Toward personalized lymphoma immunotherapy: Identification of common
driver mutations recognized by patient CD8\(^+\) T cells

Running title: Immune recognition of driver mutations in lymphoma

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**Key words:** tumor immunology, lymphoma, oncogenes, mutanome, CD8+ T cells, next-generation sequencing, personalized medicine

**Financial support:** This work was supported by the Canadian Cancer Society (grant #701789), the British Columbia Cancer Foundation (BCCF), and the Whittle Family Multiple Myeloma Research Fund. JSN received funding from the Lymphoma Foundation Canada. ZLB is the recipient of a Canadian Institutes of Health Research (CIHR) New Investigator Award and a Michael Smith Foundation for Health Research Scholar Award. RDG, MAM, and JMC are supported by a Terry Fox Research Institute New Frontiers in Cancer Program Project Grant (#1023). JMC receives research funding from Genome British Columbia, Genome Canada, CIHR, and BCCF. RDM is supported by start-up funds provided by the BCCF Innovator Support Fund and a CIHR New Investigator Award.

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Conflicts of Interest: The authors declare no competing financial interests.

Word count: 4082 words (abstract: 248 words)

Figures and Tables: 2 tables, 4 figures, 4 supplemental tables, 3 supplemental figures

Reference count: 50
Statement of Translational Relevance

This study provides the first systematic demonstration that follicular lymphoma patients harbor circulating CD8$^+$ T cells specific for driver mutations commonly found in this malignancy. The results support the concept of using next-generation sequencing to identify functionally important, tumor-specific target antigens for immunotherapy, providing a new approach to the treatment of this currently incurable disease.

Abstract

**Background:** A fundamental challenge in the era of next-generation sequencing (NGS) is to design effective treatments tailored to the mutational profiles of tumors. Many newly discovered cancer mutations are difficult to target pharmacologically; however, T cell-based therapies may provide a valuable alternative owing to the exquisite sensitivity and specificity of antigen recognition. To explore this concept, we assessed the immunogenicity of a panel of genes that are common sites of driver mutations in follicular lymphoma (FL), an immunologically sensitive yet currently incurable disease. **Methods:** Exon capture and NGS were used to interrogate tumor samples from 53 FL patients for mutations in 10 frequently mutated genes. For 13 patients, predicted mutant peptides and proteins were evaluated for recognition by autologous peripheral blood T cells after *in vitro* priming. **Results:** Mutations were identified in 1-5 genes in 81% (43/53) of tumor samples. Autologous, mutation-specific CD8$^+$ T cells were identified in 23% (3/13) of evaluated cases. T cell responses were directed toward putative driver mutations in CREBBP and MEF2B. Responding T cells showed exquisite specificity for mutant versus wild type proteins and recognized lymphoma cells expressing the appropriate mutations. Responding T cells appeared to be from the naïve repertoire, as they were found at low frequencies and only at
single time points in each patient. **Conclusions:** FL patients harbor rare yet functionally competent CD8\(^+\) T cells specific for recurrent mutations. Our results support the concept of using NGS to design individualized immunotherapies targeting common driver mutations in FL and other malignancies.
Introduction

A fundamental challenge in cancer genomics is to design effective treatments that exploit the mutational profile of tumors. Next-generation sequencing (NGS) studies have revealed that tumors can contain tens to thousands of mutations (1). While some mutations affect common cellular pathways, there is often extensive mutational heterogeneity within and between patients (2). Adding to the challenge, many mutations or aberrant pathways are difficult to target pharmacologically because of either fundamental biochemical obstacles or a lack of selective agents.

An alternative strategy is to use mutation profiles to design personalized T cell-based therapies. This approach relies on the exquisite sensitivity and specificity of the adaptive immune system. T cells can detect even single amino acid changes in mutated proteins; therefore, any non-synonymous mutation has the potential to generate a new T cell epitope. Moreover, T cells can recognize mutations in both intracellular and extracellular proteins, regardless of protein structure or function. Finally, T cells can be used to target multiple mutant proteins simultaneously. Thus, T cell-based therapies offer an attractive approach to target the tumor genome.

