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The AP-1 -BATF and -BATF3 module is essential for growth, survival and TH17 / ILC3 skewing of anaplastic large cell lymphoma

Running title: BATF and BATF3 in ALCL

Nikolai Schleussner[1,2*], Olaf Merkel[3,4*], Mariantonia Costanza[1,2,4], Huan-Chang Liang[3,4], Franziska Hummel[1,2], Chiara Romagnani[5,6], Pawel Durek[5], Ioannis Anagnostopoulos[7], Michael Hummel[7,8], Korinna Jöhrens[7], Antonia Niedobitek[1,2], Patrick R. Griffin[9], Roberto Piva[10], Henrike L. Sczakiel[1,2], Wilhelm Woessmann[4,11], Christine Damm-Welk[4,11], Christian Hinze[1,12], Dagmar Stoiber[13,14], Bernd Gillissen[2], Suzanne D. Turner[4,15], Eva Kaergel[1], Linda von Hoff[1], Michael Grau[16,17], Georg Lenz[16,17], Bernd Dörken[1,2,8], Claus Scheidereit[1], Lukas Kenner[3,4,14,18], Martin Janz[1,2], and Stephan Mathas[1,2,4,8]

[1]Max-Delbrück-Center for Molecular Medicine, 13125 Berlin, Germany; [2]Hematology, Oncology, and Tumor Immunology, Charité – Universitätsmedizin Berlin, 12200 Berlin, Germany; [3]Institute of Clinical Pathology, Medical University of Vienna, Vienna, Austria; [4]European Research Initiative on ALK-related malignancies (ERIA); [5]German Rheumatism Research Centre, A Leibniz Institute, 10117 Berlin; [6]Medical Department I, Charité - Universitätsmedizin Berlin, 12203 Berlin, Germany; [7]Institute of Pathology, Charité – Universitätsmedizin Berlin, 10117 Berlin; [8]German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany; [9]The Scripps Research Institute, Jupiter, Florida 33458; [10]Department of Molecular Biotechnology and Health Sciences, Center for Experimental Research and Medical Studies, University of Torino, Torino, Italy; [11]NHL-BFM Study Centre and Department of Paediatric Haematology and Oncology, Justus-Liebig-University, Giessen, Germany; [12]Department of Nephrology, Charité – Universitätsmedizin Berlin, 12200 Berlin, Germany; [13]Institute of Pharmacology, Center for Physiology and Pharmacology, Medical University Vienna, Vienna, Austria; [14]Ludwig Boltzmann Institute for Cancer Research (LBI-CR), Vienna, Austria; [15]Department of Pathology, University of Cambridge, Cambridge CB21QP, UK; [16]Translational Oncology, Department of Medicine A, Albert-Schweitzer-Campus 1, University Hospital Münster, 48149 Münster, Germany; [17]Cluster of Excellence EXC 1003, Cells in Motion, 48149 Münster, Germany; [18]University of Veterinary Medicine, Vienna, Austria. [*]These authors contributed equally

Address correspondence to:
Stephan Mathas, MD; Max-Delbrück-Center for Molecular Medicine and Charité – Universitätsmedizin Berlin, Hematology, Oncology and Tumor Immunology; Robert-Rössle-Str. 10; D-13125 Berlin, Germany; email: stephan.mathas@charite.de; Tel.: +49.30.94062863; Fax: +49.30.94063124,
or

Lukas Kenner, MD; Ludwig Boltzmann Institute for Cancer Research and Department for Experimental Pathology and Laboratory Animal Pathology, Medical University Vienna and Veterinarian Medical University Vienna; Währinger Gürtel 18-20, 1090 Vienna, Austria; email: lukas.kenner@meduniwien.ac.at; Tel.: +43.1.4040051760; Fax: +43.1.4040051930.

ABSTRACT

Transcription factor AP-1 is constitutively activated and IRF4 drives growth and survival in ALK$^+$ and ALK$^-$ Anaplastic Large Cell Lymphoma (ALCL). Here we demonstrate high-level expression of BATF and BATF3 in ALCL, irrespective of the ALK-status. Both BATFs bind classical AP-1 motifs and interact with in ALCL deregulated AP-1 factors. Together with IRF4, they co-occupy AP-1-IRF composite elements (AICE), differentiating ALCL from non-ALCL. Gene-specific inactivation of BATFs by CRISPR/Cas9 or siRNAs, or global AP-1 inhibition by the dominant-negative A-Fos results in ALCL growth retardation and/or cell death *in vitro* and *in vivo*. Furthermore, the AP-1-BATF module establishes TH17 / innate lymphoid cell type 3 (ILC3)-associated gene expression in ALCL, including marker genes such as *AHR*, *IL17F*, *IL22*, *IL26*, *IL23R*, *IL18R1* and *RORγt*. Elevated IL-17A and IL-17F levels were detected in pretreatment sera of a subset of children and adolescents with ALK$^+$ ALCL. Finally, pharmacological inhibition of RORC as single treatment leads to cell death in ALCL cell lines, and, in combination with the ALK inhibitor crizotinib, enforces death induction in ALK$^+$ ALCL. Our data highlight the crucial role of AP-1 / BATFs for ALCL biology and lead to the concept that ALCL might originate from ILC3 cells.

INTRODUCTION

Transcription factor (TF) activities and their regulated gene expression programs are crucial determinants of hematopoietic malignancies[1,2,3]. One example of lymphoid neoplasms with distinct dysregulated TF activities is anaplastic large cell lymphoma (ALCL).[4,5] The current WHO classification of lymphoid neoplasms distinguishes two ALCL entities: ALK-positive (ALK^+) ALCL, which is in most cases characterized by t(2;5)(p23;q35) creating the oncogenic NPM-ALK fusion protein, and ALK-negative (ALK^-) ALCL lacking translocations involving the *ALK* gene.[6] Both entities belong to the subgroup of peripheral T cell lymphomas (PTCL). Whereas the oncogenic activity of NPM-ALK is considered as causative of ALK^+ ALCL,[7] the pathogenesis of ALK^- ALCL is despite recent progress[8,9] less clarified.

