NPM-ALK Oncogenic Tyrosine Kinase Controls T-Cell Identity by Transcriptional Regulation and Epigenetic Silencing in Lymphoma Cells

Chiara Ambrogio,1,2 Cinzia Martinengo,1 Claudia Voena,1,2 Fabrizio Tondat,2 Ludovica Riera,3 Paola Francia di Celle,3 Giorgio Inghirami,1,2,4 and Roberto Chiarle1,2

1Center for Experimental Research and Medical Studies and 2Department of Biomedical Sciences and Human Oncology, University of Torino. 3ASO “San Giovanni Battista,” Torino, Italy and 4Department of Pathology and New York Cancer Center, New York University School of Medicine, New York, New York

Abstract

Transformed cells in lymphomas usually maintain the phenotype of the postulated normal lymphocyte from which they arise. By contrast, anaplastic large cell lymphoma (ALCL) is a T-cell lymphoma with aberrant phenotype because of the defective expression of the T-cell receptor and other T-cell-specific molecules for still undetermined mechanisms. The majority of ALCL carries the translocation t(2;5) that encodes for the oncogenic tyrosine kinase NPM-ALK, fundamental for survival, proliferation, and migration of transformed T cells. Here, we show that loss of T-cell-specific molecules in ALCL cases is broader than reported previously and involves most T-cell receptor–related signaling molecules, including CD3ε, ZAP70, LAT, and SLP76. We further show that NPM-ALK, not the kinase-dead NPM-ALKK210R, downregulated the expression of these molecules by a STAT3-mediated gene transcription regulation and/or epigenetic silencing because this downregulation was reverted by treating ALCL cells with 5-aza-2-deoxycytidine or by knocking down STAT3 through siRNA. At the same time, NPM-ALK fusion protein is an oncogenic tyrosine kinase that plays a key role in the pathogenesis of ALCL, being essential for their survival and growth both in vitro and in vivo and sustaining the activated phenotype of neoplastic T cells through several pathways, including the Rho family GTPases (14, 15).

Introduction

T-cell lymphomas are thought to originate from T lymphocytes that are chronically stimulated before acquiring a fully transformed phenotype (1). Chronic stimulation requires the engagement of the T-cell receptor (TCR) complex and TCR-related downstream signaling molecules and plays an important role in some T-cell lymphomas (2, 3). After the acquisition of a transformed phenotype, T cells maintain the expression of most T-cell–specific antigens, thus allowing the classification into different subtypes of T lymphomas (4). In vitro studies on T-cell lines, such as Jurkat E6 cells, have shown that transformed T cells remain sensitive to TCR engagement and activate TCR-dependent signaling pathways in a pattern similar to normal T cells (5). In agreement with these findings, mutant Jurkat lines that lack surface expression of αβ-TCR heterodimers or molecules in the TCR signaling cascade, such as LCK, ZAP70, LAT, SLP76, or VAV1, show defects in downstream TCR signaling, such as Ca2+ mobilization and the activation-associated cytoskeletal rearrangements. Cytoskeletal rearrangements lead to changes in cell motility and cell shape, thus conferring to T cells an activated morphology. These effects are mainly mediated by the Rho family GTPases (6).

Anaplastic large cell lymphoma (ALCL) is a specific subtype of non-Hodgkin’s lymphomas classified among mature T-cell neoplasms (4). However, the majority of ALCL lack αβ-TCR heterodimer, CD4, CD8, and CD3ε, showing a loss of T-cell phenotype that is quite unusual among lymphomas and forced the classification of many cases of ALCL as “null” phenotype despite the presence of TCR rearrangements (4, 7, 8). This loss of lineage-specific markers closely resembles the lack of B-cell–specific phenotype in Hodgkin’s lymphoma. Tumor cells in Hodgkin’s lymphoma originate from mature B cells that lost the B-cell phenotype as a result of aberrant expression of transcriptional regulators, such as ABF-1 and Id2 (9). In contrast, in ALCL, the loss of T-cell phenotype remains still unexplained, and despite the lack of the TCR complex and related signaling molecules, ALCL cells display morphology, hypermotility, and cytoskeletal rearrangements comparable with an activated T cells (10).

