Transgenic αβ TCR tonic signaling is leukemogenic while strong stimulation is leukemia-suppressive

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Abstract

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

Introduction

The pre-T cell receptor (TCR) and αβ TCR are critical protein complexes for T cell development. Pre-TCR expression in $CD4^{-}CD8^{-}$ double-negative (DN) thymocytes induces their survival, proliferation and maturation through the β-selection checkpoint,[1,2] whereas surface expression of the αβ TCR in $CD4^{+}CD8^{+}$ double-positive (DP), thymocytes drives thymic positive and negative selection to establish major histocompatibility complex (MHC) restriction and eliminate autoreactive thymocytes.[3] The pre-TCR, TCR and CD3 proteins are frequently expressed in T-cell acute lymphoblastic leukemia (T-ALL).[4] Although T-ALL is genetically a very heterogeneous disease, with mutations detected in multiple genes, a number of genetic alterations have been recurrently reported for T-ALL, including *CDKN2A* loss of function, *NOTCH1*-activating mutations, and mutations in JAK/STAT signaling pathway genes (e.g. *IL7R*, *JAK3*, *JAK1*, and *STAT5B*).[5] Mutations in components of the TCR signaling machinery are rare,[5] but the Lck kinase, a key member of the pre-TCR and TCR signaling pathways, has been shown to be activated in T-ALL and put forward as a potential therapeutic target.[6–9] These observations hint that pre-TCR signaling can promote human T cell leukemogenesis. In this line, genetic inactivation of the pre-TCR complex (e.g. CD3ε, pTα or Rag1/2 deficiency) was shown to delay T-ALL development in several mouse models.[10–14] In contrast, the impact and function of TCR signaling in T-ALL development and progression remains controversial. While transgenic TCR expression was shown to promote mouse T-ALL development in cooperation with STAT5 overexpression or deficiency of p53 or Tpl2 proteins,[15–17] expression of endogenous αβ TCR or MHC-dependent antigen presentation were not required for leukemogenesis in the ETV6::JAK2 or TAL/LMO transgenic mouse models.[13,14] Indicating that TCR could rather have an antagonistic action in T-ALL, T-ALL onset in a *Pten*-deficient mouse model was accelerated by TCR genetic inactivation and delayed by expression of transgenic TCR.[18] In this line, it was also reported that strong agonist TCR stimulation of mouse ETV6::JAK2-driven T-ALL or patient T-ALL was anti-leukemic and induced a transcriptional program similar to that involved in thymocyte negative selection.[19] Overall, these observations suggest that, depending on the oncogenic drivers of leukemia and degree of TCR stimulation, the TCR signaling complex might play either a leukemia-promoting or a leukemia-suppressive role.

Here, our aim was to investigate the role of basal versus strong TCR signaling in T-ALL leukemogenesis. In normal T cells, TCR recognition of self-peptides presented by MHC (self-pMHC) induces tonic signaling, while agonist peptides induce strong TCR signaling. Using the Marilyn transgenic TCR mouse strain, specific for the H-Y male antigen, we show that tonic TCR signaling led to *Notch1*-mutated T-ALL exclusively in

females. MHC-dependent agonist antigenic stimulation prevented spontaneous T-ALL development in males, induced apoptosis of female leukemic cells and hampered their leukemogenic potential. Our results support the notion that αβ TCR tonic signaling promotes T cell precursor malignant transformation and that strong signaling suppresses leukemia.

Results

Transgenic expression of an αβ TCR specific for the H-Y male antigen drives leukemia development in female mice

To study the effect of TCR expression in leukemia development, we resorted to Marilyn transgenic mice, which express an MHC class II-restricted transgenic TCR (TCR-Vα1.1/Vβ6) specific for the H-Y male antigen (HY-TCR henceforth).[20] In this model, male thymocytes expressing the HY-TCR are exposed to the cognate antigen (DBY peptide), through MHC class II presentation, and are eliminated by apoptosis. In contrast, HY-TCR-expressing female thymocytes undergo positive selection and differentiate as mature $CD4^+$ T cells. To verify if the transgenic Marilyn HY-TCR promoted leukemogenesis, we bred transgenic mice expressing the ETV6::JAK2 fusion protein in lymphoid cells with Marilyn mice. As expected,[13] all ETV6::JAK2 transgenic mice developed T-ALL expressing CD8, CD25, CD24, CD3ε and TCRβ (not shown). Remarkably, co-expression of the Marilyn HY-TCR with ETV6::JAK2 accelerated spontaneous T-ALL development in females (median survival of 10.5 weeks versus 14 weeks; Figure 1A). This suggests that HY-TCR tonic signaling, which is induced by TCR interaction with self-peptides presented by MHC, cooperates with the ETV6::JAK2 transgene in driving leukemogenesis.

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

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

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

course (Supplementary Figures 3A,B). For a better characterization of the hematological disease developing in different Marilyn females, we performed immunophenotyping by flow cytometry. To define the T cell maturation status, we probed expression of CD24, which is downregulated upon thymocyte maturation, CD5, which is upregulated after thymocyte positive selection, and CD44, which is expressed in memory T cells.[21,22] Within the TL subgroup, five mice (nos. 2, 6, 22, 33 and 40) presented thymic T cells with immature phenotype, characterized by surface expression of CD90.2, TCR-Vβ6, and CD5, low levels of CD4 and CD8, high CD24 levels, and CD44 negativity (Figure 2 and Supplementary Table 1). Such immunophenotype was recapitulated in leukemic cells disseminated to the spleen, LNs, and bone marrow (Supplementary Figure 4 and not shown). Two other TL cases (nos. 9 and 29) presented a more mature T cell immunophenotype, characterized by $CD4^{+}CD8^{-}$TCR-$V\beta6^{+}CD5^{+}$ leukemic cells with CD44 expression (Figure 2). These cells were also present in spleen and lymph nodes (Supplementary Figure 4). Finally, and rather unexpectedly, two TL cases (nos. 3 and 34) presented a large proportion of B220-positive B cells, concomitantly with TCR-$V\beta6^{+}CD5^{+}CD44^{+}$ CD4 single-positive (SP) and CD8SP T cells (Figure 2). Of note, TL cases with immature immunophenotype, but not those with mature phenotype, could reinitiate disease in recipient syngeneic mice (Supplementary Table 1), suggesting that only the former were malignantly transformed.

