Aging of Preleukemic Thymocytes Drives CpG Island Hypermethylation in T-cell Acute Lymphoblastic Leukemia

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ABSTRACT
Cancer cells display DNA hypermethylation at specific CpG islands in comparison with their normal healthy counterparts, but the mechanism that drives this so-called CpG island methylator phenotype (CIMP) remains poorly understood. Here, we show that CpG island methylation in human T-cell acute lymphoblastic leukemia (T-ALL) mainly occurs at promoters of Polycomb Repressor Complex 2 (PRC2) target genes that are not expressed in normal or malignant T cells and that display a reciprocal association with H3K27me3 binding. In addition, we reveal that this aberrant methylation profile reflects the epigenetic history of T-ALL and is established already in preleukemic, self-renewing thymocytes that precede T-ALL development. Finally, we unexpectedly uncover that this age-related CpG island hypermethylation signature in T-ALL is completely resistant to the FDA-approved hypomethylating agent decitabine. Altogether, we provide conceptual evidence for the involvement of a preleukemic phase characterized by self-renewing thymocytes in the pathogenesis of human T-ALL.

SIGNIFICANCE: We developed a DNA methylation signature that reveals the epigenetic history of thymocytes during T-cell transformation. This human signature was recapitulated by murine self-renewing preleukemic thymocytes that build an age-related CpG island hypermethylation phenotype, providing conceptual evidence for the involvement of a preleukemic phase characterized by self-renewing thymocytes in the pathogenesis of human T-ALL.

INTRODUCTION
During the last decade, aberrant DNA methylation has been identified as a hallmark of human cancer and several studies have highlighted the promising potential of DNA methylation as a clinically or diagnostically relevant biomarker (1). In comparison with their putative normal healthy counterparts, cancer cells generally display DNA hypermethylation at specific CpG islands, but the actual mechanism that drive this so-called CpG island methylator phenotype (CIMP) remains poorly understood (2). Despite the fact that DNA hypomethylating agents are actively used in the clinic for the treatment of some hematologic malignancies, their putative effects on leukemia-specific DNA methylation signatures and gene expression remain unclear (1, 2).

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic cancer for which prognostically relevant CIMP subtypes have also been described (3). Indeed, based on the methylation status of about 1,000 promoter-associated CpG sites, CIMP+ T-ALLs were associated with a better event-free and overall survival as compared to CIMP− leukemias (3). Notably, these findings were recently confirmed in independent pediatric (4) and adult (5) T-ALL cohorts, further reinforcing the idea that aberrant DNA methylation might act as a clinically relevant biomarker in human T-ALL. However, the mechanism driving the CIMP in T-cell leukemia has remained elusive so far.

In this study, we performed a comprehensive DNA methylation analysis of normal and malignant T cells in both human and murine settings in an attempt to increase our understanding on the origin of aberrant DNA methylation in T-ALL.

RESULTS
A CpG Island and Open Sea DNA Methylation Signature in Human T-ALL
Previous studies have shown that molecular genetic subtypes of human T-ALL are associated with an arrest at specific
stages of normal human T-cell differentiation (6, 7). Here, we performed DNA methylation profiling using the 850k EPIC array platform on 109 primary T-ALLs (refs. 8; Supplementary Table S1) and 10 stages of sorted human thymocytes (9, 10), reflecting the normal counterparts of this disease (refs. 6, 7; Supplementary Table S2). Using unsupervised clustering of the 5,000 most variably methylated CpGs, we built a methylation-based signature that we termed COSMe (CpG island and Open Sea Methylation), referring to CpGs located inside CpG islands (n = 2,713), CpGs located outside CpG islands (n = 1,245), and CpGs that flank CpG islands (CpG shores and shelves, n = 1,042; Supplementary Table S3). This COSMe signature divides T-ALLs in two methylation-based categories, that is, COSMe type I and type II (Fig. 1A; Supplementary Table S3), based on three main clusters of CpGs (cluster A, B, and C). The subdivision of T-ALLs in COSMe-I and COSMe-II is dominated by sites located in cluster A, which are mainly located at CpG islands and largely correspond to the CIMP classification that has previously been established in T-ALL (refs. 3; Fig. 1A; Supplementary Table S3). In line with previous reports on the clinical relevance of CIMP status in both pediatric (3, 4) and adult (5) T-ALL, we also observed a significantly higher cumulative incidence of relapse in the CIMP− T-cell leukemias from our cohort treated according to the ALL IC-BFM 2002/2009 protocol (P = 0.04; Supplementary Fig. S1; Supplementary Tables S4 and S5).

At the genetic level, the COSMe subtypes were differentially enriched for genetic defects previously associated with T-ALL biology and CIMP status (refs. 7, 8, 11; Fig. 1A; Supplementary Fig. S2). COSMe-I T-ALLs were significantly enriched for TAL1 rearrangements, whereas COSMe-II T-ALLs mainly consisted of leukemias with aberrant activation of TLX1, TLX3, NKX2.1, or HOXA (Fig. 1A; Supplementary Fig. S2; Supplementary Table S6; ref. 8). In addition, other genetic defects, which have previously been associated with the TAL1 gene expression cluster (7), were also more prevalent in COSMe-I T-ALLs, including 6q deletions (P < 0.01) and PTEN deletions (P = 0.071; Fig. 1A; Supplementary Fig. S2; Supplementary Table S6). In contrast, COSMe-II T-ALLs showed enrichment for genetic aberrations previously associated with double-negative or early-cortical T-ALLs (12, 13), including 5q deletions and loss-of-function alterations targeting WT1, CTCF, PRC2 (EZH2, SUZ12, or EED), as well as leukemias displaying aberrant activation of HOXA genes (Supplementary Fig. S3A and S3B). In contrast, there was a trend for higher prevalence of 6q deletions, loss-of-function alterations targeting PTEN, as well as T-ALLs showing aberrant expression of NKX2.1 in COSMe-II cluster B+ leukemias (Supplementary Fig. S3A and S3B).

Finally, and in contrast to clusters A and B, cluster C CpG methylation was more heterogeneous in normal T cells and both COSMe-I T-ALL subtypes (Fig. 1A).

