

1 **Transgenic $\alpha\beta$ TCR tonic signaling is leukemogenic while strong stimulation is**
2 **leukemia-suppressive**

3 Telmo A. Catarino,^{1,2,3} Ivette Pacheco-Leyva,^{1,2} João L. Pereira,^{1,2} Marina Baessa,^{1,2}
4 Nuno R. dos Santos^{1,2,4}

5

6 ¹i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto,
7 Portugal.

8 ²Institute of Molecular Pathology and Immunology of the University of Porto
9 (IPATIMUP), Porto, Portugal.

10 ³Graduate Program in Areas of Basic and Applied Biology, Instituto de Ciências
11 Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

12 ⁴Centre for Biomedical Research (CBMR), University of Algarve, Faro, Portugal.

13

14 Running title: Dual function for $\alpha\beta$ TCR in leukemogenesis

15

16 Text word count: 4304

17 Number of figures: 4

18 Number of references: 42

19

20 **Abstract**

21 The pre-T cell receptor (TCR) and TCR complexes are frequently expressed in
22 T-cell acute lymphoblastic leukemia (T-ALL), an aggressive T cell precursor
23 malignancy. Although mutations in TCR components are infrequent in T-ALL, earlier
24 research indicated that transgenic $\alpha\beta$ TCR expression in mouse T cell precursors
25 promoted T-ALL development. However, we recently found that stimulation of TCR
26 signaling in T-ALL induced leukemic cell apoptosis and suppressed leukemia. Our aim
27 was to elucidate if a given $\alpha\beta$ TCR complex has a dual role in leukemogenesis
28 depending on the nature of the stimulus. We demonstrate that transgenic expression of
29 the Marilyn $\alpha\beta$ TCR, specific for the H-Y male antigen presented by major
30 histocompatibility complex class II, triggers T-ALL development exclusively in female
31 mice. This T-ALL exhibited *Notch1* mutations, *Cdkn2a* copy number loss, immature
32 immunophenotype and infiltrated both lymphoid and non-lymphoid organs.
33 Furthermore, leukemic cells expressed surface CD5, a marker of tonic TCR signaling.
34 T-ALL efficiently developed in *Rag2*-deficient Marilyn transgenic females, indicating
35 that *Rag2*-mediated recombination is not implicated in this T-ALL model. Remarkably,
36 exposure of Marilyn female T-ALL to male antigen in recipient mice resulted in T-ALL
37 apoptosis and prolonged mouse survival. These findings underscore that the same $\alpha\beta$
38 TCR complex has a dual role in T-ALL in that its tonic stimulation is leukemogenic,
39 while strong stimulation is leukemia-suppressive.

40

41 Introduction

42 The pre-T cell receptor (TCR) and $\alpha\beta$ TCR are critical protein complexes for T cell
43 development. Pre-TCR expression in CD4⁻CD8⁻ double-negative (DN) thymocytes
44 induces their survival, proliferation and maturation through the β -selection
45 checkpoint,^{1,2} whereas surface expression of the $\alpha\beta$ TCR in CD4⁺CD8⁺ double-positive
46 (DP), thymocytes drives thymic positive and negative selection to establish major
47 histocompatibility complex (MHC) restriction and eliminate autoreactive thymocytes.³
48 The pre-TCR, TCR and CD3 proteins are frequently expressed in T-cell acute
49 lymphoblastic leukemia (T-ALL).⁴ Although T-ALL is genetically a very heterogeneous
50 disease, with mutations detected in multiple genes, a number of genetic alterations
51 have been recurrently reported for T-ALL, including *CDKN2A* loss of function,
52 *NOTCH1*-activating mutations, and mutations in JAK/STAT signaling pathway genes
53 (e.g. *IL7R*, *JAK3*, *JAK1*, and *STAT5B*).⁵ Mutations in components of the TCR signaling
54 machinery are rare,⁵ but the Lck kinase, a key member of the pre-TCR and TCR
55 signaling pathways, has been shown to be activated in T-ALL and put forward as a
56 potential therapeutic target.⁶⁻⁹ These observations hint that pre-TCR signaling can
57 promote human T cell leukemogenesis. In this line, genetic inactivation of the pre-TCR
58 complex (e.g. CD3 ϵ , pT α or Rag1/2 deficiency) was shown to delay T-ALL
59 development in several mouse models.¹⁰⁻¹⁴ In contrast, the impact and function of TCR
60 signaling in T-ALL development and progression remains controversial. While
61 transgenic TCR expression was shown to promote mouse T-ALL development in
62 cooperation with STAT5 overexpression or deficiency of p53 or Tpl2 proteins,¹⁵⁻¹⁷
63 expression of endogenous $\alpha\beta$ TCR or MHC-dependent antigen presentation were not
64 required for leukemogenesis in the ETV6::JAK2 or TAL/LMO transgenic mouse
65 models.^{13,14} Indicating that TCR could rather have an antagonistic action in T-ALL, T-
66 ALL onset in a *Pten*-deficient mouse model was accelerated by TCR genetic
67 inactivation and delayed by expression of transgenic TCR.¹⁸ In this line, it was also
68 reported that strong agonist TCR stimulation of mouse ETV6::JAK2-driven T-ALL or
69 patient T-ALL was anti-leukemic and induced a transcriptional program similar to that
70 involved in thymocyte negative selection.¹⁹ Overall, these observations suggest that,
71 depending on the oncogenic drivers of leukemia and degree of TCR stimulation, the
72 TCR signaling complex might play either a leukemia-promoting or a leukemia-
73 suppressive role.

74 Here, our aim was to investigate the role of basal versus strong TCR signaling
75 in T-ALL leukemogenesis. In normal T cells, TCR recognition of self-peptides
76 presented by MHC (self-pMHC) induces tonic signaling, while agonist peptides induce
77 strong TCR signaling. Using the Marilyn transgenic TCR mouse strain, specific for the

78 H-Y male antigen, we show that tonic TCR signaling led to *Notch1*-mutated T-ALL
79 exclusively in females. MHC-dependent agonist antigenic stimulation prevented
80 spontaneous T-ALL development in males, induced apoptosis of female leukemic cells
81 and hampered their leukemogenic potential. Our results support the notion that $\alpha\beta$ TCR
82 tonic signaling promotes T cell precursor malignant transformation and that strong
83 signaling suppresses leukemia.

