Gene regulation and suppression of type I interferon signaling by STAT3 in diffuse large B cell lymphoma

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STAT3 is constitutively activated in many cancers and regulates gene expression to promote cancer cell survival, proliferation, invasion, and migration. In diffuse large B cell lymphoma (DLBCL), activation of STAT3 and its kinase JAK1 is caused by autocrine production of IL-6 and IL-10 in the activated B cell–like subtype (ABC). However, the gene regulatory mechanisms underlying the pathogenesis of this aggressive lymphoma by STAT3 are not well characterized. Here we performed genome-wide analysis and identified 2,251 STAT3 direct target genes, which involve B cell activation, survival, proliferation, differentiation, and migration. Whole-transcriptome profiling revealed that STAT3 acts as both a transcriptional activator and a suppressor, with a comparable number of up- and down-regulated genes. STAT3 regulates multiple oncogenic signaling pathways, including NF-κB, a cell-cycle checkpoint, PI3K/AKT/mTORC1, and STAT3 itself. In addition, STAT3 negatively regulates the lethal type I IFN signaling pathway by inhibiting expression of IRF7, IRF9, STAT1, and STAT2. Inhibition of STAT3 activity by ruxolitinib synergizes with the type I IFN inhibitor lenalidomide in diffuse large B cell lymphoma in vitro and in a xenograft mouse model. Therefore, this study provides a mechanistic rationale for clinical trials to evaluate ruxolitinib or a specific JAK1 inhibitor combined with lenalidomide in ABC DLBCL.

RESULTS

Genome-Wide Analysis Identifies STAT3 Transcriptional Target Genes in ABC DLBCL Cells. To identify STAT3 target genes genome-wide, we performed STAT3 ChIP-seq in the ABC DLBCL cell lines TMD8 and OCI-Ly10, in which high levels of STAT3 phosphorylation were detected by immunoblot analysis (Fig. L4). Since STAT3 activity was sufficiently inhibited by the JAK1/JAK2 inhibitor AZD1480 (Fig. L4) and this inhibitor was used for H3Y41-P ChIP-seq analysis (3), AZD1480-treated cells served as a control for STAT3 ChIP-seq experiments. Using the model-based analysis

Significance

We demonstrate that STAT3 is a critical transcriptional regulator of the activated B cell–like subtype of diffuse large B cell lymphoma (ABC DLBCL), the most common, aggressive, non-Hodgkin lymphoma. By genome-wide assessment, we have identified target genes of STAT3. Gene regulation by STAT3 in ABC DLBCL accentuates survival signaling pathways while dampening the lethal type I interferon pathway. Knowledge of these STAT3-regulated genes has led to our demonstration that a small-molecule inhibitor in the JAK1/STAT3 signaling pathway synergizes with the type I interferon inducer lenalidomide, suggesting a new therapeutic strategy for ABC DLBCL, a subtype that is particularly difficult to treat and has poor prognosis.


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of ChIP-seq (MACS) for peak calling (16), we identified a total of 11,487 STAT3-binding sites (peaks) in TMD8 cells and 22,856 in OCI-Ly10 cells compared with the AZD1480-treated control sample (Fig. 1B and Dataset S1). Specificity of these STAT3-binding
sites was confirmed by the MEME motif enrichment analysis (15), with a similar distribution pattern of STAT3 motifs between the two cell lines (Fig. 1C).

Based on genomic loci of these peaks, we mapped near a protein-coding gene within a window extending from −15 kb 5′ of the transcriptional start site to the 3′ end of any annotated transcript...
associated with the gene, as for our previous study (3). We identified 4,746 potential STAT3 target genes in TMD8 cells and 6,058 in OCI-Ly10 cells, with an overlap of 2,251 genes between the two cell lines (Fig. 1D and Dataset S1). Considering these overlapped genes as common STAT3 targets in ABC DLBCL, we performed PANTHER gene ontology analysis (17). The results revealed that these common target genes were enriched for biological processes that include B cell activation, proliferation, differentiation, cell-cycle progression, stress response, cell migration, and metabolism (Fig. 1E), suggesting an important role for STAT3 in the pathogenesis of ABC DLBCL.