Albeit both ALCL entities show differences with respect to genomic alterations or gene and miRNA expression levels,[10-12] phenotypically, both ALCL entities are highly similar and share biological and molecular key aspects.[13-15] This points to common pathogenic mechanisms. In particular, the deregulated TF programs in both ALCL entities overlap. They share STAT3 and NOTCH1 activation as well as high-level IRF4 and MYC expression and activity.[8,15-18] Moreover, we previously revealed a unique constitutive activation of AP-1 in ALK^+ and ALK^- ALCL, with the main constituents JUNB, JUN, FRA2 and the interacting basic region leucine zipper TF ATF3 (refs. 14, 19, 20). Several lines of evidence point towards a crucial role of these factors in ALCL biology: NPM-ALK induces JUNB and JUN,[21-23] genomic gains of the *JUNB* and *FRA2* loci are found in ALCL,[14,24] inhibition of AP-1 in ALK^+ ALCL results in cell cycle arrest and cell death,[19,22,25] and deletion of JUNB and JUN in mouse models impairs NPM-ALK-driven lymphomagenesis.[26] Finally, expression of the AP-1 interacting TF BATF3 distinguishes ALCL from other PTCL.[27]

BATFs, comprising BATF, BATF2 and BATF3, belong to the family of basic leucine zipper TFs which modulate transcription primarily by interaction with JUN proteins.[28] The

lack of a transactivation domain,[28] redundancy of the various BATFs,[29] and the number of interaction partners with positive or negative regulatory functions make the functional characterization of BATFs challenging. Whereas initially thought to act as transcriptional inhibitors, recent work highlighted positive regulatory functions of BATFs in particular within the lymphoid lineage.[28-30] This is exerted among others by mutual enforcement of DNA binding and combinatorial target gene regulation. In particular, IRF4 and BATF enhance each other´s DNA binding ability,[31] and they cooperatively bind to so-called AP-1-IRF composite elements (AICEs).[29,31,32] JUNB is the key interaction partner in these complexes. Moreover, STAT3, IRF4, JUNB and BATF TFs act in feed-forward loops and initiate the fate of T helper 17 (TH17) cells by priming the chromatin landscape of T cells towards that of TH17 cells, which subsequently enforces expression of the key TH17 TF RORC2 (murine RORγt).[33] Regarding this TF network and TH17-associated genes, characteristic features are shared with innate lymphoid type 3 (ILC3) cells.[34]

Given the role of BATF TFs in this regulatory network and expression of STAT3, IRF4, JUNB and BATF3 in ALCL we investigated expression and function of BATFs in ALCL.

MATERIALS AND METHODS

Cell lines, culture conditions and transfections

ALCL (Karpas-299 [named as K299], SU-DHL-1, DEL, JB6, SUP-M2, all ALK^+; Mac-1, Mac-2A, FE-PD, DL40, all ALK^-), T cell leukemia-derived (Jurkat, KE-37, Molt-14, H9) and HEK293 cell lines were cultured as described.[14] Where indicated, 1 μg/ml doxycycline (Sigma), the ALK-inhibitor crizotinib (Selleckchem), the RORC antagonists SR2211, SR1903 (both in-house generated, laboratory PRG) and GSK805 (Calbiochem), or DMSO control was added. For transient transfections and generation of A-Fos-inducible cells see **Supplementary Methods**.

RNA preparation and PCR analyses

RNA preparation, cDNA synthesis and semi-quantitative RT-PCR analyses were performed as described.[14] Primers are listed in **Supplementary Table 1**.

Preparation of protein extracts, Western blotting (WB), electrophoretic mobility shift assay (EMSA), and co-immunoprecipitation (CoIP) assays

Protein preparation, WB, EMSA and CoIPs were performed as described.[14] EMSA oligonucleotides are listed in **Supplementary Table 1**. Antibodies are indicated in **Supplementary Methods**. CoIP was performed as described[14] using 1,000 μg of protein with 2 μg of JUNB (sc-5052), BATF (sc-100974) or BATF3 (sc-398902; all Santa Cruz) antibody or isotype control (MAB002). Immunoblotting was performed using anti-JUNB (sc-8051), anti-BATF (8638S), anti-BATF3 (AF7437, R&D Systems), anti-BATF3 (sc-398902; Santa Cruz), and anti-β-actin antibody.

Chromatin immunoprecipitation (ChIP) assays and real-time PCR analyses

ChIP assays were performed in two biological replicates using ChIP-validated BATF (#8638; Cell Signaling), BATF3 (#AF7437; R&D Systems) and JUNB (#3753; Cell Signaling) antibodies according to a modified Millipore protocol. Primer sequences used for qPCR analyses are indicated in **Supplementary Table 2**. For detailed information see **Supplementary Methods**.

DNA constructs

For the pRTS-1 (ref. 36)-based inducible A-Fos expression vector, *A-Fos* was amplified from a CMV500-based construct[37] by use of primers A-Fos *Xba*I s 5´-GCTCTAGAAAGCTCCACCATGGACTACAAG and A-Fos *Xba*I as 5´-GCTCTAGAGAAGCTTGAATTAATCAGG, ligated into the *Xba*I site of pUC19, and mobilized by SfiI digestion for cloning into pRTS-1. CMV500-based A-Fos for constitutive expression has been described.[37] For *BATF*, *BATF3*, *RORC1* and *RORC2* expression constructs and lentiviral sgRNA and BATF and BATF3 constucts refer to **Supplementary Methods**.

siRNA-mediated knock-down of BATF and BATF3

Accell siRNAs were obtained from Dharmacon (**Supplementary Table 1**) and passively transfected into K299, JB6 and Mac-1 cells using RPMI1640 and 1% FCS. Cells were cultivated at 500 – 750 nM for 72 hours. Functional assays were performed in standard medium.

