Reduced TET2 function leads to T-cell lymphoma with follicular helper T-cell-like features in mice

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TET2 (Ten Eleven Translocation 2) is a dioxygenase that converts methylcytosine (mC) to hydroxymethylcytosine (hmC). TET2 loss-of-function mutations are frequently found in subtypes of T-cell lymphoma that harbor follicular helper T (Tfh)-cell-like features, such as angioimmunoblastic T-cell lymphoma (30–83%) or peripheral T-cell lymphoma, not otherwise specified (10–49%), as well as myeloid malignancies. Here, we show that middle-aged Tet2 knockout (Tet20/0) mice exhibit Tfh-like cell overproduction in the spleen compared with control mice. The Tet2 knockout mice eventually develop T-cell lymphomas with Tfh-like features after a long latency (median 67 weeks). Transcriptome analysis revealed that these lymphoma cells had Tfh-like gene expression patterns when compared with splenic CD4-positive cells of wild-type mice. The lymphoma cells showed lower hmC densities around the transcription start site (TSS) and higher mC densities at the regions of the TSS, gene body and CpG islands. These epigenetic changes, seen in Tet2 insufficiency-triggered lymphoma, possibly contributed to predated outgrowth of Tfh-like cells and subsequent lymphomagenesis. The mouse model described here suggests that TET2 mutations play a major role in the development of T-cell lymphoma with Tfh-like features in humans.

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INTRODUCTION
In mammalian cells, three TET (Ten Eleven Translocation) proteins, TET1, TET2 and TET3, function as methylcytosine dioxygenases, commonly converting methylcytosine (mC) to hydroxymethylcytosine (hmC), which is thought to be an essential intermediate in both active and passive demethylation processes. Furthermore, hmC is also believed to serve as an alternative epigenetic mark to mC in the regulation of gene expression. Nevertheless, its biological function remains unclear.

TET2 mutations are frequently found in myeloid malignancies (myelodysplastic syndrome, 14–26%; myeloproliferative neoplasm, 7.6–37% and acute myeloid leukemias, 12–43%). TET2 loss-of-function mutations are associated with aberrant DNA methylation patterns in myeloid malignancies. Of interest here, TET2 mutations are extremely frequent in subtypes of T-cell lymphoma such as angioimmunoblastic T-cell lymphoma (AITL, 30–83%) and peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS, 10–49%). AITL is thought to emerge from follicular helper T (Tfh) cells, based on findings from gene-expression profiling and immunohistochemical staining. Tfh cells exist in the follicles of lymph nodes and spleen, and interact with follicular B cells and antigen-presenting cells. B-cell leukemia/lymphoma 6 (Bcl6) and v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (cMaf) encode key transcription factors governing Tfh differentiation and proliferation. Tfh cells express co-stimulatory molecules such as programmed cell death 1 (PD1) and inducible T-cell costimulator (Icos), as well as chemokine receptors, such as chemokine (C-X-C motif) receptor 5 (Cxc5) on the cell surface. PTCL-NOS is a group of heterogeneous T-cell lymphomas, some of which also show Tfh-like features.

Thus far, Tet2 function has been assessed in various knockout/knockdown mice. Common phenotypes seen following Tet2 loss are increased frequency of the lineage-negative, Sca1-positive and c-Kit-positive (LSK) fraction, enhanced competitive repopulation capacity and skewed differentiation toward myeloid lineages. Some Tet2-knockout mice are reported to develop myeloid malignancies, resembling chronic myelomonocytic leukemia (CML). Tet2-knockout mice eventually develop T-cell lymphoma with Tfh-like features.

Here, we show that Tet2-knockdown mice develop T-cell lymphoma with Tfh-like features. Comprehensive gene expression analysis and DNA methylation and hydroxymethylation analysis revealed epigenetic changes in lymphoma cells.