84 **Results**

85 **Transgenic expression of an $\alpha\beta$ TCR specific for the H-Y male antigen drives**
86 **leukemia development in female mice**

87 To study the effect of TCR expression in leukemia development, we resorted to Marilyn
88 transgenic mice, which express an MHC class II-restricted transgenic TCR (TCR-
89 $V\alpha 1.1/V\beta 6$) specific for the H-Y male antigen (HY-TCR henceforth).²⁰ In this model,
90 male thymocytes expressing the HY-TCR are exposed to the cognate antigen (DBY
91 peptide), through MHC class II presentation, and are eliminated by apoptosis. In
92 contrast, HY-TCR-expressing female thymocytes undergo positive selection and
93 differentiate as mature CD4⁺ T cells. To verify if the transgenic Marilyn HY-TCR
94 promoted leukemogenesis, we bred transgenic mice expressing the ETV6::JAK2 fusion
95 protein in lymphoid cells with Marilyn mice. As expected,¹³ all ETV6::JAK2 transgenic
96 mice developed T-ALL expressing CD8, CD25, CD24, CD3 ϵ and TCR β (not shown).
97 Remarkably, co-expression of the Marilyn HY-TCR with ETV6::JAK2 accelerated
98 spontaneous T-ALL development in females (median survival of 10.5 weeks versus 14
99 weeks; Figure 1A). This suggests that HY-TCR tonic signaling, which is induced by
100 TCR interaction with self-peptides presented by MHC, cooperates with the ETV6::JAK2
101 transgene in driving leukemogenesis.

102 To determine whether HY-TCR tonic signaling alone could induce T cell
103 malignant transformation, we monitored a cohort of both Marilyn females and males
104 until 18 months of age. Remarkably, while most females developed fatal hematological
105 disease (median survival of 58 weeks), none of the males did (Figure 1B). Macroscopic
106 analyses showed that Marilyn females developed enlarged lymphoid (thymus, spleen
107 and lymph nodes (LNs)) and non-lymphoid organs (mainly liver and kidneys) (Figure
108 1C; Supplementary Figure 1), together with high number of blood lymphoblasts (Figure
109 1D). Histological analyses revealed massive lymphoid cell infiltration of liver, kidneys,
110 lungs and bone marrow (Supplementary Figure 2). These data indicate that tonic (in
111 females) but not strong (in males) transgenic HY-TCR signaling is sufficient to induce
112 leukemia and lymphoma development.

113

114 **Transgenic HY-TCR expression induced T-cell disease with variable**
115 **immunophenotype**

116 Macroscopic analysis revealed two distinct groups of diseased Marilyn females: a
117 subset with thymic lymphoma (TL; 9 cases) and a subset characterized by
118 splenomegaly and lymphadenopathy without thymic lymphoma (non-TL; 7 cases;
119 Figure 1E). TL females developed leukemia slightly faster and presented higher total
120 lymphoid organ disease burden than non-TL females, suggestive of a more aggressive

121 disease course (Supplementary Figures 3A,B). For a better characterization of the
122 hematological disease developing in different Marilyn females, we performed
123 immunophenotyping by flow cytometry. To define the T cell maturation status, we
124 probed expression of CD24, which is downregulated upon thymocyte maturation, CD5,
125 which is upregulated after thymocyte positive selection, and CD44, which is expressed
126 in memory T cells.^{21,22} Within the TL subgroup, five mice (nos. 2, 6, 22, 33 and 40)
127 presented thymic T cells with immature phenotype, characterized by surface
128 expression of CD90.2, TCR-V β 6, and CD5, low levels of CD4 and CD8, high CD24
129 levels, and CD44 negativity (Figure 2 and Supplementary Table 1). Such
130 immunophenotype was recapitulated in leukemic cells disseminated to the spleen, LNs,
131 and bone marrow (Supplementary Figure 4 and not shown). Two other TL cases (nos.
132 9 and 29) presented a more mature T cell immunophenotype, characterized by
133 CD4⁺CD8⁻TCR-V β 6⁺CD5⁺ leukemic cells with CD44 expression (Figure 2). These cells
134 were also present in spleen and lymph nodes (Supplementary Figure 4). Finally, and
135 rather unexpectedly, two TL cases (nos. 3 and 34) presented a large proportion of
136 B220-positive B cells, concomitantly with TCR-V β 6⁺CD5⁺CD44⁺ CD4 single-positive
137 (SP) and CD8SP T cells (Figure 2). Of note, TL cases with immature
138 immunophenotype, but not those with mature phenotype, could reinitiate disease in
139 recipient syngeneic mice (Supplementary Table 1), suggesting that only the former
140 were malignantly transformed.

141 Non-TL Marilyn females exhibited a mixture of B220⁺ B cells and CD90.2⁺TCR-
142 V β 6⁺CD5⁺CD44⁺ CD4SP cells in lymphoid organs (Figure 2 and Supplementary Figure
143 4 and Table 1). Analysis of CD69 expression, a marker of TCR stimulation,²¹ showed
144 that TCR-V β 6⁺ cells from mixed B and T lymphomas were CD69-positive, indicating
145 they experienced recent TCR stimulation (Supplementary Figure 5). This suggests that
146 mixed B and T disease found in Marilyn female mice was driven by continuous
147 stimulation of the HY-TCR transgene expressed in mature CD4SP or CD8SP T cells.
148 Immature-type leukemic cells did not express CD69, suggesting that their proliferation
149 was likely driven by oncogenic mechanisms rather than TCR stimulation.

150 We conclude that while one third (5 of 16) of Marilyn TCR transgenic females
151 developed a transplantable immature disease, akin to T-ALL, marked by pronounced
152 thymic enlargement, the remaining diseased mice developed a non-transplantable and
153 heterogeneous mature T and B nonmalignant lymphoproliferation affecting the thymus,
154 spleen, lymph nodes and other organs, with statistically significant longer delay
155 (median survival of 40 versus 60 weeks; Supplementary Figure 6).

156

157 **Marilyn T-ALL presents *Notch1* PEST domain mutations and genetic copy**
158 **number alterations**

159 To determine whether Marilyn T-ALL was associated with secondary genetic
160 alterations, we first sequenced the *Notch1* exon 34 (PEST domain), where most
161 murine T-ALL *Notch1* mutations have been found.^{23,24} Four out of 5 Marilyn T-ALL
162 cases analyzed carried exon 34 frameshift mutations (Supplementary Figure 7 and
163 Table 1), while none were detected in cases of mixed T and B cell disease
164 (Supplementary Table 1). Additionally, by performing low-coverage whole-genome
165 sequencing, we found that T-ALL samples had near-diploid genomes, with only a few
166 copy number alterations: a chromosome 15 trisomy (case no. 6), a chromosome 10
167 trisomy (case no. 33) (Supplementary Figure 8A), and loss of the *Cdkn2a* locus
168 (chromosome 4) in three cases (nos. 2, 22 and 40) (Supplementary Figure 8B). In
169 contrast, no consistent copy number alterations were found in 2 cases of mixed T and
170 B cell disease (Supplementary Figure 8A). Deletions of the *Tcra/d*, *Tcrb* and *Tcrq* loci
171 were also found in Marilyn T-ALL (Supplementary Figure 8C), indicating that these loci
172 underwent genetic rearrangements and that leukemia arose from post- β -selection
173 thymocytes with rearranged TCR loci. As expected from their T lineage origin, T-ALL
174 cases did not exhibit consistent copy number alterations in the immunoglobulin *Ighm* or
175 *Igk* loci (Supplementary Figure 8C). The detection of clonal copy number genetic
176 alterations in Marilyn T-ALL bulk populations further underscores their malignant nature.
177 Given the high frequency of *NOTCH1*-activating mutations and *CDKN2A* inactivation in
178 human T-ALL,⁵ our findings show that the Marilyn transgenic HY-TCR T-ALL
179 recapitulates key cellular and molecular features of the human disease.