The majority of ALCL are characterized by chromosomal translocations involving the anaplastic lymphoma kinase (ALK) gene (11) and are classified into a category separated from the ALK–ALCL in the recently updated WHO classification (4). Most frequently, ALCL carry the t(2;5)(p23;q35) translocation that fuses the ALK gene to the nucleophosmin (NPM) gene, resulting in the aberrant expression of the chimeric protein NPM-ALK (12). The NPM-ALK fusion protein is an oncogenic tyrosine kinase that plays a key role in the pathogenesis of ALCL, being essential for their survival and growth both in vitro and in vivo (13) and sustaining the activated phenotype of neoplastic T cells through several pathways, including the Rho family GTPases (14, 15). We showed previously that this phenotype depends on the kinase activity of NPM-ALK, which activates VAV1 and Cde42 (10).

Here, we show that NPM-ALK suppressed CD3ε, ZAP70, LAT, and SLP76 expression. Inhibition of ALK kinase activity or treating ALCL cells with DNA methyltransferase (DNMT) inhibitors
restored CD3ε, ZAP70, LAT, and SLP76 expression, thus showing transcriptional repression and/or epigenetic silencing of these molecules in ALCL. This regulation was dependent on STAT3, a major mediator of NPM-ALK function, because their expression was restored by STAT3 knockdown in ALCL cells. Thus, our data show that deregulated tyrosine kinase activity can profoundly modify the phenotype of transformed T lymphocytes.

Materials and Methods

Tissue samples, reagents, cell lines, and culture. Formalin-fixed, paraffin-embedded tissues of ALCL and peripheral T-cell lymphoma (PTCL) were obtained from the Surgical Pathology Unit of the University of Torino and used for the construction of tissue microarrays. For immunohistochemical stainings, sections from tissue microarrays were incubated with the following primary antibodies: ALK (ALK-1; DAKO), CD3ε (NCL-CD3-Ps1; Novocastra), ZAP70 (clone 2F3.2; Upstate), LAT (FL-233; Santa Cruz Biotechnology), and SLP76 (Cell Signaling). The reactions were developed as described previously (16). For the Western blot and DNA analysis, we selected fresh or frozen samples with >70% of ALK+ cells among reactive cells from the tissue bank from the Surgical Pathology Unit of the University of Torino. Human lymphoid cells SU-DHL1, JB6, Karpas-299 (NPM-ALK+), CEM, Jurkat, MAC-1, ST-4, and PFS82 (ALK−) were maintained in RPMI 1640 containing 10% FCS. ALK inhibitors CEP-14083 and CEP-26939 were kindly provided by Cephalon (17). Methyltransferase inhibitor 5-aza-2-deoxycytidine (DAC) was purchased from Sigma-Aldrich. Inducible short hairpin RNAs (shRNA) cells were obtained by transduction of pLV-TKRRAB vector followed by pLVTHM vectors (kindly provided by Dr. D. Trono) containing the shRNA cassette as described previously (16).

Cell lysis, immunoprecipitation, and immunoblotting antibodies. Total cellular proteins were extracted and cell lysates were used for Western blotting or immunoprecipitation as described previously (14). The following antibodies were used in the study: anti-ALK (Zymed), anti-phospho-Tyr (PY20; Transduction Lab), anti-ZAP70 (Upstate), anti-LAT (FL-233; Santa Cruz Biotechnology), anti-Nck 1/2 (Santa Cruz Biotechnology), anti-LCK (Cell Signaling), anti-SLP76 (Cell Signaling), anti-VAV1 (R775; Cell Signaling), anti-Cd4 (DAKO), anti-Cd8 (DAKO), anti-Cd3ε (NCL-CD3-Ps1; Novocastra), anti-α-tubulin (B-5-1-1; Sigma-Aldrich), anti-STAT3 (Cell Signaling), anti-phospho-STAT3 (Cell Signaling), and anti-DNMT1 and anti-DNMT3a (Imgenex). Secondary antibodies were purchased from Amersham.

