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CD30 Regulation of IL-13–STAT6 Pathway in Breast Implant–Associated Anaplastic Large Cell Lymphoma

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Abstract

Background: Breast implant–associated anaplastic large cell lymphoma (BIA-ALCL) is a rare, usually indolent CD30+ T-cell lymphoma with tumor cells, often surrounded by eosinophils, expressing IL-13 and pSTAT6.

Objectives: The aim of this study was to understand the unique tumor pathology and growth regulation of BIA-ALCL, leading to potential targeted therapies.

Methods: We silenced CD30 and analyzed its effect on IL-13 signaling and tumor cell viability. IL-13 signaling receptors of BIA-ALCL cell lines were evaluated by flow cytometry and pSTAT6 detected by immunohistochemistry. CD30 was deleted by CRISPR/Cas9 editing. Effects of CD30 deletion on transcription of IL-13 and IL-4, and phosphorylation of STAT6 were determined by real-time polymerase chain reaction and western blotting. The effect of CD30 deletion on p38 mitogen-activated protein kinase (MAPK) phosphorylation was determined. Suppression of IL-13 transcription by a p38 MAPK inhibitor was tested. Tumor cell viability following CD30 deletion and treatment with a pSTAT6 inhibitor were measured in cytotoxicity assays.

Results: BIA-ALCL lines TLBR1 and TLBR2 displayed signaling receptors IL-4Rα, IL-13Rα1 and downstream pSTAT6. Deletion of CD30 by CRISPR/Cas9 editing significantly decreased transcription of IL-13, less so Th2 cytokine IL-4, and phosphorylation of STAT6. Mechanistically, we found CD30 expression is required for p38 MAPK phosphorylation and activation, and IL-13–STAT6 signaling was reduced by an inhibitor of p38 MAPK in BIA-ALCL tumor cells. Tumor cell viability was decreased by silencing of CD30, and a specific inhibitor of STAT6, indicating STAT6 inhibition is cytotoxic to BIA-ALCL tumor cells.

Conclusions: These findings suggest reagents targeting the IL-13 pathway, pSTAT6 and p38 MAPK, may become useful for treating BIA-ALCL patients.

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INTRODUCTION

Breast implant–associated anaplastic large cell lymphoma (BIA-ALCL) is a CD30+ ALK– peripheral T-cell lymphoma occurring infrequently in women with textured breast implants.[1,2] CD30 is a constitutive marker of BIA-ALCL and has been used for targeted immunotherapy.[3,4] As reported by Laurent et al, infiltrative BIA-ALCL is associated with an inflammatory infiltrate including numerous eosinophils.[5] Cytokines are signaling peptides that allow intercellular communication, serve as autocrine/paracrine growth factors, and shape the microenvironment. Previous studies have demonstrated select cytokines in BIA-ALCL tumor tissues and delayed seromas, likely contributing to the pathogenesis of this lymphoma.[6] Anaplastic cells express interleukin 13 (IL-13) in most cases.[2,7] IL-13 is a prominent Th2 cytokine released by many cell types, especially by T helper type 2 (Th2) cells. The effects of IL-13 on immune cells are similar to those of IL-4 as both cytokines share a common receptor subunit (the α subunit), although IL-13 plays a predominant role in allergic inflammation. In a mouse model, IL-13 was found to be an important mediator of allergic asthma, inducing eosinophil recruitment to airways in an IL-5- and CCL11/eotaxin-dependent manner.[8] IL-13 also promotes IL-4-independent isotype switching of B cells to produce immunoglobulin E (IgE).[9] Accordingly, we described IgE decorating the surface of mast cells in capsules infiltrated by BIA-ALCL.[2]

Because IL-13 plays a prominent role in BIA-ALCL, we reasoned it would be important to understand how its expression is regulated. In a murine system, ligation of CD30 on CD4+ effector T cells was found to induce IL-13 production independent of T-cell antigen receptor (TCR) engagement.[10] IL-13 production induced by CD30 engagement was dependent on TRAF2 and p38. Like the murine system, the anaplastic cells of BIA-ALCL are commonly CD4+ and lack a surface TCR.[11] We therefore studied CD30 regulation of IL-13 signaling in BIA-ALCL and found a link to phosphorylation of STAT6. We show that IL-13 expression is regulated by CD30 and that silencing of CD30 suppresses the IL-13 signaling pathway, including pSTAT6, in BIA-ALCL.

METHODS

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the corresponding author's IRB (Lifespan, Providence, RI).

Histopathology

Formalin-fixed, paraffin-embedded tissue sections on charged slides were analyzed from 10 patients with infiltrative BIA-ALCL; MD Anderson TNM Stage IC, 2 patients; Stage IIA, 4 patients; Stage III, 4 patients.[12] Tissues were stained with hematoxylin-eosin and Giemsa, the latter to better visualize eosinophils.

Immunohistochemistry

Following antigen retrieval by steam heat (90°C), 5-µm paraffin sections were stained with (1 µg/mL) anti-human IL-13 rabbit polyclonal antibody (catalog no. 9576; Abcam, Cambridge, UK); 1:50 dilution of rabbit anti-pSTAT6 (Tyr641) (SAB4300038; Sigma-Aldrich, St Louis, MO); and 1:40 concentration of Ber-H2 anti-CD30 mouse IgG1-κ monoclonal antibody (Sigma-Aldrich). Antibody reactions were detected with CTS002 anti-mouse, CDS005 anti-rabbit kits (R&D Systems, Minneapolis, MN). Primary antibodies were omitted in negative controls.

