

1 **Potent but transient immunosuppression of T-cells is a general feature of**
2 **CD71⁺ erythroid cells**

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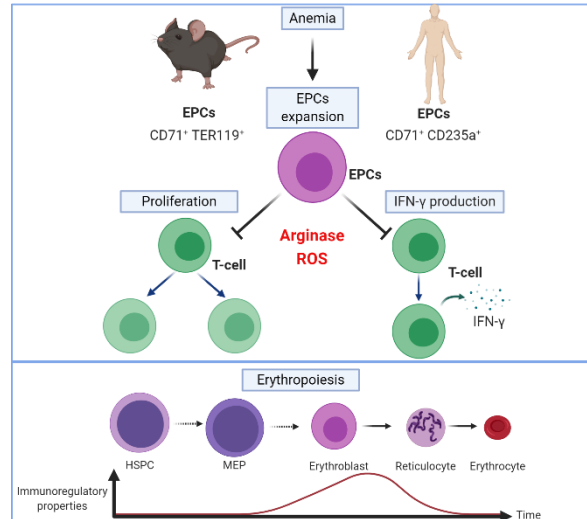
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50 **Abstract**

51 CD71⁺ erythroid cells (CECs) have been
52 recently recognized in both neonates and
53 cancer patients as potent
54 immunoregulatory cells. Here, we show
55 that in mice early-stage CECs expand in
56 anemia, have high levels of arginase 2
57 (ARG2) and reactive oxygen species



58 (ROS). In the spleens of anemic mice, CECs expansion-induced L-arginine depletion
59 suppresses T-cell responses. In humans with anemia, CECs expand and express
60 ARG1 and ARG2 that suppress T-cells IFN-γ production. Moreover, bone marrow
61 CECs from healthy human donors suppress T-cells proliferation. CECs differentiated
62 from peripheral blood mononuclear cells potently suppress T-cell activation,
63 proliferation, and IFN-γ production in an ARG- and ROS-dependent manner. These
64 effects are the most prominent for early-stage CECs (CD71^{high}CD235a^{dim} cells). The
65 suppressive properties disappear during erythroid differentiation as more
66 differentiated CECs and mature erythrocytes lack significant immunoregulatory
67 properties. Our studies provide a novel insight into the role of CECs in the immune
68 response regulation.

69 **Introduction**

70 CD71⁺ erythroid cells (CECs) normally reside in the bone marrow and are progenitors
71 and precursors to over 2×10^{11} of oxygen-transporting red blood cells (RBCs)
72 generated per day¹. In mice, when steady-state erythropoiesis becomes insufficient
73 to meet increased tissue oxygen demands, CECs are released from the bone marrow
74 to the circulation and expand in the extramedullary hematopoietic sites. In humans,
75 increased RBCs damage or loss of blood is compensated by increased erythropoietic
76 activity in the bone marrow. Recent studies revealed an unexpected complexity of
77 CECs functions. CECs arose as a relevant population of cells regulating immunity²⁻⁵.
78 Initially, CECs were reported to suppress both innate and humoral immune
79 responses in neonates^{4,6,7} and it was suggested that their immunomodulatory
80 functions are restricted to early life events⁴. However, further studies revealed a
81 crucial role of CECs in the regulation of multiple phenomena such as fetomaternal
82 tolerance⁸, immune response in cancer patients^{9,10}, systemic inflammation in colitis¹¹,
83 and anti-viral response in human immunodeficiency virus (HIV) infection¹², as well as
84 SARS-CoV-2-induced disease (COVID-19)¹³. It has been reported that CD45⁺ CECs
85 induced by advanced tumors inhibit CD8⁺ and CD4⁺ T-cell proliferation and impair
86 antimicrobial immunity¹⁰. Interestingly, the authors demonstrated that CECs from
87 mice with acute hemolytic anemia, induced by systemic phenylhydrazine (PHZ)
88 administration, are not immunosuppressive as compared with CECs from tumor-
89 bearing mice¹⁰. This could lead to the conclusion that only CECs in newborns and
90 patients with advanced cancer have robust immunosuppressive properties. In this
91 study, we provide evidence that CECs in anemic mice do have immunoregulatory
92 properties, but PHZ used to induce hemolysis affects the mechanisms of immune
93 suppression used by these cells masking their phenotype. Moreover, we

94 comprehensively elucidate the role of CECs in the regulation of immune response in
95 both mice and humans and demonstrate that immunomodulatory properties of CECs
96 are robust but transient and disappear during their maturation.

97 **Results**

98 **Early-stage CECs expand in the spleens of anemic mice**

99 We initially compared the expansion of CECs in 3 days old neonatal and adult
100 anemic mice (Fig. 1a). Non-hemolytic anemia (NHA) was induced by phlebotomy and
101 hemolytic anemia (HA) was induced either by administration of PHZ (HA-PHZ) or
102 anti-TER119 antibodies (HA-TER119) (see Supplementary Fig. 1 for hematological
103 parameters of these mice). Since in mice stress erythropoiesis rely on the
104 erythropoietic activity of the spleen^{14,15}, we assessed CECs expansion in this organ.
105 CECs expanded in the spleens of anemic mice as compared with controls but were
106 significantly less frequent than in neonatal mice (Fig. 1b). However, CECs numbers
107 in the spleen were substantially higher in anemic mice than in neonates or controls
108 (Fig. 1c). The percentage of CECs increased also in the blood of anemic mice
109 (Supplementary Fig. 2a) but remained unchanged in the bone marrow
110 (Supplementary Fig. 2b). Recent studies indicated that CECs at the earliest stages of
111 differentiation express CD45 and more potently suppress immune response^{9,10}. The
112 proportion of CD45⁺ to CD45⁻ CECs was the highest in HA-PHZ mice and the lowest
113 in neonatal mice (Fig. 1d). Analysis of developmental stages of CECs based on cell
114 size and CD44 levels (Fig. 1e)¹⁶ revealed enrichment of less differentiated CECs in
115 anemic mice compared to non-anemic controls (Fig. 1f, Supplementary Fig. 2c).
116 These early-stage CECs expressed CD45 (Fig. 1g,h) and were predominantly
117 erythroid progenitors before enucleation (Fig. 1i).

118

119 **The T-cell immune response is impaired in anemic mice**

120 Next, we sought to determine whether the expansion of early-stage CECs induced by
121 anemia might impair the function of the immune system. To this end, we assessed
122 selected functionalities of myeloid cells, B-cells, and T-cells in control and anemic
123 mice. In contrast to neonatal mice^{4,6}, production of TNF- α by splenic CD11b⁺ cells
124 after stimulation with heat-killed *E. coli* (HKEc) (Supplementary Fig. 3a,b) or the
125 concentration of anti-ovalbumin (OVA) IgG antibodies after OVA-ALUM immunization
126 (Supplementary Fig. 3c,d) was unimpaired in adult anemic mice as compared with
127 healthy controls. Intriguingly, we found that the proliferation of adoptively transferred
128 SIINFEKL-specific OT-I T-cells in response to OVA stimulation was decreased in the
129 spleen of NHA mice compared to healthy controls (Fig. 2a,b).

130 Since the expansion of CD71⁺ cells was the most substantial in the spleens of
131 anemic mice (Supplementary Fig. 3e) and the ratio of CECs number to T-cells
132 number was significantly increased in anemia (Supplementary Fig. 3f,g), we
133 hypothesized that CECs might be responsible for T-cells suppression. Indeed, CECs
134 isolated from the spleens of both HA and NHA anemic mice (Fig. 2c, Supplementary
135 Fig. 4a) suppressed the proliferation of CD4⁺ T-cells that were activated with anti-
136 CD3/CD28 beads (Fig. 2d). Altogether, these data document a rather selective
137 impairment of T-cell response by CECs in anemic mice.

138

139 **Murine CECs have high ROS levels and express ARG2**

140 Both ROS generation and expression of L-Arg-degrading enzymes were previously
141 identified as the effectors of the immunoregulatory activity of neonatal CECs^{4,17}.

142 Accordingly, we found that both cytoplasmic and nuclear ROS levels were higher in
143 anemia-induced CECs as compared with mature RBCs (Fig. 3a, Supplementary Fig.
144 5a,b). ROS reached the highest values in the CECs at the earliest stages of their
145 maturation (Supplementary Fig. 5c,d) i.e. in CD45⁺ CECs (Supplementary Fig. 5e,f).
146 Interestingly, in contrast to human CECs¹³, ROS levels in murine CECs, including
147 CD45⁺ CECs, were significantly lower than in the cells of non-erythroid lineages such
148 as myeloid cells or T-cells (Fig. 3b).

149 Murine CECs expressed ARG2, a mitochondrial arginase isoform (Fig. 3c,d), but had
150 almost undetectable cytosolic ARG1 based on intracellular staining (Fig. 3e) as well
151 as enhanced Yellow Fluorescent Protein (eYFP) signal in reporter B6.129S4-
152 Arg1^{tm1Lky}/J mice that express eYFP under *Arg1* promoter (Fig. 3f,g). Similar to ROS,
153 the levels of ARG1 and ARG2 were the highest in early-stage CECs and
154 consequently decreased during maturation (Supplementary Fig. 6a-g). Intriguingly,
155 while the level of ARG2 (Fig. 3c), as well as the percentage of ARG2⁺ CECs, were
156 similar in all groups (Fig. 3d), the fraction of ARG1⁺ cells was substantially higher in
157 HA-PHZ mice as determined by intracellular staining (Fig. 3e). This finding seems
158 counterintuitive considering that ARG-dependent degradation of L-arginine leads to
159 T-cell suppression^{18,19}, and we did not observe the suppression of T-cells in HA-PHZ
160 mice *in vivo* (Fig. 2b). Moreover, CECs from HA-PHZ mice exerted the weakest
161 suppressive effects on T-cells proliferation (Fig. 2d). Increased expression of ARG1
162 in HA-PHZ CECs was further confirmed by ARG1 mRNA detection (Supplementary
163 Fig. 7a) and in reporter B6.129S4-Arg1^{tm1Lky}/J mice (Fig. 3g,h) indicating that flow
164 cytometry findings were not artifactual. HA-PHZ CECs had increased expression of
165 ARG2 mRNA as compared with NHA CECs (Supplementary Fig. 7b), but no increase
166 in ARG2 protein levels was observed (Fig. 3d). Surprisingly, despite robust

167 upregulation of ARG1 levels, total arginase activity in both CECs isolated from HA-
168 PHZ mice and CECs-conditioned medium was lower even than that in CECs from
169 NHA mice (Fig. 3i,j). Moreover, CECs cultured *ex vivo* in the presence of PHZ
170 strongly upregulated ARG1 expression (Fig. 3k,l). Then, we sought to confirm
171 whether PHZ is responsible for the attenuation of CECs immunoregulatory
172 properties. Indeed, we found that CECs isolated from NHA lose their suppressive
173 effects on T-cells proliferation in the presence of PHZ (Supplementary Fig. 8a,b).

174

175 **PHZ targets arginase and suppresses its activity**

176 Increased expression with a concomitant decrease in arginase activity suggested an
177 interaction between PHZ and arginase. Further studies showed that indeed PHZ
178 inhibits the activity of recombinant human ARG1 and ARG2, with an IC₅₀ of 1017 μM
179 and 61 μM, respectively (Fig. 4a). However, PHZ did not affect the production of nitric
180 oxide (NO) by nitric oxide synthase, which is also using L-arginine as a substrate
181 (Fig. 4b). To elucidate how PHZ interacts with ARG1 and ARG2 a molecular docking
182 simulation was carried out with PHZ, L-arginine as well as 2-amino-6-borono-2-(2-
183 (piperidin-1-yl)ethyl)hexanoic acid (ABH) that is a strong ARG1 inhibitor²⁰. PHZ binds
184 to the active sites of all arginases, where it forms several polar interactions involving
185 D128, D232, or T246 (Supplementary Fig. 9a). Thus, it may block the entry of other
186 molecules to the active site. However, predicted binding energies suggest that
187 among the tested ligands PHZ has the weakest affinity for arginases, and thus a
188 significant concentration of this compound may be required to induce any biological
189 effect, which indeed is the case *in vivo*. The transient nature of interactions between
190 PHZ and arginases was also confirmed by a short 100 ns MD simulation
191 (Supplementary Fig. 9b,c). The ligand remained bound to the active site for only 15-

192 30% of the simulation time, despite its initial placement inside the ligand-binding
193 pocket. The analysis of electrostatic surface potential revealed the presence of a
194 large, negatively charged area around the substrate-binding pocket of ARG1 that
195 likely plays a role in attracting positively charged L-arginine to the catalytic site (Fig.
196 4c). Since PHZ in the presence of oxygen leads to the formation of free radicals and
197 hydrogen peroxide²¹, we hypothesized that decreased ARG activity in CECs from
198 HA-PHZ mice might emerge due to non-specific non-covalent interactions of PHZ
199 with the catalytic pocket of ARG1 that leads to oxidative changes in the enzyme,
200 decreased activity, and compensatory increase in its expression. Indeed, the
201 incubation of recombinant ARG1 with PHZ in the presence of oxygen led to a
202 significant increase in the carbonylation of the enzyme. However, this effect was only
203 slightly reduced by concomitant incubation with N-acetylcysteine (ROSi) (Fig. 4d).
204 Moreover, ROS scavengers did not prevent ARG1 induction by PHZ *in vivo*
205 (Supplementary Fig. 10a,b) nor *in vitro* (Supplementary Fig. 10c,d). Thus, we
206 demonstrated that PHZ targets ARG leading to the diminishment of CECs
207 immunoregulatory properties, however, the exact mechanism that would explain
208 PHZ-mediated inhibition of ARG activity remains elusive.

209

210 **CECs degrade L-Arg and produce ROS leading to the suppression of T-cells**

211 Due to the interaction between PHZ and arginases, we chose NHA as a model of
212 anemia-induced CECs for further studies. We found that CD4⁺ T-cells stimulated with
213 anti-CD3/CD28 beads in the presence of CECs showed downregulation of activation
214 markers CD25 and CD69, which was less pronounced for CD62L (Fig. 5a). Both
215 arginase inhibitor (ARGi, OAT-1746, a membrane-permeable, potent inhibitor of both
216 arginase isoforms²²⁻²⁴) and ROS inhibitor (ROSi, N-acetylcysteine) nearly completely

217 restored the proliferation of T-cells that was inhibited by co-culture with CECs
218 isolated from NHA mice (Fig. 5b), similar to CECs isolated from neonates
219 (Supplementary Fig. 11). Likewise, CECs-conditioned medium had a suppressive
220 effect on T-cell proliferation, and supplementation with either of L-arginine or ARGi
221 restored T-cell proliferation to percentages akin to the control group (Fig. 5c).

222 To confirm that early-stage CECs that have the highest ROS levels (Supplementary
223 Fig. 5) and ARG expression (Supplementary Fig. 6) have the most potent
224 suppressive effects on T-cells, we isolated the fraction of nucleated cells (erythroid
225 progenitors, developmental stages I-III, Fig. 1i) from CECs using density gradient
226 centrifugation. We found that the whole CECs population suppressed CD4⁺ T-cells
227 proliferation by 43% while isolated nucleated CECs (nCECs) completely inhibited it
228 (Fig. 5d), confirming that they are responsible for the suppressive effects.

229 At a 1:10 of T-cells to CECs ratio, similar to that observed in anemia (Supplementary
230 Fig. 3f,g), CECs completely suppressed the proliferation of CD4⁺ (Fig. 5e) and CD8⁺
231 T-cells (Fig. 5f). Further studies revealed that the expansion of CECs in anemic mice
232 leads to the substantial increase of the total arginase activity (Fig. 5g). This effect
233 was caused by an increased ARG2 but not ARG1 levels in the spleen (Fig. 5h-j).
234 Even though the concentration of L-arginine was only slightly decreased in the serum
235 of anemic mice (Supplementary Fig. 12), their splenic CD4⁺ T-cells and CD8⁺ T-cells
236 had decreased levels of CD3ζ (Fig. 5k,l), a marker of L-arginine T-cell starvation^{25,26}.
237 Accordingly, *ex vivo* stimulation of T-cells with anti-CD3/CD28 beads in the presence
238 of CECs resulted in a decrease in CD3ζ, which was prevented by ARGi and
239 completely restored by the combination of ARGi and ROSi (Fig. 5m,n). Noteworthy,
240 the decrease in CD3ζ was not observed in the lymph nodes of anemic mice, where
241 CECs are a relatively rare population (Supplementary Fig. 13a-c). Altogether, these

242 results show that CECs suppress T-cells response in anemic mice *via* both arginase
243 and ROS and their local accumulation in the spleen impairs T-cell immunity.