To further validate these findings, we subsequently profiled an independent cohort of 14 patients with T-ALL and two thymocyte subsets (CD34+ and CD4+CD8+) by EPIC sequencing (EPIC-seq), an alternative, sequencing-based DNA methylation profiling method (Supplementary Fig. S4A–S4C). Clustering using the COSMe signature (Supplementary Table S3) confirmed the presence of COSMe-I and COSMe-II T-ALLs, with TAL1-rearranged T-ALLs being exclusively present in the COSMe-I subtype (Supplementary Fig. S4A). Furthermore, in this series, COSMe-II T-ALLs consisted of immature ETP-ALL as well as TLX1+, TLX3+, NKX2.1+, or HOXA+ leukemias. In addition, within COSMe-II T-ALLs, the lowest levels of cluster B methylation were also present in immature ETP-ALLs and a HOXA+ T-ALL (Supplementary Fig. S4A).

Altogether, we here show robust COSMe classification as a more elaborate version of the previously established CIMP classification in T-ALL, using different DNA profiling methods and two independent leukemia patient cohorts.

Figure 1. DNA methylation profiling in normal and malignant T cells. A, Unsupervised clustering of mean-centered methylation score ($\beta$ values) of the 5,000 most variably methylated CpGs in 109 T-ALL cases and 10 subsets of developing thymocytes (two biological replicates each), with indication of the three main clusters of CpG probes and their location with respect to CpG context. T-ALL subtypes and genetic defects of all T-ALL cases are indicated below the heatmap. Mean $\beta$ values of cluster A (B) and cluster C (A) in a T-ALL cohort (GSE110637) plotted separately for TAL1-rearranged T-ALLs (enriched in COSMe-I) and T-ALLs without such rearrangements (enriched in COSMe-II). Normal thymocytes (GSE151079) were included as healthy controls. Genes in the NODCH1 pathway were used as positive reference value for T-ALL F, ChiP-seq enrichment in clusters A, B, and C for HK277me3 in CD34+ HSPCs (GSM2277818) and in the JURKAT T-ALL cell line (GSM2279072) and for PU.1 in CD34+ HSPCs (GSM1816090) and the TALL-1 cell line transfected with FLAG-PU.1 (GSE128837). Data publicly available from the Gene Expression Omnibus. C, COSMe signature in paired primary-relapsed T-ALL samples (21). Row-scaled $\beta$ values of CpG methylation are shown.
DNA Methylation Uncovers the Premalignant History of T-ALL

A

Cluster A

Cluster B

Cluster C

T-ALL subtypes

Genetic defects

Genetic defects

Alteration present

Mutation present

No alteration present

No information available

B

Cluster A CpG sites

DNAm ($\beta$ value)

C

Cluster B CpG sites

DNAm ($\beta$ value)

D

CpG position in genome

Cluster A

Cluster B

Cluster C

Percentage

0

50

100

Promoter

Exon

Intron

UTR

Intergenic

E

Gene expression

Human T-ALLs

F

H3K27me3

PU.1

HSPCs JURKAT

HSPCs TALL-1

G

Status

Patient

Status

Primary T-ALL

Relapsed T-ALL

COSMe

Cluster A

Cluster B

Cluster C

CpG relation

CpG islands

Open Sea CpGs

Flanking CpGs

Distance (Kb)

CpG

CpG

CpG

CpG

-2.5

0.0

2.5

-2.5

0.0

2.5

HSPCs
Characterization of CpG Clusters that Define COSMe in T-ALL

To understand which genes or regulatory pathways are potentially affected by COSMe methylation in T-ALL, we performed enrichment (14) and gene expression analysis (15, 16) of transcripts associated with the CpG clusters mentioned above. Of note, cluster A sites were mostly located in gene promoters, while cluster B and C CpG sites were mostly located in gene bodies or intergenic regions (Fig. 1D). Cluster A CpG sites were enriched for promoters of PRC2 targets (Supplementary Fig. S5), which uniformly showed very low expression in T-ALLs as well as normal thymocytes (Fig. 1E). Using publicly available chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq) data, we confirmed the presence of the repressive H3K27me3 histone mark at these cluster A CpG sites in both human CD34+ hematopoietic stem/precursor cells (HSPC) and the T-ALL cell line JURKAT (ref. 17, Fig. 1F).

Notably, cluster B CpG sites did not show enrichment for repressive histone modifications (Supplementary Fig. S5) and were associated with genes higher expressed in T-ALL compared with normal developing T cells (Fig. 1E). Cluster B sites showed significant enrichment for PU.1 (SPI1) binding motifs (Supplementary Fig. S5), which corresponded to specific binding of PU.1 (18, 19) at these exact loci in CD34+ HSPCs and the Flag-tag PU.1–transfected T-ALL cell line TALL-1 (ref. 20; Fig. 1F). Of note, during normal T-cell differentiation, we observed a significant inverse correlation between cluster B PU.1–binding site methylation and SPI1 (which encodes PU.1) expression (Supplementary Fig. S6).

Cluster C CpG sites, characterized by a heterogeneous pattern of DNA methylation, also showed higher expression in T-ALL as compared with normal T cells (Fig. 1E). Cluster C sites showed significant enrichment for genes specifically expressed in T-ALL cell lines, which was not the case for cluster A- or cluster B–associated transcripts (Supplementary Fig. S7A–S7C). More specifically, cluster C sites include CpGs associated with genes known to be involved in T-ALL disease and/or normal T-cell differentiation, such as TLX3, LCK, CDKN2B, BCL2L11, MEF2C, BCL11B, RAG1, RAG2, CD1, CD28, and the TCR loci (Supplementary Table S3). Thus, cluster C CpG sites are enriched near genes with known roles in normal and malignant T-cell development.

Finally, to evaluate the progression of COSMe methylation from diagnosis to relapse, we investigated paired primary and relapsed T-ALL cases that were previously profiled by EPIC arrays (21). Out of 9 patients analyzed, we found that 6 patients were classified as COSMe-I and 3 patients as COSMe-II at diagnosis (Fig. 1G). Patients at relapse still clustered together with the corresponding primary samples, suggesting that COSMe status is largely conserved from diagnosis to relapse (Fig. 1G). Nevertheless, patients with COSMe-I did show a significant increase in cluster A methylation from diagnosis to relapse (Supplementary Fig. S8).