Driver mutations (mutations that give cells a proliferative or survival advantage) represent the most attractive targets for any therapeutic modality. Indeed, T cells that recognize the common drivers BCR-ABL and mutant KRAS have been identified in patient blood samples, and peptide-based vaccines against these mutations have shown promise in the clinic (3-6). With the advent of NGS, it is now feasible to extend this concept to additional driver mutations, even those that are unique to individual patients. Indeed, many cancer clinics are now using “panel” approaches wherein commonly mutated genes are sequenced to guide the design of individualized therapies.
Although immunological targeting of mutations offers several potential advantages, there are two major issues to consider. First, for a given mutation to be recognized by the immune system, several criteria must be met: (a) a peptide containing the mutant residue must be generated by the antigen processing machinery; (b) the mutant peptide must bind with sufficient affinity to one or more of the patient’s HLA alleles; and (c) the patient must harbor T cells that recognize the mutant peptide-HLA complex (7). Owing to these factors, studies in melanoma and other cancers have found that only about 0.5-4% of non-synonymous mutations spontaneously trigger T cell recognition (8-11). A second major issue is immune editing, whereby the immune system eliminates cells expressing specific antigens (12). Since driver mutations are generally present at tumor initiation, their prolonged exposure to the adaptive immune system may make them particularly susceptible to immune editing. If this were the case, the only driver mutations present at diagnosis would be those that are immunologically “silent” (i.e., not processed or presented by the patient’s HLA complex or recognized by their T cell repertoire).

To investigate these issues in human cancer, we assessed the immunogenicity of a panel of genes that are common sites of driver mutations in follicular lymphoma (FL). FL is considered an immunologically sensitive disease, since tumor infiltrating lymphocytes (TIL) are associated with increased patient survival (13, 14) and up to 30% of patients experience spontaneous tumor regressions (15). Moreover, at least 28 commonly mutated genes have been identified in FL, involving such processes as histone modification (e.g. CREBBP, EP300, EZH2, MEF2B, MLL2), signal transduction (e.g. CARD11, CD79B, STAT6), cell cycle regulation (e.g. CCND3, PIM1), and cellular differentiation (e.g. FOXO1, IRF4) (16-26). For the majority of these genes, the specific mutations are unique to individual patients. Here we demonstrate that FL patients harbor
CD8$^+$ T cells with exquisite specificity for driver mutations in their tumors. Our results support the concept of using NGS data to design individualized immunotherapies for FL and other malignancies.

**Materials and Methods**

**Biospecimens and processing of samples**

All specimens and clinical data were collected with written informed consent under protocols approved by the University of British Columbia-British Columbia Cancer Agency Research Ethics Board. Tumor tissue was obtained from biopsy specimens and cryopreserved or fixed in formalin and processed in paraffin. Peripheral blood mononuclear cells (PBMC) were purified from annual 200 ml blood draws using Ficoll-Paque PLUS (GE Healthcare) and cryopreserved in nitrogen vapor freezers. The frequencies of major immune cell subsets were determined by flow cytometry (Table S1).

**DNA sequencing**

Genomic DNA was isolated from tumor specimens and matched PBMC using the AllPrep DNA/RNA Mini kit (Qiagen). Targeted exon capture and sequencing was performed at Canada’s Michael Smith Genome Sciences Centre, Vancouver, Canada (Supplemental Methods). High-resolution HLA class I typing of patient samples was performed using sequence-based approaches (27). HLA class I typing of healthy donor samples was performed using PCR-SSOP (ProImmune).

**Epitope predictions and peptide libraries**
Epitope predictions were performed using NetMHCpan, version 2.8 (28). For 13 patients with adequate PBMC availability and at least one predicted epitope, all peptides with a predicted IC$_{50}$ binding score <1000 nM (and wild type counterparts as required) were synthesized and purified commercially (ThinkPeptides and Genscript), reconstituted in 80% DMSO, and stored at -80°C.

**In vitro T cell priming**

Monocyte-derived dendritic cells were pulsed with peptides and cultured with autologous PBMC to activate peptide-specific CD8$^+$ T cells (Supplemental Methods). Stimulations were performed in 96-well plates with 1.5 x10$^5$ PBMC/well and an average of 413 wells or 6.2x10$^7$ PBMC/patient. After a second round of stimulation using irradiated, peptide-pulsed autologous PBMC as antigen presenting cells (APC), T cells were screened for reactivity by interferon-gamma (IFN-γ) ELISPOP (Supplemental Methods). The frequency of responding T cells was determined by counting the number of positive wells and dividing by the number of input cells. Positive cultures were subjected to the Rapid Expansion Protocol (REP) (29). In some cases, T cells were cloned by limiting dilution following FACS-based enrichment of peptide-stimulated cells expressing the activation marker CD137 (4-1BB).

