The effect of CARD11 mutation and HBZ expression accelerated lymphoproliferative diseases and formed the pathological basis for adult T-cell leukemia/lymphoma

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The effect of *CARD11* mutation and *HBZ* expression accelerated lymphoproliferative diseases and formed the pathological basis for adult T-cell leukemia/lymphoma

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Abstract

Adult T-cell leukemia/lymphoma (ATL) is caused by human T-cell leukemia virus type 1 (HTLV-1). In addition to *HTLV-1 bZIP factor (HBZ)*, a leukemogenic antisense transcript of HTLV-1, abnormalities of genes involved in TCR-NF- κ B signaling such as *CARD11* have been detected in about 90% of patients. Utilizing mouse models with T cell-specific CARD11(E626K) expression and/or CD4⁺ T cell-specific *HBZ* expression, namely CARD11(E626K)^{CD4⁺Cre} mice, *HBZ* transgenic (Tg) mice, and CARD11(E626K)^{CD4⁺Cre};*HBZ*Tg compound mice, we clarified the effect of these genes on pathogenesis. Both CARD11(E626K)^{CD4⁺Cre} and *HBZ*Tg mice developed lymphoproliferative disease, and the compound mice showed accelerated disease and increased CD4⁺ T cells *in vivo*. CARD11(E626K) and *HBZ* cooperatively activated the NF- κ B non-canonical pathway, IRF4 targets, and cell proliferation. Most of the KEGG and HALLMARK gene sets, which are enriched in acute-type ATL, were also enriched in the compound mice, indicating that these genes cooperatively form the pathological basis for ATL development.

Introduction

Adult T-cell leukemia/lymphoma (ATL) is an aggressive peripheral T-cell lymphoma that develops in about 5% of people infected with human T-cell leukemia virus type 1 (HTLV-1), usually after decades of clinical latency¹⁻⁵. Viral oncogenes encoded by HTLV-1, namely *tax* and *HTLV-1 bZIP factor (HBZ)*, are thought to play a major role in ATL development^{6,7}. Tax possesses transforming activity *in vitro*, and Tax transgenic (Tg) mice develop cancer *in vivo*^{8,9}. However, Tax is highly immunogenic, and its expression is suppressed and disrupted in many ATL cells^{10,11}. In contrast, *HBZ*, which is encoded in the minus strand of the HTLV-1 genome, is transcribed in all ATL cases, and HBZ promotes ATL cell proliferation *in vivo* and induces T-cell lymphoma and inflammation *in vivo*¹²⁻¹⁵.

The development of ATL involves not only viral oncogenes, but also presumably the accumulation of gene mutations in T cells during the long latency period from HTLV-1 infection in infancy to ATL onset later in life⁴. We previously reported that about 90% of ATL patients possessed mutations in genes involved in T-cell receptor (TCR)–NF- κ B signaling and downstream or associated pathways¹⁶. Among them, mutations in *CARD11*, a cytoplasmic scaffolding protein required for antigen

receptor-induced NF- κ B activation in both T and B cells, were detected in 24% of ATL patients¹⁶. The most frequently observed CARD mutation, comprising 17% of the total, was the E626K gain-of-function mutation¹⁶. Gain-of-function mutations in *CARD11* were also reported in 9.6% of patients with diffuse large B-cell lymphoma (DLBCL) of the activated B-cell (ABC) subtype, and mice harboring active *CARD11* mutations in B cells developed aggressive B-cell lymphoproliferation, leading to early postnatal lethality¹⁷⁻¹⁹.

Here we generated a mouse model involving the conditional expression of a CARD11(E626K) gain-of-function mutant, which is the most common mutation observed in ATL patients¹⁶, and demonstrated that CARD11 activation in CD4⁺ T cells induced lymphadenopathy and T-cell infiltration of many organs. We also showed that the combination of HBZ expression and CARD11 mutation cooperatively accelerated lymphoproliferative disease, affected the expressions of many genes, and served as the molecular basis of acute-type ATL.

Materials and Methods

Mouse models

CARD11 mutant mice were generated by inserting cDNA containing the *Card11*E626K

mutation, the homologue of *CARD11*(E626K), into the *Rosa26* vector, preceded by a *loxP*-flanked (FL) stop sequence (Supplemental figure 1A). The resulting vector was electroporated into C57BL/6J embryonic stem (ES) cells. The recombinant ES cells were injected into ICR blastocysts, and subsequent chimera breeding resulted in *CARD11*(E626K)^{stop^{FL}} mice. Crossing these mice with *CD4*-Cre Tg mice²⁰ resulted in *CARD11*(E626K)^{*CD4*-Cre} mice. To generate *HBZ* Tg mice, *HBZ* cDNA was cloned from the ATL cell line Su9T²¹. The clones were inserted into a cassette vector containing a mouse *CD4* promoter/enhancer (Supplemental figure 1B)²². Electroporation and subsequent chimera breeding resulted in *HBZ* Tg. Crossing *CARD11*(E626K)^{*CD4*-Cre} and *HBZ* Tg mice resulted in *CARD11*(E626K)^{*CD4*-Cre};*HBZ* Tg mice. Animal studies were performed in accordance with the University of Miyazaki Ethics Committee. The materials used in the experiments are shown in Supplemental table 1.

Gene expression profiling of mouse samples

Splenic CD4⁺CD44⁺CD62L⁻ effector/memory T cells (Tem) (3×10^4 cells per mouse) were sorted from 4 types of mice: wild type (WT), *CARD11*(E626K)^{*CD4*-Cre}, *HBZ* Tg, and *CARD11*(E626K)^{*CD4*-Cre};*HBZ* Tg (n = 3 for each mouse type). Total RNA extraction, cDNA library preparation, sequencing, and gene set enrichment analysis (GSEA)²³⁻²⁵

were performed as described in the Supplemental methods. A false discovery rate (FDR) ≤ 0.25 was considered significant.

Comparison of gene expression signatures enriched in mutant mice and acute-type ATL samples

To assess the extent to which the mouse models mimicked the molecular pathogenesis of ATL, we compared mouse and human GSEA results for the KEGG and HALLMARK gene sets, using their normalized enrichment score (NES) and FDR values. Human gene expression data were obtained from the healthy donors (n = 3) and acute-type ATL patients (n = 21) included in our previous report (EGAS00001001296)¹⁶. Using these expression data, GSEA of human samples was performed with the same protocol used with mouse samples, including the same gene sets and the same FDR cutoff value. To interpret the single and compound effects of the CARD mutant and *HBZ* expression on ATL pathogenesis, each gene set in the mouse and human GSEA results was linked by name, plotted according to the mouse and human NES values, and highlighted according to the mouse and human FDR values. This was done for each combination of mouse genotype (WT / CARD11(E626K)^{CD4}Cre / *HBZ*Tg / CARD11(E626K)^{CD4}Cre;*HBZ*Tg) and human condition (Healthy / Acute-type ATL).