Non-TL Marilyn females exhibited a mixture of $B220^{+}$ B cells and $CD90.2^{+}$TCR-$V\beta6^{+}CD5^{+}CD44^{+}$ CD4SP cells in lymphoid organs (Figure 2 and Supplementary Figure 4 and Table 1). Analysis of CD69 expression, a marker of TCR stimulation,[21] showed that TCR-$V\beta6^{+}$ cells from mixed B and T lymphomas were CD69-positive, indicating they experienced recent TCR stimulation (Supplementary Figure 5). This suggests that mixed B and T disease found in Marilyn female mice was driven by continuous stimulation of the HY-TCR transgene expressed in mature CD4SP or CD8SP T cells. Immature-type leukemic cells did not express CD69, suggesting that their proliferation was likely driven by oncogenic mechanisms rather than TCR stimulation.

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

155 **Marilyn T-ALL presents *Notch1* PEST domain mutations and genetic copy number**
156 **alterations**
157 To determine whether Marilyn T-ALL was associated with secondary genetic
158 alterations, we first sequenced the *Notch1* exon 34 (PEST domain), where most murine
159 T-ALL *Notch1* mutations have been found.[23,24] Four out of 5 Marilyn T-ALL cases
160 analyzed carried exon 34 frameshift mutations (Supplementary Figure 7 and Table 1),
161 while none were detected in cases of mixed T and B cell disease (Supplementary Table
162 1). Additionally, by performing low-coverage whole-genome sequencing, we found that
163 T-ALL samples had near-diploid genomes, with only a few copy number alterations: a
164 chromosome 15 trisomy (case no. 6), a chromosome 10 trisomy (case no. 33)
165 (Supplementary Figure 8A), and loss of the *Cdkn2a* locus (chromosome 4) in three cases
166 (nos. 2, 22 and 40) (Supplementary Figure 8B). In contrast, no consistent copy number
167 alterations were found in 2 cases of mixed T and B cell disease (Supplementary Figure
168 8A). Deletions of the *Tcra/d*, *Tcrb* and *Tcrg* loci were also found in Marilyn T-ALL
169 (Supplementary Figure 8C), indicating that these loci underwent genetic rearrangements
170 and that leukemia arose from post-β-selection thymocytes with rearranged TCR loci. As
171 expected from their T lineage origin, T-ALL cases did not exhibit consistent copy number
172 alterations in the immunoglobulin *Ighm* or *Igk* loci (Supplementary Figure 8C). The
173 detection of clonal copy number genetic alterations in Marilyn T-ALL bulk populations
174 further underscores their malignant nature. Given the high frequency of *NOTCH1*-
175 activating mutations and *CDKN2A* inactivation in human T-ALL,[5] our findings show that
176 the Marilyn transgenic HY-TCR T-ALL recapitulates key cellular and molecular features
177 of the human disease.
178
179 **HY-TCR-induced T-ALL is not dependent on *Rag2* recombinase expression**
180 Since several diseased Marilyn females developed B cell lymphoproliferation, we
181 generated Marilyn mice with *Rag2* constitutive knockout to block B cell development,
182 while maintaining HY-TCR-dependent T-cell development. This approach
183 simultaneously assessed whether Rag-mediated gene recombination plays a role in HY-
184 TCR-induced T cell leukemogenesis. Marilyn HY-TCR cohorts with (Marilyn;*Rag2*$^{+/-}$) or
185 without Rag2 protein (Marilyn;*Rag2*$^{-/-}$) were generated in another animal facility
186 (i3S/Porto). Surprisingly, most Marilyn;*Rag2*$^{+/-}$ female mice (78%) did not develop
187 disease, with the exception of two mice with splenomegaly at nearly 18 months of age,
188 which was composed of B and myeloid cells (Figure 3A and not shown). Although half
189 of Marilyn;*Rag2*$^{-/-}$ males developed hematological splenic disease after 1 year of age (4
190 cases of myeloid proliferation and 1 case of *Notch1*-mutated TCR-Vβ6^{+} CD4^{-}CD8^{-}
191 splenic lymphoma), nearly all Marilyn;*Rag2*$^{-/-}$ females developed lymphoma with much

192 earlier onset than males (median survival of 46 and 70 weeks, respectively; Figure 3A).
193 All diseased Marilyn;*Rag2*$^{-/-}$ females presented thymic lymphoma, of variable size,
194 frequently accompanied by enlarged spleen, LNs, liver and kidneys (Figure 3B;
195 Supplementary Figure 9A). Leukemias from Marilyn;*Rag2*$^{-/-}$ females were mostly
196 composed of CD4$^{+/low}$CD8$^{+/low}$ immature T cells expressing TCR-Vβ6, high levels of
197 CD24, CD44 negativity and variable expression of CD25 and CD5 (Figure 3C, panel i,
198 and Supplementary Table 2). A similar immunophenotype was detected across different
199 affected organs, suggesting that diseased cells disseminated from the thymus to other
200 organs (Figure 3C, panel ii). The pathological and immunophenotypic features, together
201 with the observed 60% frequency of *Notch1* exon 34 frameshift mutations and ability to
202 reinitiate disease in syngeneic recipients (Supplementary Table 2), indicate that
203 Marilyn;*Rag2*$^{-/-}$ females developed T-ALL. To assess the degree of leukemic cell
204 infiltration in different Marilyn;*Rag2*$^{-/-}$ organs, we performed histological analysis.
205 Lymphoblasts were conspicuous in the thymus and spleen, and infiltrated with variable
206 extent, the liver, kidneys, lungs and bone marrow (Supplementary Figure 9B).
207 Interestingly, immunohistochemical analyses of thymus histological sections showed
208 that while Marilyn T-ALL cells were highly proliferative, with conspicuous Ki67 staining,
209 they maintained a high level of apoptosis, as gauged by cleaved caspase 3 staining
210 (Figure 3D). In sum, these data confirm that Marilyn HY-TCR tonic signaling drives T-
211 ALL and demonstrated that Rag recombinase-mediated DNA rearrangements are not
212 implicated.
213
214 **Hematological disease in HY-TCR transgenic females is associated with microbial**
215 **status**
216 Marilyn females in the UAlg/Faro animal facility had higher incidence of
217 hematological disease than the Marilyn;*Rag2*$^{+/-}$ females in the i3S/Porto facility. Since
218 opportunistic microorganisms were detected in the UAlg but not the i3S facility (see
219 Materials and methods), we posit that an environmental input present only at the UAlg
220 facility increased susceptibility for lymphoid disease. Considering that bacteria are
221 frequently present in tumors, and can be detected by immunohistochemical methods,[25]
222 we probed lymphomas obtained in both facilities for the presence of bacterial-derived
223 lipopolysaccharide (LPS). Indeed, Marilyn lymphomas derived from the UAlg facility
224 displayed more conspicuous LPS immunostaining than Marilyn;*Rag2*$^{-/-}$ lymphomas
225 derived from the i3S facility (Supplementary Figure 10A). Of note, the pattern of LPS
226 staining in Marilyn splenic lymphomas overlapped with B220^{+} B cell areas
227 (Supplementary Figure 10B). These data suggest that higher bacterial exposure could
228 have promoted hematological diseases in Marilyn females.

