Leukemia cell-rhabdovirus vaccine: personalized immunotherapy for acute lymphocytic leukemia

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Research Article

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Treating acute lymphoblastic leukemia (ALL) involves prolonged multi-agent radio-chemotherapy. While children are often cured, they develop significant late adverse treatment-related health effects. The majority of adult patients unfortunately succumb to the disease. Evidence derived from recipients of allogeneic hematopoietic stem cell grafts support the concept that ALL is susceptible to immune attack but treatment-related toxicity limits its broad application. Robust, yet non-toxic leukemia-specific immunotherapy could improve outcomes for all patients. Using several murine models of ALL, we test the immunotherapeutic potential of leukemia cells infected with engineered attenuated rhabdoviruses (iLOV). These preclinical studies demonstrate the potency and durability of the anti-leukemic immune response induced by iLOV. This is the first time an oncotropic virus has been successfully employed in clinically relevant orthotopic models using syngeneic acute leukemia cells. This novel immunotherapeutic has the potential to advance towards early-phase clinical trials for patients with ALL.
Abstract

Purpose: Acute lymphocytic leukemia (ALL) remains incurable in most adults. It has been difficult to provide effective immunotherapy to improve outcomes for the majority of patients. Rhabdoviruses induce strong anti-viral immune responses. We hypothesized that mice administered \textit{ex-vivo} rhabdovirus-infected ALL cells (iLOV) would develop robust anti-leukemic immune responses capable of controlling ALL.

Experimental Design: Viral protein production, replication and cytopathy were measured in human and murine ALL cells exposed to attenuated rhabdovirus. Survival following injection of graded amounts of ALL cells was compared between cohorts of mice administered $\gamma$-irradiated rhabdovirus-infected ALL cells (iLOV) or multiple control vaccines to determine key immunotherapeutic components and characteristics. Host immune requirements were assessed in immunodeficient and bone marrow transplanted mice or by adoptive splenocyte transfer from immunized donors. Anti-leukemic immune memory was ascertained by second leukemic challenge in long-term survivors.

Results: Human and murine ALL cells were infected and killed by rhabdovirus; this produced a potent anti-leukemia vaccine. iLOV protected mice from otherwise lethal ALL by developing durable leukemia-specific immune-mediated responses ($P<0.0001$), which required an intact cytotoxic T-cell compartment. Pre-existing anti-viral immunity augmented iLOV potency. Splenocytes from iLOV-vaccinated donors protected 60% of naïve recipients from ALL challenge ($P=0.0001$). Injecting leukemia cells activated by, or concurrent with, multiple TLR agonists could not reproduce the protective effect of iLOV. Similarly, injecting uninfected irradiated viable, apoptotic or necrotic leukemia cells with/without concurrent rhabdovirus administration was ineffective.

Conclusion: Rhabdovirus-infected leukemia cells can be used to produce a vaccine that induces robust specific immunity against aggressive leukemia.
Introduction

Acute lymphoblastic leukemia (ALL) is an aggressive hematopoietic malignancy characterized by rapid accumulation of lymphoblasts in the marrow with suppression of hematopoiesis (1). Children are treated with prolonged multi-agent radio-chemotherapy, achieving at least 80% long-term survival (2), but this is associated with frequent late adverse effects including secondary malignancies, various chronic medical problems, psychological and cognitive impairments (3,4). While adults with ALL frequently obtain complete remission, most relapse with only a third surviving 5 years from diagnosis (2,5). Immunologically mediated graft versus leukemia (GvL) effects are responsible, in part, for improved outcomes in the minority of patients eligible for allogeneic HSCT (6–12). Unfortunately, the benefits of GvL are difficult to separate from the detrimental effects of graft versus host disease (13). Thus, measures that reduce the intensity or duration of chemotherapy without compromising disease control would improve the quality of life for survivors of childhood ALL, while more potent therapies are required for curing adult ALL. ALL is amenable to immunotherapy as demonstrated by the effectiveness of GvL, consequently administering vaccines targeting residual leukemic cells remaining after induction chemotherapy may help achieve these goals.

Leukemia cells often harbor unique antigens with immunogenic potential. For instance, the presence of autologous CD8+ and CD4+ T-cell immune responses to peptides derived from the leukemia-specific antigen NPM1mut has been shown in patients with AML (14). The use of adjuvants or mechanisms that up-regulate cell-surface immune activation molecules, improve the intrinsic immunogenicity and therapeutic potential of leukemia cell vaccines (15–17). Indeed, allogeneic HSCT recipients with advanced high-risk ALL and AML were shown to generate tumor specific responses and survive longer following treatment with autologous leukemia cells mixed with syngeneic skin fibroblasts expressing CD40L and IL-2 from adenoviral vectors (18). Phase I/II clinical studies of idiotype and dendritic cell (DC)-based vaccines have shown a modest survival benefit for patients with...
Conrad et al  iLOV: Personalized Immunotherapy for ALL acute and chronic lymphomas (19). Unfortunately, available immunotherapeutic technologies suffer from inefficient recognition and processing of tumor peptide(s), leading to suboptimal anti-tumor T-cell responses in-vivo (20). These challenges limit the potential impact of current immune-based anti-cancer therapies.

