T-cell Receptor Signaling Activates an ITK/NF-κB/GATA-3 axis in T-cell Lymphomas Facilitating Resistance to Chemotherapy

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Abstract

Purpose: T-cell lymphomas are a molecularly heterogeneous group of non-Hodgkin lymphomas (NHL) that account for a disproportionate number of NHL disease-related deaths due to their inherent and acquired resistance to standard multiagent chemotherapy regimens. Despite their molecular heterogeneity and frequent loss of various T-cell–specific receptors, the T-cell antigen receptor is retained in the majority of these lymphomas. As T-cell receptor (TCR) engagement activates a number of signaling pathways and transcription factors that regulate T-cell growth and survival, we examined the TCR's role in mediating resistance to chemotherapy.

Experimental Design: Genetic and pharmacologic strategies were utilized to determine the contribution of tyrosine kinases and transcription factors activated in conventional T cells following TCR engagement in acquired chemotherapy resistance in primary T-cell lymphoma cells and patient-derived cell lines.

Results: Here, we report that TCR signaling activates a signaling axis that includes ITK, NF-κB, and GATA-3 and promotes chemotherapy resistance.

Conclusions: These observations have significant therapeutic implications, as pharmacologic inhibition of ITK prevented the activation of this signaling axis and overcame chemotherapy resistance. Clin Cancer Res; 23(10); 2506–15. ©2016 AACR.

Introduction

T-cell lymphoproliferative disorders are derived from mature (post-thymic) T cells and account for approximately 10% of non-Hodgkin lymphomas (NHL). In contrast to many aggressive B cell–derived NHLs that are highly curable with modern multiagent chemotherapy regimens, acquired chemotherapy resistance is frequently observed in the majority of similarly treated T-cell lymphoma (TCL) patients (1, 2). Despite initial chemosensitivity, primary refractory disease and cross-resistance to alternative agents is frequently observed, thus explaining the high mortality rates observed with these aggressive NHLs (3, 4). Therefore, improved understanding of chemotherapy resistance mechanisms and the development of novel therapeutic strategies to overcome them are needed.

Despite their proliferative capacity, resistance to chemotherapy, and survival in vivo, the establishment of long-term TCL cell lines has been challenging, as malignant T cells undergo spontaneous apoptosis during in vitro culture (5, 6). Therefore, chemotherapy resistance is unlikely explained by intrinsic resistance mechanisms alone, but is likely explained by the provision of extrinsic growth and survival signals by nonneoplastic cells within the tumor microenvironment (TME). Every milestone in the lifecycle of a conventional T cell is regulated by professional antigen-presenting cells (APC). Professional APCs provide ligands for antigen, costimulatory, and cytokine receptors, the provision of which may similarly promote T-cell lymphomagenesis and resistance to chemotherapy, as engagement of these receptors activates signaling pathways and transcription factors, including NF-κB, that promote cell growth and survival and have been previously implicated in TCI pathogenesis and chemotherapy resistance (7–10). This hypothesis is further supported by the observation that professional APCs, including lymphoma-associated macrophages and dendritic cells, directly promote the growth and survival of malignant T cells and are abundant constituents of the TME in most TCLs (5, 6, 11).

Collectively, these observations suggest that TCLs are regulated by lineage-specific pathways, like the antigen receptor, that are engaged by professional APCs. Therefore, we sought to investigate the extent to which T-cell receptor (TCR) engagement regulates TCI growth, survival, and particularly chemotherapy resistance. In doing so, we observed that engagement of the TCR on primary malignant T cells activates an ITK/NF-κB/GATA-3 axis that
Promotes chemotherapy resistance but is abrogated by the BTK/ITK inhibitor ibrutinib.