CRISPR/Cas9-mediated deletion of BATF and BATF3 in ALCL cell lines

The Cas9 containing plasmid lentiCRISPR v2 was a gift from F. Zhang (Addgene, Cambridge, MA, USA). gRNAs for *BATF* and *BATF3* were designed using E-CRISP program version 5.2 (www.e-crisp.org/E-CRISP/index.html) and targeted the second exon of

BATF and the first of *BATF3* (see **Supplementary Table 1**). For cloning of gRNAs into lentiCRISPR v2, lentiviral packaging, transduction and clone isolation as well as GFP-labeled BATF and BATF3 double knock-outs monitored over time see **Supplementary Methods**.

Murine xenograft experiments

A murine xenograft model was established by injecting $8x10^5$ K299 WT, BATF KO or BATF3 KO cells into both flanks of 7-9 weeks old NSG mice (NCI, Frederick, MD). Xenograft studies were approved by the institutional review board.

Immunohistochemistry (IHC) and mRNA extraction of primary lymphoma cases

For IHC analyses, BATF (sc-100974) or BATF3 antibody (sc-162246; both Santa Cruz) were applied 1:200. Bound antibody was visualized by APAAP and FastRed (DAKO). mRNA extraction of frozen lymphoma samples was approved by the Local Ethics Committee of the Charité – Universitätsmedizin Berlin and performed in compliance with the Declaration of Helsinki.

Processing and analysis of microarray data; gene set enrichment analysis (GSEA) and principal component (PC) analysis

For generation of TH17 and ILC3 signatures, microarray data for TH17, ILC3 and TH1 cells were obtained from GEO (GSE78897).[34] Human primary ALCL and PTCL data were obtained from GEO (GSE65823, GSE6338, GSE19069) **(PMID 26463425, 17304354, 19965671)**. ILC3 microarray data were obtained from GEO (GSE43409) (**PMID 27156452**). For microarray analyses of the cell lines, RNA processing and hybridization to Human Genome U133 Plus 2.0 arrays (Affymetrix) were performed according to the manufacturer´s recommendation. For processing details and GSEA and PC analyses refer to **Supplementary Methods**.

Additional Materials and Methods.

Detailed methodology is described in the **Supplementary Materials and Methods**.

RESULTS

Characterization of BATF-containing DNA binding complexes and physical interactions of BATF and BATF3 with JUNB in ALCL

To identify BATF-containing TF complexes in ALCL, we first analyzed AP-1 DNA binding activity at the classical AP-1 5´-TGA[G/C]TCA-3´ motif (**Supplementary Figure 1A**, upper panel). As in our previous studies,[14,15,19] we used a panel of ALK$^+$ and ALK$^-$ ALCL and T cell-derived control cell lines (from hereon referred to as *non-ALCL* cell lines). We verified an ALCL-restricted AP-1 DNA binding activity (**Supplementary Figure 1A**, upper panel) and high-level JUNB and IRF4 expression (**Supplementary Figure 1A**, lower panels).[15,19] Supershift analyses revealed DNA binding of the AP-1/FOS members JUNB and FRA2 as previously demonstrated,[14,19] and in addition strong BATF binding (**Figure 1A**), whereas BATF3 was only weakly detectable (data not shown). IRF4 did not bind to this motif. In other cellular systems, BATF-JUN drives gene expression together with IRFs from AP-1 IRF composite elements (AICEs), comprising among others 5´-IRF/AP-1- 3´ or 5´-IRF/NNNN/AP-1-3´ motifs.[29,31,32] The DNA binding activity at these AICEs differing in structure (0- and 4-bp spacing) and TF binding affinity was strong in ALCL, whereas it was absent in non-ALCL cell lines (**Figure 1A**, right panel). Supershift analyses demonstrated binding of FRA2 (weaker compared to the classical AP-1 motif), JUNB and, more prominently, of BATF as well as BATF3 and IRF4 (**Figure 1B**, left panel). By immunoprecipitations, we detected protein-protein interactions between BATF and BATF3 with JUNB specifically in ALCL cell lines (**Figure 1B**, right panel, and **Supplementary Figure 1B**). We did not detect an interaction with IRF4 (data not shown).

High-level expression of BATF and BATF3 in ALCL

The distinct DNA binding activities of BATF and BATF3 in ALCL indicated cell type-specific expression. Indeed, *BATF* mRNA expression was largely restricted to, and

BATF3 mRNA was exclusively expressed in ALCL cell lines (**Figure 1C**, upper left, and **Supplementary Figure 1C**). According to our microarray data from the various cell lines, *BATF2* was not expressed (**Supplementary Table XY**). We confirmed high expression of both BATFs at the protein level in all ALCL cell lines, whereas they were hardly detectable in any of the non-ALCL cell lines (**Figure 1C**, lower left). Some of the ALK$^-$ cell lines showed the highest BATF expression levels which might be reflected by the somewhat stronger DNA binding activity at the AICE_IL12RB site (see Figure 1A) in the respective cell lines.

Immunohistochemistry of BATF and BATF3 in human lymphoma specimens demonstrated nuclear localization (**Figure 1C**, right). Regarding BATF, among 70 non-ALCL B- and T-NHL, none of the mantle cell (MCL; 0/7), follicular (FL; 0/11) and Burkitt´s lymphomas (BL; 0/11) expressed BATF. 15 of 20 DLBCL showed varying numbers of positive lymphoma cells. All CLL cases (9/9; only in proliferative centers), 2/2 NLPHL and 9/9 PTCL (NOS) stained positive for BATF. We concluded that BATF expression is associated with distinct lymphoma sup-types and subpopulations. Importantly, strong staining was observed in 16/16 ALCL (7 ALK$^+$ / 9 ALK$^-$ cases) (**Figure 1C**, upper row) and 8/8 classical Hodgkin lymphoma (cHL) cases.