**In vitro transcription and RNA transfection**

Mutant and wild type $EZH2$ cDNA, as well as wild type $CCND3$ and $MEF2B$ cDNA were provided by the Genome Sciences Centre. A mini-gene encoding 96 amino acids of CREBBP fused with a C-terminal flag-tag was synthesized commercially (Life Technologies). All sequences were cloned into pENTR/D-TOPO (Invitrogen). To recreate patient-specific mutations, wild type $CCND3$, $CREBBP$, and $MEF2B$ sequences were subjected to site-directed mutagenesis using the
Transformer Site-Directed Mutagenesis kit (Clontech). All sequences were cloned into the pST1-Gateway destination vector (30) downstream of the T7 promoter (Invitrogen) and verified by Sanger sequencing (Genscript). Plasmids were linearized after the poly(A) sequence with Pmel (New England Biolabs) and subjected to in vitro transcription using the mMessage mMachine T7 Ultra Kit (Ambion). RNA integrity was confirmed by gel electrophoresis. Lymphoma lines (BCWM.1 (31) and Karpas 422 (32)) and CD40-activated B cells (Supplemental Methods) (3 × 10⁶ cells) were electroporated with 1-18 μg in vitro-transcribed RNA (ivtRNA) using the Amaza Cell Line Nucleofector Kit L (Lonza, program X-001). BCWM.1 does not have an endogenous MEF2B^{D83A} mutation (unpublished data), and Karpas 422 does not have an endogenous CREBBP^{R1446C} mutation (33). Recombinant protein expression was confirmed by Western blotting (Supplemental Methods).

Flow cytometry

T cell lines were pulsed with the relevant minimal peptide (10μM) (to serve as targets) and incubated at a 1:1 ratio with unpulsed T cells (effectors) overnight at 37°C. Cells were stained with APC-labeled anti-human CD137 (BD Pharmingen, clone 4B4-1) and FITC-labeled anti-human CD8 (BD Pharmingen, clone RPA-T8) and analyzed using a BD Influx flow cytometer and FlowJo software.

Results

Mutation profiling

The patient cohort consisted of 53 FL patients, whose clinical characteristics are shown in Table S2. Using genomic DNA from biopsy specimens, we performed exon capture and NGS of 10 genes frequently mutated in non-Hodgkin lymphomas: CARD11, CCND3, CD79B, CREBBP,
Non-synonymous mutations were identified in samples from 81% of patients (Table S3), with a mean of 1.7 mutated genes per case (range: 1-5). In accord with previous studies (16), the most frequently mutated genes were CREBBP, EZH2, and MEF2B, with mutations occurring in 20, 17, and 10 patients, respectively (Figure 1A). FOXO1 has been reported as recurrently mutated in DLBCL (35) but not FL, yet it was the fourth most commonly mutated gene in our cohort. This may be partly attributable to poor coverage of FOXO1 in previous NGS data owing to high GC content (RDM and RDG, unpublished observation). The relative abundance of each mutation within a given tumor was inferred from the variant allele frequency (VAF; Table 1).

Detection of T cell responses against mutant peptides

Epitope predictions were performed for each patient’s mutation(s) against all 6 of their HLA class I alleles (Table S4). Using an IC\(_{50}\) cut-off of 1000 nM, predicted epitopes were identified for 37/43 patients (Figure S1), with a mean of 3.1 predicted epitopes per mutation and 5.3 predicted epitopes per patient (range: 0-26 epitopes). Based on PBMC availability and the presence of at least one predicted epitope, 13 patients were selected for in vitro immunogenicity experiments. The clinical characteristics and treatment protocols for this subcohort were representative of the larger cohort (Table S2, Figure S2).

To assess whether predicted epitopes (Figure 1B) could elicit T cell responses, patient PBMC from all available time points were subjected to two rounds of in vitro stimulation with autologous APC pulsed with a pool of purified, synthetic peptides corresponding to each patient’s predicted epitopes. Stimulations were performed in 96-well plate micro-cultures containing an initial aliquot of 150,000 PBMC/well, and an average of 6.2 x 10\(^7\) PBMC (413 wells) were...
screened per patient. By IFN-γ ELISPOT, T cell responses were detected in samples from 7/13 patients (Table 1). The frequency of responding T cells ranged from 1:5 x 10^6 to 1:9.7 x 10^7 PBMC (Table 1). All positive T cell cultures were expanded, and reactivity to mutant peptides was confirmed by ELISPOT.

