I-131-Anti-CD45 Radioimmunotherapy Effectively Targets and Treats T-Cell Non-Hodgkin Lymphoma

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Abstract:
Radioimmunotherapy (RIT) options for T-cell lymphomas (T-NHL) are limited. We evaluated anti-CD45-RIT in human (h) and murine (m) T-NHL. CD45 was highly expressed on hT-NHL patient samples (median $2.3 \times 10^5$ antigen binding capacity [ABC] units /cell) and hT-NHL cell lines ($3.4 \times 10^5$ CD45 ABC units/cell). Biodistribution studies in hTNHL xenografts showed that $^{131}$I-labeled BC8 (anti-hCD45) delivered 154% (p=.01) and 237% (p=.002) more radioiodine to tumor sites over control antibodies at 24h and 48h, respectively. Importantly, tumor sites targeted with $^{131}$I-BC8 exhibited 2.5 (p=.05), 3.0 (p=.007), and 3.6 (p=.07)-fold higher $^{131}$I retention over the non-target organs of lungs, liver, and kidneys, respectively (24h). Since the clinical use of anti-hCD45 would target both T-NHL and other hematolymphoid tissues, we evaluated the ability of anti-mCD45 to target mT-NHL. mT-NHL grafts targeted with anti-mCD45 correspondingly retained 5.3 (p<.001), 5.4 (p<.001), and 8.7 (p<.001) times the radioactivity in tumor sites compared to non-target organs of lung, liver and kidney. $^{131}$I-labeled BC8 therapy yielded improved complete remission rates (75% vs 0%, p<.0001) and progression-free survivals (median 23d vs 4.5d, p<.0001) compared to controls. These data indicate that the high CD45 expression of T-NHL allows reliable tumor targeting and disease control supporting anti-CD45 RIT for T-NHL patients.
Introduction:

T-cell non-Hodgkin lymphomas (T-NHL) encompass a heterogeneous group of high-risk diseases characterized by inferior response rates, remission durations and survivals as compared to their B-NHL counterparts. Radioimmunotherapy (RIT) has emerged as one of the most efficacious new treatment approaches for B-NHL, yet for T-NHL this strategy has been thwarted in part due the lack of a successful, widely applicable radioimmunoconjugate for this antigenically-diverse group of malignancies. CD45, a panhematopoietic antigen, represents an attractive target for RIT based on its lack of shedding or internalization and its reported expression by the vast majority of T-NHL. The broad hematopoietic expression of CD45, though requiring hematopoietic stem cell transplantation (HSCT), also carries the theoretical advantage of amplifying the radiation dose to minimal disease sites via the 'crossfire effect' from targeting adjacent CD45 bearing cells.

We hypothesize and test in a preclinical model the potential efficacy of anti-CD45 RIT for the treatment of T-NHL. In a succession of experiments, we first demonstrate that CD45 is reliably expressed in high-concentrations on T-NHL cell lines and patient samples to facilitate targeting. Furthermore, we illustrate that anti-CD45 RIT results in preferential radiation exposure to tumor sites and limits exposure to non-target tissues in both xenogeneic and syngeneic systems. Finally, we show that anti-CD45 RIT yields effective tumor control and improved progression-free survival (PFS) in pre-clinical models. These findings
set the stage for translating this strategy into a clinical application of anti-CD45 RIT for patients with T-NHL.

Materials and Methods

Cells

The human T-NHL lines CCRF-CEM, HUT-78 and Karpas 299 were purchased from American Type Culture Collection (ATCC, Bethesda, MD). All cell lines were kept in log growth phase in RPMI1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin (GIBCO, Carlsbad, CA) and 1% 100X L-Glutamine (GIBCO, Carlsbad, CA). Cell viability was kept at >95% as measured by trypan blue exclusion. Patient samples were obtained using University of Washington institutional review board approved methods.

Antibodies and Radiolabeling

BC8 (a murine anti-human IgG1) was produced from a hybridoma using a hollow fiber bioreactor system in the Biological Production Facility at the Fred Hutchinson Cancer Research Center (Seattle, WA). BHV-1 (a murine IgG1, isotype matched non-binding control for BC8) was produced using the ascities method and purified using a HiTrap protein G column (GE Healthcare, Piscataway, NJ) 16. 30F11 (rat anti-murine IgG2b) was purchased from BD Pharmingen (San Jose, CABC8). BHV-1 and 30F11 were iodinated with Na131I or Na125I (Perkin Elmer, Boston, MA) by the chloramine T method as previously described 17.