Materials and Methods

Patient samples and cell lines

Normal donors and TCL patients at the University of Michigan Comprehensive Cancer Center (Ann Arbor, MI) were considered for study participation. Study approval was granted by the Institutional Review Board of the University of Michigan, in accordance with U.S. federal regulations and the Declaration of Helsinki. TCL patients with Sezary syndrome, n = 6; or systemic peripheral TCL, not otherwise specified (PTCL, NOS), n = 1] with extensive leukemic involvement, representing >80% of peripheral blood lymphocytes by flow cytometry, as determined by clinical flow cytometry and reported in the electronic medical record, were preferentially selected for isolation and in vitro studies, after providing informed consent. Normal T cells and TCL cells were isolated from peripheral blood mononuclear cells by density centrifugation (Ficoll—Hypaque, GE Healthcare) and subjected to positive selection using CD4 magnetic beads (Miltenyi Biotec). Isolation and purity of TCL cells was examined by multicolor flow cytometry. Malignant T cells were recognized by their expression of T-cell markers (CD3, CD4, and/or CD8) and the aberrant loss of other T cell–associated antigens (most commonly CD7), as per EORTC and ISCL guidelines (12). Purity was assessed by flow cytometry and was >93% (Supplementary Fig. S1). Lymphoma cells were used immediately following isolation for the experiments described. Monocytes were isolated in a similar fashion using CD14 magnetic beads. Coculture experiments were performed as described previously (5), with the addition of functional grade anti-HLA-DR/DP/DQ (clone Tu39, BD Biosciences) or an isotype control. Cells were cocultured for 7 to 10 days prior to assessing Ki67 expression on CD4+CD7+ malignant T cells. The TCL cell lines (T8ML-1, H9, MyLa, and SU-DHL-1) used in this study were mycoplasma free and independently validated, as described previously (11, 13). T8ML-1 cells were derived from a patient with refractory PTCL, NOS. These cells are CD3+CD8+ and are immunophenotypically identical to the patient’s primary tumor. Clonality was confirmed by multiplex PCR, and the expression of Vβ12.2/16/Cα and Vβ15.1/21/Cβ2 TCR chains was confirmed as reported previously (13). Primary cells and cell lines were grown at 37°C in 5% CO2 in RPMI1640 (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals), 10 mmol/L HEPES, 1 mmol/L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. For T8ML1 and its derivative cell lines, 100 IU/mL recombinant human IL2 was also added. To engage TCR signaling, anti-CD3/CD28 Dynabeads (Life Technologies) were cocultured with TCL cells for various times (5 minutes for phosphorylation events, examined by Western blot analysis; 1 hour for NF-κB nuclear localization, by ELISA and Western blot analysis; 24 hours for NF-κB target gene mRNA quantitation, cytokine measurement by ELISA, or assessment of T-cell activation and GATA-3 expression by flow cytometry). Chemotherapeutic agents were obtained from Selleckchem or the University of Michigan Pharmacy.

Translational Relevance

The acquisition of chemotherapy-resistant disease, observed in approximately 25% of peripheral T-cell lymphoma patients treated with standard regimens, remains a clinical challenge, as the mechanism(s) driving the emergence of resistant clones is poorly understood. We demonstrate that engagement of the T-cell receptor (TCR) on malignant T cells culminates in NF-κB activation and the upregulation of GATA-3 expression, both of which regulate the growth and survival of conventional T cells, and were shown to promote chemotherapy resistance in malignant T cells. ITK regulates the formation of the TCR signalosome; thus, its pharmacologic inhibition significantly impaired TCR signaling and chemotherapy resistance in T-cell lymphoma cells. Therefore, ITK inhibition, or similar therapeutic strategies that inhibit TCR-dependent signaling, is a novel and promising therapeutic strategy that may overcome the challenge of chemotherapy resistance in the T-cell lymphomas.