**Identification of minimal peptides, HLA restrictions, and cross-reactivity**

For the seven patients demonstrating responses, each positive T cell response was further evaluated to determine the underlying mutation, minimal peptide, relevant HLA allele, and extent of cross-reactivity to corresponding wild type peptides. T cell responses were directed against peptides corresponding to mutations in four genes: *CCND3*, *CREBBP*, *EZH2*, and *MEF2B* (Table 2, Figure 2A). From these genes, a total of 15 minimal peptides were recognized, along with 7 extended peptides containing these minimal peptides. Each peptide was recognized by between one and seven T cell lines. To assess specificity, T cell lines were screened for reactivity against mutant and corresponding wild type peptides at concentrations ranging from 0.01 to 10,000 nM. 11/15 T cell lines exhibited exquisite specificity for the mutant peptide over the corresponding wild type peptide (Table 2, Figure 2B-E), whereas the remainder cross-reacted to wild type peptides (Table 2). To determine HLA restriction, T cell lines were incubated with peptide-pulsed B-lymphoblastoid cell lines (LCL) matched at 0-6 HLA class I alleles (Supplemental Methods). HLA restriction was successfully determined for 9/15 peptides, and in each case the actual and predicted HLA alleles matched (Table 2, Figure 2F).

**T cell recognition of processed mutant proteins**

Since surgery is not part of standard care for most FL patients, sufficient autologous target cells for tumor recognition experiments were not available. Instead, we evaluated T cell
recognition of mutant proteins after ectopic expression in autologous B cells and HLA-matched lymphoma cell lines. Constructs encoding full-length wild type and mutant versions of CCND3, EZH2, and MEF2B were generated. CREBBP was too large to be expressed as a full-length protein, so instead it was expressed as a 96 amino acid polypeptide with the mutated residue located centrally. Constructs were transfected as ivtRNA into autologous CD40L-stimulated B cells. T cell lines were tested by IFN-γ ELISPOT for recognition of cells expressing the relevant mutant gene, the wild type counterpart, an irrelevant gene, or no exogenous gene. For some patients, multiple T cell lines exhibited the same peptide reactivity pattern; in such cases, the T cell line with the highest apparent affinity was evaluated. T cell lines from 3/7 patients exhibited reactivity against B cells expressing the relevant mutant protein (Table 2, Figure 3). In each case, no significant cross-reactivity was seen against the corresponding wild type or irrelevant proteins (Table 2, Figure 3). All of these T cell lines had shown specificity for mutant over wild type peptides in the experiments described above. Conversely, none of the T cell lines that had exhibited cross-reactivity to wild type peptides were able to recognize full-length proteins (Table 2). By flow cytometry we confirmed that 2/2 tested bona fide mutation-specific responses were mediated by CD8+ rather than CD4+ T cells (Figure S3). The third bona fide response could not be tested by flow cytometry because of the low frequency of mutation-specific cells in the T cell line. However, this T cell line recognized a 9-mer peptide with a strong predicted binding affinity for HLA-B*07:02 (IC50: 4 nM), so it is also likely to represent a CD8+ T cell response.

The three bona fide T cell responses were rare in terms of both precursor frequency and temporal dynamics. For patient IROL017, the T cell response to MEF2B^{D83A} was detected at a frequency of 1:2.9 x 10^7 PBMC (1:7.3 x 10^6 CD8+ T cells) in a pre-treatment blood sample and was not found in any of three post-treatment samples (Figure S2). For patient IROL054, the T cell
response to MEF2B<sup>D83G</sup> was detected at a frequency of 1:3.4 x 10<sup>7</sup> PBMC (1:6.8 x 10<sup>6</sup> CD<sup>+</sup> T cells) in a blood sample taken during clinical remission (3 years post-chemotherapy, 1.5 years post-maintenance rituximab therapy) and was not found at earlier or later time points (Figure S2).

Finally, for patient IROL046, the T cell response to CREBBP<sup>R1446C</sup> was detected at a frequency of 1:4.2 x 10<sup>6</sup> PBMC (1:1.4 x 10<sup>6</sup> CD<sup>+</sup> T cells) in a blood sample taken during clinical remission (6 years post-chemotherapy, 4 years post-maintenance rituximab therapy) and was not found at 3 earlier (post-treatment) time points (Figure S2). These low precursor frequencies suggest that the T cells may have been derived from the naïve repertoire.

