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Mutation-specific control of BCR-ABL T315I positive leukemia with a recombinant yeast-based therapeutic vaccine in a murine model

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ABSTRACT

Chromosomal translocations generating the *BCR-ABL* oncogene cause chronic myeloid leukemia (CML) and a subset of acute lymphoblastic leukemia. The BCR-ABL^{T315I} mutation confers drug resistance to FDA-approved targeted therapeutics imatinib mesylate, dasatinib, and nilotinib. We tested the ability of a recombinant yeast-based vaccine expressing the T315I-mutated BCR-ABL antigen to stimulate an anti-BCR-ABL^{T315I} immune response. The yeast-based immunotherapy significantly reduced or eliminated BCR-ABL^{T315I} leukemia cells from the peripheral blood of immunized animals and extended leukemia-free survival in a murine model of BCR-ABL^{+} leukemia compared to animals receiving sham injection or yeast expressing ovalbumin. With immunization, leukemic cells harboring BCR-ABL^{T315I} were selectively eliminated after challenge with a mixed population of BCR-ABL and BCR-ABL^{T315I} leukemias. In summary, yeast-based immunotherapy represents a novel approach against the emergence of cancer drug resistance by the pre-emptive targeted ablation of tumor escape mutants.

1. Introduction

The *BCR-ABL* oncogene results from a chromosomal translocation at position t(9:22)(q34;q11) [1,2]. *BCR-ABL* is causal for chronic myelogenous leukemia (CML) and present in 20–40% of adults with acute lymphoblastic leukemia (ALL) [3–6]. CML and ALL result in a massive accumulation of clonal myeloid or lymphoid progenitor cells respectively. Untreated CML results in death at a median of 3 years after diagnosis, while the more aggressive ALL results in death at a median of 11 months after diagnosis, even with chemotherapy [6,7]. The targeted BCR-ABL kinase inhibitor, imatinib mesylate (IM), achieves a complete cytogenetic response at 18 months in 76% of patients with CML [8–11]. However, after 3.5 years, ~16% of chronic phase CML patients relapse despite IM therapy. Most of these relapses are due to BCR-ABL point mutations that prevent IM binding and restore aberrant kinase activity [12,13]. The T315I mutation is a particularly relevant target, as this "gatekeeper" mutation occurs with the highest incidence (16%, [14–16]) and is the only point mutation causing cross-resistance to both IM and second generation kinase inhibitors [17–19].

Investigational approaches for drug-resistant leukemia treatment involve small molecule inhibitors effective against BCR-ABL^{T315I} and other drug escape mutations, inhibitors targeting effectors downstream of BCR-ABL, or drugs with epigenetic targets [20,21]. An attractive alternative would be to activate T cell immunity in CML or ALL patients against tumor cells expressing leukemia-specific or leukemia-associated antigens [22]. The mutated BCR-ABL^{T315I} protein is a tumor specific antigen, and thus is not subject to central self-tolerance mechanisms [23,24]. The activation of T cell immunity against IM escape mutants in combination with IM treatment could suppress or eliminate the emergence of resistant variants.

Immunotherapy with whole heat-killed recombinant Tarmogen® yeast delivering the target antigen activates a potent innate response to the yeast vector, plus an adaptive T cell immune response to the target antigen. Thus, immune recognition of the target antigen is directly coupled to the innate danger response against the yeast vector and uptake by phagocytic dendritic cells (DC), macrophages and neutrophils. Another advantage of this immunotherapy approach is the delivery of target polypeptides whose processing by host DCs affords a full spectrum of epitopes derived from the polypeptide, facilitating presentation of and response to epitopes relevant for each person's HLA (MHC-I and MHC-II) receptor repertoire.

Immunization with GI-4000 Tarmogen® yeast elicits mutation-selective killing of tumors harboring the mutated Ras protein in a carcinogen-induced murine lung tumor model [25,26]. GI-4000

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Tarmogen® yeast delivering mutated Ras polypeptides are being tested in clinical trials in patients with pancreatic cancer, non-small cell lung cancer, and colorectal cancer. In those trials, the tumors are genotyped to establish the presence of *ras* mutations and to administer the GI-4000 Tarmogen® yeast product that would activate T cell immunity against the matched mutant Ras protein (e.g. ClinicalTrials.gov Identifier: NCT00300950). Given the immune activation against mutated Ras proteins using Tarmogen® yeast immunotherapy, we tested whether this approach would elicit T cell responses against the T315I mutation in BCR-ABL, for the purpose of preventing or limiting BCR-ABL^{T315I} IM drug-resistance.

2. Materials and methods

2.1. MHC-binding peptide prediction algorithms and REVEAL assay

Eighty-one overlapping 8–10 amino acid peptides spanning BCR-ABL^{T315I} and two other BCR-ABL IM point mutations, E255K and M351T, were analyzed for potential CD8$^+$ T cell epitopes. The MHC-binding peptide prediction algorithms, Arbmatrix [27], libscore [28], smm [29], arb [27], bimas [30], SYFPEITHI [31] and ann [32], were used to predict binding to the C57BL/6 and BALB/c MHC class I alleles; H-2D^b, H-2K^b, H-2D^d, H-2K^d and H-2L^d. Prediction software algorithms with accuracy scores estimated to be higher than 66.5% for any given allele were used for these analyses (Supplementary Fig. 1). The 62 highest scoring peptides were synthesized. These were evaluated using the REVEAL® *in vitro* H-2D^b and H-2K^b MHC class I-binding assays performed by Proimmune.