180

181 **HY-TCR-induced T-ALL is not dependent on *Rag2* recombinase expression**

182 Since several diseased Marilyn females developed B cell lymphoproliferation, we
183 generated Marilyn mice with *Rag2* constitutive knockout to block B cell development,
184 while maintaining HY-TCR-dependent T-cell development. This approach
185 simultaneously assessed whether Rag-mediated gene recombination plays a role in
186 HY-TCR-induced T cell leukemogenesis. Marilyn HY-TCR cohorts with (Marilyn;*Rag2*^{+/-})
187 or without *Rag2* protein (Marilyn;*Rag2*^{-/-}) were generated in another animal facility
188 (i3S/Porto). Surprisingly, most Marilyn;*Rag2*^{+/-} female mice (78%) did not develop
189 disease, with the exception of two mice with splenomegaly at nearly 18 months of age,
190 which was composed of B and myeloid cells (Figure 3A and not shown). Although half
191 of Marilyn;*Rag2*^{-/-} males developed hematological splenic disease after 1 year of age (4
192 cases of myeloid proliferation and 1 case of *Notch1*-mutated TCR-V β 6⁺ CD4⁻CD8⁻
193 splenic lymphoma), nearly all Marilyn;*Rag2*^{-/-} females developed lymphoma with much

194 earlier onset than males (median survival of 46 and 70 weeks, respectively; Figure 3A).
195 All diseased Marilyn;*Rag2*^{-/-} females presented thymic lymphoma, of variable size,
196 frequently accompanied by enlarged spleen, LNs, liver and kidneys (Figure 3B;
197 Supplementary Figure 9A). Leukemias from Marilyn;*Rag2*^{-/-} females were mostly
198 composed of CD4^{+/low}CD8^{+/low} immature T cells expressing TCR-Vβ6, high levels of
199 CD24, CD44 negativity and variable expression of CD25 and CD5 (Figure 3C, panel i,
200 and Supplementary Table 2). A similar immunophenotype was detected across
201 different affected organs, suggesting that diseased cells disseminated from the thymus
202 to other organs (Figure 3C, panel ii). The pathological and immunophenotypic features,
203 together with the observed 60% frequency of *Notch1* exon 34 frameshift mutations and
204 ability to reinitiate disease in syngeneic recipients (Supplementary Table 2), indicate
205 that Marilyn;*Rag2*^{-/-} females developed T-ALL. To assess the degree of leukemic cell
206 infiltration in different Marilyn;*Rag2*^{-/-} organs, we performed histological analysis.
207 Lymphoblasts were conspicuous in the thymus and spleen, and infiltrated with variable
208 extent, the liver, kidneys, lungs and bone marrow (Supplementary Figure 9B).
209 Interestingly, immunohistochemical analyses of thymus histological sections showed
210 that while Marilyn T-ALL cells were highly proliferative, with conspicuous Ki67 staining,
211 they maintained a high level of apoptosis, as gauged by cleaved caspase 3 staining
212 (Figure 3D). In sum, these data confirm that Marilyn HY-TCR tonic signaling drives T-
213 ALL and demonstrated that Rag recombinase-mediated DNA rearrangements are not
214 implicated.

215

216 **Hematological disease in HY-TCR transgenic females is associated with** 217 **microbial status**

218 Marilyn females in the UAlg/Faro animal facility had higher incidence of hematological
219 disease than the Marilyn;*Rag2*^{+/-} females in the i3S/Porto facility. Since opportunistic
220 microorganisms were detected in the UAlg but not the i3S facility (see Materials and
221 methods), we posit that an environmental input present only at the UAlg facility
222 increased susceptibility for lymphoid disease. Considering that bacteria are frequently
223 present in tumors, and can be detected by immunohistochemical methods,²⁵ we
224 probed lymphomas obtained in both facilities for the presence of bacterial-derived
225 lipopolysaccharide (LPS). Indeed, Marilyn lymphomas derived from the UAlg facility
226 displayed more conspicuous LPS immunostaining than Marilyn;*Rag2*^{-/-} lymphomas
227 derived from the i3S facility (Supplementary Figure 10A). Of note, the pattern of LPS
228 staining in Marilyn splenic lymphomas overlapped with B220⁺ B cell areas
229 (Supplementary Figure 10B). These data suggest that higher bacterial exposure could
230 have promoted hematological diseases in Marilyn females.

231

232 **The transgenic HY-TCR is functional in Marilyn T-ALL cells and its stimulation**
233 **delays leukemogenesis**

234 Given that antigenic stimulation prevented spontaneous T-ALL development in male
235 HY-TCR mice, we set out to determine the response of female HY-TCR leukemic cells
236 to TCR stimulation. Marilyn T-ALL cells readily increased CD69 and CD5 expression
237 and enlarged in size when stimulated *in vitro* with plate-bound CD3 antibody, which
238 directly activates the TCR signaling complex, or phorbol 12-myristate-13-acetate (PMA)
239 and ionomycin, triggering downstream activation of protein kinase C and intracellular
240 calcium pathways (Supplementary Figure 11). Previous research demonstrated that
241 agonist TCR stimulation can induce T-ALL apoptosis *in vitro*.¹⁹ However, Marilyn
242 leukemic cells underwent extensive apoptosis *in vitro*, impeding determination if TCR
243 stimulation induced cell death. To assess the impact of TCR stimulation *in vivo*, Marilyn
244 T-ALL cells were injected in syngeneic male and female mice and disease progression
245 followed by peripheral blood collection. Marilyn T-ALL cells were found in the blood of
246 females, but not males, as early as 30 days post-injection (Figure 4A). Most female
247 recipients rapidly developed fatal T-ALL, while male recipients often failed to develop
248 leukemia and therefore survived longer (Figure 4B). In addition, females infused with
249 Marilyn T-ALL presented significantly larger spleen and kidneys (Figure 4C), and more
250 extensive BM leukemia infiltration (Figure 4D), than male recipients euthanized
251 simultaneously. Furthermore, male spleens showed higher levels of cleaved caspase 3
252 immunostaining than female spleens (Figure 4E), indicating that delayed
253 leukemogenesis in males was caused by apoptosis induction.