**Whole-Transcriptome Profiling Reveals That STAT3 Acts as both a Transcriptional Activator and a Repressor in ABC DLBCL.** To determine genes that are directly regulated by STAT3, we performed the whole-transcriptome analysis by RNA-seq in the above TMD8 and OCI-Ly10 cell lines.

**Fig. 3.** STAT3 suppresses type I IFN signaling in ABC DLBCL. (A) STAT3 is recruited to regulatory regions of STAT1, STAT2, IRF7, and IRF9, as shown by read density tracks (DMSO controls in red, AZD1480-treated samples in green). (B) Heat maps show expression changes of type I IFN pathway genes in TMD8 and OCI-Ly10 cells after 2 d of knockdown of STAT3 by two shRNAs. STAT3-binding genes are shown in red. (C) Immunoblot analysis of the indicated proteins in TMD8, OCI-Ly10, and the control cell line SUDHL7 after 2 d of knockdown of STAT3 by shRNA. (D) Immunoblot analysis of the indicated proteins in TMD8 and HBL1 cells after 2 or 4 d of retroviral expression of constitutively activated STAT3 (STAT3-C).
OCI-Ly10 cell lines. We knocked down STAT3 by two shRNAs from our previous study (7). As shown in Fig. 24, 53% (2,495/4,746) of the STAT3 target genes in TMD8 cells and 68% (4,146/6,058) in OCI-Ly10 cells changed their expression when STAT3 was knocked down. Of note, the number of down-regulated genes was comparable to that of up-regulated genes, suggesting that
STAT3 functions as both a transcriptional activator and a repressor in ABC DLBCL cells.

Next, we performed gene set enrichment analysis (GSEA) to identify the signaling pathways in which these up-regulated and down-regulated genes are involved. The results revealed significant enrichment in gene signature of multiple signaling pathways (Fig. S1), including the PI3K/AKT/mTORC1 (Fig. S2), E2F/G2M cell-cycle checkpoint (Fig. S3), IL6-JAK-STAT3, NF-kB, and type I IFN signaling pathways. Consistent with previous studies (4, 5, 14), STAT3 is involved in the positive feedback regulation of the IL-6/IL-10 signaling and shows crosstalk with NF-kB signaling pathways in ABC DLBCL cells (Fig. 2 B–D).

We verified SOCS3, a negative regulator of JAK-STAT signaling (18), as a target gene of STAT3 (Fig. 2 D). More significantly, 16 of the genes down-regulated by STAT3 shRNAs are direct STAT3 targets, including TNFAIP8, TRAF1, CD44, CD69, BCL3, FOS, SGK1, NF-kB2, and STAT3 itself (Fig. 2 B and C).

**STAT3 Suppresses Type I IFN Signaling in ABC DLBCL.** In ABC DLBCL, production of the proapoptotic cytokine IFNβ can result from oncogenic MYD88 mutations (11). This type I IFN signaling is suppressed by the transcription factors IRF4 and SPIB, which repress IRF7 expression to prevent IFNβ transcription and TYK2 activation (12). Our recent work demonstrated that IRF4 and SPIB are epigenetic targets of JAK1 due to H3Y41 phosphorylation, but IRF4 expression is not regulated by STAT3 (3). These findings along with the above RNA-seq analysis prompted us to investigate whether STAT3 directly targets critical genes in the IFNβ signaling pathway. It is known that, in response to IFNβ, STAT1 and STAT2 are phosphorylated, together with IRF9, to form the tripartite transcription factor IFN-stimulated factor gene 3 (ISGF3), which binds to distinct IFN-stimulated elements of genomic DNA for gene transcription (19, 20).

Indeed, STAT3 ChIP-seq data displayed peaks in the promoter region, near the transcription start sites of STAT1, IRF7, and IRF9, and a peak in the enhancer region of STAT1. These peaks were significantly reduced after AZD1480 treatment, suggesting that they are direct targets of STAT3 (Fig. 3 A). Increased expression of STAT1, STAT2, and IRF9 was observed when STAT3 was knocked down by two different shRNAs in both TMD8 and OCI-Ly10 cell lines (Fig. 3 B).