Microarray gene expression profiling and statistical analysis

Microarray profiling was used to determine gene expression changes following TCR engagement. T8ML-1 and 3 TCL patients’ (2 Sezary syndrome, 1 PTCL, NOS) primary cells were used as study subjects. Briefly, at various time points (4, 12, or 24 hours) following stimulation, total RNA was extracted and submitted to the University of Michigan DNA Sequencing Core for processing, QC, and analysis of the data. The RNA samples were processed using the NuGen WT Pico Kit and were hybridized to the Human Gene ST 2.1 arrays and were scanned on the GeneAtlas system. Probe intensity values were converted to expression values using the robust multiarray average (RMA) method for each experiment independently. This was implemented in the R statistical environment using the “oligo” package of Bioconductor (14, 15). This technique includes a quantile normalization step effectively making the distribution of probe intensities the same across the chips. For this reason, density plots were used to confirm that no sample had a different distribution in intensities. RMA log-transformed expression values were used for downstream analysis. The affyPLM package of Bioconductor was also used to fit probe level models and plot boxplots of the normalized unscaled SEs. These boxplots showed very little variation. Finally, we used PCA plots to confirm there were no major outliers. All plots together suggested these data were of very good quality. We fit linear models to the data using the limma package of Bioconductor (16, 17). We limited the analysis to “main” probe sets as defined by Affymetrix and filtered out the probe set with a variance less than 0.05. A gene-by-gene update algorithm was used to help identify unique and interesting patterns. This was developed to provide a list of genes that were differentially expressed between groups or unique to a given sample. Functional/pathway enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; david.ncifcrf.gov).

Results

The TCR regulates gene expression in primary malignant T cells

Monocytes and their progeny promote the expansion and long-term survival of malignant T cells during in vitro culture (5, 6).
Therefore, this experimental system was initially employed to interrogate the role of the TCR in stimulating the proliferation of malignant T cells. To do so, malignant CD4⁺ T cells and autologous monocytes were isolated from TCL patient PBMCs. T cells were either cultured alone or cocultured with autologous monocytes or an antagonistic MHC class II mAb (isotype control) and T-cell proliferation determined by Ki67 expression, as indicated. TCR stimulation was performed using anti-CD3/CD28 beads and proliferation determined by Ki67 expression, as indicated in the representative examples shown. 

Figure 1. The TCR stimulates proliferation and activates gene expression in TCLs. A, Primary TCL cells were cultured for 7 days in the presence or absence of autologous monocytes with an isotype control (iso) or blocking MHC class II antibody (anti-class II), as indicated. TCL proliferation was determined by Ki67 expression, and the percentage of CD4⁺ CD7⁻ TCL cells that were Ki67⁺ is indicated in the gates shown in a representative FACs analysis (SS, side scatter). B, TCL proliferation in the presence of autologous monocytes was independently determined (n = 4) and was expressed as the fold increase in Ki67 expression compared with TCL cells cultured alone (mean ± SEM). C, The primary TCL cell line T8ML-1 and primary TCL cells were cultured in the presence or absence of anti-CD3/CD28 beads and proliferation determined by Ki67 expression, as indicated in the representative examples shown. D, The percentage of Ki67⁺ TCL cells (mean ± SEM) cultured alone (open histograms) or with beads (gray histograms) is shown for T8ML-1 (in technical replicates) and for primary TCL cells (n = 3). E, Unsupervised hierarchical clustering highlighting changes (log₂-transformed fold change) in gene expression over time upon bead stimulation is shown for 3 TCL patients (Sezary syndrome; TCL, NOS). F, Venn diagram showing substantial overlap in differentially expressed genes among the 3 TCL patients examined. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
expression were observed following stimulation of T8ML-1 cells (Supplementary Fig. S3), as 19% of differentially expressed genes identified in T8ML-1 were shared among all three primary samples, whereas 59% of genes identified were shared with at least one primary sample. Therefore, we concluded that TCR engagement on malignant T cells has a profound impact on gene expression and conceivably activates relevant pathways involved in conferring resistance to chemotherapy. We next sought to confirm these findings in independent primary TCL specimens. To do so, cytokine production and the inducible expression of T-cell activation markers were examined. We preferentially selected gene products that were identified in the gene expression profiling dataset, including those that are inductively expressed following TCR activation of conventional T cells, and examined their expression following TCR engagement in T8ML-1 (SupplementaryFig. S4) and primary TCL cells (Supplementary Fig. S5). As anticipated, TCR engagement in T8ML-1 and primary TCL cells led to the phosphorylation of proximal tyrosine kinases, including Zap-70 and IL2-inducible T-cell kinase (ITK; Supplementary Fig. S5A and S5B), culminating in a significant increase in IL2, IL13, IL10, and IFNγ production (Supplementary Fig. SSC), and the upregulation of T-cell activation markers (Supplementary Fig. SSD and SSE).