143

144 **Statistical Analysis**

145 Differences between groups in terms of numbers and frequencies of cells were analyzed
146 by ANOVA complemented with the post-hoc Tukey–Kramer test. Differences in animal
147 survival, assessed by the Kaplan–Meier method, were analyzed by the log-rank test.
148 Differences between frequencies of lymphadenopathy were assessed by Fisher’s exact
149 test with Benjamini–Hochberg correction. $p < 0.05$ indicated statistical significance.

150

151 **Results**

152 **Generation of CARD11(E626K)^{CD4⁺Cre} and *HBZ*Tg mice**

153 To investigate constitutively active CARD11 signaling in CD4⁺ T cells *in vivo*, we
154 generated CARD11(E626K)^{CD4⁺Cre} mice; this mutation is the homologue of human
155 *CARD11*(E626K), the most commonly observed mutation in ATL patients. In contrast
156 to WT CD4⁺ T cells, CD4⁺ T cells from CARD11(E626K)^{CD4⁺Cre} mice demonstrated
157 phosphorylation of CARD11 Ser652 and NF-κB p65 Ser536, and an increased amount
158 of cleaved BCL-10 (Supplemental figure 2A). Subsequently, increments of nuclear p50,
159 RelA p65, and nuclear p52 were observed, confirming the activation of both the
160 canonical and non-canonical NF-κB pathways *in vivo*.

*HBZ*Tg mice were generated by *HBZ* cDNA expression under a mouse *CD4* promoter (Supplemental figure 1B). Western blotting detected HBZ in CD4⁺ T cells from *HBZ*Tg mice, but not in those from WT mice (Supplemental figure 2B).

Crossing CARD11(E626K)^{CD4-Cre} and *HBZ*Tg mice resulted in CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice. About half of the CARD11(E626K)^{CD4-Cre} mice died at 6 or more months after birth (Figure 1A). *HBZ*Tg mice began to die at 10 months after birth. Most CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice died within 8 months after birth.

Both CARD11(E626K)^{CD4-Cre} and *HBZ*Tg mice developed lymphoproliferative disease

CARD11(E626K)^{CD4-Cre} mice did not show an increase in splenic nucleated cells (Figure 1B). At 6 months after birth, the splenic histological architecture in CARD11(E626K)^{CD4-Cre} mice was almost intact, but the interface between white and red pulp was uneven and obscure (Figure 1C). This was attributed to focal infiltration of CD3⁺ T cells from the white pulp periarteriolar region into the red pulp. At 12 months after birth, areas of white pulp were decreased in number and size, while those of red pulp were larger. The interface had become disrupted. CD3⁺ T cells had diffusely infiltrated both the shrunken white pulp and the expanding red pulp.

Most CARD11(E626K)^{CD4⁺Cre} mice developed lymphadenopathy. However, the swollen lymph nodes (LNs) were not big, with a mean diameter of about 3 mm. Lymphadenopathy was observed in 12 of 18 mice (67%) at 6 months after birth, and in 12 of 14 mice (86%) at 12 months after birth (Figure 2A). Slightly shrunken follicles, a thickened cortex, and an expanded paracortex with CD3⁺ T-cell infiltration were observed at 6 months after birth (Figure 2B). The interface between the cortex and paracortex was obscured by infiltrating T cells. At 12 months, the normal lymph node architecture was barely present in CARD11(E626K)^{CD4⁺Cre} mice. The cortex had become thickened and the follicles had disappeared. The paracortex was expanded by CD3⁺ T cells, and the interface was disrupted.

The most affected organ in CARD11(E626K)^{CD4⁺Cre} mice was the lung. Focal lymphocyte infiltration was observed at 6 months; both T and B cells had infiltrated the perivascular interstitium and occasionally the alveolar septa. Over time this infiltration increasingly consisted of CD3⁺ T cells, which formed diffuse lung lesions with thickening of the interstitium and alveolar septa at 12 months (Figure 3).

*HBZ*Tg mice did not exhibit splenomegaly. Histological examination at 6 months after birth revealed that the spleen was almost normal; however, at 12 months after birth, the white pulp exhibited expansion (Figure 1B, C). CD3⁺ T cells had caused

expansion of both white and red pulp, which made their interface disrupted.

Lymphadenopathy was obvious at 12 months after birth in *HBZ*Tg mice (Figure 2A). The frequency of lymphadenopathy at 12 months after birth was 67%, which was similar to that in a previous report¹⁵. The normal LN architecture was destroyed. The follicles were shrunken, the cortex was thickened, and the paracortex was expanded (Figure 2B). CD3⁺ T cells were mainly observed in the paracortex, and they infiltrated the cortex.

As in *CARD11(E626K)^{CD4⁺Cre}* mice, the most affected organ in *HBZ*Tg mice was the lung. Diffuse infiltration of lymphocytes was observed at 6 months, and lymphocytes comprising mainly T cells invaded the perivascular interstitium and alveolar septa. The alveolar space was filled with exudate at 6 months after birth (Figure 3). These abnormalities were much more pronounced at 12 months after birth. Severe lung findings were observed, with exudate and thickening of the interstitium and alveolar septa. These features, except for the exudate, resembled those in *CARD11(E626K)^{CD4⁺Cre}* mice.

The combination of *CARD11* mutation and *HBZ* expression accelerated the development of lymphoproliferative disease

215 Mice with the combination of *CARD11* mutation and *HBZ* expression
 216 developed more aggressive lymphoproliferative disease than that seen in
 217 *CARD11(E626K)^{CD4^{Cre}}* or *HBZ*Tg mice. As most *CARD11(E626K)^{CD4^{Cre}}*; *HBZ*Tg mice
 218 died within 8 months after birth, the subsequent analysis was performed at 6 months
 219 after birth. Mild splenomegaly and an increased number of nucleated cells were
 220 observed in *CARD11(E626K)^{CD4^{Cre}}*; *HBZ*Tg mice (Figure 1B, 1C). In the spleen, the
 221 splenic architecture was destroyed and the interface between white and red pulp was
 222 disrupted. CD3⁺ T cells were diffusely present throughout the spleen.

223 Lymphadenopathy was detected in 17 of 20 (85%) *CARD11(E626K)^{CD4^{Cre}}*; *HBZ*
 224 Tg mice (Figure 2). The normal LN architecture was destroyed; follicles had almost
 225 disappeared, the cortex was thickened, and the paracortex was expanded. CD3⁺ T cells
 226 were observed mainly in the paracortex and had massively infiltrated the cortex,
 227 leading to the highly disrupted interface.

228 The most affected organ in *CARD11(E626K)^{CD4^{Cre}}*; *HBZ*Tg mice was the lung.
 229 The lung interstitium and alveolar septa were thickened with infiltrating cells
 230 consisting mainly of lymphocytes (Figure 3). CD3⁺ T cells had accumulated around
 231 capillary blood vessels. The alveolar space had filled with exudate, and subsequently
 232 with macrophages, and consequently almost no air spaces were observed.