Cell Lines

Experiments were done with TLBR1 and TLBR2 cell lines derived from BIA-ALCL; Mac-1 and Mac-2A cells derived from early and advanced cutaneous ALCL; and MOLT-4, a T- lymphoblastic lymphoma cell line. All cell lines were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) + 10% fetal bovine serum (Hyclone Defined; Hyclone, Logan, UT) + penicillin/streptomycin (Invitrogen). All cell lines were grown in a 5% CO_2 incubator at 37°C, and were in log phase growth when experiments were started.

Flow Cytometry

Cells (10^6) from BIA-ALCL cell lines TLBR1 and TLBR2 were labeled with mouse monoclonal fluorochrome-labeled antibodies against IL-4Rα (CD124; R&D Systems Clone #25463 or isotype-matched control antibody, R&D Systems #IC002P), anti-human IL-13Rα1 (CD213) antibody (clone SS12B; BioLegend, San Diego, CA), and anti-human isotype-matched control (R&D Systems #IC002P), and analyzed for detection of surface receptors with a Beckman Coulter CyAn flow cytometer (Brea, CA, USA). Dead cells were excluded with Zombie Violet dye (BioLegend). At least 5×10^5 cells were counted for each experiment. The Overton cumulative histogram subtraction method was used to determine the percentage of cells positive as determined by the fluorescence intensity being greater than that of the isotope control.

Generation of the Inducible Cas9 Expression Cell Lines

ALCL cell lines Mac-1, Mac-2A, TLBR1, and TLBR2 were transduced with an inducible Cas9 vector, single-cell cloned, and tested for Cas9 cutting efficiency and inducibility. CRISPR/Cas9 ALCL lines were transduced with CD30 or control single-guide RNAs (sgRNAs) along with green

Table 1. Single-Guide RNA Sequences Used in This Study

Name	Sequence
Control	GTAGCGAACGTGTCCGGCGT
CD30 #1	AAGTCACGCAGGCTGTACAG
CD30 #2	TTGCAGATTCCAGACCCAGG

fluorescent protein (GFP). The CD30 sgRNAs targets CD30 sequences whereas the control sgRNA contains nontargeting gRNA sequences that do not recognize any sequence in the human or mouse genome. The sgRNA sequences used in this study are listed in Table 1.

Lentiviral Production and Transduction

Lentivirus was produced by triple transfection of HEK-293FT cells with a lentiviral transfer vector and the packaging plasmids psPAX2 and pMD2.G. Transfection was performed with TransIT-293 transfection reagent as recommended by the manufacturer (Mirus Bio, Madison, WI). The viral supernatant was collected 48 hours following transfection, filtered through a 0.45-μm filter, and added to target cells.

Western Blotting

Cell pellets were lysed in the modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.25% deoxycholic acid, 1 mM EDTA) supplemented with a protease inhibitor tablet and a phosphatase inhibitor tablet (Roche, Basel, Switzerland), 1 mM dithiothreitol, 1 mM Na_3VaO_4, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were measured with a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Total proteins were separated on 4% to 12% sodium dodecylsulfate–polyacrylamide gels and transferred to nitrocellulose membranes.

Real-time Quantitative Polymerase Chain Reaction

Total RNA was extracted from ALCL cell lines with an RNeasy mini kit (Qiagen, Venlo, The Netherlands). Total RNA was reverse transcribed with random primers and SuperScript III Reverse Transcriptase (Invitrogen). The resultant cDNA was analyzed with an ABI7500 polymerase chain reaction instrument (Applied Biosystems). Gene expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase for all samples. The quantitative polymerase chain reaction primers used are listed in Table 2.

Table 2. Quantitative Polymerase Chain Reaction Primers Used in This Study

	Forward	Reverse
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG
IL-4	CCAACTGCTTCCCCCTCTG	TCTGTTACGGTCAACTCGGTG
IL-13	GAAGGCTCCGCTCTGCAAT	TCCAGGGCTGCACAGTACA

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Cell Viability MTS Assay

Cells were seeded in triplicate 96-well tissue culture plates at 15,000 cells/well in 150 μL of media under various treatment conditions. After 48 or 72 hours of treatment, 20 μL of CellTiter Aqueous One cell proliferation reagent (Promega, Madison, WI) was added to each well and mixed for 5 minutes. The absorbance was read on a Spark multimode microplate reader (Tecan, Mannedorf, Switzerland) after 2- to 4-hour incubation. The background was subtracted by means of a media-only control. All experiments were performed in triplicate.

RESULTS

Pathology

Anaplastic cells infiltrating capsules were found in small clusters and individually scattered in a background of fibrosis and inflammatory cells, including lymphocytes, plasma cells, neutrophils, mast cells, and eosinophils, which were often most numerous in the vicinity of anaplastic cells. Immunohistochemistry revealed anaplastic cells stained for cytoplasmic IL-13 in 9 of 10 cases and nuclear pSTAT6 in all cases (Figure 1).

BIA-ALCL Cell Lines Express Signaling Receptors IL-4Rα1 and IL-13Rα1

Nearly 30% of viable TLBR1 cells expressed surface receptors IL-4Rα and IL-13Rα1; a similar proportion of TLBR2 cells expressed IL-13Rα1 and nearly 65% were positive for IL-4Rα (Figure 2A). Immunocytochemistry of cytospins revealed staining of 92% of TLBR1 and 80% of TLBR2 cells for nuclear pSTAT6 (Figure 2B).