DNA constructs. Wild-type NPM-ALK was cloned in the plasmid vector pCDNA3 (Invitrogen) at HindIII/Xhol sites and stably transduced into 293T-Rex Tet-On cells using Effectene reagents as described by the manufacturer (Qiagen). Pallino retroviral vectors containing NPM-ALK or NPM-ALK(W210R) were described previously (14). ALK and STAT3-specific shRNAs have been described previously (18, 19). To obtain STAT3 and NPM-ALK constructs resistant to specific shRNAs, STAT3 and NPM-ALK were mutated into SMUT and ALKMUT constructs by PCR-based mutagenesis (Stratagene). Primer sequences for mutagenesis were SMUT-forward 5′-CAACCTCAAGAGGACGCAAATTTAAG and ALKMUT-forward 5′-GGGGAGGACTATCACGGTTAGGAGTTGC.

The mutants were then subcloned into Pallino at HindIII/Xhol sites for retrovirus production.

Retrovirus and lentivirus production and cell infection. Retroviruses and lentiviruses for cell transduction were obtained as described previously (16). Cells were analyzed for the efficiency of transduction by enhanced green fluorescent protein content on a FACSCalibur flow cytometer (Becton Dickinson). When the efficiency of infection was ~80%, cells were sorted on a MoFlo High-Performance Cell Sorter (DAKO Cytomation) to normalize both the intensity of fluorescence and the percentages of transduced cells. Cell cycle analyses and TMRM stainings were done as described (18).

Semi-quantitative and quantitative RT-PCR analysis. CDNA was prepared from different lymphoma cell lines and semi-quantitative PCRs were done using the following primers: ZAP70 forward 5′-TGCGGTAGGAG and reverse 5′-ACATGGTGACCTCATGC; LAT forward 5′-ACAGGTTGCGGACCTAG and reverse 5′-CGTCAGTACTCATCAATGG; SLP76 forward 5′-AAAGGCGAAGGAGGCA and reverse 5′-GGACCTCTCTCCTGCTTGGAG; and CD3 forward 5′-TGGGACAGGAGGTTGAGG and reverse 5′-TTGACGGTTGAGGACGTTGAGGACG. Quantitative PCR was done using Quantitect SYBR Green PCR kit (Qiagen). Optimization of the quantitative PCR was done according to the manufacturer’s instructions but scaled down to 15 μl reaction. Nucleotide primers were used at 10 μmol/l for the detection and quantification of LAT. For quantitative PCR, the LAT primers used were forward 5′-ACTACGACTGCTGCCCATC and reverse 5′-CCGAGGCTTCAGTCAA.

Bisulfite modification, PCR, and sequencing. The methylation status of the 5′-region of ZAP70 was determined by bisulfite sequencing. Genomic DNA was extracted, denatured, and modified using CpGnome DNA Modification kit (Chemicon) and then PCR-amplified using the following primers: ZAP70 5′-TTTTATATTGATGTTGAAAATTGG and ZAP70 3′-TATCAGAAAACAAACACTCATT (20). PCR products were purified and cloned into the TA Cloning Vector System (Invitrogen). DNA from individual bacterial colonies was then automatically sequenced with SP6 and T7 primers.

Mice and in vivo experiments. NPM-ALK transgenic mice have been described previously (21). Mice were treated properly and ethically in accordance with European Community guidelines.

Results

ALCL expresses low to undetectable levels of most TCR complex–related molecules. To understand how broad the loss of TCR complex–related molecules is in ALCL, we analyzed their expression in primary lymphoma cases. By immunohistochemical stainings, the loss of CD3ε, ZAP70, LAT, and SLP76 was shown in the majority of the ALCL cases in contrast to other T-cell lymphomas, such as PTCL, where normal levels were found. Among ALCL, ALK+ cases showed the highest frequency of loss of TCR complex–related molecules (Fig. Lf; Table 1). We obtained similar results by analyzing protein expression in both ALK+ and ALK– human transformed T-cell lines. Most ALK+ ALCL lymphoma cells expressed undetectable or very low levels of CD3ε, ZAP70, LAT, SLP76, and LCK proteins compared with ALK− cell lines or peripheral blood mononuclear cells obtained from healthy donors. In contrast, the expression of molecules further downstream the TCR complex, such as Nck and VAV1, was unchanged (Fig. Lg). The analysis of mRNA levels by RT-PCR showed that ALK+ ALCL cell lines had either undetectable or very low levels of transcripts of these genes (Fig. 1C).