254 To address whether the leukemia-delaying effect of agonist-induced TCR
255 stimulation in Marilyn T-ALL cells was dependent on MHC class II presentation, Marilyn
256 T-ALL cells were infused in immunodeficient syngeneic and allogenic males and
257 females. In contrast to the delayed T-ALL development in syngeneic males, as
258 compared to female counterparts (Figure 4F, left panel), no significant difference
259 between allogeneic males and females was observed (Figure 4F, right panel). These
260 findings indicate that the T-ALL-suppressive effect of the male antigen was dependent
261 on restricted MHC class II molecules. To confirm that delayed Marilyn leukemogenesis
262 in male mice was due to male antigen presentation, we next treated syngeneic
263 immunodeficient females infused with Marilyn T-ALL with the DBY cognate peptide.
264 Indeed, DBY administration significantly delayed T-ALL development in females
265 (Figure 4F). Overall, these data indicate that strong agonistic HY-TCR signaling
266 suppresses leukemogenesis.

267

268 Discussion

269 In this report, we addressed the functional role of TCR signaling intensity in mouse T-
270 ALL. We show that transgenic Marilyn HY-TCR tonic signaling not only had a
271 synergistic effect with the ETV6::JAK2 fusion kinase, but also was sufficient to induce
272 T-ALL on its own. Furthermore, our data show that the TCR expressed in Marilyn T-
273 ALL cells responded to agonist stimuli, inducing apoptosis and leading to
274 leukemogenesis suppression. Thus, these results support the notion that tonic TCR
275 signaling can be leukemogenic, and strong signaling emanating from the same TCR is
276 anti-leukemic.

277 Tonic signaling is generated by relatively weak TCR interactions with self-pMHC
278 ligands and is important for controlling survival of naive T cells and allow their
279 responsiveness to foreign antigens.²⁶ Thymocytes expressing transgenic TCRs against
280 foreign antigens (such as the male antigen for females) are subjected to self-pMHC-
281 mediated tonic signaling during their maturation, namely at the positive selection stage.
282 The notion that TCR signaling can be leukemogenic has been earlier described, and
283 transgenic TCR mouse strains other than the Marilyn strain, were shown to lead to
284 spontaneous T-ALL development with variable latency and penetrance.²⁷⁻³⁰ It is thus
285 likely that tonic signaling originates pro-survival or pro-proliferative genetic programs
286 that can cooperate with stochastic oncogenic genetic alterations, such as *Notch1*-
287 activating mutations and *Cdkn2a* deletions. *Cdkn2a* inactivation in particular was
288 reported to facilitate the malignant transformation of immature T cells,^{31,32} so it is
289 possible that loss of *Cdkn2a* function may cooperate with TCR tonic signaling in DN
290 thymocytes.

291 The finding that tonic TCR signaling can promote malignant transformation finds
292 parallels in other systems. In fact, tonic B cell receptor signaling, defined as antigen-
293 independent cell-autonomous signaling, has been shown to drive B cell
294 lymphomagenesis³³⁻³⁵ and to be therapeutically targetable.³⁶ By the same token, tonic
295 TCR signaling could be a targetable mechanism in human T-ALL, as suggested by the
296 frequent activation of Lck kinase (a mediator of TCR signaling) in T-ALL and by the
297 therapeutic potential of Lck inhibitors.⁶⁻⁹ Lck kinase activation in T-ALL was attributed
298 to constitutive pre-TCR signaling found in a subset of patients.⁶ Since TCR and CD3
299 proteins are also frequently expressed in T-ALL,⁴ further studies are warranted to
300 determine the role of TCR signaling in human T-ALL pathogenesis.

301 Although Rag recombinase-mediated mutagenic events can contribute to
302 leukemia development,³⁷ that was not the case for Marilyn T-ALL. In fact, T-ALL
303 developed with higher penetrance and much faster in Marilyn;*Rag2*^{-/-} females than in
304 Marilyn;*Rag2*^{+/-} females generated in the same animal facility. Since Rag2 deficiency

305 arrests thymocyte development at the DN3 stage, these results suggest that ectopic
306 TCR expression at the immature DN3 stage in the absence of more mature thymocytes
307 has increased leukemogenic potential, in line with the finding that early expression of
308 mature TCR in DN thymocytes promotes T-ALL development.³⁰

309 Rag2-sufficient, Marilyn mouse cohorts housed in different animal facilities led
310 to unexpected different rates of T-ALL incidence. Indeed, Marilyn T-ALL frequency was
311 much higher in females housed at the UAlg/Faro facility than females housed at the
312 i3S/Porto facility. Since several microorganisms (*Helicobacter* spp., *Pasteurella*
313 *pneumotropica* and murine norovirus) were detected in the UAlg facility, but not in the
314 i3S facility, increased exposure to pathogenic microbiota could have promoted a
315 microenvironment favorable for transgenic TCR-induced leukemogenesis. This notion
316 is supported by the finding of higher levels of LPS staining in lymphomas from UAlg
317 facility mice than in lymphomas from i3S facility mice. Early research revealed that B-
318 ALL in *Pax5* heterozygous mice was initiated only upon exposure to common
319 pathogens.³⁸ Future studies should, therefore, determine whether the presence of
320 microorganisms promotes TCR-induced leukemogenesis and whether TCR interactions
321 with MHC molecules presenting non-cognate foreign peptides generate pro-
322 leukemogenic basal TCR signaling.

323 Although T-ALL in Marilyn females developed in the presence of self-pMHC, we
324 found that MHC-dependent TCR signaling was not required for its maintenance.
325 Indeed, secondary leukemia development was not affected by lack of MHC-bound self-
326 peptides in recipient allogenic mice. These results contrast with the finding that
327 continuous self-pMHC-mediated TCR signaling was necessary for lymphoma cell
328 growth in a TCR-expressing reprogrammed T cell model.³⁹ Of note, expression of a
329 transgenic TCR recognizing a peptide from the murine survivin protein was also shown
330 to result in increased TCR signaling and to induce T-ALL.²⁹ In this case, the specific
331 (self) antigen, survivin, was expressed constitutively, which contrasts with the absence
332 of the HY-TCR cognate peptide (DBY) in Marilyn females. Our data together with
333 previous reports demonstrate that the TCR signaling complex promotes T-ALL, and
334 that this ability very much depends on the TCR affinity to antigen.

335 Finally, we showed that Marilyn T-ALL cells had a functional TCR and that *in*
336 *vivo* TCR stimulation of transplanted female Marilyn T-ALL cells was associated with
337 apoptosis and impaired Marilyn T-ALL leukemogenesis. These observations
338 corroborate previous reports showing that mouse or human T-ALL underwent
339 apoptosis upon activation of the TCR/CD3 signaling complex.^{19,28,40} These findings
340 suggest that, similarly to thymocytes undergoing thymic selection, TCR activation with
341 low-affinity or high-affinity antigens will stimulate differentially the TCR signaling

342 pathway and result in cell survival or death.⁴¹ Future research should determine the
343 TCR signaling regulators that dictate if a particular antigenic stimulus promotes or
344 antagonizes T-ALL.