CD30 Is Required for IL-13 Expression and Phosphorylation of STAT6 in BIA-ALCL

We hypothesized that CD30 regulated IL-13 production as in murine CD4+ effector cells.[10] To test this hypothesis, we silenced CD30 in cell lines of cutaneous ALCL (Mac-1/

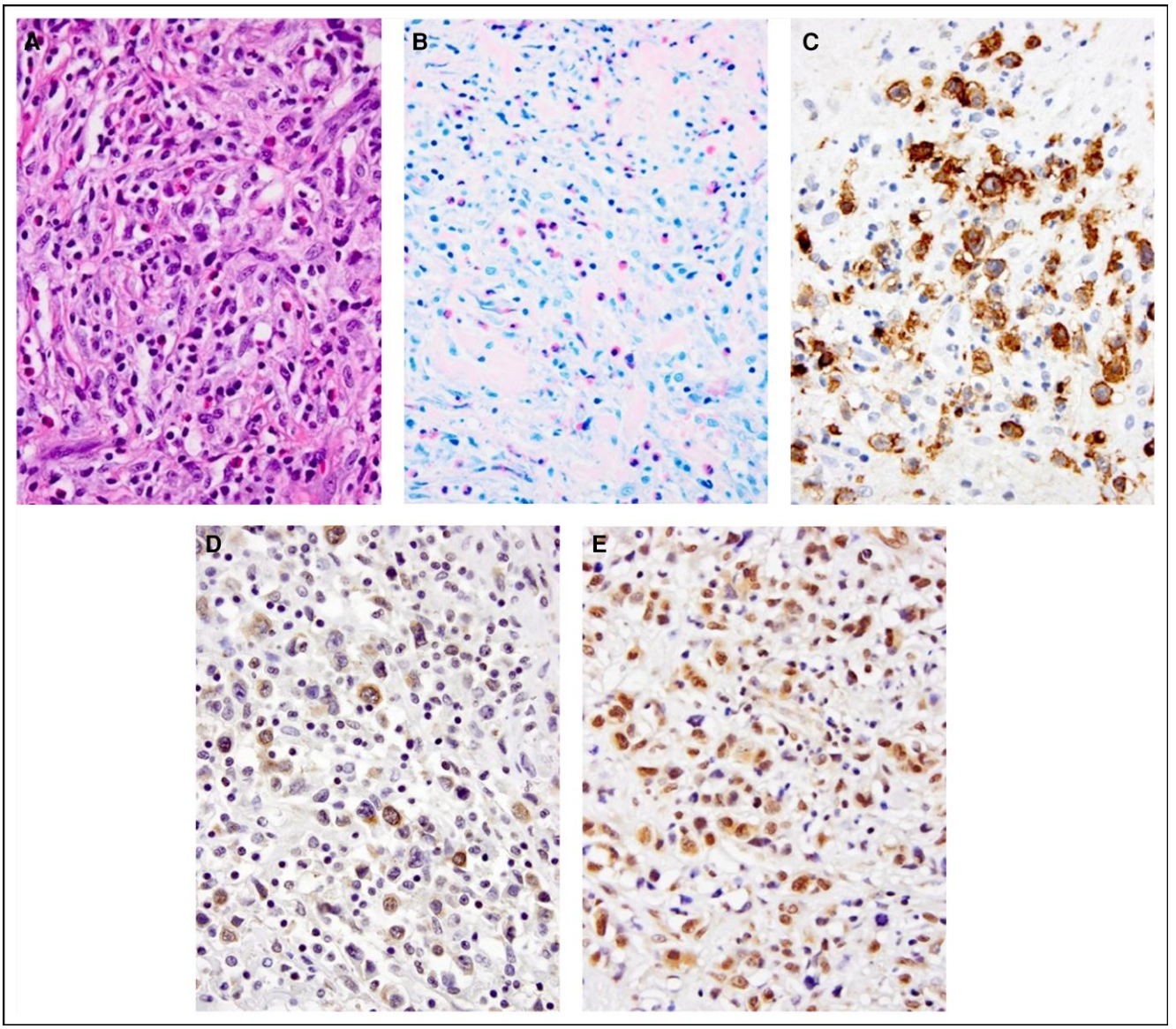

Figure 1. Pathology of breast implant capsule infiltrated by breast implant–associated anaplastic large cell lymphoma. (A) Hematoxylin-eosin stain showing anaplastic cells surrounded by inflammatory cells including eosinophils. (B) Giemsa stain highlighting eosinophils. (C) CD30 stains anaplastic cells. (D) Anaplastic cells stain for IL-13. (E) Anaplastic cells stain for pSTAT6. Original magnification, 600×.

Mac-2A) and in BIA-ALCL cell lines TLBR1 and TLBR2 by means of the CRISPR/Cas9 gene-editing method. Mac-1 cells do not produce IL-4 or IL-13. Silencing CD30 in Mac-2A cells decreased IL-4 mRNA by 40% but IL-13 by only 5% to 10%. The BIA-ALCL cells lines TLBR1 and TLBR2 do not express IL-4, but IL-13 mRNA was suppressed by 45% to 55% (in TLBR1) and by 80% to 95% (in TLBR2), confirming strong regulation by CD30 (Figure 3A).

