Histone acetylation and DNA demethylation of T cells result in an anaplastic large cell lymphoma-like phenotype

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ABSTRACT

A characteristic feature of anaplastic large cell lymphoma is the significant repression of the T-cell expression program despite its T-cell origin. The reasons for this down-regulation of T-cell phenotype are still unknown.
To elucidate whether epigenetic mechanisms are responsible for the loss of the T-cell phenotype, we treated anaplastic large cell lymphoma and T-cell lymphoma/leukemia cell lines (n=4, each) with epigenetic modifiers to evoke DNA demethylation and histone acetylation. Global gene expression data from treated and untreated cell lines were generated and selected, and differentially expressed genes were evaluated by real-time reverse transcriptase polymerase chain reaction and western blot analysis. Additionally, histone H3 lysine 27 trimethylation was analyzed by chromatin immunoprecipitation. Combined DNA demethylation and histone acetylation of anaplastic large cell lymphoma cells was not able to reconstitute their T-cell phenotype. Instead, the same treatment induced in T cells: (i) an up-regulation of anaplastic large cell lymphoma-characteristic genes (e.g. *ID2*, *LGALS1*, *c-JUN*), and (ii) an almost complete extinction of their T-cell phenotype including CD3, LCK and ZAP70. In addition, suppressive trimethylation of histone H3 lysine 27 of important T-cell transcription factor genes (*GATA3*, *LEF1*, *TCF1*) was present in anaplastic large cell lymphoma cells, which is in line with their absence in primary tumor specimens as demonstrated by immunohistochemistry. Our data suggest that epigenetically activated suppressors (e.g. ID2) contribute to the down-regulation of the T-cell expression program in anaplastic large cell lymphoma, which is maintained by trimethylation of histone H3 lysine 27.

Introduction

Anaplastic large cell lymphoma (ALCL), which was first described by Stein and colleagues in 1985, is a T-cell neoplasm that accounts for approximately 26% of all T-cell lymphomas and 2% of all adult non-Hodgkin's lymphomas in western countries.[1] The World Health Organization classification of Tumors of Hematopoietic and Lymphoid Tissues defines three distinct subtypes of ALCL:[2] (i) anaplastic lymphoma kinase–positive (ALK^+)-ALCL which represents about 60% of ALCL and expresses an oncogenic ALK-fusion protein, resulting from a translocation most commonly involving the nucleophosmin (*NPM*) and *ALK* genes (translocation 2;5). (ii) ALK^--ALCL, which is morphologically indistinguishable from ALK^+-ALCL, but lacks t(2;5) and thus ALK expression; and (iii) a primary cutaneous ALK^--ALCL, which arises primarily in the skin and lacks both ALK expression and t(2;5).[2] Although ALCL was described for the first time more than 25 years ago, little is yet known about the pathogenesis of this disease, especially the ALK^- cases.[3,4]

Interestingly, ALCL show several similarities with classical Hodgkin's lymphoma (cHL) which, however, derives genotypically from mature B cells. Both lymphoma entities share some cytomorphological features and the consistent expression of the CD30 antigen, a member of the tumor necrosis factor receptor superfamily which, in normal lymphoid tissues, is restricted to few activated T and B cells.[5,6] A very striking feature of cHL is the dramatic loss of the B-cell phenotype. This is in contrast to other types of B-cell lymphoma, in which the B-cell phenotype is usually preserved.[7,8] Similar to the extinction of the B-cell phenotype in cHL, down-regulation of the T-cell gene expression program is frequently observed in the tumor cells of ALCL. Furthermore, in analogy to absent immunoglobulin expression in cHL, T-cell receptors (TCR) are often not expressed in ALCL despite *TCR* genes being rearranged. This may contribute to the dysregulation of intracellular signaling pathways controlling T-cell activation and survival.[9,10]

Epigenetic alterations apparently play an important role in the down-regulation of B-cell-specific genes in cHL since DNA demethylation and histone acetylation of B-cell lines induce up-regulation of cHL typical genes and extinction of the B-cell expression program. Among the epigenetically up-regulated genes, suppressors of lineage fidelity (e.g. *ID2*) are present, which suggests that the loss of B-cell phenotype is largely mediated by indirect mechanisms and not a direct consequence of the epigenetic treatment.[11,12] This concept is strongly supported by the findings of our previous work which demonstrated that transfection of B cells with *ID2* expression constructs led to a dramatic down-regulation of B-

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cell characteristic genes.[13] Interestingly, we demonstrated that B-cell characteristic genes such as *CD19* and *CD79a* as well as the B-cell transcription factor *PAX5* have additional suppressive trimethylation of histone H3 lysine 27 (H3K27) in cHL which is absent in B cells.[12]

Here we show that DNA demethylation and histone acetylation of T-cell lines induced an ALCL-like phenotype, whereas the same treatment of ALCL cells did not restore the expression of T-cell genes and did not switch off the ALCL-associated genes. Furthermore, we demonstrate that the promoters of important T-cell transcription factor genes (*GATA3*, *TCF1* and *LEF1*)[14] were silenced by H3K27 trimethylation, a finding in harmony with their absence in primary ALCL tissue specimens.