MATERIALS AND METHODS
Mice
Tet2 gene trap mice, in which a poly-A trapping cassette containing the β-galactosidase/neomycin resistance gene is inserted into the second intron, were purchased from TransGenic Inc. (Kumamoto, Japan). Mice were genotyped by tail DNA PCR using the primers listed in Supplementary Table 7. Mice were backcrossed > 8 times onto a C57BL/6 background. Experiments were performed according to the Guide for Care and Use of Laboratory Animals at the University of Tsukuba.
Gene expression array analysis

Gene expression analysis was carried out with samples from CD4+ cells from lymphoma-developing Tet2\textsuperscript{gt/gt} mice or from wild-type (WT) mice with GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA), according to the manufacturer’s instructions. The Gene Expression Omnibus (GEO) accession number for the microarray data reported in this paper is GSE52430. See Supplementary Methods for more information.

MeDIP and hMeDIP sequencing

MeDIP and hMeDIP sequencing protocols were performed as described, with minor modifications.\textsuperscript{27} The DNA Data Bank of Japan (DDBJ) accession numbers are DRA001275 and DRA001277. See Supplementary Methods for more information. See Supplementary information for more methods.

RESULTS

Decreased Tet2 function significantly increases the number of Thf-like cells in the spleen

We analyzed homozygous Tet2 (hereafter named Tet2\textsuperscript{gt/gt}) mice harboring a gene-trap vector in the Tet2 second intron (Supplementary Figure S1a).\textsuperscript{31} We reproduced various findings described in previous papers using the same mice, such as 80% decrease in Tet2 mRNA levels and 50% decrease in hmC levels in fetal liver (FL) lineage-negative cells (Supplementary Figures S1b and c), and enhanced repopulating activity in FL LSks cells only after secondary transplantation (Supplementary Figures S2a and b).\textsuperscript{30} Tet2\textsuperscript{gt/gt} mice were born and grew almost normally at a frequency of a half the expected Mendelian ratio (Tet2\textsuperscript{gt/gt} : Tet2\textsuperscript{+/gt} : Tet2\textsuperscript{+/+} = 32:124:70). There were no significant differences in appearance, complete blood cell counts and proportions of granulocytes, T cells and B cells in the peripheral blood among Tet2\textsuperscript{gt/gt}, heterozygous (Tet2\textsuperscript{+/gt}) and Tet2\textsuperscript{+/+} mice during the period between 40 and 60 weeks of age (Supplementary Figures S3a and b).

When evaluated at 40-60 weeks old, the spleen weights of Tet2\textsuperscript{gt/gt} mice were significantly higher than those of Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice (179.8 ± 72.3 mg, 97.0 ± 10.6 mg and 108.5 ± 26.1 mg, respectively) (Figure 1a). One of the 10 Tet2\textsuperscript{gt/gt} mice developed marked splenomegaly (>300 mg). Hematoxalin-Eosin (HE) staining demonstrated preserved follicular structures, having enlarged germinal centers in some Tet2\textsuperscript{gt/gt} mice (Figure 1b).

Next we used flow cytometry to analyze splenocyte phenotypes and observed no differences in proportions of CD4+ T cells and B220+ B lymphocytes between Tet2\textsuperscript{gt/gt}, Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice, although the proportion of CD3+ T cells was marginally, but significantly, decreased in Tet2\textsuperscript{gt/gt} mice compared with those of Tet2\textsuperscript{+/+} mice. The population of Gr1+Mac1+ granulocytes was slightly, but significantly, increased in Tet2\textsuperscript{gt/gt} mice compared with that of Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice (Figure 1c). The absolute number of cells was increased in all the fractions of Tet2\textsuperscript{gt/gt} mice compared with those of Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice (Figure 1d). Furthermore, we observed significant increases in the ratios of CD4+CD44+PD1+ fraction in Tet2\textsuperscript{gt/gt} mice compared with those of Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice (Figures 1e and f). The ratio of CD4+PD1+CXcr5+ fraction was significantly higher in Tet2\textsuperscript{gt/gt} mice than that in Tet2\textsuperscript{+/+} mice (Figure 1f). The absolute numbers of CD4+CD44+PD1+ and CD4+PD1+CXcr5+ fractions were significantly increased in Tet2\textsuperscript{gt/gt} compared with that in Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice (Figure 1g). PD1 is a co-stimulatory molecule expressed on T cells, which are in fact marked by co-expression of CD4, PD1 and CXcr5 in mice.\textsuperscript{33} In a similar manner to the analysis above, immunofluorescent staining of spleen tissues also indicated an increase of Thf-like cells in Tet2\textsuperscript{gt/gt} mice (Figure 1h).