Immunoblot analysis confirmed that protein levels of STAT1, STAT2, IRF7, and IRF9 were all increased by STAT3 shRNA in these two ABC DLBCL cell lines but not in the control GCB cell line SUDEHL7 (Fig. 3 C). In addition, phosphorylation of STAT1 and STAT2 was remarkably increased in the STAT3 shRNA expressing ABC DLBCL cells (Fig. 3 C). To further validate these results, we used the constitutively activated form of STAT3 (STAT3-C) with activating mutations (A661C and N663C) in the SH2 domain (21). Retroviral expression of STAT3-C in OCI-Ly10 and HBL1 ABC DLBCL cells reduced IRF7 and IRF9 expression and completely removed STAT1 phosphorylation (Fig. 3 D). Taken together, these data suggest that STAT3 activity blocks the type I IFN signaling pathway by inhibiting expression of multiple essential signaling components, including STAT1, STAT2, IRF7, and IRF9.

**Synergism Between STAT3 Inhibition and Lenalidomide in Growth Inhibition of ABC DLBCL.** Lenalidomide, an active agent in ABC DLBCL, induces type I IFN response by down-regulation of IRF4 and SPIB, which otherwise inhibit IRF7 expression (12, 22). Given partial inhibition of IRF4 expression by lenalidomide (12) and that IRF4 is not a direct target of STAT3, we hypothesized that inhibition of STAT3 activity augments IFNβ production and synergizes with lenalidomide in killing ABC DLBCL cells. To test this hypothesis, we performed an in vitro survival assay in TMD8 and OCI-Ly10 cells when STAT3 shRNA was induced for expression in the presence of lenalidomide. We used the GCB DLBCL cell line SUDEHL7 as a control. As shown in Fig. 4 A, after 3 d of culture, both STAT3 shRNA and lenalidomide significantly reduced cell viability in the two ABC DLBCL cell lines but not in the control. Of note, expression of STAT3 shRNA increased lenalidomide-mediated cytotoxicity in these ABC DLBCL cultures (Fig. 4 A). A reduction in viable cells was mainly due to inhibition of cell proliferation (Fig. S3 B) although a slight increase in apoptosis was observed (Fig. S4). Quantitative PCR analysis confirmed that IFNβ expression was significantly increased in cells that expressed STAT3 shRNA and were treated with lenalidomide, consistent with the above survival assay demonstrating a cytotoxic synergism between STAT3 shRNA and lenalidomide (Fig. 4 B).

To further examine the above synergistic effect, we used ruxolitinib, a clinically used JAK1 and JAK2 inhibitor (23), to...
inhibit STAT3 activity. Immunoblot analysis confirmed dose-dependent inhibition of STAT3 phosphorylation in both TMD8 and OCI-Ly10 cell lines (Fig. 4C). As expected, we observed a synergism between ruxolitinib and lenalidomide in killing these cells (Fig. 4D). Cell-cycle analysis revealed that a combination of the two drugs increased G1 phase population (Fig. 4E), suggesting inhibition of cell proliferation. More importantly, our xenograft analysis in the OCI-Ly10 cell line demonstrated that cotreatment of ruxolitinib and lenalidomide caused nearly complete tumor growth inhibition during the period of treatment, but the single drug treatment achieved only partial inhibition (Fig. 4F). Thus, these data suggest that the ruxolitinib and lenalidomide combination is a potential therapeutic strategy for ABC DLBCL cases.

Discussion

Deregulation of the JAK-STAT signaling pathway, such as constitutive activation of STAT3, plays a pathogenic role in many hematologic malignancies (24–26). In DLBCL, STAT3 is activated in the ABC subtype by IL-6/IL-10 and JAK1 to promote cancer cell survival (3–7). STAT3 activity is also associated with a poor prognosis in DLBCL (27, 28). Here, we conducted genomewide assessment and established a working model of STAT3 in the pathogenesis of ABC DLBCL (Fig. 5). STAT3 acts as both a transcriptional activator and a suppressor. Gene set enrichment analysis revealed that genes regulated by STAT3 are involved in several oncogenic signaling pathways, including NF-κB, PISK/AKT/mTORC1, cell-cycle checkpoint, and STAT3 itself. Notably, STAT3 suppresses expression of STAT1, STAT2, IRF7, and IRF9, all of which are critical transcription factors in the type I IFN pathway. Thus, gene regulation by STAT3 in ABC DLBCL accentuates the survival signaling pathways while dampening the lethal type I IFN pathway.