Neoplastic transformation is associated with defects of cellular antiviral defenses allowing selective infection of cancer cells by diverse families of attenuated viruses (21–23). Rhabdoviruses, such as the engineered VSVd51 with deletion at methionine-51 of the matrix protein, and MG1, a recombinant Maraba virus with cooperative attenuating mutations in its matrix protein (L123W) and glycoprotein (Q242R), complement the interferon signaling defects in cancer cells. These specific mutations attenuate virulence and increase their tropism towards malignant cells (24,25). Vesicular Stomatitis Virus (VSV) can be rendered incapable of spreading between cells by creating mutants with deletion of the viral glycoprotein gene (VSV_ΔG) required for final virion assembly and egress (26). The biology of these enveloped single stranded negative-sense RNA viruses has been reviewed (24). Infection of tumor cells by these oncotropic viruses initiates a chain of events causing peri-tumor inflammation, activation of natural killer cells, macrophage-mediated innate immune attack, as well as induction of adaptive anti-tumor immune responses (21,27,28).

Rhabdoviruses are capable of infecting leukemia cells in-vitro, however viral replication is quite limited in these cells (29). This hinders their use as direct cytolytic agents for treatment of ALL. However, using immunocompetent murine models of ALL, we show that a vaccine composed of syngeneic leukemia cells infected ex-vivo with rhabdovirus (immunotherapy by Leukemia-Oncotropic Virus or iLOV) generates a potent and durable anti-leukemia effect that is specifically directed towards the leukemia cell used to produce this vaccine.
Materials and Methods

Reagents

Blasticidin and Zeocin™, used for VSVd51ΔG production, were purchased from Invitrogen (Carlsbad, CA). The toll-like receptor (TLR) 3 agonist polyinosinic-polycytidylic acid (poly I:C) and TLR4 agonist lipopolysaccharide (LPS) were purchased from Sigma Aldrich (St. Louis, MO). Anti-mouse CD40-APC, Propidium iodide, 7-AAD viability-staining solutions and Annexin V Apoptosis Detection Kit APC were obtained from eBioscience (San Diego, CA). Anti-mouse CD19-FITC, CD3-PE, CD4-PerCP, CD8-PerCPCy5.5, biotin anti-mouse CD252 (Ox40L), and PE-streptavidin were obtained from BD Bioscience (Franklin Lakes, NJ).

Tumor cells

L1210 and EL4 murine lymphoblastic cell lines, from ATCC (Manassas VA), were maintained in suspension culture, Dulbecco’s modified Eagle’s medium-high glucose (DMEM) (HyClone, Logan, UT), with 10% fetal calf serum (FCS) (CanSera, Etobicoke, ON) at 37°C and 5% CO2. Cells were routinely split every 2-4 days to maintain concentration between 0.5-1.0 x 10^6 cells/mL. The Jurkat human acute T-cell lymphoblastic leukemia cell line, from ATCC, the human acute immunoblastic B-cell line OCI-Ly-18, kind gift of Dr. Hans Messner (Ontario Cancer Institute), and the human acute T-cell lymphoblastic cell line A301, kind gift from Dr. Thomas Folks of the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, were maintained in similar culture conditions. The Vero cell line, from ATCC, was maintained as adherent cell cultures in DMEM and 10% FCS. Vero cells were used for virus propagation, detection or enumeration of infectious viral particles and for viral-neutralization antibody assays. T-Rex™-293 cells (Invitrogen, Carlsbad, CA) were used for manufacturing of VSVd51ΔG virus.
Mice

DBA/2, C57BL/6, athymic nude *nu/nu* and B6D2F1 hybrid mice (all 6-8 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA) and housed in a biosafety unit at the University of Ottawa, accredited by the Canadian Council on Animal Care (CCAC). Institutional guidelines and review board for animal care (The Animal Care and Veterinary Service of the University of Ottawa) approved all animal studies.