IL-13 engagement of the IL-14/IL-13 Type II receptor complex normally results in homodimerization and phosphorylation of STAT6. We therefore postulated that silencing CD30 would abrogate phosphorylation of STAT6 in BIA-ALCL tumor cells. As expected, silencing CD30 had no effect on pSTAT6 in Mac-1 cells which do not express IL-13 or IL-4. pSTAT6 was partially suppressed in Mac-2A cells in which CD30 silencing reduced IL-13 mRNA by less than 10%; IL-4 likely contributes to partial phosphorylation of STAT6 by Mac-2A cells. In contrast, pSTAT6 was largely suppressed by silencing CD30 in TLBR1 or TLBR2 cells in which IL-13mRNA was reduced by 45% to 55% or by 80% to 95% (Figure 3B, left). The ability of the CD30 sgRNAs to inhibit CD30 expression was confirmed by

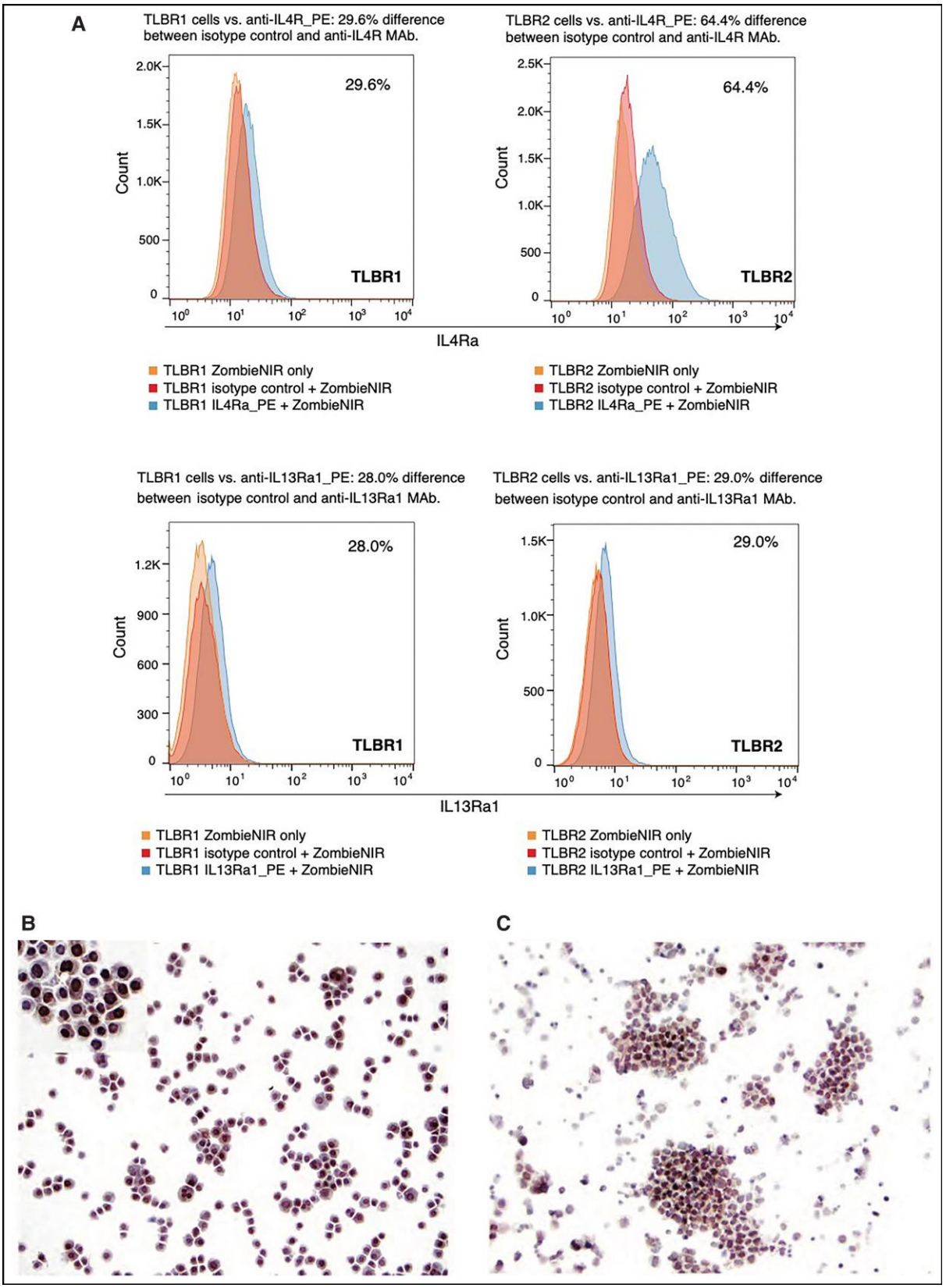

Figure 2. (A) Breast implant–associated anaplastic large cell lymphoma cell lines express signaling receptors IL-4rα and IL-13Rα1. Immunostaining of nuclear pSTAT6 in TLBR1 (B) and TLBR2 (C) cytospins. Original magnification, 200×; upper left inset in TLBR1, 400×. Details of flow cytometry and immunohistochemistry are described in the Methods section.