229

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

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

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

265

266 **Discussion**

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

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

287 The finding that tonic TCR signaling can promote malignant transformation finds
288 parallels in other systems. In fact, tonic B cell receptor signaling, defined as antigen-
289 independent cell-autonomous signaling, has been shown to drive B cell
290 lymphomagenesis [33–35] and to be therapeutically targetable.[36] By the same token, tonic
291 TCR signaling could be a targetable mechanism in human T-ALL, as suggested by the
292 frequent activation of Lck kinase (a mediator of TCR signaling) in T-ALL and by the
293 therapeutic potential of Lck inhibitors.[6–9] Lck kinase activation in T-ALL was attributed to
294 constitutive pre-TCR signaling found in a subset of patients.[6] Since TCR and CD3
295 proteins are also frequently expressed in T-ALL,[4] further studies are warranted to
296 determine the role of TCR signaling in human T-ALL pathogenesis.

297 Although Rag recombinase-mediated mutagenic events can contribute to
298 leukemia development,[37] that was not the case for Marilyn T-ALL. In fact, T-ALL
299 developed with higher penetrance and much faster in Marilyn;*Rag2*$^{-/-}$ females than in
300 Marilyn;*Rag2*$^{+/-}$ females generated in the same animal facility. Since Rag2 deficiency
301 arrests thymocyte development at the DN3 stage, these results suggest that ectopic TCR
302 expression at the immature DN3 stage in the absence of more mature thymocytes has

303 increased leukemogenic potential, in line with the finding that early expression of mature
304 TCR in DN thymocytes promotes T-ALL development.[30]
305 Rag2-sufficient, Marilyn mouse cohorts housed in different animal facilities led to
306 unexpected different rates of T-ALL incidence. Indeed, Marilyn T-ALL frequency was
307 much higher in females housed at the UAlg/Faro facility than females housed at the
308 i3S/Porto facility. Since several microorganisms (*Helicobacter* spp., *Pasteurella*
309 *pneumotropica* and murine norovirus) were detected in the UAlg facility, but not in the
310 i3S facility, increased exposure to pathogenic microbiota could have promoted a
311 microenvironment favorable for transgenic TCR-induced leukemogenesis. This notion is
312 supported by the finding of higher levels of LPS staining in lymphomas from UAlg facility
313 mice than in lymphomas from i3S facility mice. Early research revealed that B-ALL in
314 *Pax5* heterozygous mice was initiated only upon exposure to common pathogens.[38]
315 Future studies should, therefore, determine whether the presence of microorganims
316 promotes TCR-induced leukemogenesis and whether TCR interactions with MHC
317 molecules presenting non-cognate foreign peptides generate pro-leukemogenic basal
318 TCR signaling.
319 Although T-ALL in Marilyn females developed in the presence of self-pMHC, we
320 found that MHC-dependent TCR signaling was not required for its maintenance. Indeed,
321 secondary leukemia development was not affected by lack of MHC-bound self-peptides
322 in recipient allogenic mice. These results contrast with the finding that continuous self-
323 pMHC-mediated TCR signaling was necessary for lymphoma cell growth in a TCR-
324 expressing reprogrammed T cell model.[39] Of note, expression of a transgenic TCR
325 recognizing a peptide from the murine survivin protein was also shown to result in
326 increased TCR signaling and to induce T-ALL.[29] In this case, the specific (self) antigen,
327 survivin, was expressed constitutively, which contrasts with the absence of the HY-TCR
328 cognate peptide (DBY) in Marilyn females. Our data together with previous reports
329 demonstrate that the TCR signaling complex promotes T-ALL, and that this ability very
330 much depends on the TCR affinity to antigen.
331 Finally, we showed that Marilyn T-ALL cells had a functional TCR and that *in vivo*
332 TCR stimulation of transplanted female Marilyn T-ALL cells was associated with
333 apoptosis and impaired Marilyn T-ALL leukemogenesis. These observations corroborate
334 previous reports showing that mouse or human T-ALL underwent apoptosis upon
335 activation of the TCR/CD3 signaling complex.[19,28,40] These findings suggest that, similarly
336 to thymocytes undergoing thymic selection, TCR activation with low-affinity or high-
337 affinity antigens will stimulate differentially the TCR signaling pathway and result in cell
338 survival or death.[41] Future research should determine the TCR signaling regulators that
339 dictate if a particular antigenic stimulus promotes or antagonizes T-ALL.