244 To further study the role of ARG2 in the modulation of immune response by CECs,
245 we assessed the suppressive effects of CECs isolated from anemic mice lacking
246 functional *Arg2* gene (*Arg2*^{-/-}, *Arg2*^{tm1W^{eo}/J} mice²⁷). *Arg2*^{-/-} mice had effective stress
247 erythropoiesis (Supplementary Fig. 14a). Despite a slightly increased percentage of
248 ARG1⁺ CECs compared to wild-type mice (*Arg2*^{+/+}) (Supplementary Fig. 14b), no
249 significant changes in total ARG1 levels were observed in these cells (Supplementary
250 Fig. 14c). In contrast to wild-type mice, expansion of CECs in the spleen of anemic
251 *Arg2*^{-/-} mice was not associated with a significant decrease in CD3ζ in T-cells (Fig.
252 6a,b). Moreover, CECs isolated from *Arg2*^{-/-} mice had substantially diminished
253 suppressive effects on T-cell proliferation as compared with *Arg2*^{+/+} CECs (Fig. 6c),
254 confirming a critical role of ARG2 in the regulation of T-cells function by murine
255 CECs.

256

257 **CECs expand in the blood of anemic individuals and suppress IFN-γ** 258 **production by T-cells**

259 Then, we sought to investigate the role of CECs in anemic patients (Supplementary
260 Table 1, Supplementary Table 2). The percentages of CECs (CD71⁺CD235a⁺) in the
261 peripheral blood were substantially increased in anemic patients compared to non-
262 anemic control individuals (Fig. 7a,b). The number of CECs in the blood (Fig. 7c)
263 reversely correlated with hemoglobin concentration (Fig. 7d) and was the highest in
264 patients with moderate and severe anemia (Fig. 7e).

265 In anemic patients, CECs constituted a substantial fraction of peripheral blood
266 mononuclear cells (PBMCs) (Fig. 7f,g) and were predominantly at the latest stages of
267 differentiation with a very small percentage of CD45⁺ CECs (Supplementary Fig.
268 15a,b). We found that the production of IFN- γ in response to CD3/CD28 stimulation
269 was suppressed in T-cells from anemic individuals when compared to non-anemic
270 controls (Fig. 7h, i). However, T-cells proliferation (Supplementary Fig. 15c,d) or the
271 production of TNF- α by myeloid cells in response to killed bacteria (Supplementary
272 Fig. 15e) were comparable in anemic patients and control individuals.

273

274 **CECs from human bone marrow suppress T-cells proliferation**

275 Since the expansion of CECs in the peripheral blood of anemic individuals was not
276 associated with the suppression of T-cells proliferation, we investigated the
277 immunoregulatory properties of CECs from the healthy human bone marrow (Fig.
278 8a). CECs in the bone marrow are enriched with early-stage CECs (Supplementary
279 Fig. 16a,b) and are predominantly CD45⁺ (Supplementary Fig. 16c,d). Similar to
280 murine CECs, their counterparts in the human bone marrow express ARG2 (Fig. 8b-
281 d). Importantly, human erythroid cells also express ARG1 (Fig. 8b-d). The expression
282 of both ARG isoforms was higher in CD45⁺ than in CD45⁻ CECs (Supplementary Fig.
283 16e,f). CECs isolated from human bone marrow (Supplementary Fig. 16g)
284 significantly suppressed proliferation of both CD4⁺ and CD8⁺ T-cells (Fig. 8e,f).
285 These effects were diminished by the ARGi, confirming arginase-dependent effects
286 of human CECs.

287

288 **Erythroleukemia-derived erythroid cell lines suppress T-cells in an ARG- and**
289 **ROS-dependent mechanism**

290 Further, we investigated the immunoregulatory properties of model human
291 erythroleukemia-derived erythroid cell lines: K562, HEL92.1.7, and TF-I. These cells
292 express multiple erythroid-lineage markers, including CD71 and CD235a (Fig. 9a),
293 and spontaneously differentiate into erythroblast-like cells. We found that similarly to
294 primary CECs, erythroid cells have substantial arginase activity (Supplementary Fig.
295 17a), express both ARG1 (Supplementary Fig. 17b) and ARG2 (Supplementary Fig.
296 17c), and have high ROS levels (Supplementary Fig. 17d). Notably, all examined
297 types of erythroid cells potently suppressed proliferation of human CD4⁺ (Fig. 9b-e)
298 and CD8⁺ (Fig. 9f-i) T-cells in an ARG- and ROS-dependent manner (Fig. 9e,i,
299 Supplementary Fig. 18a,b).

300

301 **Suppression of T-cells function is a general feature of erythroid cells which**
302 **disappears during their maturation**

303 Our results demonstrated that T-cell suppression is a common feature of both murine
304 and human CECs. Apparently the immunoregulatory properties were the most potent
305 at the earliest stages of differentiation when the levels of ROS, ARG1, and ARG2 are
306 the highest. Therefore, we next sought to establish a model of *ex vivo* differentiation
307 of erythroid cells. To this end, CECs were expanded and differentiated from PBMCs
308 of healthy human donors (Supplementary Fig. 19a). PBMC-derived CECs expressed
309 erythroid markers, including CD71, CD235a, CD36, and CD49d, and had high
310 expression of CD44 and CD45 (Supplementary Fig. 19a,b). Similar to their bone
311 marrow counterparts, PBMCs-derived CECs had high levels of both ARG1 and

312 ARG2 (Supplementary Fig. 20a,b). Moreover, isolated PBMC-derived CECs (Fig.
313 10a) potently suppressed both CD4⁺ and CD8⁺ human T-cell proliferation (Fig.
314 10b,c).

315 Next, we aimed to study the possible changes in immunoregulatory properties of
316 erythroid cells during differentiation into RBC. First, we investigated whether
317 hematopoietic stem and progenitor cells (HSPCs) exert immunosuppressive effects.
318 Mobilized hematopoietic stem cells obtained from peripheral blood (peripheral blood
319 stem cells, PBSCs, Supplementary Fig. 21a) had high ARG1 and ARG2 levels
320 (Supplementary Fig. 21b) and included only a small percentage of CECs
321 (Supplementary Fig. 21c). Despite high arginase expression, peripheral blood stem
322 cells had no impact on T-cell proliferation (Supplementary Fig. 21d,e).

323 Then, using continuous CECs culture, we demonstrated that CECs differentiated
324 from PBMCs (Fig. 10d) exert robust, but transient suppressive properties, that
325 disappear during erythroid differentiation (Fig. 10e-g). We found that of all CECs
326 developmental stages, cells at the stage of CD71^{high} CD235a^{mid} most strongly
327 inhibited T-cells proliferation (Fig. 10f,g). Moreover, these cells potently suppressed
328 T-cells activation based on the CD25 and CD69 levels (Supplementary Fig. 22a,b) as
329 well as inhibited IFN- γ production by T-cells (Supplementary Fig. 22c). The
330 suppression depended on both ARG and ROS since only the combination of ARGi
331 and ROSi significantly diminished suppressed T-cell activation, IFN- γ production
332 (Supplementary Fig. 22c), and proliferation (Supplementary Fig. 23a,b). Loss of the
333 suppressive properties corresponded with a decrease in CD71 (Fig. 10h), an
334 increase in CD235a (Fig. 10i) as well as a decrease in CD49d (Fig. 10k) levels, the
335 latter being a marker of the transition to the reticulocyte stage^{28,29}. Subsequent CECs
336 differentiation resulted in a complete loss of suppressive effects on T-cells.

337 Further, we observed that induction of erythroid differentiation of K562 cells by
338 sodium butyrate³⁰ (Supplementary Fig. 24a) was associated with a decrease of
339 immunosuppressive effects on T-cells (Supplementary Fig. 24b). These differentiated
340 cells had decreased ARG2, but not ARG1 levels (Supplementary Fig. 24c), and
341 decreased total arginase activity as compared with non-differentiated K562 cells
342 (Supplementary Fig. 24d). Downregulation of ARG2 was most probably caused by
343 mitophagy, a crucial process during erythroid differentiation³¹, as evidenced by the
344 decreased signal from a mitochondrial probe in differentiated K562-erythroid cells
345 (Supplementary Fig. 24e). Similar changes in ARG expression were also detected in
346 primary murine (Supplementary Figure 6b-e) and human CECs (Supplementary
347 Figure 20b). Finally, we demonstrated that mature RBCs obtained from healthy
348 donors had no impact on T-cell proliferation (Supplementary Fig. 25a-c). Altogether,
349 we show that human CECs possess robust, but transient suppressive properties that
350 are most potent in the earliest developmental stages and disappear during erythroid
351 cell maturation.

352

353 **Discussion**

354 In this study, we demonstrate that suppression of T-cells is a general feature of
355 murine and human CECs. Anemic CECs *via* arginases and ROS suppress
356 proliferation and production of IFN- γ by T-cells. Using continuous human erythroid
357 cell culture, we show that the immunoregulatory properties of CECs are transient and
358 disappear during maturation.

359 Recent studies have broadened our understanding of the many roles played
360 by CECs expanded by different triggers³. Immunoregulatory functions of CECs were

361 reported for the first time in neonates that are characterized by a physiological
362 abundance of CECs⁴. Neonatal CECs suppress anti-bacterial immunity *via* ARG2 by
363 decreasing the production of proinflammatory cytokines by myeloid cells⁴ and by
364 suppressing antibody production in response to *B. pertussis*⁶. We found that in adult
365 mice anemia induced the expansion of early-stage CECs that had the highest
366 expression of ARG2. Neither ARG2-expressing CECs nor recombinant ARG1
367 suppressed the production of TNF- α from myeloid cells. However, arginases seem to
368 primarily impair T-cells by decreasing their activation and proliferation³². Accordingly,
369 we observed decreased proliferation of adoptively transferred OT-I cells in the spleen
370 of anemic mice, which was reflected *ex vivo* in the co-culture of murine T-cells with
371 CECs. Expansion of CECs in the spleen of anemic mice resulted in the increased
372 ARG activity in the spleen leading to the L-arginine starvation of T-cells, decreased
373 levels of CD3 ζ , and suppressed proliferation. Moreover, human CECs expressed
374 both ARG1 and ARG2 and suppressed T-cell proliferation in an ARG-dependent
375 manner. Thus, expansion of ARG-expressing CECs in anemia may induce immune
376 suppression, similar to the expansion of ARG-expressing myeloid cells in cancer³³
377 and during pregnancy³⁴.

378 CECs were also reported to modulate immune response *via* ROS in tumor-
379 bearing mice and cancer patients¹⁰. We found that ROSi restored T-cell proliferation
380 in co-culture with CECs from anemic mice to a similar extent as ARGi. Importantly,
381 ROS also may decrease CD3 ζ in T-cells³⁵. However, ROSi restored CD3 ζ decreased
382 by CECs only in combination with ARGi, which confirms that ARG cooperates with
383 ROS in CECs to induce T-cells hyporesponsiveness to proliferative triggers.

384 Importantly, we demonstrated that previously described lack of
385 immunosuppressive capacities of CECs in anemic mice¹⁰ resulted from the

386 interaction between PHZ used to induced anemia and ARGs. PHZ-induced hemolytic
387 anemia is one of the most commonly used models of anemia. PHZ leads to the
388 formation of ferrihemoglobin from oxyhemoglobin and production of free radicals that
389 disrupt the interactions between hem and globin chains leading to the formation of
390 Heinz bodies and hemolysis²¹. However, PHZ-induced CECs are less effective in
391 suppressing T-cell proliferation as compared with CECs isolated from neonatal or
392 other anemic mice. We show that PHZ targets ARGs, critical immunomodulating
393 enzymes. It needs to be considered in future studies that the interaction between
394 PHZ and ARGs may have considerable effects on the obtained results.

395 Anemia correlates with worse outcomes in many diseases, including
396 pneumonia³⁶ or cancer³⁷. Moreover, preoperative anemia is associated with an
397 increased risk of infection and mortality in patients undergoing surgery^{38,39}. We
398 demonstrated that CECs expand in anemic patients and may suppress the
399 production of cytokines by T-cells. A small fraction of CD45⁺ CECs in the peripheral
400 blood of our cohort subject is consistent with a recent report on CECs in systemic
401 juvenile idiopathic arthritis patients⁴⁰. In line with our results, a recent study showed
402 that anemia status influences the blood transcriptome with enrichment of erythrocyte
403 differentiation genes as well as ARG1 in anemic children, but decreased signatures
404 of CD4⁺ T-cell activation and differentiation⁴¹. It remains unknown to which extent
405 CECs are responsible for immune suppression and whether in these conditions
406 supplementation of iron, vitamin B12, or administration of erythropoiesis-stimulating
407 agents including EPO may restore immune response.

408 Erythropoiesis is a continuum of developmental states that gives mature red
409 blood cells from a hematopoietic stem cell (HSC) and is strictly regulated by multiple
410 factors⁴². Recent studies demonstrated that immunomodulatory properties are strong

411 in early-stage CD45⁺ CECs in contrast to more mature CD45⁻ CECs that lack these
412 capacities^{9,10,17,43}. However, other studies also reported the immunomodulatory role
413 of CD45⁻ CECs^{40,44}, which suggests that CD45 alone may not be a reliable marker of
414 immunosuppressive CECs. Here, we showed that human CECs acquire
415 immunomodulatory properties during erythroid differentiation and are the most potent
416 in an early stage characterized by the CD71^{high} CD235a^{mid} phenotype. Further
417 erythroid maturation is associated with the disappearance of the suppressive
418 properties. These early-stage CECs are relatively rare in the peripheral blood of
419 anemic individuals, which may be a reason for the lack of T-cell proliferation
420 suppression. In contrast, human bone marrow is enriched in early-stage CECs that
421 suppress T-cells proliferation. Nonetheless, the suppression of T-cells by bone
422 marrow CECs was substantially weaker than that of CD71^{high} CD235a^{mid} CECs from
423 *ex vivo* culture. This may be explained by the fact that in human bone marrow CECs
424 at the earliest stages of differentiation are still much less abundant than late-stage
425 CECs^{28,45}.

426 The exact role of transient immunomodulatory properties of CECs remains
427 elusive. It was suggested that expansion of CECs in neonates provides tolerance to
428 harmless antigens, including the commensal microbiota⁴, and minimize damage
429 caused by inflammation in the intestines⁴, liver⁷, and lungs⁴⁶ during the first days of
430 postnatal life. In adults, the role of CECs seems to be similar. Recent studies
431 demonstrated that stress erythropoiesis is a key inflammatory response⁴⁷, therefore,
432 expansion of CECs may prevent progression to chronic inflammation. Indeed,
433 transfer of CECs suppressed inflammatory response and attenuated the wasting
434 syndrome in murine models of colitis¹¹. In cancer, which is characterized by a chronic
435 inflammation⁴⁸, CECs substantially expand and suppress immune response

436 facilitating tumor growth and increasing the susceptibility to pathogens¹⁰. On the
437 other hand, impaired immunoregulatory properties of CECs may exacerbate the
438 damage caused by inflammation⁴⁹. Moreover, CECs by suppressing production of
439 IFN- γ , a crucial inflammatory cytokine and a potent inhibitor of erythropoiesis^{50,51},
440 may allow maintenance of erythropoiesis and play a role in preventing systemic
441 inflammation.

442 Importantly, most of the knowledge on CECs has been build based on murine
443 models⁵. However, several crucial divergences between mice and humans may limit
444 the translational character of CECs studies in mice, that include differences in stress
445 erythropoiesis⁵². In mice, stress erythropoiesis primarily takes place in the spleen and
446 relies on the expansion of early-stage CECs⁵³. Thus, suppressive CECs may interact
447 with immune cells in the spleen, which is also an active immune organ⁵⁴. We
448 observed that T-cells in anemic mice are rather locally suppressed in the spleen,
449 while not affected in the lymph nodes, similar to the conditions described for
450 neonates⁴. In contrast, in humans stress erythropoiesis primarily involves the bone
451 marrow, and expansion of CECs in extramedullary sites is rather occasional^{5,52}.
452 Moreover, CECs at the earliest stages of differentiation are relatively rare in healthy
453 individuals⁴⁵. Thus, it seems that CECs may have the most significant
454 immunoregulatory role under conditions that are characterized by the impaired
455 erythroid differentiation and robust enrichment of early-stage CECs, that include
456 cancer⁵⁵.