BATF3 showed a more restricted expression pattern. 16/16 ALCL (7 ALK$^+$ / 9 ALK$^-$ cases) (**Figure 1C**, lower row) and 8/8 cHL cases strongly stained positive, whereas among 70 B- and T-NHL (20 DLBCL, 10 MC, 9 CLL, 11 FL, 8 BL, 9 PTCL, 2 LPHL) only 1 CLL was BATF3-positive. Taken together, the simultaneous abundant expression of BATF and BATF3 was unique to ALCL and cHL.

CRISPR/Cas9-mediated deletion and siRNA knock-down of BATF and/or BATF3 in ALCL

We next defined the role of BATF and BATF3 in ALCL by gene-specific inactivation by CRISPR/Cas9-mediated knock-out (KO) in K299, SUP-M2 and Mac-1 cells (**Figures 2A** and **2B** and **Supplementary Figure 2A**). Interestingly, following BATF3

deletion BATF was upregulated. This phenomenon was also observed in SUP-M2 cells, in which BATF was virtually absent at baseline. Deletion of BATF or BATF3 in K299 (**Figure 2A**, left) or BATF3 in SUP-M2 cells (**Figure 2A**, center) resulted in sustained growth retardation, but did not alter the growth of Mac-1, in which we however observed the strongest counter-regulation of BATF and BATF3 (**Figure 2A, right**).

We were unable to generate BATF and BATF3 double KO cells in any of the ALCL cell lines, which suggested that complete loss of both BATFs is lethal to ALCL. However, to monitore the loss of CRISPR/Cas9-mediated BATF and BATF3 double knock-out cells we applied a strategy in which a GFP-coupled BATF-targeting guide-RNA was transduced in BATF3 single KO cells (**Figure 2B,** left and center, and **Supplementary Figure 2A**). To this end, BATF3 single KO K299 and Mac-1 cells described above were used as background, and transduced with GFP-labeled vectors carrying either BATF-targeting guide-RNA or, as a control, a non-targeting guide-RNA. In Mac-1 cells, despite the absence of an effect of either BATF or BATF3 single knock-out (see Figure 2A), transduction of BATF3 single KO cells with guide-RNA targeting BATF led to a rapid loss of GFP-positive cells over time. A similar effect was observed in K299 cells. The less pronounced effect compared to Mac-1 cells was most likely due to the growth retardation already observed in BATF3 single KO cells (see Figure 2A). Finally, in a xenotransplanted NSG mouse model, K299 BATF KO cells produced significantly smaller tumors compared to WT cells (**Figure 2B,** right), with a similar tendency for BATF3 KO tumors.

In a complementary approach we performed siRNA-mediated knock-down of both *BATF*s (**Figure 2C** and **Supplementary Figures 2B** and **2C**). Single knock-down of BATF (**Figure 2C**, left) did not alter viability of Mac-1 cells, whereas single knock-down of BATF3 (**Figure 2C**, center) moderately inhibited growth and induced cell death of Mac-1 cells. Importantly, simultaneous knock-down of both BATFs resulted in strong growth inhibition and apoptotic cell death induction (**Figure 2C**, right). This synergistic effect reflected our

inability to generate double *BATF* and *BATF3* KO clones using CRISPR techniques. Similar results were obtained with K299 cells (**Supplementary Figure 2C**). To demonstrate specificity of the toxic effects following simultaneous BATF and BATF3 knock-down, we showed reversion of toxicity by ectopic expression of BATF and BATF3 and concomitant use of siRNAs targeting the untranslated regions of the respective mRNAs (**Supplementary Figure 3A**). Together, these results further demonstrated the requirement of BATFs for growth and apoptosis protection of ALCL cells.

Induction of cell death following global abrogation of AP-1 DNA binding activity in ALCL

In an independent approach we globally inhibited AP-1 by a dominant repressor of AP-1 and leucine zipper TFs such as BATFs, named A-Fos.[37] These experiments complemented our approaches targeting BATFs, as BATF proteins alone have only low DNA binding affinity and require JUN proteins for heterodimer formation and DNA binding (**LIT**). We generated doxycycline (Dox)-inducible A-Fos FE-PD cells (**Figure 3A**), in which AP-1 was virtually abolished following Dox addition (**Figure 3A**, right panel). This strongly inhibited cell growth (**Figure 3B**, left panel) and induced cell death (**Figure 3B**, right panel). Similar results were obtained following transient A-Fos expression in K299 (**Supplementary Figure 3B**). These data indicated that constitutive AP-1 / BATF activity is essential for viability of ALK^+ and ALK^- ALCL cells.

ALCL shows a gene expression pattern characteristic for TH17 and ILC3 cells

JUN-BATF heterodimers, IRF4 and STAT3 coordinate a TH17 gene expression program.[33] As all these TFs are activated in ALCL we hypothesized that they impose a cellular fate resembling TH17 differentiation in these cells. Indeed, ALCL cell lines consistently expressed TH17-associated genes (**Figure 4A**). In particular, *AHR*, *IL1R1*, *IL4R*, *IL18R1*, *IL22*, *IL23R*, and *IL26* expression was a unifying feature of all ALCL cell lines.

