Tumorigenesis and Neoplastic Progression

IL-21 Contributes to JAK3/STAT3 Activation and Promotes Cell Growth in ALK-Positive Anaplastic Large Cell Lymphoma

Jennifer Dien Bard,* Pascal Gelebart,*
Mona Anand,* Zoulika Zak,* Samar A. Hegazy,*
Hesham M. Amin,[†] and Raymond Lai*

From the Department of Laboratory Medicine and Pathology,*
Cross Cancer Institute and University of Alberta, Edmonton,
Canada; and the Department of Hematopathology,[†] the
University of Texas M.D. Anderson Cancer Center,
Houston, Texas

Interleukin (IL)-21 has been reported to both stimulate cell growth and promote survival in benign lymphoid cells and several types of hematopoietic neoplasms. It induces JAK3/STAT3 signaling, a biologically important cellular pathway activated in most cases of anaplastic lymphoma kinase (ALK)-expressing anaplastic large cell lymphoma (ALK⁺ALCL). Therefore, we hypothesize that IL-21 may contribute to JAK3/STAT3 activation and cell growth in ALK⁺ALCL. By reverse transcription-PCR, we found consistent expression of IL-21 receptor (IL-21R) in all ALK⁺ALCL cell lines and frozen tumors examined. IL-21 was also consistently expressed in ALK⁺ALCL tumors, although its mRNA was detectable in only one of three cell lines tested. By immunohistochemistry, we examined 10 paraffin-embedded ALK⁺ALCL tumors; all cases were positive for both IL-21 and IL-21R in these neoplastic cells. IL-21 signaling is biologically significant in ALK⁺ALCL since the addition of recombinant IL-21 enhanced the activation of JAK3/STAT3 and significantly increased cell growth in ALK⁺ALCL cell lines. However, small interfering RNA down-regulation of IL-21R significantly decreased both STAT3 activation and cell growth. IL-21R expression is not linked to nucleophosmin-ALK since forced expression of nucleophosmin-ALK and small interfering RNA down-regulation of nucleophosmin-ALK did not significantly change the expression of either IL-21R or IL-21. Our findings thus support the enhancement of JAK3/STAT3 activation and cell growth in ALK⁺ALCL via IL-21 signaling. These results further support the concept that constitutive activation of STAT3 in these tumors

is multifactorial. (Am J Pathol 2009, 175:825–834; DOI: 10.2353/ajpath.2009.080982)

Anaplastic lymphoma kinase-expressing anaplastic large-cell lymphoma (ALK⁺ALCL) is a subtype of T/null-cell non-Hodgkin's lymphoma characterized by a constellation of pathological and clinical features.¹ The aberrant expression of ALK in most of these tumors is the result of the reciprocal chromosomal translocation, t(2;5)(p23;q35), which leads to the fusion of the *nucleophosmin (NPM)* gene at 5q35 with the *anaplastic lymphoma kinase (ALK)* gene at 2p23.^{2,3} It is widely accepted that NPM-ALK directly contributes to lymphomagenesis.¹ Accumulating data suggest that NPM-ALK mediates lymphomagenesis by virtue of its constitutively active tyrosine kinase activity that is embedded in the ALK portion of this fusion protein. Through their interactions with NPM-ALK, multiple signaling proteins are phosphorylated at various tyrosine residues and they become constitutively activated.^{4–8} JAK3/STAT3 is a well-characterized signaling pathway in ALK⁺ALCL. JAK3 is pathogenetically important in ALK⁺ALCL, since inhibition of JAK3 reduces the ALK tyrosine kinase activity, down-regulates STAT3 activation, and induces apoptosis and G₁ cell-cycle arrest in ALK⁺ALCL cell lines.⁴ One of the JAK3 downstream me-

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J.D.B. and P.G. contributed equally to this work.

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Address reprint requests to Raymond Lai, M.D., Ph.D., Department of Laboratory Medicine and Pathology, Cross Cancer Institute and University of Alberta, 11560 University Avenue, Edmonton, Alberta, Canada T6G 1Z2. E-mail: raymond.lai@cancerboard.ab.ca.