Overall, our results indicate that reduced Tet2 function causes outgrowth of Thf-like cells in the spleen at 40–60 weeks old. Extramedullary hematopoiesis in Tet2-knockout/dowm mice, as described by others,\textsuperscript{45,62,67,73} was not apparent in our analysis, although there was a tendency towards increase in erythroid precursor cells and a statistically significant increase in LSK cells in the spleens of Tet2\textsuperscript{gt/gt} mice compared with mice of other genotypes (Supplementary Figure S4a). Tet2\textsuperscript{gt/gt} mice develop T-cell lymphoma with Thf-like features

We next analyzed Tet2\textsuperscript{gt/gt} mice older than 60 weeks and found that five in seven mice developed marked splenomegaly, multiple swollen lymph nodes and multiple nodules in the liver and lungs (median age, 67 weeks old) (Figures 2a–c). HE staining showed that follicular structures in spleens of tumor-developing Tet2\textsuperscript{gt/gt} mice were completely destroyed due to the invasion of large pleomorphic cells exhibiting irregular-shaped nuclei (Figure 2c). These findings implied that Tet2\textsuperscript{gt/gt} mice developed lymphoma. We performed immunofluorescent staining to examine the phenotype of the infiltrating cells, demonstrating that most of the cells expressed CD4+, PD1+ and CXcr5+ (Figure 2d). To confirm whether the tumors were T-cell lymphoma, we examined the T-cell receptor (TCR) rearrangement pattern using DNA from CD4+ T cells in tumor-developing spleens and lymph nodes, and found that the tumor-derived CD4+ T cells demonstrated distinct rearrangement patterns in TCR VB1/BJ2. Furthermore, tumor-derived CD4+ T cells from the spleen and swollen lymph nodes of a mouse showed identical TCR rearrangement patterns (Figure 2e, related to Table 1). Thus, we concluded that Tet2\textsuperscript{gt/gt} mice developed T-cell lymphoma.

Next, we characterized the lymphoma-developing Tet2\textsuperscript{gt/gt} mice. There were no significant differences in white blood cell count and hemoglobin concentration in the peripheral blood of Tet2\textsuperscript{gt/gt}, Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice, while platelet count in lymphoma-developing Tet2\textsuperscript{gt/gt} mice was significantly lower than that of Tet2\textsuperscript{+/+} mice (Figure 3a). The spleen weight of lymphoma-developing Tet2\textsuperscript{gt/gt} mice was significantly higher than that of Tet2\textsuperscript{+/+} and Tet2\textsuperscript{+/} mice (1466 ± 105 mg, 1103 ± 101 mg, and 165 ± 83 mg, respectively) (Figure 3b). In the spleens, the CD4+ helper T-cell fractions were significantly increased in Tet2\textsuperscript{gt/gt} mice compared with those of Tet2\textsuperscript{+/gt} and Tet2\textsuperscript{+/+} mice (Figure 3c). The B220+ B-cell fraction was significantly decreased in Tet2\textsuperscript{gt/gt} mice compared with that of Tet2\textsuperscript{+/+} mice. There was no significant difference in Gr1+/Mac1+ myeloid cell fraction. The CD4+ T cells were mostly positive for CD44 and PD1, and to a lesser extent for CXcr5 (Figure 3d). The percentage of CD4+PD1+CXcr5+ Thf-like cell fraction was significantly higher than that of mice with the other genotypes. There were no differences in the erythroid precursor and LSK fraction in the spleens between the aged Tet2\textsuperscript{+/+} and Tet2\textsuperscript{+/+} mice (Supplementary Figure S5b).