NPM-ALK downregulates the expression of TCR complex–related molecules in vitro and in vivo. The high frequency of loss of T-cell signaling molecules in ALCL prompted us to investigate the possibility that the oncogenic NPM-ALK tyrosine kinase, expressed in the majority of ALCL, could cause this phenotype. First, we investigated whether NPM-ALK could directly control the expression of these molecules in vitro. We transduced the ALK− cell line MAC-1 with a retroviral vector encoding NPM-ALK or the kinase-dead NPM-ALK(W210R) as control. MAC-1 cells transduced with NPM-ALK showed a downmodulation of ZAP70, SLP76, and protein levels (Fig. 24), in contrast to cells transduced with the inactive NPM-ALK(W210R), thus indicating a direct effect of the NPM-ALK tyrosine kinase activity on the regulation of the expression of proximal molecules downstream of the TCR. CD3ε regulation could not be addressed because these cells do not express it (Fig. 1B). Next, we asked whether NPM-ALK could also affect the expression of these molecules in vivo. To this end, we analyzed NPM-ALK transgenic mice that spontaneously develop lymphomas with a high frequency (21). In these mice, primary NPM-ALK...
lymphomas showed significant downregulation of ZAP70, LAT, and SLP76 proteins compared with wild-type thymocytes (Fig. 2B and C). This downregulation was evident in NPM-ALK+ T cells only after transformation. In fact, pretumoral thymocytes and splenic T cells displayed a phenotype and expression of proximal molecules downstream of the TCR comparable with control wild-type mice (ref. 21; data not shown). Altogether, our data indicate that the phenotype of primary human ALCL cells can be reproduced both in vitro and in vivo and that it is dependent on the tyrosine kinase activity of NPM-ALK.

Table 1. Immunohistochemical expression of TCR-related molecules in ALCL

<table>
<thead>
<tr>
<th></th>
<th>ALK</th>
<th>CD3*</th>
<th>ZAP70*</th>
<th>SLP76*</th>
<th>LAT*</th>
</tr>
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<tbody>
<tr>
<td>ALK+ ALCL</td>
<td>29/29 (100%)</td>
<td>6/29 (21%)</td>
<td>5/29 (17%)</td>
<td>8/29 (27%)</td>
<td>7/29 (24%)</td>
</tr>
<tr>
<td>ALK- ALCL</td>
<td>0/23 (0%)</td>
<td>9/23 (39%)</td>
<td>11/23 (48%)</td>
<td>11/23 (48%)</td>
<td>5/23 (22%)</td>
</tr>
<tr>
<td>PTCL</td>
<td>0/10 (0%)</td>
<td>10/10 (100%)</td>
<td>10/10 (100%)</td>
<td>10/10 (100%)</td>
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ALK+ ALCL vs PTCL:
- ALK*: $P < 0.0001 (\chi^2)$
- CD3*: $P < 0.0001 (\chi^2)$
- ZAP70*: $P < 0.0001 (\chi^2)$
- SLP76*: $P < 0.0001 (\chi^2)$
- LAT*: $P < 0.0001 (\chi^2)$

