vaccine. *Eur J Hum
527 Genet*. 2013 Sep;21(9):1000–6.

- 528 13. Carr EJ, Dooley J, Garcia-Perez JE, Lagou V, Lee JC, Wouters C, et al. The cellular
529 composition of the human immune system is shaped by age and cohabitation. *Nat Immunol.*
530 2016 Apr;17(4):461–8.
- 531 14. Kaczorowski KJ, Shekhar K, Nkulikiyimfura D, Dekker CL, Maecker H, Davis MM, et al.
532 Continuous immunotypes describe human immune variation and predict diverse responses.
533 *PNAS.* 2017 Jul 10;201705065.
- 534 15. Farber DL, Netea MG, Radbruch A, Rajewsky K, Zinkernagel RM. Immunological
535 memory: lessons from the past and a look to the future. *Nat Rev Immunol.* 2016
536 Feb;16(2):124–8.
- 537 16. Karlsson EK, Kwiatkowski DP, Sabeti PC. Natural selection and infectious disease in
538 human populations. *Nat Rev Genet.* 2014 Jun;15(6):379–93.
- 539 17. Boehm T. Evolution of vertebrate immunity. *Curr Biol.* 2012 Sep 11;22(17):R722-732.
- 540 18. Cicin-Sain L, Smyk-Pearson S, Smyk-Pearson S, Currier N, Byrd L, Koudelka C, et al. Loss
541 of naive T cells and repertoire constriction predict poor response to vaccination in old
542 primates. *J Immunol.* 2010 Jun 15;184(12):6739–45.
- 543 19. Huster KM, Stemberger C, Gasteiger G, Kastenmüller W, Drexler I, Busch DH. Cutting
544 edge: memory CD8 T cell compartment grows in size with immunological experience but
545 nevertheless can lose function. *J Immunol.* 2009 Dec 1;183(11):6898–902.
- 546 20. Boots M, Bowers RG. The evolution of resistance through costly acquired immunity. *Proc*
547 *Biol Sci.* 2004 Apr 7;271(1540):715–23.
- 548 21. Graw F, Magnus C, Regoes RR. Theoretical analysis of the evolution of immune memory.
549 *BMC Evol Biol.* 2010;10:380.
- 550 22. Wodarz D. Evolution of immunological memory and the regulation of competition between
551 pathogens. *Curr Biol.* 2003 Sep 16;13(18):1648–52.
- 552 23. Antia R, Pilyugin SS, Ahmed R. Models of immune memory: on the role of cross-reactive
553 stimulation, competition, and homeostasis in maintaining immune memory. *Proc Natl Acad*
554 *Sci USA.* 1998 Dec 8;95(25):14926–31.
- 555 24. Jiang J, Lau LL, Shen H. Selective depletion of nonspecific T cells during the early stage of
556 immune responses to infection. *J Immunol.* 2003 Oct 15;171(8):4352–8.
- 557 25. Gray D. A role for antigen in the maintenance of immunological memory. *Nat Rev*
558 *Immunol.* 2002 Jan;2(1):60–5.
- 559 26. Vezys V, Yates A, Casey KA, Lanier G, Ahmed R, Antia R, et al. Memory CD8 T-cell
560 compartment grows in size with immunological experience. *Nature.* 2009 Jan
561 8;457(7226):196–9.
- 562 27. Capece T, Kim M. The Role of Lymphatic Niches in T Cell Differentiation. *Mol Cells.* 2016
563 Jul;39(7):515–23.

- 564 28. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune
565 defence. *Nat Rev Immunol*. 2016 Feb;16(2):79–89.
- 566 29. Junt T, Scandella E, Ludewig B. Form follows function: lymphoid tissue microarchitecture
567 in antimicrobial immune defence. *Nat Rev Immunol*. 2008 Oct;8(10):764–75.
- 568 30. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell
569 differentiation: Human memory T-cell subsets. *Eur J Immunol*. 2013 Nov 1;43(11):2797–
570 809.
- 571 31. Prabhu SB, Rathore DK, Nair D, Chaudhary A, Raza S, Kanodia P, et al. Comparison of
572 Human Neonatal and Adult Blood Leukocyte Subset Composition Phenotypes. *PLoS ONE*.
573 2016;11(9):e0162242.
- 574 32. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the Human
575 Immunology Project. *Nat Rev Immunol*. 2012 Feb 17;12(3):191–200.
- 576 33. Mostafavi S, Ortiz-Lopez A, Bogue MA, Hattori K, Pop C, Koller D, et al. Variation and
577 genetic control of gene expression in primary immunocytes across inbred mouse strains. *J*
578 *Immunol*. 2014 Nov 1;193(9):4485–96.
- 579 34. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and
580 visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics*. 2009;10:48.
- 581 35. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene
582 lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44–57.
- 583 36. Bergmann B, Grimsholm O, Thorarinsdottir K, Ren W, Jirholt P, Gjertsson I, et al. Memory
584 B cells in mouse models. *Scand J Immunol*. 2013 Aug;78(2):149–56.
- 585 37. Zuccarino-Catania GV, Sadanand S, Weisel FJ, Tomayko MM, Meng H, Kleinstein SH, et
586 al. CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent
587 of antibody isotype. *Nat Immunol*. 2014 Jul;15(7):631–7.
- 588 38. Lai D, Zhu J, Wang T, Hu-Li J, Terabe M, Berzofsky JA, et al. KLF13 sustains thymic
589 memory-like CD8⁺ T cells in BALB/c mice by regulating IL-4-generating invariant natural
590 killer T cells. *J Exp Med*. 2011 May 9;208(5):1093–103.
- 591 39. Chiu B-C, Martin BE, Stolberg VR, Chensue SW. Cutting edge: Central memory CD8 T
592 cells in aged mice are virtual memory cells. *J Immunol*. 2013 Dec 15;191(12):5793–6.
- 593 40. Li S, Xie Q, Zeng Y, Zou C, Liu X, Wu S, et al. A naturally occurring CD8⁺CD122⁺ T-cell
594 subset as a memory-like Treg family. *Cellular & Molecular Immunology*. 2014
595 Jul;11(4):326.
- 596 41. Sprent J, Boehmer HV, Nabholz M. Association of immunity and tolerance to host H-2
597 determinants in irradiated F1 hybrid mice reconstituted with bone marrow cells from one
598 parental strain. *J Exp Med*. 1975 Aug 1;142(2):321–31.