In the clinical setting, AITL patients often show polyclonal hypergammaglobulinemia. Serum immunoglobulin levels in both CD4+ and CD8+ T cells in tumor-developing spleens and lymph nodes, and found that the tumor-derived CD4+ T cells demonstrated distinct rearrangement patterns in TCR VB1/BJ2. Furthermore, tumor-derived CD4+ T cells from the spleen and swollen lymph nodes of a mouse showed identical TCR rearrangement patterns (Figure 2e, related to Table 1). Thus, we concluded that Tet2\textsuperscript{gt/gt} mice developed T-cell lymphoma.

Taken together, these results indicate that Tet2\textsuperscript{gt/gt} mice develop T-cell lymphomas that exhibit features characteristic of Thf-like cells, but these T-cell lymphomas do not show pathological or clinical findings relevant to human AITL. PTCL-NOS with Thf-like features\textsuperscript{13} might be the closest counterpart.

Lymphoma cells exhibit gene expression profiles similar to those of Thf cells

Gene-expression profiles of CD4+ cells derived from splenic tumors of Tet2\textsuperscript{gt/gt} mice versus CD4+ cells from normal WT spleen (hereafter designated lymphoma cells versus control CD4+ cells, respectively) were evaluated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) resource\textsuperscript{34} and Gene Set Enrichment Analysis (GSEA).\textsuperscript{35} When 903 upregulated genes (> 2 folds) and 896 downregulated genes (< 0.5 folds) were searched, the KEGG pathway relevant to human AITL. PTCL-NOS with Thf-like features\textsuperscript{13} might be the closest counterpart.
For the GSEA, the Tfh-upregulated gene set was unavailable in BIOCARTA and thus had to be newly made by selecting 21 genes based on previous microarray analyses. The Th1/Th2 pathway gene sets were adopted from BIOCARTA for GSEA analysis. The Tfh-upregulated genes were significantly enriched in lymphoma cells compared with control CD4+ cells (P-value 0.0020, FDR Q-value 0.0027, NES 1.869) (Figure 4a). In contrast, genes associated with Th1/2 differentiation were enriched in control CD4+ cells (P-value 0.0169, FDR Q-value 0.0169, NES -1.702) (Figure 4b).

To validate the Tfh-like signature in lymphoma cells, we

Figure 1. Outgrowth of Tfh-like cells in spleens of 40- to 60-week-old Tet2gt/gt mice. (a) Spleen weights of Tet2+/+ (n = 10), Tet2+/gt (n = 3) and Tet2gt/gt (n = 10) mice (mean ± s.d.). *P < 0.05, **P < 0.01. (b) Hematoxylin-Eosin (HE) staining of sections from spleen. Representative figures were shown. Black bars indicate 500 μm. (c) Cell surface analysis of splenocytes in Tet2+/+ (n = 10), Tet2+/gt (n = 3) and Tet2gt/gt (n = 10) mice (mean ± s.d.). *P < 0.05. (d) Absolute numbers of splenocytes in each fraction (Tet2+/+ n = 6, Tet2+/gt n = 3, Tet2gt/gt n = 6) (mean ± s.d.). *P < 0.05, **P < 0.01. (e) Representative figures of CD4+CD44+PD1+ and CD4+PD1+Cxcr5+ fractions in the spleen of 40- to 60-week-old Tet2+/+, Tet2+/gt and Tet2gt/gt mice. (f) Percentages of CD4+CD44+PD1+ and CD4+PD1+Cxcr5+ fractions in the spleen of Tet2+/+ (n = 9), Tet2+/gt (n = 3) and Tet2gt/gt (n = 9) mice (mean ± s.d.). *P < 0.01. (g) Absolute numbers of CD4+CD44+PD1+ and CD4+PD1+Cxcr5+ cell fractions in the spleen of Tet2+/+ (n = 6), Tet2+/gt (n = 3) and Tet2gt/gt (n = 6) mice (mean ± s.d.). *P < 0.05, **P < 0.01. (h) Immunofluorescent staining of 40- to 60-week-old spleen in Tet2+/+, Tet2+/gt and Tet2gt/gt. CD4 (green), PD1 (red) and Cxcr5 (purple) were stained with counter staining of DAPI. White bars indicated 20 μm.
performed real-time PCR for representative Tfh-associated mRNAs, such as Bcl6, cMaf, PD1, Icos and Cxcr5. Transcript levels of all these molecules were significantly higher in lymphoma than control CD4+ cells (Figure 4c). In contrast, transcript levels of Tbx21 and Gata3, which encode master transcription factors regulating Th1 and Th2 differentiation, respectively, were comparable or slightly lower in lymphoma (Figure 4d). In addition, immunofluorescent staining revealed many cells were co-stained for CD4 and Bcl6 or cMaf in lymphoma-developing spleen tissue (Figure 4e).