Given the absence of a T cell receptor (TCR) rearrangement in approx. 14% of ALCL cases,[38] we reasoned that ALCL cells could be derived alternatively from ILC3. These cells are characterized by the absence of BCR or TCR gene rearrangements and, compared to TH17 cells, an overlapping but distinct gene expression pattern.[34]

To follow the idea that a TH17- or ILC3-like signature was an inherent feature of the overall ALCL expression pattern, we performed gene set enrichment analyses (GSEA) with our cell line panel (**Figure 4B**, upper panels). We defined based on published gene expression data[34] a TH17 and ILC3 signature, using the top 100 up- or downregulated genes compared to TH1 cells. Our ALCL cell lines showed significant enrichment for genes upregulated in TH17 cells (**Figure 4B**, left upper panel) and an even more prominent enrichment for the ILC3 signature, as indicated by the normalized enrichment score (NES) (**Figure 4B**, right upper panel). Consistently, principal component analysis (PCA) of ALK$^+$ (K299, DEL, JB6), ALK$^-$ (FE-PD, Mac-2A) and control (T; Jurkat, KE-37, Molt-14, H9) samples based on the top 100 differentially expressed genes between TH17 and ILC3 and TH1 signatures revealed a clear separation of ALCL and control samples along PC1 as judged by visual inspection as well as Welch´s-Test of the PC1 score (**Figure 4B**, lower panels). Moreover, ALCL cells were localized closer to additionally projected ILC3, again more significant for ILC3 signatures (*P* values ≤ 1.6E-9 for TH17 signature based PCA and 9.5E-17 for ILC3). Taken together, these analyses supported the concept of ALCL skewing towards a TH17 / ILC3 signature, and suggested an in-between or pending localization of ALCL between ILC3 and TH17 phenotypes with stronger skewing towards an ILC3 phenotype.

Expression of TH17 / ILC3 genes in primary ALCL; IL-22, IL-17A and IL-17F are secreted by ALCL cell lines; IL-17A and IL-17F are detectable in ALCL patients

We next aimed to confirm the expression of selected TH17- and ILC3-associated genes in primary lymphomas. In primary ALCL, TH17- / ILC3-associated genes were much

stronger or even exclusively expressed at the mRNA level in the majority of cases compared to the primary NHL controls including cases of PTCL-NOS (**Figure 4C** and **Supplementary Figure 4A**). Moreover, we detected IL-22 secretion in the three ALK^{-} cell lines with the highest *IL22* mRNA expression (**Supplementary Figure 4B**) and IL-17A and IL-17F secretion in various ALCL cell lines, correlating with mRNA expression (**Supplementary Figure 4B**). In primary ALCL, IL-17A was measurable by a cytometric bead array in three of 21 pretreatment serum/plasma samples of ALK^{+} ALCL patients and one healthy control (**Supplementary Figure 4C**). IL-17A was undetectable in patients in remission, and IL-17A levels in ALCL patients did not differ significantly from the other groups (P = 0.48). High levels of IL-17F were detected in four of the 21 ALCL patients, whereas no healthy control or patient in remission contained measurable IL-17F (**Supplementary Figure 4C**). Even though there was only a tendency towards a higher mean IL-17F level in ALK^{+} ALCL patients (P = 0.08), these data indicated a specific IL-17F up-regulation and secretion in a subset of ALK^{+} ALCL patients. We did not detect a significant correlation between detection of IL-17 and clinical or biological characteristics or treatment outcome of the respective lymphoma patients (data not shown).

Recruitment of BATF, BATF3 and JUNB to regulatory regions of TH17 / ILC3 genes and downregulation of TH17 / ILC3 genes following AP-1 inhibition in ALCL

To substantiate a direct regulation of TH17 / ILC3-associated genes by AP-1 complexes containing BATF or BATF3 we performed chromatin immunoprecipitations (ChIP) of BATF and BATF3 and as well as JUNB, which is the main interaction partner of BATF and BATF3 in these complexes. We analyzed promoter or enhancer regions of *IL1R1*, *IL12RB*, *IL17A*, *IL18R1*, *IL22*, *IL23R*, and *IL26* with the ALCL cell lines K299, JB6 and Mac-2A and the control cell line Jurkat (**Figure 4D** and **Supplementary Figure 5**). Most of the regulatory regions showed a strong BATF, BATF3 and JUNB recruitment in ALCL cells,

which was not observed in Jurkat cells. Functionally, we confirmed the involvement of AP-1 factors in the upregulation of TH17 / ILC3 genes by their expression analyses upon A-Fos-mediated AP-1 / BATF inhibition in K299 cells. Expression of *AHR*, *CCL20*, *IL4R*, *IL17A*, *IL22*, *IL23R* and *IL26* decreased following AP-1 inhibition (**Figure 4E**). **XY Target genes after double KO.**

Primary ALCL show a gene expression pattern characteristic for ILC3, while taking an intermediate position between two groups of PTCL when compared for the TH17 signature genes.

To globally approach the concept of TH17 / ILC3 skewing of primary ALCL we performed GSEA with microarray data of an extended number of primary ALCL and PTCL used in previous studies (**LIT**; **Figure 5**). A correlation analysis of gene expression of all samples (bracket **[a])** revealed two distinct PTCL clusters, one cluster positioned in bracket **[b]**, and one type positioned in bracket **[c]** of **Figure 5A**. In an overall analysis including all ALCL and PTCL samples (**Figure 5A,** bracket **[a]**) we found an enrichment of the ILC3 signature in ALCL (NES -1.5603; FDR 0.0209; **Figure 5B**, center top panel). This was in contrast to the TH17 signature, which did not show an enrichment in ALCL but was non-significantly enriched in PTCL (NES 1.1495; FDR 0.1939; **Figure 5B**, center bottom panel). The GSEA between both PTCL clusters revealed a strong enrichment of the TH17 signature in PTCLs positioned in bracket **[a]** compared to those positioned in bracket **[c]** (NES 3.2556; FDR 0.0; data not shown). These TH17-like PTCL in bracket [a] also showed an enrichment of the TH17 signature when compared to ALCL (NES 2.0998; FDR 0.0; **Figure 5B**, left bottom). The ALCL, however, showed an enrichment of the TH17 signature when compared to the PTCLs positioned in bracket **[c]** (NES -1.5021; FDR 0.0286; **Figure 5B**, right bottom). Remarkably, the ILC3 signature was enriched when compared to any of the PTCL clusters separately (**Figure 5B**, upper row). We concluded, that gene expression pattern characteristic

for ILC3 is a common feature of ALCL, while, regarding TH17 signature genes, ALCL take an intermediate position between PTCL with or without expression of TH17 genes. These data further supported our concept raised in the cell line panel, in which ALCL showed the strongest skewing towards ILC3 gene expression (see Figure 4B).