340 **Methods**

341 **Mice**

342 HY-TCR Marilyn transgenic mice (B6.Cg-Tg(TcraH-Y,TcrbH-Y)1Pas), on a
343 CD45.1 background, were obtained from Jocelyne Demengeot (IGC, Oeiras). Marilyn
344 mice were bred with EμSRα-ETV6::JAK2 (B6.Cg-Tg(Emu-ETV6/JAK2)71Ghy)
345 transgenic mice[13], or *Rag2* (B6.129S6-Rag2^{tm1Fwa}) knockout mice. The ETV6::JAK2
346 transgene was kept in hemizygosity. *Foxn1*$^{nu/nu}$ (Nude) C57BL/6 and CBA mice were
347 bred at the i3S facility. Mice were maintained at the UAlg/Faro and i3S/Porto barrier
348 animal facilities, under 12 h light/dark cycles and with food and water *ad libitum*. HY-TCR
349 Marilyn mice and ETV6::JAK2;Marilyn double transgenic were bred at the UALg animal
350 facility, while the Marilyn;*Rag2*$^{+/-}$ and Marily;*Rag2*$^{-/-}$ were bred at the i3S animal facilty.
351 Microorganism screening detected opportunistic pathogens (*Helicobacter* spp.,
352 *Pasteurella pneumotropica* and murine norovirus) in the UAlg, but not i3S, experimental
353 rooms. All experimental procedures were approved by the i3S and CBMR/UAlg ethics
354 committees and Portuguese authorities (*Direção-Geral de Agricultura e Veterinária*) and
355 followed recommendations from the European Commission (Directive 2010/63/UE) and
356 the local Portuguese authorities (*Decreto-Lei* n°113/2013). Both female and male mice
357 were used for all experiments. Mice were monitored for signs of disease (e.g. dyspnea,
358 lethargy, enlarged lymph nodes and enlarged abdomen) and killed by CO_2 inhalation
359 when reaching predefined experimental endpoints. Mice of different genotypes from the
360 same litter were kept together in the same cages and monitoring for signs of disease
361 was done blindly. Adult mice that were euthanized without leukemia were censored in
362 Kaplan-Meier survival curves.

363

364 **Mice genotyping**

365 The following primers were used for genotyping. HY-TCR Marilyn transgene: 5'-
366 CGAGAGGAACCTGGGAGCTGT-3' and 5'-TGCTGTCTGTACCACCAGAAATAC-3';
367 *Rag2*: 5'-TGTCCCTGCAGATGGTAACA-3', 5'-CCTTTGTATGAGCAAGTAGC-3', 5'-
368 CTATTCGGCTATGACTGGG-3' and 5'-AAGGCGATAGAAGGCGATG-3'; *Cdkn2a*: 5'-
369 GTGATCCCTCTACTTTTTCTTCTGACTT-3', 5'-CGGAACGCAAATATCGCAC-3' and
370 5'-GAGACTAGTGAGACGTGCTACTTCCA-3'.

371

372 ***In vivo* experiments**

373 For mouse leukemia transplantation assays, 0.5-2 x 10^6 leukemic cells collected
374 from diseased female Marilyn mice were intravenously injected in the tail vein of recipient
375 8-12-week-old C57BL/6, C57BL/6 Nude or CBA Nude mice of the indicated sex, and
376 regularly monitored through peripheral blood detection of leukemic cells

377 (CD45.1^{+}CD45.2^{+}TCR-Vβ6^{+}). Female *Rag2*$^{-/-}$ mice infused with Marilyn T-ALL cells were
378 treated intraperitoneally once a week for two weeks with 0.01 mg of DBY peptide
379 (NAGFNSNRANSSRSS; cat. no. AS-61046; Eurogentec).
380
381 **Flow cytometry**
382 Single-cell suspensions were prepared from lymphoid organs using cell strainers,
383 washed with FACS buffer (phosphate-buffered saline (PBS) with 3% fetal bovine serum
384 (FBS) and 10 mM NaN_3), and incubated for 30-45 min with fluorochrome-labeled
385 antibodies in FACS buffer. The following Biolegend antibodies were used: CD25-
386 fluoresceine isothiocyanate (FITC) (clone PC61), TCRβ-phycoerithrin (PE) (clone H57-
387 597), CD44-PE/Cyanine5 (clone IM7), CD90.2-PE-Cyanine7 (clone 30-H12), CD24-
388 allophycocyanin (APC) (clone M1/69), CD4-APC/Cyanine7 (clone GK1.5), CD8α-Pacific
389 Blue (clone 53-6.7), Gr1-PE (clone RB6-8C5), CD127-PE/Cyanine5 (clone A7R34),
390 B220-APC/Cyanine7 (clone RA3-6B2), CD24-Pacific Blue (clone M1/69), CD90.2-FITC
391 (clone 30-H12), TCR-Vb6-PE (clone RR4-7), TCR-Vb5.1, 5.2-PE (clone MR9-4), CD5-
392 APC (clone 53-7.3), CD69-FITC (clone H1.2F3), CD62L-APC (clone MEL-14), CD45.1-
393 APC (clone A20) and CD45.2-Peridinin Chlorophyll Protein (PerCP)-Cyanine5.5 (clone
394 104). Immunostained cells were washed twice with FACS buffer and incubated in PBS
395 with 10 mM NaN_3. Cell viability was determined using the Zombie Aqua Fixable Viability
396 Kit (Invitrogen). Samples were acquired using BD FACS Calibur, CANTO II or Accuri C6
397 and analyzed using FlowJo software.
398
399 ***Ex-vivo* T-ALL cell culture**
400 Marilyn T-ALL cells were cultured ex-vivo for 16 h in RPMI medium supplemented
401 with 10% FBS, 1% penicillin-streptomycin and 50 μM of 2-mercaptoethanol, all from
402 Gibco. For *ex vivo* stimulation of Marilyn T-ALL cells, plates were coated with 10 μg/ml
403 anti-CD3 (145-2C11, Biolegend) for 2 h at 37°C, and then plates were washed with PBS.
404 Ten ng/ml of PMA and 250 ng/ml Ionomycin (both from Sigma-Aldrich) treatments were
405 performed for 18 hours.
406
407 ***Notch1* mutation detection**
408 Genomic DNA from mouse leukemia samples was isolated using the GeneJET
409 Genomic DNA purification kit (Thermo Fisher Scientific), following the manufacturer's
410 instructions. DNA was used for PCR amplification of two segments of *Notch1* exon 34:
411 primer pair 5'-GCTCCCTCATGTACCTCCTG-3' and 5'-TAGTGGCCCCATCATGCTAT-
412 3', generating a predicted amplicon of 904 bp, and primer pair 5'-