Lymphoma cells exhibit genome-wide aberrant methylation and hydroxymethylation patterns

To examine potential epigenetic changes in lymphoma cells, we performed methylated DNA immunoprecipitation (MeDIP) and hydroxymethylated DNA immunoprecipitation (hMeDIP) combined with high-throughput sequencing, using three independent samples for lymphoma and control CD4+ cells. In the gene-oriented analysis, the average level of mC showed a similar pattern in the lymphoma cells and control CD4+ cells, while the
hmC level was slightly reduced in lymphoma cells. Focused around the TSS, the average mC level showed a slight decrease just downstream of the TSS, but an increase toward its gene body region in lymphoma cells. In comparison, the decrease of average hmC level was remarkable between TSS and at 5 k bp downstream of TSS in lymphoma cells (Figure 5a). We then examined the distribution of the highly enriched regions of mC and hmC. The regions in which the Model-based analysis for ChIP-Seq (MACS) score was >5 were considered to be highly enriched regions. Within these regions, methylation at the TSS regions (TSS ± 1 k bp, TSS ± 5 k bp), gene bodies and CpG islands was increased in lymphoma cells in comparison with the control CD4+ cells (P=0.013, 0.006, 0.006 and 0.022, respectively). Hydroxymethylation was decreased at the TSS regions in lymphoma cells.

Table 1. Characteristics of aged Tet2gt/gt mice (n = 7 more than 60 weeks old)

| No  | 1   | 2   | 3   | 4   | 5   | 6   | 7   |
|-----|-----|-----|-----|-----|-----|-----|-----|
| Spleen weight (mg) | 433 | 1615| 1437| 656 | 3189| 136 | 167 |
| Macroscopic lymphoma involvement | +   | +   | +   | +   | +   | +   | -   |
| Microscopic lymphoma involvement | +   | +   | +   | +   | +   | +   | -   |
| TCRβ rearrangement | +   | +   | +   | +   | +   | +   | -   |

Lymphoma involvement includes swollen lymph nodes or organ lesion such as the liver or lung.

Figure 3. Characteristics of lymphoma-developing Tet2gt/gt mice. (a) White blood cell (WBC) counts, hemoglobin (Hb) concentrations and platelet (Plt) counts in aged Tet2+/+ (n = 5), aged Tet2+/gt (n = 3) and lymphoma-developing Tet2gt/gt (n = 4) mice over 60 weeks of age. Black bars indicate mean values. *P < 0.05. (b) Spleen weights of Tet2+/+ (n = 5), Tet2+/gt (n = 3) and lymphoma-developing Tet2gt/gt (n = 5) and non-lymphoma-developing Tet2gt/gt mice (n = 2). Black bars indicate mean values. *P < 0.05, **P < 0.01. (c) Cell surface analysis of splenocytes in Tet2+/+ (n = 5), Tet2+/gt (n = 3) and lymphoma-developing Tet2gt/gt (n = 4) mice (mean ± s.d.). *P < 0.05. (d) Representative figures of cell surface analyses of the spleen from Tet2+/+ mice (upper), and spleen and lymph node (LN) from lymphoma-developing Tet2gt/gt mice (middle and lower) over 60 weeks old. The graph shows the percentages of Tfh-like fractions (Tet2+/+ n = 5, Tet2+/gt n = 3 and Tet2gt/gt n = 4; mean ± s.d.). *P < 0.05, **P < 0.01.
compared with the control CD4+ cells (P = 0.018 and 0.006, respectively) (Figure 5b).