Reciprocal DNA Methylation and H3K27me3 Association in COSMe-I and COSMe-II T-ALLs

As described above, COSMe cluster A CpG sites are located in the promoters of PRC2 target genes that uniformly show low expression across all genetic subtypes of human T-ALL. PRC2 is a methyltransferase that primarily produces H3K27me3, a mark of transcriptionally silent chromatin. To validate the enrichment for PRC2 targets at cluster A CpGs, we profiled H3K27me3 in 3 COSMe-I and 3 COSMe-II human T-ALLs using ChIPmentation (22).

Remarkably, at cluster A sites, H3K27me3 was found more abundant in COSMe-I T-ALLs, which have lower levels of DNA methylation at these sites, as compared with COSMe-II T-ALLs (Fig. 2A). To further study this apparent anticorrelation between DNA methylation and H3K27me3, a differential analysis was conducted comparing H3K27me3 of COSMe-II with COSMe-I T-ALLs (Fig. 2B). This revealed that the majority of differential regions (88%) had significantly lower levels of H3K27me3 in COSMe-II compared with COSMe-I T-ALLs (Fig. 2B), as also shown in a genome browser view for the genes ESRRG and DKK2 as representative examples (Supplementary Fig. S9). Genes with differential H3K27me3 were generally low but not differentially expressed between different T-ALL subtypes (Supplementary Fig. S10). Forty-four percent of all genes with differential H3K27me3 in this comparison (Fig. 2B) were also present in cluster A, underlining the reciprocal association between DNA methylation and H3K27me3 at these sites. High levels of cluster A H3K27me3 also corresponded to low levels of cluster A DNA methylation in 4 patients for which paired EPICseq and H3K27me3 profiles were available (Fig. 2C). In line with this, core components of the PRC2 complex showed higher expression in TAL1-rearranged T-ALLs (EZH2, EED, $p_{\text{adj}} < 0.05$, SUZ12 not significant; Supplementary Fig. S11), which are most often COSMe-I T-ALLs displaying low levels of cluster A methylation. In contrast, deletions of PRC2 members were significantly more prevalent in COSMe-II T-ALLs, whereas amplifications were exclusively found in COSMe-I T-ALLs (Supplementary Fig. S2).

Finally, from all differential H3K27me3 regions, those that overlapped with CpG islands did show a differential DNA methylation pattern (111 regions, 50%), whereas the methylation of Open Sea sites was not affected by the presence of differential H3K27me3 (Fig. 2D).

Altogether, these data collectively show a reciprocal association between DNA methylation and H3K27me3 levels at cluster A CpG islands in human T-ALL.

Cluster A CpG Island Methylation Defines the Proliferative History of Human T-ALL

Cluster A CpG sites are mainly located in promoters of PRC2 target genes that are differentially covered by H3K27me3 between COSMe-I and II T-ALL subtypes. Interestingly, DNA hypermethylation at PRC2-enriched CpG sites has previously been associated with increased age and proliferative history in both hematopoietic stem cells and T-ALL (11, 23). Given this, we predicted the mitotic age of our T-ALL patient cohort using the Epigenetic Timer of Cancer (Epitoc; ref. 24) and this, we predicted the mitotic age of our T-ALL patient cohort using the Epigenetic Timer of Cancer (Epitoc; ref. 24) and identified a strong and significant correlation with the level of cluster A methylation, which was not observed for cluster B or cluster C (Fig. 3A).

Furthermore, using the Horvath (25) age predictor, which employs methylation of a set of CpGs to predict a person’s actual age in years, an increase in epigenetic age of primary T-ALL samples was observed in comparison with the
actual age at diagnosis (Fig. 3B; Supplementary Table S7). A significant interaction between the patient’s age and the Horvath age was indeed observed (t test, \( P < 2e-16 \)). However, although the age at diagnosis was already significantly different between COSMe-I and -II T-ALL patients (Fig. 3C; \( P = 0.02506 \)), the predicted Horvath and Epitoc age showed a much better segregation between the two T-ALL subtypes (Fig. 3B and C; \( P < 2.2e-16 \) and 3.85e-16). Thus, cluster A methylation defines the proliferative history of T-ALL, with COSMe type I having a shorter history of proliferation in comparison with COSMe type II T-ALL.

Proliferation during the time frame from primary diagnosis to relapsed T-ALL disease should also lead to an increase in mitotic age. Indeed, a significant increase in Epitoc age was also observed by comparing 9 paired diagnosis and relapse samples (ref. 21; Fig. 3D). However, the increase in age and methylation was considerably more pronounced in COSMe-I T-ALLs (\( \Delta \text{Epitoc} 0.11, P = 0.0020 \)) as compared with COSMe-II
T-ALLs (ΔEpitoc = 0.015, P = 0.0522), yet there was no significant difference between the time of relapse between both entities (Supplementary Table S8; P = 0.2619). From this limited analysis (21), COSMe-I T-ALLs seem to proliferate at a faster pace than COSMe-II T-ALLs between diagnosis and relapse.

**Proliferation of Preleukemic Thymocytes Drives the Aging CpG Island Methylation Signature in T-ALL**

As shown above, cluster A CpG island methylation positively correlates with the proliferative history and mitotic age of human T-ALL cells. To investigate whether two distinct trajectories toward T-ALL development might underlie the observed discrepancy in epigenetic age between COSMe-I and COSMe-II T-ALLs (Fig. 3B and C), we used two known T-ALL mouse models that might recapitulate these features.

First, we investigated whether CD2-Lmo2 transgenic (CD2-Lmo2tg) mice (26) could function as a model for COSMe-II T-ALLs. This in vivo T-ALL mouse model has a long disease latency and an immature T-ALL phenotype reminiscent of T-ALLs inside the COSMe-II subgroup. In CD2-Lmo2tg mice, a long-term self-renewing thymocyte population has been observed many months before tumor development. In contrast to wild-type (WT) control mice, in which the thymus is continuously replenished by progenitor cells from the bone marrow, these preleukemic CD2-Lmo2tg thymocytes are...
self-sustaining from young age. Therefore, CD2-Lmo2\(\text{tg}\) thymocytes should undergo a gradual aging process in the months prior to malignant transformation.