345 **Methods**

346 **Mice**

347 HY-TCR Marilyn transgenic mice (B6.Cg-Tg(TcraH-Y,TcrbH-Y)1Pas), on a CD45.1
348 background, were obtained from Jocelyne Demengeot (IGC, Oeiras). Marilyn mice
349 were bred with E μ SR α -ETV6::JAK2 (B6.Cg-Tg(Emu-ETV6/JAK2)71Ghy) transgenic
350 mice¹³, or *Rag2* (B6.129S6-*Rag2*^{tm1Fwa}) knockout mice. The ETV6::JAK2 transgene
351 was kept in hemizyosity. *Foxn1*^{nu/nu} (Nude) C57BL/6 and CBA mice were bred at the
352 i3S facility. Mice were maintained at the UAlg/Faro and i3S/Porto barrier animal
353 facilities, under 12 h light/dark cycles and with food and water *ad libitum*. HY-TCR
354 Marilyn mice and ETV6::JAK2;Marilyn double transgenic were bred at the UALg animal
355 facility, while the Marilyn;*Rag2*^{+/-} and Marilyn;*Rag2*^{-/-} were bred at the i3S animal facility.
356 Microorganism screening detected opportunistic pathogens (*Helicobacter* spp.,
357 *Pasteurella pneumotropica* and murine norovirus) in the UAlg, but not i3S,
358 experimental rooms. All experimental procedures were approved by the i3S and
359 CBMR/UAlg ethics committees and Portuguese authorities (*Direção-Geral de*
360 *Agricultura e Veterinária*) and followed recommendations from the European
361 Commission (Directive 2010/63/UE) and the local Portuguese authorities (*Decreto-Lei*
362 n°113/2013). Both female and male mice were used for all experiments. Mice were
363 monitored for signs of disease (e.g. dyspnea, lethargy, enlarged lymph nodes and
364 enlarged abdomen) and killed by CO₂ inhalation when reaching predefined
365 experimental endpoints. Mice of different genotypes from the same litter were kept
366 together in the same cages and monitoring for signs of disease was done blindly. Adult
367 mice that were euthanized without leukemia were censored in Kaplan-Meier survival
368 curves.

369

370 **Mice genotyping**

371 The following primers were used for genotyping. HY-TCR Marilyn transgene: 5'-
372 CGAGAGGAACCTGGGAGCTGT-3' and 5'-TGCTGTCTGTACCACCAGAAATAC-3';
373 *Rag2*: 5'-TGTCCCTGCAGATGGTAACA-3', 5'-CCTTTGTATGAGCAAGTAGC-3', 5'-
374 CTATTCGGCTATGACTGGG-3' and 5'-AAGGCGATAGAAGGCGATG-3'; *Cdkn2a*: 5'-
375 GTGATCCCTCTACTTTTTCTTCTGACTT-3', 5'-CGGAACGCAAATATCGCAC-3' and
376 5'-GAGACTAGTGAGACGTGCTACTTCCA-3'.

377

378 **In vivo experiments**

379 For mouse leukemia transplantation assays, 0.5-2 x 10⁶ leukemic cells collected from
380 diseased female Marilyn mice were intravenously injected in the tail vein of recipient 8-
381 12-week-old C57BL/6, C57BL/6 Nude or CBA Nude mice of the indicated sex, and

382 regularly monitored through peripheral blood detection of leukemic cells
383 (CD45.1⁺CD45.2⁺TCR-V β 6⁺). Female *Rag2*^{-/-} mice infused with Marilyn T-ALL cells
384 were treated intraperitoneally once a week for two weeks with 0.01 mg of DBY peptide
385 (NAGFNSNRANSSRSS; cat. no. AS-61046; Eurogentec).

386

387 **Flow cytometry**

388 Single-cell suspensions were prepared from lymphoid organs using cell strainers,
389 washed with FACS buffer (phosphate-buffered saline (PBS) with 3% fetal bovine
390 serum (FBS) and 10 mM NaN₃), and incubated for 30-45 min with fluorochrome-
391 labeled antibodies in FACS buffer. The following Biolegend antibodies were used:
392 CD25-fluoresceine isothiocyanate (FITC) (clone PC61), TCR β -phycoerithrin (PE)
393 (clone H57-597), CD44-PE/Cyanine5 (clone IM7), CD90.2-PE-Cyanine7 (clone 30-
394 H12), CD24-allophycocyanin (APC) (clone M1/69), CD4-APC/Cyanine7 (clone GK1.5),
395 CD8 α -Pacific Blue (clone 53-6.7), Gr1-PE (clone RB6-8C5), CD127-PE/Cyanine5
396 (clone A7R34), B220-APC/Cyanine7 (clone RA3-6B2), CD24-Pacific Blue (clone
397 M1/69), CD90.2-FITC (clone 30-H12), TCR-V β 6-PE (clone RR4-7), TCR-V β 5.1, 5.2-PE
398 (clone MR9-4), CD5-APC (clone 53-7.3), CD69-FITC (clone H1.2F3), CD62L-APC
399 (clone MEL-14), CD45.1-APC (clone A20) and CD45.2-Peridinin Chlorophyll Protein
400 (PerCP)-Cyanine5.5 (clone 104). Immunostained cells were washed twice with FACS
401 buffer and incubated in PBS with 10 mM NaN₃. Cell viability was determined using the
402 Zombie Aqua Fixable Viability Kit (Invitrogen). Samples were acquired using BD FACS
403 Calibur, CANTO II or Accuri C6 and analyzed using FlowJo software.

404

405 ***Ex-vivo* T-ALL cell culture**

406 Marilyn T-ALL cells were cultured *ex-vivo* for 16 h in RPMI medium supplemented with
407 10% FBS, 1% penicillin-streptomycin and 50 μ M of 2-mercaptoethanol, all from Gibco.
408 For *ex vivo* stimulation of Marilyn T-ALL cells, plates were coated with 10 μ g/ml anti-
409 CD3 (145-2C11, Biolegend) for 2 h at 37°C, and then plates were washed with PBS.
410 Ten ng/ml of PMA and 250 ng/ml Ionomycin (both from Sigma-Aldrich) treatments were
411 performed for 18 hours.