457 Our findings might be of relevance in better understanding the mechanisms
458 underlying suppressed cell-mediated immunity and anti-bactericidal capacity of
459 leucocytes⁵⁶ and the impaired of T-cell mediated immunity in anemic children⁵⁷.

460 Moreover, our study suggests that CECs may be a crucial regulator of immune
461 response in different disease conditions.

462 **Methods**

463 **Reagents**

464 Recombinant human ARG1 was obtained from Biolegend (San Diego, CA, USA),
465 recombinant murine Arg1 was obtained from Cloud-Clone Corp., arginase inhibitor
466 OAT-1746 was synthesized at OncoArendi Therapeutics, Warsaw, Poland. All other
467 reagents, if not otherwise stated, were obtained from SigmaAldrich.

468

469 **Cell lines**

470 K562, HEL92.1.7 and TF-I cell lines were purchased from American Type Culture
471 Collection (ATCC). Cells were cultured in RPMI-1640 medium supplemented with
472 10% heat-inactivated fetal bovine serum (FBS, HyClone), 2 mM L-glutamine
473 (SigmaAldrich) 100 U/ml penicillin and 100 µg/ml streptomycin (SigmaAldrich) at
474 37°C in a humidified atmosphere of 5% CO₂ in the air. Additionally, TF-I cells medium
475 contained 2 ng/ml of recombinant human GM-CSF (R&D Systems). Cells have been
476 cultured for no longer than 4 weeks after thawing and were regularly tested for
477 *Mycoplasma* contamination using PCR technique and were confirmed to be negative.

478

479 **Human samples**

480 Human PBMCs used as a source of CD4⁺ and CD8⁺ T-cells and used for the
481 expansion and differentiation of PBMC-derived CECs were isolated from buffy coats
482 obtained commercially from the Regional Blood Centre in Warsaw, Poland. PBMCs
483 were isolated by density gradient centrifugation method using Histopaque[®]-1077
484 (SigmaAldrich) or Lymphoprep[™] (STEMCELL Technologies), according to the
485 manufacturer's protocols. All donors were males between the ages of 18 and 45
486 years old. Donors were screened for general health and qualified by the physician for

487 blood donation. All donors had negative clinical laboratory tests for HIV-1, HIV2,
488 hepatitis B, and hepatitis C and hematology values within normal ranges.

489 Peripheral blood samples were obtained from patients hospitalized in the
490 Central Teaching Clinical Hospital, Medical University of Warsaw or treated in the
491 Outpatient Clinic of Central Teaching Clinical Hospital, Medical University of Warsaw,
492 Warsaw. The study was conducted in accordance with the Declaration of Helsinki.
493 The study was approved by the Bioethical Committee of the Medical University of
494 Warsaw (KB/8/2021). Patients with or without anemia based on WHO diagnostic
495 criteria⁵⁸ were enrolled in the study. Patients with proliferative diseases, including
496 cancer, were excluded. The blood samples were obtained by venipuncture and
497 subjected to complete blood count evaluation. The remaining blood was used for
498 further examination. Flow cytometry was performed as described below.
499 CountBright™ Absolute Counting Beads (Thermo Fisher Scientific) were used for
500 CECs counting. PBMCs were purified from whole blood of anemic and non-anemic
501 patients by density separation using Lymphoprep (STEMCELL Technologies).

502 Human bone marrow samples were commercially obtained from Lonza
503 Walkersville, Inc. Aspirates were withdrawn from bilateral punctures of the posterior
504 iliac crests. Every 100 ml of bone marrow was collected into syringes containing 10
505 ml of heparin (Porcine Intestinal Mucosa) Sodium Injection (~100 units heparin per ml
506 bone marrow). Bone marrow samples were obtained from both healthy males (n=6)
507 and healthy non-pregnant females (n=3) US-based donors between the ages of 23
508 and 45 years old. Samples were collected after obtaining permission for their use in
509 research applications by informed consent or legal authorization. All donors were
510 screened for general health and negative medical history for heart disease, kidney

511 disease, liver disease, cancer, epilepsy, and blood or bleeding disorders. All donors
512 had negative clinical laboratory tests for HIV-1, HIV2, hepatitis B, and hepatitis C.

513 Mobilized peripheral blood stem cells (PBSCs) were obtained from familial
514 donors from the material remaining after allogeneic stem cell transplantation. PBSCs
515 were mobilized with the granulocyte colony-stimulating factor (G-CSF) and isolated
516 from the donor peripheral blood using apheresis according to the standard clinical
517 protocol. Informed consent was obtained from PBSCs donors. Collected PBSCs were
518 subjected to density gradient centrifugation using Lymphoprep (STEMCELL
519 Technologies) to remove dead cells and debris, washed three times with RPMI
520 medium, and used for the analysis.

521

522 **Mice**

523 Wild-type C57BL/6 mice, both male and female, 8 to 14-week-old were obtained from
524 the Animal House of the Polish Academy of Sciences, Medical Research Institute
525 (Warsaw, Poland). Transgenic mice of C57BL/6 genetic background, B6.129S4-
526 $Arg1^{tm1Lky}/J$ (YARG Mice co-expressing Arg1 and enhanced yellow fluorescent
527 protein (eYFP)⁵⁹, stock #015857), C57BL/6-Tg(TcraTcrb) 1100Mjb/J (OT-I, TCR
528 transgenic mice producing OVA peptide-specific CD8⁺ T cells⁶⁰, stock #003831) and
529 $Arg2^{tm1Weo}/J$ ($Arg2^{-/-}$, Arg2 functional knockout²⁷), were purchased from the Jackson
530 Laboratories and bred at the animal facility of the Department of Immunology,
531 Medical University of Warsaw. Mice were housed in controlled environmental
532 conditions in specific-pathogen-free (SPF) conditions (breeding cages, OT-I mice) or
533 conventional (others) animal facility of the Department of Immunology, Medical
534 University of Warsaw, with water and food provided ad libitum. For mice genotyping,
535 DNA was isolated with DNeasy Blood & Tissue Kit (QIAGEN) according to the

536 manufacturer's instructions. The concentration and purity of DNA were determined
537 using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). PCR
538 reaction was performed using OneTaq[®] 2× Master Mix with Standard Buffer (New
539 England Biolabs). Primers sequences, PCR, and agarose electrophoresis conditions
540 were set according to genotyping protocols available on The Jackson Laboratory
541 website (<https://www.jax.org>). The experiments were performed in accordance with
542 the guidelines approved by the II Local Ethics Committee in Warsaw (approval No.
543 WAW2/117/2019 and WAW2/143/2020) and in accordance with the requirements of
544 the EU (Directive 2010/63/EU) and Polish (Dz. U. poz. 266/15.01.2015) legislation.

545

546 **Animal anemia models**

547 To induce non-hemolytic anemia (NHA) mice were phlebotomized 4 and 2 days
548 before harvest. At least 100 µl of blood was collected each time. To induce hemolytic
549 anemia (HA), mice were injected intraperitoneally (i.p.) three days before harvest with
550 50 mg per kg body weight of phenylhydrazine (PHZ) hydrochloride solution (HA-PHZ)
551 or mice were injected intravenously (i.v.) six days before harvest with 45 µg of anti-
552 TER119 monoclonal antibody (TER-119, BioXCell) into the caudal vein. For the
553 analysis of stress erythropoiesis, mice were injected i.v. with 30 µg of anti-TER119
554 monoclonal antibody (TER-119, BioXCell) into caudal vein followed by the monitoring
555 of RBC count in the peripheral blood. For complete blood count, blood was collected
556 into EDTA-coated tubes from inferior palpebral veins and examined using Sysmex
557 XN-2000 Hematology Analyzer. The parameters of complete blood counts and
558 reference intervals⁶¹ are presented in Supplementary Fig. 1. For the analysis of
559 amino acid concentration, blood was collected into IMPROMINI[®] Gel & Clot Activator
560 Tubes. Tubes were gently inverted five times to mix the clot activator and incubated

561 for 30 min at room temperature (RT) followed by centrifugation for 10 min at 1000 x g
562 at 4°C. Serum was collected and stored at -20°C until analysis. L-arg concentration in
563 the serum was determined with ultra-performance liquid chromatography-tandem
564 mass spectrometry (UPLC-MS/MS) method on Waters Xevo TQ-S mass
565 spectrometer equipped with Waters Acquity UPLC chromatograph (Waters) in the
566 Mass Spectrometry Lab at the Institute of Biochemistry and Biophysics, Polish
567 Academy of Sciences, Warsaw, Poland.

568

569 **Antibodies**

570 Fluorophore- or biotin-conjugated antibodies specific for mouse cell-surface antigens
571 and cytokines were as follows: anti-CD71 (8D3, NovusBio; R17217, eBioscience),
572 anti-TER119 (TER-119, BioLegend), anti-CD45.2 (104, BD Biosciences), anti-CD45
573 (30-F11, BioLegend), anti-CD44 (IM7, BioLegend), anti-CD3e (145-2C11,
574 eBioscience), anti-CD4 (GK1.5, eBioscience; RM4-5. eBioscience), anti-CD8a (53-
575 6.7, eBioscience), anti-CD69 (H1.2F4, eBioscience), anti-CD25 (PC61.5,
576 eBioscience), anti-CD62L (MEL-14, Invitrogen), anti-CD11b (M1/70, BioLegend),
577 anti-CD11c (HL3, BD Bioscience), anti-CD3 zeta (H146-968, Abcam), anti-IFN- γ
578 (XMG1.2, eBioscience), anti-TNF- α (MP6-XT22, eBioscience), anti-Arg1 (polyclonal,
579 IC5868P/F, R&D Systems), anti-Arg2 (ab81505, Abcam), goat anti-rabbit IgG
580 (Invitrogen).

581 Fluorophore- or biotin-conjugated antibodies specific for human cell-surface antigens
582 and cytokines were as follows: anti-CD71 (CY1G4, BioLegend, DF1513, NovusBio),
583 anti-CD235a (HI264, BioLegend), anti-CD44 (IM7, BioLegend), anti-CD25 (BC96,
584 eBioscience), anti-CD69 (FN50, e Bioscience), anti-CD45 (HI30, BD Bioscience),
585 anti-CD49d (9F10, eBioscience), anti-CD36 (NL07, eBioscience), anti-CD34 (561,

586 BioLegend), anti-CD3 (OKT3, eBioscience), anti-CD4 (RPA-T4, eBioscience), anti-
587 CD8a (RPA-T8, eBioscience), anti-IFN- γ (4S.B3, BioLegend), anti-TNF- α (MAb11,
588 BD Bioscience), anti-Arg1 (polyclonal, IC5868P/F, R&D Systems), anti-Arg2
589 (ab137069, Abcam), goat anti-rabbit IgG (Invitrogen).

590

591 **Flow cytometry analysis**

592 Flow cytometry was performed on FACSCanto II (BD Biosciences) or Fortessa X20
593 (BD Biosciences) operated by FACSDiva software. For data analysis Flow Jo v10.6.1
594 software (TreeStar) or BD FACSDiva software (BD Biosciences) were used. Murine
595 whole blood was collected from the inferior palpebral vein to EDTA-coated tubes.
596 Spleens were isolated from mice and mechanically dispersed by pressing gently
597 through a 70 μ m nylon cell strainer using a rubberized 1 ml syringe piston. Murine
598 bone marrow was isolated from the femur by the centrifugation method. Briefly,
599 femurs were dissected, followed by the removal of any muscle or connective tissue.
600 The condyles and epiphysis were removed and a cleared bone was placed in
601 microcentrifuge tubes followed by centrifugation at 2500 x g for 30 sec. Bone marrow
602 cells were filtered through a 70 μ m nylon strainer and used for further analysis. No
603 erythrocyte lysis was performed in flow cytometry analysis or experiments that
604 involved analysis or isolation of CECs or RBCs. If only other types of cells were
605 analyzed using flow cytometry, erythrocytes were lysed using ACK (Ammonium-
606 Chloride-Potassium) Lysing Buffer (Thermo Fisher Scientific), according to the
607 manufacturer's protocol.

608 For cell surface staining, cells were stained with Zombie NIR[™], Zombie UV[™] or
609 Zombie Aqua[™] Fixable Viability Kit (BioLegend), blocked on ice with 5% normal rat
610 serum in FACS buffer (PBS; 1% BSA, 0.01% sodium azide), and then incubated for

611 30 min on ice with fluorochrome-labeled antibodies. Fluorochrome-conjugated
612 antibodies used for the staining are listed above. After washing in FACS buffer, cells
613 were immediately analyzed.

614 For nucleus staining, cells were incubated before cell surface staining with Hoechst
615 33342 Fluorescent Stain (Invitrogen) at a final concentration of 1 $\mu\text{g/ml}$ in Dulbecco's
616 phosphate-buffered saline (DPBS) for 30 min, followed by a wash in DPBS. For
617 mitochondrial staining, cells were incubated before cell surface staining with
618 MitoSpy™ Red CMXRos (BioLegend) at a final concentration of 50 nM in RPMI
619 medium at 37°C for 30 min, followed by a wash in FACS buffer.

620 For intracellular staining, membrane-stained cells were fixed using Fixation Buffer for
621 30 min at RT, followed by a wash with permeabilization buffer, and staining with an
622 antibody diluted in permeabilization buffer for 30 min at RT (Intracellular Fixation &
623 Permeabilization Buffer Set, eBioscience). For anti-ARG2 indirect intracellular
624 staining, cells were fixed using Fixation Buffer for 30 min at RT, followed by a wash
625 with permeabilization buffer, and staining with anti-ARG2 antibody for 1 h at RT,
626 followed by a wash with permeabilization buffer and staining with fluorochrome-
627 conjugated goat anti-rabbit IgG for 30 min at RT. Gating strategies used to analyze
628 the flow cytometry data are presented in Supplementary Figures 26-44.

629

630 **IFN- γ and TNF- α production assay of murine cells**

631 Murine splenocytes were isolated from anemic or healthy mice. Cells were plated in
632 round-bottomed 96-well plates (1×10^6 cell per well) in L-arginine-free RPMI-medium
633 (SILAC RPMI-medium, Thermo Fisher Scientific) supplemented with 10% dialyzed
634 FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$

635 streptomycin, 40 mg/L L-lysine, and 150 μ M L-arginine (all from Sigma-Aldrich).
636 Splenocytes were stimulated with Heat Killed *E. coli* 0111:B4 (HKEc, InvivoGen) at
637 the concentration 1×10^6 cells per ml or Dynabeads Mouse T-Activator CD3/CD28
638 (ratio 1:2, Thermo Fisher Scientific) in the presence of protein transport inhibitor (BD
639 GolgiStop™) for 6 hours. Then, cells were stained with cell surface antigens-binding
640 antibodies, followed by fixation, permeabilization, and intracellular staining for IFN- γ
641 and TNF- α . Flow cytometry was performed on Fortessa X20 (BD Biosciences). Cell
642 viability after culture in the presence of protein transport inhibitor was >80%.

643

644 **IFN- γ and TNF- α production assay of human cells**

645 Human PBMCs were isolated from the peripheral blood of anemic or non-anemic
646 patients. Cells were plated in round-bottomed 96-well plates (1×10^6 cell per well) in L-
647 arginine-free RPMI-medium (SILAC RPMI-medium, Thermo Fisher Scientific)
648 supplemented with 10% dialyzed FBS (Thermo Fisher Scientific), 2 mM glutamine,
649 100 U/ml penicillin, 100 μ g/ml streptomycin, 40 mg/L L-lysine, and 150 μ M L-arginine
650 (all from Sigma-Aldrich). PBMCs were stimulated with Heat Killed *E. coli* 0111:B4
651 (HKEc, InvivoGen) at the concentration 1×10^6 cells per ml for TNF- α assessment in
652 myeloid cells or Dynabeads Human T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher
653 Scientific) for IFN- γ assessment in T-cells in the presence of protein transport
654 inhibitor (BD GolgiStop™) for 12 hours. Then, cells were stained with cell surface
655 antigens-binding antibodies, followed by fixation, permeabilization, and intracellular
656 staining for IFN- γ and TNF- α . Flow cytometry was performed on Fortessa X20 (BD
657 Biosciences). Cell viability after culture in the presence of protein transport inhibitor
658 was >93%.