Second, we investigated whether Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) mice could function as a model for COSMe-I T-ALL. This mouse T-ALL model has a short disease latency and results in the development of more mature murine T-cell leukemias. In addition, PTEN mutations and deletions are also more often observed in human COSMe-I T-ALLs.

To study this, we isolated full thymus from CD2-Lmo2\(\text{tg}\) and Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) mice and littermate controls at different time points before and after leukemia development and performed DNA methylation profiling by reduced representation bisulfite sequencing (RRBS; \(n = 4\) biological replicates per condition). The most variably methylated CpGs between CD2-Lmo2\(\text{tg}\) and Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) thymocytes and blasts (Supplementary Table 59) were more often situated in CpG islands (Fig. 4A, cluster 1) than in Open Sea–enriched regions (Fig. 4A, clusters 2 and 3). The CpG methylation in cluster 1 was highly increased in preleukemic and leukemic CD2-Lmo2\(\text{tg}\) mice, but not in Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) mice isolated before leukemia development. In leukemic Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) mice, cluster 1 methylation was only moderately increased compared with CD2-Lmo2\(\text{tg}\) samples at the same stage of disease manifestation.

Notably, cluster 1 CpGs were mostly situated in promoter regions (Fig. 4B) of lowly expressed genes (Fig. 4C) and displayed enrichment for PRC2 target genes and H3K27me3 (27, 28) at these sites (Supplementary Fig. S12; Fig. 4D), thus showing similarity with the hypermethylation phenotype of T-ALLs in the human cluster A. In contrast, we did not find any evidence in these mouse models for potential overlap between murine clusters 2 or 3 and the human COSMe cluster B.

To further confirm similarities between murine cluster 1 and human cluster A, we subsequently looked at cross-species overlap at the gene level. Notably, 726 of 2,248 CpG sites in mouse cluster 1 overlapped with human gene orthologs in cluster A (overlap significant at \(P < 0.0001\), exact hypergeometric probability). These sites, which we termed cluster A\(^{\text{mm}}\) sites (Supplementary Table S10), were equally significantly enriched for PRC2 target genes (\(P_{\text{adj}} < 0.0001\)).

Cluster A\(^{\text{mm}}\) regions showed a gradual increase in methylation with aging in CD2-Lmo2\(\text{tg}\) thymocytes but remained constant in corresponding WT mice from 8, 16, and 24 weeks old (Fig. 4E). Already in 8-week-old CD2-Lmo2\(\text{tg}\) thymocytes, a strong increase in methylation could be observed compared with WT control cells of the same age. In contrast, in Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) mice at 8 weeks, no difference in cluster A\(^{\text{mm}}\) methylation was detected. We found that cluster A\(^{\text{mm}}\) methylation did not further increase in fully transformed CD2-Lmo2\(\text{tg}\) leukemia, sacrificed on average at 35.75 ± 8.28 weeks, compared with preleukemic CD2-Lmo2\(\text{tg}\) at 24 weeks (Fig. 4E), whereas only a moderate increase in cluster A\(^{\text{mm}}\) methylation was detected in leukemic Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) mice (sacrificed on average at 18 ± 3.83 weeks). Finally, the epigenetic age, calculated using the mouse-specific calculation method of Petkovich and colleagues (29), increased over time in preleukemic thymocytes of the CD2-Lmo2\(\text{tg}\) mice, which was not the case in the Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) model (Fig. 4F).

Thus, preleukemic, self-renewing thymocytes display a murine CpG island DNA hypermethylation signature that recapitulates features of the CpG island hypermethylation phenotype that we observed in human COSMe-II T-ALL. Therefore, CD2-Lmo2\(\text{tg}\) might serve as a bona fide model for COSMe-II T-ALL development. In contrast, this CpG island hypermethylation phenotype was not observed in preleukemic thymocytes of Lck-Cre\(^{e6+/}\)Pten\(^{fl/fl}\) mice, suggesting that this murine model is more similar to COSMe-I human T-ALL.

Furthermore, based on these results, COSMe-I and COSMe-II T-ALLs might have followed a different trajectory toward leukemia, marked by the absence or presence of a thymic self-renewing population that preceded leukemia development.

**Age-Related CpG Island Hypermethylation Is Resistant to the FDA-Approved Hypomethylating Agent Decitabine**

DNA hypomethylating agents such as azacitidine and decitabine are approved for myelodysplastic syndrome and acute myeloid leukemia (30). Interestingly, few publications have also investigated the possible use of DNA hypomethylating agents for the treatment of human T-ALL. One ETP-ALL and T-ALL patient responded to decitabine and achieved complete response in a phase I clinical trial (31). In addition, durable remissions have been reported for few other cases of (early) T-cell precursor ALL treated with decitabine as monotherapy (32–34) or in combination with the BCL-2 inhibitor venetoclax (35, 36). The largely perturbed DNA methylation profile observed in T-ALL further supports the rationale of using DNA hypomethylating agents for the treatment of this disease. However, the actual mechanism of action that could explain the antileukemic properties of these hypomethylating agents and their putative effect on aberrant DNA methylation in T-ALL, as exemplified by the COSMe phenotype, has remained largely unclear.

To address these questions, we first evaluated the antileukemic properties of decitabine in a preclinical setting using four different primary human T-ALL patient-derived xenograft (PDX) models with variable genetic backgrounds (Supplementary Table S11A–S11H). Notably, 10 days of decitabine (5 days on, 2 days off, 0.5 mg/kg) significantly improved leukemia-free survival for all PDX samples analyzed, including a sample that originated from a STIL-TAL1-mature T-ALL at second relapse (Fig. 5A). As expected, this improved survival coincided with a significant decrease in blast percentage in the peripheral blood, as shown for one PDX T-ALL (Supplementary Fig. S13).