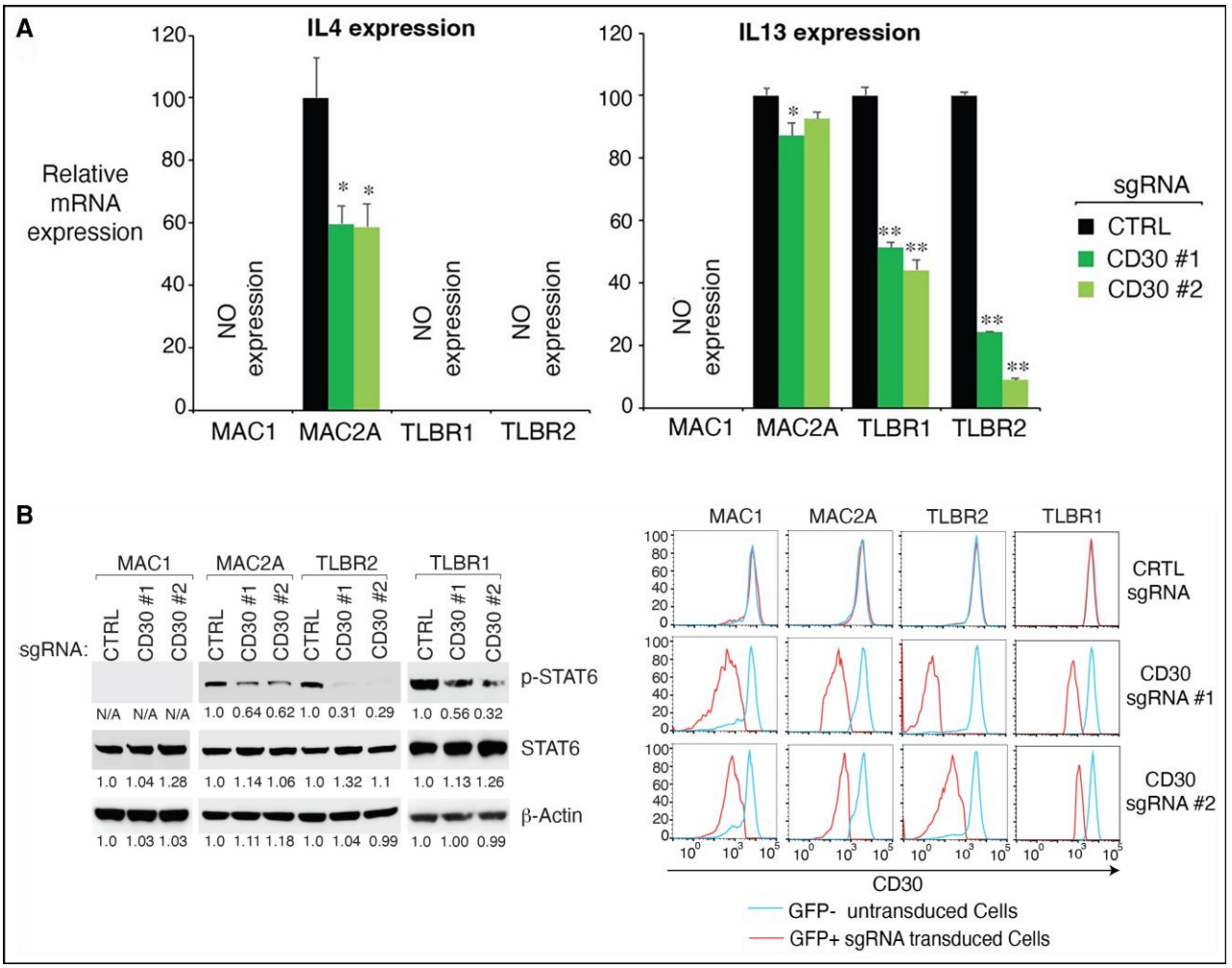

Figure 3. CD30 regulates IL-13 transcription and the phosphorylation of STAT6 in breast implant–associated ALCL cells. (A) ALCL lines were transduced with control or CD30 sgRNAs along with a GFP marker. IL-13 and IL-4 gene expression were measured by real-time polymerase chain reaction. Error bars denote SEM of triplicates. (B) Bottom: indicated ALCL lines were transduced with CD30 or control sgRNAs, selected, and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. The relative signal intensity of each band was determined by densitometric analysis and normalized to the control sgRNA conditions in each cell line. Bottom: indicated ALCL lines were transduced with CD30 or control sgRNAs along with GFP, induced to expression for 4 days. Surface CD30 expression in nontransduced (GFP–) cells and in sgRNA-transduced (GFP+) cells was measured by flow cytometry. ALCL, anaplastic large cell lymphoma; GFP, green fluorescent protein; sg, single guide.

fluorescence-activated cell sorting analysis of the surface CD30 level in transduced cell lines (Figure 3B, right). Therefore, CD30 expression is required for the transcription of IL-13, and to lesser extent of IL-4, and for the phosphorylation of STAT6 in BIA-ALCL.