413 ATAGCATGATGGGGCCACTA-3' and 5'-CTTCACCCTGACCAGGAAAA-3', generating
414 a predicted amplicon of 893 bp. PCR products were Sanger sequenced using the same
415 primers at i3S Genomics (Porto, Portugal) or CCMAR *Serviços de Biologia Molecular*
416 (Faro, Portugal).
417
418 **Low-coverage whole genome sequencing**
419 Genomic DNA was used for copy number analysis performed as described
420 previously[42] using a HiSeq 4000 (Illumina) sequencer in a single-read 50-cycle run mode.
421 For copy number analysis, sequencing data was aligned with the mouse genome
422 (mm10) as reference and further analyzed using QDNAseq (RRID:SCR_003174)
423 package (version 1.12) in R software. A bin size of 15 kb was used.
424
425 **Histology and immunohistochemistry**
426 For histological and immunohistochemistry analysis, formalin-fixed paraffin-
427 embedded tissues were sectioned with 4 μm thickness. Hematoxylin and eosin staining
428 was performed using standard procedures. For immunohistochemistry, heat-mediated
429 antigen retrieval was performed for 35-40 min with citrate-based antigen retrieving
430 solution (cat. no. H-3300, Vector Laboratories), for cleaved-caspase 3, Ki67 and LPS
431 immunodetection, or 10 μM EDTA, pH 8.0, and 0.05% Tween 20 in PBS, for B220 or
432 CD3 immunodetection. Endogenous peroxidase was inactivated with 3% H_2O_2 in
433 methanol, and nonspecific antibody binding was blocked using Ultravision Protein-block
434 (Thermo Fisher Scientific). Sections were incubated overnight with rat anti-B220 (1:200,
435 clone RA3-6B2, Biolegend), anti-CD3 (1:200, ab5690, Abcam), anti-cleaved-caspase 3
436 (1:200, 9661, Cell Signaling), Ki67 (1:500, ab15580, Abcam) or LPS (1:100, WN1222-5,
437 Hycult Biotech) at 4°C. For B220 detection, slides were incubated sequentially with
438 avidin/biotin blocking system (Biolegend), goat anti-rabbit secondary antibody
439 (Biovision), and streptavidin-horseradish peroxidase (HRP) (Enzo Biochem). For CD3,
440 Ki67 and LPS detection, HRP-conjugated rabbit/mouse secondary antibody (Agilent)
441 was used. 3,3′-Diaminobenzidine chromogen (Agilent) was used as detection reagent.
442 For quantification of percentage of cleaved caspase 3-positive cells, five images were
443 taken from different areas of each tissue section.
444
445 **Statistics**
446 Statistical analysis was performed with GraphPad Prism 6.0 software
447 (RRID:SCR_002798). Log-rank test was used to compare survival of different groups.
448 Unpaired student's t-test was used for comparisons between two groups. Welch's
449 correction was used when variances were different. Mann-Whitney test was used to

compare white blood cell (WBC) count. Detection of CD45.1$^+$ T-ALL cells in the peripheral blood of males and females was compared using two-way ANOVA. Sample numbers are indicated in figure legends. $P<0.05$ was considered statistically significant.

Data Sharing Statement

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

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

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

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593 specimens by shallow whole-genome sequencing with identification and exclusion
594 of problematic regions in the genome assembly. *Genome Res* **24**, 2022–32
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596
597

598 **Figure legends**

599

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

615

616 **Figure 2. Immunophenotypic analysis of HY-TCR leukemic cells.** Flow cytometry
617 analysis of thymus-derived leukemic cells from representative Marilyn mice presenting
618 immature T cell phenotype, based on CD44 negativity and high CD24 expression (nos.
619 22, 33 and 40), mature T cell phenotype, based on CD44 positivity and CD24
620 intermediate/low expression (nos. 9 and 29), and mixed B and T cell phenotype, based
621 on B220 and CD90.2 expression (nos. 25 and 34). In CD44 and CD24 histogram plots,
622 unstained cells were used as negative control (grey shades). Healthy HY-TCR female
623 total thymocyte immunostaining is shown as control. TL, thymic lymphoma.

624

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

633 staining of representative thymic lymphoma sections from Marilyn;*Rag2*$^{-/-}$ and
634 Marilyn;*Rag2*$^{+/+}$ diseased mice. Black scale bars: 500 μm; green scale bar: 200 μm.
635
636 **Figure 4. HY-TCR T-ALL antigenic stimulation *in vivo* hampers leukemia**
637 **development.** (A) Percentage of CD45.1^{+} T-ALL cells detected by flow cytometry in the
638 peripheral blood (PB) of male and female C57BL/6 mice infused with female HY-TCR T-
639 ALL cells. *P* value was obtained with two-way ANOVA. (B) Kaplan-Meier leukemia-free
640 survival curve for the same mice as in (A), which is representative of two experiments
641 performed with different primary leukemias. *P* value obtained with log-rank test. (C)
642 Spleens and kidneys (top panels) and respective organ weights (bottom panels) for male
643 (n=5) and female (n=6) C57BL/6 mice 21 days after infusion with HY-TCR T-ALL cells.
644 (D) Percentage of CD45.1^{+}CD90.2^{+} HY-TCR T-ALL cells present in the bone marrow of
645 males and females shown in (C). *P* values in (C) and (D) were obtained from unpaired *t*
646 tests with Welch's correction. (E) Representative sections and quantification of anti-
647 cleaved caspase 3 immunohistochemistry for spleens from females and males shown in
648 (C,D; n=3). *P* value from unpaired *t* test. (C-E) Graphs show mean and SEM. (F) Kaplan-
649 Meier leukemia-free survival curves for syngeneic C57BL/6 nude or allogeneic CBA
650 nude male and female mice injected with female Marilyn T-ALL cells. Number of mice
651 and *P* values from log-rank test are indicated. (G) Kaplan-Meier leukemia-free survival
652 curves for syngeneic *Rag2*$^{-/-}$ females infused on day 0 with female Marilyn T-ALL cells
653 and treated once a week with DBY peptide (0.01 mg; n=5) or Control (n=4). Gray shade
654 represents treatment duration of 2 weeks.