Design and Methods

Cell cultures

Four anaplastic large cell lymphoma (ALCL) cell lines of T-cell origin [FE-PD (ALK^-), JB6 (ALK^+), Karpas 299 (ALK^+) and SU-DHL-1 (ALK^+)] and four T-cell lymphoma/leukemia cell lines (CCRF-CEM, Jurkat, MOLT-3 and MOLT-4) were employed in this study. The latter four cell lines are designated as "T-cell lines" throughout the manuscript given their immunophenotype of mature T cells and because of their rearranged *TCR* genes (*data not shown*). All cell lines were cultured in RPMI 1640 (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Combined 5-aza-2'-deoxycytidine/trichostatin A treatment

The four ALCL-derived cell lines and four T-cell lines were treated with 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 1 μM for 6 days. 5-aza-dC and medium were replaced on days 2 and 5. On day 5 cells were additionally incubated for 24 h with 625 nM trichostatin A (TSA) (Sigma-Aldrich). Cells were harvested on day 6.[11]

RNA isolation and microarray analysis

For both microarray analysis and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis, total RNA was isolated from untreated and 5-aza-dC/TSA-treated cells using the RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For real-time RT-PCR, total RNA was additionally treated with DNase for 20 min at room temperature. The quality of the RNA was controlled using the Bioanalyzer (Agilent, Santa Clara, CA, USA) and exclusively high quality RNA (RIN≥8) was used for further analysis. Affymetrix GeneChip hybridization (HG-U133A) was performed with 5 μg total RNA according to the manufacturer's recommendations. CEL files of all experiments were generated with the GCOS 1.3 software (Affymetrix) and are available via the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (*www.ncbi.nlm.nih.gov/geo*) under accession number GSE26101.

Microarray data analysis

Analysis of the microarray data was performed using R and Bioconductor. All arrays were normalized using robust multichip average.[15,16] A moderated t-test was used for statistical evaluation. Probe sets were defined as differentially expressed if they had a fold change of at least 2 and a Benjamini-Hochberg (BH) corrected *P*-value less than 0.05 (after the moderated t-test). All probe set identifiers were mapped to Entrez gene identifiers using Biomart.[17,18] Genes lacking a validated or reviewed entry in RefSeq were removed from the full list of 46,875 Entrez genes, leading to a set of 18,546 Entrez genes associated with 24,885 RefSeq entries. Venn diagrams were calculated using the Entrez identifiers corresponding to the differentially expressed probe sets. We used a single-sided Fisher's exact test to determine the significance of overlaps ($P \leq 0.01$). Transcripts in significant intersections were subjected to a hierarchical clustering analysis based on the gene expression data, using Pearson's correlation as a similarity measure and complete linkage.

Enrichment analysis of biological annotations

To identify enriched biological annotations, particularly enriched pathways, 5-aza-dC/TSA up- and down-regulated genes in T-cell and ALCL cell lines were analyzed using Genomatix Software (*http://www.genomatix.de*, Munich, Germany).

Real-time reverse transcriptase polymerase chain reaction analysis

TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) were used to transcribe total RNA in cDNA with random hexamer primers. Real-time RT-PCR was performed with SYBR Green PCR-Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR (Applied Biosystems) using the PCR parameters recommended by the manufacturer. The housekeeping gene succinate dehydrogenase complex, subunit A (*SDHA*) was amplified in parallel with each gene of interest. Relative mRNA quantification was calculated using the comparative $\Delta\Delta C_T$ method.[19] All primers were purchased from Eurofins MWG (Ebersberg, Germany). Primer sequences are listed in *Online Supplementary Table S1*.

DNA isolation and global quantification of methylated DNA

DNA was isolated from untreated and 5-aza-dC/TSA-treated cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. To determine global DNA methylation, DNA samples were denatured with heat and equal amounts of DNA (200 ng) from untreated and 5-aza-dC/TSA-treated cells were spotted onto a nylon Hybond N+ membrane (Amersham Bioscience). After air-drying, ultraviolet cross-linking and blocking over night at 4°C, the membrane was incubated for 1 h with an anti-5-methylcytosine monoclonal mouse antibody (clone 33D3, Diagenode, Liège, Belgium) (1:250). Subsequently, a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Amersham Biosciences) was applied for 1 h and antibody binding was made visible by enhanced chemiluminescence (ECL, Amersham Biosciences) according to the manufacturer's instructions. To ensure equal spotting of total DNA, the blots were stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.2). The intensity of 5-methylcytosine staining in the immunodot blots was analyzed using Fusion Advance Capt software (Version 16.02) (Peqlab, Erlangen, Germany). The values for each sample were averaged and the standard error was calculated from five independent blots. The values for 5-aza-dC/TSA-treated samples were adjusted relative to the untreated counterpart.

Western blot analysis

Whole-cell protein lysates from untreated and 5-aza-dC/TSA-treated cells were separated on 12% sodium dodecylsulfate polyacrylamide gels (Invitrogen, San Diego, CA, USA) and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA) by electroblotting. Membranes were then placed in primary antibody solution at 4°C overnight (information on primary antibodies is available in *Online Supplementary Table S2*). After incubation for 1.5 h with a secondary antibody conjugated with horseradish peroxidase (anti-mouse

Ig or anti-rabbit Ig; Amersham Biosciences) binding was detected by enhanced chemiluminescence (ECL, Amersham Biosciences) according to the manufacturer's instructions.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments with an anti-histone H3 K27 trimethylation antibody (07-499, Cell Signaling Technology, Danvers, MA, USA) were performed as described previously.[12] Successful enrichment of immunoprecipitated DNA was confirmed by real-time DNA-PCR by comparison with the negative control (input DNA). Primer sequences are given in *Online Supplementary Table S3*.