diators is STAT3, a family member of latent transcription factors activated in response to cytokines and growth factors.^{9,10} Both JAK3 and STAT3 are constitutively activated in ALK⁺ALCL.^{9,11,12} STAT3 is oncogenic when it becomes constitutively activated,¹³ a phenomenon found in many types of human cancer.^{14–17} STAT3 is known to promote oncogenesis by modulating the expression of many important regulatory proteins involved in apoptosis and cell cycle, such as c-Jun, c-Myc, Bcl-xL, Bcl-2, Mcl-1, survivin, cyclins, p21, and p27.^{9,18–21} Accumulating evidence supports the concept that NPM-ALK mediates its oncogenic effects via STAT3 activation,^{6,11,12,20} and blockade of STAT3 in ALK⁺ALCL cell lines results in significant apoptosis and cell cycle arrest.²³ While NPM-ALK plays a direct role in activating STAT3, sustained activation of this protein appears to be multifactorial in ALK⁺ALCL; multiple previous studies have revealed these mechanisms including those related to Src and the loss of various negative feedback systems such as SHP1, a tyrosine phosphatase.^{4,24–26} As stated above, JAK3, the physiological activator of STAT3, also contributes to STAT3 activation in ALK⁺ALCL. One of our previous studies suggests that activation of JAK3 in these tumors can be attributed to autocrine cytokine stimulation, namely interleukin (IL)-9.²⁴

IL-21, a newly discovered cytokine, is expressed exclusively by CD4-positive T cells and known to regulate the functions of T-cells, B-cells, natural killer cells, and myeloid cells.^{27,28} IL-21 is considered a class I cytokine and it has a significant homology to IL-2, IL-4, and IL-15.²⁹ All of the class I cytokines, including IL-9, IL-15, and IL-21, have receptors that contain the IL-2 common γ -chain (γ_c). Their biological importance is highlighted by the phenotype identified in the JAK3-deficient severe combined immunodeficient mice, and the X-linked severe combined immunodeficient mice, which carry a mutated γ_c gene.^{30–32} IL-21-mediated cell signaling requires heterodimerization of its receptor complex, consisting of γ_c and IL-21R, which is normally expressed on B-cells, T-cells, and natural killer cells.²⁸ IL-21 induces activation of both JAK1 and JAK3, which then initiate STAT1 and STAT3 signal transduction and stimulate various cellular responses in a cell-type specific manner.^{27,33–35} For example, IL-21 has a pro-apoptotic effect on B-cells,³⁶ but a proliferative effect on T cells.^{37,38} In hematological malignancies, while IL-21 promotes cell growth and proliferation in myeloma and adult T-cell leukemia,^{39–41} it has been shown to induce apoptosis in follicular lymphoma.³⁹

In view of the biological relevance of JAK3/STAT3 in ALK⁺ALCL, and the link between IL-21 and the JAK3/STAT3 signaling pathway, we hypothesize that IL-21 may contribute to JAK3/STAT3 activation and promote cell growth in ALK⁺ALCL, perhaps in a similar manner as IL-9.²⁴ The biological significance of IL-21 has not been previously examined in ALK⁺ALCL. In our initial study using oligonucleotide microarrays to study the cytokine expression in ALK⁺ALCL, we found that both IL-21R and IL-21 are frequently expressed in this cell type (unpublished data). In this study, we comprehensively evaluated the expression of IL-21 and IL-21R in ALK⁺ALCL cell

lines and tumors. We then assessed the biological effects of IL-21 in ALK⁺ALCL cells, with a focus on the JAK3/STAT3 signaling.

Materials and Methods

ALK⁺ALCL Cell Lines

Three well-described ALK⁺ALCL cell lines including SU-DHL-1, Karpas 299, and SUP-M2 were used in this study. Hepatocellular carcinoma cell line HepG2 was included as a negative control for IL-21 and a positive control for IL-21R. Breast cancer cell line MDA-MB-231 (American Type Culture Collection, Manassas, VA) was included as a negative control for both IL-21 and IL-21R. Jurkat, a T cell leukemia cell line, was used for the NPM-ALK gene transfection experiments. ALK⁺ALCL cell lines and Jurkat cells were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) whereas HepG2 and MDA-MB-231 were maintained in Dulbecco's Modified Eagle's medium (Sigma-Aldrich). Both types of culture media were enriched with 10% fetal bovine serum (Gibco, Grand Island, NY) and antibiotics (10,000 units/ml penicillin G, 10,000 μ g/ml streptomycin, Gibco). All cells were grown at 37°C in 5% CO₂. For the cytokine experiment, cells at a concentration of 1×10^6 cells/ml were washed twice with PBS, pH 7.5, and serum-starved for 16 hours. Cells were treated with 10 ng/ml of recombinant IL-21 (rIL-21)(Sigma) for 30 minutes and harvested for Western blot studies.