Antigen density of cell lines and patient samples
All samples were evaluated on a modified 4-laser, 10-color Becton-Dickinson LSRII flow cytometer utilizing the following laser-fluorochrome combinations: 1) 405 nm violet laser (1 color) – Pacific Blue (PB); 2) 488 nm blue laser (5 colors) – fluorescein isothiocyanate (FITC), Phycoerythrin (PE), PE-Texas Red (ECD/PE-TR), PE- Cyanine-5 (PE-Cy5), Pe-Cy5.5, and PE-Cy7; 3) 594 nm yellow laser (1 color) -- AlexaFluor 594; and 4) 633 nm red laser (3 colors) – allophycocyanin (APC), APC-AlexaFluor 700 (APC-A700), and APC-Cy7.

CD45 antigen density (measured as antigen binding capacity [ABC]/cell) on neoplastic cells from human blood, bone marrow, and lymph node suspension samples and cell lines were performed according to the manufacturer’s protocol (www.bangslabs.com). The microbeads (Quantum™ Simply Cellular® anti-Mouse IgG, Bangs Laboratories, Inc, Fishers, IN) employed in these studies are coated with known quantities of goat anti-mouse IgG which, when mixed with saturating quantities of mouse anti-CD45-PE, produced a standard to measure CD45 density on the CD45-PE-labeled cells of interest.

To create the standard curve, 1 drop of blank and each of the labeled components (Bead 1,2,3 and 4), were individually added to 50 µL of PBS\BSA. 20 µl (titered previously to determine amount of antibody required for saturation) of anti-CD45-PE antibody (clone J33, Beckman Coulter, Miami, FL) was added to all mixtures except for the blank. After 30 min incubation in the dark, 2 mL of PBS\BSA were added to each tube, and the tubes were centrifuged (>3500 RPM
for 5 min). The pellets were then washed twice with PBS/BSA, centrifuged, and re-suspended in 100 µl of PBS/BSA for analysis.

For each patient specimen or cell line, a custom combination of fluorescently labeled antibodies were used to uniquely identify the neoplastic T cell population based on the previously determined neoplastic immunophenotype (example, CD2-FITC, CD34-ECD, CD8-PECy5.5, CD3-PECy7, CD4-A594, CD7-APC, and CD5-APC-Cy7). To approximately 300,000 cells, the custom antibody combinations and anti-CD45-PE were added, and the mixture incubated for 15 minutes at 25 °C in 100 μl RPMI, lysed and fixed with 0.15 M ammonium chloride (Polysciences), pH 4.8, containing 0.25% formaldehyde for 15 min, washed with 3 ml PBS/BSA, and incubated with 0.1 ml of PBS prior to analysis. Standards and patient samples or the cell line cells were then evaluated by flow cytometry. For the standards, a plot of mean fluorescent intensity (MFI) in the PE channel vs. log (ABC) defined a standard curve. The antigen density of the patient specimen or cell line was determined from this standard curve after the MFI of the neoplastic population was measured. Both the cell lines and the patient specimens were measured in triplicate.

In vivo studies

Biodistribution: For all mouse studies female athymic nude mice, aged 6-8 weeks, were purchased from Haralan Sprague-Dawley (Indianapolis, IN) and were housed and cared for according to protocols approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee. CCRF-CEM (2 x 10^7) or Karpas (2 x 10^7) cells were injected
subcutaneously in the right flank and palpable xenografts developed within ~10 days. EL-4 (1 x 10^7) cells were injected as above and palpable xenografts developed within 3 days.

Groups of at least 5 mice were co-injected with 200µg ^131^I-BC8 and ^125^I-BHV-1 or ^125^I-30F11 labeled with 10 µCi of radioisotope via the tail vein. The mice were euthanized 24 or 48 hours after radiolabeled antibody injection, and blood, normal organs (lungs, liver, spleen, kidneys, stomach, large intestine and small intestine) and tumors were harvested and weighed. Residual radioactivity in the harvested tissues was determined via gamma counter measurements. Using an aliquot of the injectate, the percent-injected dose of ^131^I and ^125^I per gram (%ID/g) of blood, tumor and normal organs was calculated and corrections for radioactive decay and crossover were made. Tumor-to-normal organ ratios of absorbed radioactivity were calculated from these data.