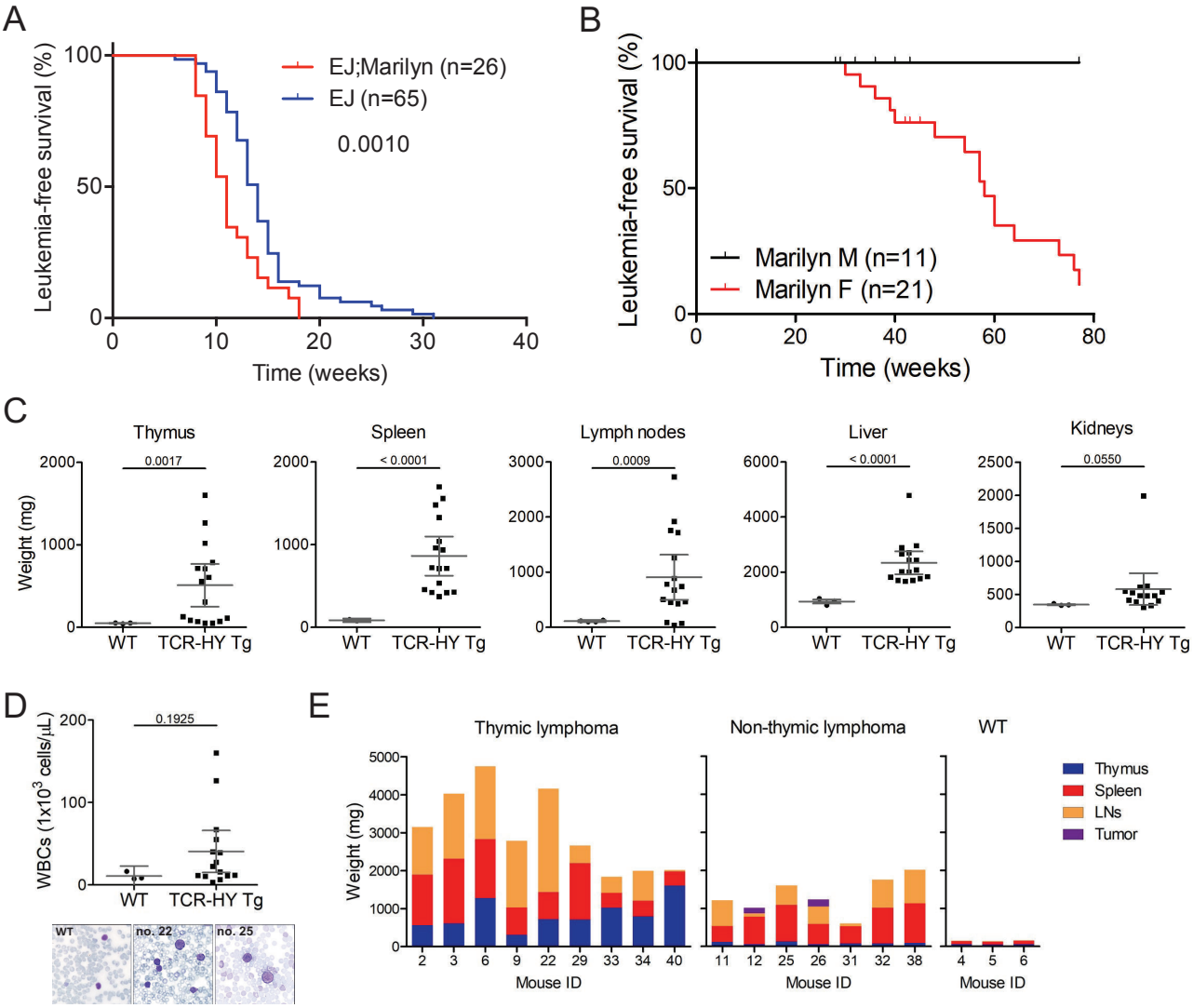
A
Leukemia-free survival (%)
100
50
0
EJ;Marilyn (n=26)
EJ (n=65)
0.0010
0
10
20
30
40
Time (weeks)
B
Leukemia-free survival (%)
100
50
0
Marilyn M (n=11)
Marilyn F (n=21)
0
20
40
60
80
Time (weeks)
C
Thymus
Spleen
Lymph nodes
Liver
Kidneys
Weight (mg)
0.0017
< 0.0001
0.0009
< 0.0001
0.0550
WT
TCR-HY Tg
D
WBCs (1x10^3 cells/µL)
0.1925
WT
TCR-HY Tg
WT
no. 22
no. 25
E
Thymic lymphoma
Non-thymic lymphoma
WT
Weight (mg)
Thymus
Spleen
LNs
Tumor
2 3 6 9 22 29 33 34 40
11 12 25 26 31 32 38
4 5 6
Mouse ID