To ensure that transfection resulted in physiologically relevant protein expression levels, RNA titration experiments were performed. IROL017 B cells were transfected with 1-18 μg of RNA encoding MEF2B<sup>D83A</sup>. By Western blot, 9 μg of RNA gave rise to a similar level of protein expression as seen for endogenous MEF2B in the DB lymphoma line (Figure 4A). By ELISPOT, IROL017 T cells recognized cells transfected with as little as 3 μg of RNA (Figure 4B), indicating that recognition was in the physiological range. In an analogous experiment, IROL046 T cells could recognize autologous B cells transfected with as little as 1 μg of RNA encoding CREBBP<sup>R1446C</sup> (Figure 4C).

To determine whether lymphoma cells had the capacity to process and present the antigens of interest, we expressed the same constructs in HLA-matched lymphoma cell lines, where available. The bona fide epitope recognized by T cells from patient IROL017 corresponded to a peptide from MEF2B<sup>D83A</sup> presented by HLA-B*40:01; therefore, the HLA-B*40:01-positive lymphoma cell line, BCWM.1 was transfected with RNA encoding mutant MEF2B. Similarly, T cells from patient IROL046 recognized a peptide from CREBBP<sup>R1446C</sup> presented by HLA-
B*07:02; therefore, the HLA-B*07:02-positive lymphoma line Karpas 422 was transfected with RNA encoding mutant CREBBP. In both cases, patient T cells recognized lymphoma lines expressing the appropriate mutant protein (Figure 4D,E), demonstrating that these mutant epitopes can be processed and presented by the MHC class I pathway in lymphoma cells to confer recognition by CD8+ T cells.

Discussion

We systematically investigated the extent to which autologous CD8+ T cells recognize patient-specific driver mutations in FL. By interrogating a panel of 10 genes in 53 FL cases, we identified mutations in 81% of cases. Of relevance to immunotherapy, T cells specific for bona fide mutant epitopes were identified in 23% (3/13) of patients. One of the corresponding mutations (CREBBP^{R1446C}) is a well-established driver mutation in FL (18). The other two mutations (MEF2B^{D38A} and MEF2B^{D38G}) involve a commonly mutated residue in MEF2B (19); these too are likely to represent driver mutations based on amino acid similarities to the well-characterized MEF2B^{D83V} driver mutation (36). Mutation-specific T cells were present at very low frequencies in peripheral blood, yet they could be expanded to large numbers using clinically feasible methods. Thus, driver mutations represent attractive target antigens for T cell-based immunotherapy of FL and related malignancies.

Our study used an in vitro priming approach that, in theory, interrogated both the naive and activated T cell repertoires of cancer patients for mutation-specific responses. Encouragingly, our findings suggest that a higher proportion of mutations can be recognized by autologous T cells than previously thought based on analyses of pre-existing T cell responses alone (37). For example, in anecdotal studies of melanoma patients who responded well to TIL therapy or
ipilimumab, TIL were shown to recognize only about 2% of mutations identified by whole exome sequencing (8, 9, 38). Similarly, in a study of three ovarian cancer patients, we found that TIL recognized only 1.3% (1/78) of mutations (11). Finally, in a non-small cell lung cancer patient who responded to pembrolizumab, only one mutation was recognized out of 226 putative neoantigens (39). In contrast, by using in vitro priming in the present study, we identified bona fide T cell responses to 11% (3/27) of mutant proteins in 23% (3/13) of FL patients tested. Likewise, in two patients with chronic lymphocytic leukemia, in vitro priming revealed T cell responses against 12% (3/25) of mutations tested (although MHC class I processing and presentation was assessed for only one of these peptides) (40). Thus, although the sample sizes are small in each of these studies, the combined results suggest that, by using sensitive methods that allow detection of naïve and/or rare T cells, one can identify T cell responses to a larger proportion of mutations than previously appreciated. This conclusion is supported by murine studies, where vaccination with mutant peptides can prime CD4+ and CD8+ T cell responses to approximately 30% of selected mutations (41, 42).