Antibodies, Western Blot Analysis, and Co-Immunoprecipitation

Western blot analysis was performed using standard techniques. Briefly, the cells were lysed in a buffer (20 mmol/L Tris-HCl, pH 7.5, 2% SDS, 10% glycerol, 5.0 mmol/L EDTA, 40.0 μ g/ml leupeptin, 1 μ mol/L pepstatin, 1 mmol/L 4-[2-aminoethyl]-benzenesulfonyl fluoride), and centrifuged at 15,000 $\times g$ for 15 minutes at 4°C. The supernatant was removed and 50 to 100 μ g of protein was run on SDS polyacrylamide gel. After protein transfer, the nitrocellulose membranes were blocked with 5% milk in a buffer containing 20 mmol/L Tris-HCl and 150 mmol/L NaCl (pH 7.6), and then incubated with primary antibodies overnight followed by a 1-hour incubation with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The membranes were washed in PBS with 0.05% Tween-20 for 30 minutes between steps. Proteins were detected using the enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL). Antibodies used in this study included anti-STAT3 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated STAT3 (pSTAT3, 1:500, Santa Cruz), anti-phosphorylated STAT1 (pSTAT1, 1:500, Santa Cruz), anti-STAT1 (1:500, Santa Cruz), anti-phosphorylated JAK3 (pJAK3, 1:500, Santa Cruz), anti-JAK3 (1:500, Santa Cruz), and anti- β -actin (1:3000, Sigma). The method used for co-immunoprecipitation between NPM-ALK and IL-21R has been described previ-

Table 1. Primer Sequence and PCR Conditions

	Sense primers	Antisense primers	Product size (bp)
IL-21	5'-GGCAACATGGAGAGGATTG-3'	5'-AAGCAGGAAAAAGCTGACCA-3'	240
IL-21R	5'-TCTACATGCTGAAGGGCAAG-3'	5'-TCACTCCATTCACTCCAGGT-3'	315
GAPDH	5'-AAGGTCATCCCTGAGCTGAA-3'	5'-CCCTGTTGCTGTAGCCAAAT-3'	316
NPM-ALK	5'-GTTTCAGGGCCAGTGCATATT-3'	5'-ATGATCAGGGCTTCCATGAG-3'	379

ously.^{4,23} An anti-ALK antibody (Santa Cruz) was used to pull down NPM-ALK present in cell lysates and an anti-IL-21R antibody (Sigma) was used for immunoblotting.

Immunofluorescence Staining and Confocal Microscopy

Immunofluorescence was performed using standard techniques. Briefly, 1×10^6 cells grown on coverslips in a 6-well plate were fixed with 4% paraformaldehyde in PBS. Cells were rinsed with PBS, permeabilized with PBS-0.5% triton X-100 for 5 minutes, and rinsed twice with PBS. Cells were then incubated with 30 μ l of anti-IL-21R (1:50, Sigma) overnight, followed by washing with PBS. After incubation with 25 μ l of Alexa 488 goat anti-rabbit secondary antibody (1:250) for 1 hour, cells were washed with PBS and mounting media (Sigma) was added to the slides. Cells were visualized and imaged with a Zeiss, LSM 510 confocal microscope (Oberkochen, Germany) at the Cross Cancer Institute imaging facility. Argon laser with a 488 nm wavelength was used to visualize IL-21R at $\times 40$ objective and images were analyzed using the Zeiss LSM 5 image browser. IgG antibody used in place of anti-IL-21R served as the negative control.

Flow Cytometric Detection of IL-21R in ALK⁺ ALCL Cell Lines

ALK⁺ ALCL cells were fixed in the CytoFix Buffer from Becton Dickinson Biosciences (Franklin Lakes, NJ), washed in cold PBS, centrifuged, and re-suspended in the fluorescein-activated cell sorting buffer purchased from Becton Dickinson. Cells were incubated with primary antibodies for 60 minutes at 4°C in the dark, and washed twice using cold buffer between incubations. The following antibodies were used: unconjugated mouse IgG1 as the isotype control (10 μ g/ml, Santa Cruz biotechnology), unconjugated mouse anti-human IL-21R (IgG1, 10 μ g/ml, clone MAB9911, R&D Systems, Minneapolis, MN), and phycoerythrin-conjugated rat anti-mouse antibody (IgG1, 5 μ g/ml, Becton Dickinson). Fluorescein-activated cell sorting analyses were performed using the FACScan (Becton Dickinson) and accompanying CELLQuest software as per manufacturer's guidelines.

Reverse Transcription-PCR

Total cellular RNA was extracted from SU-DHL-1, Karpas 299, SUP-M2, HepG2, MDA-MB-231, as well as four randomly chosen frozen ALK⁺ ALCL tumors, using TRIzol extraction method (Gibco). Reverse transcription (RT)

was performed using 500 ng total RNA in the first strand cDNA synthesis reaction with superscript reverse transcriptase as recommended by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). Primer pairs were designed to detect IL-21 and IL-21R (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR was performed by adding 1 μ l RT product in a 24 μ l reaction mix, containing 1 $\times$ PCR buffer, 200 μ mol/L of each dNTPs, oligonucleotide primer, and 0.2 U AmpliTaq polymerase. The primer sequences and PCR cycles are shown in Table 1. For DNA amplification, cDNA was denatured at 94°C for 1 minute, and then subjected to primer annealing at 60°C for 1 minute, and then subjected to DNA extension at 72°C for 1 minute for 35 cycles in a thermal cycler (Applied Biosystems, Foster City, CA). Amplified products were analyzed by DNA gel electrophoresis in 1% agarose and visualized by the α Imager 3400 (α Innotech, San Leandro, CA).