Next, we merged the two gene lists, the first for upregulated gene expression and the second for aberrant deposition of methylation or hydroxymethylation. Generally, DNA methylation in the promoter region is closely associated with the inactivation of gene expression. In contrast, aberrant accumulation of methylation in the gene body is known to be associated with upregulated transcription. 39 The tumor cells in our T-cell lymphoma mouse model showed Tfh-like cell outgrowth and lymphoma cells showed upregulation in the Tfh-related genes. Thus, we selected 2555 genes (upper 10% of total genes) showing greater mC density at the gene body regions in lymphoma cells compared with the control CD4+ cells (mean difference of tag counts by MACS > 7). Independently, we selected 2500 highly expressed genes (upper 10% of total genes), and the top 903 genes with an expression fold of > 2 in lymphoma cells compared with control CD4+ cells. In these two groups, 448 and 118 genes, respectively, fulfilled both criteria of mC increase in gene body and fold change in gene expression. Gene ontology (GO) analysis picked up several enriched terms associated with oncogenesis, such as regulation of cell death and anti-apoptosis for the 448 genes (Supplementary Table S3), and cell division, cell cycle and cell cycle process (Supplementary Table S4) for the 118 genes (Figure 5c).

We similarly selected 2639 genes (upper 10% of total genes) (mean difference of tag counts by MACS < -3) showing lower hmC density at the TSS ± 1 kbp regions in lymphoma cells compared with the control CD4+ cells (P = 0.018 and 0.006, respectively) (Figure 5b).

Next, we merged the two gene lists, the first for upregulated gene expression and the second for aberrant deposition of methylation or hydroxymethylation. Generally, DNA methylation in the promoter region is closely associated with the inactivation of gene expression. In contrast, aberrant accumulation of methylation in the gene body is known to be associated with upregulated transcription. 39 The tumor cells in our T-cell lymphoma mouse model showed Tfh-like cell outgrowth and lymphoma cells showed upregulation in the Tfh-related genes. Thus, we selected 2555 genes (upper 10% of total genes) showing greater mC density at the gene body regions in lymphoma cells compared with the control CD4+ cells (mean difference of tag counts by MACS > 7). Independently, we selected 2500 highly expressed genes (upper 10% of total genes), and the top 903 genes with an expression fold of > 2 in lymphoma cells compared with control CD4+ cells. In these two groups, 448 and 118 genes, respectively, fulfilled both criteria of mC increase in gene body and fold change in gene expression. Gene ontology (GO) analysis picked up several enriched terms associated with oncogenesis, such as regulation of cell death and anti-apoptosis for the 448 genes (Supplementary Table S3), and cell division, cell cycle and cell cycle process (Supplementary Table S4) for the 118 genes (Figure 5c).

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Figure 5. Genome-wide mC and hmC analysis of CD4+ spleen cells from Tet2+/+ and lymphoma-developing Tet2gt/gt mice. (a) Average levels of mC (upper) and hmC (lower) in CD4+ splenocytes from Tet2+/+ (n = 3) and lymphoma-developing Tet2gt/gt (n = 3) mice. Left graphs indicate gene-oriented distribution of mC and hmC; right graphs indicate mC and hmC distribution around the transcription start site (TSS). TSS, transcription termination site. Black allows indicate the regions at differences between lymphoma cells and control cells. (b) Distribution of mC and hmC enriched regions (MACS score $\geq 5$) at TSS $\pm 1$ kbp, TSS $\pm 5$ kbp, gene body regions and CpG islands in CD4+ splenocytes from Tet2+/+ (n = 3) and lymphoma-developing Tet2gt/gt (n = 3) mice. (c) The top 2555 genes showing increased methylation at the gene body in lymphoma cells compared with control CD4+ cells were selected, as were the top 2500 and 903 genes showing higher expression in lymphoma compared with control CD4+ cells; 448 and 118 genes were common to both groups, respectively. Gene ontology (GO) analysis was performed using these genes. (d) The top 2639 genes showing decreased hydroxymethylation in the gene body in lymphoma cells compared with control CD4+ cells were selected, as were the top 2500 and 903 genes showing higher expression in lymphoma compared with control CD4+ cells. 248 and 57 genes were common to both groups, respectively. GO analysis was performed using these genes.
compared with the control CD4+ cells, in light of a recent report demonstrating that hmC at the promoter region negatively regulates gene expression.