Bisulfite conversion, polymerase chain reaction, TOPO TA cloning and sequencing

Bisulfite conversion of 2 μg DNA from untreated and 5-aza-dC/TSA-treated MOLT-3 and Jurkat cells and four primary ALCL cases was carried out using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions.

Bisulfite-modified DNA was amplified by PCR using the primers designed for detection of the methylation status in the promoter region of *ID2* (forward: 5' TGAATTTTAGGAAGGTGAGTTT 3'; reverse: 5' CTCCAAAAAAAAACAATATTCAAA 3'). Cycling conditions were as follows: 10 min at 95 °C, followed by three cycles of 30 s at 59 °C, 35 s at 72 °C and 30 s at 95 °C, followed by 42 cycles of 30 s at 56 °C, 35 s at 72 °C and 30 s at 95 °C and a final 10 min extension step at 72 °C.

PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega GmbH, Mannheim, Germany) and subcloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen). Sequencing was performed with vector-specific primers on an ABI 3130 sequencer and data were analyzed using AB DNA sequencing analysis software version 5.3.1 (Applied Biosystems).

Immunohistochemistry

The expression of RYBP, GATA3, TCF1 and LEF1 was determined by immunohistochemistry of primary tumor specimens [the use of patients' material was approved by the ethical review board of the Charité (EA4/085/07)]. Paraffin sections (4 μm) of 15 primary ALCL and 16 primary cHL were cooked for 2 min in citrate buffer (pH 6.0) in order to evoke antigen retrieval.[20] After incubation with the primary antibodies (information on primary antibodies is available in *Online Supplementary Table S2*) and extensive washing, the bound antibodies were made visible by the streptavidin-biotin-alkaline phosphatase method with FastRed as the chromogen (all from DAKO, Glostrup, Denmark).[21]

Results

General impact of 5-aza-2'-deoxycytidine/trichostatin A treatment

The success of our 5-aza-dC/TSA treatment was analyzed by western blotting (TSA-mediated acetylation) and by immuno-dot blot analysis (5-aza-dC-mediated DNA demethylation). All 5-aza-dC/TSA-treated cells demonstrated a very strong global increase of H3K9 acetylation (*Online Supplementary Figure S1A, B*) and a significant global decrease in DNA methylation as compared to their untreated counterparts (*Online Supplementary Figure S1C*).

To determine the overall impact of the combined 5-aza-dC/TSA treatment on the gene expression profile, all treated T- and ALCL cell lines (n=8) were compared to all untreated cell lines (n=8) after Affymetrix GeneChip (HG-U133A) hybridization. This led to the identification of 1238 differentially expressed genes, the majority of which were down-regulated by the treatment (1012 genes = 82%) whereas 18% (226 genes) were up-regulated. Twelve cancer/testis genes, e.g. members of the GAGE and MAGE group, were among the 226 up-regulated genes. Since the expression of cancer/testis genes is epigenetically controlled, the up-regulation of these genes can be regarded as a positive control for our 5-aza-dC/TSA treatment.[22]

In order to identify cell type-specific effects of the 5-aza-dC/TSA treatment, T-cell lines and ALCL cell lines were analyzed separately. The comparison of gene expression profiles of treated and untreated T-cell lines led to the identification of 1646 genes which were affected by 5-aza-dC/TSA treatment. In contrast, only 654 genes were found to be differentially expressed in ALCL cell lines after 5-aza-dC/TSA treatment. A comparison of the two sets of differentially expressed genes revealed that 455 genes were affected in both T-cell and ALCL cell lines (*Online Supplementary Figure S2*) and were thus excluded from further analysis since they do not represent cell-type specific differences. This led to the identification of 1191 (1646 – 455 = 1191) genes specifically affected in T-cell lines (245 up-regulated and 946 down-regulated genes) and 199 (654 – 455 = 199) genes specifically affected in ALCL cell lines (70 up-regulated and 129 down-regulated genes) (*Online Supplementary Table S4*). Thus, 5-aza-dC/TSA treatment has a considerably stronger impact on gene expression in T-cell lines than in ALCL cell lines.

Differentially expressed genes in T-cell and anaplastic large cell lymphoma cell lines

To establish a gene expression signature which characteristically describes the cell type-specific differences between the T-cell lines (CCRF-CEM, Jurkat, MOLT-3 and MOLT-4) and the ALCL cell lines (FE-PD, JB6, Karpas 299 and SU-DHL-1) we compared their gene expression profiles (untreated cells). This led to the identification of 683 genes, 406 of which were found to be up-regulated in T-cell lines (designated "T-cell characteristic genes") and 277 in ALCL cell lines (designated "ALCL-characteristic genes") (*Online Supplementary Table S5*). These gene lists were employed to test the impact of the 5-aza-dC/TSA treatment on T-cell- and ALCL-characteristic genes.