Figure 1

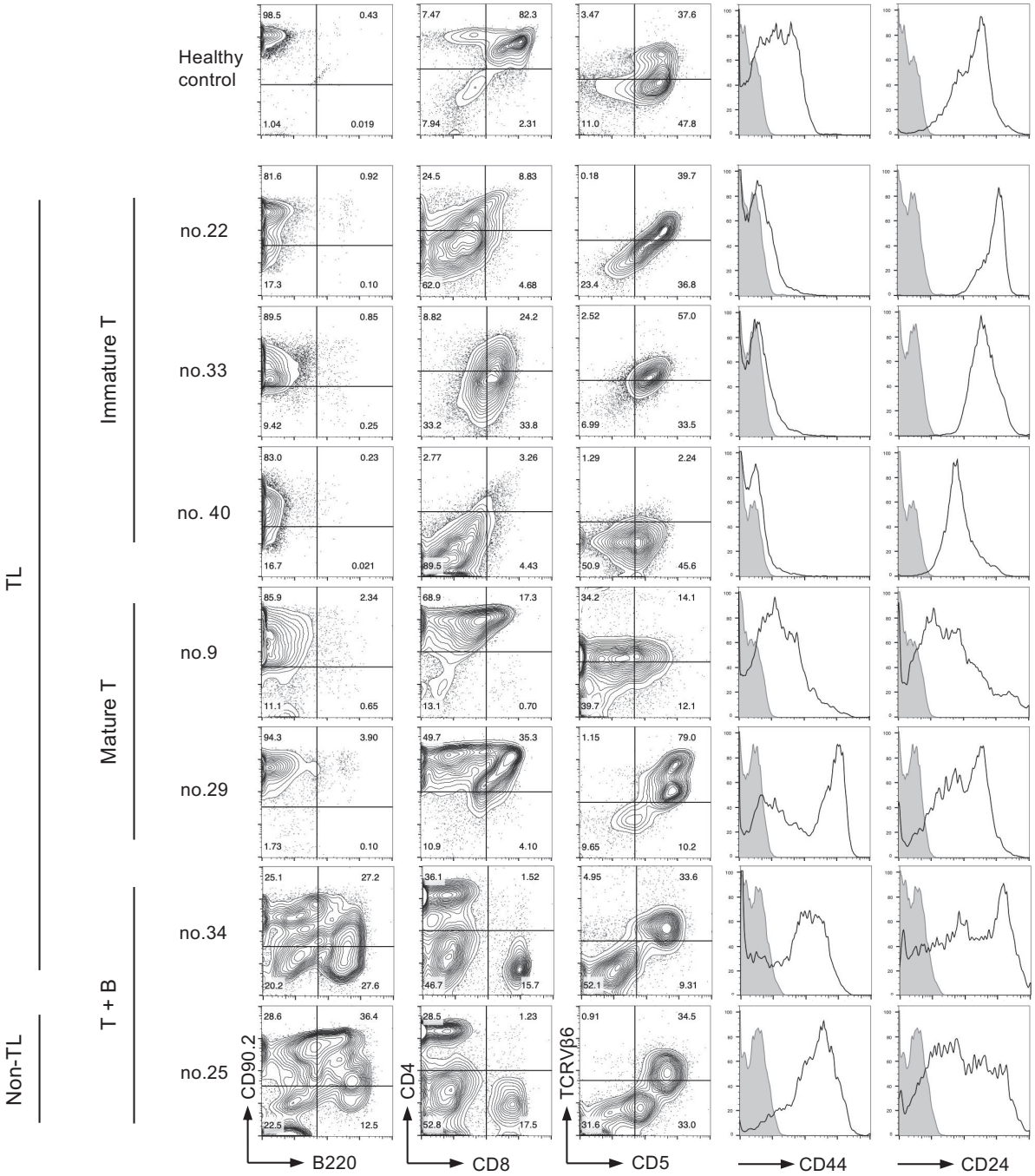
Healthy control
no.22
no.33
no. 40
no.9
no.29
no.34
no.25
TL
Non-TL
Immature T
Mature T
T + B
CD90.2
B220
CD4
CD8
TCRVβ6
CD5
CD44
CD24

Figure 2

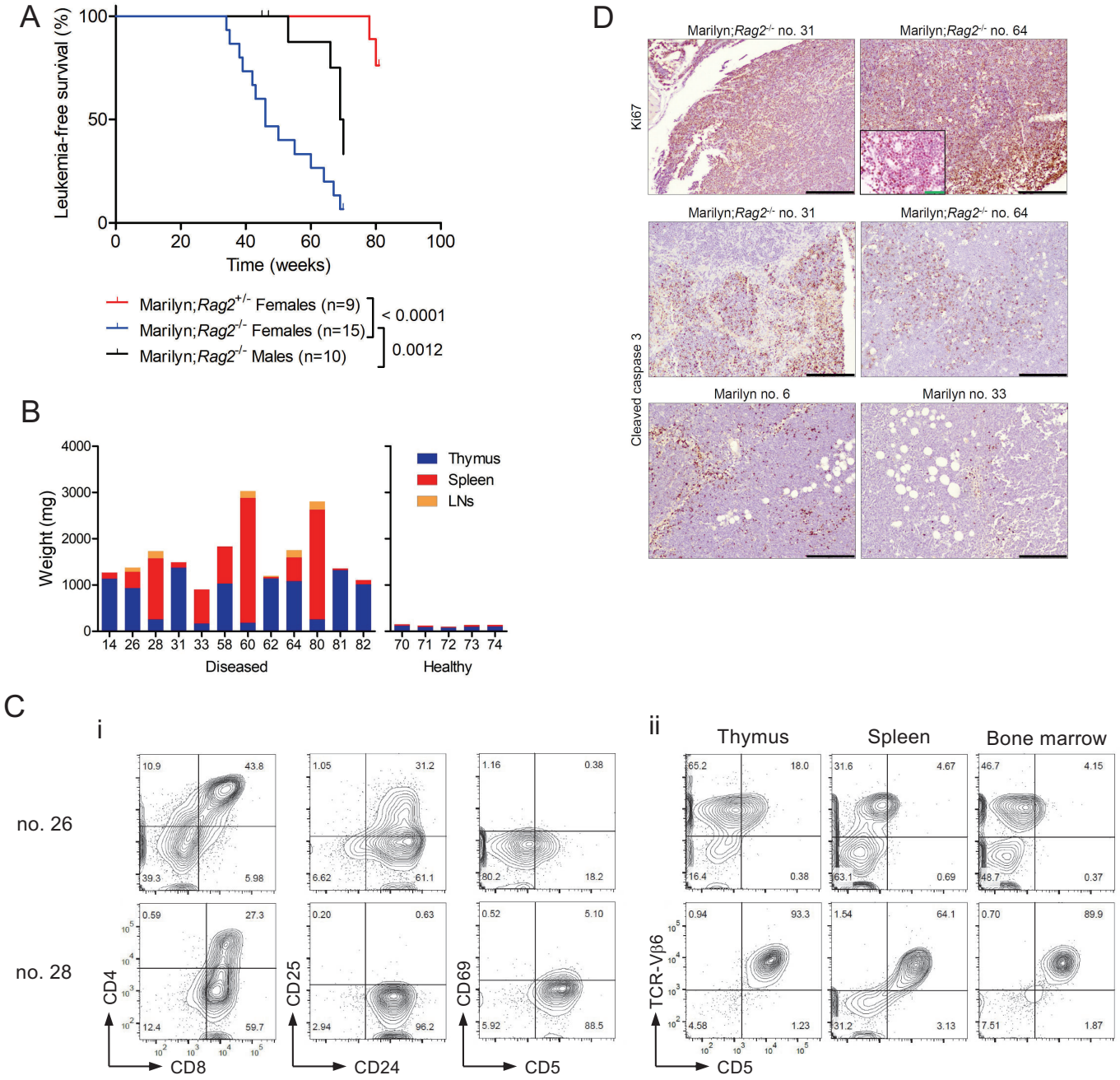
A
Leukemia-free survival (%)
100
50
0
0
20
40
60
80
100
Time (weeks)
Marilyn;Rag2+/- Females (n=9)
Marilyn;Rag2-/- Females (n=15)
Marilyn;Rag2-/- Males (n=10)
< 0.0001
0.0012
B
Weight (mg)
4000
3000
2000
1000
0
14 26 28 31 33 58 60 62 64 80 81 82
Diseased
70 71 72 73 74
Healthy
Thymus
Spleen
LNs
C
i
no. 26
no. 28
CD4
CD8
CD25
CD24
CD69
CD5
ii
Thymus
Spleen
Bone marrow
TCR-Vβ6
CD5
D
Ki67
Cleaved caspase 3
Marilyn;Rag2-/- no. 31
Marilyn;Rag2-/- no. 64
Marilyn no. 6
Marilyn no. 33