Quantitative PCR

Using the TRIzol extraction method (Gibco), total cellular RNA was extracted from cells. RT was performed using 500 ng total RNA in the first strand cDNA synthesis reaction with superscript reverse transcriptase as recommended by the manufacturer (Invitrogen). Real-time PCR was performed using SYBR green (Superarray, Fredericks, MD), and three primer sets were used in this study (IL-21R, NPM-ALK, and GAPDH) (Table 1). The PCR conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were processed on an ABI 9700 HT system (Applied Biosystems, Inc., Foster City, CA). Results were examined using SDS 2.2 software, and the relative expression levels of IL-21R (or NPM-ALK) were calculated by normalizing the cycle threshold values of IL-21R (or NPM-ALK) with those of GAPDH. Fold differences between cells treated with IL-21R small interfering (si)RNA (or NPM-ALK siRNA) versus those treated with scrambled siRNA were calculated.

Cell Growth Assays

To determine changes in the number of viable cells due to rIL-21 treatment, daily cell counts were obtained. For cell counting, 25,000 cells were plated in 24-well culture plates with a medium containing 5% fetal bovine serum. rIL-21 of 10 ng/ml was added daily and cells were counted daily using trypan blue (Gibco). For the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI), 5000 cells transfected with ei-

Figure 1. Expression of the IL-21 and IL-21 receptor in ALK⁺ALCL cell lines and tumors. **A:** RT-PCR studies demonstrated the presence of *IL-21R* mRNA in all three ALK⁺ALCL cell lines. *IL-21* was only detected in Karpas 299. HepG2 was included as a positive control for IL-21R and a negative control for IL-21. Breast cancer cell line MDA-MB-231 was included as a negative control for both IL-21R and IL-21. GAPDH was included as an internal control. **B:** Confocal immunofluorescence microscopy staining of ALCL cell lines with antibodies directed against IL-21R, differential interference contrast (DIC), or control IgG demonstrated the membranous localization of IL-21R in Karpas 299 cells. **C:** Flow cytometric studies confirmed the cell surface expression of IL-21R in all three ALK⁺ALCL cell lines. **D:** RT-PCR studies demonstrated the presence of *IL-21* and *IL-21R* mRNA in four ALK⁺ALCL patient tumors. HepG2 was included as a positive control for IL-21R and a negative control for IL-21. A breast cancer cell line MDA-MB-231 was included as a negative control for IL-21R. GAPDH was included as an internal control. **E:** Immunohistochemical staining of paraffin-embedded tissue sections confirmed the expression of IL-21 (left panel) and IL-21R (right panel) in a case of ALK⁺ALCL tumors. **(A)** and **(C)** demonstrate staining at $\times 20$ magnification, and **(B)** and **(D)** demonstrate staining at $\times 40$ magnification. For IL-21, the neoplastic cells showed cytoplasmic staining; the adjacent benign B-cell areas were largely nonreactive. IL-21R demonstrated a cytoplasmic/membranous staining pattern. The adjacent benign B-cell areas also were largely nonreactive. Negative controls for IL-21 and IL-21R were provided by omitting the primary antibody or using an isotype control antibody (not shown).

ther IL-21R siRNA or scrambled siRNA were seeded in 96-well culture plates. Cell growth was then measured colorimetrically at 450 nm using a microplate reader (Bio-rad Life Science Research Group, Hercules, CA) and absorbance values were normalized using Microplate Manager 5.2.1 (Biorad).

Transfection of siRNA to Knock Down IL-21R and NPM-ALK

Karpas 299 cells (1×10^6 cells in 1 ml of culture medium) were transfected with 100 pmol of IL-21R or scrambled siRNA (Qiagen). The IL-21R target sequence is 5'-CCCGGTCATCTTTTCAGACCCA-3' and the scrambled

sequence is 5'-UGGGUCUGAAAGAUGACCG-3'. Cells were harvested 24 hours after the transfection. Specific targeting of *NPM-ALK* genes was performed by transient transfection of the cells with SMARTpool-designed siRNA, and the siCONTROL non-targeting siRNA was used as a negative control (Dharmacon, Lafayette, CO). In both cases, Amaxa transfection instrument (program, A-30) and the Nucleofector solution V (Amaxa, Cologne, Germany) were used.