Expression of RORC2 (RORγt) in ALCL; RORC inhibition results in cell death induction in ALCL and synergizes with ALK-inhibitors

TH17 and ILC3 cells are characterized by a unique expression of RORC2, also known as RORγt.[39-41] The distinguishing feature of RORC2 from RORC1 is a different 5´ coding sequence, resulting in a molecular weight decrease of approx. 2 kDa (**Supplementary Figure 5C**, left).[42] *RORC2* was expressed in 5 of 8 ALCL cell lines, whereas it was absent in the controls (**Figure 6A**, upper left). *RORC1* was expressed in most of the cell lines, although with stronger expression in all ALCL cell lines. SU-DHL-1 lacked *RORC* expression (**Figure 6A**, left panel). At the protein level, we confirmed RORC overexpression and RORC2 restriction to ALCL cell lines (**Figure 6A**, lower left). Furthermore, robust *RORC2* mRNA expression was detectable in a subfraction of primary ALCL lymph node specimens in contrast to NHL control specimens, including 5 cases of PTCL-NOS (**Figure 6A**, center, and **Supplementary Figure 5C**, right). Functionally, *RORC2* expression decreased following AP-1 / BATF inhibition by A-Fos (**Figure 6A**, right panel), which again supported the link between AP-1 / BATF activity and TH17 / ILC3 gene expression.

Finally, we investigated the effect of pharmacological RORC inhibition in ALCL. Treatment of the ALCL cell lines K299, JB6 and Mac-2A with the inhibitory RORC modulators SR2211 (ref. 43) and SR1903 (a close analog of SR2211) resulted in a decrease in viable cells over time (**Figure 6B**, upper row). No effect was observed in cell lines without (KE-37) or low level (Jurkat) RORC expression and in FE-PD cells with RORC1 but no RORC2 expression (**Figure 6B**, lower row). Moreover, we investigated the effect of the

RORC inhibitors SR2211, SR1903 and GSK805 (ref. 44) in combination with ALK inhibition in the ALK$^+$ ALCL cell lines K299, DEL and JB6 (**Figure 6C**). These experiments were performed at concentrations at which the ALK inhibitor crizotinib or the RORC antagonists SR2211, SR1903 and GSK805 alone induced no or only moderate cell death. Remarkably, the combination of crizotinib with RORC inhibitors enhanced cell death induction in ALK$^+$ ALCL cell lines.

DISCUSSION

We demonstrate here that ALK$^+$ and ALK$^-$ ALCL are characterized by an unprecedented activation of AP-1 family and the leucine zipper TFs BATF and BATF3. Even though BATF expression is found in other lymphoma entities than ALCL, the simultaneous high-level expression of both BATFs is a particular feature of ALCL. This is in accordance with the fact that BATF3 expression distinguishes ALCL from other PTCL.[27] BATF and BATF3 thus add a new layer of complexity to deregulated AP-1 in ALCL.[14,19,20] Remarkably, even though NPM-ALK induces JUNB,[22] ALK$^+$ and ALK$^-$ ALCL neither differ in their global AP-1 activity nor in expression of distinct AP-1 factors (refs. 14, 19, 20 and this work). These data support our hypothesis that ALK$^+$ and ALK$^-$ ALCL share a common pathogenic mechanism.[45,46] In favour of this hypothesis, recent work demonstrated a high similarity of the epigenome between ALK$^+$ and ALK$^-$ ALCL.[45]

AP-1 forms homo- or heterodimers and exerts cell-type and differentiation stage-specific functions,[47] and thereby activates or inhibits transcription.[47] These interactions make experimental approaches to single AP-1 factors distinctly challenging. Furthermore, AP-1 effects certainly differ between transient and the long-term activation observed in ALCL, as it is known for e.g. varying temporal NF-κB activation.[48] Despite these challenges, we present evidence that BATF and BATF3 are essential components of the TF network in ALCL. First, CRISPR/Cas9-guided single deletion of BATF or BATF3 in ALCL cell lines caused growth retardation *in vitro* and *in vivo*. Our inability to generate BATF and BATF3 double KO cells indicates the lethality of combined BATF deletion to ALCL. Second, we observed a comparable phenotype with siRNA-mediated knock-down of BATF and/or BATF3, in which the combined knock-down resulted in pronounced growth arrest and cell death induction. The cross-regulation detected in our cell lines and in genetically manipulated mice and a functional redundancy[28,29] might attenuate effects of targeting single BATFs. BATFs modify transcriptional activity by interaction with AP-1 TFs like JUNB and JUN[28], both highly

activated in ALCL.[19,25] In line with their concerted activity, global AP-1 inhibition caused death of ALCL cells (this work and ref. 19).