233

234 **Increment of CD4⁺ T cells, Tem, and regulatory T cells (Treg) in CARD11(E626K)^{CD4⁺}**
235 **^{Cre};HBZTg mice**

236 As ATL is a neoplasm of CD4⁺ T lymphocytes, and both CARD11(E626K) and
237 *HBZ* were expressed in the CD4⁺ T cells in our mice, we next evaluated the absolute
238 numbers of CD4⁺ cells and their subsets, namely CD4⁺CD44⁺CD62L⁻ Tem and
239 CD4⁺CD25⁺Treg in the body of each mouse as the sum of the numbers in whole bone
240 marrow and whole spleen ^{26,27} (Figure 4). In CARD11(E626K)^{CD4⁺Cre} mice, the numbers
241 of CD4⁺ T cells, Tem, and Treg in the body were not increased compared with WT mice.
242 In *HBZ*Tg mice, an increment of CD4⁺ T cells and Tem in the body was observed at 12
243 months after birth. *HBZ* expression had no effect on the number of Treg. In
244 CARD11(E626K)^{CD4⁺Cre};HBZTg mice, the numbers of CD4⁺ T cells, Tem, and Treg were
245 increased compared with WT, CARD11(E626K)^{CD4⁺Cre}, and *HBZ*Tg mice; this indicated
246 a cooperative positive effect of *CARD11* mutation and *HBZ* expression on the
247 proliferation of each cell type.

248 Even though the number of Treg in the body was not increased in
249 CARD11(E626K)^{CD4⁺Cre} mice, an increment of Treg was observed in the LNs and lung of
250 CARD11(E626K)^{CD4⁺Cre} mice (Supplemental figures 3 and 4). In *HBZ*Tg mice at 12

months after birth, Tem cells were prominent in LNs and lung, consistent with the increased number of Tem in the body. In CARD11(E626K)^{CD4⁺Cre}; *HBZ*Tg mice, both Tem and Treg infiltrated the LNs and lung. In LNs of CARD11(E626K)^{CD4⁺Cre}; *HBZ*Tg mice, about 50% of CD4⁺ cells were Tem (Supplemental figure 3). Cell proliferation was assessed by Ki-67 staining. Ki-67–positive cells comprised about 50% of LN cells in CARD11(E626K)^{CD4⁺Cre}; *HBZ*Tg mice, and this value was much higher than in the LNs of CARD11(E626K)^{CD4⁺Cre} and *HBZ*Tg mice, indicating the cooperative positive effect of CARD11 mutation and HBZ expression on LN cell proliferation (Supplemental figure 4).

Gene expression changes due to the CARD11 mutant and *HBZ* expression

To understand the basis for the phenotypes induced by the CARD11 mutant and *HBZ* expression *in vivo*, we performed gene expression profiling of Tem derived from WT, CARD11(E626K)^{CD4⁺Cre}, *HBZ*Tg, and CARD11(E626K)^{CD4⁺Cre}; *HBZ*Tg mice. Unsupervised hierarchical clustering of the global gene expression signatures revealed two main branches in the gene expression hierarchy (Supplemental figure 5A). WT and CARD11(E626K)^{CD4⁺Cre} samples comprised one branch, and *HBZ*Tg and CARD11(E626K)^{CD4⁺Cre}; *HBZ*Tg samples comprised the other. This indicated

that in CARD11(E626K)^{CD4-Cre};*HBZ*Tg Tem, *HBZ* expression had greater effects on the global gene expression pattern than the CARD11 mutant. Principal component analysis also showed that the CARD11 mutant and *HBZ* expression induced distinct transcriptomes (Supplemental figure 5B).

CARD activates the NF-κB signaling pathway. GSEA showed heavy enrichment of NF-κB canonical pathway gene sets²⁵ in CARD11(E626K)^{CD4-Cre} mice, but not in *HBZ*Tg or CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice (Figure 5A, Supplemental figure 6). NF-κB non-canonical pathway gene sets^{24,28} were significantly enriched in CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice, and they were nonsignificantly enriched in both CARD11(E626K)^{CD4-Cre} and *HBZ*Tg mice. *HBZ* mRNA and its transcript directly bind to the promoter regions of many genes. The selective apoptosis- and proliferation-related genes directly regulated by *HBZ* mRNA²⁹ were enriched in both *HBZ*Tg and CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice, and the enrichment of *HBZ* target gene sets³⁰, including indirect ones, were also observed in both mice (Supplemental figure 7).

The gene set enrichment pattern in Tem differed drastically between CARD11(E626K)^{CD4-Cre} and *HBZ*Tg mice (Figure 5B). Gene sets related to the NF-κB canonical pathway, cytokine signaling, and inflammation were heavily enriched in CARD11(E626K)^{CD4-Cre} mice, while those associated with cell adhesion and metabolism

287 were heavily enriched in *HBZ*Tg mice. CARD activation and *HBZ* expression might
 288 cooperatively affect global gene expression. In *CARD11(E626K)^{CD4^{Cre}};HBZ*Tg mice,
 289 there was greater enrichment of gene sets related to the NF-κB non-canonical pathway,
 290 IRF4 targets, proliferation, T-cell differentiation, and cell adhesion than in
 291 *CARD11(E626K)^{CD4^{Cre}}* and *HBZ*Tg mice. Positive enrichment of proliferation-related
 292 gene sets was also confirmed in *CARD11(E626K)^{CD4^{Cre}};HBZ*Tg mice relative to *HBZ*
 293 Tg mice and *CARD11(E626K)^{CD4^{Cre}}* mice, highlighting the cooperative effect between
 294 *CARD11(E626K)^{CD4^{Cre}}* and *HBZ*. We next compared the enriched KEGG and
 295 HALLMARK gene sets between human samples obtained from acute-type ATL
 296 patients¹⁶ and samples from *CARD11(E626K)^{CD4^{Cre}}*, *HBZ*Tg, and *CARD11(E626K)^{CD4^{Cre}}*
 297 *CD4^{Cre};HBZ*Tg mice. As shown in Figure 6A, about one-third of KEGG and HALLMARK
 298 gene sets were enriched in human acute-type ATL samples but not in healthy controls.
 299 Fewer than 15% of the enriched gene sets from both the KEGG and HALLMARK
 300 databases were also enriched in *CARD11(E626K)^{CD4^{Cre}}* and *HBZ*Tg mice. In contrast,
 301 *CARD11(E626K)^{CD4^{Cre}};HBZ*Tg mice demonstrated enrichment of 74% and 89% of the
 302 enriched gene sets from the two databases, respectively. The remaining gene sets that
 303 were only enriched in human acute-type ATL samples but not in *CARD11(E626K)^{CD4^{Cre}}*
 304 *CD4^{Cre};HBZ*Tg mice included many involved in metabolism (Supplemental table 2). In

addition, the NOTCH signaling pathway was only enriched in human acute-type ATL samples and not in Tem from CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice (Figure 6B). Since there were many mutations besides those involving the TCR-NF-κB pathway in acute-type ATL patients, these other mutations might be responsible for the activation of the other signaling pathways that were enriched in human ATL samples but not in CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice.