TCR-Independent CD30 Signaling in BIA-ALCL Is Mediated by Phosphorylated p38 MAPK

In the murine system of CD4+ effector T cells, TCR-independent CD30-mediated production of IL-13 is triggered by association of CD30 with TRAF2 and subsequent activation of p38 MAPK. In our previous study we compared the interaction of CD30 with TRAF2 and TRAF3 in TLBR2 cells with the ALK+ systemic ALCL line DEL.[13] Because CD30-mediated production of IL-13 in murine effector cells required activation of p38 MAPK, we next tested effects of silencing CD30 on phosphorylation of p38 MAPK. As shown in Figure 4A, silencing CD30 abrogated phosphorylation of p38 MAPK in TLBR1, TLBR2, and Mac-2A cells. We then investigated the role of p38 MAPK in the IL-13–STAT6 signaling cascade. Using a specific p38 MAPK inhibitor, p38 MAPK inhibition significantly

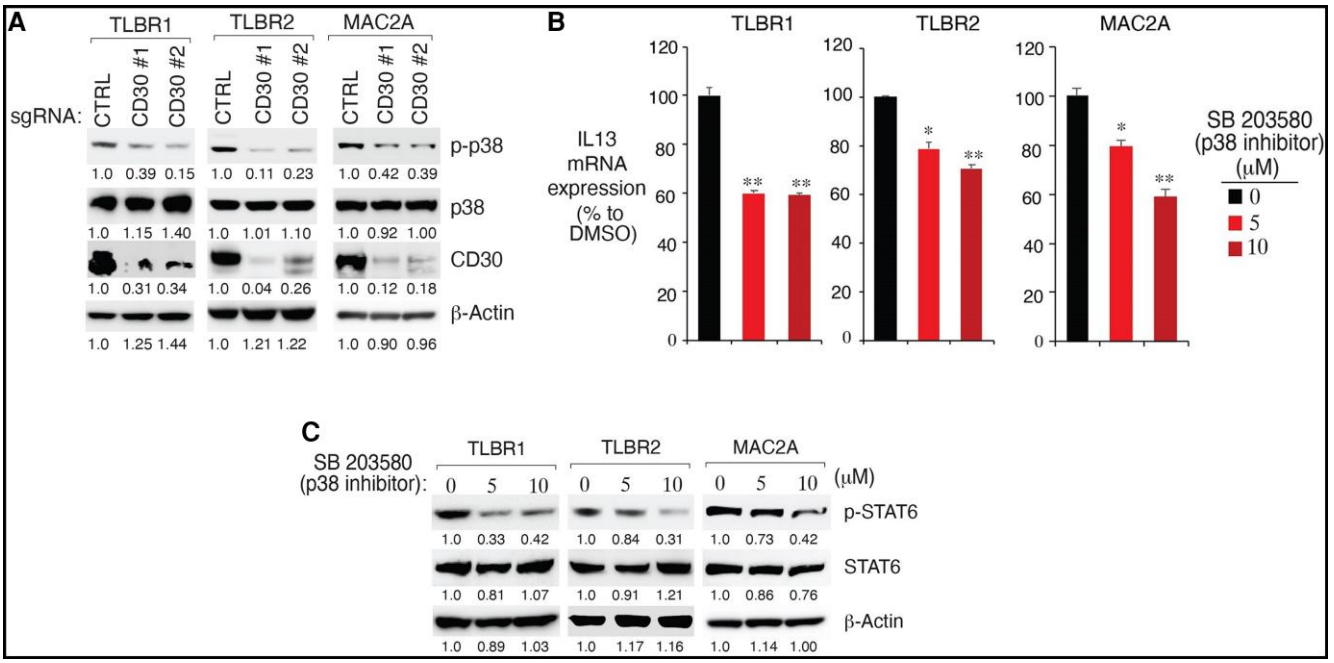

Figure 4. T-cell antigen receptor–independent CD30 signaling in breast implant–associated anaplastic large cell lymphoma is mediated by p38 MAPK. (A) TLBR1, TLBR2, and Mac-2A cells were transduced with CD30 or control sgRNAs and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. The relative signal intensity of each band was determined by densitometric analysis, and normalized to the control sgRNA conditions in each cell line. (B) TLBR1, TLBR2, and Mac-2A cells were treated with p38 MAPK inhibitor SB203580 at the indicated concentrations for 24 hours, and IL-13 gene expression was measured by real-time polymerase chain reaction. Error bars denote standard error of the mean of triplicates. (C), TLBR1, TLBR2, and Mac-2A cells were treated with p38 MAPK inhibitor SB203580 at the indicated concentrations for 24 hours, and lysates were analyzed by immunoblotting for the indicated proteins. The relative signal intensity of each band was determined by densitometric analysis and normalized to untreated conditions for each cell line. sg, single-guide.

reduced IL-13 transcription in BIA-ALCL cell lines TLBR1 andTLBR2, as well as in the cutaneous ALCL cell line Mac-2A (Figure 4B), suggesting that p38 MAPK plays an important role in transmitting signals in these tumor cells. In support, p38 MAPK inhibitor treatment strongly inhibited STAT6 phosphorylation in TLBR1, TLBR2, and Mac-2A cells (Figure 4C). Thus, p38 MAPK appears to regulate the IL-13–STAT6 signaling cascade in BIA-ALCL.

Silencing CD30 and Inhibition of STAT6 Are Cytotoxic to BIA-ALCL

Silencing CD30 was highly toxic to ALK– BIA-ALCL lines TLBR1 and TLBR2 (Figure 5A), in line with its role in STAT6 activation (Figure 3B). Inhibition of STAT6 has been shown to reduce viability of tumor cells in cutaneous T-cell lymphoma and CD30+ cutaneous ALCL.[14,15] Therefore, we tested the hypothesis that inhibition of STAT6 activity would decrease viability of BIA-ALCL tumor cells. A specific inhibitor of STAT6, AS1517499, decreased survival of BIA-ALCL cell lines TLBR1 and TLBR2 up to 80%, but had almost no effect on the T-lymphoblastic cell line MOLT-4 (Figure 5B). Thus, STAT6 inhibitor is specifically effective in BIA-ALCL. The data statement is available as an Supplemental Appendix online at www.aestheticsurgeryjournal.com.