TCR engagement activates NF-κB and decreases responsiveness to chemotherapy in TCLs

TCR engagement culminates in the activation of signaling cascades and transcription factors, including NF-κB, that are required for the homeostatic survival of conventional T cells. Similarly, TCR engagement led to the activation of downstream kinases, including ITK and PLC-γ (Supplementary Fig. S5A and S5B), in TCL. Examination of NF-κB (p65) nuclear localization (Fig. 2A and Supplementary Fig. S5F), DNA binding (Fig. 2B), and expression of NF-κB target genes (Fig. 2C) demonstrated that NF-κB is activated upon TCR engagement in TCL cells. As NF-κB regulates the expression of many genes involved in T-cell growth and survival, TCL cell growth and viability were examined following TCR engagement. TCR engagement significantly enhanced the growth and viability of cultured TCL cells, conferring decreased susceptibility to chemotherapy (Fig. 2D). T8ML-1 cells were treated with either a microtubule-targeting agent (vincristine) or with a histone deacetylase inhibitor (romidepsin) at concentrations approximating the IC50. A significant increase in TCL cell viability was observed in bead-stimulated T8ML-1 cells. Primary TCL cells failed to proliferate in culture and were consequently not susceptible to the cell cycle-dependent agent vincristine (data not shown). Therefore, primary TCL cells were treated with romidepsin alone, whereby TCR stimulation was observed to provide a similar degree of protection (Fig. 2D). Sotrastaurin, a PKC-δ inhibitor, was utilized to block NF-κB activation following TCR engagement and was found to significantly inhibit TCR-mediated chemotherapy resistance in T8ML-1 and in most primary TCL cells (Supplementary Fig. S6). Collectively, these findings demonstrate that TCR activation, as may be provided by MHC-expressing APC within the TME, supports the growth and survival of malignant T cells. Improved understanding of the mechanisms involved in TCR-mediated resistance to chemotherapy may unveil novel therapeutic strategies that abrogate resistance.

TCR-mediated activation of NF-κB and chemotherapy resistance are ITK dependent

ITK, by regulating the spatiotemporal localization of the TCR signalosome (21), is a critically important mediator of TCR signaling and is commonly expressed (22), if not overexpressed (23), in TCLs. Therefore, its localization to the cell membrane...
following CD3 cross-linking in primary TCL cells may not be surprising (Supplementary Fig. S7). To examine its role in TCL cells, an shRNA-mediated gene targeting approach was adopted in T8ML-1 cells, and ITK knockdown was confirmed (Supplementary Fig. S8). Upon ITK knockdown, TCR-dependent cytokine production (Supplementary Fig. S9A), upregulation of activation-dependent cell surface receptors (Supplementary Fig. S9B), and NF-κB activation (Supplementary Fig. S9C) were significantly impaired. Therefore, the extent to which ITK loss abrogated TCR-mediated chemotherapy resistance was examined. In its absence, resistance to vincristine (Fig. 3A) and romidepsin (Fig. 3B) following TCR engagement was significantly impaired in T8ML-1 cells. Ibrutinib, an irreversible inhibitor of ITK (24), and its homolog BTK were used to pharmacologically inhibit ITK (Supplementary Fig. S10A), T-cell activation (Supplementary Fig. S10B and S10C), and NF-κB activation (Supplementary Fig. S11A–S11C) in primary TCL cells. Consistent with these results, ibrutinib significantly impaired TCR-mediated resistance to romidepsin in primary TCL cells (Fig. 3C). As ibrutinib inhibits murine ITK, we next examined the extent to which ibrutinib abrogates chemotherapy resistance in vivo using T8-28 cells, a spontaneously generated murine TCL expressing an intact TCR (25). In vitro studies were challenging, as approximately 90% of T8-28 cells undergo apoptosis within 48 hours of in vitro culture and must be maintained by serial passage in vivo. Nonetheless, TCR engagement inhibited spontaneous apoptosis of T8-28 cells during in vitro culture and was abrogated by ibrutinib (Supplementary Fig. S12). In this model, lymphoma-bearing mice develop massive hepatosplenomegaly secondary to lymphomatous involvement (Supplementary Fig. S13; ref. 25). Consistent with results obtained in primary human TCL cells, ibrutinib treatment alone had no significant impact on T8-28 progression. In contrast, ibrutinib administration significantly improved the responses observed with conventional chemotherapeutic agents (vincristine and cyclophosphamide), further implicating ITK in mediating resistance to chemotherapy (Fig. 3D and Supplementary Fig. S14).