Finally, we examined 2 recently reported oncogenic transcriptional programs that induced proliferation and survival signals in ATL: the BATF3/IRF4/HBZ transcriptional network, and feed-forward regulatory loop genes driven by IRF4 and NF-κB^{31,32}. As shown in Figure 6C, the BATF3/IRF4/HBZ transcriptional network was enriched in both CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice and human acute-type ATL samples. On the other hand, genes coordinately upregulated by IRF4 and NF-κB were not enriched in CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice, but were positively enriched in human acute-type ATL samples.

Discussion

We showed here that a gain-of-function mutation in CARD11 induced lymphoproliferative disease *in vivo*, and this disease become more severe under

323 concurrent *HBZ* constitutive expression. *CARD11* mutation and *HBZ* expression
 324 cooperatively increased the numbers of CD4⁺ T cells, Tem, and Treg *in vivo*, and
 325 activated NF-κB non-canonical pathway, IRF4 targets, and cell proliferation. These
 326 findings, and the fact that many of the KEGG and HALLMARK gene sets that were
 327 enriched in acute-type ATL patient samples were also enriched in *CARD11*(E626K)^{CD4⁺}
 328 ^{Cre};*HBZ*Tg mice, indicate that the combination of *CARD11* mutation and *HBZ*
 329 expression forms the pathological basis for ATL development.

330 *CARD11*(E626K)^{CD4⁺Cre} mice developed lymphadenopathy and T-cell organ
 331 invasion, and exhibited shorter survival. The majority of infiltrating cells were Tem,
 332 which is consistent with previous reports that ATL clones are preserved in T memory
 333 stem cells (Tscm) and that the majority of ATL cells are conventional memory T cells³³.
 334 However, the degree of leukocytosis was mild and the spleen was only minimally
 335 affected. This phenotype induced by the constitutive activation of *CARD11* in T cells is
 336 very different from that in B cells. A mouse model that conditionally expressed the
 337 human DLBCL-derived *CARD11*(L225LI) mutant in B cells developed a rapidly lethal
 338 lymphoproliferative disorder with massive splenomegaly associated with a drastic
 339 increment in B-cell number¹⁹. Our observations might indicate that the *in vivo*
 340 signaling and other effects caused by constitutive activation of *CARD11*/BCL10/MALT1

(CBM) signaling differ between B and T cells, although the *CARD11* mutation is frequently observed in both B-cell malignancies, as the DLBCL ABC type, and in T-cell malignancies, as ATL. These ideas are consistent with the clinical features of the B-cell expansion with NF- κ B and T-cell anergy (BENTA) syndrome, which is caused by germline gain-of-function mutations in *CARD11*³⁴. Patients with BENTA syndrome exhibit splenomegaly and an increased number of B cells in PB, but a normal number of T cells^{35,36}.

As *HBZ* was constitutively expressed in many ATL cases and *HBZ*Tg mice were reported to develop lymphoma and systemic inflammation^{15,37}, we next examined the effect of constitutive *HBZ* expression on *CARD11*(E626K)^{CD4^{Cre}} mice. The phenotype of our *HBZ*Tg mice resembled that in previous reports^{15,37}. Lymphadenopathy was observed in 66.7% of *HBZ*Tg mice at 12 months after birth. In a report by Satou, 37.8% of *HBZ*Tg mice developed T-cell lymphoma after 16 months¹⁵. In our *HBZ*Tg mice, the increment of CD4⁺ T cells and Tem in the body was not observed at 6 months after birth, but became prominent at 12 months after birth. A long period of time might be required for the development of lymphoma in *HBZ*Tg mice.

CARD11(E626K)^{CD4^{Cre}};*HBZ*Tg mice exhibited more aggressive

lymphoproliferative disease than CARD11(E626K)^{CD4⁺Cre} mice and *HBZ*Tg mice. Most CARD11(E626K)^{CD4⁺Cre};*HBZ*Tg mice died within 8 months after birth. The numbers of CD4⁺ T cells, Tem, and Treg in the body increased due to the cooperative effect of *CARD11* mutation and *HBZ* expression. There was significant infiltration of lymphocytes into the perivascular region and alveolar septum of the lung, causing almost complete obliteration of the alveolar space in CARD11(E626K)^{CD4⁺Cre};*HBZ*Tg mice. These mice demonstrated lymphadenopathy and complete destruction of the normal LN architecture. These observations support the theory that constitutive CARD11 activation and *HBZ* expression have a cooperative effect.

Even though CARD11(E626K)^{CD4⁺Cre} and *HBZ*Tg mice had similar phenotypes, their gene set enrichment patterns were drastically different. NF-κB canonical pathway gene sets were enriched in CARD11(E626K)^{CD4⁺Cre} mice. It was reported that activation of the NF-κB canonical pathway was selectively inhibited by *HBZ* expression³⁸. In line with this, the gene set associated with this pathway was negatively enriched in *HBZ*Tg mice, but the finding was not statistically significant. The negative impact of *HBZ* on the NF-κB canonical pathway might negate the enrichment of these gene sets in CARD11(E626K)^{CD4⁺Cre};*HBZ*Tg mice. Non-canonical pathway gene sets were not enriched in either CARD11(E626K)^{CD4⁺Cre} or *HBZ*Tg mice,

although their enrichment was demonstrated in CARD11(E626K)^{CD4^{Cre}};HBZTg mice. This might be due to the cooperative effect of CARD11 activation and HBZ expression. These observations support the idea that CARD11(E626K)^{CD4^{Cre}};HBZTg mice mimic ATL, as it was reported that freshly isolated ATL cells display a high expression level of NF-κB-inducing kinase as well as nuclear translocation of p52 and RelB, indicating activation of the NF-κB non-canonical pathway³⁹. In addition to the NF-κB pathway, cytokine signaling cascades were heavily enriched in CARD11(E626K)^{CD4^{Cre}} mice. On the other hand, gene sets related to adhesion or migration, proliferation, and cancer or leukemia were heavily enriched in HBZTg mice. In accordance with the most severe phenotype in CARD11(E626K)^{CD4^{Cre}};HBZTg mice, CARD11 mutation and HBZ expression cooperatively activated many signaling cascades, including those associated with gene sets related to the NF-κB non-canonical pathway, IRF4 targets, T-cell differentiation, cell proliferation, and adhesion or migration.