659 For the analysis of the effect of PBMC-derived CECs on T-cells activation and IFN- γ
660 production, PBMCs were isolated from the peripheral blood healthy blood donor.
661 CD4⁺ or CD8⁺ T-Cells were isolated from PBMC using EasySep™ Human CD4⁺ or
662 CD8⁺ T-Cell Isolation Kit (STEMCELL Technologies) according to the manufacturer's
663 protocols. CD4⁺ or CD8⁺ T-Cells were plated in round-bottomed 96-well plates (2×10⁴
664 cell per well) in L-arginine-free RPMI-medium (SILAC RPMI-medium, Thermo Fisher
665 Scientific) supplemented with 10% dialyzed FBS (Thermo Fisher Scientific), 2 mM
666 glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 40 mg/L L-lysine, and 150 μ M
667 L-arginine (all from Sigma-Aldrich). T-cells were stimulated with Dynabeads Human
668 T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher Scientific) in the presence of PBMC-
669 derived CECs (ratio 1:2, 4×10⁵ CECs per well). The arginase inhibitor OAT-1746
670 (1500 nM), or N-acetylcysteine (200 μ M) were added as indicated in the figures. In
671 these concentrations, ARGi and ROSi had no effects on T-cells nor CECs viability.
672 Human T-cells were incubated for 72 h at 37°C in 5% CO₂. Protein transport inhibitor
673 (BD GolgiStop™) was added for the last 12 hours. Then, cells were stained with cell
674 surface antigens-binding antibodies, followed by fixation, permeabilization, and
675 intracellular staining for IFN- γ and TNF- α . Flow cytometry was performed on
676 Fortessa X20 (BD Biosciences). Cell viability after culture in the presence of protein
677 transport inhibitor was >86%.

678

679 ***In vivo* OVA immunization and analysis of the humoral response**

680 Control and NHA mice were immunized with albumin from chicken egg white (OVA,
681 Ovalbumin) from Sigma (Grade VII). Each mouse received 25 μ g of OVA with
682 Imject™ Alum Adjuvant (ALUM, Thermo Fisher Scientific) at a ratio of 1:1 in the final
683 volume of 100 μ l per mouse administered i.p. After 14 days, mice were challenged

684 once again with the same dose of OVA-ALUM. NHA mice were divided into three
685 groups. NHA before mice were phlebotomized before first immunization, NHA boost
686 mice were phlebotomized before second OVA immunization, and NHA both were
687 phlebotomized before first and second immunization (see Supplementary Fig. 3c).
688 Untreated mice received Imject™ Alum Adjuvant without OVA. Blood was obtained
689 from mice 14 days after the second immunization, plasma was isolated and stored at
690 -80°C. The concentration of anti-OVA IgG antibodies was determined using Anti-
691 Ovalbumin IgG1 (mouse) ELISA Kit (Cayman Chemical).

692

693 ***In vivo* proliferation assay**

694 OVA (SIINFEKL)-specific CD8⁺ T cells were isolated from the spleen and lymph
695 nodes of healthy 6-8-week old OT-I mice using EasySep™ Mouse CD8⁺ T-Cell
696 Isolation Kit (STEMCELL Technologies) according to the manufacturer's protocols.
697 Isolated OT-I cells labeled with CTV for 20 min at 37°C at a final concentration of 2.5
698 µM in PBS, washed and transferred into the caudal tail vein of host C57BL/6 mice at
699 a cell number of 7×10⁶ in 150 µl of PBS. Twenty-four hours post OT-I T-cells
700 inoculation, host mice were challenged with 7.5 µg of full-length OVA protein (grade
701 V, Sigma Aldrich) injected into the caudal tail vein. Three mice from controls were
702 injected only with PBS (negative control). On day 3 post OVA immunization, spleens
703 were harvested, mashed through a 70 µm nylon strainer, stained with OVA-specific
704 MHC tetramers (iTAg Tetramer/PE-H-2 K^b OVA (SIINFEKL), MBL Inc., WA, USA) to
705 detect OT-I CD8⁺ T-cells, followed by anti-CD3 and anti-CD8 staining, and analyzed
706 for proliferation by flow cytometry. The gate for proliferating cells (CTV^{low}) was set
707 using unstimulated negative control. OT-I T-cells with lower fluorescence of CTV than
708 non-proliferating T-cells were identified as proliferating cells.

709

710 **Murine T-cell proliferation assay**

711 Murine T-cells were isolated from spleens of healthy 6-8-week old C57BL/6 mice
712 using EasySep™ Mouse CD4⁺ or CD8⁺ T-Cell Isolation Kit (STEMCELL
713 Technologies) according to the manufacturer's protocols. CECs were isolated from
714 the spleens of anemic mice using the EasySep™ Release Mouse Biotin Positive
715 Selection Kit (STEMCELL Technologies) according to the manufacturer's protocols.
716 Biotin-conjugated anti-CD71 antibodies (anti-mouse clone 8D3, NovusBio,) were
717 used at a final concentration of 1 µg/ml. CECs purity was >80%. For cell proliferation
718 assay, T-cells were labeled with Cell Trace Violet (CTV) dye (Thermo Fisher
719 Scientific) for 20 min at 37°C at a final concentration of 2.5 µM in PBS. Next, the
720 labeled T-cells were plated in round-bottomed 96-well plates (5×10⁴ cell per well) in
721 L-arginine-free RPMI-medium (SILAC RPMI-medium, Thermo Fisher Scientific)
722 supplemented with 10% (v/v) dialyzed FBS (Thermo Fisher Scientific), 2 mM
723 glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% (v/v) MEM non-essential
724 amino acids solution (Thermo Fisher Scientific), 50 µM 2-Mercaptoethanol
725 (Thermofisher Scientific), and 150 µM L-arginine and 40 mg/L L-lysine (SigmaAldrich).
726 Proliferation was triggered by the stimulation with Dynabeads Mouse T-Activator
727 CD3/CD28 (ratio 1:2, Thermo Fisher Scientific). The arginase inhibitor OAT-1746
728 (ARGi, 500 nM), L-arginine (1000 µM), or N-acetylcysteine (ROSi, 100 µM) were
729 added as indicated in the figures. In these concentrations, ARGi and ROSi had no
730 effects on T-cells nor CECs viability. Murine CECs were added to the wells in a 1:2
731 ratio (1×10⁵ CECs per well). Murine T-cells were incubated for 72 h at 37°C in 5%
732 CO₂. Then, cells were harvested, stained with live/dead Zombie dye (Biolegend),
733 anti-CD3 and anti-CD4 or anti-CD8 antibody, and analyzed by flow cytometry. The

734 gate for proliferating cells was set based on the unstimulated controls. Cell
735 autofluorescence was determined using CTV-unstained controls. Percentages of
736 proliferating cells were calculated using the FlowJo Software v10.6.1 (Tree Star).

737

738 **Human T-cell proliferation assay**

739 Human T-cells were isolated from peripheral blood mononuclear cells (PBMC)
740 isolated from buffy coats commercially obtained from the Regional Blood Centre in
741 Warsaw, Poland using EasySep™ Human CD4⁺ or CD8⁺ T-Cell Isolation Kit
742 (STEMCELL Technologies) according to the manufacturer's protocols. CECs were
743 isolated from human bone marrow aspirates or PBMC derived CECs culture using
744 EasySep™ Release Mouse Biotin Positive Selection Kit (STEMCELL Technologies)
745 according to the manufacturer's protocols. Biotin-conjugated anti-CD71 antibodies
746 (anti-human clone DF1513, NovusBio) were used at a final concentration of 1 µg/ml.
747 CECs purity was >80%. For cell proliferation assay, T-cells were labeled with Cell
748 Trace Violet (CTV) dye (Thermo Fisher Scientific) for 20 min at 37°C at a final
749 concentration of 2.5 µM in PBS. Next, the labeled T-cells were plated in round-
750 bottomed 96-well plates (2×10⁴ cell per well) in L-arginine-free RPMI-medium (SILAC
751 RPMI-medium, Thermo Fisher Scientific) supplemented with 10% (v/v) dialyzed FBS
752 (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml
753 streptomycin, 1% (v/v) MEM non-essential amino acids solution (Thermo Fisher
754 Scientific), 50 µM 2-Mercaptoethanol (Thermo Fisher Scientific), and 150 µM L-
755 arginine and 40 mg/L L-lysine (SigmaAldrich). Proliferation was triggered by the
756 stimulation with Dynabeads Human T-Activator CD3/CD28 (ratio 1:2, Thermo Fisher
757 Scientific). The arginase inhibitor OAT-1746 (1500 nM), or N-acetylcysteine (200 µM)
758 were added as indicated in the figures. In these concentrations, ARGi and ROSi had

759 no effects on T-cells nor CECs viability. Human CECs were added to the wells in a
760 1:2 ratio (4×10^5 CECs per well). T-cells were incubated for 120 h at 37°C in 5% CO₂.
761 Then, cells were harvested, stained with live/dead Zombie dye (Biolegend), anti-CD3
762 and anti-CD4 or anti-CD8 antibody, and analyzed by flow cytometry. The gate for
763 proliferating cells was set based on the unstimulated controls. Cell autofluorescence
764 was determined using CTV-unstained controls. Percentages of proliferating cells
765 were calculated using the FlowJo Software v10.6.1 (Tree Star).

766

767 **CECs-conditioned medium (CM)**

768 Conditioned medium was obtained by culturing CECs in the arginine-free RPMI-
769 medium (SILAC RPMI-medium, Thermo Fisher Scientific) supplemented with 150 µM
770 L-arginine at the density 1×10^6 cells/ml for 24h. Cells were centrifuged and the
771 supernatant was collected and immediately frozen at -80°C. After thawing, the
772 supernatant was filtered through a 0.45 µm Syringe Filter (Wenk LabTec) and was
773 used in experiments a 1:1 ratio with 150 µM L-arginine RPMI SILAC medium.

774

775 **Reactive oxygen species (ROS) detection**

776 The level of ROS in cells was determined using CellROX Green Reagent (Thermo
777 Fisher Scientific) or 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). Cells were
778 stained with CellROX at a final concentration of 5 µM or DCFDA at a final
779 concentration of 10 µM in pre-warmed PBS for 30 minutes at 37°C, followed by three
780 washes with PBS. H₂O₂-treated cells served as positive controls. For some
781 experiments, cells stained with CellROX or DCFDA were further stained with

782 fluorochrome-labeled antibodies on ice. Stained cells were acquired on Fortessa X20
783 flow cytometer (BD Biosciences).

784

785 **Arginase activity assay and Griess test**

786 Recombinant enzymes (ARG1 and ARG2) to study ARGi were produced at
787 OncoArendi Therapeutics in *E. coli* expression system and purified by the FPLC
788 method. The proteins were purified by FPLC and stored at -80°C in the storage buffer
789 containing: 20 mM Tris pH 8.0, 100 mM NaCl, 10 mM DDT and 10% glycerol. The
790 basic assay buffer was composed of 100 mM sodium phosphate buffer, 130 mM
791 sodium chloride, 1 mg/mL BSA, pH 7.4. The enzymatic reaction was carried out in
792 the presence of 200 µM MnCl₂ (cofactor) and 10 mM or 20 mM L-arginine
793 hydrochloride (for hARG1 or hARG2, respectively), mixed at the final volume of 25
794 µL. Basic developing buffer contained 50 mM boric acid, 1 M sulfuric acid, 0.03%
795 (m/v) Brij[®] 35 detergent. PHZ or ABH was diluted in basic assay buffer at the volume
796 of 50 µL. The recombinant enzyme was diluted in a basic assay buffer at the volume
797 of 25 µL. The reaction was performed at the final volume of 100 µL. Developing
798 mixture included freshly prepared equal volume mixture of developing solution A (4
799 mM o-phthaldialdehyde) and solution B (4 mM N-(1-naphthyl)ethylenediamine
800 dihydrochloride) prepared in the basic developing buffer. The compound background
801 wells contained each of the tested compounds and the substrate/cofactor mixture,
802 but not the recombinant enzyme (data were excluded from the analysis when the
803 compound background exceeded 10% of the signal obtained in the wells with
804 enzyme). The “0% activity” background wells contained only the substrate/cofactor
805 mixture. Following 1 h incubation at 37°C, the freshly prepared developing reagent
806 was added (150 µL) and the colorimetric reaction was developed (12 min at RT,

807 gentle shaking). The absorbance, proportional to the amount of the produced urea,
808 was measured at 515 nm using Tecan's Spark™ microplate reader. Data were
809 normalized by referring the absorbance values to the positive control wells (100%
810 enzyme activity). The IC₅₀ value was determined by the nonlinear regression method.
811 Arginase activity in the CECs or splenocytes lysates and cell supernatant was
812 determined using Arginase Activity Assay (Sigma) according to the manufacturer's
813 protocol.

814 To evaluate nitric oxide (NO) production as a measure of NOS (nitric oxide
815 synthase) activity, Griess Reagent System (Promega) was used according to the
816 manufacturer's protocol. Splenocytes or CECs were isolated from murine spleens
817 and were cultured in non-adherent 6-well plate 1×10^6 or 5×10^5 cells per 2 ml,
818 respectively, for 24h followed by supernatants collection and measurement of NO
819 concentration.

820

821 **Bioinformatical analysis of arginase structure**

822 The structure and predicted binding energies for the complexes of PHZ, L-arginine
823 and 2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid with both human and
824 mouse arginases were compared. The 3D models of mouse arginases were
825 proposed using available structures of human arginases (pdb|4hww and pdb|4hze for
826 ARG1 and ARG2, respectively) as templates. Both templates shared more than 87 %
827 sequence identity with their respective target. The sequence to structure alignments
828 between mouse arginases and selected templates were calculated with the muscle
829 program⁶². The 3D structure was proposed with MODELLER⁶³. Models quality was
830 assessed with the Molprobitry webserver⁶⁴. Next, both human and mouse proteins
831 were prepared for docking using the Chimera dock prep module. Molecular docking

832 was carried out with two programs - GOLD⁶⁵ and Surflex⁶⁶. The active site was
833 specified based on the position of the inhibitor present in the active site of the
834 arginase 1 (pdb|4hww). The default parameters of both programs were used.

835 To assess if PHZ remains stably bound to the active site of both human
836 arginases short molecular dynamics simulations were performed. The initial
837 configurations of ligand-protein complexes were derived from docking results for
838 PHZ. For the PHZ-arginase complexes the simulation included the following steps.
839 First protein and ligand were put in a dodecahedron box with the distance between
840 solute and a box equal to 1 nm. The 0.1 M NaCl was added to the system including
841 neutralizing counterions. After energy minimization using the steepest descent
842 algorithm, 100 ps NVT and NPT simulation were carried out. For this modified
843 Berendsen thermostat was used to maintain the temperature at 310 K using and
844 Berendsen barostat to keep the pressure at 1 atm. Positions of both protein and
845 ligand heavy atoms remained constrained. During the following 300 ps of simulation
846 time, the ligand's constraints were gradually removed. Finally, an unconstrained 100
847 ns simulation is performed in which Berendsen barostat was replaced by Parrinello-
848 Rahman barostat. During simulation short-range nonbonded interactions were cut off
849 at 1.4nm, with long-range electrostatics calculated using the particle mesh Ewald
850 (PME) algorithm. Bonds were constrained using the lincs algorithm. Simulations were
851 carried out with Gromacs⁶⁷ using the gromos54a7 force field, modified to include
852 parameters for Mn²⁺ ion adopted from⁶⁸. Spc model was used for water molecules.
853 Parameters for the ligand were obtained with Automated Topology Builder (ATB)⁶⁹.

854 Additional analyses were performed to assess if PHZ can migrate to the
855 arginase active site when present in solute in high concentration. For this analysis
856 protein was put in dodecahedron box with the distance between solute and a box

857 equal to 1.5 nm in which 6 PHZ molecules were placed randomly. This corresponds
858 to 0.02 M concentration of the compound. A similar simulation setup to one described
859 above was used with exception that ligand molecules remained unconstrained
860 throughout simulation.