Gene Transfection of NPM-ALK

The original *NPM-ALK* plasmid was kindly provided by Dr. Stephan W. Morris (St Jude's Children Research Hos-

Figure 2. Effects of rIL-21 on JAK3, STAT3, and STAT1. **A:** Western blot studies revealed that treatment of Karpas 299 recombinant IL-21 protein (10 ng/ml) for 30 minutes increased the levels of pJAK3 and pSTAT3. No detectable change in pSTAT1 was noted. **B:** rIL-21-induced changes in pSTAT3 and pJAK3 in the three ALK⁺ ALCL cell lines, as shown on Western blots, were quantified using densitometry.

pital, Memphis, TN) and the *NPM-ALK* fusion gene was inserted in a pCDNA vector.⁴² Transfection was performed using the Amaxa transfection instrument (Cologne, Germany) and the Nucleofector V solution (program, X-05).

ALK⁺ ALCL Tumors and Immunohistochemistry

Formalin-fixed, paraffin-embedded lymph node biopsy samples ($n = 10$) from patients with ALK⁺ ALCL were retrieved from the file at the Department of Laboratory Medicine and Pathology, Cross Cancer Institute, with the approval by the Institutional Ethics Committee. The diagnosis of these cases was based on the criteria established by the World Health Organization Classification scheme, and all cases were confirmed to express ALK by immunohistochemistry. Immunohistochemical staining for IL-21 and IL-21R was performed using standard tech-

niques. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 μ mol/L thickness were deparaffinized and hydrated. Heat-induced epitope retrieval was performed using EDTA retrieval buffer (Tris-Borate-EDTA, pH 8.0, Sigma). Slides that were previously washed in hot water were placed in the boiled EDTA and microwaved for 10 minutes, followed by a cold water wash for 5 minutes. Endogenous peroxidase was blocked using 10% H₂O₂ and methanol, followed by washing in running tap water for 5 minutes. Tissue sections were then incubated with anti-IL-21R (1:300, Capralogics, Hardwick, MA) or anti-IL-21 antibody (1:800, Capralogics) overnight in a humidified chamber at 4°C. After three washes with PBS, tissue sections were incubated with a secondary antibody for 20 minutes at room temperature using the labeled streptavidin biotin system (Dako, Glostrup, Denmark), which is a combination of anti-rabbit, anti-goat, and anti-mouse linked to biotin. After two washes with PBS, streptavidin-horseradish peroxidase complex (Dako) is added to the sections and incubated at room temperature for 20 minutes. The tissue sections were incubated with 3,3'-diaminobenzidine/H₂O₂ (Dako) for color development, using hematoxylin as a counterstain.

Statistical Analysis

The association between IL-21 and cell growth after siRNA transfection was evaluated using Student's *t*-test. A *P* value of <0.05 is considered to be statistically significant.

Results

Expression of IL-21 and IL-21R in ALK⁺ ALCL Cell Lines and Tumors

The expression of *IL-21* and *IL-21R* mRNA in three ALK⁺ ALCL cell lines was assessed using RT-PCR. As shown in Figure 1A, *IL-21* mRNA was readily detectable in Karpas 299 but not in SU-DHL-1 and SUP-M2. In contrast, all three cell lines expressed *IL-21R*. The expression of γ_c in these cells has been previously reported by our group.²⁴ HepG2 cells served as the positive control and MDA-MB-231 served as the negative control for *IL-21R*. Both of these cell lines served as the negative controls for *IL-21*. To determine the subcellular localization of IL-21R, we performed immunofluorescence staining and confocal microscopy. As shown in Figure 1B, IL-21R was localized predominantly to the cell membrane of Karpas 299, SU-DHL-1, and SUP-M2 cells. In keeping with these findings, the cell surface expression of IL-21R in all three ALK⁺ ALCL cell lines was confirmed using flow cytometry (Figure 1C).

To evaluate the expression of *IL-21* and *IL-21R* mRNA in ALK⁺ ALCL tumors, RT-PCR was performed using frozen tumor tissues. All of these four tumors were previously confirmed to contain largely neoplastic cells by histological examination. As shown in Figure 1D, all four tumors had detectable *IL-21* and *IL-21R*; while the *IL-21R* expression levels were relatively equal among all four

Figure 3. IL-21 modulated cell proliferation in ALK⁺ALCL cell lines. **A:** Treatment of SU-DHL-1 and Karpas 299 cell line with recombinant IL-21 protein induced a significant increase in the cell number on day 3 for SU-DHL-1, as assessed by the trypan blue exclusion assay. A significant increase in the cell number was also detected on day 4 for Karpas 299 cells. Triplicate experiments were performed. **B:** MTS assay was used to assess changes in the number of viable cells in three ALK⁺ALCL cell lines treated with rIL-21. On day 5, all three ALK⁺ALCL cell lines showed a significant increase in the number of viable cells. Triplicate experiments were performed.

tumors, the *IL-21* level was appreciably lower in tumor 1 and 2, as compared with that of tumors 3 and 4. HepG2 cells served as the positive control for *IL-21R*. MDA-MB-231 served as the negative control for *IL-21R*; both of these cell lines were negative for *IL-21*.