2.2. Computer modeling

Crystal structures were used for generating the H-2K^b/nanomeric peptide model (pdb 1VAC [33]) and the H-2K^b/octameric peptide model (2VAB [34]) by using the swiss pdb viewer program [35]. The initial amino acid conformations of the peptides were manually adjusted to avoid clashes. Energy minimization refinement was performed on the peptides in CNS [36]. The H-2K^b molecules were fixed in the refinement. The final models were inspected in O [37] on Silicon Graphics OCTANE2 Workstations.

2.3. Animals

Wild-type BALB/c and C57BL/6 mice were purchased from the National Cancer Institute or bred at the University of Colorado Denver School of Medicine. The Institutional Animal Care and Use Committee approved all experiments using mice.

2.4. Primary leukemia generation

Murine stem cell viruses (MSCV) were prepared by transient transfection of Pheonix-E cells together with pCL-Eco (encodes Gag, Pol and Env), and titered on fibroblasts using GFP or RFP as the marker BCR-ABL expression. Bone marrow cells were harvested from C57BL/6 or BALB/c mice and enriched for CD117$^+$ cells by MACS® (Miltenyi Biotec, Bad Gladback, Germany). The cells were first cultured overnight in DMEM plus 10% defined fetal bovine serum with the cytokines hSCF (gift from Dr. Chris Hogan, UC Denver), hIL-11, mIL-3, and hIL-6 (PeproTech, Rocky Hill, New Jersey, USA). The cells were infected for 6 h with 6 μL/mL Polybrene (Sigma) and supernatants containing MSCV-ires-GFP or MSCV-ires-RFP viruses encoding p210 *BCR-ABL* or p210 *BCR-ABL^{T315I}* (the drug-resistant mutant was a gift from Drs. Michael Deininger and Brian Druker, Oregon Health Sciences University). The cells were then transplanted intravenously into sublethally irradiated (3 Gy) mice using an X-ray source (RadSource RS2000 irradiator). Infection efficiencies were determined after culturing aliquots of transduced cells under HSC conditions for 48–72 h [38]. Primary leukemias expressing BCR-ABL or BCR-ABL^{T315I} developed with similar kinetics between 3 and 8 weeks. Transplanted mice were monitored for disease development, as judged by increasing percentages of GFP or RFP expressing cells with blast morphology in peripheral blood of transplanted animals, as well as symptoms, such as reduced mobility, hunching, and labored breathing. Moribund animals were sacrificed and leukemias were characterized and stored at −80 °C. Automated hematocrit analysis of peripheral blood was performed using a Cell-Dyn 1700 System (Abbott, Abbott Park, IL, USA).

2.5. Yeast vaccine

Immunotherapy constructs were made by subcloning the *BCR-ABL* junction, SH3, SH2 and kinase domains by blunt end ligation into the pBlueScript SKII-® vector (Fermentas) and introducing point mutations using sequential QuikChange Site Directed Mutagenesis Kit® (Stratagene). To avoid triggering a xenogeneic immune response of the human Abl protein by the murine immune system, two amino acids were altered to match the mouse *ABL* sequence ortholog (Fig. 1a). The altered *ABL* sequence spanning just downstream of the junction epitope through the region encoding the three escape mutant epitopes was cloned into the Tarmogen® expression plasmid under control of a strong constitutive promoter (Fig. 1a). A C-terminal hexahistidine tag was added for detection and quantification. The cloned DNA inserts were bidirectionally sequenced and the plasmid was transfected into *S. cerevisiae* yeast (W303alpha) using the EZ yeast transfection kit (Zymo research). Transfectants were selected on solid medium lacking uracil and growth of cultures was in medium containing 20 g/L glucose, 6.7 g/L yeast nitrogen base, and 2 mg/mL each of adenine, histidine, tryptophan, and leucine. Liquid yeast cultures were grown to mid-exponential phase, harvested by centrifugation, washed once in PBS, and heat inactivated at 56 °C for 1 h. The yeast were washed three times in PBS and re-suspended in PBS at 20 YU/mL (1 YU = 10^7 Tarmogen® yeast cells). Tarmogen® yeast (5 YU) were injected subcutaneously at 2 sites per immunization. Dosing was administered 10, 8, 6 and 1 weeks prior to leukemia challenge. The parental yeast vector expressing an irrelevant antigen, chicken ovalbumin (yeast^{OVA}) was used as a control immunogen [39].

2.6. Detection of Abl T315I protein by western blotting

The expression of Abl protein by yeast^{T315I} was quantified by western blotting using an anti-(His)$_6$ tag monoclonal antibody. Additional positive identification of the expressed protein was conducted by immunoblotting with a monoclonal antibody recognizing the SH2 domain of the Abl protein (Mouse Anti-ABL Monoclonal Antibody 554148, BD Pharmingen).

2.7. Challenge with primary leukemias

Primary leukemia cells (1.7×10^5 GFP$^+$ or RFP$^+$ cells) suspended in 200 μL IMDM/1% FBS were used for challenging vaccinated or control recipient mice, which resulted in leukemia development in most control recipients between 10 and 14 days post-transfer. Challenged mice were monitored at least once per day for secondary leukemia development as described above.

2.8. Flow cytometric analysis

Tail blood, spleen and bone marrow cells were isolated, and the contributions of BCR-ABL expressing cells to hematopoiesis as