*Cases with >10% positive cells were considered as positive cases.
NPM-ALKK210R. Seventy-two hours after infection, the percentages of retrovirus or retroviruses containing NPM-ALK or the kinase-dead mutant were obtained in TS and SU-DHL1 cells where the NPM-ALK protein was knocked down by an inducible shRNA against NPM-ALK. Off-target effects were excluded by rescuing the expression of ALK with the retrovirus construct ALKALKMUT, which carries three-nucleotide substitutions in the region targeted by the shALK. Analysis by flow cytometry showed that DAC treatment increased the percentages of unmethylated CpG islands in both TS and SU-DHL1 lines (Supplementary Fig. S1D). In keeping with these observations, the ZAP70 intron 1-exon 2 boundary region was hypermethylated in DNA extracted from three cases of primary ALK+ ALCL (Supplementary Fig. S2B). Finally, we asked whether NPM-ALK could be directly responsible for the hypermethylation of the ZAP70 intron 1-exon 2 boundary region. We sequenced DNA purified from MAC-1 transduced with NPM-ALK or kinase-dead NPM-ALKK210R retroviruses. The ZAP70 intron 1-exon 2 boundary region showed increased methylation on transduction with NPM-ALK but not with the kinase-dead NPM-ALKK210R, thus suggesting a direct role of NPM-ALK kinase in the transcriptional repression of ZAP70 through the hypermethylation of its intron 1-exon 2 boundary region (Fig. 3D).

Overall, these results showed that inhibition for a short time (<12 h) of the ALK kinase activity allows the reexpression of LAT, suggesting a transcriptional regulation for this protein. On the other hand, CD3ε, ZAP70, and SLP76, but also LAT, are mostly regulated through gene hypermethylation.

CD3ε, ZAP70, LAT, and SLP76 expression in ALCL is controlled by NPM-ALK through transcriptional repression or gene hypermethylation. As shown in Fig. 1C, ALCL cell lines lack the mRNA transcripts of TCR complex-related molecules. To test whether in ALCL the constitutive NPM-ALK kinase activity is responsible for the lack of proteins and mRNA transcripts of those genes, we inhibited NPM-ALK kinase activity with the specific kinase inhibitor CEP-14083 (17) in TS and SU-DHL1 cells, two ALK+ lines derived from primary human ALCL. After 12 h of NPM-ALK activity inhibition, we detected increased mRNA levels (Supplementary Fig. S1A) and protein levels only for LAT (Fig. 3A), whereas CD3ε, ZAP70, and SLP76 proteins remained undetectable. No changes of these molecules were observed when the ALK– cell line Jurkat was treated with the same inhibitor (Fig. 3A). Similar results were obtained in TS and SU-DHL1 cells where the NPM-ALK protein was knocked down by an inducible shRNA against NPM-ALK (shALK). Off-target effects were excluded by rescuing the expression of ALK with the retrovirus construct ALKALKMUT, which carries three-nucleotide substitutions in the region targeted by the ALK shRNA, thus becoming resistant to the ALK shRNA downmodulation (Supplementary Fig. S1B and C). Unfortunately, longer times could not be analyzed because ALCL cells start to undergo apoptosis when the ALK kinase activity is inhibited for >12 to 14 h (17). We also tried to replicate these experiments by culturing some primary samples from ALCL patients with the ALK inhibitor. Most of the samples were not informative due to the low percentage of ALK+ cells among reactive T lymphocytes, and because of the poor spontaneous survival of ALK+ cells isolated ex vivo, survival further decreased by the ALK inhibitor treatment. However, in one primary sample rich of ALK+ cells, we observed a modest but consistent increase in LAT protein expression after treatment with the specific ALK kinase inhibitor CEP-14083. This inhibitor, as expected, abrogated ALK phosphorylation in the sample (Supplementary Fig. S1D).