Therapy: T-NHL xenografts were established as above. When tumors were measurable (~100mm³), groups of at least 9 mice each were injected via tail vein with 200µg BC8 labeled with 300 or 400 µCi of ^131^I or BHV-1 labeled with 300 µCi of ^131^I. Tumor size and body weight were measured sequentially approximately thrice weekly following injections. Tumor progression was scored following two consecutive increases in tumor volume. Mice were euthanized when they experienced weight loss > 20% or tumor size > 16 mm x 16 mm x 9 mm per animal health guidelines.
**Statistical Considerations**

Continuous variables with normal distributions were compared using the students T-test. Simple proportions were evaluated using the Chi-square or Fisher’s Exact test as appropriate. Time dependent variables (survival, disease progression, etc) we estimated using the method of Kaplan and Meier and comparisons between these estimates were made using the log-rank test.

**Results:**

*CD45 is readily expressed on T-NHL lines and patient samples*

We first quantified the surface density of CD45 on T-NHL lines and T-NHL patient tumor samples in order to determine if CD45 expression would be sufficient to serve as a target for RIT and if subsequent animal models utilizing T-NHL lines would be representative of clinical T-cell lymphoma. The antigen density (ABC) of CD45 was determined for T-NHL lines (CCRF-CEM, Karpas 299, HUT-78) and bone marrow, blood and lymph node samples from patients with known T-NHL (T-lymphoblastic leukemia/lymphoma [T-LBL], Anaplastic Large Cell Lymphoma [ALCL], Peripheral T-cell lymphoma [PTCL], Mycosis Fungoides [MF], and NK/T-cell lymphoma) as described above. CD45 was expressed in high copy numbers on the surface of human T-NHL samples and T-NHL cell lines with a median of $3.4 \times 10^5$ ABC units/cell on T-NHL lines (CCRF-CEM, Karpas 299, HUT-78) and $2.3 \times 10^5$ ABC units/cell on 9 patient derived T-NHL specimens (**Figure 1**). Taken together, these data indicate that the high
CD45 expression in cell lines recapitulates most patient-derived T-NHL and, thus, should represent a viable target for RIT.

*Anti-human CD45 RIT targets human T-NHL xenografts*

We next determined if the high-expression of CD45 in T-NHL could be utilized to target human T-NHL xenografts in mouse models. Groups of ≥5 mice with palpable human T-NHL (Karpas 299, CCRF-CEM) xenografts were co-injected via the tail vein with 200µg $^{131}$I-BC8 (anti-CD45) and 200µg $^{125}$I-BHV-1 (control) labeled with 10 µCi of radioisotope followed by tumor and normal organ harvest at 24 and 48 hours. CCRF-CEM xenografts targeted with $^{131}$I-BC8 demonstrated 154% (p=.01) and 237% (p=.002) more radioiodine retention at 24h and 48h, respectively (*Figures 2a and b*). Likewise, when Karpas-299 xenografts were targeted with BC8, they retained 223% (p=.002) and 313% (p=.0002) more radioiodine than the isotype-matched control BHV1 (*Figures 2c and d*). More importantly, tumor sites retained more activity than the critical non-target organs. For example, T-NHL (CCRF-CEM) xenografts exhibited 2.5 (p=.05), 3.0 (p=.007), and 3.6 (p=.07)-fold higher $^{131}$I retention compared to the lungs, liver, and kidneys, respectively at 24 hours (*Figure 3a*). Similar preferential tumor targeting was observed at 48 hours and in Karpas 299 xenografts (*Figures 3b - d*).

*Anti-murine CD45 RIT targets murine T-NHL*

Since CD45 is also expressed on normal hematopoietic cells, we hypothesized that the inability of BC8 to bind mCD45 in the spleen and marrow may have favorably influenced our biodistribution results in the above xenograft
models. To address this question in a model that would more directly replicate the likely clinical use of this agent, namely expression of CD45 both on T-NHL sites and normal hematolymphoid tissues, we evaluated the ability of anti-CD45 RIT to preferentially target tumor sites in a syngeneic model. Mice with palpable EL-4 (mT-NHL) xenografts were injected with 200 µg of $^{131}$I-30F11 (anti-mCD45) followed by harvest of tumor sites and normal organs as above. Hematopoietic target sites (tumor, marrow, and spleen) retained 14-42% of the injected I-131 dose/g tissue as compared to 1.2-4.2% in non-target tissues (Figure 4a) despite the broad expression of CD45 on hematolymphoid tissues. This preferential targeting by anti-CD45 RIT in a syngeneic model translated into favorable tumor-to-normal organ ratios of radioisotope retention indicating that 3.6-11.8 times more radiation would be delivered to tumor sites as compared to non-target tissues at 24 hrs (Figure 4b). The precise ratios of %ID/g and p-values comparing target to non-target sites are detailed in table 1.