412

413 ***Notch1* mutation detection**

414 Genomic DNA from mouse leukemia samples was isolated using the GeneJET
415 Genomic DNA purification kit (Thermo Fisher Scientific), following the manufacturer's
416 instructions. DNA was used for PCR amplification of two segments of *Notch1* exon 34:
417 primer pair 5'-GCTCCCTCATGTACCTCCTG-3' and 5'-TAGTGGCCCCATCATGCTAT-

418 3', generating a predicted amplicon of 904 bp, and primer pair 5'-
419 ATAGCATGATGGGGCCACTA-3' and 5'-CTTCACCCTGACCAGGAAAA-3',
420 generating a predicted amplicon of 893 bp. PCR products were Sanger sequenced
421 using the same primers at i3S Genomics (Porto, Portugal) or CCMAR *Serviços de*
422 *Biologia Molecular* (Faro, Portugal).

423

424 **Low-coverage whole genome sequencing**

425 Genomic DNA was used for copy number analysis performed as described previously⁴²
426 using a HiSeq 4000 (Illumina) sequencer in a single-read 50-cycle run mode. For copy
427 number analysis, sequencing data was aligned with the mouse genome (mm10) as
428 reference and further analyzed using QDNAseq (RRID:SCR_003174) package
429 (version 1.12) in R software. A bin size of 15 kb was used.

430

431 **Histology and immunohistochemistry**

432 For histological and immunohistochemistry analysis, formalin-fixed paraffin-embedded
433 tissues were sectioned with 4 μ m thickness. Hematoxylin and eosin staining was
434 performed using standard procedures. For immunohistochemistry, heat-mediated
435 antigen retrieval was performed for 35-40 min with citrate-based antigen retrieving
436 solution (cat. no. H-3300, Vector Laboratories), for cleaved-caspase 3, Ki67 and LPS
437 immunodetection, or 10 μ M EDTA, pH 8.0, and 0.05% Tween 20 in PBS, for B220 or
438 CD3 immunodetection. Endogenous peroxidase was inactivated with 3% H₂O₂ in
439 methanol, and nonspecific antibody binding was blocked using Ultravision Protein-
440 block (Thermo Fisher Scientific). Sections were incubated overnight with rat anti-B220
441 (1:200, clone RA3-6B2, Biolegend), anti-CD3 (1:200, ab5690, Abcam), anti-cleaved-
442 caspase 3 (1:200, 9661, Cell Signaling), Ki67 (1:500, ab15580, Abcam) or LPS (1:100,
443 WN1 222-5, Hycult Biotech) at 4 °C. For B220 detection, slides were incubated
444 sequentially with avidin/biotin blocking system (Biolegend), goat anti-rabbit secondary
445 antibody (Biovision), and streptavidin-horseradish peroxidase (HRP) (Enzo Biochem).
446 For CD3, Ki67 and LPS detection, HRP-conjugated rabbit/mouse secondary antibody
447 (Agilent) was used. 3,3'-Diaminobenzidine chromogen (Agilent) was used as detection
448 reagent. For quantification of percentage of cleaved caspase 3-positive cells, five
449 images were taken from different areas of each tissue section.

450

451 **Statistics**

452 Statistical analysis was performed with GraphPad Prism 6.0 software
453 (RRID:SCR_002798). Log-rank test was used to compare survival of different groups.
454 Unpaired student's t-test was used for comparisons between two groups. Welch's

455 correction was used when variances were different. Mann-Whitney test was used to
456 compare white blood cell (WBC) count. Detection of CD45.1⁺ T-ALL cells in the
457 peripheral blood of males and females was compared using two-way ANOVA. Sample
458 numbers are indicated in figure legends. $P < 0.05$ was considered statistically significant.

459

460 **Data Sharing Statement**

461 The data that support the findings of this study are available from the corresponding
462 author, upon reasonable request. Raw unaligned sequencing reads (fastq-format) that
463 support the findings of this study have been deposited in the SRA.

464

465 **Acknowledgments**

466 We thank Jocelyne Demengeot (IGC, Oeiras) and Nuno L. Alves (i3S, Porto) for
467 providing Marylin transgenic mice. We thank members of i3S Intercellular
468 Communication and Cancer group for fruitful discussions about this work. The authors
469 acknowledge the support the Animal Facility, Translational Cytometry and Histology,
470 Electron Microscopy (member of the Portuguese Platform of Bioimaging; PPBI-POCI-
471 01-0145-FEDER-022122) and Genomics i3S Scientific Platforms. This work was
472 supported by a fellowship (FAZ Ciencia prize) from Fundação AstraZeneca (Lisbon,
473 Portugal). This work was supported by European Regional Development Fund (ERDF),
474 through COMPETE 2020 - Operational Program for Competitiveness and
475 Internationalisation, Portugal2020, and *Fundação para a Ciência e a Tecnologia* (FCT;
476 POCI-01-0145-FEDER-007274; PTDC/MED-ONC/32592/2017), and by ERDF, through
477 the Norte Portugal Regional Program (NORTE2020), Portugal2020 (NORTE-01-0145-
478 FEDER-000029) and HEALTH-UNORTE (NORTE-01-0145-000039). T.A.C. was
479 recipient of FCT (PD/BD/114129/2015) and *Liga Portuguesa Contra o Cancro – Núcleo*
480 *Regional do Norte* fellowships. J.L.P. (SFRH/BD/147979/2019) and M.B.
481 (2021.06024.BD) were recipients of FCT fellowships.

482

483 **Contribution:** T.A.C. designed, performed and analyzed experiments, made the
484 figures and wrote the paper; I.P.-L., J.L.P., and M.B. performed experiments; N.R.S.
485 designed the study, performed experiments, made the figures and wrote the paper.

486

487 **Conflict-of-interest disclosures:** No competing financial interests to declare.

488

489 **Correspondence:** Nuno R. dos Santos, i3S - Instituto de Investigação e Inovação em
490 Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal; e-
491 mail: nunos@i3s.up.pt