Although T cell-based targeting of driver mutations offers several advantages over pharmacological strategies, our findings highlight two major challenges that necessitate an individualized approach. First, despite the existence of commonly affected genes and pathways, FL demonstrates a considerable inter-patient mutational heterogeneity. For example, by interrogating 10 commonly mutated genes, we identified mutations in 81% of cases. About half (46%) of these mutations were located in hotspots in 8 genes; however, the remaining mutations were unique to individual cases. Thus, NGS will be required to identify the relevant mutations in each patient’s tumor. Second, as expected from basic immunological principles, the patient’s HLA status was a critical determinant of the immunogenicity of mutations (7). For example, of the two
cases with a CREBBP^{R1446C} substitution, a corresponding T cell response was detected in only the HLA-B*07-02-positive individual. The prediction algorithm used here worked well in the case of CREBBP, as it gave a better binding score for the HLA-B*07-02 allele (IC_{50}: 4 nM) than for the HLA alleles from the other patient (IC_{50} scores: 177 - 976 nM). In fact, applying an IC_{50} cut-off of 165 nM would have correctly identified the 3 bona fide epitopes, while identifying only 30 irrelevant peptides (5 of which elicited responses to peptides that were not processed). The fact that all three bona fide epitopes had predicted IC_{50} values \leq 165 nM indicates that, despite being rare, the mutation-reactive T cells identified are expected to have T cell receptor (TCR) affinities within a therapeutically relevant range. Given the importance of HLA haplotypes, clinical trials will likely be more effective if candidate epitopes are subjected to \textit{in vitro} immunological assays before being selected for clinical use. The need for empirical testing should diminish as protein processing and epitope prediction algorithms continue to improve.

Despite the need for an individualized approach overall, our results also illustrate that recurrent mutations occasionally give rise to shared epitopes presented by common HLA alleles. For example, the CREBBP^{R1446C} mutation discussed above has a prevalence of 4.3-13\% in FL (Table S3) (18), and the corresponding HLA-B*07-02 allele is present in 30\% of American Caucasians (43). Therefore, the mutant epitope we identified may be a relevant target in up to 4\% of FL patients. The two remaining bona fide epitopes were derived from substitutions at D83 of MEF2B, which occur in 4.2-5.7\% of FL cases (Table S3) (19); although D83V is the most common substitution at this site, the alanine and glycine substitutions found here are also recurrent mutations (19). The two corresponding HLA alleles (HLA-B*40 and HLA-A*03) are present in 13\% and 21\% of American Caucasians, respectively (43), and are predicted to bind mutant epitopes containing D83V, D83A and D83G with equivalent affinity. Based on these estimates,
substitutions at D83 of MEF2B may give rise to CD8+ T cell epitopes in approximately 1.9% of FL patients. Thus, in this relatively small study, 3/3 bona fide epitopes we identified have the potential to serve as shared epitopes with therapeutic relevance to additional FL patients. With further study, it may be possible to create an inventory of shared mutant epitopes that could partly alleviate the need for entirely personalized mutation targeting.

Taking into consideration mutation frequency, epitope predictions, and in vitro stimulation results, we estimate that approximately 1/6 FL patients would be amenable to T cell-based therapy targeting this panel of 10 mutations. The number of eligible patients could be increased in several ways. There are at least 18 other common driver mutations in FL that could be added to the panel used here (16, 19). Moving beyond driver mutations, the average lymphoma contains approximately 100 mutations (1, 16), which could be readily identified by whole exome sequencing. Finally, the proportion of eligible patients could be increased by assessing both CD8+ and CD4+ (T helper 1, or Th1) T cell responses. In the present study, we elected to focus on CD8+ T cell responses owing to the greater accuracy of epitope prediction algorithms for MHC class I compared to class II. However, CD4+ T cell responses also play important roles in anti-tumor immunity. Indeed, recent studies have shown a high prevalence of mutation-specific CD4+ T cell responses in murine tumor models (42) and human melanoma patients (44). Moreover, infusion of TIL enriched for a mutation-specific CD4+ Th1 clone led to a dramatic clinical response in a patient with cholangiocarcinoma (10).

One can envision several therapeutic strategies for inducing immune responses to patient-specific driver mutations. Arguably the simplest strategy would be checkpoint blockade using antibodies to inhibitory proteins such as PD-1 and CTLA-4. In support of this, ipilimumab appears
preferentially to induce new T cell responses rather than to enhance pre-existing responses (45). Moreover, immune checkpoint blockade appears to be more efficacious against tumors with high mutation load (39, 46). However, the increasing use of NGS in clinical practice offers an opportunity to generate more precise anti-tumor immune responses. For example, NGS data is currently being used to design personalized mutation-specific vaccines (47). However, the low T cell precursor frequencies reported here suggest that it may prove difficult to generate robust, mutation-specific T cell responses in patients through vaccination alone.