Impact of global DNA demethylation and histone acetylation on the phenotypes of T- and anaplastic large cell lymphoma cells

Next, two questions were addressed. (i) Is combined global DNA demethylation and histone acetylation able to induce an ALCL-like phenotype in T-cell lines? (ii) Is combined global DNA demethylation and histone acetylation able to reactivate T-cell-specific genes in ALCL cell lines?

(i) To determine the impact of the epigenetic treatment on T-cell lines, we compared the 245 genes up-regulated by 5-aza-dC/TSA treatment of T-cell lines and the 277 identified ALCL-characteristic genes. This comparison showed a statistically highly significant (P=2.8x10^{-15}) overlap of 29 genes (*Online Supplementary Figure S3A*) including *ID2, LGALS1, c-JUN* and *SLC2A3* (*Online Supplementary Table S6A*). Vice versa, the comparison of the 406 T-cell characteristic genes and the 946 genes down-regulated genes by 5-aza-dC/TSA treatment of T-cell lines revealed

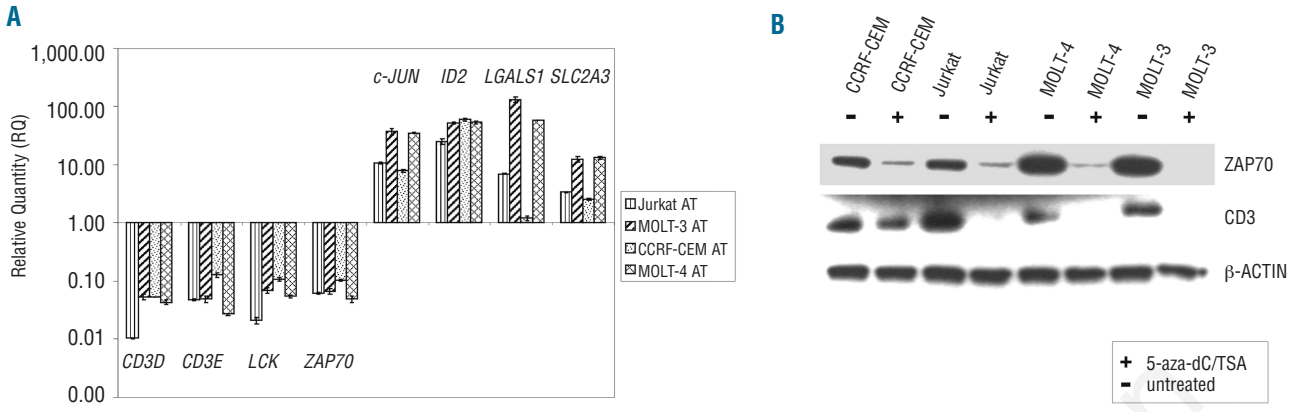

Figure 1. Up-regulation of ALCL characteristic genes and down-regulation of genes crucial for T-cell signaling upon epigenetic treatment of T-cell lymphoma cell lines with 5-aza-dC/TSA. (A) Relative mRNA expression of *CD3D*, *CD3E*, *LCK*, *ZAP70*, *ID2*, *c-JUN*, *LGALS1* and *SLC2A3* in 5-aza-dC/TSA-treated T-cell lines as analyzed by means of real-time RT-PCR. AT: 5-aza-dC/TSA treated. (B) Western blot analysis of the T-cell characteristic proteins CD3 and ZAP70 in T-cell lines treated (+) or not (-) with 5-aza-dC/TSA.

a common set of 176 genes (P=2.2x10^{-16}) (*Online Supplementary Figure S3B*; *Online Supplementary Table S6B*).

Real-time RT-PCR for selected genes was applied to independently confirm the expression changes (Figure 1A). Taken together, the epigenetic treatment of T-cell lines resulted in a dramatic change towards an ALCL-like phenotype.

To gain more insight into the biological function of the genes which were down-regulated in T-cell lines after 5-aza-dC/TSA treatment, we calculated their most overrepresented (enriched) pathways using the Genomatix Pathway System (GePS). This revealed that the TCR signaling pathway was most significantly affected in 5-aza-dC/TSA-treated T-cells (P=1.66x10^{-13}), including the down-regulation of 89 T-cell characteristic genes such as *CD3*, *LCK* and *ZAP70*. The down-regulation of selected genes crucial for T-cell signaling such as *CD3* and *ZAP70* was confirmed by real-time RT-PCR and western blotting (Figure 1A, B). All in all, epigenetic treatment of T-cell lines led to a highly significant shift of their gene expression towards an ALCL-like expression program with the typical loss of the T-cell gene expression program (Figure 2).

(ii) To examine whether 5-aza-dC/TSA treatment of ALCL cell lines is able to reactivate their repressed T-cell expression program, we compared our 406 T-cell characteristic genes with the genes specifically up-regulated in 5-aza-dC/TSA-treated ALCL cell lines. The treatment induced weakly or moderately increased expression of only six genes, which was found to be not statistically significant. Thus, combined DNA demethylation and histone acetylation is unable to reactivate the T-cell expression program in ALCL cell lines (Figure 2).