492

493 **References**

- 494 1. Shah, D. K. & Zúñiga-Pflücker, J. C. An Overview of the Intrathymic Intricacies
495 of T Cell Development. *The Journal of Immunology* **192**, 4017–4023 (2014).
- 496 2. Liu, X. *et al.* Notch-induced endoplasmic reticulum-associated degradation
497 governs mouse thymocyte β -selection. *Elife* **10**, (2021).
- 498 3. Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative
499 selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev*
500 *Immunol* **14**, 377–391 (2014).
- 501 4. Asnafi, V. *et al.* Analysis of TCR, pT alpha, and RAG-1 in T-acute lymphoblastic
502 leukemias improves understanding of early human T-lymphoid lineage
503 commitment. *Blood* **101**, 2693–2703 (2003).
- 504 5. Girardi, T., Vicente, C., Cools, J. & De Keersmaecker, K. The genetics and
505 molecular biology of T-ALL. *Blood* **129**, 1113–1123 (2017).
- 506 6. Gocho, Y. *et al.* Network-based systems pharmacology reveals heterogeneity in
507 LCK and BCL2 signaling and therapeutic sensitivity of T-cell acute
508 lymphoblastic leukemia. *Nat Cancer* **2**, 284–299 (2021).
- 509 7. Laukkanen, S. *et al.* Therapeutic targeting of LCK tyrosine kinase and mTOR
510 signaling in T-cell acute lymphoblastic leukemia. *Blood* **140**, 1891–1906 (2022).
- 511 8. Serafin, V. *et al.* Glucocorticoid resistance is reverted by LCK inhibition in
512 pediatric T-cell acute lymphoblastic leukemia. *Blood* **130**, 2750–2761 (2017).
- 513 9. Buffière, A. *et al.* Saracatinib impairs maintenance of human T-ALL by targeting
514 the LCK tyrosine kinase in cells displaying high level of lipid rafts. *Leukemia* **32**,
515 2062–2065 (2018).
- 516 10. Campese, A. F. *et al.* Notch1-dependent lymphomagenesis is assisted by but
517 does not essentially require pre-TCR signaling. *Blood* **108**, 305–310 (2006).
- 518 11. Bellavia, D. *et al.* Combined expression of pT α and Notch3 in T cell leukemia
519 identifies the requirement of preTCR for leukemogenesis. *Proceedings of the*
520 *National Academy of Sciences* **99**, 3788–3793 (2002).
- 521 12. Smith, D. P., Bath, M. L., Harris, A. W. & Cory, S. T-cell lymphomas mask
522 slower developing B-lymphoid and myeloid tumours in transgenic mice with
523 broad haemopoietic expression of MYC. *Oncogene* **24**, 3544–3553 (2005).
- 524 13. dos Santos, N. R. *et al.* Pre-TCR expression cooperates with TEL-JAK2 to
525 transform immature thymocytes and induce T-cell leukemia. *Blood* **109**, 3972–
526 3981 (2007).
- 527 14. Tremblay, M. *et al.* Modeling T-cell acute lymphoblastic leukemia induced by
528 the SCL and LMO1 oncogenes. *Genes Dev* **24**, 1093–1105 (2010).

- 529 15. Liao, M.-J. *et al.* No Requirement for V(D)J Recombination in p53-Deficient
530 Thymic Lymphoma. *Mol Cell Biol* **18**, 3495–3501 (1998).
- 531 16. Kelly, J. A. *et al.* Stat5 Synergizes with T Cell Receptor/Antigen Stimulation in
532 the Development of Lymphoblastic Lymphoma. *Journal of Experimental*
533 *Medicine* **198**, 79–89 (2003).
- 534 17. Tsatsanis, C. *et al.* Tpl2 and ERK transduce antiproliferative T cell receptor
535 signals and inhibit transformation of chronically stimulated T cells. *Proceedings*
536 *of the National Academy of Sciences* **105**, 2987–2992 (2008).
- 537 18. Gon, S. *et al.* Fit $\alpha\beta$ T-cell receptor suppresses leukemogenesis of Pten-
538 deficient thymocytes. *Haematologica* **103**, 999–1007 (2018).
- 539 19. Trinquand, A. *et al.* Triggering the TCR Developmental Checkpoint Activates a
540 Therapeutically Targetable Tumor Suppressive Pathway in T-cell Leukemia.
541 *Cancer Discov* **6**, 972–85 (2016).
- 542 20. Lantz, O., Grandjean, I., Matzinger, P. & di Santo, J. P. γ chain required for
543 naïve CD4+ T cell survival but not for antigen proliferation. *Nat Immunol* **1**, 54–
544 58 (2000).
- 545 21. Bendelac, A., Matzinger, P., Seder, R. A., Paul, W. E. & Schwartz, R. H.
546 Activation events during thymic selection. *J Exp Med* **175**, 731–42 (1992).
- 547 22. Azzam, H. S. *et al.* CD5 expression is developmentally regulated by T cell
548 receptor (TCR) signals and TCR avidity. *J Exp Med* **188**, 2301–11 (1998).
- 549 23. O’Neil, J. *et al.* Activating Notch1 mutations in mouse models of T-ALL. *Blood*
550 **107**, 781–785 (2006).
- 551 24. Lin, Y.-W., Nichols, R. A., Letterio, J. J. & Aplan, P. D. Notch1 mutations are
552 important for leukemic transformation in murine models of precursor-T
553 leukemia/lymphoma. *Blood* **107**, 2540–2543 (2006).
- 554 25. Nejman, D. *et al.* The human tumor microbiome is composed of tumor type-
555 specific intracellular bacteria. *Science (1979)* **368**, 973–980 (2020).
- 556 26. Cho, J.-H. & Sprent, J. TCR tuning of T cell subsets. *Immunol Rev* **283**, 129–
557 137 (2018).
- 558 27. Brabb, T., Rubicz, R., Mannikko, V. & Goverman, J. Separately expressed T
559 cell receptor α and β chain transgenes exert opposite effects on T cell
560 differentiation and neoplastic transformation. *Eur J Immunol* **27**, 3039–3048
561 (1997).
- 562 28. Strzadala, L., Miazek, A., Matuszyk, J. & Kisielow, P. Role of thymic selection in
563 the development of thymic lymphomas in TCR transgenic mice. *Int Immunol* **9**,
564 127–38 (1997).