We selected two PDX T-ALLs for DNA methylation profiling representative for a COSMe-I and a COSMe-II T-ALL based on the genetics (PDX#1 and PDX#2). For this, 7 days after start of the treatment, leukemic blasts from control and decitabine-treated animals - (0.5 mg/kg, \(n = 3\) each group) were collected from the spleen. We performed differential methylation analysis comparing decitabine treatment with vehicle control for each PDX in vivo. As expected, a very large number of regions showed a significant decrease in DNA methylation, but a substantial portion of CpGs also displayed an increase in methylation, especially those located in CpG islands (Fig. 5B; DESeq2 \(P_{\text{adj}} < 0.05\)). Despite these global changes in DNA methylation, decitabine did not drastically affect DNA methylation.
A. Mean-centered methylation scores (β values) of the most variably methylated CpGs in RRBS profiling of aging (8, 16, and 24 weeks) preleukemic and fully transformed CD2-Lmo2tg mice (Tg), 8-week-old and fully transformed Lck-CreTg/+Ptenfl/fl mice (KO), and littermate controls (WT), with indication of three main subclusters and their relation to CpG context. Data from 4 mice per condition.

B. Percentage of CpGs located in different genomic categories for each of the clusters defined in A. UTR, untranslated region.

C. Per sample mean expression of genes in the clusters defined in A shown in CD2-Lmo2tg mice (Limma log 2 normalized counts, microarray data, GSE49164) or in Lck-CreTg/+Ptenfl/fl (FPKM RNA sequencing, GSE115346). Notch1 pathway genes are shown as positive reference values.

D. ChIP-seq of H3K27me3 in murine clusters 1 to 3 (A) in CD34+ HSPCs (GEO: GSM4067369) and mouse T-ALL (GEO: GSM4067369).

E. Methylation at 726 cluster 1 CpGs (defined above in A) that overlap with genes in COSMe cluster A (defined in Fig. 1A, now termed cluster Aimm sites) in aging preleukemic and transformed CD2-Lmo2tg, Lck-CreTg/+Ptenfl/fl, and control mice.

F. Relative epigenetic age estimation for (pre)leukemic thymocytes from CD2-Lmo2tg, Lck-CreTg/+Ptenfl/fl, and control mice.
Figure 5. DNA methylation profiling of T-ALL PDXs treated with decitabine in vivo. A, Mice engrafted with T-ALL patient samples were treated two cycles with vehicle or decitabine (DAC, 0.5 mg/kg body weight) for 5 consecutive days followed by 2 days off and followed for survival analysis. Kaplan–Meier analysis of leukemia-free survival is shown (log-rank Mantel–Cox test) for each of the 4 PDX samples analyzed. B, Mice engrafted with T-ALL patient samples were treated with vehicle or decitabine (0.5 mg/kg body weight) for 5 consecutive days. Seven days after treatment initiation, leukemic blasts from control and decitabine-treated animals (n = 3 each group) were collected from the spleen and used for DNA methylation profiling with EPIC arrays. Number of significantly differentially methylated CpGs is shown for each PDX comparing decitabine-treated with vehicle-treated samples, with respect to their location inside different genomic categories. Red bars indicate more methylation after decitabine treatment, whereas blue bars mark a decrease in methylation upon treatment. CpGI, CpG island; DNAm, DNA methylation. C, β values of COSMe CpGs in PDX#1 and PDX#2 T-ALLs with decitabine or vehicle treatment. Methylation at the 5,000 COSMe CpG sites was used to determine COSMe-I and COSMe-II T-ALL subtypes. D, RNA-seq of samples described in A. Volcano plots show log₂ fold change of protein-coding gene expression upon decitabine treatment for PDX T-ALL#1 and PDX T-ALL#2. Genes with FDR < 0.05 are represented in blue (Deseq2). E, Preranked GSEA of PDX#1 and PDX#2 in decitabine versus control conditions.
in the 5,000 human COSMe CpGs, neither in PDX#1, which we determined to be a COSMe-type I cluster B* T-ALL, nor in the COSMe type II cluster B* PDX#2 (Fig. 5C).

To obtain additional insights in the mechanism of action of hypomethylating agents in T-ALL, we used the same samples (PDX T-ALL#1 and #2, 5 days, 0.5 mg/kg, n = 3 each group) for RNA sequencing (RNA-seq). Decitabine treatment resulted in significant differential expression of 456 (PDX T-ALL#1) and 886 (PDX T-ALL#2) protein-coding genes (Deseq2, Padj < 0.05; Supplementary Table S12A and S12B). Of these differentially expressed genes, 361 (PDX#1) and 348 (PDX#2) genes also showed a significant decrease in methylation at one or more CpGs (Supplementary Table S13A and S13B). However, the transcriptome is less disturbed than one would expect, as decitabine induced profound genome-wide DNA hypomethylation, which globally followed CpG density (Supplementary Fig. S14A and S14B). The genes downregulated by decitabine more frequently had CpG islands in their promoters (57%) compared with upregulated or randomly selected genes (both 23%, χ² test, P < 0.00001).

As decitabine did not revert the COSMe methylation signature, we further looked at the gene expression changes induced by this hypomethylating agent to explain its anti-leukemic properties. Both PDX samples showed significant upregulation of tumor suppressor genes (TSG) upon decitabine treatment, including BCL2L11 (BIM), BBC3 (PUMA), and BMF (Supplementary Table S12A and S12B). In addition, multiple known T-ALL oncogenes were significantly downregulated by in vivo decitabine treatment, including MYC, HES1, and BCL2 in PDX#1 and NOTCH3, GFI1, IL7R, CISH, MYB, and TOX in PDX#2 (Fig. 5D). Enrichment analysis revealed that decitabine induced a global downregulation of MYC target genes in both PDX samples, as exemplified by pre-ranked gene set enrichment analysis (GSEA; ref. 37; Fig. 5E; Supplementary Table S14). To further validate these findings, we performed additional RNA-seq on five human T-ALL cell lines (LOUCY, PER117, PEER, MOLT16, and TALL-1) following in vitro decitabine treatment (1 μmol/L, 48 hours). The differentially expressed genes from both PDX samples significantly overlapped with the differential data from the cell lines (33% in PDX#1 and 35% in PDX#2). Indeed, also in these cell lines, significant upregulation of TSGs like BMF and BBC3 (PUMA) and coordinate downregulation of the MYC pathway upon treatment with decitabine were observed (Supplementary Fig. S15A and S15B; Supplementary Table S15A–S15F).