Next, we analyzed whether the lack of TCR complex-related molecules in ALCL cells was due to gene hypermethylation. We treated TS and SU-DHL1 cells with the methyltransferase inhibitor DAC for up to 8 days. Indeed, DAC treatment restored the mRNA levels as well as protein expression of CD3ε, ZAP70, SLP76, and LAT (Fig. 3B and C). Because ZAP70 gene hypermethylation of the intron 1-exon 2 boundary region has been described to correlate with ZAP70 protein expression in chronic lymphocytic leukemia cells (20), we sequenced this region of the ZAP70 gene after bisulfite modification of DNA obtained from ALCL cells. We found that 8 CpG islands in the ZAP70 genomic region spanning from 223 to 384 bp after the transcription starting site, which are thought to regulate ZAP70 expression (20), were hypermethylated in TS and SU-DHL1 lines but not in MAC-1 (Fig. 3D). Moreover, treatment with DAC increased the percentages of unmethylated CpG islands in both TS and SU-DHL1 lines (Supplementary Fig. S2A). Because ZAP70 intron 1-exon 2 boundary regions were hypermethylated in DNA extracted from three cases of primary ALK+ ALCL (Supplementary Fig. S2B), we asked whether NPM-ALK could be directly responsible for the hypermethylation of the ZAP70 intron 1-exon 2 boundary region. We sequenced DNA purified from MAC-1 transduced with NPM-ALK or kinase-dead NPM-ALKK210R retroviruses. The ZAP70 intron 1-exon 2 boundary region showed increased methylation on transduction with NPM-ALK but not with the kinase-dead NPM-ALKK210R, thus suggesting a direct role of NPM-ALK kinase in the transcriptional repression of ZAP70 through the hypermethylation of its intron 1-exon 2 boundary region (Fig. 3D).

CD3ε, ZAP70, LAT, and SLP76 expression in ALCL is regulated by STAT3. Next, we investigated the mechanisms by which NPM-ALK regulates gene methylation. Because NPM-ALK mediates most of its transcriptional effects through STAT3 (22) and STAT3 has been shown to be involved in the epigenetic silencing of some genes in ALCL (23), we tested whether STAT3 was involved in the NPM-ALK–mediated regulation of TCR complex–related molecules. We transduced TS and SU-DHL1 cells with shRNA specific for STAT3 (19) and found that mRNA and protein levels for CD3ε, ZAP70, LAT, and SLP76 were upregulated when STAT3 was knocked down (Fig. 4A-C). A quantitative RT-PCR showed a 5-fold increase of LAT mRNA in lymphoma cells silenced for STAT3 expression (Fig. 4B). To exclude off-target effects of the shRNA...
against STAT3, we rescued the expression of STAT3 with a mutated (S3MUT) construct, which carries three point mutations in the region targeted by the shRNA. When shRNA-infected cells were rescued for STAT3 expression by complementation with S3MUT expressed by a retrovirus, none of the considered genes were upregulated either at the mRNA or at the protein level, indicating a specific effect of STAT3 downmodulation on their expression (Supplementary Fig. S3A and B). In keeping with these findings, TS and SU-DHL1 cells in which STAT3 was knocked down showed decreased CpG island hypermethylation in the ZAP70 intron 1-exon 2 boundary region (Fig. 4D). These data indicate that STAT3 is a fundamental regulator of the epigenetic silencing induced by NPM-ALK in ALCI cells.

NPM-ALK controls the expression of DNMT1 through STAT3. Inherited and de novo epigenetic methylation patterns are mediated by the DNMTs, which methylate newly synthesized CpG sequences during DNA replication or modify unmethylated DNA (24, 25). Protein levels of methyltransferases are thought to be responsible for changes in the methylation status of many genes (26). Therefore, we asked whether NPM-ALK could regulate the expression of DNMT1. To this end, we infected the ALK− Jurkat, CEM, and MAC-1 cell lines with NPM-ALK or NPM-ALKK210R retroviruses and analyzed the expression levels of DNMT1 by Western blot. After 72 h of culture, the forced expression of NPM-ALK caused a significant increase in DNMT1 expression (Fig. 5A), whereas DNMT1 and also DNMT3a were downregulated when NPM-ALK was knocked down in ALCI (Fig. 5B), thus suggesting an involvement of multiple methyltransferases in NPM-ALK−mediated silencing of T-cell–specific molecules. Off-target effects of the ALK shRNA were ruled out by reconstituting ALCI cells with a shRNA-resistant NPM-ALK mutant (Supplementary Fig. S4). STAT3 mediates many NPM-ALK−dependent effects, induces hypermethylation of different genes (23),