Concurrent up-regulation of many genes in epigenetically treated B- and T-cell lines

Since the result of the epigenetic treatment of T-cell lines strongly resembles previous findings in B-cell lines, we compared a set of 435 genes which we found to be up-regulated in 5-aza-dC/TSA-treated B-cell lines[12] with the 245 genes epigenetically up-regulated in T-cell lines described in this work. One hundred and ten genes were up-regulated in both cell types (P=7.78 x10^{-97}). These up-regulated genes included several genes known to be expressed in cHL and ALCL cells (e.g. *ID2*, *HSPA1A*, *LGALS1*, *OPTN*, *c-JUN*, *AAK1*[23-25] and *RYBP* (Table 1, *Online Supplementary Figure S4* and Sanchez-Beato *et al.*[26]).

Analysis of ID2 promoter methylation

To demonstrate that 5-aza-dC/TSA treatment of T cells directly activates the lineage fidelity suppressor *ID2*, we studied the methylation status of CpG dinucleotides in the *ID2* promoter. Our bisulfite sequencing results show that the *ID2* promoter was demethylated after epigenetic treatment (Figure 3). Furthermore, we studied the DNA methylation status of the *ID2* promoter in four primary ALCL cases by bisulfite sequencing. In agreement with our concept, we found that the CpG islands of the *ID2* promoter were - as a prerequisite for gene activation - largely demethylated (Figure 3).

Silencing of T-cell transcription factors by H3K27 trimethylation

To evaluate the role of H3K27 trimethylation in the silencing of the T-cell immunophenotype in ALCL we performed quantitative real-time PCR with DNA-fragments obtained after ChIP with an antibody against H3K27 trimethylation for six T-cell related genes. Interestingly, H3K27 trimethylation was present in promoters of T-cell transcription factor genes such as *GATA3*, *LEF1* and *TCF1* in ALCL cells whereas T cells were devoid of this suppressive histone mark. In contrast, genes of the T-cell signaling pathway such as *CD3E*, *CD3G* and *ZAP70* showed no H3K27 trimethylation in ALCL and T-cell lines (Figure 4 A) indicating that the down-regulation of these genes in ALCL cells is mediated by other mechanisms. To validate these cell line findings we carried out immunostaining for the T-cell transcription factors GATA3, LEF1 and TCF1 in 15 primary ALCL cases. In all cases at least one of these transcription factors was absent from the tumor cells (Table 1) supporting a model that H3K27 trimethylation-mediated silencing of important T-cell transcription factors in ALCL also occurs *in vivo* (Figure 4B).

Discussion

The cellular origin of ALCL tumor cells was uncertain for a long time since most lineage specific markers are absent.[10] Only when molecular studies identified their rearranged *TCR* genes was the T-cell origin of ALCL tumor cells established.[9] This is very similar to the situation in cHL, the tumor cells of which lack almost all B-cell features but are derived from B cells as demonstrated by their rearranged immunoglobulin genes.[27,28] The extinction of the B-cell identity of cHL is closely associated with epigenetic alterations, especially epigenetically activated genes (e.g. suppressors of the lineage fidelity such as *ID2*) which contribute to an indirect down-regulation of B-cell antigens.[11,13]

The reason for the loss of the T-cell identity in ALCL is still unknown. We hypothesized that, in analogy to cHL, epigenetic alterations are involved or responsible for the loss of the T-cell identity in ALCL. To unravel this situation we investigated whether DNA demethylation and histone acetylation are able: (i) to down-regulate the T-cell program and to up-regulate ALCL-characteristic genes in T cells and (ii) to restore the T-cell phenotype of ALCL cells.

Combined DNA demethylation and histone acetylation of anaplastic large cell lymphoma cells is unable to restore the T-cell phenotype

Our gene expression profiling showed no significant up-regulation of T-cell characteristic genes by 5-aza-dC/TSA treatment of ALCL cells. Thus, no re-establishment of the T-cell phenotype was induced in ALCL by this treatment. Only a faint and statistically insignificant increase of some T-cell genes was observed in one ALCL cell line (SU-DHL-1; *Online Supplementary Table S7A,B*) which had already been reported.[29] These findings are very similar to the situation in cHL in which no re-expression of B-cell genes was induced by DNA demethylation and histone acetylation.[11]

There are two possible explanations for the inability of 5-aza-dC/TSA treatment to reactivate the cell lineage specific phenotype in ALCL and cHL: (i) suppressors of lineage fidelity such as *ID2* are already up-regulated in ALCL and cHL and are not affected by our epigenetic treatment and (ii) gene silencing by other epigenetic mechanisms such as H3K27 trimethylation is not directly affected by 5-aza-dC-mediated demethylation.

Extinction of the T-cell phenotype and up-regulation of anaplastic large cell lymphoma-characteristic genes by epigenetic treatment of T-cell lines

Gene expression analysis of the four T-cell lines (CCRF-CEM, Jurkat, MOLT-3 and MOLT-4) revealed that 1191 genes were differentially regulated after 5-aza-dC/TSA treatment. Of these, 946 were down-regulated and 245 genes were up-regulated. Analysis of the down-regulated genes for significantly enriched pathways demonstrated that genes (n=89) of the TCR signaling pathway were most strongly affected by 5-aza-dC/TSA treatment, including genes such as *CD3D* (δ), *CD3E* (ε), *CD3G* (γ), *LCK*, *ZAP70*, *LAT* and *VAV1*. This down-regulation of important factors of TCR signaling was confirmed by means of real-time RT-PCR (*CD3D, CD3E, LCK* and *ZAP70*) and western blot analysis (CD3, ZAP70) (Figure 1A, B).