Oncotropic viruses

The rhabdoviruses, MG1 and VSVd51 were propagated in Vero cells and purified as previously described (22). MG1-eGFP and VSVd51-eGFP, genetically engineered to express enhanced green fluorescent protein (eGFP) gene, were grown and purified in similar manner. VSVd51 with deletion of the Glycoprotein gene (VSVd51ΔG) was propagated in T-REx™-293 cells stably transfected with pcDNA4/TO plasmid expressing VSV Glycoprotein gene. VSVd51ΔG was grown by infecting this cell line with virus stock, 24 hrs after glycoprotein induction with 1 μg/mL tetracycline. Supernatants were collected 24 hours after infection and virus purified. For certain experiments 50 μL aliquots of viral preparation were exposed to UVC radiation (Spectrolinker™ UV Crosslinker XL-1000, Spectronics, Westbury, NY); complete inactivation was confirmed by testing for absence of cytopathic effects and infectious particles on Vero cells. Enumeration of virus particles was performed as previously described (28). Suspension cultures of human and murine leukemic cells were infected by adding virus preparations directly to culture (at 1x10⁶ cells/ml), at a multiplicity of infection (MOI) of 0.1.

Vaccine Preparations

iLOV was prepared by infecting suspension cultures of L1210 or EL4 cells at 1 x 10⁶/mL with virus at MOI of 10, and maintained at 37°C in humidified (5% CO₂) incubator. Eighteen hours after
infection, an aliquot of culture was analyzed by flow cytometry, assessing extent of infection (eGFP expression), concentration and viability. The remainder was pelleted by centrifugation at 1500, media aspirated, washed once in phosphate buffered saline (PBS) and re-suspended for final concentration of 1x10^7 cells/mL in PBS. Vaccine preparations received 30 Gy gamma-irradiation (γ-IR) (HF-320, Pantak, Branford, CT) prior to administration. Specific experiments used uninfected leukemia cell vaccine prepared analogously and injected alone, co-injected with MG1 (MOI 10) in a separate syringe or mixed with MG1 at MOI of 10, at room temperature 60 minutes prior to administration. In other experiments virus infected L1210 cells were fixed in 1% (final) paraformaldehyde (PFA) before γ-IR. In specific experiments, toll-like receptor (TLR) agonists replaced virus infection in the vaccine. Standard murine doses of either 150 μg/100 μL poly I:C or 17 μg/100μL LPS were added to L1210 cells prior to γ-IR and injection. In other experiments, 150 μg/10^6 cells poly I:C and/or 17 μg/10^6 cells LPS were add to cultures of L1210 cells for 18 hours prior to washing and γ-IR. Apoptotic leukemia cell vaccine was prepared from uninfected L1210 cell cultures. Cells were pelleted by centrifugation, washed once and resuspended in PBS at 2 x 10^6/mL. Ten ml of cell suspension was placed in a 150x25mm plate and exposed to 500 mJ/cm^2 UVC. Cells were then pelleted, resuspended in fresh media and incubated at 37°C for 4 hours prior to preparation for use in a manner analogous to iLOV. Necrotic leukemia cell vaccine was prepared by pressure disruption (1500 PSI) of washed uninfected L1210 cells at 1 x 10^7 cells/mL in a French hydraulic press (AMINCO J5-598A, Newport Scientific – Jessup, MD).

**Virus Treatment of Leukemia**

DBA/2 mice received tail-vein injections of 1x10^6 L1210 leukemia cells. Leukemic mice were treated with 100 μL PBS or PBS containing 1x10^8 plaque forming units (pfu) MG1 by tail vein injection 7, 10 and 14 days later (Supplemental Fig. S1A). Mice were euthanized upon development of
typical signs of advanced leukemia such as hind-leg paralysis, focal tumor development, significant
weight loss and/or respiratory distress.

**Immunization and leukemic challenge**

Immunization was performed by tail vein injection of 100 μL per mouse per dose of freshly
prepared iLOV, an alternative vaccine or PBS. Vaccines were administered once weekly for 3 doses,
followed one week later by intravenous tail vein injection of viable leukemia cells from suspension
cultures. Cells were pelleted by centrifugation, media aspirated, washed once in PBS and resuspended
at 1x10^7 cells/ml in PBS. Mice received a dose of 1 x 10^6 cells unless otherwise specified. Mice were
euthanized upon development of predetermined signs of advanced leukemia endpoints, (Supplemental
Fig. S1B).

**Adoptive cell transfer**

Under sterile conditions, single cell suspensions of splenocytes were prepared from donor
spleens removed from iLOV immunized or naïve DBA/2 mice using gentleMACS Dissociator
(Miltenyi Biotec, Auburn, CA) according to manufacturer’s recommendations with red blood cells
lysed by ACK lysis buffer. Donor splenocytes were pooled and 15 x 10^7 cells were injected
intravenously via tail vein into syngeneic recipients. Splenocyte recipients received a leukemia
challenge of 1x10^6 L1210 cells, administered intravenously via tail vein, one week after adoptive
transfer.