We previously reported that mutations in genes encoding components of TCR-NF-κB signaling pathways were observed in 90% of samples from patients with acute-type ATL, and the median number of driver gene mutations in these samples was 10¹⁶. In addition, constitutive HBZ expression was observed in acute-type ATL samples. We then compared the gene expressions in CARD11(E626K)^{CD4^{Cre}};HBZTg mice and acute-

395 type ATL samples, and examined the combined effects of TCR-NF- κ B signaling
396 pathway activation and *HBZ* expression on the pathogenesis of ATL. Many signaling
397 cascades that were activated in human acute-type ATL samples were also activated in
398 CARD11(E626K)^{CD4Cre};*HBZ*Tg mice, indicating that TCR-NF- κ B signaling pathway
399 activation and *HBZ* expression were together responsible for the basic molecular
400 pathogenesis of acute-type ATL. In line with this, the BATF3/IRF4/*HBZ* transcriptional
401 network³¹ was enriched in both CARD11(E626K)^{CD4Cre};*HBZ*Tg mice and human acute-
402 type ATL samples. The proliferation of an ATL cell line was reported to be dependent
403 on BATF3 and IRF4, which cooperatively drive ATL-specific gene expression, and *HBZ*
404 was reported to induce BATF3 expression and its downstream target genes, including
405 *MYC*. CARD activation and *HBZ* expression might be sufficient for the cooperative
406 induction of proliferation/survival signals driven by BATF3 and IRF4 in ATL. On the
407 other hand, genes coordinately upregulated by IRF4 and NF- κ B³², which was positively
408 enriched in human acute-type ATL samples, were not enriched in CARD11(E626K)^{CD4}
409 ^{Cre};*HBZ*Tg mice. Among 23 genes that were upregulated by IRF4 and NF- κ B, only one
410 gene, *MYC*, was among the 69 genes driven by the BATF3/IRF4/*HBZ* transcriptional
411 network. *MYC* was highly expressed in Tem from CARD11(E626K)^{CD4Cre};*HBZ*Tg mice;
412 however, overexpression of other genes, including *CCR4* and *BIRC3*, was not observed

(data not shown), suggesting that mutations besides those in the TCR-NF- κ B pathway are required for their expression.

Patients with acute-type ATL often exhibit an extremely elevated lymphocyte count in PB, widespread lymphadenopathy, splenomegaly, and lymphocyte invasion of not only the lung but also the digestive tract, liver, adrenal glands, and brain. As this phenotype is much more severe than that in *CARD11(E626K)^{CD4^{Cre}};HBZ* Tg mice, additional mutations in T cells that already harbor NF- κ B activation mutations and *HBZ* expression might be required for the explosive increase in T cell number observed in acute-type ATL patients. In our previous analysis, *CARD11* mutation was an early event in ATL development¹⁶, suggesting that later mutations occur in cells that already harbor *CARD11* mutations, thereby resulting in the full phenotype of aggressive ATL. The mutation of genes coordinately upregulated by IRF4 and NF- κ B, as well as of signaling cascade genes that are activated in human acute-type ATL samples but not in *CARD11(E626K)^{CD4^{Cre}};HBZ* Tg mice, for example the NOTCH signaling cascade gene set, might contribute to the full development of acute-type ATL.

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Competing interests

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Data availability

Gene expression datasets obtained from human samples has been deposited at the European Genome-phenome Archive (EGAD00001001411). Gene expression datasets obtained from mouse samples will be deposited as well. For data sharing requests, please contact Kazuya Shimoda (kshimoda@med.miyazaki-u.ac.jp).

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467 **References**

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576

Figure legends

Figure 1. Shortened overall survival, splenic lymphocyte increment, and splenic structural disruption in mutant mice.

(A) Kaplan–Meier survival curves of mutant mice (wild type (WT), n = 40; CARD11(E626K)^{CD4}Cre, n = 20; *HBZ*Tg, n = 20; CARD11(E626K)^{CD4}Cre;*HBZ*Tg, n = 13). The median survival times of WT, CARD11(E626K)^{CD4}Cre, *HBZ*Tg, and CARD11(E626K)^{CD4}Cre;*HBZ*Tg mice, respectively, are as follows: not reached, 13.5, 11.8, and 6.9 months after birth.

(B) Absolute numbers of nucleated cells in the spleen at 6 months after birth (wild type (WT), n = 20; CARD11(E626K)^{CD4}Cre, n = 14; *HBZ*Tg, n = 19; CARD11(E626K)^{CD4}Cre;*HBZ*Tg, n = 12) and 12 months after birth (WT, n = 11; CARD11(E626K)^{CD4}Cre, n = 5; *HBZ*Tg, n = 12). p values were calculated by the Tukey test after one-way ANOVA, and * represents p values less than 0.05.

(C) Histologic analysis of the spleen at 6 and 12 months after birth (n = 5 for each mouse type). Spleens were stained with hematoxylin and eosin (HE) and anti-CD3 and anti-B220 antibodies. The interface between white and red pulp is shown at high magnification (HE 400x). CD3⁺ T cells in red pulp are shown at high magnification

(CD3 400x). In *CARD11(E626K)^{CD4^{Cre}}* mice at 6 months after birth, the splenic architecture is almost intact (HE 40x), but the interface between white and red pulp is uneven and obscure (HE 400x). These findings are attributed to focal infiltration of CD3⁺ T cells from the periarteriolar region in the white pulp into the red pulp (CD3 40x, CD3 400x). At 12 months after birth, areas of white pulp are decreased in number and size, while those of red pulp are larger (HE 40x). The interface has become disrupted (HE 400x). CD3⁺ T cells show diffuse infiltration into both the shrunken white pulp and the expanded red pulp (CD3 40x, CD3 400x). In *HBZ*Tg mice at 6 months, the splenic architecture is almost normal; at 12 months, however, white pulp has expanded (HE 40x, HE 400x). The number of CD3⁺ T cells is larger in both white and red pulp, which disrupts their interface (CD3 40x, CD3 400x). In *CARD11(E626K)^{CD4^{Cre}};*HBZ*Tg mice at 6 months, the splenic architecture is destroyed and the interface between white and red pulp is disrupted (HE 40x, HE 400x). CD3⁺ T cells are diffusely present throughout the spleen (CD3 40x, CD3 400x).*

Figure 2. Lymphadenopathy and disruption of lymph node architecture in mutant

mice.

(A) The frequency of lymphadenopathy at 6 and 12 months after birth. Swollen lymph nodes (LNs) are not large, with a mean diameter of about 3 mm. Differences between frequencies of lymphadenopathy were assessed by Fisher's exact test with Benjamini-Hochberg correction.

(B) Histologic analysis of LNs at 6 and 12 months after birth (n=5 for each mouse type).

LNs were stained with hematoxylin and eosin (HE), and anti-CD3 and anti-B220 antibodies. The interface between the cortex and paracortex is shown with high magnification (CD3 400x). In *CARD11(E626K)^{CD4⁺Cre}* mice, slightly shrunken follicles, a thickened cortex, and an expanded paracortex with CD3⁺ T-cell infiltration are observed at 6 months after birth (HE 100x, CD3 100x). The interface between the cortex and paracortex is obscured by infiltrating T cells (CD3 400x). At 12 months, the normal lymph node architecture is barely present in *CARD11(E626K)^{CD4⁺Cre}* mice (HE 100x). The cortex has become thickened and the follicles have disappeared. The paracortex is expanded by CD3⁺ T cells, and the interface is disrupted (CD3 100x, CD3 400x). In *CARD11(E626K)^{CD4⁺Cre};HBZ*Tg mice, the normal LN architecture is destroyed; follicles have almost disappeared, the cortex is thickened, and the

628 paracortex is expanded (HE 100x, CD3 100x). CD3⁺ T cells are observed mainly in the
629 paracortex and have massively infiltrated the cortex, leading to the highly disrupted
630 interface (CD3 100x, CD3 400x).
631

Figure 3. Lymphocyte infiltration into the lung in mutant mice.