Crosstalk between the NF-κB and IL-6/IL-10/JAK1/STAT3 signaling pathways is an oncogenic process in ABC DLBCL (8, 29). Through histone H3 phosphorylation but independent of STAT3, JAK1 up-regulates expression of IRF4 and MYD88, which is required for cancer cell survival (3). The present study revealed that many other genes in the NF-κB pathway are STAT3 targets, including NF-κB2 and TRAF1. Interestingly, NF-κB2 signalling is associated with MYD88 mutations and promotes development of DLBCL (3). In a transgenic mouse model, TRAF1 is involved in lymphomagenesis mediated by constitutively activated NF-κB2 (31). These findings suggest a role for the noncanonical NF-κB pathway in the pathogenesis of ABC DLBCL. Disruption of oncogenic loops between the NF-κB and JAK1/STAT3 signaling pathways by their small-molecule inhibitors produces the synergistic cytotoxicity in ABC DLBCL (3, 4, 32).

Several biochemical studies have found a phenomenon of STAT3-mediated suppression of IFN antiviral responses in immune cells (33–36). In ABC DLBCL, the type I IFN signaling pathway, which can be activated by the MYD88 L265P mutation, is proapoptotic to the cancer cells (11) (Fig. 5). Our integrated genomic analysis elucidates the molecular mechanisms of STAT3 in suppression of this lethal pathway in ABC DLBCL: active STAT3 prevents the cancer cells from producing IFNβ through inhibition of IRF7 expression and also suppresses transcription of STAT1, STAT2, and IRF9 (ISGF3 complex) to block IFNβ signaling.

The multilayer suppression of IFNβ signaling by STAT3 is one of the major mechanisms by which autocrine IL-6/IL-10 signaling prevents cancer cell death. In addition, this cytokine signaling inhibits IFNβ production through the JAK1-mediated epigenetic mechanism; that is, JAK1 targets histone H3 to induce expression of IRF4 and SPIB, which form a transcription complex to inhibit IRF7 expression (3, 12). The type I IFN signaling pathway has emerged as an effective therapeutic target in DLBCL. Recent clinical trials have revealed that lenalidomide treatment alone or combined with immunochemotherapy achieved promising efficacy in DLBCL (37–39). However, remission after a single lenalidomide treatment lasted for only 6 mo (37), suggesting that combinations of targeted agents that inhibit distinct survival pathways will be necessary. Our findings demonstrated that STAT3 regulates expression of genes that involve multiple oncogenic pathways and suppresses genes in the type I IFN signaling pathway. Inhibition of STAT3 activity by ruxolitinib synergizes with lenalidomide in growth inhibition of ABC DLBCL cells in vitro and in a xenograft mouse model. Therefore, this study provides a mechanistic rationale for clinical trials of ruxolitinib or a specific JAK1 inhibitor and lenalidomide in ABC DLBCL.

Materials and Methods

Full details of the methods used and data analysis are presented in SI Materials and Methods.

Cell Lines and Culture. All doxycycline-inducible human DLBCL cell lines that express the bacterial tetracycline repressor were engineered as described previously (40). Doxycycline (20 ng/ml) was used to induce the expression of genes or shRNAs of interest. All cultures were routinely tested for mycoplasma contamination.

ChIP-Seq Analysis. ChIP-enriched DNA samples were used to create adapter-ligated libraries for massively parallel sequencing with the Ovation Ultralow Library System V2 (NuGen Technologies) following the manufacturer’s protocol. ChIP-Seq data are available at https://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE106844).

RNA-Seq Analysis. Total RNA was extracted using RNeasy plus mini kit (Qiagen) according to the manufacturer’s protocol. RNA-seq libraries were prepared by using the Illumina TruSeq stranded mRNA LT sample preparation kit (Illumina). Sequencing was performed on Illumina Hiseq 2500 at 50-bp length. RNA-seq data are available at https://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE106844).

Xenografts. The xenograft tumor model of human ABC DLBCL lymphoma was established by s.c. injection of OCI-Ly10 cells into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Jackson Labs). The tumor growth was monitored by measuring tumor size in two orthogonal dimensions. All animal experiments were approved by the National Cancer Institute Animal Care and Use Committee (NCI ACUC) and were performed in accordance with NCI ACUC guidelines.

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