861

862 **Protein carbonylation assay**

863 The carbonyl content of proteins was determined in a 2,4-DNPH reaction. Five μg of
864 murine ARG1 (Cloud-System Corp) was resuspended in 400 μl of distilled water and
865 incubated with PHZ (10 μM), PHZ (10 μM) with NAC (10 mM), H_2O_2 (10 mM) or
866 water (negative control) as indicated in the Fig. 3d for 1 hour at 37°C. Proteins were
867 precipitated with 10% TCA. The precipitates were treated with either 2N HCl alone
868 (control) or 2N HCl containing 5 mg/ml 2,4-DNPH at RT for 30 min. The resulting
869 hydrazones were precipitated in 10% TCA and then washed three times with ethanol-
870 ethyl acetate (1:1). Final precipitates were dissolved in 8 M guanidine chloride. Equal
871 amounts of proteins were separated on 4-12% SDS-polyacrylamide gel (Bio-Rad),
872 transferred onto nitrocellulose membranes (Bio-Rad) blocked with TBST [Tris-
873 buffered saline (pH 7.4) and 0.05% Tween 20] supplemented with 5% non-fat milk.
874 Anti-DNP antibodies (Life Diagnostics, Inc) at concentration 1 U/ml were used for
875 overnight incubation at 4°C. After washing with TBST, the membranes were
876 incubated with horseradish peroxidase-coupled secondary antibodies (Jackson
877 ImmunoRes.). The reaction was developed using SuperSignal™ West Femto
878 Maximum Sensitivity Substrate (ThermoFisher Scientific) and imaged using
879 ChemiDoc Touch Gel Imaging System (Bio-Rad). Densitometry was done using
880 ImageJ software.

881

882 **RNA isolation from CECs, reverse transcription, and quantitative polymerase**
883 **chain reaction**

884 Total RNA was isolated from CECs isolated from murine spleens using RNeasy Mini
885 Kit (Qiagen). RNA was subjected to reverse transcription using the GoScript™
886 Reverse Transcriptase system (Promega). All qPCRs were performed in MicroAmp
887 Fast Optical 96 Well Reaction Plates (Thermo Fisher Scientific) using
888 Applied Biosystems 7500 Fast Real-Time PCR System with 7500 Software V2.0.6
889 (Thermo Fisher Scientific). Samples were assayed in triplicates. Primers sequences
890 used in the study: ARG1 forward 5'-CTCCAAGCCAAAGTCCTTAGAG-3', reverse 5'-
891 AGGAGCTGTCATTAGGGACATC-3', ARG2 forward 5'-
892 AGGAGTGGAATATGGTCCAGC-3', reverse 5'-GGGATCATCTTGTGGGACATT-3',
893 and GAPDH forward 5'-GAAGGTGGTGAAGCAGGCATC-3', reverse 5'-
894 GCATCGAAGGTGGAAGAGTGG-3' as an endogenous control. The mean Ct values
895 of a target gene and endogenous control were used to calculate relative expression
896 using the $2^{-\Delta Ct}$ method.

897

898 **Western blot**

899 Splenocytes lysates were prepared using Cell Lysis Buffer (#9803, Cell Signaling
900 Technology) supplemented with protease inhibitors (Roche) according to the
901 manufacturer's protocol. Total protein concentration was assessed using Pierce BCA
902 Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of whole-cell protein
903 lysates samples were boiled in Laemmli loading buffer, separated on 4-12% SDS-
904 polyacrylamide gel (Biorad), transferred onto nitrocellulose membranes (Bio-Rad)
905 blocked with TBST [Tris-buffered saline (pH 7.4) and 0.05% Tween 20]
906 supplemented with 5% non-fat milk. Anti-Arg1 antibodies (polyclonal, GTX109242,

907 GeneTex) at dilution 1:2000 or anti-Arg2 antibodies (polyclonal, ab81505, Abcam) at
908 dilution 1:1000 were used for overnight incubation at 4°C. After washing with TBST,
909 the membranes were incubated with horseradish peroxidase-coupled secondary
910 antibodies (Jackson ImmunoResearch). The reaction was developed using
911 SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermofisher Scientific)
912 and imaged using ChemiDoc Touch Gel Imaging System (Bio-Rad). After imaging,
913 bound antibodies were removed from membranes using Restore™ PLUS Western
914 Blot Stripping Buffer (Thermofisher Scientific), followed by blocking with TBST
915 supplemented with 5% non-fat milk. Next, the membranes were incubated with anti-
916 β -Actin (A5060, Santa Cruz) conjugated with peroxidase. Densitometric
917 quantifications were done using ImageJ software.

918

919 **Erythroid cells differentiation**

920 CECs were differentiated from human peripheral blood mononuclear cells (PBMC)
921 according to the protocol by Heshusius et al.⁷⁰ with modifications. Human PBMC
922 were purified from buffy coats from healthy donors by density separation using
923 Lymphoprep (STEMCELL Technologies). PBMC were seeded at 1×10^6 cells/mL in
924 erythroid differentiation-promoting medium based on StemSpan™ Serum-Free
925 Expansion Medium (SFEM) supplemented with human recombinant EPO (2 U/ml,
926 Roche), human recombinant stem cell factor (25 ng/ml, R&DSystems),
927 dexamethasone (1 μ M, SigmaAldrich), human recombinant insulin (10 ng/ml,
928 SigmaAldrich), L-Glutamine (2 mM, SigmaAldrich), iron-saturated holo-transferrin (20
929 μ g/ml, Sigma-Aldrich), sodium pyruvate (1 mM, Gibco), MEM non-essential amino
930 acids (1% v/v, Gibco), bovine serum albumin (0.1% m/v, SigmaAldrich), EmbryoMax
931 Nucleosides (1% v/v, Merck), and 100 U/ml penicillin and 100 μ g/ml streptomycin

932 (SigmaAldrich). The expansion and differentiation of CECs were assessed by flow
933 cytometry.

934

935 **Statistics and Reproducibility**

936 Data are shown as means \pm SD or means \pm SEM, as indicated in the figure legends.
937 Graphpad Prism 8.4.3 (GraphPad Software) was used for statistical analyses. Data
938 distribution was tested using the Shapiro-Wilk test, D'Agostino & Pearson test, and
939 Kolmogorov-Smirnov test. Statistical analyses of three or more groups were
940 compared using one-way analysis of variance (ANOVA) or Brown-Forsythe ANOVA
941 followed by Tukey's, Dunnett's, or Bonferroni's multiple comparisons test or Kruskal-
942 Wallis test followed by Dunn's multiple comparisons test. Repeated measures
943 ANOVA with Sidak's or Holm-Sidak's post-hoc tests were used to analyze the
944 differences in paired samples. Statistical analyses of two groups were compared
945 using unpaired *t*-test, paired *t*-test, or Mann-Whitney test. Methods of statistical
946 analyses are defined in every figure legend. *P*-value of less than 0.05 was
947 statistically significant. Each experiment was performed in technical duplicates or
948 triplicates. The number of biological replicates for each experiment is mentioned in
949 the figure legends.

950

951 **Data availability**

952 Source data underlying the graphs and charts presented in the figures are available
953 in the Supplementary Data. Uncropped western blots are provided in the
954 Supplementary Data file. Any remaining information can be obtained from
955 corresponding authors upon reasonable request.

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968

969 **Authorship Contributions**

970 TM.G. designed and supervised the study, conducted the experiments, analyzed the
971 data, and wrote the manuscript. A.S. participated in *in vivo* studies. Z.R. participated
972 in *in vitro* experiments. M.L. and D.P. performed molecular docking and molecular
973 dynamics simulations, K.K. performed real-time qPCR and participated in *in vitro*
974 experiments, M.M. and O.C. collected and provided human blood samples, A.R.-L.
975 performed analysis of murine blood, M.J. participated in *in vitro* experiments, P.P and
976 M.M.G. carried out arginase activity assays, R.B. designed and synthesized OAT-
977 1746, M.W. bred and provided *Arg2^{-/-}* mice, A.T and G.B. collected and provided
978 HSPCs. J.G. conceived, designed and supervised the study, provided funding and
979 wrote the manuscript. D.N. provided funding, performed *in vivo* studies, designed and

980 supervised the study, and wrote the manuscript. All authors edited and approved the
981 final manuscript.

982 **Competing interests**

983 P.P., M.M.G., and R.B. are employees of OncoArendi Therapeutics, Warsaw,
984 Poland.

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1210

Figures

1211

1212 **Figure 1. Anemia induces CECs expansion in the spleen**

1213 **a**, Representative plots of CD71⁺TER119⁺ CECs of total live cells in the spleens of
1214 control, anemic, and 3-days old neonatal mice. **b**, The frequency of CD71⁺TER119⁺
1215 CECs of total splenocytes in control (n=10), control-IgG (n=7), anemic (NHA, n=13;
1216 HA-PHZ, n=9; HA-TER119, n=8), and 3-days old neonatal mice (n=5). *P* values were
1217 calculated with Kruskal-Wallis test with Dunn's post-hoc test. **c**, Numbers of
1218 CD71⁺TER119⁺ CECs in the spleens of control (n=4), anemic (NHA, n=4; HA-PHZ,
1219 n=4; HA-TER119, n=4), and neonatal (n=4) mice. *P* values were calculated with one-
1220 way ANOVA with Dunnett's post-hoc test. **d**, Percentages of CD45.2⁻ and CD45.2⁺
1221 cells within CECs (CD71⁺TER119⁺) population (n=5). Representative plot of CD45
1222 levels in CECs and TER119⁻ cells in the spleen of NHA mouse. **e**, Gating strategy for
1223 CECs developmental stages based on CD44 expression and cells size¹⁶. **f**,
1224 Developmental stages of CECs in control mice (n=9), NHA mice (n=13), HA-PHZ
1225 (n=9), HA-TER119 (n=5), and neonatal mice (n=5). **g**, Representative plot of CD71
1226 and CD45 levels in CECs in the spleen of NHA mouse and analysis of developmental
1227 stages of CD45⁺ CECs and CD45⁻ CECs. **h**, Percentages of CD45⁺ CECs in different
1228 developmental stages in the spleens of NHA mice (n=5). Histograms show the
1229 fluorescence of CD45 – BV711. Red blood cells (RBCs) are presented as a negative
1230 control. **i**, Percentages of nucleated CECs (Hoechst 33342⁺) in different
1231 developmental stages in the spleens of NHA mice (n=5). Histograms show the
1232 fluorescence of Hoechst 33342 – INDO-1. Red blood cells (RBCs) are presented as
1233 a negative control. Data show means ± SD. Each point in **b-d,h,i** represents data
1234 from individual mice. *n* values are the numbers of mice used to obtain the data. The
1235 source data underlying Fig.1b-d,f,h,i are provided as a Supplementary Data file.

1236

1237 **Figure 2. Anemic mice have impaired T-cell immune response**

1238 **a**, Schematic presentation of the experimental setting. T-cells isolated from OT-I mice
1239 were labeled with CellTraceViolet (CTV) and adoptively transferred to anemic and
1240 healthy control mice and stimulated with OVA. **b**, Percentage of proliferating (CTV^{low})
1241 OT-I T-cells in the spleen of NHA mice (n=8), HA-PHZ mice (n=8), and healthy
1242 controls (n=5). Histograms show the fluorescence of CTV (CellTraceViolet) – V450 of
1243 OT-I T-cells. *P* values were calculated with one-way ANOVA with Tukey's post-hoc
1244 test. **c**, Representative plot of CD71 and TER119 levels in isolated CECs. Additional
1245 plots are presented in Supplementary Fig. 4. **d**, Proliferation triggered by
1246 α CD3/ α CD28 in CTV-labelled CD4⁺ T-cells co-cultured with CECs isolated from the
1247 spleens of NHA (n=8), HA-PHZ (n=8), or HA-TER119 (n=4) mice. T-cell:CECs ratio
1248 was 1:2. Representative proliferation histograms of α CD3/ α CD28-stimulated CD4⁺ T-
1249 cells co-cultured with CECs. Histograms show the fluorescence of CTV
1250 (CellTraceViolet) – V450. *P* values were calculated with one-way ANOVA with
1251 Dunnet's post-hoc test. Data show means \pm SD. Each point in **b,d** represents data
1252 from individual mice. *n* values are the numbers of mice. The source data underlying
1253 Fig. 2b,d are provided as a Supplementary Data file.

1254

1255 **Figure 3. CECs from anemic mice express ARG2 and have high levels of ROS**

1256 **a**, Mean Fluorescence Intensity (MFI) of CellROX Green – FITC in CECs
1257 (CD71⁺TER119⁺) and RBCs (CD71⁻TER119⁺) of control (n=6), NHA (n=6), and HA-
1258 PHZ (n=6) mice. Histograms show representative fluorescence of CellROX Green –
1259 FITC in CECs and RBCs from the spleens of the NHA mouse. *P* values were

1260 calculated with unpaired *t*-test. **b**, Mean Fluorescence Intensity (MFI) of CellROX
1261 Green – FITC in CECs (CD71⁺TER119⁺), CD45⁺ CECs (CD45⁺CD71⁺TER119⁺),
1262 leukocytes (CD45⁺TER119⁻), T-cells (CD45⁺CD3e⁺), and myeloid cells
1263 (CD45⁺CD11b⁺) (n=18). *P* values were calculated with Friedman's test with Dunn's
1264 post-hoc test. **c**, Percentages of ARG2⁺ CECs in control mice (n=11), anemic mice
1265 (NHA, n=5; HA-PHZ, n=11; HA-TER119, n=11), neonatal mice (n=5), and isotype
1266 control-IgG-treated mice (control-IgG, n=7) based on intracellular staining. **d**, Mean
1267 Fluorescence Intensity (MFI) of ARG2-APC in CECs from control mice, anemic mice
1268 (NHA, HA-PHZ, HA-TER119), neonatal mice, and isotype control-IgG-treated mice
1269 (each group n=5). Histograms show the representative fluorescence of ARG2 – APC
1270 in CECs in different groups and in anti-ARG2-unstained controls. **e**, Percentages of
1271 ARG1⁺ CECs based on intracellular staining (n=5). *P* values were calculated with
1272 one-way ANOVA with Dunnet's post-hoc test and with an unpaired *t*-test for HA-
1273 TER119. **f**, Percentages of YFP⁺ CECs in reporter B6.129S4-Arg1^{tm1Lky}/J mice
1274 (controls n=4, NHA n=8, HA-PHZ n=8, neonatal n=5, control-IgG n=4, HA-TER119
1275 n=8). *P* values were calculated with one-way ANOVA with Dunnet's post-hoc test and
1276 with an unpaired *t*-test for HA-TER119. **g**, Mean Fluorescence Intensity (MFI) of YFP
1277 – FITC in CECs of reporter B6.129S4-Arg1^{tm1Lky}/J mice in control mice (n=4), anemic
1278 (NHA n=8, HA-PHZ n=4, HA-TER119 n=8), neonatal mice (n=5) and isotype control-
1279 IgG-treated mice (n=4). *P* values were calculated with one-way ANOVA with
1280 Dunnet's post-hoc test. **h**, Representative fluorescence of ARG1 – YFP in CECs in
1281 reporter B6.129S4-Arg1^{tm1Lky}/J control mice and anemic mice (NHA, HA-PHZ).
1282 Background fluorescence of YFP in CECs from wild-type C57Bl/6 mice presented as
1283 a negative YFP control. **i,j**, Total arginase activity in CECs lysates (**i**, n=8) or in the
1284 supernatants from CECs cultures (**j**, n=8). *P* values were calculated with one-way

1285 ANOVA with unpaired *t*-test. **k,l**, Percentages of ARG1⁺ CECs (**k**) isolated from the
1286 spleens of B6.129S4-Arg1^{tm1Lky}/J incubated with diluent or PHZ (100 μM for 24h)
1287 (n=3) and representative fluorescence of ARG1 – YFP (**l**). *P* values were calculated
1288 with unpaired *t*-test. Data show means ± SD. Each point in **a-g, i-k** represents data
1289 from individual mice. *n* values are the numbers of mice used to obtain the data. The
1290 source data underlying Fig. 3a-g, i-k are provided as a Supplementary Data file.

1291

1292 **Figure 4. Phenylhydrazine targets arginase, inhibits its activity, and induces**
1293 **oxidative damage**

1294 **a**, Inhibition curves for recombinant human ARG1 and ARG2, and IC₅₀ values for
1295 PHZ (n=2) and 2(S)-amino-6-boronohexanoic acid (ABH). **b**, NO production from
1296 CECs and whole splenocytes population isolated from NHA (n=4) and HA-PHZ (n=4)
1297 mice. *P*-values were calculated with an unpaired *t*-test. **c**, The electrostatic surface
1298 potential of the human ARG1. The potential was calculated with APBS and projected
1299 onto the molecular surface of the protein. The figure was prepared with UCSF
1300 Chimera. **d**, Carbonylation of ARG1 in the presence of PHZ (n=3). Representative
1301 blot (left) and densitometric analysis done with ImageJ software (right). The negative
1302 lane represents ARG1 precipitates treated with 2N HCl alone. *P*-values were
1303 calculated with Ordinary one-way ANOVA with Dunnett's multiple comparisons test.
1304 Data show means ± SD. Each point in **b** represents data from individual mice. *n*
1305 values are the numbers of mice used to obtain the data or the number of biological
1306 replicates of *in vitro* experiments. The source data underlying Fig.4a, 4b, 4d are
1307 provided as a Supplementary Data file.