Histologic analysis of the lung at 6 and 12 months after birth (n=5 for each mouse type). Lungs were stained with hematoxylin and eosin (HE), as well as anti-CD3 and anti-B220 antibodies. Lymphocyte infiltration into the lung perivascular interstitium (upper panel) and alveolar septa (lower panel) is observed in mutant mice at 6 and 12 months. In *CARD11(E626K)^{CD4^{Cre}}* mice, focal lymphocyte infiltration is observed at 6 months (HE 100x); both T and B cells have infiltrated the perivascular interstitium and occasionally the alveolar septa (HE 400x, CD3 400x, B220 400x). This infiltration became increasingly dominated by CD3⁺ T cells over time, and formed diffuse lung lesions with thickening of the interstitium and alveolar septa at 12 months. In *HBZ*Tg mice, diffuse infiltration of lymphocytes is observed at 6 months (HE 100x); lymphocytes comprising mainly T cells have invaded the perivascular interstitium and alveolar septa (HE 400x, CD3 400x, B220 400x). The alveolar space is filled with exudate at 6 months after birth (HE 400x). These abnormalities are much more pronounced at 12 months after birth. Severe lung findings with exudate and thickening of the interstitium and alveolar septa are observed. These features, except for exudate, resemble those in *CARD11(E626K)^{CD4^{Cre}}* mice. In *CARD11(E626K)^{CD4^{Cre}}*; *HBZ*Tg mice,

650 the lung interstitium and alveolar septa are thickened by infiltrating cells consisting
651 mainly of lymphocytes (HE100x, HE400x, CD3 400x). CD3⁺ T cells have accumulated
652 around capillary blood vessels. The alveolar space was initially filled with exudate, and
653 subsequently with macrophages, and consequently almost no air spaces are observed.

654

655

Figure 4. Increased numbers of CD4⁺ T cells and their subsets per body in mutant

mice.

Absolute numbers of CD4⁺CD8⁻ T cells, CD4⁺CD44⁺CD62L⁻ effector/memory T cells

(Tem), and CD4⁺CD25⁺ regulatory T cells (Treg) per body in wild type (WT) (n = 11),

CARD11(E626K)^{CD4⁺Cre} (n = 10), *HBZ*Tg (n = 12), and CARD11(E626K)^{CD4⁺Cre};*HBZ*Tg

(n = 14) mice at 6 months after birth (A), and those in WT (n = 11), CARD11(E626K)^{CD4⁺}

^{Cre} (n = 6), and *HBZ*Tg (n = 11) mice at 12 months after birth (B). In

CARD11(E626K)^{CD4⁺Cre} mice, the numbers of CD4⁺ T cells, Tem, and Treg in the body

are not increased compared with WT mice. In *HBZ*Tg mice, increments of CD4⁺ T cells

and Tem in the body are observed at 12 months after birth. In CARD11(E626K)^{CD4⁺}

^{Cre};*HBZ*Tg mice, the numbers of CD4⁺ T cells, Tem, and Treg are increased compared

with WT, CARD11(E626K)^{CD4⁺Cre}, and *HBZ*Tg mice. p values were calculated by the

Tukey test after one-way ANOVA, and *, *, and *** represent p values less than 0.05,

0.01, and 0.001, respectively.

Figure 5. Activation of the NF- κ B signaling pathway in CARD11(E626K)^{CD4-Cre} mice and the cooperative gene expression change driven by CARD mutation and *HBZ* expression in CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice.

(A) Gene set enrichment analysis (GSEA) of CD4⁺CD44⁺CD62L⁻ Tem in CARD11(E626K)^{CD4-Cre}, *HBZ*Tg, and CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice, compared with wild type (WT) mice (n = 3 for each mouse type). Enrichment plots of the NF- κ B-related gene set are shown with normalized enrichment scores (NESs) and false discovery rates (FDRs). The NFKB-1 gene set (SignatureDB²⁵), whose expressions are dependent on the canonical pathway molecule I κ B kinase beta, is positively enriched only in CARD11(E626K)^{CD4-Cre} mice; in contrast, the GO_NIK_NF_KAPPAB_SIGNALING gene set (Molecular Signatures Database²⁴), whose expressions are dependent on the non-canonical pathway molecule NF- κ B-inducing kinase (NIK), is positively enriched only in CARD11(E626K)^{CD4-Cre};*HBZ*Tg mice.

(B) Heatmap overview of gene sets showing significant enrichment in one or more mutant Tem compared to WT Tem. Comparisons of results are shown in the following order: CARD11(E626K)^{CD4-Cre} vs. WT, *HBZ*Tg vs. WT, CARD11(E626K)^{CD4-Cre};*HBZ*Tg

689 vs. WT, CARD11(E626K)^{CD4^{Cre}};*HBZ*Tg vs. *HBZ*Tg, and CARD11(E626K)^{CD4^{Cre}};*HBZ*
690 Tg vs. CARD11(E626K)^{CD4^{Cre}} (n = 3 for each mouse type). CARD11(E626K)^{CD4^{Cre}} mice
691 demonstrate enrichment in gene sets related to the NF-κB canonical pathway, cytokine
692 signaling, and inflammation. *HBZ*Tg mice show enrichment in gene sets related to cell
693 adhesion or migration, proliferation, cancer or leukemia, and metabolism. The
694 CARD11(E626K)^{CD4^{Cre}};*HBZ*Tg mice exhibit enrichment of gene sets related to the NF-
695 κB non-canonical pathway, IRF4 targets (SignatureDB²⁵), proliferation, T-cell
696 differentiation, cell adhesion/migration/chemotaxis, and metabolism. Positive
697 enrichment of proliferation-related gene sets were also confirmed in comparisons
698 between CARD11(E626K)^{CD4^{Cre}};*HBZ*Tg mice and either *HBZ*Tg mice or
699 CARD11(E626K)^{CD4^{Cre}} mice.

700

Figure 6. Gene expression signatures in CARD11(E626K)^{CD4^{Cre}};HBZTg mice

recapitulate many of those in human acute-type ATL samples.