**Bone marrow transplantation and vaccination**

Eight-week-old DBA/2 mice received total body irradiation (TBI) using 2 fractions of 450 cGy
given 2 hours apart. Under aseptic conditions, 10^6 bone marrow cells, collected from flushed femurs of
8-week-old B6D2F1 donors, were intravenously infused into each TBI-treated recipient. On day 43, cohorts ($n=5-6$/group) of bone marrow transplantation (BMT) recipient DBA/2 mice and healthy B6D2F1 mice, received the first of 3 weekly iLOV vaccinations. On day 63, a leukemia challenge of $1 \times 10^7$ L1210 cells was administered to all mice, including parallel-unimmunized cohorts ($n=5$/group). Additional cohorts of unimmunized TBI-treated DBA/2 and naïve B6D2F1 were euthanized at day 43 for enumeration of the major lymphocyte compartments by flow cytometry.

**Flow cytometry**

Leukemia cell infections were evaluated by flow cytometric analysis of 10,000 cells using Quanta SC (Beckman Coulter). A 500 μL aliquot of infected cells was stained with 5μL PI (1mg/mL) approximately 30 minutes before data acquisition. All vaccine preparations were analyzed by flow cytometry in similar fashion to allow dose standardization and quality control of virus expression. For analysis of apoptosis and necrosis, acquisition was performed on CyAn ADP (Beckman Coulter) using Annexin V apoptosis detection kit APC and cell viability dye 7-AAD, with minimum of 50,000 cells counted. Cell-surface expression of activation/co-stimulation molecules CD40 and Ox40 ligand (CD252) on L1210 cells incubated for 18 hours with either MG1 (MOI 10), LPS (17 μg/10^6 cells) or Poly I:C (150 μg/10^6 cells) was analyzed on CyAn ADP (minimum of 60,000 cells acquired) and performed in triplicate with background mean fluorescence intensity (MFI) of unstained cells under each condition subtracted from the MFI of stained cells. Single cell suspensions of splenocytes were collected from unimmunized TBI-treated DBA/2 and naïve age-matched B6D2F1 mice and total cells/spleen were measured by trypan blue exclusion. Enumeration of B-cell (CD19+), T-cell (CD3+/CD4+, and CD3+/CD8+) and NK-cell (NK1.1+) subpopulations was performed on CyAn ADP, counting a minimum of 60,000 cells, performed in technical duplicates for each biologic replicate. Data
analysis performed with Kaluza software version 1.1 (Beckman Coulter) and Cell Lab Quanta Analysis (Beckman Coulter).

Statistics

Survival curves were generated using product limit (Kaplan-Meier) method and comparisons were performed using log-rank (Mantel-Cox) test, all P values are two-tailed. Elsewhere, data presented as mean + SEM with significance determined by Welch’s corrected T-test. Statistical significance was determined at level of $P < 0.05$. Analyses were performed using Prism 5 software (GraphPad Software, La Jolla CA).

Results

MG1 infects leukemia lines in-vitro but is ineffective in halting leukemia progression in-vivo

We first wished to explore whether mice with disseminated ALL could be successfully treated by systemic delivery of live oncotropic rhabdovirus. We first established that MG1 was able to infect and kill various murine and human leukemia cell lines in-vitro at low MOI (Fig. 1A, 1B, S2). L1210 leukemia cells demonstrate considerable permissiveness to MG1 infection and result in efficient, rapid cytolysis yet virus production is modest over 24-40 hours incubation (Fig. 1C). Next, a cohort of leukemia-bearing mice was given $1 \times 10^8$ pfu of MG1-eGFP daily every 3 days for 3 doses. This was unable to prevent disease progression (Fig. 1D). Organs were recovered from all mice at endpoint. Following homogenization, an aliquot of each organ was co-cultured for 15 hours on Vero cells and the monolayer was then scanned under a fluorescence microscope for presence of green fluorescenting cells. Homogenates from the brain and liver of a single mouse, that died 3 days following the second virus injection, contained MG1 and resulted in GFP positivity on the Vero monolayer. Despite the ability of
MG1 to infect and kill leukemic cells in-vitro, the oncolytic effect of live MG1 was ineffective at controlling leukemia at doses large enough to cause toxicity in leukemia-bearing hosts.

**MG1 infected leukemia cells (iLOV) acts as a vaccine, eliminating otherwise lethal acute leukemia**

Barriers to systemic replication-competent virotherapy for leukemia may include inadequate cytolysis at tolerable doses and rapid tumor kinetics that outpace establishment of anti-tumor immune responses. To address the latter issue, mice were administered 3 weekly doses of γ-IR virus-infected L1210 cells (MG1-iLOV). This was followed, a week later, by injection of viable L1210 cells. Mice that received MG1-iLOV demonstrated greater than 90% long term survival following challenge with viable leukemia cells compared to untreated control mice which reproducibly reached leukemic end-points with median survival of 18 days (Fig. 2A), confirming that iLOV was able to establish highly protective anti-tumor responses. However, when leukemic challenge was administered one day prior to the MG1-iLOV vaccination series, 100% of control mice succumbed to leukemia, whereas 50% of mice that received MG1-iLOV survived (Fig. 2B), illustrating that iLOV is able to induce a protective effect even in the presence of early-disseminated leukemia. This incomplete protection is likely due to rapid growth of L1210 leukemia in this aggressive tumor model, which outstrips the development of anti-tumor responses.