Adoptive T cell therapy (10, 48) allows one to circumvent issues of low precursor frequency, cross-reactivity, and immune suppression by isolating and expanding T cells from peripheral blood to produce defined T cell products with high specificity for target mutations. Recent adoptive cell therapy trials using TIL specifically selected for mutation reactivity (or retrospectively shown to be mutation-reactive) underscore the clinical feasibility and efficacy of this strategy (8-10). Moreover, in the setting of lymphoma, adoptive transfer of T cells engineered to express CD19-specific chimeric antigen receptors (CARs) is showing promise in early clinical trials (49, 50). Notably, CD19-CAR T cell therapy can induce prolonged B cell aplasia, neurological toxicities, and cytokine release syndrome, in part due to destruction of the normal B cell compartment. These risks could potentially be mitigated by targeting tumor-specific mutations rather than the pan-B cell marker CD19. Finally, our results suggest that for the most common driver mutations in FL, such as CREBBP<sup>R146C</sup>, it may be practical to produce off-the-shelf mutation-specific TCR vectors for use in T cell engineering. TCR engineering would also be required in cases where mutation-specific T cells fail to expand to sufficient quantities for ACT. Irrespective of the precise approach, our results provide proof-of-concept for T cell-based targeting of common driver mutations in FL and other malignancies.
Acknowledgments

We thank the study participants and referring clinicians for specimens and clinical data; Adria Devlieger, Tim Turcotte, and Natalie Kinloch for technical assistance; and Drs. Richard Moore, John Webb, and Robert Holt for advice. Canada's Michael Smith Genome Sciences Centre provided sequencing data.

References

Table 1: Summary of T cell responses against pools of peptides corresponding to patient-specific mutations

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<thead>
<tr>
<th>Patient</th>
<th>Mutated gene</th>
<th>*Mutant VAF in tumor</th>
<th># of peptides with IC50&lt;1000nM</th>
<th># of stimulated wells</th>
<th># of peptide-reactive wells</th>
<th>**Peptide reactive T cell frequency</th>
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*VAF: Variant allele frequency. **Frequency of peptide pool-reactive T cells in PBMC. The estimated frequency within the CD8+ T cell fraction was approximately ten-fold higher. This included T cells reactive against any peptide within the pool.
Table 2: Characterization of T cell responses against patient-specific mutations

<table>
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<tr>
<th>Patient</th>
<th>Peptides eliciting T cell response (gene name &amp; AA sequence)</th>
<th>Cross-reactivity with WT peptide</th>
<th>Predicted HLA restriction</th>
<th>Experimentally determined HLA restriction</th>
<th>Predicted mutant IC₅₀</th>
<th>Predicted WT IC₅₀</th>
<th>Response to mutant protein</th>
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<td>IROL011</td>
<td>EZH2 - FISESCGEI</td>
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<td>CREBBP - HEIHIGLEYV</td>
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<td>CREBBP - AVYHEIHIYGLY</td>
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<td>MEF2B - HESRTNTAIL</td>
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<td>MEF2B - RTNTGILETLK</td>
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* Some lines showed minimal cross reactivity while others showed none. **Not determined.
Figure Legends

Figure 1. Frequency of somatic mutations and predicted epitopes in follicular lymphoma patients. (A) Targeted exon capture and Illumina-based sequencing were used to identify mutations in 10 candidate genes in genomic DNA isolated from 53 tumor samples. The graph indicates the number of patients with at least one non-silent mutation in each gene. Mutations determined to be germline in origin were excluded from this analysis. (B) The epitope prediction algorithm NetMHCpan version 2.8 was used to identify predicted HLA class I-binding peptides with IC<sub>50</sub> scores of less than 50 nM, 500 nM, or 1000 nM from the entire cohort of patients with somatic mutations in a panel of 10 candidate genes. The 13 patients shown here were selected for immunogenicity studies based on PBMC availability and the presence of at least one predicted epitope.