(A) Normalized enrichment scores (NESs) in gene set enrichment analysis (GSEA) of the entire KEGG and HALLMARK gene sets shows that mice and humans share many of the same enriched gene sets. Each gene set is indicated by a dot and is highlighted in a different color according to the respective false discover rate (FDR) values for mice and humans, with a common cutoff value 0.25. Each plot legend indicates the frequency (number) of significant/non-significant gene sets, based on the common FDR cutoff values in both mice and humans. In the table at the bottom of each plot, the frequency (number) of pathways is indicated, based on the positive/negative values for both mouse NES (NES_m) and human NES (NES_h). Among the KEGG gene sets that were enriched in human acute-type ATL samples, 0% (0/65) and 14% (9/65) were also enriched in CARD11(E626K)^{CD4^{Cre}} mice and HBZTg mice, respectively, while 74% (48/65) were also enriched in CARD11(E626K)^{CD4^{Cre}};HBZTg mice; all of the gene sets had positive NESs in both mice and humans. Among the HALLMARK gene sets that were enriched in human acute-type ATL samples, 11% (2/18) and 11% (2/18) were also enriched in CARD11(E626K)^{CD4^{Cre}} mice and HBZTg mice, respectively, while 89%

(16/18) were also enriched in CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice; all of the gene sets had positive NESs in both mice and humans.

(B) Notch signaling is enriched in the acute-type ATL samples, but not in CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice. Enrichment plots of the KEGG Notch signaling gene set in mice and humans are shown with their NESs and FDRs.

(C) CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice partially recapitulated a transcriptional network change that is characteristic of ATL. The BATF3/IRF4/HBZ transcriptional network³¹ is enriched in both CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice and human acute-type ATL samples. On the other hand, genes coordinately upregulated by IRF4 and NF- κ B³² are not enriched in CARD11(E626K)^{CD4^{Cre}};HBZ Tg mice, but are positively activated in human acute-type ATL samples. Enrichment plots of each gene set in mice and humans are shown with their NESs and FDRs.

Figures

Figure 1

Shortened overall survival, splenic lymphocyte increment, and splenic structural disruption in mutant mice. (A) Kaplan–Meier survival curves of mutant mice (wild type (WT), n = 40; CARD11(E626K)CD4-Cre, n = 20; HBZ Tg, n = 20; CARD11(E626K)CD4-Cre ;HBZ Tg, n = 13). The median survival times of WT,

CARD11(E626K)CD4-Cre , HBZ Tg, and CARD11(E626K)CD4-Cre ;HBZ Tg mice, respectively, are as follows: not reached, 13.5, 11.8, and 6.9 months after birth. (B) Absolute numbers of nucleated cells in the spleen at 6 months after birth (wild type (WT), n = 20; CARD11(E626K)CD4-Cre , n = 14; HBZ Tg, n = 19; CARD11(E626K)CD4- Cre;HBZ Tg, n = 12) and 12 months after birth (WT, n = 11; CARD11(E626K)CD4-Cre , n = 5; HBZ Tg, n = 12). p values were calculated by the Tukey test after one-way ANOVA, and * represents p values less than 0.05. (C) Histologic analysis of the spleen at 6 and 12 months after birth (n = 5 for each mouse type). Spleens were stained with hematoxylin and eosin (HE) and anti-CD3 and anti-B220 antibodies. The interface between white and red pulp is shown at high magnification (HE 400x). CD3+ T cells in red pulp are shown at high magnification 31 (CD3 400x). In CARD11(E626K)CD4-Cre mice at 6 months after birth, the splenic architecture is almost intact (HE 40x), but the interface between white and red pulp is uneven and obscure (HE 400x). These findings are attributed to focal infiltration of CD3+ T cells from the periarteriolar region in the white pulp into the red pulp (CD3 40x, CD3 400x). At 12 months after birth, areas of white pulp are decreased in number and size, while those of red pulp are larger (HE 40x). The interface has become disrupted (HE 400x). CD3+ T cells show diffuse infiltration into both the shrunken white pulp and the expanded red pulp (CD3 40x, CD3 400x). In HBZ Tg mice at 6 months, the splenic architecture is almost normal; at 12 months, however, white pulp has expanded (HE 40x, HE 400x). The number of CD3+ T cells is larger in both white and red pulp, which disrupts their interface (CD3 40x, CD3 400x). In CARD11(E626K)CD4-Cre ;HBZ Tg mice at 6 months, the splenic architecture is destroyed and the interface between white and red pulp is disrupted (HE 40x, HE 400x). CD3+ T cells are diffusely present throughout the spleen (CD3 40x, CD3 400x).

Figure 2

Figure 2

Lymphadenopathy and disruption of lymph node architecture in mutant mice. (A) The frequency of lymphadenopathy at 6 and 12 months after birth. Swollen lymph nodes (LNs) are not large, with a mean diameter of about 3 mm. Differences between frequencies of lymphadenopathy were assessed by Fisher’s exact test with Benjamini– Hochberg correction. (B) Histologic analysis of LNs at 6 and 12 months after birth (n=5 for each mouse type). LNs were stained with hematoxylin and eosin (HE), and

anti-CD3 and anti-B220 antibodies. The interface between the cortex and paracortex is shown with high magnification (CD3 400x). In CARD11(E626K)CD4-Cre mice, slightly shrunken follicles, a thickened cortex, and an expanded paracortex with CD3+ T-cell infiltration are observed at 6 months after birth (HE 100x, CD3 100x). The interface between the cortex and paracortex is obscured by infiltrating T cells (CD3 400x). At 12 months, the normal lymph node architecture is barely present in CARD11(E626K)CD4-Cre mice (HE 100x). The cortex has become thickened and the follicles have disappeared. The paracortex is expanded by CD3+ T cells, and the interface is disrupted (CD3 100x, CD3 400x). In CARD11(E626K)CD4-Cre ;HBZ Tg mice, the normal LN architecture is destroyed; follicles have almost disappeared, the cortex is thickened, and the paracortex is expanded (HE 100x, CD3 100x). CD3+ T cells are observed mainly in the paracortex and have massively infiltrated the cortex, leading to the highly disrupted interface (CD3 100x, CD3 400x).

Figure 3

Figure 3

Lymphocyte infiltration into the lung in mutant mice. Histologic analysis of the lung at 6 and 12 months after birth (n=5 for each mouse type). Lungs were stained with hematoxylin and eosin (HE), as well as anti-CD3 and anti-B220 antibodies. Lymphocyte infiltration into the lung perivascular interstitium (upper panel) and alveolar septa (lower panel) is observed in mutant mice at 6 and 12 months. In CARD11(E626K)CD4-Cre mice, focal lymphocyte infiltration is observed at 6 months (HE 100x); both T

and B cells have infiltrated the perivascular interstitium and occasionally the alveolar septa (HE 400x, CD3 400x, B220 400x). This infiltration became increasingly dominated by CD3+ T cells over time, and formed diffuse lung lesions with thickening of the interstitium and alveolar septa at 12 months. In HBZ Tg mice, diffuse infiltration of lymphocytes is observed at 6 months (HE 100x); lymphocytes comprising mainly T cells have invaded the perivascular interstitium and alveolar septa (HE 400x, CD3 400x, B220 400x). The alveolar space is filled with exudate at 6 months after birth (HE 400x). These abnormalities are much more pronounced at 12 months after birth. Severe lung findings with exudate and thickening of the interstitium and alveolar septa are observed. These features, except for exudate, resemble those in CARD11(E626K)CD4-Cre mice. In CARD11(E626K) CD4-Cre ;HBZ Tg mice, the lung interstitium and alveolar septa are thickened by infiltrating cells consisting mainly of lymphocytes (HE100x, HE400x, CD3 400x). CD3+ T cells have accumulated around capillary blood vessels. The alveolar space was initially filled with exudate, and subsequently with macrophages, and consequently almost no air spaces are observed.