To test whether the protective effects of iLOV were mediated through development of anti-tumor immunity, MG1-iLOV was administered to athymic nude or immunocompetent DBA/2 mice once weekly for 3 doses. Treated and untreated nude mice died from leukemia a median of 18 and 23 days respectively following injection of viable L1210 cells. In contrast, immunocompetent mice that received iLOV rejected L1210 cells (Fig. 3A). To further examine the immune requirements of the host, a BMT model was employed. After receiving myeloablative TBI, DBA/2 mice were administered...
10^6 rescue marrow cells from B6D2F1 donors. Following a 43-day recovery, absolute numbers of lymphocyte populations were compared between unimmunized BMT recipients versus healthy donors. While NK-cells, CD3+/4+ T-cells and B-cells were similar in number between the groups; CD3+/8+ T-cells were significantly reduced in the BMT recipients (Fig 3B). This depletion of cytotoxic T-lymphocytes (CTLs) was associated with a reduction in long-term protective immunity against a leukemic challenge by approximately 40% in a parallel cohort of iLOV-vaccinated BMT recipients (Fig 3B). The additional control cohorts of unimmunized BMT recipients and unimmunized B6D2F1 mice all reached typical endpoints by day 28. In a separate experiment, we wished to examine the effect of adoptive splenocyte transfer from long-term iLOV-protected mice to naïve recipients. Accordingly, 17 mice that received MG1-iLOV and survived between 211-349 days following leukemic challenge were used as splenocyte donors. Pooled donor splenocytes were administered to 8 naïve DBA/2 recipients followed 7 days later by injection of viable L1210 cells. Long-term survival was observed in 63% of recipients, while control mice that received the same number of splenocytes from untreated donors were unable to reject leukemic challenge (Fig. 3C). Collectively, these observations indicate an intact thymocyte compartment mediates the anti-leukemic protection afforded by iLOV and CTLs are critical for optimal effect.

To examine the strength of the immune response that develops following iLOV, cohorts of unimmunized and MG1-iLOV treated mice were challenged with increasing amounts of viable L1210 cells. The LD_{50} of unimmunized mice was approximately 4.9x10^4 cells while the LD_{50} for MG1-iLOV vaccinated mice was estimated to be 3.8x10^6 cells. Thus, iLOV was able to protect mice against an almost 100 fold larger inoculum of leukemia than would be spontaneously rejected by unimmunized mice (Fig. 3D). The durability of such a response is particularly critical as the ability to prevent leukemic recurrence may wane over time. iLOV-treated mice that survived a primary leukemic challenge were administered a second L1210 leukemia challenge either 100, 134, or 255 days after...
initial L1210 challenge. The majority of mice were able to reject this additional leukemic challenge, but there may be a time-dependent decline in the ability to reject a late secondary leukemic challenge (Fig. 3E).

The effectiveness of iLOV treatment was not limited to a single rhabdovirus, leukemic cell line or mouse strain. Survival following leukemic challenge was observed when animals were administered iLOV prepared using a different rhabdovirus – VSVd51, indicating that the protective effects were independent of the specific rhabdovirus (Supplemental Fig. S3). Similarly, mice survived an otherwise lethal challenge with EL4, a T lymphoma cell line, when MG1-iLOV prepared using these cells was used to vaccinate syngeneic C57BL/6 mice (Supplemental Fig. S4). To examine the specificity of the anti-tumor protection afforded by iLOV, two cohorts of B6D2F1 hybrid mice were administered 3 weekly doses of MG1-L1210 iLOV. One cohort was subsequently challenged with viable $1 \times 10^7$ L1210 cells while the other received $1 \times 10^7$ EL4 cells. Mice that received the L1210-based iLOV were protected from L1210 challenge, while survival of EL4 challenged mice was identical to unimmunized mice challenged with EL4 (Fig. 3F). The specificity of the immune response was further examined using reciprocal immunization combinations of EL4-iLOV or L1210-iLOV in cohorts of both DBA/2 and C57BL/6 mice. To control for immune recognition of leukemia cells based on the MHC disparity alone, additional cohorts of C57BL/6 and DBA/2 mice were immunized with $\gamma$-IR L1210 or $\gamma$-IR EL4 cells respectively. All groups were subsequently challenged with viable leukemia cells syngeneic to the breed. Only mice that received iLOV produced using syngeneic leukemia cells were protected (Supplemental Figure S5). Together, these observations suggest that iLOV induces anti-tumor immunity functionally restricted to the specific antigenic profile of the leukemic cell used to produce iLOV rather than commonly expressed leukemic antigens.
Virus infection is critical to induction of iLOV-mediated anti-leukemic immunity