Figure 3. CD3ε, ZAP70, LAT, and SLP76 expression is controlled by NPM-ALK−mediated transcriptional repression or gene hypermethylation. A, TS, SU-DHL1, and Jurkat cells were treated with 300 nmol/L of the specific ALK kinase inhibitor CEP-14083 for 12 h and then lysed and immunoblotted for the indicated antibodies. Results are from one experiment and representative of five independent experiments. B and C, TS and SU-DHL1 cells were treated for the indicated time points with 1 μmol/L DAC and then analyzed either by RT-PCR on cDNAs (B) or by immunoblotting (C) for the expression of the indicated mRNA and proteins, respectively. Jurkat cells were used as controls. Results are from one experiment and representative of three independent experiments. D, DNAs purified from TS and SU-DHL1 cells and from MAC-1 cells untransduced or transduced with NPM-ALK or NPM-ALKK210R were treated with bisulfite. Sequences were obtained from the region that surrounds the intron 1-exon 2 boundary of the ZAP70 gene and contains 8 CpG islands. The locations of the analyzed CpG sites within the CpG island are shown. Arrow, transcription starting site (TSS); filled circles, methylated CpG sites; open circles, unmethylated sites.
and regulates the transcription of the DNMT1 gene in some malignant T lymphocytes given that STAT3 binds in vitro to two STAT3 SIE/GAS-binding sites identified in promoter 1 and enhancer 1 of the DNMT1 gene (27). Therefore, we asked whether STAT3 could regulate the expression of DNMT1 in ALCL cells. We knocked down STAT3 by transducing TS and SU-DHL1 cells with STAT3-specific shRNA lentivirus and observed a concomitant decrease of DNMT1 protein levels, whereas NPM-ALK and VAV1 expressions did not change (Supplementary Fig. S5). In contrast, DNMT1 expression levels did not change when cells were reconstituted with a mutant STAT3 resistant to the shRNA (Supplementary Fig. S3B). Altogether, these results indicated that NPM-ALK regulates the expression of DNMT1 through a STAT3-dependent pathway, similarly to a STAT3-dependent DNMT1 regulation shown in some ALK–lymphoma cell lines (27). However, other factors different from STAT3 could be involved in DNMT1 regulation in ALCL, because the knockdown of STAT3 had only a limited effect on DNMT1 levels and NPM-ALK was still able to upregulate DNMT1 in cells already expressing high basal levels of STAT3 phosphorylation, such as the MAC-1 cell line.

Discussion

In this article, we present experimental evidence that the tyrosine kinase activity of NPM-ALK induces the loss of TCR complex–related signaling molecules, therefore heavily influencing the T-cell phenotype of transformed cells and the T-cell identity in ALCL. Thus, we provide a molecular explanation for the “null” phenotype of the majority of ALCL that has been a long standing puzzle for this subtype of lymphoma (4). More importantly, these data show for the
first time that deregulated tyrosine kinase activity can control the phenotype of transformed T cells by inducing a broad loss of T-cell antigens. We also show that this loss is broader in ALCL than in other T-cell lymphomas (4, 8). Interestingly, abnormal tyrosine kinase activity has been recently shown in variable proportion in other types of T-cell lymphomas, most likely due to platelet-derived growth factor receptor overexpression and activation (28). Therefore, aberrant tyrosine kinase activity could contribute as well to the partial loss of T-cell phenotype observed in a fraction of other T-cell lymphomas. We also expect that this regulation of gene hypermethylation by NPM-ALK could be extensive and involve more genes than those described in the present article. To this end, we are currently investigating through genome-wide analysis the methylation pattern induced by NPM-ALK or STAT3 in ALCL.