**GATA-3 expression in TCLs is regulated by the TCR**

The transcription factor GATA-3 promotes the homeostatic survival of conventional T cells in a TCR- and NF-κB–dependent manner (26). We have previously shown that GATA-3 is expressed in various TCL subtypes, including the most common TCL in North America, that is, PTCL, NOS (10, 11). PTCLs, NOS are clinically and molecularly heterogeneous, but the expression of GATA-3 and its gene targets identifies a distinct subset of these aggressive TCLs. While recognizing that the TCR and NF-κB regulate the expression of many progrowth and survival genes, these previous observations suggested that GATA-3 may have a significant role in mediating chemotherapy resistance following TCR engagement in TCL. Therefore, GATA-3 expression was examined following TCR stimulation in T8ML-1 and primary TCL cells. Following TCR engagement, a significant increase in GATA-3 expression was observed (Fig. 4A and B). GATA-3 upregulation following TCR engagement was significantly inhibited by ibrutinib, thus implicating ITK in TCR-mediated GATA-3 upregulation (Fig. 4C and D). The observed increase in GATA-3 transcripts following TCR engagement of T8ML-1 cells (Supplementary Fig. S15A) and inhibition by sotrastaurin (Supplementary Fig. S15B) collectively implicate an NF-κB–driven increase in GATA-3 transcription as a mechanism for TCR-mediated GATA-3 upregulation (26). However, a similar increase in GATA-3 transcription was not uniformly observed in primary TCL cells (Supplementary Fig. S15C), implicating an additional posttranscriptional mechanism of GATA-3 regulation. Significant differences in the stability of GATA-3 mRNA (Supplementary Fig. S15D) or protein (Supplementary Fig. S15E) were not observed.
GATA-3 confers chemotherapy resistance

Given these findings, and to determine the extent to which they may be generalizable, we sought to examine the potential role of GATA-3 in mediating chemotherapy resistance in cutaneous TCLs (CTCL), as these lymphomas classically express GATA-3 and its gene targets (28, 29), including Th2-associated cytokines (11, 30, 31), and are clinically resistant to conventional chemotherapeutic agents (32). Therefore, cell growth and viability was examined using CTCL cell lines (i.e., H9 and MyLa) stably transduced with either nontargeting (scr) or GATA-3–specific shRNA (G3, G5). Cells were cultured with or without beads, as indicated, in the presence of vincristine (6 nmol/L). Cell viability was determined 48 hours later and is reported relative to cells cultured without vincristine. The fold increase in viability between unstimulated and bead-stimulated cells is shown below the x-axis. F, GATA-3 was overexpressed in T8ML-1 transduced with ITK-targeting shRNA (I4, I5) following transduction with a vector containing full-length GATA-3 (GATA-3) or an empty vector (EV) control. Cells were cultured alone or in the presence of vincristine (6 nmol/L), and viability was determined 48 hours later. Viability is reported relative to cells cultured without vincristine. *, P < 0.05; **, P < 0.01; ***, P < 0.001 in unpaired two-sided Student t test.