1308

1309 **Figure 5. CECs degrade L-Arg and produce ROS leading to the suppression of**
1310 **T-cells**

1311 **a**, Proliferation and surface markers in α CD3/ α CD28-stimulated CD4⁺ T-cells co-
1312 cultured with CECs isolated from NHA mice (n=4) at a ratio 1:2 (T-cells:CECs). *P*-
1313 value was calculated with an unpaired t-test. **b**, Effects of ARGi (OAT-1746, 500 nM)
1314 and ROSi (N-acetylcysteine, 100 μ M) on the proliferation of α CD3/ α CD28-stimulated
1315 CD4⁺ T-cells co-cultured with CECs isolated from the spleens of NHA mice (n=4) at a
1316 ratio 1:2 (T-cells:CECs). Representative proliferation histograms of α CD3/ α CD28-
1317 stimulated CD4⁺ T-cells co-cultured with CECs in the presence of ARGi or ROSi.
1318 Histograms show the fluorescence of CTV (CellTraceViolet) – V450. *P*-value was
1319 calculated with one-way ANOVA with Bonferroni's post-hoc test. **c**, Effects of L-
1320 arginine supplementation (1000 μ M) or ARGi (OAT-1746, 500 nM) on the
1321 proliferation of α CD3/ α CD28-stimulated CD4⁺ T-cells cultured in full medium or in
1322 CECs-conditioned medium (CM) (n=3). *P*-value was calculated with one-way ANOVA
1323 with Bonferroni's post-hoc test. **d**, Proliferation of α CD3/ α CD28-stimulated CD4⁺ T-
1324 cells co-cultured with total CECs population or with nucleated CECs (nCECs) isolated
1325 using density-gradient centrifugation from NHA mice (n=5) at a ratio 1:2 (T-
1326 cells:CECs). Histograms show the fluorescence of CTV (CellTraceViolet) – V450. *P*-
1327 value was calculated with repeated measures ANOVA with Holm-Sidak's post-hoc
1328 test. **e,f**, Proliferation of α CD3/ α CD28-stimulated CD4⁺ T-cells (**e**) or CD8⁺ T-cells (**f**)
1329 co-cultured with CECs isolated from NHA mice (n=4) at a ratio 1:10 (T-cells:CECs).
1330 *P*-value was calculated with paired t-test. **g**, Arginase activity of the splenocytes
1331 lysate of control and anemic mice calculated per μ g of total protein based on
1332 bicinchoninic acid (BCA) protein assay. *P*-value was calculated with an unpaired t-
1333 test. **h**, The level of ARG1 and ARG2 in the splenocytes lysate of control (n=4) and

1334 anemic mice (n=4). β -actin showed as a loading control. **i,j** Relative density of ARG1
1335 (**i**) and ARG2 (**j**) compared to β -actin. *P*-value was calculated with an unpaired t-test.
1336 **k,l**, The level of CD3 ζ in CD4⁺ (**k**) and CD8⁺ (**l**) T-cells in the spleen of control (n=4)
1337 and anemic mice (n=4) based on intracellular staining. *P*-value was calculated with
1338 an unpaired t-test. **m,n**, The levels of CD3 ζ in CD4⁺ (**m**) and CD8⁺ (**n**) α CD3/ α CD28-
1339 stimulated T-cells in the presence of CECs isolated from NHA mice (n=4) based on
1340 intracellular staining. *P*-value was calculated with one-way ANOVA with Bonferroni's
1341 post-hoc test. Data show means \pm SEM (a-f, m,n) or means \pm SD (h,i-l). Each point in
1342 **a-g, i-n** represents data from individual mice. *n* values are the numbers of mice used
1343 to obtain the data or the number of biological replicates in *in vitro* experiments. The
1344 source data underlying Fig. 5a-n are provided as a Supplementary Data file.

1345

1346 **Figure 6. CECs from *Arg2*^{-/-} mice have impaired immunoregulatory properties**

1347 **a**, The levels of CD3 ζ in spleen CD4⁺ (**a**) and CD8⁺ (**b**) T-cells in control (n=4) and
1348 anemic mice (n=4) based on intracellular staining. Histograms show the fluorescence
1349 of CD3 ζ – FITC in CD4⁺ (**a**) and CD8⁺ (**b**) T-cells. *P*-values were calculated with one-
1350 way ANOVA with Tukey's post-hoc test. **c**, Proliferation of α CD3/ α CD28-stimulated
1351 CD4⁺ T-cells co-cultured with CECs isolated from NHA *Arg2*^{-/-} mice or NHA wild-type
1352 *Arg2*^{+/+} mice at a 1:4 ratio (T-cells:CECs). Representative proliferation histograms of
1353 α CD3/ α CD28-stimulated CD4⁺ T-cells co-cultured with CECs isolated from *Arg2*^{-/-}
1354 mice or wild-type *Arg2*^{+/+} mice in the presence of ARGi or ROSi. Histograms show
1355 the fluorescence of CTV (CellTraceViolet) – V450. *P*-values were calculated with
1356 one-way ANOVA with Bonferroni's post-hoc test. Data show means \pm SD (**a,b**) or
1357 means \pm SEM (**c**). Each point in **a-c** represents data from individual mice. *n* values
1358 are the numbers of mice used to obtain the data or the number of biological

1359 replicates in *in vitro* experiments. The source data underlying Fig. 6a-c are provided
1360 as a Supplementary Data file.

1361

1362 **Figure 7. CECs expand in the blood of anemic patients and suppress T-cells**
1363 **response**

1364 **a**, Percentages of live CD71⁺CD235a⁺ CECs in the whole blood of non-anemic
1365 (controls, n=42) and anemic patients (n=41). *P*-value was calculated with the Mann-
1366 Whitney test. **b**, Representative dot plots of CECs in the blood of non-anemic and
1367 anemic patients. **c**, CECs count per μ l of blood in controls (n=42) and anemic
1368 patients (n=41). *P*-value was calculated with the Mann-Whitney test. **d**, Correlation of
1369 the number of CECs per μ l of blood and hemoglobin concentration (n=82). The
1370 correlation was calculated with Spearman *r*. **e**, CECs count per μ l of blood in non-
1371 anemic controls (n=42) and patients with mild (n=7), moderate (n=32), and severe
1372 (n=2) anemia. *P*-values were calculated with Kruskal-Wallis test with Dunn's post-hoc
1373 test. **f,g**, Percentages of CECs in the fraction of peripheral blood mononuclear cells
1374 (PBMC) in controls (n=12) and anemic patients (n=13) (**f**) and representative dot
1375 plots of CECs (**g**). *P*-value was calculated with the Mann-Whitney test. **h**, PBMCs of
1376 controls (n=12) and anemic patients (n=13) were stimulated with α CD3/ α CD28 for
1377 12h in the presence of a protein transport inhibitor. IFN- γ levels were determined by
1378 intracellular staining. *P*-values were calculated with an unpaired *t*-test. **i**,
1379 Representative plots of IFN- γ levels in unstimulated or α CD3/ α CD28-stimulated
1380 CD3 ϵ ⁺ T-cells from PBMCs of anemic patients or non-anemic controls. Isotype
1381 control-stained cells are shown as a negative control. Data show means \pm SD. Each
1382 point in **a,c-f,h** represents data from individual patients. *n* values are the numbers of

1383 patients used to obtain the data or the number of biological replicates in *in vitro*
1384 experiments. The source data underlying Fig.7a, 7c-f, 7h are provided as a
1385 Supplementary Data file.

1386

1387 **Figure 8. CECs from human bone marrow express ARG1 and ARG2 and**
1388 **suppress T-cells proliferation**

1389 **a**, Representative plots of CD71⁺ and CD235⁺ CECs in the aspirate of human bone
1390 marrow of total live cells. **b**, Percentages of ARG1⁺ and ARG2⁺ CECs in the human
1391 bone marrow based on intracellular staining. **c**, Mean Fluorescence Intensity (MFI) of
1392 ARG1-PE and ARG2-APC in CECs. **d**, Representative histograms of ARG1 and
1393 ARG2 levels in CECs from human bone marrow. Fluorescence-minus-one (FMO) is
1394 shown as unstained controls. **e,f**, Proliferation triggered by α CD3/ α CD28 of CTV-
1395 labelled CD4⁺ (**e**) and CD8⁺ (**f**) T-cells co-cultured with CECs isolated from the
1396 human bone marrow. T-cell to CECs ratio was 1:2 (n=9). The proliferation of T-cells
1397 in co-culture with CECs was calculated as a percentage of maximum proliferation
1398 (100%) of T-cells that was triggered by α CD3/ α CD28 antibodies and cultured without
1399 CECs. Representative histograms shows CTV – BV421 fluorescence of CD4⁺ (**e**) or
1400 CD8⁺ (**f**) T-cells co-cultured with CECs at a 1:2 ratio in the presence of ARGi (OAT-
1401 1746, 500 nM). *P*-values were calculated with Friedman's test with Dunn's post-hoc
1402 test. Data show means \pm SD (**b,c**) or means \pm SEM (**e,f**). *n* values are the numbers
1403 of individual patients used to obtain the data or the number of biological replicates in
1404 *in vitro* experiments. The source data underlying Fig.8b, 8c, 8e, 8f are provided as a
1405 Supplementary Data file.

1406

1407 **Figure 9. Erythroleukemia-derived erythroid cell lines suppress T-cells in an**
1408 **ARG- and ROS-dependent mechanism**

1409 **a**, The levels of CD71 and CD235a in erythroleukemia-derived erythroid cell lines. **b-**
1410 **d**, Proliferation of CTV-labelled CD4⁺ T-cells triggered by α CD3/ α CD28 co-cultured
1411 with K562 (**b**), HEL92.1.7 (**c**) and TF-I (**d**) erythroid cell lines at different ratios (T-
1412 cells:CECs) (n=3). *P*-values were calculated with repeated measures ANOVA with
1413 Dunnett's post-hoc test. **e**, Representative histograms of CTV – BV421 fluorescence
1414 of CD4⁺ T-cells co-cultured with erythroid cells at a 1:2 ratio in the presence of ARGi
1415 (OAT-1746, 1.5 μ M) and ROSi (NAC, 200 μ M) (n=2). Statistical analyses are
1416 provided in Supplementary Fig. 18a. **f-h**, Proliferation of CTV-labelled CD8⁺ T-cells
1417 triggered by α CD3/ α CD28 co-cultured with K562 (**f**), HEL92.1.7 (**g**), or TF-I (**h**)
1418 erythroid cells at different ratios (n=3). *P*-values were calculated with repeated
1419 measures ANOVA with Dunnett's post-hoc test. **i**, Representative histograms of CTV
1420 – BV421 fluorescence of CD8⁺ T-cells co-cultured with erythroid cell lines at a 1:2
1421 ratio in the presence of ARGi (OAT-1746, 1.5 μ M) and ROSi (NAC, 200 μ M) (n=2).
1422 Statistical analyses are provided in Supplementary Fig. 18b. Data show means \pm
1423 SEM. *n* values are the numbers of biological replicates in *in vitro* experiments. The
1424 source data underlying Fig.9b-d, 9f-h are provided as a Supplementary Data file.

1425

1426 **Figure 10. Suppression of T-cells is a general feature of erythroid cells that**
1427 **diminishes with CECs maturation**

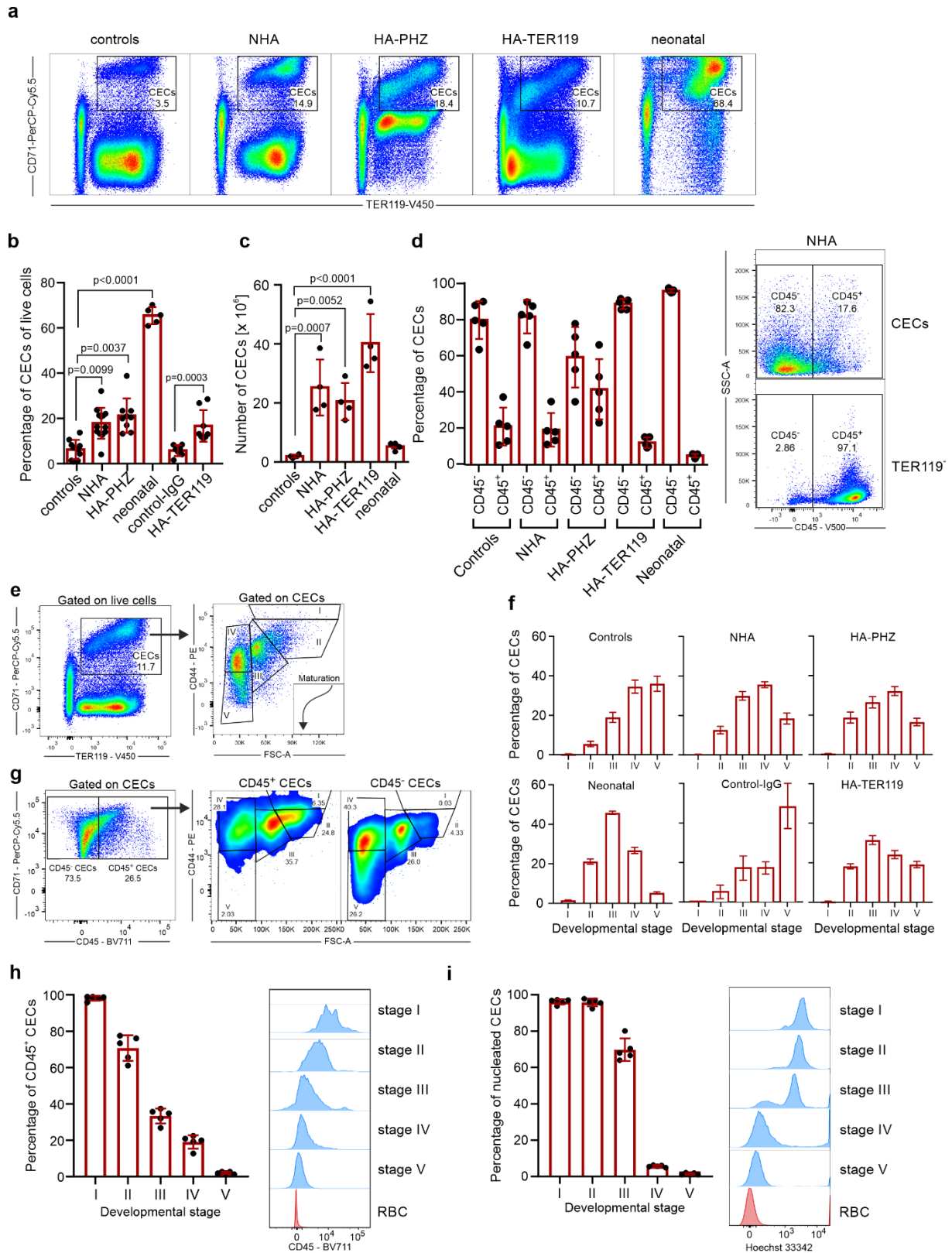
1428 **a**, Representative plot of isolated CECs differentiated from PBMCs. **b,c**, Proliferation
1429 of CTV-labelled CD4⁺ (**b**) and CD8⁺ (**c**) T-cells triggered by α CD3/ α CD28 co-cultured
1430 with CECs differentiated from PBMCs (n=4). *P*-values were calculated with one-way

1431 ANOVA with Dunnett's post-hoc test. **d**, Representative density plots of CECs
1432 differentiation from PBMCs based on CD71 and CD235a expression. **e**, Proliferation
1433 of CTV-labelled CD4⁺ triggered by α CD3/ α CD28 co-cultured with CECs-PBMCs at
1434 different developmental stages at 1:4 ratio. **f,g**, Relative proliferation of CD4⁺ (**f**) and
1435 CD8⁺ (**g**) T-cells co-cultured with CECs-PBMCs at different time points at a 1:4 ratio.
1436 *P*-values were calculated with one-way ANOVA with Bonferroni's post-hoc test. **h,i,j**,
1437 Levels of CD71 (**h**), CD235a (**i**), and CD49d (**j**) during erythroid differentiation from
1438 PBMCs. Data show means \pm SD. Each point in **b,c, f-j** represents data from
1439 individual patients. *n* values are the numbers of individual patients used to obtain the
1440 data or the number of biological replicates in *in vitro* experiments. The source data
1441 underlying Fig.10b, 10c, 10f-j are provided as a Supplementary Data file.