The loss of T-cell identity induced by NPM-ALK might also involve other molecules that are characteristic of T lymphocytes. ALCL cells treated with DAC showed upregulation also of CD4 and CD5, whereas CD2 and CD8 remained unchanged (data not shown), thus suggesting that ALK might have an even broader effect on the phenotype of transformed T cells. The mechanisms by which NPM-ALK induces this loss of T-cell–specific molecules has been mostly clarified in this work. Both in vitro experiments and transgenic mice showed that NPM-ALK downmodulated the protein levels of CD3ε, ZAP70, LAT, and SLP76, all key molecules involved in the propagation of the TCR signaling (5). LAT appears to be regulated by both transcriptional repression and gene hypermethylation, because mRNA and protein were reexpressed after a short inhibition of NPM-ALK tyrosine kinase activity as well as after prolonged inhibition of DNMTs. In contrast, CD3ε, ZAP70, and SLP76 were primarily regulated by gene hypermethylation. This regulation is mostly dependent on STAT3, which activation is already known as a fundamental step in NPM-ALK–mediated transformation of lymphocytes (Fig. 6; ref. 22). In contrast to the well-known STAT3 functions in lymphomas by regulating gene transcription (29), evidences for STAT3-mediated gene methylation are limited. STAT3 has been recently recognized to control the hypermethylation, and thereby the transcription, of few other genes in ALCL, such as STAT5 and SHP-1. Both STAT3-mediated silencing of STAT5 and SHP-1 contribute to ALCL cell growth as well as to the maintenance of high NPM-ALK and JAK3 protein and phosphorylation levels (23, 30, 31). We now expand this function and add a new role for constitutively activated STAT3 in contributing to the T-cell identity of ALCL by gene hypermethylation. It is likely that these effects are the result of the constantly deregulated STAT3 activation observed in ALCL. However, an interesting and physiologically relevant question could be whether a constitutive STAT3 signaling regulates the phenotype and expression of reactive T cells as well, thus modulating the intensity of T-cell–mediated immune response by controlling the expression of TCR-related molecules. Furthermore, it would be interesting to study whether the phenotype of other tumors, such as solid cancers, is modified by a constantly deregulated STAT3 activation.

Similarly to ALK+ ALCL, a partial loss of TCR complex–related molecules was observed in ALK− ALCL, a different subtype of ALCL that has been recently indicated as a distinct provisional entity of lymphoma in the new WHO classification (ref. 4; Table 1). No specific oncogenic abnormalities have been described thus far in ALK− ALCL, thus leaving their pathogenesis quite obscure (4). Intriguingly, ALK− ALCL display STAT3 and STAT5 phosphorylation by yet unknown mechanisms in a fraction of cases (32). Despite lacking the mechanisms of STAT activation in such cases, it is conceivable that the loss of the T-cell phenotype could result from a deregulated STAT activation similarly to what we described for ALK+ ALCL, but this possibility remains to be investigated.

Figure 5. NPM-ALK controls the expression of DNMT1 through STAT3. A, Jurkat, CEM, and MAC-1 cells were transduced with the retrovirus containing NPM-ALK or the kinase-dead NPM-ALK<sup>KD120</sup>. Seventy-two hours after infection, the percentages of transduced cells were >75% in enhanced green fluorescent protein expression as assessed by flow cytometry. Cells were lysed and immunoblotted for the indicated antibodies. Results are from one experiment and representative of three independent experiments. B, TS and SU-DHL1 cells transduced with lentiviruses expressing an inducible shALK were treated with 1 μg/mL doxycycline for 96 h as described in Supplementary Fig. S2 and then lysed and immunoblotted for the indicated antibodies. The samples are the same as shown in Supplementary Fig. S1C. Bottom, densitometric analysis of the indicated bands.

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Oncogenic Tyrosine Kinase and T-Cell Identity

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A still unresolved question is whether the loss of TCR signaling molecules could give a biological advantage to ALCL cells. Following TCR engagement and activation, normal effector T cells undergo activation-induced cell death on ligation of their TCRs via a Fas-dependent apoptotic pathway (33). ALCL cells display the phenotype of experienced activated T cells because they express CD45RO, CD25, and MHC molecules (1). However, they are resistant to Fas-induced apoptosis despite a strong Fas expression (34). Thus, the lack of TCR signaling in ALCL cells could result in some biological advantages such as an increased resistance to activation-induced cell death pathways that are functional in non-transformed T cells, but further studies are needed to validate this hypothesis. A possible set of experiments could be based on the forcing the expression of some of the molecules that are down-regulated by NPM-ALK to ask whether their presence could have an influence of ALCL cell growth or survival.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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