following TCR engagement, suggesting that GATA-3 upregulation following TCR stimulation may be attributed, at least in part (27), to the global increase in mRNA translation that was observed (Supplementary Fig. S15F). To examine the role of GATA-3 in TCR-mediated chemotherapy resistance, shRNA-mediated loss-of-function studies were performed. Cells transduced with GATA-3–targeting shRNA failed to upregulate GATA-3 following TCR engagement (Supplementary Fig. S16A), and chemotherapy resistance was curtailed (Fig. 4E). Conversely, chemotherapy resistance in ITK-deficient T8ML-1, which also fail to upregulate GATA-3 following TCR engagement (Supplementary Fig. S16B), was partially restored upon TCR-independent GATA-3 overexpression (Supplementary Fig. S16C and Fig. 4F).

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GATA-3 expression was appreciated in biopsies obtained at the time of relapse after treatment with anthracycline-based multiagent chemotherapy (i.e., CHOP/CHOEP) was examined (Fig. 5H). Following tumor engraftment, mice were either untreated (closed symbols) or treated with weekly cyclophosphamide and vincristine (as indicated by the arrows) and tumor volumes determined. Untreated tumors previously treated with cyclophosphamide/vincristine either untreated (closed symbols) or treated with single-agent vincristine (as indicated by the arrows) and tumor volumes determined. H9 19301 tumors previously treated with cyclophosphamide/vincristine subsequently relapsed (shown in C) and were analyzed for GATA-3 expression by IHC (“posttreatment”). For comparison, GATA-3 expression in established H9 scramble, 273991, and 19301 tumors (“pretreatment”) is shown on the left. F and G, The extent of GATA-3 expression was determined by IHC in serial patient biopsies obtained at the time of diagnosis and at the time of relapse after treatment (representative example shown in F). G, GATA-3 expression, as a percentage of total TCL cells, in paired samples (n = 5) is shown. H, PTCL, NOS patients treated with CHOP or CHOP were stratified by GATA-3 expression, and the rate of primary refractory disease reported (mean ± 95% confidence interval is shown). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Collectively, these results strongly suggest that GATA-3 promotes chemotherapy resistance in TCL and that mediators of TCR-dependent signaling, including ITK, promote its expression and are thus attractive therapeutic targets.

Figure 5. GATA-3 confers resistance to chemotherapy in TCLs. A and B, The CTCL cell lines H9 and MyLa were transduced with nontargeting (scr) or GATA-3-targeting shRNA (19301). H9 (A) and MyLa (B) were cultured with or without vincristine (VCR) and cell viability (reported as a percent of untreated cells) determined 48 hours later. C, H9 transduced with nontargeting (scr, ○/●) or GATA-3-targeting shRNA 273991 (●/●) or 19301 (Δ/Δ), were injected subcutaneously into the shoulders and flanks of NSG mice. Following tumor engraftment, mice were either untreated (closed symbols) or treated with weekly cyclophosphamide and vincristine (as indicated by the arrows) and tumor volumes determined (n = 12). D, In a similar fashion, NSG mice were engrafted with MyLa cells transduced with nontargeting (scr, ○/●) or GATA-3-targeting shRNA 19301/Δ/Δ and either untreated (closed symbols) or treated with single-agent vincristine (as indicated by the arrows) and tumor volumes determined. E, H9 19301 tumors previously treated with cyclophosphamide/vincristine subsequently relapsed (shown in C) and were analyzed for GATA-3 expression by IHC (“posttreatment”). For comparison, GATA-3 expression in established H9 scramble, 273991, and 19301 tumors (“pretreatment”) is shown on the left. F and G, The extent of GATA-3 expression was determined by IHC in serial patient biopsies obtained at the time of diagnosis and at the time of relapse after treatment (representative example shown in F). G, GATA-3 expression, as a percentage of total TCL cells, in paired samples (n = 5) is shown. H, PTCL, NOS patients treated with CHOP or CHOEP were stratified by GATA-3 expression, and the rate of primary refractory disease reported (mean ± 95% confidence interval is shown). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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Discussion