When the same 2500 and 903 highly expressed genes were analyzed, 248 and 57 genes, respectively, fulfilled both criteria of hmC increase at TSS − 1 kbp and fold change in gene expression (Figure 5d). GO analysis picked up enriched terms such as immune response and regulation of lymphocyte activation (Supplementary Table S5), and cell death and death (Supplementary Table S6) for the 248 and 57 genes, respectively.

These analyses suggest that the gene list overlapping highly methylated genes at the gene body and upregulated genes in lymphoma cells is linked to oncogenesis, and that the gene list overlapping less hydroxymethylated genes at TSS and upregulated genes in lymphoma cells is linked to the immune system.

Bcl6 was one of the 448 genes associated with mC change that arose in our analyses of the Tfh-related genes enriched in lymphoma cells. MeDIP sequencing of the Bcl6 locus revealed a markedly increased mC density in the first intron in lymphoma compared with the control CD4+ cells (Figure 6a). The change in the mC density in the first intron was validated by quantitative MeDIP (qMeDIP) (Figure 6b). hmC analysis as described above revealed PD1 and Cxcr5 to be among the Tfh-related genes. hmC peaks were decreased in TSS ± 1 kbp of PD1 and Cxcr5 genes.
mRNA expression and methylation status in EL4 cells, a mouse expression occurs in T-lineage lymphoma cells, we examined Bcl6. To determine whether methylation-dependent regulation of decreases mRNA expression in EL4 cells frequently coexisting mutations with T cells. This result supports the notion that the methylated cytosine decreased in a decitabine dose-dependent manner (Figure 6e).

Decitabine-induced demethylation of the first intron of Bcl6 decreases mRNA expression in EL4 cells. To determine whether methylation-dependence regulation of Bcl6 expression occurs in T-lineage lymphoma cells, we examined Bcl6 mRNA expression and methylation status in EL4 cells, a mouse T-cell lymphoma cell line. Bisulfite sequencing revealed that almost all cytosines found in CpG sequences were methylated at the Bcl6 almost all cytosines found in CpG sequences were methylated at the first intron in lymphoma relative to control cells (Figure 6c), corresponding to the MeDIP sequencing (Figure 6a).

Table 2. Validated gene list of exome sequencing with paired-normal control (No.5)

| Annotated gene | Mutation type | Ref Seq | Nucleotide change | Amino-acid change | VAF |
|----------------|---------------|---------|------------------|-------------------|-----|
| Cadm2          | Missense      | NM_001145977 | c.A512T         | p.Y171F           | 0.133333 |
| Angel2         | Missense      | NM_021421  | c.G1349A         | p.S450N           | 0.178571 |
| Skn10          | Missense      | NM_00177668 | c.C375A         | p.T132N            | 0.147059 |
| Taat5          | Missense      | NM_001009574 | c.G8880C       | p.A294P           | 0.150327 |
| Bcl6           | Missense      | NM_0009744 | c.G1049A         | p.S330N           | 0.179487 |

Abbreviation: VAF, variant allele frequency.

(Supplementary Figures S5a and S5c). Quantitative hMeDIP also showed tendencies of hmC decrease in TSS ± 1 k bp of both genes, but there was no statistical significance (Supplementary Figure S6b and S6d).

These results prompted us to further analyze the first intron of the Bcl6 gene, which has been reported as an intronic silencer region. Bcl6 int-1-S, the silencing activity of which depends on its methylation status in a human B-cell lymphoma cell line. Bcl6 plays a definitive role for the development of Th cells. Bisulfite sequencing revealed an increase in the density of modified cytosine at the first intron in lymphoma relative to control cells (Figure 6c), corresponding to the MeDIP sequencing (Figure 6a).