We examined whether the cellular and viral components of iLOV could be individually effective at inducing protective anti-tumor immunity. Mice administered 3 doses of γ-IR ex-vivo MG1 infected L1210 cells (iLOV) survived subsequent administration of an otherwise lethal dose of L1210 cells, while all mice that received γ-IR uninfected L1210 cells prior to leukemic challenge succumbed with median survival that was not significantly different from unimmunized mice that received the same leukemic challenge dose. Furthermore, the virus must infect the cell for iLOV to be effective as 3 weekly separate co-injections of γ-IR L1210 cells and MG1, or the administration of 3 weekly doses of γ-IR L1210 cells mixed with MG1 at room temperature for 1 hour prior to injection, were unable to prevent the lethality of a subsequent L1210 leukemia challenge (Fig. 4A).

In-vitro, leukemic cells exposed to UV-inactivated MG1-eGFP virus did not express GFP nor developed cytopathology. In contrast, L1210 cells exhibit green fluorescence following in-vitro exposure to live spread-incompetent VSVd51ΔG-eGFP, and delayed cytolysis occurs as long as 72 hours after infection (Fig. 4B). Immunization with iLOV produced by infection of L1210 cells with VSVd51ΔG protected 80% of mice from the lethal effects of subsequent L1210 challenge (Fig. 4C), indicating that iLOV is effective even if the virus is incapable of fully completing its lifecycle. Administration of iLOV produced with UV-inactivated MG1 prior to challenge with viable L1210 leukemia was unable to prevent death from fulminant leukemia in 80% of mice. iLOV preparations fixed with PFA after virus infection but immediately prior to γ-IR were capable of protecting immunized mice just as effectively as freshly produced, unfixed iLOV preparations (Fig. 4D). PFA-fixed iLOV preparations did not contain detectable viable MG1 in a standard plaque assay. Thus in this model, live rhabdovirus was used for the manufacturing of iLOV but it was not critical at the time of administration.
MG1 infected leukemia cells exhibit superior immunogenicity

The protective effects induced by injection of ex-vivo virus infected leukemia cells cannot be mimicked solely by activation of anti-viral defense pathways in leukemia cells used for immunization. L1210 cells were cultured for 18 hours with either MG1, poly I:C, LPS, or both TLR agonists and inactivated virus, prior to γ-IR and injection. Activation of L1210 cells by virus or TLR agonists was confirmed by measuring cell-surface expression of the B-cell immunopotentiating activation molecules CD40 and CD252 by flow cytometry (Supplemental Figure S6). Mice that received 3 weekly injections of these TLR-agonist preparations succumbed to subsequent injection of L1210 cells, in contrast to mice that received MG1-iLOV (Fig. 5A). Similarly, pulsed stimulation of host innate immunity by direct injection of either poly I:C or LPS concurrently with 3 weekly injections of γ-IR L1210 cells was also incapable of protecting animals from subsequent injection of viable L1210 cells (Fig. 5B). The protective effects induced by injection of ex-vivo virus infected leukemia cells cannot be mimicked solely by the presence of apoptotic or necrotic cells that are contained in iLOV preparations (Fig. 5C). Apoptosis was induced in L1210 cells by UV irradiation (Supplemental Fig. S7A) while parallel samples of L1210 were pressure disrupted into cellular necrosis (Supplemental Fig. S7B). Cohorts of mice received 3 weekly injections of either MG1-iLOV, UV-irradiated apoptotic L1210, or pressure-disrupted necrotic L1210 followed by challenge of viable L1210 leukemia. Mice that received UV-irradiated or pressure disrupted L1210 expired due to leukemia in contrast to the mice that received MG1-iLOV. Administration of 3 weekly injections of apoptotic or necrotic L1210 cells mixed with MG1 virus just prior to injection, were similarly ineffective (Fig. 5D, E).

Pre-existing anti-viral immunity does not impair development of anti-leukemia immunity

We wondered whether pre-existing anti-viral immunity to the rhabdovirus component would modulate iLOV efficacy as anti-viral responses have impaired the efficacy of other vector-based
vaccines (30). Accordingly, mice were injected $10^7$ pfu MG1 by tail vein to generate anti-viral immunity. Prior to receiving MG1, mice did not manifest serum virus-neutralizing antibody while the titer of MG1 neutralizing antibody was $\geq 1:800$ in serum of mice 10 days following administration of virus. Three doses of MG1-iLOV were administered starting 18 days after MG1 injection. The survival of MG1 immunized mice was no different than a cohort of mice that received MG1-iLOV without preceding MG1 inoculation when challenged with $1 \times 10^6$ L1210 cells (Fig. 6A). However, when L1210 challenge was increased 10 fold, mice immunized against MG1 prior to MG1-iLOV treatment had a significant survival advantage over mice that received MG1-iLOV treatment alone (Fig. 6B). These results suggest iLOV efficacy is not dampened but indeed may be augmented following development of anti-viral immunity.