1442

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Figure 1.

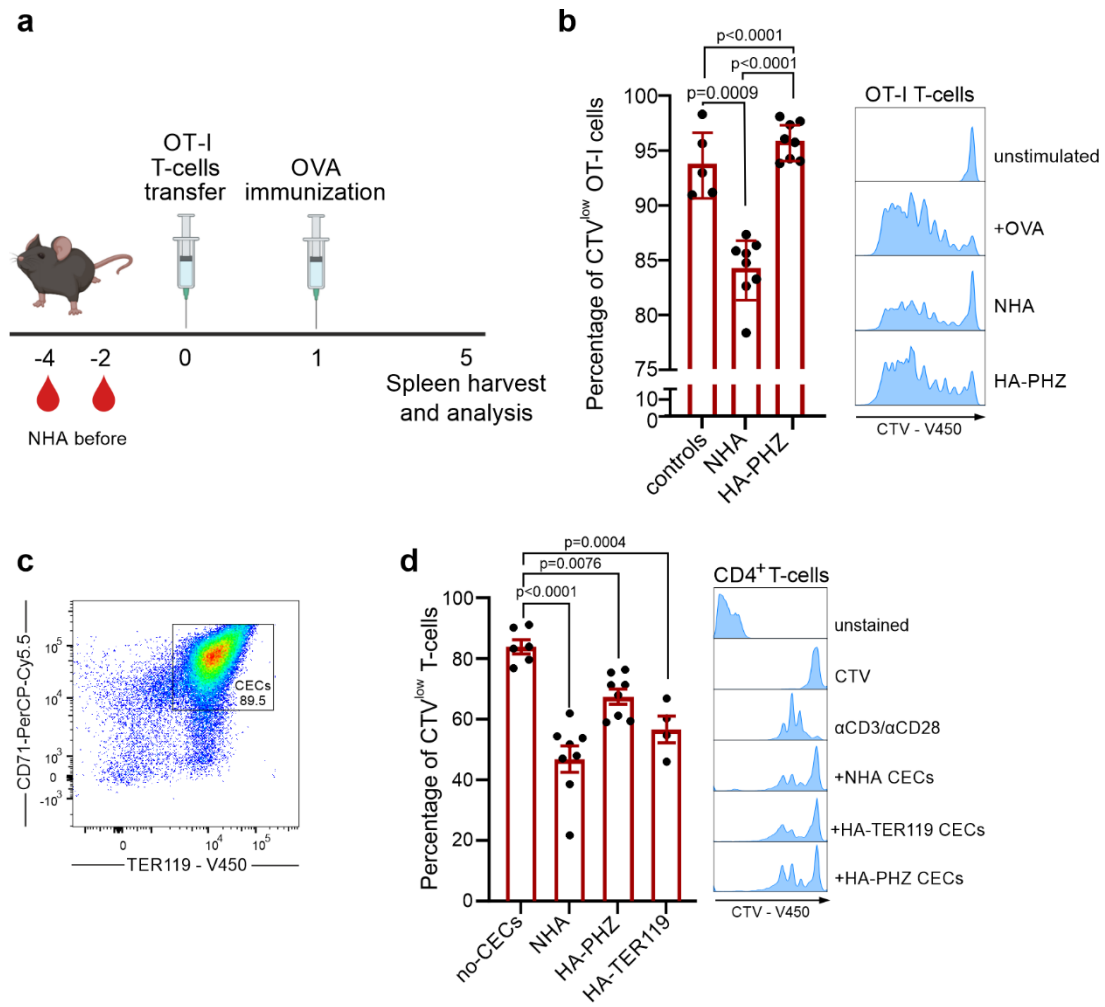


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Figure 2.

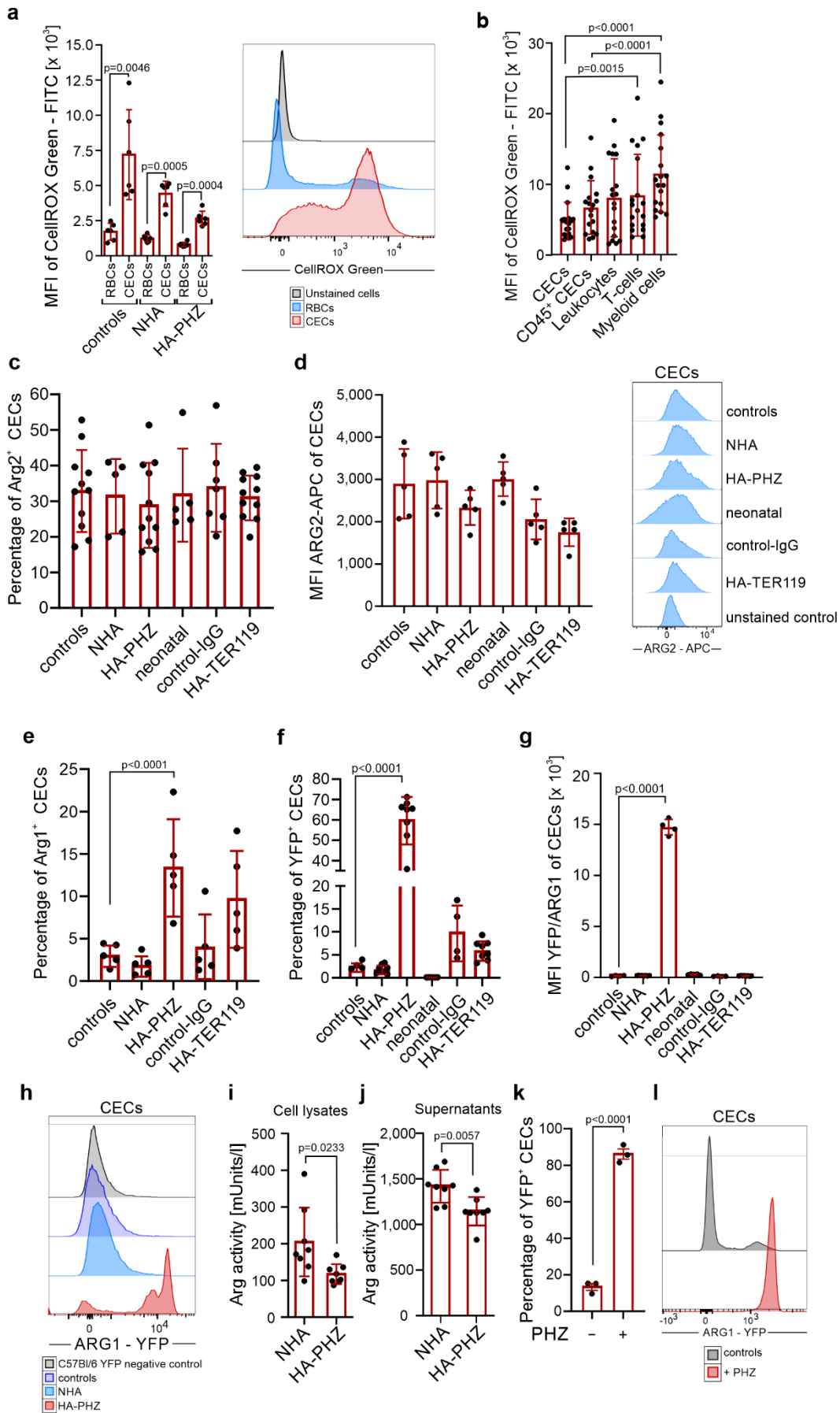


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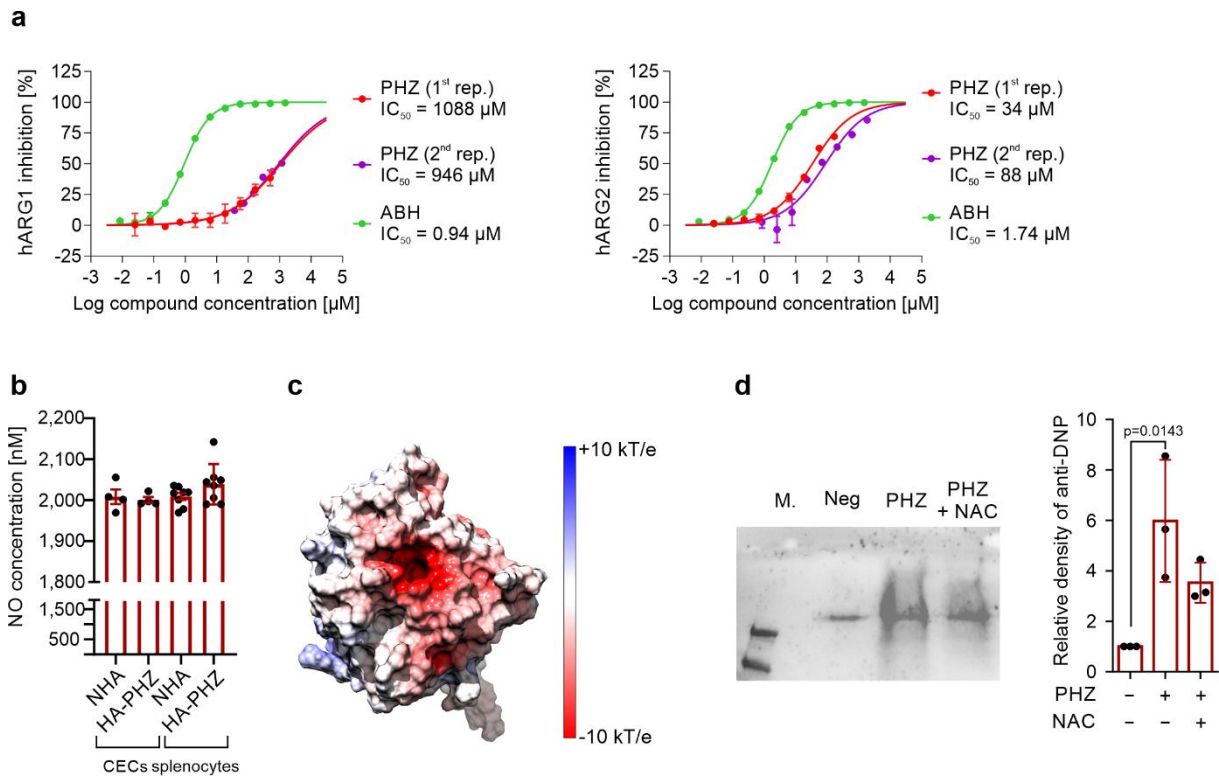
Figure 3.



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Figure 4.

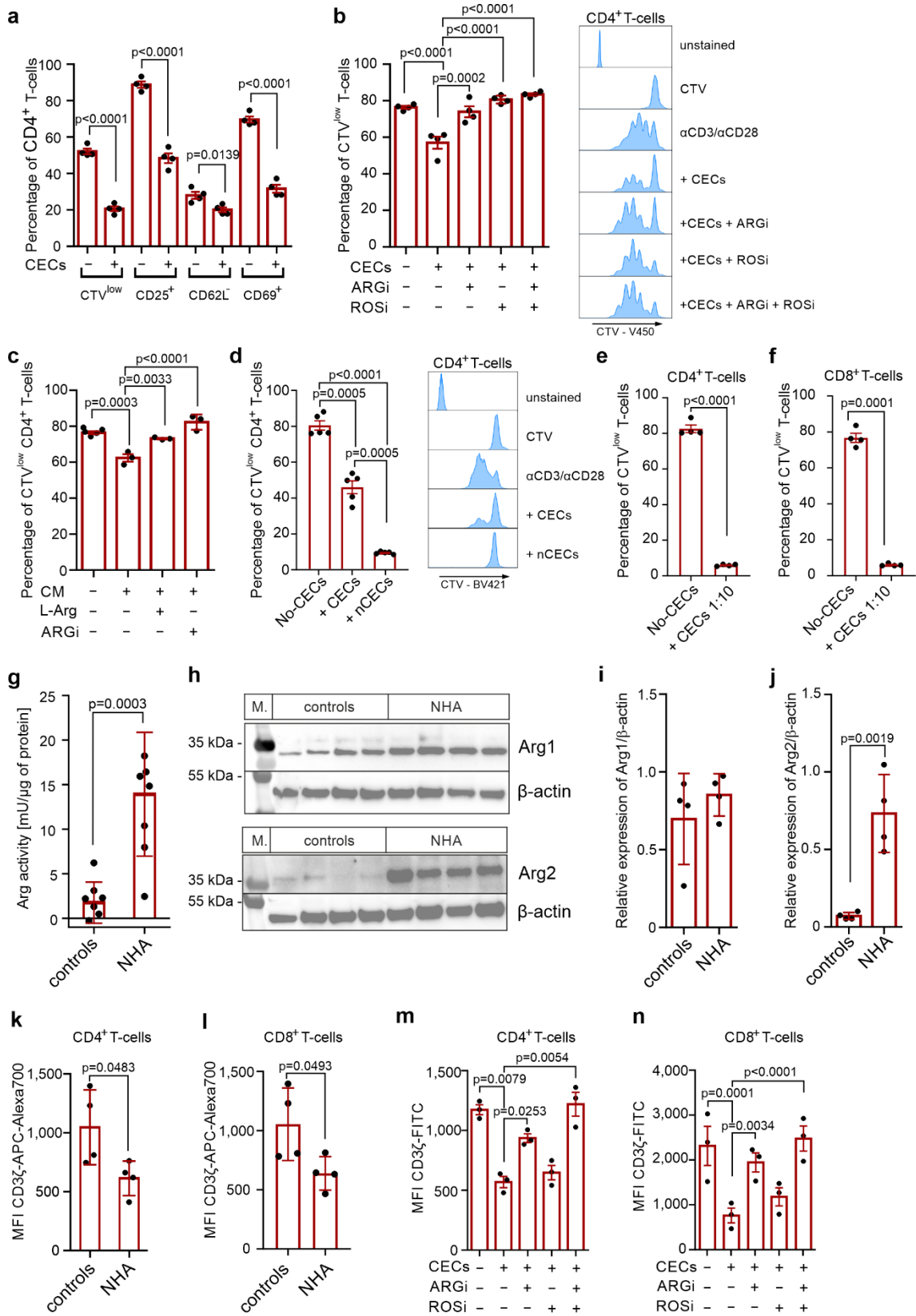


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Figure 5.