Frequently coexisting mutations with TET2 mutation in hematologic malignancies are not detected by exome and targeted sequencing. To examine additional mutations in lymphoma cells, we performed exome sequencing in the lymphoma cells developed in TET2/KO mice (sample No.5 in Table 1). Five gene mutations were identified and validated by Sanger sequencing, but none of these mutations have been reported in connection with human peripheral T-cell lymphoma (Table 2). In contrast, Flt3, Npm1, Dnmt3a, Idh2 and Rhoa mutations, which are known to coexist with TET2 mutations in various human hematologic malignancies, were not identified by targeted sequencing in tumor sample numbers 1 to 5 (Supplementary Figure S7).

**DISCUSSION**

Here, we report a new mouse model of T-cell lymphoma with Thf-like features and a long latency following proliferation of non-neoplastic Thf-like CD4⁺ T cells. Reduced Tet2 function possibly contributes to perturbed conversion of mC to hmC, an essential intermediary in the demethylation process. Genome-wide methylation analysis of lymphoma cells revealed an abnormal accumulation of mC. Further analysis suggested that one of the important targets for Tfh-like cell outgrowth might be an increase of methylation level at Bcl6 int1-S.

Heterozygous mutations in the Roquin gene, which encodes a protein that binds to and reduces the stability of Icos mRNA, also promotes T-cell lymphoma accompanied by Thf features in mice. Roquin mutations, however, have not been identified in human AITL samples. Given this, our mouse model recapitulates human T-lymphomagenesis more closely than the Roquin model. Nevertheless, other characteristic of human AITL, such as polyclonal hypergamaglobulinemia, proliferation of high endothelial venules, and infiltration of eosinophils and B cells, were not seen. Our model mimics PTCL-NOS with Thf-like features in humans.

Bcl6 has been reported to direct Thf-cell differentiation. Bcl6-deficient T cells fail to develop into Th cells and cannot sustain germal center responses, whereas forced expression of Bcl6 in CD4⁺ T cells promotes expression of Cxcr5 and PD1, which encodes Thf-cell markers. Furthermore, transgenic mice constitutively expressing Bcl6 protein in lymphocytes develop B- and T-cell lymphomas. In this model, lymphoma development required a long latency period, and the incidence was markedly enhanced by administration of N-ethyl-N-nitrosourea (ENU), which is known to induce DNA mutations. In this paper we have shown that TET2/KO mice develop lymphoma with a longer latency, similar to Bcl6 transgenic mice. Thus, impaired Tet2 or enhanced Bcl6 in isolation might induce a premalignant state, but be insufficient to induce lymphoma development in mice. Given that TET2 somatic mutations occur in 5.6% of elderly females with skewed X-inactivation without hematological malignancies, additional mutations may be required for lymphoma development. We recently reported that 38 out of 46 (82.6%) clinical AITL samples had mutations in TET2 throughout the coding region, and that 32 out of the 46 (69.5%) samples had mutations in both TET2 and ras homology family A (RHOA). The RHOA mutations were accumulated in amino-acid position 17. Other groups also reported the high frequency of RHOA mutations in AITL patients. In our mouse lymphoma samples, however, the G17 RHOA mutation was not detected, as well as any other frequently coexisting mutations with TET2 mutations in human hematological malignancies. Genomic evolution of T-cell lymphoma in our mouse model was different from that in humans.

DNA derived from AML samples harboring mutations in IDH1/2, which regulates TET2 function, is hypermethylated. DNA from diffuse large B-cell lymphoma samples harboring TET2 mutations also shows hypermethylation. In contrast, hydroxymethylation status has not been widely assessed in any hematologic malignancies. It is known that hmC is particularly enriched at TSS regions of genes in ES cells, suggesting that hmC functions in transcriptional regulation. The marked hmC reduction at TSS regions of lymphoma cells reported here suggests that impaired TET2 function might alter transcription via reduction in the hmC epigenetic mark, in addition to impaired demethylation.

In summary, we conclude that TET2/KO mice develop T-cell lymphoma with Thf-like features with epigenetic changes. Hypermethylation of Bcl6 int1-S possibly contributes to the upregulation of Bcl6 and outgrowth of Thf-like cells. Demethylating agents, such as azacitidine and decitabine, are reportedly effective for treating MDS. These agents may also possibly target T-cell lymphomas, particularly those that harbor mutations in epigenetic regulators.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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BLOOD CANCER JOURNAL
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