To further support that the expression of *IL-21R* and *IL-21* is indeed derived from the neoplastic lymphoid cells, we used immunohistochemistry applied to formalin-fixed and paraffin-embedded tissues of ten ALK⁺ALCL tumors. The staining for *IL-21* is illustrated in Figure 1E (left panel). The staining was readily detectable in all ALK⁺ALCL cases. The neoplastic cells showed relatively intense cytoplasmic staining; the adjacent B-cell areas had no definitive *IL-21* staining. For *IL-21R*, we were able to detect staining in the neoplastic cells in all 10 cases, as illustrated in Figure 1E (right panel), the neoplastic cells showed a cytoplasmic/membranous staining pattern of *IL-21R*. The adjacent benign B-cell areas had no detectable *IL-21R* by immunohistochemistry. We also assessed *IL-21* and *IL-21R* staining in reactive tonsils (not shown); all have lymphoid cell compartments showed no definitive staining using our immunohistochemical technique. These findings strongly suggest that both *IL-21* and *IL-21R* are expressed at substantially higher levels in ALK⁺ALCL when compared with benign lymphoid cells.

IL-21 Modulates the JAK3/STAT3 Signaling

Since the previous studies have reported a role for *IL-21* in activating JAK3 and STAT3,^{28,40–43} we sought to determine whether *IL-21* contributes to the activation of this signaling pathway in ALK⁺ALCL cells. All three ALK⁺ALCL cell lines were serum-starved for 16 hours followed by treatment with 10 ng/ml rIL-21 protein for 30 minutes. As shown in Figure 2, A and B, *IL-21* stimulation for 30 minutes led to an appreciable increase in pSTAT3

and pJAK3. We next assessed if *IL-21* induces activation of STAT1, another STAT protein that has been reported to be activated by *IL-21* in some cell types.^{27,33–35} With the same experimental conditions, we found no detectable change in the level of pSTAT1.

IL-21 Treatment Increases Cell Growth of ALK⁺ALCL Cell Lines

To assess the biological effects of *IL-21*, we treated ALK⁺ALCL cell lines with 10 ng/ml of rIL-21. SU-DHL-1 and Karpas 299 cells were grown in media containing reduced fetal bovine serum (ie, 5%) for 16 hours, followed by daily treatment with 10 ng/ml rIL-21 for 5 days. Cell count was performed daily using the trypan blue exclusion assay. As shown in Figure 3A, triplicate experiments revealed a significant increase in the number of viable cells observed on day 3 for SU-DHL-1 and on day 4 for Karpas 299 cells. The delayed cell growth response in Karpas 299 is most likely due to the fact that Karpas 299, but not SU-DHL-1, produces endogenous *IL-21*. Morphological examination of these cell samples, either the negative controls or cells treated with rIL-21, did not show any features of apoptosis (not shown). To further confirm the cell proliferative effects of *IL-21* in these cells, we performed MTS assay; as shown in Figure 3B, addition of rIL-21 to the ALK⁺ALCL cell lines resulted in a significant increase in the number of viable cells on day 5.

To confirm the biological importance of *IL-21* signaling in ALK⁺ALCL, we used siRNA to down-regulate the expression of *IL-21R* in Karpas 299, the only cell line that express both *IL-21* and *IL-21R* in this study. RT-PCR was performed 24 hours after siRNA transfection and a substantial decrease in *IL-21R* was demonstrated in cells transfected with *IL-21R* siRNA but not scrambled siRNA

Figure 4. IL-21R down-regulation by siRNA decreased pSTAT3 and cell growth of Karpas 299 cells. **A:** RT-PCR revealed *IL-21R* down-regulation in Karpas 299 cells treated with IL-21R siRNA. Cells treated with scrambled siRNA were used as a negative control. GAPDH served as the internal control. **B:** Compared with Karpas 299 cells transfected with scrambled siRNA, Karpas 299 cells transfected with *IL-21R1* siRNA showed a down-regulation of the pSTAT3 expression level. Cell lysates were prepared 24 hours after transfection. The STAT3 and β -actin expression levels were equal in both lanes. **C:** MTS assay revealed a significant decrease in cell growth in Karpas 299 cells transfected with *IL-21R* siRNA compared with cells transfected with scrambled siRNA (normalized to 100%). Triplicate experiments were performed.