The widespread expression of both the TCR and its associated kinases and adaptor proteins (23, 38, 39), in conjunction with the biased TCR Vβ usage that is observed in selected T-cell lymphoproliferative disorders (40–42), suggests that malignant T cells exploit the growth and survival signals provided by the TCR. This hypothesis is further supported by the growing appreciation that tyrosine kinases classically associated with TCR signaling in conventional T cells are recurrently mutated in a minority of TCLs (reviewed in ref. 43). However, for the majority of TCLs lacking such activating mutations, signaling may require engagement by antigenic peptide/MHC complexes provided by constituents of the TME, particularly professional APCs, which are particularly abundant constituents of the TME in most TCLs (5, 6, 11). Our findings are consistent with this hypothesis, as are previous observations demonstrating that survival of malignant T cells when cocultured with immature dendritic cells or monocytes/macrophages is abrogated by either clonotypic TCR- or MHC-blocking antibodies (6, 44). Nonetheless, the hypothesis that TCLs exploit TCR-dependent signals upon recognition of peptide/MHC in vivo, and the extent to which ITK, NF-κB, and GATA-3 are involved, will require future studies in mouse models. Positively selected T cells emerging from the thymus have signif-

GATA-3 are involved, will require future studies in mouse models.

In the current study, antibody-mediated cross-linking of the TCR was utilized, and in contrast to "tonic" signaling, more closely resembles TCR activation by high-affinity antigenic peptides leading to "active" TCR signaling. Recurrent genomic alterations in the TCL, including the loss of negative feedback mechanisms or gain of positive regulators, may lower the threshold for TCR activation and thus potentiate low-affinity interactions with peptide/MHC normally associated with "tonic" signaling (47–52). Although previous observational studies demonstrating biased usage of specific TCR-Vβ chains implicate the TCR in disease pathogenesis (40, 41), most TCLs are not associated with T cell–mediated autoimmunity or chronic infections. With the possible exception of bacterial superantigen-mediated engagement of the TCR in Sezary syndrome (42, 53), potential antigenic drivers remain largely speculative (54).

ITK is a critically important component of the proximal TCR signalosome (21). Therefore, we exploited the BTK/ITK inhibitor ibrutinib as a means to pharmacologically inhibit TCR signaling and NF-κB activation in primary TCL (24). Ibrutinib significantly abrogated both TCR-dependent activation of NF-κB and chemotherapy resistance. Although the findings reported here implicate TCR- and ITK-dependent GATA-3 expression in chemotherapy resistance, GATA-3 is not uniformly expressed in all TCL subtypes, including those that may be driven by the antigen receptor. Therefore, it seems quite likely that alternative GATA-3–independent pathways may also contribute to chemotherapy resistance following TCR engagement. The observation that genetic or pharmacologic approaches to inhibit ITK uniformly impairs TCR-mediated GATA-3 upregulation has significant clinical implications. Whether similar strategies targeting ITK, or other mediators of TCR signaling, will be effective strategies to inhibit GATA-3 expression and overcome acquired chemotherapy resistance warrants further study in well-designed clinical trials. Inter-