**Discussion**

We show that live attenuated rhabdoviruses are able to infect and kill leukemia cells in-vitro but are incapable of treating mice with established systemic leukemia. An alternative approach, injecting mice with $\gamma$-IR virus-infected leukemia cells, or iLOV, controls leukemic progression. This effect is mediated by development of a robust adaptive anti-tumor immunity, wherein CTLs are essential for optimal efficacy. The immune response is specifically directed against the cell used to produce iLOV. It is longstanding, protecting the animal from repeat leukemic challenge more than 8 months following immunization. Both cellular and viral components of the vaccine are necessary. In this model, infection of viable leukemia cells with a transcription-competent oncotropic rhabdovirus leads to the induction of protective immune responses; however virus spread is not essential. Furthermore, the immune effects are not simply a consequence of administering $\gamma$-IR leukemic cells responding to noxious stimuli or due to simultaneous injection of $\gamma$-IR leukemic cells during nonspecific or viral provocation of the
host’s innate immune system. In preclinical models, iLOV appears to be an effective immunotherapy for ALL.

It has been suggested that at the interface between infected tumor cells and immune system, viral pathogen associated molecular patterns (PAMPs) ligate various toll-like receptors (TLRs) in host dendritic cells (DCs), leading to NF-κB or IRF3 activation, DC maturation and licensing for expansion of tumor specific T-cells (31). However, the effectiveness of iLOV is not the result of presenting leukemia cells to an immune system stimulated by systemic administration of TLR agonists or viable oncotropic virus. TLRs have been shown to activate caspases leading to apoptotic cell death (32) and constituents of dead or dying cells are immunomodulatory (33). For example, phosphatidylserine externalized to the outer membrane during apoptosis activates both DCs and T-cells (34). Necrotic cell debris provokes inflammation and innate immune system activation through pattern recognition receptor ligation of danger associated molecular patterns (DAMPs) (35,36). In this model, the use of UV-inactivated MG1 virus with or without concurrent activation through TLR-ligation using poly I:C and LPS is insufficient to increase the cell’s recognition by the immune system. iLOV contains apoptotic and necrotic cells, however these alone or combined with live MG1 virus are insufficient to induce protective anti-leukemia immunity. These results suggest that additional pathways beyond PAMP or DAMP signaling may be activated and responsible for induction of an effective anti-leukemia immune response.

Vesiculovirus infections induce marked anti-viral immune responses. When used as priming or boosting agent, immunization with recombinant VSV expressing a tumor antigen prolonged survival and reduced tumor burden in cancer-bearing mice (37,38). Nonetheless, viruses encoding a single or few antigen targets induce an oligoclonal immune response. Further, the paucity of known TAAs along with their weak immunogenicity and variable expression on individual cancers (39), ultimately limits widespread applicability of this approach. Alternate approaches are being developed to create
personalized anti-cancer vaccines. Recently, the mutanome, the sum total of somatic mutations in a tumor, was shown to encode multiple tumor-specific immunogenic peptides (40). Vesiculovirus-expressed cDNA libraries, optimally derived from xenogeneic tumor cells, have been reported to induce anti-tumor immunity in animal models, although varying degrees of autoimmunity were observed (41). At the present time, substantial effort would be required to manufacture personalized vaccines based upon these methods.

Similar to the above methodology, our cell-based vaccine harnesses the entire library of immunologically recognized epitopes (33) in the leukemic mutatome without the need for involved recombinant DNA or “–omic” technology. The cell-based nature of iLOV may confer additional advantages, as the vaccine would contain the extensive array of aberrant post-translational modified proteins common in tumors, which could serve to broaden the epitope library iLOV presents to the immune system, resulting in a potentially more robust immune response (42–44).

While the effectiveness of iLOV requires a virus, the neoplasia-selective replication of the attenuated virus, limited viral replication in leukemia cells, and the development of neutralizing anti-viral antibodies following first injection contribute to the inherent safety of this therapeutic. The small risk of uncontrolled viral replication can be mitigated by using viral mutants incapable of spreading, treating the preparation with paraformaldehyde, or by pre-immunizing the patient against the virus, none of which diminish effectiveness of the anti-tumor response. In contrast to other viral-based anti-cancer therapies (30,45), pre-existing anti-viral immunity increases the potency of iLOV.