(Figure 4A). The reduced protein expression of IL-21R was further supported by our flow cytometry studies (supplemental Figure S1, see <http://ajp.amjpathol.org>). Quantitative RT-PCR was also performed and a significant down-regulation of IL-21R expression was confirmed with triplicate experiments (average fold difference = 7.6, *P* value, 0.0025). Correlating with these changes, pSTAT3 was substantially decreased in cells transfected with IL-21R siRNA compared with cells transfected with scrambled siRNA (Figure 4B). Using the same experimental conditions, we assessed if the cell growth was affected by IL-21R down-regulation. Thus, we performed triplicate experiments using the MTS assay in cells transfected with IL-21R siRNA. At 72 hours after transfection, the proliferation of cells transfected with IL-21R siRNA was significantly lower than that of the negative control sample (*P* = 0.028, Figure 4C).

Figure 5. NPM-ALK is not related to the expression of IL-21 or IL-21R. **A:** Gene transfection of *NPM-ALK* into Jurkat cells did not result in expression of *IL-21R* detectable by RT-PCR. Lane (a) was a negative control in which no DNA template was added to the PCR reaction mixture. Lane (b) and (c) showed the results from cells transfected with an empty vector or *NPM-ALK*, respectively. Karpas 299 (lane d) served as a positive control for IL-21R. **B:** Western blots showed the expression of NPM-ALK in Jurkat cells transfected with *NPM-ALK*, but not those transfected with an empty vector. Karpas 299 cells were used as a positive control.

IL-21 and IL-21R Expression Is Not Regulated by NPM-ALK

Lastly, we determined if NPM-ALK plays any direct role in regulating the expression of IL-21R. As shown in Figure 5, A and B, gene transfection of *NPM-ALK* into Jurkat cells, a T-cell leukemia cell line that does not express IL-21R, did not result in expression of this receptor detectable by RT-PCR. Furthermore, down-regulation of NPM-ALK in Karpas 299 using siRNA, which resulted in a sevenfold decrease in the expression of *NPM-ALK* as assessed by quantitative RT-PCR, did not significantly change the expression level of IL-21 (supplemental Figure S2, see <http://ajp.amjpathol.org>). Lastly, using co-immunoprecipitation and ALK⁺ALCL cell lines, we did not detect a physical interaction between NPM-ALK and IL-21R (not shown).

Discussion

The rationale for performing this study is based on our previous finding that JAK3 is constitutively activated in

ALK⁺ALCL, and we believe that this finding is suggestive of a role of cytokine stimulation in the pathogenesis of these tumors. With this assumption, we started to investigate the possible role of various cytokines that normally activate JAK3. JAK3 is an interleukin receptor-bound tyrosine kinase in which activation is limited to a small number of interleukins that recruit the IL-2 common γ_c to their receptors. Thus, we have focused on those interleukins whose signaling requires the γ_c chain, and they include IL-2, IL-9, IL-15, and IL-21.^{30,32} Previously, we have described evidence to support the existence of the IL-9 autocrine stimulatory pathway in ALK⁺ALCL. Specifically, blockade of IL-9 stimulation using a neutralizing antibody inhibits JAK3/STAT3 activation, accompanied by decreased cell growth and tumorigenicity in ALK⁺ALCL cell lines.²⁵ In this study, we examined IL-21, a recently described type I cytokine produced exclusively by activated CD4-positive T cells. IL-21 has been described to have profound but heterogeneous biological effects in B-cells, T-cells, and natural killer cells.^{29,33,44,45} Importantly, IL-21 is known to activate JAK3 in benign lymphoid cells.^{29,34} As described above, we were able to detect consistent expression of *IL-21* and *IL-21R* in our cytokine oligonucleotide array studies of ALK⁺ALCL cells. With this background, we hypothesize that IL-21 is a contributing factor for JAK3/STAT3 activation and pathogenesis of ALK⁺ALCL.

Our overall results are supportive of this hypothesis. Specifically, addition of rIL-21 enhanced JAK3/STAT3 activation and significantly increased cell growth in ALK⁺ALCL cell lines. In further support of this hypothesis, siRNA down-regulation of IL-21R showed the opposite biological effects in these cell lines. We believe that the IL-21 signaling likely contributes to JAK3/STAT3 activation and cell growth *in vivo*, since IL-21 and IL-21R were detectable in all 4 tumors by RT-PCR. While we favor that IL-21 stimulation is largely owing to autocrine stimulation, based on our observation that IL-21 was found in the neoplastic cell population, we cannot completely exclude the possibility that the infiltrating reactive T-cells may also contribute to the production of IL-21 intratumorally. We also cannot completely exclude the possibility that a small subset of ALK⁺ALCL tumors does not produce IL-21 and the presence of this cytokine in these tumors is largely attributed to the nonneoplastic T-cells. The existence of these IL-21 nonproducing ALK⁺ALCL may explain our observations that some ALK⁺ALCL cell lines did not produce IL-21 *in vitro*. Alternatively, it is not uncommon to see the properties (eg, cytokine requirement) of cell lines change as they go through an increasing number of passages. Despite the fact that IL-21 expression was not produced by SU-DHL-1 and SUP-M2, the IL-21 signaling pathway is intact and functional in these cells, since addition of rIL-21 consistently activated JAK3/STAT3.