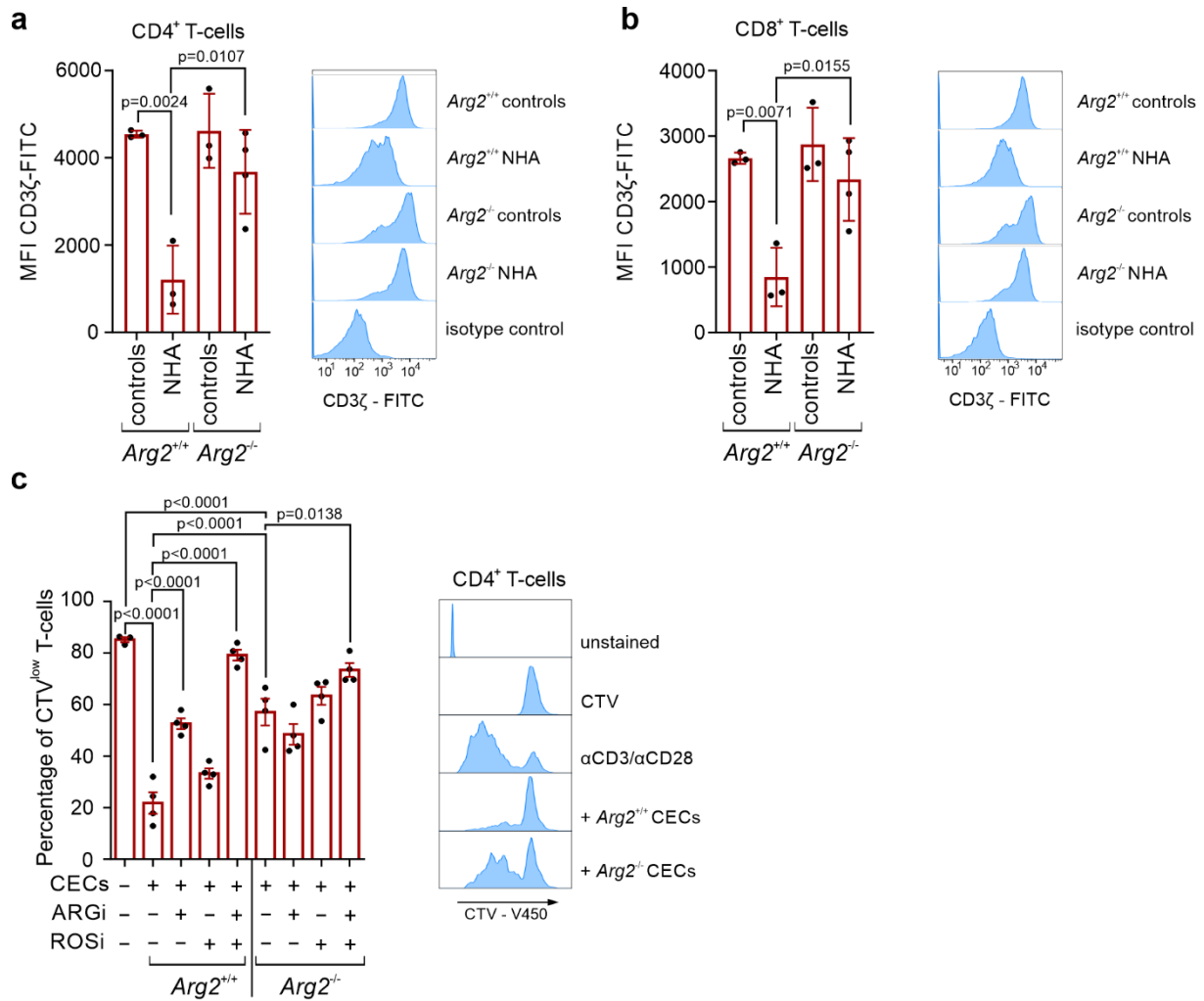


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Figure 6.

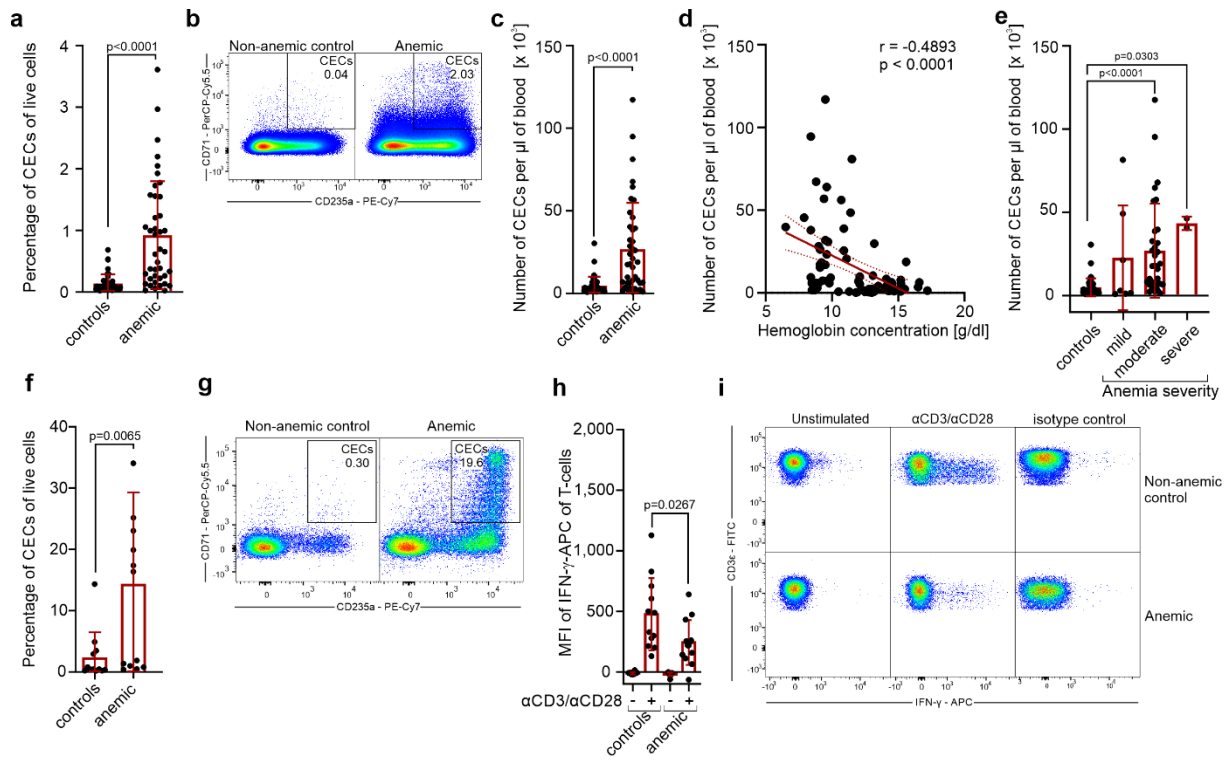


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Figure 7.

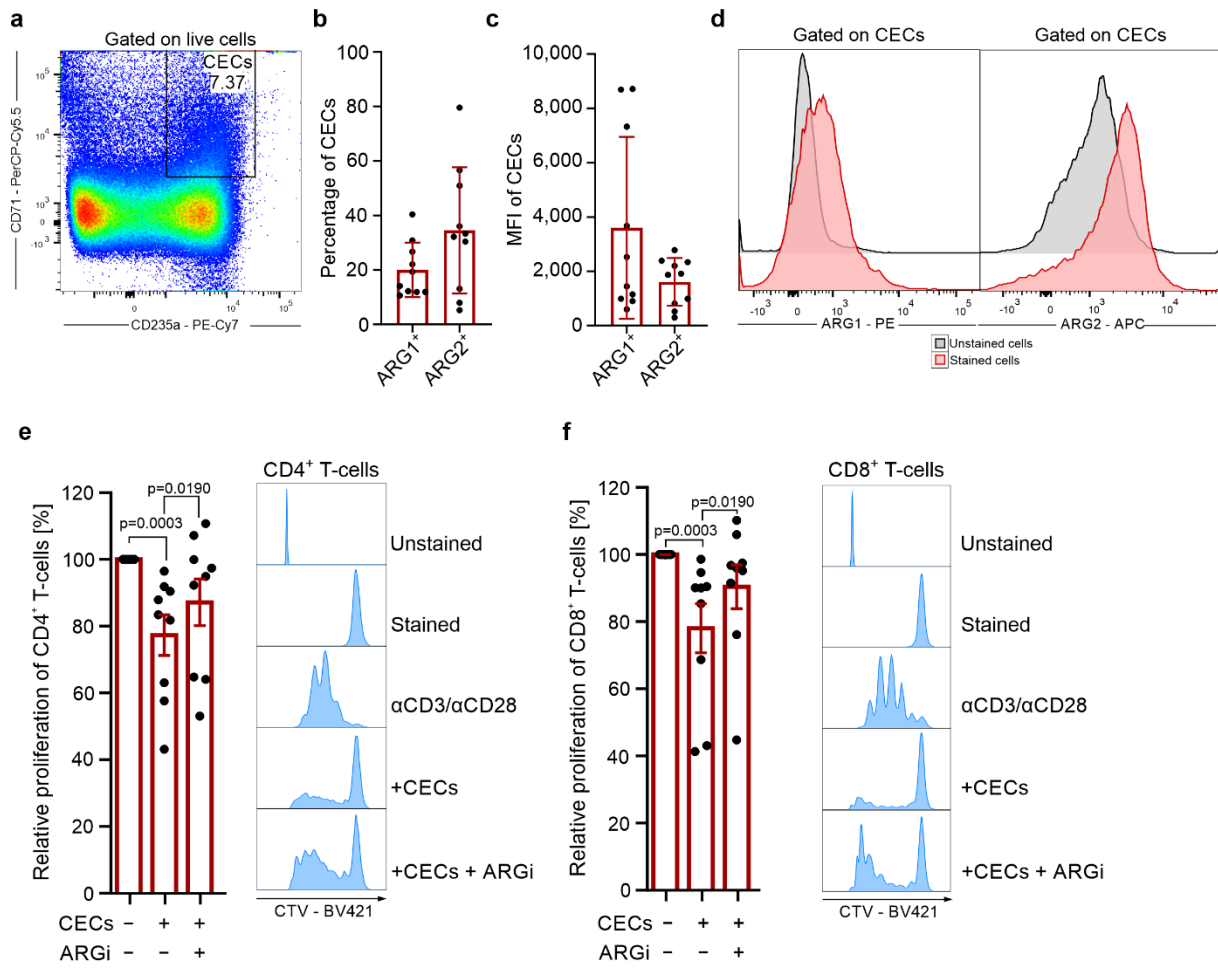


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Figure 8.

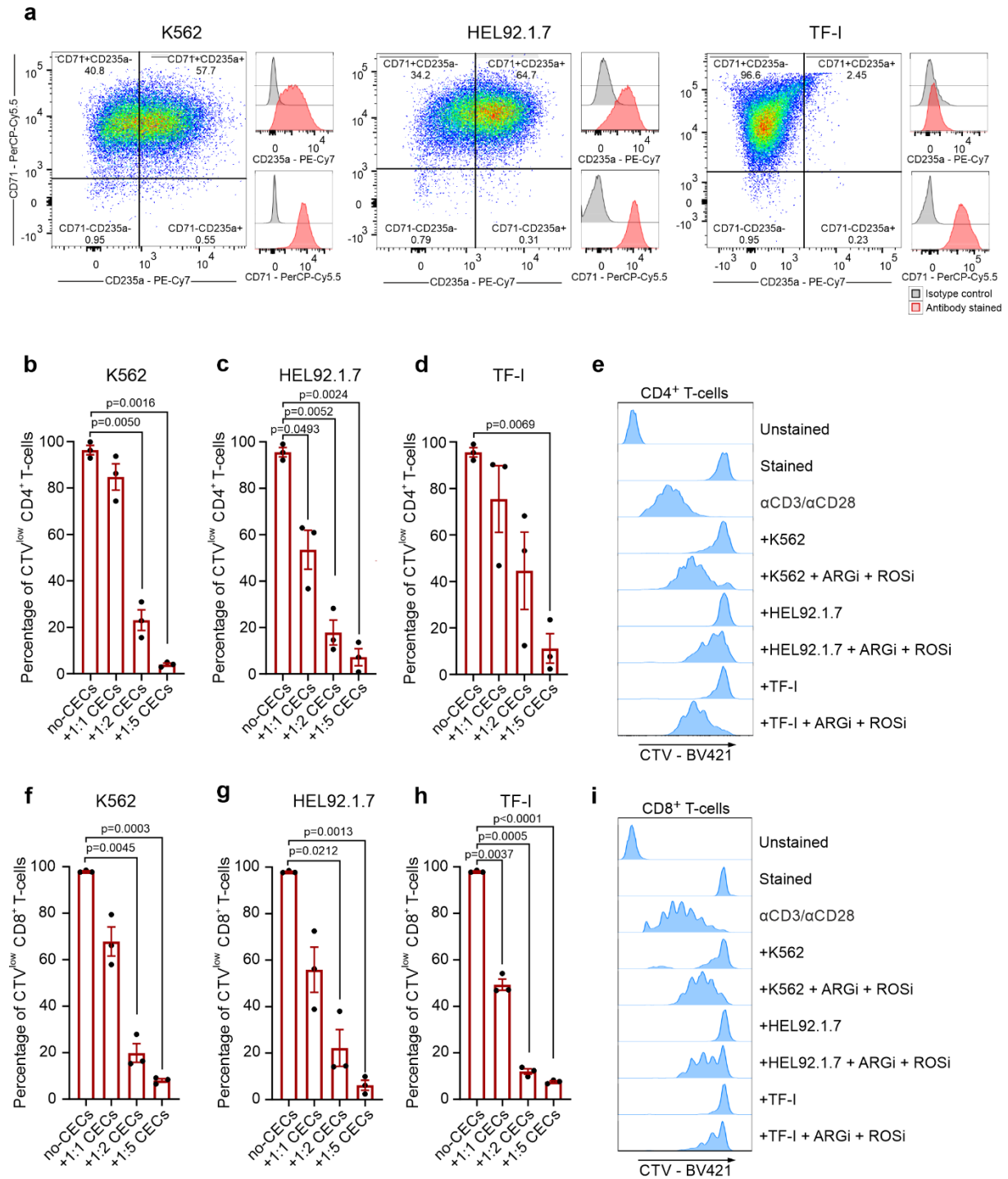


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Figure 9.

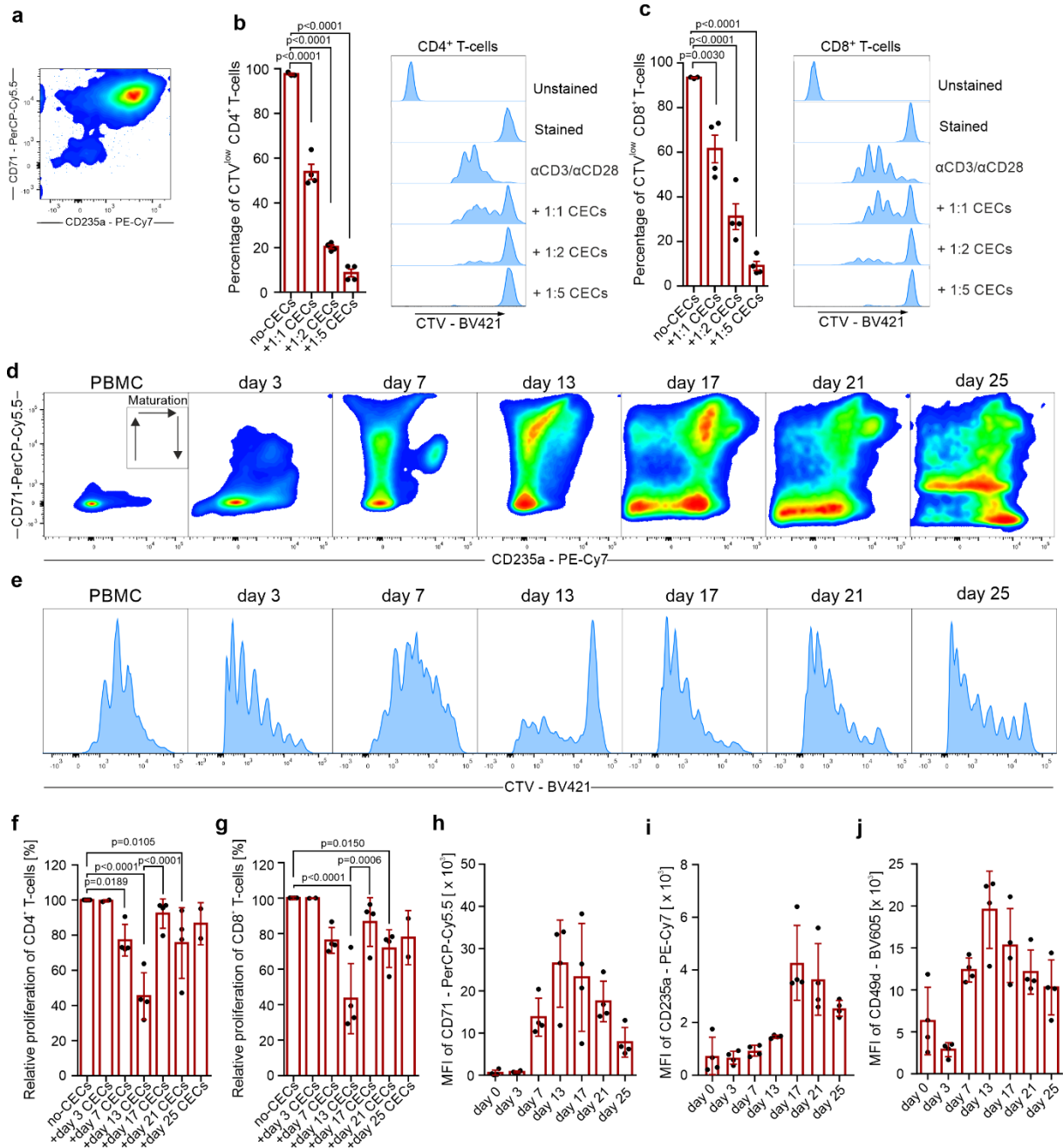


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Figure 10.



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