- 599 42. Gao EK, Lo D, Sprent J. Strong T cell tolerance in parent----F1 bone marrow chimeras
600 prepared with supralethal irradiation. Evidence for clonal deletion and anergy. *J Exp Med.*
601 1990 Apr 1;171(4):1101–21.
- 602 43. Sim B-C, Aftahi N, Reilly C, Bogen B, Schwartz RH, Gascoigne NRJ, et al. Thymic
603 skewing of the CD4/CD8 ratio maps with the T-cell receptor α -chain locus. *Current*
604 *Biology.* 1998 Jun 4;8(12):701-S3.
- 605 44. Zhao Y, Ohdan H, Manilay JO, Sykes M. NK cell tolerance in mixed allogeneic chimeras. *J*
606 *Immunol.* 2003 Jun 1;170(11):5398–405.
- 607 45. Burel JG, Qian Y, Arlehamn CL, Weiskopf D, Zapardiel-Gonzalo J, Taplitz R, et al. An
608 Integrated Workflow To Assess Technical and Biological Variability of Cell Population
609 Frequencies in Human Peripheral Blood by Flow Cytometry. *The Journal of Immunology.*
610 2017 Jan 9;1601750.
- 611 46. Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, et al. Distribution
612 and compartmentalization of human circulating and tissue-resident memory T cell subsets.
613 *Immunity.* 2013 Jan 24;38(1):187–97.
- 614 47. Rathore DK, Nair D, Raza S, Saini S, Singh R, Kumar A, et al. Underweight full-term
615 Indian neonates show differences in umbilical cord blood leukocyte phenotype: a cross-
616 sectional study. *PLoS ONE.* 2015;10(4):e0123589.
- 617 48. Schaub B, Liu J, Höppler S, Schleich I, Huehn J, Olek S, et al. Maternal farm exposure
618 modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin*
619 *Immunol.* 2009 Apr;123(4):774-782.e5.
- 620 49. Paparo L, di Costanzo M, di Scala C, Cosenza L, Leone L, Nocerino R, et al. The influence
621 of early life nutrition on epigenetic regulatory mechanisms of the immune system.
622 *Nutrients.* 2014 Nov;6(11):4706–19.
- 623 50. Angela M, Endo Y, Asou HK, Yamamoto T, Tumes DJ, Tokuyama H, et al. Fatty acid
624 metabolic reprogramming via mTOR-mediated inductions of PPAR γ directs early activation
625 of T cells. *Nature Communications.* 2016 Nov 30;7:ncomms13683.

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627

628 **Supporting information:**

629 **Figure S1: Gating strategy of human B and T memory subsets**

630 **Figure S2: Intra-individual variance in immune subsets across one year (4 time points).**

631 Each dot indicates frequency of immune subset at a time point in an individual donor. Individual
632 donors are connected by lines. (n=45; only 10 donors shown for the sake of clarity).

633 Quantification and p-values shown in figure 1.

634 **Figure S3: A and B: NK cell depletion in chimera recipients post-irradiation. Dots represent data**

635 from individual mice. **C and D:** Reconstitution efficiency in B6.SJL-CBA/CaJ chimera (C) and

636 BALB/cJ-SJL/J chimera (D). Y-axis indicates ratio of injected CD45.1/CD452 cells to

637 reconstituted CD45.1/CD452 cells for the cell subset indicated. Dots indicate mean and error bars

638 indicate 95% confidence interval. P-values are as indicated.

639 **Figure S4: Quantification of EMCD4 and CMCD4 frequencies and counts in multiple**

640 **strains of mice examined (A to D)** (Statistical quantification for these comparisons are tabulated

641 in supplementary table S5). Boxplots show median and interquartile range. Upper whisker

642 extends till the highest value that is within 1.5 times the interquartile range from 3rd quartile.

643 Lower whisker extends till the lowest value that is within 1.5 times the interquartile range from

644 1st quartile. Outliers are shown as dots. Each group consisted of > 10 mice. Cell counts are

645 shown as number in millions.

646 E: mean EMCD4 and CMCD4 of each strain plotted together. CBA/CaJ and SJL/J show

647 differences in EMCD4 frequency (x-axis), but not in CMCD4 frequency (y-axis).

648 F- Correlations between EMCD4 and EMCD8 frequencies with mice from all strains pooled

649 together. Each dot represents a mouse (n=92). Correlation coefficient (spearman) and p-values

650 are indicated.

651 **Table S5: Statistical analysis of data shown in Figure S4**

- 652 **Table S6: List of differentially expressed genes in naïve CD4 T cells between SJL/J and**
653 **CBA/CaJ and gene enrichment analysis results**