Figure 2. Representative results of in vitro immunogenicity experiments. Dendritic cells were loaded with predicted minimal peptides corresponding to patient-specific mutations and used to stimulate autologous T cells in PBMC samples from serial blood draws. After further stimulation with peptide-pulsed irradiated PBMC, T cells were screened for reactivity against the corresponding patient’s peptide pool by IFN-γ ELISPOT. Positive cultures were then expanded for additional analysis using a polyclonal expansion protocol. (A) Results of deconvolution experiments for IROL017 T cell lines. Numerous T cell lines responded to multiple minimal peptides corresponding to CREBBp<sup>L1454H</sup> and MEF2B<sup>D83A</sup>. Minimal peptides are underlined. (B) Representative ELISPOT wells for IROL017 REP225 T cells showing specific recognition of a mutant MEF2B peptide and no recognition of the corresponding wild type peptide (peptides at 10 nM). Controls include T cells without peptide (DMSO) and PHA stimulation. (C-E)
Representative ELISPOT results from peptide titration experiments using mutant peptides (black lines) and corresponding wild type peptides (grey lines) showing response patterns for T cell lines from three patients. Asterisks indicate values that were too numerous to count. Error bars represent standard deviation of triplicate wells. (F) Representative HLA restriction results for the IROL017 REP225 T cell line showing positive responses to all HLA-B*40:01-positive peptide-pulsed LCL but not LCL lacking HLA-B*40:01. Shaded boxes indicate HLA alleles shared between each donor and patient IROL017. Peptide-pulsed LCL from the first three donors elicited responses above background, while the last five did not.

**Figure 3. Mutant epitopes are naturally processed and presented by HLA to patient CD8\(^+\) T cells.** Representative ELISPOT results showing (A-C) reactivity or (D) lack of reactivity against autologous B cells expressing patient-specific mutant proteins. Negative controls include mock-transfected cells, as well as cells expressing irrelevant proteins. Positive controls include peptide-pulsed cells. Data is also shown for cells expressing wild type proteins. Asterisks indicate values that were too numerous to count. Error bars represent standard deviation of triplicate wells. Results are representative of 2-3 independent experiments.

**Figure 4. Patient T cells recognize mutant proteins expressed by lymphoma cell lines and at endogenous levels.** (A-C) T cell responses were assessed by ELISPOT using autologous B cells transfected with different quantities of RNA encoding mutant proteins. (A) Western blots show ectopic (tagged) MEF2B expression by IROL017 B cells (upper, faint band) and endogenous MEF2B expression by the lymphoma cell line, DB (lower, faint band). A MEF2B-negative cell line, DOHH2 was included as a negative control. Actin was included as a loading control. (B)
IROL017 and (C) IROL046 RNA titration ELISPOT results. Error bars represent standard deviation of triplicate wells. Results are representative of 2 independent experiments. (D-E) Representative ELISPOT results showing reactivity against autologous B cells and lymphoma cell lines expressing patient-specific mutant proteins. (D) IROL017 and (E) IROL046 T cell responses against transfected autologous B cells were compared with those against transfected HLA-matched lymphoma cell lines. The IROL017 T cell line REP225 initially displayed alloreactivity, so it was cloned to generate IROL017 REP445 prior to performing tumor recognition experiments. Negative controls include mock-transfected cells. Positive controls include peptide-pulsed cells. Asterisks indicate values that were too numerous to count.
A

Number of patients

CREBBP  EZH2  MEF2B  FOXO1  CARD11  PIM1  EP300  IRF4  CCND3  CD79B

Mutant gene

B

Epitope prediction (IC50) scores

<1000 nM
<500 nM
<50 nM

# of peptides

IROL046  IROL017  IROL032  IROL026  IROL041  IROL011  IROL054  IROL093  IROL003  IROL074  IROL012  IROL086  IROL025

Patients
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<th>Mutant Protein</th>
<th>Peptide Sequence</th>
<th># of Responding T cell Lines</th>
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<td>AVYHEIHI</td>
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**B**  
IROL017 REP225 - MEF2B (D83A)

- DMSO
- Mutant peptide
- WT peptide
- PHA

**C**  
IROL054 REP200

**D**  
IROL017 REP225

Peptide concentration (nM)

**E**  
IROL046 REP224

Peptide concentration (nM)

**F**  

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<th>HLA-A*02:01</th>
<th>HLA-B*07:01</th>
<th>HLA-B*40:01</th>
<th>HLA-C*02:01</th>
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Clin Cancer Res Published OnlineFirst December 2, 2015.

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