**Anti-CD45 RIT controls T-NHL tumor growth and improves survival**

We next sought to determine if the above biodistribution results could translate into improved tumor control and survival. Mice with palpable T-NHL xenografts (CCRF-CEM) were randomly assigned to receive 200µg of BC8 labeled with either 300 or 400µCi of $^{131}$I, 200µg of BHV-1 (control) labeled with 300µCi $^{131}$I, or no treatment. Tumor dimensions and survival were tracked. By day 9, complete remissions (CR) were attained in 90% of mice treated with 400 µCi of $^{131}$I-BC8 and 67% of mice that received 300 µCi of $^{131}$I-BC8 (Figure 5a). In contrast, none of the untreated control mice or mice that received 300 µCi of $^{131}$I-
BHV-1 achieved CR (p<0.0001). This improved tumor control translated into longer progression-free survivals (PFS) for the BC8 treated mice versus the controls (median PFS 23 days BC8 vs 4.5 days controls, log-rank p<.0001 [Figure 5b]). All 20 mice in the 2 control groups required euthanasia due to tumor growth, whereas only 4 of 20 of the mice in experimental arms needed to be sacrificed due to disease progression and unmaintained remissions persisted for over 72 days following therapy. A dose-response relationship was noted between the 300 µCi and 400 µCi $^{131}$I-BC8 groups, but since transplant was not used to abrogate the expected hematologic toxicity of high-doses of radiodine, the 400 µCi $^{131}$I-BC8-treated animals experienced inferior PFS due to non-relapse mortality from the expected pancytopenia as evidenced by petechiae, pallor, and weight loss.

Discussion:

The results of this study are the first illustration of the potential efficacy of anti-CD45 RIT for the treatment of T-NHL. In this series of experiments, we initially demonstrated that CD45 was expressed in high-copy number in both T-NHL cell lines and a spectrum of T-NHL patient samples. The surface density of CD45 was reliably expressed on the majority of T-NHLs at or above the concentration expected for CD20 in B-NHL, indicating that sufficient antigen should be available for successful targeting $^{19}$. Interestingly, two of 3 LBL specimens exhibited significantly lower CD45 expression, suggesting that CD45 may not be optimal for targeting precursor T-cell neoplasms $^{20}$. 
The subsequent murine studies confirmed that this strategy can yield higher retained activity and, thus, radiation exposure in CD45-bearing tumor sites, while sparing normal non-target tissues. It is this favorable tumor-to-normal organ ratio that provides the therapeutic index of RIT. This therapeutic index was maintained even in the more clinically relevant and scientifically rigorous syngeneic tumor model where both CD45-bearing T-NHL sites and normal CD45-bearing hematolymphoid tissues were targeted. Despite the broad expression of CD45, tumor sites, spleen, and marrow retained 3.6-11.3 times the radioactivity as the highest non-target organ at 24 hrs. These ratios appeared superior to published biodistribution data when targeting CD20 in B-NHL and translated into $^{131}$I dose related improved response rates and remission durations$^6,16$.

Unlike with the B-NHLs, targeted therapies for T-NHLs are less broadly utilized. This may be in part due the heterogeneity of ideal antigens expressed in these diseases and the potential requirement to develop novel agents for each group of T-cell malignancies$^13$. The first commercially available MoAb targeting antigens found on some T-NHLs was alemtuzumab (Campath 1H). Alemtuzumab targets CD52, an antigen expressed on $\sim$50% of T-NHLs and can induce responses of 6-12 months in duration in 36-55% of patients with relapsed T-NHL or MF$^{21,22,23}$. A second targeted agent directed against the IL-2 receptor (CD25), denileukin diftitox (Ontak®), has been approved for the treatment of MF and has been shown to yield response rates of 30% lasting a median of 6.9 months$^{24}$. Further improvement on targeted therapies for T-NHL have been
limited, though pilot data exist regarding the use of anti-CD30 antibodies in ALCL as well as $^{90}$Y-anti-CD25, $^{90}$Y-anti-CD5, and $^{131}$I-antiCD5 antibodies in T-cell leukemia/lymphoma$^{25-28}$. Unfortunately, to date there are no FDA-approved anti-T-NHL radioimmunoconjugates. More importantly, most T-NHL RIT programs in development may be limited to a subset of T-lymphoid malignancies due to the varied antigen expression (eg CD5, CD25, CD30) in these diseases$^{13,29}$. Thus, a pan-T-cell antigen target would be ideal to develop a successful, widely applicable RIT-based approach for T-NHL.