Figure 4

(A) Absolute number of cell fractions per body at 6 months after birth

(B) Absolute number of cell fractions per body at 12 months after birth

Figure 4

Increased numbers of CD4⁺ T cells and their subsets per body in mutant mice. Absolute numbers of CD4⁺CD8⁺ T cells, CD4⁺CD44⁺CD62L⁻ effector/memory T cells (Tem), and CD4⁺CD25⁺ regulatory T cells (Treg) per body in wild type (WT) (n = 11), CARD11(E626K)CD4-Cre (n = 10), HBZ Tg (n = 12), and CARD11(E626K)CD4-Cre;HBZ Tg (n = 14) mice at 6 months after birth (A), and those in WT (n = 11), CARD11(E626K)CD4-Cre (n = 6), and HBZ Tg (n = 11) mice at 12 months after birth (B). In

CARD11(E626K) CD4-Cre mice, the numbers of CD4+ T cells, Tem, and Treg in the body are not increased compared with WT mice. In HBZ Tg mice, increments of CD4+ T cells and Tem in the body are observed at 12 months after birth. In CARD11(E626K) CD4- Cre ;HBZ Tg mice, the numbers of CD4+ T cells, Tem, and Treg are increased compared with WT, CARD11(E626K) CD4-Cre , and HBZ Tg mice. p values were calculated by the Tukey test after one-way ANOVA, and *, *, and *** represent p values less than 0.05, 0.01, and 0.001, respectively

Figure 5

Activation of the NF- κ B signaling pathway in CARD11(E626K)CD4-Cre mice and the cooperative gene expression change driven by CARD mutation and HBZ expression in CARD11(E626K)CD4-Cre ;HBZ Tg mice. (A) Gene set enrichment analysis (GSEA) of CD4+CD44+CD62L- Tem in CARD11(E626K)CD4-Cre, HBZ Tg, and CARD11(E626K)CD4-Cre ;HBZ Tg mice, compared with wild type (WT) mice (n = 3 for each mouse type). Enrichment plots of the NF- κ B- related gene set are shown with normalized enrichment scores (NESs) and false discovery rates (FDRs). The NFKB-1 gene set (SignatureDB25), whose expressions are dependent on the canonical pathway molecule I κ B kinase beta, is positively enriched only in CARD11(E626K)CD4-Cre mice; in contrast, the GO_NIK_NF_KAPPAB_SIGNALING gene set (Molecular Signatures Database24), whose expressions are dependent on the non-canonical pathway molecule NF- κ B- inducing kinase (NIK), is positively enriched only in CARD11(E626K)CD4-Cre ;HBZ Tg mice. (B) Heatmap overview of gene sets showing significant enrichment in one or more mutant Tem compared to WT Tem. Comparisons of results are shown in the following order: CARD11(E626K)CD4-Cre vs. WT, HBZ Tg vs. WT, CARD11(E626K)CD4-Cre ;HBZ Tg vs. WT, CARD11(E626K)CD4-Cre ;HBZ Tg vs. HBZ Tg, and CARD11(E626K)CD4-Cre ;HBZ Tg vs. CARD11(E626K)CD4-Cre (n = 3 for each mouse type). CARD11(E626K)CD4-Cre mice demonstrate enrichment in gene sets related to the NF- κ B canonical pathway, cytokine signaling, and inflammation. HBZ Tg mice show enrichment in gene sets related to cell adhesion or migration, proliferation, cancer or leukemia, and metabolism. The CARD11(E626K)CD4-Cre ;HBZ Tg mice exhibit enrichment of gene sets related to the NF- κ B non-canonical pathway, IRF4 targets (SignatureDB25), proliferation, T-cell differentiation, cell adhesion/migration/chemotaxis, and metabolism. Positive enrichment of proliferation-related gene sets were also confirmed in comparisons between CARD11(E626K)CD4-Cre ;HBZ Tg mice and either HBZ Tg mice or CARD11(E626K)CD4-Cre mice

Figure 6

Gene expression signatures in CARD11(E626K)CD4-Cre ;HBZ Tg mice recapitulate many of those in human acute-type ATL samples. (A) Normalized enrichment scores (NESs) in gene set enrichment analysis (GSEA) of the entire KEGG and HALLMARK gene sets shows that mice and humans share many of the same enriched gene sets. Each gene set is indicated by a dot and is highlighted in a different color according to the respective false discover rate (FDR) values for mice and humans, with a common cutoff value 0.25. Each plot legend indicates the frequency (number) of significant/non-significant gene sets, based on the common FDR cutoff values in both mice and humans. In the table at the bottom of each plot, the frequency (number) of pathways is indicated, based on the positive/negative values for both mouse NES (NES_m) and human NES (NES_h). Among the KEGG gene sets that were enriched in human acute-type ATL samples, 0% (0/65) and 14% (9/65) were also enriched in CARD11(E626K)CD4-Cre mice and HBZ Tg mice, respectively, while 74% (48/65) were also enriched in CARD11(E626K)CD4-Cre ;HBZ Tg mice; all of the gene sets had positive NESs in both mice and humans. Among the HALLMARK gene sets that were enriched in human acute-type ATL samples, 11% (2/18) and 11% (2/18) were also enriched in

CARD11(E626K)CD4-Cre mice and HBZ Tg mice, respectively, while 89% 40 (16/18) were also enriched in CARD11(E626K)CD4-Cre ;HBZ Tg mice; all of the gene sets had positive NESs in both mice and humans. (B) Notch signaling is enriched in the acute-type ATL samples, but not in CARD11(E626K)CD4-Cre ;HBZ Tg mice. Enrichment plots of the KEGG Notch signaling gene set in mice and humans are shown with their NESs and FDRs. (C) CARD11(E626K)CD4-Cre ;HBZ Tg mice partially recapitulated a transcriptional network change that is characteristic of ATL. The BATF3/IRF4/HBZ transcriptional network³¹ is enriched in both CARD11(E626K)CD4-Cre ;HBZ Tg mice and human acutetype ATL samples. On the other hand, genes coordinately upregulated by IRF4 and NF- κ B2 are not enriched in CARD11(E626K)CD4-Cre ;HBZ Tg mice, but are positively activated in human acute-type ATL samples. Enrichment plots of each gene set in mice and humans are shown with their NESs and FDRs.

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