estingly, single-agent ibrutinib did not significantly impair TCL growth in our studies (e.g., Fig. 3D). This may be explained by the bidirectional relationship between ITK and GATA-3 in T-cell activation and differentiation. Studies performed in ITK-deficient mice demonstrate that ITK is required for optimal Th2-biased immunity, as ITK qualitatively regulates TCR signaling and negatively regulates the expression of T-bet (55). Conversely, established Th2 cells are increasingly dependent upon ITK, as GATA-3 promotes the selective downregulation of RLK, a TEC family kinase that is, at least partially, redundant with ITK and is not sensitive to ibrutinib. Therefore, it is noteworthy that ITK inhibition was associated with decreased GATA-3 expression in primary TCLs (Fig. 4C and D) and in T8M1-1 cells (Supplementary Fig. S16B). These observations may imply significant heterogeneity among PTCL subtypes in their dependence upon ITK and susceptibility to ibrutinib. Impaired GATA-3 expression alone does not inhibit TCL growth (e.g., Fig. 5C and D), despite diminishing chemotherapy resistance. The mechanism(s) underlying these apparently discordant, but not unprecedented, observations is poorly understood. In contrast, the selection pressure imposed by chemotherapy favors the outgrowth of "GATA-3 high" cells (Fig. 5E and G), but the emergence of these resistant clones may be subverted upon ITK inhibition. Whether the emergence of "GATA-3 high" cells is associated with an increased ITK/RLK ratio and increasing sensitivity to ibrutinib, although biologically plausible, was not examined. We cannot exclude the possibility that ibrutinib's effects are, at least partially, indirect and due to increased host antitumor immunity in the T8-28 model. Activation of NF-κB is commonly observed in T cells and, not surprisingly, promotes resistance to chemotherapy (7, 8). A myriad of NF-κB target genes regulate T-cell growth and survival and likely collaborate in conferring chemotherapy resistance to their malignant counterparts. Nonetheless, the data we present clearly implicate GATA-3 in chemotherapy resistance, but the GATA-3 target genes responsible are unknown. Gene expression profiling studies performed following GATA-3 knockdown failed to clearly identify a single candidate gene, including those involved in multidrug resistance (data not shown). The number of GATA-3–binding sites identified in the human genome (>7,000 in Th2 cells) rivals that observed for c-myc in Burkitt lymphoma (56). Therefore, it may be naïve to imagine that GATA-3 regulates chemotherapy resistance by regulating a single gene target or that its role in chemotherapy resistance is not influenced by the context, including the cell-of-origin, in which it is expressed. In addition, GATA-3 may regulate chemotherapy resistance in a non–cell-autonomous manner, as its target genes may functionally polarize constituents of the TME, particularly lymphoma–associated macrophages (11).

We initially hypothesized that ITK, by activating NF-κB, transcriptionally regulates GATA-3 (26). Although ITK-dependent NF-κB activation was demonstrated, a significant increase in GATA-3 transcription was not uniformly observed following TCR activation of primary TCL cells, implicating an additional posttranscriptional mechanism of regulation. The collective data imply that inductive GATA-3 expression following TCR engagement may be translationally regulated. The GATA-3 5′-UTR is GC rich and is likely amenable to "cap-dependent," and mTOR-regulated, translational control (data not shown). This is pertinent, as TCR activation leads to mTOR activation, which has recently been implicated in regulating GATA-3 expression and Th2 differentiation (27, 57). More importantly, mTOR-dependent genes were

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enriched in GATA-3–positive, but not GATA-3–negative, TCL (10). In contrast to TCR-inducible GATA-3 expression, basal expression of GATA-3, as in conventional T cells, appeared to be NF-kB dependent, as its inhibition with the PKC-θ inhibitor sotrastaurin reproducibly impaired basal GATA-3 expression in TCL (data not shown; ref. 58).

In summary, activation of the TCR in TCL culminates in ITK-dependent NF-κB activation, GATA-3 upregulation, and chemotherapy resistance. As GATA-3 regulates chemoresistance in these lymphomas, ITK inhibition, or similar therapeutic strategies that inhibit TCR-dependent signaling and GATA-3 expression, is a novel and promising therapeutic strategy that may overcome the challenge of chemotherapy resistance in these aggressive lymphomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Wang, Y. Lu, R.A. Wilcox
Development of methodology: T. Wang, Y. Lu, C.M. Zamalloa, R.A. Wilcox
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Correction: T-cell Receptor Signaling Activates an ITK/NF-κB/GATA-3 Axis in T-cell Lymphomas Facilitating Resistance to Chemotherapy

In this article (Clin Cancer Res 2017;23:2506–15), which was published in the May 15, 2017, issue of Clinical Cancer Research (1), the name of an author was incorrectly listed as Carlos Murga Zamalloa; it should be Carlos Murga-Zamalloa. The Publisher regrets this error.

Reference

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