Apart from the interaction of BATFs with AP-1, we describe a composite DNA binding activity at AICEs with IRF4. Such combinatorial activity coordinates TH17-instructive genes expression.[30,32,33] Our comprehensive analysis of TH17 genes suggests a restriction to ALCL, and their expression depends at least in part on AP-1 / BATFs. In ALK$^+$ ALCL, expression of some TH17 genes has been reported,[49-51] and NPM-ALK-induced miR-135b enforces IL-17 production.[51] However, our data demonstrate that TH17 gene expression is a unifying feature of ALK$^+$ and ALK$^-$ ALCL.

Furthermore, the ILC3 gene set, which overlaps with that of TH17 cells,[34] is more strongly enriched in ALCL, compared to the TH17 gene set. This is of particular relevance regarding the cellular origin of ALCL and opens a new view on ALCL pathogenesis. Only recently, germline TCR configuration has been reported in 14% of ALCL.[38] The absence of BCR or TCR rearrangements is as much a hallmark of ILC3 as the lack of typical B or T cell markers.[52,53] ILC3 are enriched in human tonsils and the intestinal lamina propria, but also circulate in the peripheral blood.[52] Whereas nearly all known hematopoietic cell types give rise to malignancies, no ILC3-derived neoplasm is known so far. We propose that a subfraction of ALCL originates from ILC3. The expression of key ILC3 genes, the lack of B or T lymphoid surface markers as well as the lack of a genomic lymphoid fingerprint in a fraction of ALCL is in accordance with such a hypothesis. Alternatively, the deregulated TF network might superimpose a TH17 / ILC3 cellular fate on ALCL cells with a more mature T cell origin, irrespective of the particular cell of origin at the beginning of the transformation process.

Apart from these implications, our work provides new aspects for targeted treatment strategies for ALCL. Due to their involvement in autoimmune and inflammatory diseases, inhibitors of TH17 cells are developed.[54,55] The potential of TH17 interference to ameliorate

such diseases in preclinical mouse models led to clinical trials. For example, IL17-neutralizing antibodies and small compounds targeting RORC are evaluated in inflammatory skin diseases.[56,57] Interference with TH17 gene activity might thus provide a treatment strategy for ALCL, as RORC-inhibitory small compounds partially induce cell death of ALCL cell lines. Such targeted treatment strategies are not only required for ALK^{-} ALCL, but also for ALK^{+} ALCL patients. Among those, treatment with ALK inhibitors exerts long-term disease control,[58] but is obviously unable to eradicate the respective lymphoma clone.[59] The synergistic activity of RORC inhibitors together with ALK inhibitors might represent a possible strategy to eradicate such persisting ALCL cell populations. In addition, our work provides the basis for future studies exploring interference with e.g. IL-17 and IL-26 circuits and for further evaluation of TH17-related cytokines as diagnostic and prognostic markers for ALCL, as also suggested by an independent study.[49]

Overall, we identify high-level BATF and BATF3 as essential components of the transcription factor and gene regulatory network in ALCL and demonstrate their pathogenic and therapeutic relevance. Furthermore, we provide the hypothesis that a subset of ALCL originates from ILC3, a finding that has to be elaborated in future studies.

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FIGURE LEGENDS

Figure 1. ALCL-specific BATF and BATF3 binding at AP-1 and AICE sites; co-immunoprecipitation of BATFs and JUNB; BATF and BATF3 expression in ALCL. (**A**) Left panel, EMSA of complexes bound to AP-1 TRE without (-) or with addition of specific antibodies, or isotype control (IC). Positions of the AP-1 complex, supershifts (ss), and a non-specific band (n.s.) are indicated. Right panel, IRF/AP-1 DNA binding at AICE (AICE_Bcl11b; AICE; AICE_IL12RB) analyzed by EMSA. Underlined, IRF motif; bold, AP-1 motif; grey, intervening bases. The free probe of one representative EMSA is shown. (**B**) Left panel, EMSA by use of AICE_Bcl11b, performed as in (A). Right, JUNB and BATF co-immunoprecipitations. Whole cell extracts were immunoprecipitated (IP) with anti-JUNB (upper panels), anti-BATF (lower panels) or isotype controls (IC). (Co-) immunoprecipitated proteins were detected by immunoblotting (WB). β-actin and input extracts were analyzed as controls. (**C**) Left, BATF and BATF3 were analyzed in lymphoma cell lines at mRNA level by RT-PCR (upper panel) and at protein level by immunoblotting of nuclear extracts (lower panel). *GAPDH* and PARP1 were analyzed as controls. Right, representative examples of BATF and BATF3 immunohistochemistry (IHC) of primary lymphomas. Upper row, BATF IHC of an ALK$^+$ ALCL (a), an ALK$^-$ ALCL (b), and a mantle cell lymphoma [MCL; (c)] case. Lower row, BATF3 IHC of an ALK$^+$ ALCL (d), an ALK$^-$ ALCL (e), and a DLBCL (f) case.

Figure 2. CRISPR/Cas9-mediated deletion and siRNA-mediated knock-down of BATF and BATF3 in ALCL. (**A**) CRISPR/Cas9-mediated deletion of BATF and/or BATF3 in K299 (left), SUP-M2 (center) and Mac-1 (right) cells. Upper panels, immunoblotting of wild-type (WT), control-treated (CRISPR CTL), BATF KO and/or BATF3 KO cells for BATF and BATF3. Note the compensatory increase of BATF expression following BATF3-deletion. Lower panels, cell numbers are shown over time. (**B**) Left and center, BATF3 single

knock-out K299 (left) and Mac-1 (center) cells were transduced with a GFP-labeled vector encoding BATF targeting guide RNA (BATF KO) or non-targeting control (CRISPR CTL). The percentage of GFP-positive cells was monitored over time and is indicated as GFP fold change. Right, xenotransplantation of K299 WT, BATF KO and BATF3 KO cells on NSG mice. Tumor weight at day 14 is shown in gram (g). Right, representative examples of tumors at day 14. (**C**) siRNA-mediated knock-down of BATF and/or BATF3 in Mac-1. Cells were treated with control siRNAs (siCTL #1 and siCTL #2), siRNAs targeting BATF (siBATF #1 and siBATF #2; left panels) or BATF3 (siBATF3 #1 and siBATF3 #2; center), or respective combinations (right panels). Knock-down was confirmed by immunoblotting (WB) (top panels). Cell numbers (upper graphs), [^{3}H]-thymidine incorporation (center) and percentage of Annexin V-positive cells (lower graphs) are shown over time. **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