- 565 29. Cui, Y. *et al.* Thymic expression of a T-cell receptor targeting a tumor-
566 associated antigen coexpressed in the thymus induces T-ALL. *Blood* **125**,
567 2958–2967 (2015).
- 568 30. Laffey, K. G. *et al.* Early expression of mature $\alpha\beta$ TCR in CD4 – CD8 – T cell
569 progenitors enables MHC to drive development of T-ALL bearing NOTCH
570 mutations. *Proceedings of the National Academy of Sciences* **119**, (2022).
- 571 31. Volanakis, E. J., Williams, R. T. & Sherr, C. J. Stage-specific Arf tumor
572 suppression in Notch1-induced T-cell acute lymphoblastic leukemia. *Blood* **114**,
573 4451–4459 (2009).
- 574 32. Catarino, T. A. *et al.* Cdkn2a inactivation promotes malignant transformation of
575 mouse immature thymocytes before the β -selection checkpoint. *Exp Hematol*
576 **116**, 30–36 (2022).
- 577 33. Minden, M. D. *et al.* Chronic lymphocytic leukaemia is driven by antigen-
578 independent cell-autonomous signalling. *Nature* **489**, 309–312 (2012).
- 579 34. Schmitz, R. *et al.* Burkitt lymphoma pathogenesis and therapeutic targets from
580 structural and functional genomics. *Nature* **490**, 116–120 (2012).
- 581 35. Havranek, O. *et al.* Tonic B-cell receptor signaling in diffuse large B-cell
582 lymphoma. *Blood* **130**, 995–1006 (2017).
- 583 36. Szydowski, M. *et al.* FOXO1 activation is an effector of SYK and AKT inhibition
584 in tonic BCR signal-dependent diffuse large B-cell lymphomas. *Blood* **127**, 739–
585 748 (2016).
- 586 37. Bianchi, J. J., Murigneux, V., Bedora-Faure, M., Lescale, C. & Deriano, L.
587 Breakage-Fusion-Bridge Events Trigger Complex Genome Rearrangements
588 and Amplifications in Developmentally Arrested T Cell Lymphomas. *Cell Rep*
589 **27**, 2847-2858.e4 (2019).
- 590 38. Martín-Lorenzo, A. *et al.* Infection Exposure Is a Causal Factor in B-cell
591 Precursor Acute Lymphoblastic Leukemia as a Result of Pax5 -Inherited
592 Susceptibility. *Cancer Discov* **5**, 1328–1343 (2015).
- 593 39. Serwold, T. *et al.* T-cell receptor-driven lymphomagenesis in mice derived from
594 a reprogrammed T cell. *Proceedings of the National Academy of Sciences* **107**,
595 18939–18943 (2010).
- 596 40. Tran Quang, C. *et al.* Preclinical efficacy of humanized, non-Fc γ R-binding anti-
597 CD3 antibodies in T-cell acute lymphoblastic leukemia. *Blood* **136**, 1298–1302
598 (2020).
- 599 41. dos Santos, N. R., Ghysdael, J. & Tran Quang, C. The TCR/CD3 complex in
600 leukemogenesis and as a therapeutic target in T-cell acute lymphoblastic
601 leukemia. *Adv Biol Regul* **74**, 100638 (2019).

602 42. Scheinin, I. *et al.* DNA copy number analysis of fresh and formalin-fixed
603 specimens by shallow whole-genome sequencing with identification and
604 exclusion of problematic regions in the genome assembly. *Genome Res* **24**,
605 2022–32 (2014).
606

607 **Figure legends**

608

609 **Figure 1. HY-TCR transgene expression drives leukemia development.** (A,B)
610 Kaplan-Meier leukemia-free survival curves of ETV6::JAK2 (male and female together)
611 and female ETV6::JAK2;Marilyn transgenic mice (A) and male (M) and female (F)
612 Marilyn transgenic mice (B). Log-rank test *P* values are shown in graphs. (C) Organ
613 weights of Marilyn females with leukemia/lymphoma compared to those of 7 month-old
614 wild-type (WT) females (n=3), or 10-week-old WT males for lymph nodes (n=4). For
615 Marilyn females n=16, except n=14 for kidneys. (D) White blood cell (WBC) counts of
616 Marilyn (n=15) and WT (n=3) females. Lower panels show blood smears of
617 representative WT and diseased Marilyn females. *P* values in (C) were obtained from
618 unpaired *t* tests with Welch's correction, and in (D) from Mann-Whitney test. Mean and
619 standard error of the mean (SEM) are shown. (E) Histogram representation of tumor
620 burden in lymphoid organs (thymus, spleen, and lymph nodes (LNs)) of Marilyn
621 females with or without thymic lymphoma. Female no. 12 had a mediastinal lymphoid
622 tumor, while no. 26 had lymphoid tumors on the duodenum wall. Each column
623 represents a mouse. Wild-type (WT) female mice were used as control.

624

625 **Figure 2. Immunophenotypic analysis of HY-TCR leukemic cells.** Flow cytometry
626 analysis of thymus-derived leukemic cells from representative Marilyn mice presenting
627 immature T cell phenotype, based on CD44 negativity and high CD24 expression (nos.
628 22, 33 and 40), mature T cell phenotype, based on CD44 positivity and CD24
629 intermediate/low expression (nos. 9 and 29), and mixed B and T cell phenotype, based
630 on B220 and CD90.2 expression (nos. 25 and 34). In CD44 and CD24 histogram plots,
631 unstained cells were used as negative control (grey shades). Healthy HY-TCR female
632 total thymocyte immunostaining is shown as control. TL, thymic lymphoma.

633

634 **Figure 3. Transgenic HY-TCR expression in *Rag2*^{-/-} mice drives development of T-
635 ALL with thymic lymphomas.** (A) Kaplan-Meier leukemia-free survival curve for the
636 indicated genotypes and number of mice (n). Log-rank test *P* values are indicated. (B)
637 Histogram representation of lymphoid organ weight (thymus, spleen and lymph nodes
638 (LNs)) of Marilyn females with leukemia (left graph) and healthy (right graph). Each
639 column represents a mouse. (C) Flow cytometry immunostaining of cells from the
640 thymus (i) and indicated organs (ii) of two representative Marilyn;*Rag2*^{-/-} leukemic mice
641 with the indicated surface markers. (D) Immunohistochemical Ki67 and cleaved-

642 caspase 3 staining of representative thymic lymphoma sections from Marilyn;*Rag2*^{-/-}
643 and Marilyn;*Rag2*^{+/+} diseased mice. Black scale bars: 500 μm; green scale bar: 200 μm.
644

645 **Figure 4. HY-TCR T-ALL antigenic stimulation *in vivo* hampers leukemia**
646 **development.** (A) Percentage of CD45.1⁺ T-ALL cells detected by flow cytometry in
647 the peripheral blood (PB) of male and female C57BL/6 mice infused with female HY-
648 TCR T-ALL cells. *P* value was obtained with two-way ANOVA. (B) Kaplan-Meier
649 leukemia-free survival curve for the same mice as in (A), which is representative of two
650 experiments performed with different primary leukemias. *P* value obtained with log-rank
651 test. (C) Spleens and kidneys (top panels) and respective organ weights (bottom
652 panels) for male (n=5) and female (n=6) C57BL/6 mice 21 days after infusion with HY-
653 TCR T-ALL cells. (D) Percentage of CD45.1⁺CD90.2⁺ HY-TCR T-ALL cells present in
654 the bone marrow of males and females shown in (C). *P* values in (C) and (D) were
655 obtained from unpaired *t* tests with Welch's correction. (E) Representative sections and
656 quantification of anti-cleaved caspase 3 immunohistochemistry for spleens from
657 females and males shown in (C,D; n=3). *P* value from unpaired *t* test. (C-E) Graphs
658 show mean and SEM. (F) Kaplan-Meier leukemia-free survival curves for syngeneic
659 C57BL/6 nude or allogeneic CBA nude male and female mice injected with female
660 Marilyn T-ALL cells. Number of mice and *P* values from log-rank test are indicated. (G)
661 Kaplan-Meier leukemia-free survival curves for syngeneic *Rag2*^{-/-} females infused on
662 day 0 with female Marilyn T-ALL cells and treated once a week with DBY peptide (0.01
663 mg; n=5) or Control (n=4). Gray shade represents treatment duration of 2 weeks.