Several studies showed that DNA methylation can alter the binding of CTCF to influence the three-dimensional architecture of the genome (38–40); therefore, we compared the genes downregulated by decitabine in both PDX and T-ALL cell lines with gene expression in CTCF-depleted acute leukemia (41). Interestingly, we observed a very significant enrichment for downregulated genes in both datasets by GSEA (Supplementary Fig. S16), providing a potential mechanism for decitabine-induced downregulation of the MYC pathway in human T-ALL.

**DISCUSSION**

In this study, we directly compared the genome-wide DNA methylation landscape in human T-ALL with human thymocytes covering the complete trajectory of normal T-cell development. This simultaneous DNA methylation analysis of T-cell leukemias with their putative cell of origin empowered us to distinguish cell-of-origin methylation profiles from leukemia-specific alterations at CpG islands and Open Sea sites. Integration of DNA methylation profiles with gene expression signatures and ChIP-seq allowed us to obtain novel insights in the functional relevance of differentially methylated CpG sites between normal and malignant T cells, and to distinguish two trajectories toward T-ALL development.

We built a DNA methylation–based signature for T-ALL, termed COSMe. COSMe comprises three clusters that can divide T-ALLs in two subgroups, COSMe type I and COSMe type II, which both have unique characteristics, as summarized in Table 1. First, COSMe cluster A sites were closely related to the CpG sites that have previously been used to determine CIMP status in T-ALL (42) and showed low expression in both normal developing thymocytes and human T-ALLs. An inverse correlation between cluster A DNA methylation and H3K27me3 was observed. This interplay could explain the low expression of cluster A genes across all genetic subtypes of human T-ALLs, as repression
is maintained by different mechanisms in COSMe-I (PRC2-mediated repression) and COSMe-II T-ALLs (DNA methylation-mediated repression). This phenomenon has previously been observed in murine Ezh2 knockout ETP-ALL (43) that displayed increased DNA methylation. In ETP-ALL, EZH2 inactivating events were previously linked to oncogenic active stem and progenitor cell genes (44). Also, in our cohort, inactivating alterations targeting PRC2 members were more prevalent in COSMe-II T-ALL. However, the relation between PRC2 and DNA methylation is complex, as PRC2 has been shown to recruit both DNMTs (45) and TET enzymes (46). Therefore, PRC2 might potentially affect both methylation as well as demethylation, depending on the cellular context. However, the mechanism and functional relevance of these different levels of H3K27me3 between human COSMe-I and COSMe-II T-ALLs remains to be established. In addition, additional studies will be required to further unravel how these epigenetic alterations exactly create a permissive landscape for T-ALL transformation or if they are just the result of specific cell-intrinsic antitumor mechanisms.

Next, our study linked DNA hypermethylation in COSMe cluster A to the proliferative history of the cancer cells, thereby confirming (11) that COSMe-II T-ALLs are epigenetically older and displayed a longer mitotic history as compared with COSMe-I T-ALL. In line with this, we showed that relapsed COSMe-I T-ALLs displayed a larger increase in epigenetic age during their progression from diagnosis to relapsed disease. The younger mitotic age in combination with a faster rate of cellular proliferation might potentially contribute to the more aggressive nature of COSMe-I T-ALLs as compared with COSMe-II leukemias.

Furthermore, we could recapitulate these methylation features distinguishing fast and aggressive COSMe-I from the slower-transforming COSMe-II T-ALLs in two distinct T-ALL mouse models: the spontaneous CD2-Lmo2δtg/δfl/fl mouse model, which mimics immature T-ALL development and has a long disease latency, and the fast-transforming Lck-Creδtg/δfl/fl and CD2-Lmo2δtg mice, as a model of more mature human T-ALL, where PTEN deletions are more abundant. Notably, these findings establish Lck-Creδtg/δfl/fl and CD2-Lmo2δtg as models to further study COSMe-I and COSMe-II T-ALL cluster A methylation in vivo, but also provide evidence that a preleukemic self-renewing thymocyte population might also exist in human COSMe-II T-ALL. Indeed, in CD2-Lmο2δtg mice, but not in Lck-Creδtg/δfl mice, a preleukemic state has been described in which thymic precursors gain self-renewing potential prior to full malignant T-cell transformation. The preleukemic cells gain DNA methylation at these sites before transformation from 8 to 24 weeks, which was not observed in the corresponding aging thymocytes of Lck-Creδtg/δfl mice or littermate controls. The methylation at these sites did not further increase after full transformation of the preleukemic cells, suggesting that aberrant methylation at these sites is mainly derived from the preleukemic history of these tumor cells.

Besides cluster A CpG island methylation as a surrogate marker for the epigenetic age and replicative history of tumor cells, cluster B sites enabled additional classification of COSMe type-II T-ALL into two categories based on the methylation level of specific Open Sea–enriched CpG sites. Interestingly, these cluster B loci displayed significant enrichment for the binding motif of PU.1, a transcription factor critically involved in early T-cell development (47).

Of note, we could show that these cluster B sites are able to distinguish immature from more mature T-ALLs within the COSMe-II leukemias. Furthermore, immunophenotypically validated ETP-ALLs, which are derived from the most immature T-cell precursors, also showed the lowest levels of cluster B methylation in the validation cohort. Whereas conclusive immunophenotypic data were missing to accurately define ETP-ALLs in the initial T-ALL cohort, these data do collectively suggest that both immature T-ALL and ETP-ALL could be distinguished from other T-ALLs based on the absence of cluster B methylation within COSMe-II leukemias.