DISCUSSION

CD30 is expressed predominantly on normal human CD4+ cells producing Th2 cytokines.[16] CD30 also is a marker of tumor cells in ALCL but there is limited information about its role in cytokine regulation in ALCL. Here we show that CD30 regulates IL-13 expression in BIA-ALCL. Unlike normal T cells and most other peripheral T-cell lymphomas, neoplastic cells in ALCL lack expression of a surface TCR. In this regard, our results are remarkably similar to what has been observed in nonmalignant mouse effector CD4+ T cells.[10] In the murine system, TCR-independent CD30-mediated production of IL-13 is triggered by association of CD30 with TRAF2 and subsequent activation of p38 MAPK but independent of CD30-induced activation of NF-κB. Similar to the murine system, our studies in BIA-ALCL revealed an association of CD30 with TRAF2,[17] and showed that CD30-mediated production of IL-13 is triggered by the phosphorylation of p38 MAPK. Accordingly, inhibition of p38 MAPK downregulated components of the IL-13 signaling pathway.

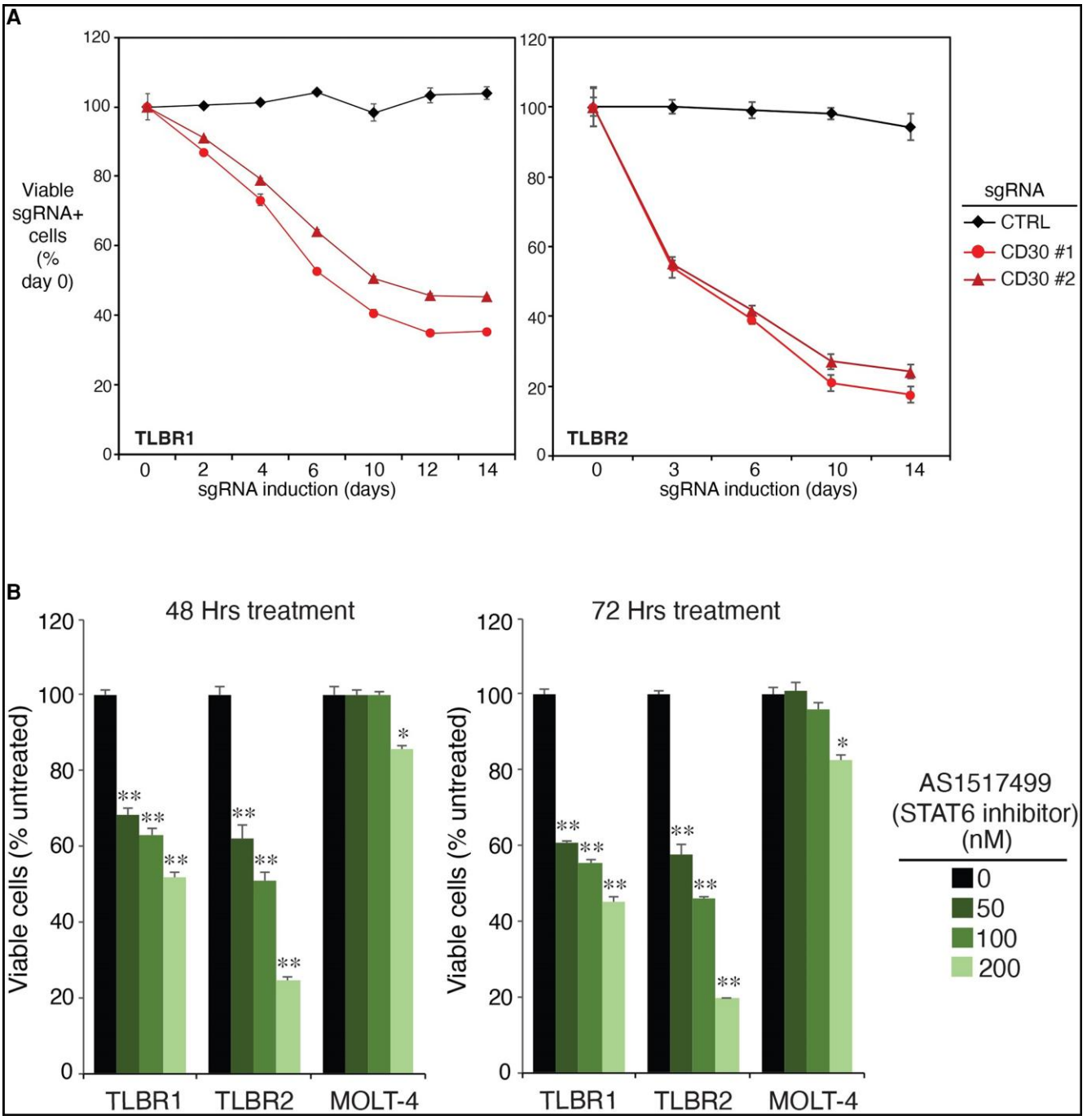

Figure 5. Silencing CD30 and STAT6 inhibitor treatment are cytotoxic to breast implant–associated anaplastic large cell lymphoma. (A) TLBR1 and TLBR2 cell lines were transduced with CD30 or control sgRNAs along with green fluorescent protein. The fraction of viable sgRNA-expressing cells relative to the total viable cell fraction at indicated times following induction of the indicated sgRNAs was normalized to Day 0 values. Error bars denote standard deviation. (B) Indicated lines were treated with STAT6 inhibitor at the indicated concentrations for 48 or 72 hours. Viability was measured by the MTS assay and normalized to dimethylsulfoxide-treated cells. Error bars denote the standard error of the mean of triplicates. sg, single-guide.