Figure 3. Inhibition of global AP-1 activity by its dominant-negative A-Fos in FE-PD cells. (**A**) Inducible A-Fos expression abrogates constitutive AP-1. Left, following Dox addition for 48 h, > 80% of cells were GFP-positive. Cells were analyzed by transmitted light microscopy (upper panel) and UV fluorescence at 280 nm (lower panel). Right, immunoblotting (WB) for A-Fos expression (upper panel; FLAG antibody) and EMSA for DNA binding to TRE site (center, top) and BCL_11b AICE (center, bottom) after Dox addition. β-actin and Sp1 DNA binding were analyzed as controls. Two independent experiments (#1 and #2) are shown. (**B**) Reduced [^{3}H]-thymidine incorporation (left panel) and increased cell death (right panel) following A-Fos induction. Left, data of triplicates from two independent experiments (#1 and #2) are represented as means ± SD. Right, the percentage of viable cells measured by PI staining and flow cytometry is shown over time. *, $P < 0.05$; ***, $P < 0.001$; n.s., not significant.

Figure 4. TH17 / ILC3 gene expression in ALCL and link to the deregulated BATF/BATF3/AP-1 activity. (**A**) mRNA expression of TH17 / ILC3-associated genes and, as control, *GAPDH* were analyzed by RT-PCR. (**B**) More global approaches to TH17 / ILC3 gene expression in ALCL. Upper panels, GSEA of differentially expressed genes between ALCL cell lines (K299, SU-DHL-1, DEL, JB6, FE-PD, Mac-2A) and control (CTL) samples (Jurkat, KE-37, Molt-14, H9) based on TH17 (left panel) and ILC3 (right panel) top 100 up-regulated genes. Lower panels, PC analyses of ALK^+ (K299, SU-DHL-1, DEL, JB6) and ALK^- ALCL (FE-PD, Mac-2A) as well as CTL samples (Jurkat, KE-37, Molt-14, H9) based on 100 top differentially expressed TH17 (left) or ILC3 (right) genes, separating ALCL and CTL cell lines along the PC1 axis. PCAs were supplemented by projection of ILC3 samples.[41] n.s., not significant; *, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. (**C**) mRNA expression of TH17 / ILC3-associated genes in 12 ALCL and 5 FL as analyzed by RT-PCR. *, ALK^+ ALCL; **, ALK status not known. (**D**) BATF (upper panel) and BATF3 (lower panel) ChIP from K299 cells. Input and precipitated DNA were amplified by qPCR for the indicated promoter or enhancer regions. Data of two biological replicates were combined and are shown as mean ± SD. (**E**) Inhibition of AP-1 leads to down-regulation of TH17 / ILC3 genes. A-Fos or Mock transfected, GFP-positive K299 cells were enriched, and mRNA expression of the indicated TH17 / ILC3 genes was analyzed by RT-PCR. Two (#1 and #2) of four independent experiments are shown. (**F**) **XY**

Figure 5. TH17 / ILC3 gene set enrichment analyses of primary ALCL and PTCL. (**A**) Pearson correlation heatmap between ALCL and PTCL samples. Samples are clustered by the Euclidean distance and separate into three major clusters. (**B**) GSEA of ALCL and PTCL samples from indicated clusters, marked by brackets underneath the heatmap shown in (A). Note, while ALCL shows an overall enrichment of the ILC3-signature (GSEA of upper row), the TH17 signature-enrichment is decreasing from left to the right.

Figure 6. Expression and inhibition of RORC2 in ALCL. (**A**) Left, analysis of RORC1 and RORC2 mRNA by RT-PCR (upper panel) and of RORC2 protein expression by immunoblotting of nuclear extracts (lower panel) in lymphoma cell lines. *GAPDH* and PARP1 were analyzed as controls. Center, *RORC2* expression in 7 ALCL, 5 FL and 5 PTCL-NOS cases as analyzed by RT-PCR. *, ALK^+ ALCL; **; ALK status not known. The *GAPDH* control of the upper panel is the same as in Figure 4C. Right, inhibition of AP-1 leads to down-regulation of *RORC2*. K299 cell were treated as in Figure 4E, and *RORC2* mRNA expression was analyzed by RT-PCR. Two (#1 and #2) out of four independent experiments are shown. The *GAPDH* control is the same as in Figure 4E (upper panels). RORC2 expression changes at the protein level in similarly treated cells were analyzed by WB (lower panels). (**B**) RORC inhibition by small compounds induces cell death of ALCL cell lines. Cells were treated with 5 μM of the RORC inhibitors SR2211 or SR1903, or the control (DMSO), and the percentage of viable cells was analyzed by PI staining. One of three independent experiments is shown. (**C**) Enforced cell death induction by crizotinib in combination with RORC inhibitors. The ALK^+ cell lines K299, DEL and JB6 cells were left untreated, or treated with DMSO control, the RORC inhibitors SR2211 (7.5 μM), SR1903 (7.5 μM) and GSK805 (7.5 μM), or 25 nM crizotinib (Crizo) alone, or the different RORC inhibitors together with crizotinib. Induction of cell death was analyzed by Annexin V-FITC / PI staining. The percentage of viable cells is shown. Experiments were performed in triplicates and results are shown as mean ± SD. One of three independent experiments is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.