Notably, a similar correlation between PU.1-binding site methylation and SPI1 expression was recently also identified in TCF7–SPI1 fusion–positive T-ALLs (4). However, the lack of cluster B methylation in this very aggressive subtype of PU.1-rearranged human T-ALL (4) is most probably caused by the aberrant SPI1 expression downstream of the fusion proto-oncogene rather than being associated with its cell of origin.

Finally, cluster C CpG sites were also enriched for Open Sea sites but showed more heterogeneity in DNA methylation between T-ALLs. Cluster C–associated transcripts showed generally lower expression in normal T-cell subsets but were clearly active in at least some T-ALL tumors and included genes involved in T-ALL disease biology (48) as well as normal T-cell differentiation. Thus, the methylation pattern at some of these cluster C Open Sea CpG sites might be T-ALL specific and potentially hold some information on the genetic abnormalities present in each individual T-ALL.

In the last part of our work, we looked for further evidence for DNA hypomethylating agents as a promising therapeutic strategy for human T-ALL. Indeed, some case reports have previously shown that the FDA-approved drug decitabine might be effective as salvage therapy for T-ALL (31) and ETP-ALL (32–34). In line with this, decitabine here displayed profound antileukemic properties in PDXs from a variety of different human T-ALLs obtained from both primary as well as relapse tumor material; however, and most unexpectedly, these antitumoral effects, which were shown to be mediated by downregulating oncogenic MYC signaling, were observed in both COSMe-I and COSMe-II samples. Indeed, decitabine did not revert the age-related human CpG island hypermethylation phenotype in vivo. Instead, this hypomethylating agent triggered a profound and genome-wide hypomethylation effect on CpGs located in Open Sea areas, and surprisingly also hypermethylation in CpG islands. The widely altered DNA methylation profile has only limited effects on the transcriptome of these cells. Decitabine-downregulated genes are reconciled by CTCF depletion, thus DNA hypomethylating agents might alter the binding capacity of CTCF as a potential mechanism to induce changes at the transcriptomic level. Several studies also show a direct link between MYC and DNMTs (49). MYC inactivation has been shown to reduce the expression of DNMT3B in T-ALL (50), and reciprocally, reduced expression of DNMT3B resulted in reduced proliferation and tumor maintenance, reflecting the effects observed
in our decitabine-treated PDX T-ALLs. Thus, downregulation of MYC or inhibition of DNMTs by decitabine might converge on counteracting T-ALL disease burden.

Altogether, our work identifies aging of preleukemic thymocytes as a driver of the CIMP in human T-ALL, revealing different trajectories toward T-cell transformation. Our work provides evidence for the involvement of preleukemic thymocytes in the pathogenesis of human T-ALL, which has previously only been reported in mouse T-ALL models so far. In addition, we provide a biological explanation for the profound differences in epigenetic age between COSMe-I and COSMe-II T-ALLs and show that the FDA-approved hypomethylation drug decitabine shows a promising increase in survival of both epigenetically young and old T-ALLs, but fails to revert age-related CpG island hypermethylation in human T-ALL xenografts. This very extensive DNA methylome dataset of murine and human T-ALL will be of importance to further increase our understanding of T-ALL disease biology, which could ultimately result in better treatment stratification and the development of novel and less toxic therapeutic strategies for the treatment of this aggressive hematologic malignancy.

METHODS

Patient Samples, Normal T Cells, and Cell Lines

DNA from 109 T-ALLs was collected from the previously characterized (8, 51) ALL IC-BFM 2002/2009 protocol. Exome sequencing was performed by Novogene. Clinical characteristics of patients treated according to both treatment regimens were not significantly different ($\chi^2$ test).

Thymocytes were isolated from postnatal thymus suspension of two donors each, as described previously (9, 16).

Cell lines were purchased from DSMZ and cultured in RPMI1640 medium (Life Technologies) supplemented with 10% or 20% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies), and 2 mmol/L L-glutamine (Life Technologies) at 37°C with 5% CO₂. CUTTL-1 and PER-117 were a kind gift from Adolfo Ferrando (Columbia University, New York, NY) and Rishi Kotecha (Telethon Kids Cancer Center, Perth, Western Australia), respectively. Cell lines were screened monthly for Mycoplasma contamination and were consistently negative.

All human samples were acquired with written informed consent according to the Declaration of Helsinki, and the studies were approved by the ethical committee review board of the Department of Pediatric Hemato-Oncology at Ghent University Hospital (Ghent, Belgium).

DNA Methylation Profiling

Human DNA methylation analysis of 109 T-ALLs was done with the Infinium HumanMethylationEPIC BeadChip array ( Illumina). DNA (250 ng) was used for bisulfite conversion by the EZ DNA Methylation Kit (Zymo Research).

DNA methylation profiling of the 14 human T-ALL validation cohort was done by TruSeq Methyl Capture EPIC-seq using 500 ng of DNA. Sequencing was done on the HiSeq3000 (PE150). Reads were trimmed using TrimGalore (v0.4.5) and aligned with Bismark (ref. 52; v0.20.0) on GRCm38.

RRBS was done on thymus isolated from CD2-Lmo2tg and Lck-CrePten−/− mice and littermate controls (n=4 mice per condition). DNA was isolated from full thymus using the QIAamp DNA Mini Kit (Qiagen). RRBS was done using MSPI digest. Bisulfite-converted DNA libraries were sequenced on Illumina NextSeq500 using the NextSeq 500/550 High Output v2 kit (SE75). Reads were trimmed using TrimGalore (v0.4.5) with -rrbs and –nondirectional and aligned with Bismark (ref. 52; v0.20.0) on GRCm38.

Illumina EPIC array and RRBS data were analyzed using RnBeads. hg19 or RnBeads.mm10, respectively (53) in R (versions >3.4). Beta values were obtained after filtering and normalization using the default preprocessing pipeline. EPIC-seq was analyzed using the package “methylkit” in R. Annotation of CpGs to the closest gene was done by ChipPeakAnno (54). Copy number variations were called from EPIC array data using the R packages “minfi” and “conumee.”