IL-13 and IL-4 signal through two receptor complexes.[18] The Type I receptor is a heterodimer of the IL-4Rα (140 kDa) and γc chain (60 kDa); the Type II receptor is comprised of IL-4Rα and IL-13Rα1 (65-70 kDa) chains. Signaling through the Type I receptor leads to activation of Janus activated kinases and downstream signaling adaptor molecules STAT6 and IRS-2, whereas signaling through the Type II receptor predominantly activates STAT6. When

STAT6 is phosphorylated it homodimerizes and translocates to the nucleus where it binds DNA promoter elements to regulate gene transcription.[18] Signaling initiated when IL-13 engages the receptor complex comprised of IL-4Rα and IL-13Rα1 was demonstrated on TLBR cell lines by flow cytometry.

STAT6 was phosphorylated (pSTAT6) in most cells of BIA-ALCL lines TLBR1 and TLBR2. pSTAT6 was also detected in cases of infiltrative BIA-ALCL including 1 Stage IIA case where IL-13 was not detected in anaplastic cells. In that case phosphorylation of STAT6 could be a result of IL-4 signaling or an activating STAT6 mutation, which is under investigation; we observed IL-4 in anaplastic cells of 4 of 8 cases of infiltrative BIA-ALCL.[2] Recurrent STAT3 mutations occur in BIA-ALCL and both TLBR1 and TLBR2 harbor STAT3 mutations.[19,20] We reported that STAT3 upregulates CD30 transcription in both ALK+ and ALK– ALCL cell lines.[17] Analyses of the CD30 gene revealed two highly conserved STAT3 binding sites in intron1, ~19 kb downstream from the transcription start site, indicating a candidate enhancer region. STAT3 bound to these two sites in TLBR2 and all other ALCL lines we tested, in addition to the transcription start site of CD30.

The current study demonstrates a possible mechanism for numerous eosinophils observed in infiltrative BIA-ALCL (Figure 1).[5] IL-13 levels are elevated in animal models of eosinophilic inflammation and in the blood and tissue of patients diagnosed with eosinophilic disorders.[21] IL-13 detected in anaplastic cells upregulates eotaxin, which recruits eosinophils.[22] Eosinophils contribute to the induction of Th2 immunity by promoting Th2 differentiation and recruiting effector Th2 cells to the site of infection.[23] Th2 cytokine genes are regulated by p38 MAPK-mediated phosphorylation of GATA3.[24] We reported Th2 skewing with GATA3 expression by tumor cells in BIA-ALCL.[2,25] IL-13 induces B cells to produce IgE which we find bound to mast cells in 5 of 8 capsules infiltrated by BIA-ALCL.[2] Activated mast cells can produce histamine,[26] which causes leaky vessels and could contribute to the common presenting feature of fluid accumulation around breast implants.

The IL-13–STAT6 pathway may be involved in growth regulation of BIA-ALCL as reported for Hodgkin lymphoma,[27] cutaneous T-cell lymphomas,[14,15] and primary effusion lymphomas.[28] Downregulation of IL-13 signaling by silencing CD30 depleted pSTAT6 and diminished the viability of BIA-ALCL tumor cells. A specific STAT6 inhibitor was cytotoxic to BIA-ALCL tumor cells. This suggests that reagents targeting the IL-13 pathway may become useful for treating BIA-ALCL patients.

Among biologic drugs widely used in treating human diseases is the humanized monoclonal antibody dupilumab which targets the IL-4Rα common to both IL-4 Type 1 and Type 2 receptor complexes. Clinical applications include asthma, atopic dermatitis, and other allergic diseases.[29]

Selective IL-13 inhibitors lebrikizumab and tralokinumab are also useful in the management of atopic dermatitis. Lebrikizumab binds to IL-13 cytokine at an epitope that overlaps with the IL-4Rα, preventing heterodimerization of IL-4α and IL13Rα subunits, and was shown to be effective in a clinical trial for treatment of adults with moderate to severe atopic dermatitis.[30] New treatment approaches for ulcerative colitis that specifically target IL-13 are also being developed.[31] Thus several biologic agents with good safety profiles currently in clinical trials could potentially be useful for treatment of BIA-ALCL.

CONCLUSIONS

We conclude that: (1) TCR-independent activation of IL-13 by CD30 mimics the effect of CD30 ligation on murine CD4+ effector T cells, potentially leading to a new model for BIA-ALCL pathogenesis; (2) IL-13/STAT6 signaling in tumor cells was reduced by inhibitors of p38 MAPK and pSTAT6; and (3) suppression of IL-13 signaling offers potential targeted therapies for BIA-ALCL.

Supplemental Material

This article contains supplemental material located online at www.aestheticsurgeryjournal.com.

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Disclosures

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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