TCR signaling is mainly achieved by the CD3 heterodimers (δε and γε) and the TCR ζ homodimer which transmit extracellular binding events to the cytoplasm via their immunoreceptor tyrosine-based activation motifs (ITAM). The subsequent phosphorylation of tyrosines of the ITAM sequence by a src family kinase, usually LCK in T cells, is the initiating event in TCR signaling. This leads to recruitment of the protein tyrosine kinase ZAP70, which phosphorylates important adapter proteins, such as linker for the activation of T cells (LAT), initiating downstream events promoting T-cell proliferation and differentiation.[30-32] Most of these important molecules required for TCR signaling are clearly down-regulated by 5-aza-dC/TSA treatment of T cells and this mainly involves genes at the proximal part of the signaling cascade. This observation is in line with the lack of TCR molecules or molecules of proximal TCR signaling in primary ALCL cases.[9]

The treatment of T-cell lines with 5-aza-dC/TSA resulted not only in a down-regulation of the T-cell phenotype but also in an up-regulation of 29 genes which are characteristically expressed in ALCL-derived cell lines and partly in primary ALCL tumor cells. The most up-regulated gene upon 5-aza-dC/TSA treatment of T-cell lines was *ID2*. ID2 is a member of the inhibitor of differentiation family of helix-loop-helix transcription factors, which could play a central role in the suppression of the T-cell phenotype in ALCL. The members of this gene family are incapable of binding directly to DNA, but they exert their activity through interaction with other helix-loop-helix transcription factors, preventing them from binding to DNA. It is thought that ID proteins have a dominant-negative effect on the transcription of lineage-specific genes.[13,33] Our data reveal that up-regulation of *ID2* after epigenetic treatment in T cells is correlated with demethylation of the *ID2* promoter. Furthermore, we showed that the *ID2* promoter is demethylated in primary ALCL cases in harmony with an up-regulation of *ID2* (Figure 3). Our finding of strong up-

Table 1. Expression of RYBP, TCF1, LEF1 and GATA3 in 15 cases of primary anaplastic large cell lymphoma (ALCL).

Case	Diagnosis	PcG protein associated	T-cell transcription factor		
		RYBP	TCF1	LEF1	GATA3
1	ALCL, ALK-	+	-	-	-
2	ALCL, ALK-	+	-	-	+
3	ALCL, ALK-	+	-	-	-
4	ALCL, ALK-	+	-	-	+
5	ALCL, ALK-	+	-	-	-
6	ALCL, ALK-	+	+	-	+
7	ALCL, ALK-	+	+	-	-
8	ALCL, ALK-	+	+	+	+
9	ALCL, ALK-	+	-	-	-
10	ALCL, ALK+	+	-	-	-
11	ALCL, ALK+	(+)	+	+	-
12	ALCL, ALK+	+	-	-	-
13	ALCL, ALK+	+	-	-	-
14	ALCL, ALK+	+	-	-	-
15	ALCL, ALK+	+	+	-	-

+ expression; (+) weak expression; - no expression. PcG: polycomb group protein.

regulation of *ID2* after epigenetic treatment is strongly supported by our previous data which demonstrated a down-regulation of T-cell genes (e.g. *CD3E*, *TCF1*, *GATA3*) by ectopic over-expression of ID2 in T-cells.[24]

Similar epigenetic mechanisms in anaplastic large cell lymphoma and classical Hodgkin's lymphoma for repression of their cell type-specific expression programs

The impact of epigenetic modifications evoked by 5-aza-dC/TSA treatment is very similar in T- and B-cell lines.[11,12] Both lose expression of their cell-type characteristic genes and concurrently up-regulate cell-type atypical genes which are found to be already consistently expressed in cHL and ALCL, e.g. the suppressor *ID2*. In addition, silencing through H3K27 trimethylation of important B-cell genes such as *CD19* and *PAX5* was identified in cHL.[12] Here we show that the promoters of important T-cell transcription factor genes (i.e. *GATA3*, *TCF1* and *LEF1*) are also silenced in ALCL cell lines by H3K27 trimethylation. In line with this *in vitro* observation is the absence of these transcription factors in the tumor cells of primary ALCL cases which – in turn – prevents the development of the full T-cell phenotype (Figure 4A, B).[14]

H3K27 trimethylation is mediated by Polycomb group proteins,[34] which are epigenetic chromatin modifiers and comprise two functionally and biochemically distinct multimeric Polycomb repressive complexes, called PRC1 and PRC2. According to the currently proposed model, PRC2 triggers transcriptional repression by inhibiting transcription initiation, whereas PRC1 maintains the repressive