Our preclinical results are particularly applicable to therapy of T-NHL where it is estimated that less than 15% of patients with more than 1 adverse factor at diagnosis will be alive and in remission at 5 years$^{3,4}$. Despite the poor prognosis, even the most refractory subtypes of T-NHL (angiocentric T-NHL and NK/T-NHL) remain radiation-sensitive with local control being attainable when absorbed doses of $\geq 40$Gy are applied$^{30}$. Likewise, HSCT has been employed to improve outcomes$^{31-33}$. Unfortunately, less than a third of patients with relapsed, chemosensitive, T-NHL will achieve long-term remission with this strategy with the major cause of failure being recurrent disease$^{31-33}$. One could hypothesize that escalation of the radiation dose to tumor sites prior to HSCT by the use of anti-CD45 RIT could deliver potentially curative radiation exposure to multifocal disease beyond that which could be administered with external beam therapy. Since CD45 is also expressed on most cells of hematopoietic origin, HSCT would be required with any use of this strategy. Dual targeting of both specific tumor sites and hematolymphoid tissues, however, may also prove advantageous for
the control of both measurable and minimal disease sites since the radiation exposure to individual tumor cells could be amplified via crossfire from targeting adjacent CD45-bearing tissues.

In conclusion, these results in conjunction with other preclinical and clinical data from our group indicate the potential efficacy of anti-CD45 RIT in T-NHL and support the rapid translation of this strategy to the clinic. We anticipate opening a clinical trial to test these hypotheses in patients with relapsed T-NHL in 2009.

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Author Contributions:
Ajay Gopal: Conceived project, designed experiments, analyzed data, wrote and edited the manuscript.
John Pagel: Designed experiments, edited the manuscript.
Jonathan Fromm: Designed experiments and analyzed data for antigen binding studies, edited the manuscript.

Shani Wilbur: Designed, performed and analyzed mouse experiments. Edited manuscript.

Oliver Press: Designed experiments, edited the manuscript.

References:


Table 1: Ratios of percent injected I-131 dose/g between target (tumor, marrow, spleen) and non-target organs following I-131-30F11 (anti-murine CD45) in a EL-4 (murine T-NHL) model (24h)

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<th>p value</th>
<th>Spleen/organ</th>
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Figure 1: Mean and standard deviations of antigen density (antigen binding capacity [ABC]) of CD45 on T-NHL patient samples (light bars) and T-NHL lines (dark bars). PTCL= peripheral T-cell lymphoma, ALCL= anaplastic large cell lymphoma, LBL= T-lymphoblastic leukemia/lymphoma, MF= mycosis fungoides, NK/T= NK/T-cell lymphoma, nasal type.
Figure 2: Biodistributions quantified as percent injected radiiodine dose/g tissue (%ID/g) of I-131 BC8 (anti-CD45, dark bars) and I-125-BHV-1 (control, light bars) in mice with CCRF-CEM (a and b) and Karpas 299 (c and d) xenografts after 24 and 48 hours.
Figure 3: Tumor-to-normal organ ratios of percent injected radioiodine/g tissue of I-131 BC8 (anti-CD45, dark bars) and I-125-BHV-1 (control, light bars) in mice with CCRF-CEM (a and b) and Karpas 299 (c and d) xenografts after 24 and 48 hours.
Figure 4: a. Percent injected I-131 dose/g tissue (%ID/g) in target (solid bars) and non-target (hatched bars) organs following I-131-30F11 (anti-murine CD45) in an EL-4 (murine T-NHL) model. b. The resultant tumor-to-normal organ ratios (24h).
Figure 5: Tumor volume (a) and progression-free survival (b) in mice bearing human T-NHL xenografts (CCRF-CEM) following no treatment, BHV1 labeled with 300µCi $^{131}$I (control), BC8 labeled with 300µCi $^{131}$I, or BC8 labeled with 400µCi $^{131}$I.
I-131-anti-CD45 radioimmunotherapy effectively targets and treats T-cell non-Hodgkin lymphoma

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