H3K27me3 Profiling

Fifty to 100,000 CD45⁺ sorted cells were fixed using 1% formaldehyde (Thermo Fisher Scientific 28906) and quenched by glycin (125 mmol/L final). Next, cell pellets were lysed in 100 µL of short-term complete lysis buffer [50 mmol/L Tris-HCl pH 8.0, 10 mmol/L EDTA, 0.25% SDS, 20 mmol/L NaBu histone deacetylase inhibitor, 1X complete protease inhibitors cocktail EDTA free (Roche, 5056489001)]. Chromatin was sheared on the Bioruptor Pico (Diagenode) using a 15 seconds–on/30 seconds–off, 7-cycle regimen. Sheared chromatin was magnetically immunoprecipitated and tagmented using the Auto ChiPmentation Kit for Histones (Diagenode, C01011010) on the IP-Star Compact Automated System (Diagenode, B03000002), according to the manufacturer’s instructions. Input DNA was decrosslinked, purified (MiniElute, Qiagen), and tagmented (Nextera DNA Library Prep, Illumina). Stripping, end repair, and library amplification were performed according to the ChiPmentation Kit guidelines. Libraries were sequenced with the NextSeq500 (SR75, High Output). Reads were trimmed by Trimmomatic and aligned to hg38 with Bowtie2 using the parameters -N 1 -k 1. Peaks were called with MACS2 with the respective input control for each patient sample.

Gene Expression Profiling

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen) and evaluated on the Agilent 2100 bioanalyzer (Agilent Technologies). Library preparation was performed using QuantSeq 3’mRNA-Seq FWD for Illumina (LEXOGEN). CDNA libraries were sequenced as described above. Reads were aligned to GRCh38 using STAR2.4.2a (55) and quantified on Gencode v24.

For visualization of RNA-seq data, EdgeR log₂-transformed normalized counts per million were plotted unless mentioned otherwise. Microarray data normalized with Limma and was log₂ transformed.

In Vivo Treatment of Xenografts

PDXs were established in female NOD/SCID γ (NSG) mice. For the initial PDX experiments, upon disease establishment, human leukemic cells were isolated from the spleen. Secondary injections were performed in randomized NSG mice (two groups of 5) and treated two cycles (5 days on, 2 days off) with vehicle-only or decitabine (0.5 mg/kg body weight).

For RNA and DNA collection, mice were randomized in two groups of 3 and treated for 5 days with vehicle only or decitabine (0.5 mg/kg body weight). At day 7, animals were sacrificed and tumor cells collected from the spleen.

The animal welfare ethical committee (Ghent University Hospital) approved all animal experiments.

CIMP Classification

CIMP CpGs (1,099) were defined by filtering (3) CIMP probes to exclude CpGs within five base pairs from European SNPs, cross-hybridizing probes, repeated regions (56), and methylation quantitative trait locus (57). Missing values were imputed using K nearest neighbor.
**COSMe Classification**

Five-thousand human COSMe CpGs were defined by most variably methylated CpGs in all normal thymocyte subsets and 109 T-ALLs. The classification of diagnosis and relapsed T-ALLs was obtained by hierarchical clustering of these 5,000 CpGs with Euclidean distance measures and clustering method “ward.D” using row scaling.

**Epigenetic Age Calculations**

The Horvath (25) and Epitoc (24) age were calculated with “cageR.” Epigenetic age in mouse samples was estimated by taking the weighted average of CpGs within the 90 CpG age classifier defined by Petkovich and colleagues (29). Only CpGs that had enough coverage (>5) after data imputation with BoostMe (58) were retained. The weighted average was scaled and used as a relative measure of epigenetic age.

**Data Analysis and Statistics**

R or GraphPad Prism 6.0 was used for statistical analyses. When applicable, normality was tested using a Shapiro–Wilk test. Publicly-available ChIP-seq data were retrieved from the ChIP-Atlas (59). Heatmaps of ChIPseq data were generated by deepTools (60) computeMatrix, and plotHeatmap with the options reference-point and missingDataAsZero.

Enrichment analyses were performed with GSEA (37, 61), DAVID (62, 63), and Enrichr (14, 64). Hierarchical clustering and heatmaps were generated with R “pheatmap” using row scaling. Differential expression was identified by DeSEQ2 (65).

**Data Availability**

All generated data was deposited in NCBI Gene Expression Omnibus (GEO) under accession number GSE155339.

**Disclosure of Potential Conflicts of Interest**

T. Lammens reports grants from vzw Kinderkankerfonds during the conduct of the study. D. Deforce reports grants from FWO during the conduct of the study. A.E. Kulozik reports grants and personal fees from Bluebird Bio outside the submitted work. D.J. Curtis reports grants from NHMRC during the conduct of the study. T. Taghon reports grants from Research Foundation - Flanders (FWO), Stichting Tegen Kanker (STK), Kinderkankerfonds (a nonprofit childhood cancer foundation under Belgian law), Cancer Research Institute Ghent (CRIG), the National Science Center Poland (2017/24/T/N/25/00359), The National Centre for Research and Development Poland (STRATEGMED3/304586/5/NCBR/2017), the Swedish Childhood Foundation (PR2018-0064), the Medical Faculty of Umeå University, the Kempe Foundation, and the Lion’s Cancer Research Foundation. R.S. Kotecha (NHMRC APP1142627) is supported by a fellowship from the National Health and Medical Research Council of Australia. M.R. Mansour is a Bloodwise Bennett Fellow. The Davé Lab was supported by R01CA207530 from the National Cancer Institute, I01BX001799 from the Department of Veterans Affairs, and the Indiana University School of Medicine Strategic Research Initiative. The computational resources and services used in this work were provided by the VSC (Flemish Supercomputer Center), funded by the Research Foundation - Flanders (FWO) and the Flemish Government–department EWI.

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Received April 17, 2020; revised August 6, 2020; accepted September 15, 2020; published first September 23, 2020.

**REFERENCES**

of enhancer repertoires drives lineage and stage-specific transcription.


Horvath S. DNA methylation age of human tissues and cell types. Genome Biol 2013;14:R115.


Aging of Preleukemic Thymocytes Drives CpG Island Hypermethylation in T-cell Acute Lymphoblastic Leukemia

Juliette Roels, Morgan Thénoz, Bronislawa Szarzynska, et al.


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