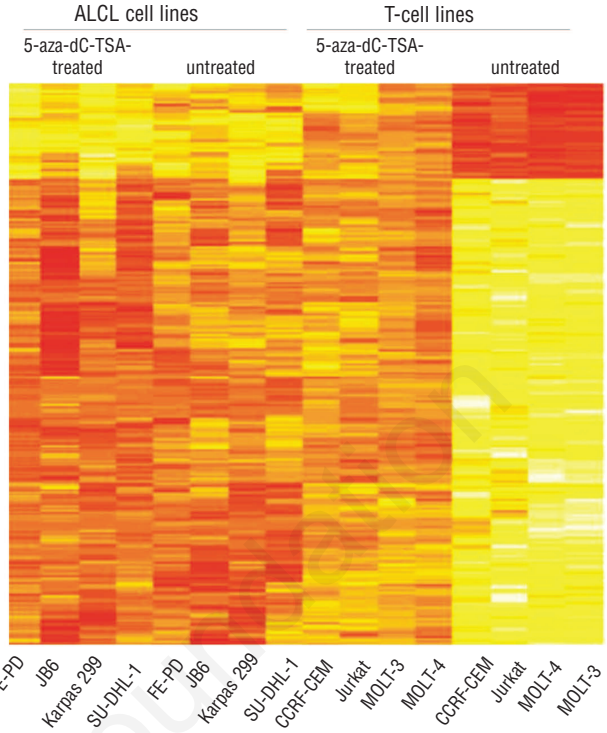

Figure 2. Hierarchical cluster analysis (Pearson's similarity and complete linkage). Expression of the 205 genes identified to be significantly regulated in 5-aza-dC/TSA-treated T cells in all 16 cell line samples in this study. The expression profile of 5-aza-dC/TSA-treated T-cell lines is clearly shifted towards an ALCL-typical phenotype, whereas ALCL cells remain largely unaffected with respect to this gene set.

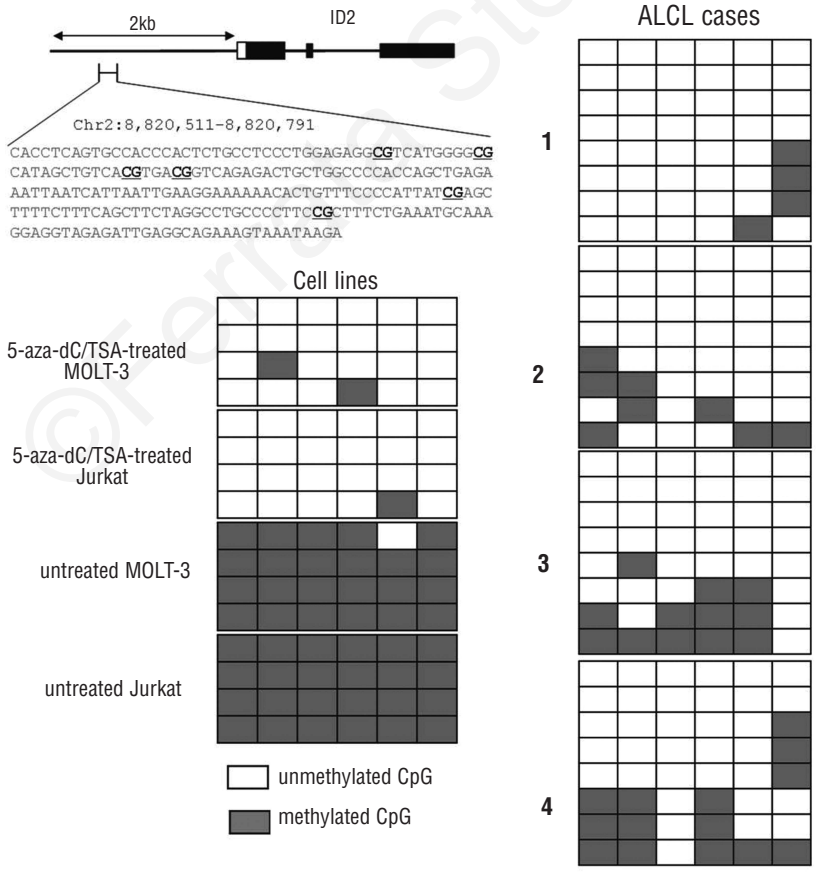

Figure 3. The ID2 promoter region is demethylated in 5-aza-dC/TSA-treated T cells and in tumor cells of primary ALCL. The methylation status of the ID2 promoter was analyzed by bisulfite sequencing. The sequenced region in the ID2 promoter contained six CpG dinucleotides. Clones were sequenced from 5-aza-dC/TSA-treated and untreated Jurkat and MOLT-3 cells and four primary ALCL cases. Every row represents a single PCR clone.