The tumor-promoting effects of IL-21 in ALK⁺ALCL are in parallel with the observations made in adult T-cell leukemia,³⁹ myeloma,⁴⁰ and classical Hodgkin's lymphoma.^{46,47} An increase in cell proliferation was observed in myeloma cells and T-cell leukemia cells when treated with rIL-21.^{39–41} Analysis of the JAK/STAT signaling pathway

was described to some extent in these papers. Brenne et al reported phosphorylation of JAK1 and STAT3, but not STAT1, after treatment of myeloma cells with rIL-21.⁴⁰ These findings are indeed in parallel with our findings regarding STAT1 and STAT3 activation. Ueda et al demonstrated STAT3 and STAT5 phosphorylation after rIL-21 treatment of T-cell leukemia cells,⁴¹ but STAT1 phosphorylation was not investigated in this study. The biological significance of IL-21-mediated STAT1 will be further discussed below.

Our data presented in this study further supports the concept that STAT3 activation in ALK⁺ALCL is multifactorial, a concept that was previously proposed.²³ These factors include NPM-ALK, the aberrancy of a tyrosine phosphatase, PP2A, to inhibit STAT3 dephosphorylation, and the absence of the protein inhibitor of activated STAT3.²³ Nevertheless, the contribution of JAK3, the physiological activator of STAT3, was not included in this study. Our findings related to IL-9 and IL-21 in activating JAK3/STAT3 and enhancing cell growth in ALK⁺ALCL not only further supports the 'multifactorial STAT3 activation' concept, but also adds a new dimension to this conceptual model. Most recently, in another line of investigation, we have provided evidence that the tumorigenicity of ALK⁺ALCL is promoted by IL-22.⁴⁷ Unlike IL-9 and IL-21, IL-22-mediated activation of STAT3 is not dependent on γ_c or JAK3. Furthermore, the functional IL-22 receptor complex, which is composed of the IL-22 receptor 1 and IL-10R2 subunits, is not fully expressed on benign lymphoid cells. The aberrant expression of IL-22 receptor 1 in ALK⁺ALCL cells is directly linked to NPM-ALK, since transfection of NPM-ALK into cells resulted in the expression of IL-22 receptor 1, thus converting from an 'IL-22 un-responsive phenotype' to an 'IL-22 responsive phenotype.' In contrast, we did not find a similar relationship between NPM-ALK and IL-21 receptor in this study. Taken together, it is increasingly evident that, while NPM-ALK mediates tumorigenesis in ALK⁺ALCL by deregulating multiple signaling pathways, aberrancies of cell signaling in these neoplastic cells can be attributed to increasing number of factors. Results from our recent studies highlights the importance of autocrine cytokine stimulation of the STAT3 signaling pathway.^{6,48,49}

Aside from activating STAT3, IL-21 signaling also has been reported to result in activation of STAT1 in some cell types.^{33,42,50} In contrast to STAT3, which promotes cell survival and cell cycle progression in many cell types, STAT1 is known to have tumor suppressing properties, namely antiproliferative and pro-apoptotic effects.^{42,51} In view of the normal functions of STAT1, we believe that the lack of IL-21 induced up-regulation of pSTAT1 in ALK⁺ALCL is significant. As discussed above, myeloma cells also fail to demonstrate STAT1 activation on IL-21 stimulation.⁴⁰ Taken together, it is tempting to speculate that the IL-21 induced cell growth is attributed to the imbalance between STAT1 and STAT3 activity. It will be of great interest to determine why STAT1 is not activated in ALK⁺ALCL or myeloma in response to IL-21.

In conclusion, we have provided the first evidence that an autocrine IL-21 stimulatory pathway exists in ALK⁺ALCL tumors. In parallel with IL-9, IL-21 signaling contributes to cell growth in ALK⁺ALCL by enhancing

JAK3/STAT3 activation and may be a potential therapeutic target for this type of cancer. These findings support the pathogenetic importance of JAK3 in these tumors. In conjunction with the results of several previous studies, it becomes increasingly evident that STAT3 activation, considered to be one of the most crucial oncogenic factors in ALK⁺ ALCL, is multifactorial.

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