Figure 3

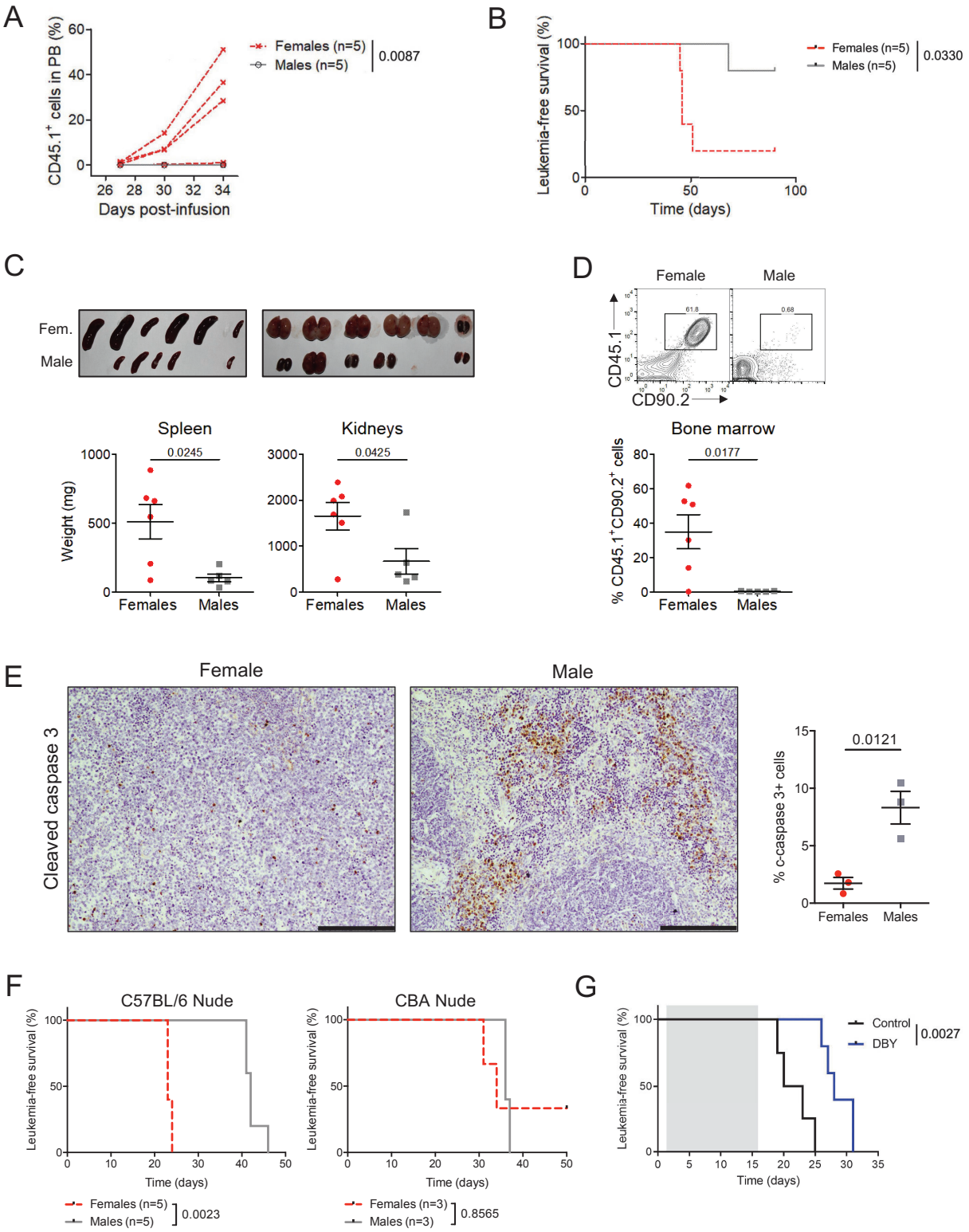
A
CD45.1+ cells in PB (%)
Days post-infusion
Females (n=5)
Males (n=5)
0.0087
B
Leukemia-free survival (%)
Time (days)
Females (n=5)
Males (n=5)
0.0330
C
Fem.
Male
Spleen
Kidneys
Weight (mg)
0.0245
0.0425
Females
Males
D
Female
Male
CD45.1
CD90.2
Bone marrow
% CD45.1+CD90.2+ cells
0.0177
Females
Males
E
Female
Male
Cleaved caspase 3
% c-caspase 3+ cells
0.0121
Females
Males
F
C57BL/6 Nude
CBA Nude
Leukemia-free survival (%)
Time (days)
Females (n=5)
Males (n=5)
0.0023
Females (n=3)
Males (n=3)
0.8565
G
Leukemia-free survival (%)
Time (days)
Control
DBY
0.0027

Figure 4