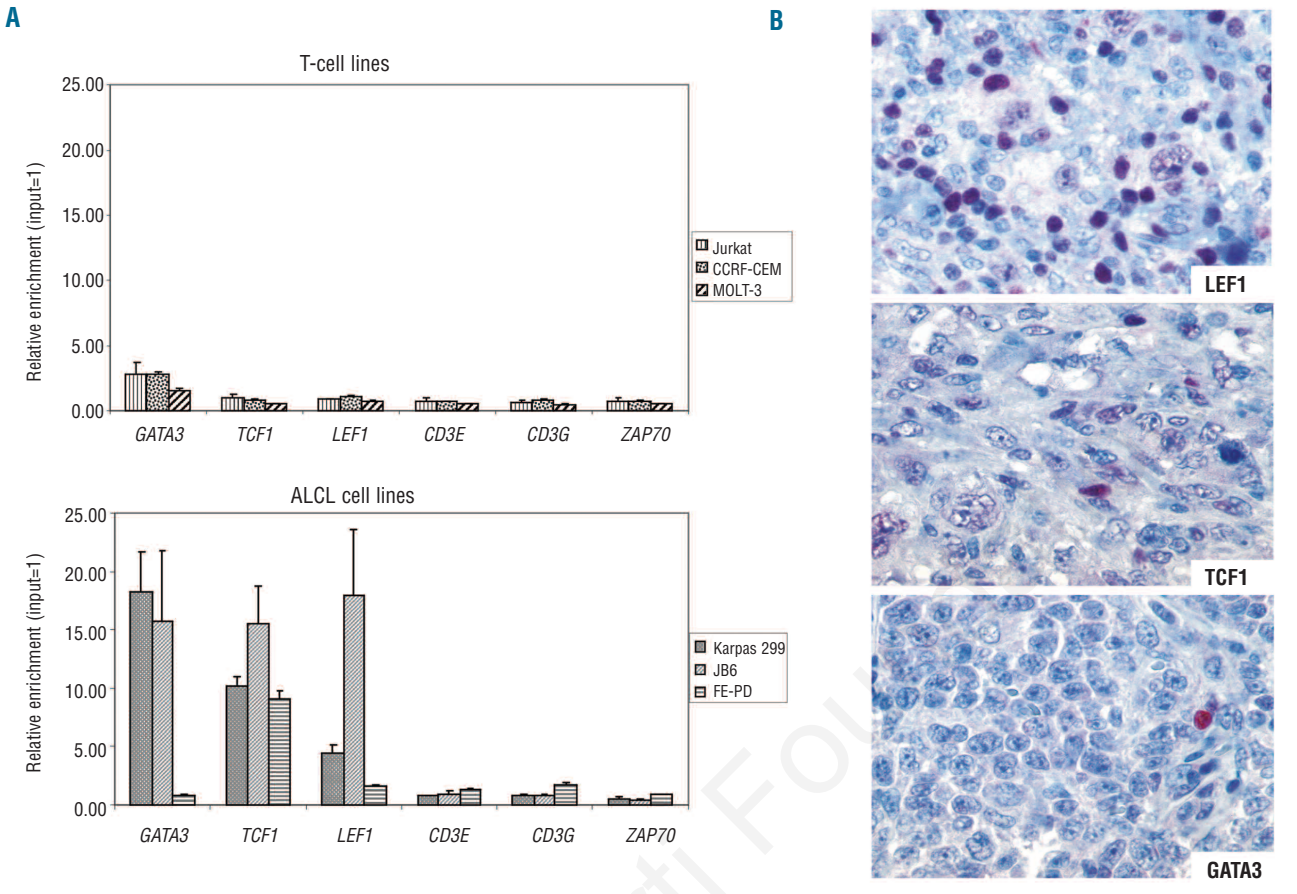

Figure 4. (A) **Promoter H3K27 trimethylation status of T-cell signaling pathway genes and T-cell transcription factor genes after ChIP and quantification by real-time DNA-PCR in T- and ALCL cell lines.** ***β-ACTIN*** **was chosen as the endogenous reference and the input was calculated relative to the ChIP input control.** (B) **Immunohistochemistry for GATA3 (original magnification, 400x), LEF1 and TCF1 (original magnification 200x) in primary ALCL cases. All three T-cell transcription factors were strongly expressed in the nuclei of the reactive T cells whereas the tumor cells displayed no or only faint expression.**

conditions.[35]

The up-regulation of *RYBP* (RING1 and YY1 binding protein) in both T- and B-cell lines upon 5-aza-dC/TSA treatment is particularly interesting. RYBP is an evolutionarily conserved protein with a zinc-finger motif, which cooperates directly with both Ring1 proteins (Ring1A and Ring1B) and with M33, two mutually interacting sets of proteins of PRC1.[36] Interestingly, RYBP is strongly overexpressed in the nuclei of the tumor cells of all cases of primary cHL and ALCL. In contrast, in all cases of cHL and ALCL, lymphoid bystander cells showed no or only faint nuclear RYBP expression (*Online Supplementary Figure S4*). However, the exact role of RYBP in this complex epigenetic regulatory network and especially in the regulation of H3K27 trimethylation is not fully understood yet.

We suggest that the same two epigenetic mechanisms are involved in the suppression of the lineage-specific expression programs of ALCL and cHL: (i) epigenetic activation of suppressors of lineage fidelity such as *ID2* evokes an indirect down-regulation of the lineage specific genes and (ii) additional silencing through H3K27 trimethylation of important transcription factors such as *PAX5* in cHL[12] and *TCF1*, *LEF1* and *GATA3* in ALCL prevents the (re-)establishment of the cell-type characteristic expression program. Interestingly, H3K27 trimethylation is not directly affected by 5-aza-dC/TSA treatment, a fact which might explain the inability of this treatment to restore cell-type characteristics in ALCL and cHL cell lines.

We conclude that most likely epigenetically activated genes such as the suppressor *ID2* contribute to the down-regulation of the T-cell phenotype during the pathogenesis of ALCL. In addition, Polycomb group protein-mediated epigenetic silencing of key T-cell transcription factors by trimethylation of H3K27 can be regarded as a failsafe mechanism to ensure the permanent silencing of the T-cell phenotype in ALCL. Based on these data, we will unravel the role of the epigenetically activated genes in the oncogenic process leading to ALCL.

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