This platform represents a feasible technology to produce a safe and effective individualized immunotherapeutic for controlling disseminated leukemia. We envision generating autologous iLOV from leukemia cells collected and stored at the time of diagnosis or relapse. Future clinical testing will determine its role in the treatment of patients with ALL.
Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. MG1 virus infects human and murine leukemia cells in-vitro, however is ineffective at halting progression of established murine leukemia in-vivo. A, Fluorescence microscopy (10X) showing expression of viral GFP at 18 hours after infection of MG1-GFP (MOI 0.1) in human T-cell leukemia lines A.301 and Jurkat, human B-cell leukemia OCI-Ly18, murine T-cell lymphoblasts EL4, and murine B-cell lymphoblasts L1210. B, Flow cytometry dot plots of the same cell lines in A, pre and 18 hours post MG1-GFP infection indicating viral gene replication and protein expression by GFP. Cell viability decreases as indicated by increased PI staining. Dot plots are representative of vesiculovirus infections of cell lines performed on several occasions. C, L1210 cell-viability (PI) versus virus enumeration by viral plaque forming units (PFU) over time following infection with MG1 at low MOI (0.1). D, Survival of DBA/2 mice that received systemic MG1 virotherapy compared to untreated DBA/2 mice for the treatment of established L1210 leukemia (n=5 each group), P = 0.19.

Figure 2. MG1-iLOV eliminates otherwise lethal leukemic challenge. A, DBA/2 mice immunized with iLOV reliably (> 90%) achieve long-term protection from L1210 leukemic challenge, as compared to unimmunized mice. Combined survival from 8 independent experiments (n=5-15 per group per experiment), P < 0.0001. B, DBA/2 mice immunized with iLOV 24 hours after high dose L1210 administration maintain survival advantage compared to unimmunized mice, (n=10 both groups), P < 0.0001

Figure 3. iLOV induces potent and durable immune-mediated leukemia-specific protection. A, Survival following leukemic challenge for iLOV-immunized or unimmunized immunocompetent DBA/2 and immunodeficient athymic mice (n=10/group). P = 0.0002, iLOV-immunized DBA/2
versus iLOV-immunized athymic mice; \( P = 0.12 \), iLOV-immunized versus unimmunized athymic mice. B, 100-day survival following L1210 challenge (10^7 cells) for iLOV-immunized DBA/2 BMT recipients \((n=6)\) and immunized B6D2F1 donors \((n=5)\). Lymphocyte enumeration by flow cytometry in unimmunized parallel cohorts \((n=2/\text{group})\), performed in triplicate \(* P \leq 0.007\). C, Survival following L1210 challenge for naïve DBA/2 recipients \((n=8)\) of pooled splenocytes from iLOV-immunized DBA/2 donors surviving 211-349 days post L1210 challenge versus naïve recipients \((n=5)\) of pooled splenocytes from unimmunized DBA/2 donors \((P = 0.0001)\), and unimmunized \((n=10)\) without splenocyte transfer \((P = 0.0001)\). D, Long-term (125 days) survival for iLOV-immunized and unimmunized DBA/2 mice \((n=5 \text{ each group})\) challenged with increasing doses of L1210 cells. E, Survival following second L1210 leukemic challenge for iLOV-immunized mice surviving 100–134 days \((n=10)\) or 255 days \((n=7)\) after first leukemic challenge versus unimmunized mice \((n=10)\); \( P = 0.0002 \) (255 day interval) and \( P < 0.0001 \) (100-134 day interval) versus unimmunized. Trend observed \((P = 0.45)\) for time-dependent decrease in survival following late second challenge. F, Survival of L1210-iLOV immunized and unimmunized B6D2F1 mice \((n=5 \text{ each group})\) following L1210 or EL4 challenge \((10^7 \text{ cells})\). \( P = 0.0133 \), iLOV-immunized L1210 challenged versus iLOV-immunized EL4 challenged mice; \( P = 0.0018 \), iLOV-immunized versus unimmunized L1210 challenged mice; \( P = 0.84 \), iLOV-immunized versus unimmunized EL4 challenged mice.

**Figure 4. Virus infection is critical to the induction of iLOV-mediated anti-leukemic immunity.**

A, Survival following leukemic challenge for DBA/2 mice immunized with iLOV versus L1210 cells alone, L1210 cells admixed with live MG1 for 1 hour at room temperature prior to injection, or unimmunized \((n=5 \text{ each group})\), all \( P = 0.0018 \); or versus L1210 cells co-injected simultaneously with live MG1 using a separate syringe \((n=5)\), \( P = 0.0027 \). B, Flow cytometry dot plots of infected L1210 cells. Cells incubated with UV-inactivated MG1 virus do not express GFP and exhibit negligible PI
staining at 18 hours. In contrast, cells infected with attenuated VSVd51ΔG-eGFP virus express GFP at 18-72 hours following infection, with viability slowly decreasing over time. Oval region indicates viable L1210 lymphoblasts. C, Survival of DBA/2 mice following L1210 leukemic challenge. Four cohorts immunized as follows; iLOV prepared using MG1 (MG1-iLOV), VSVd51 (VSVd51-iLOV), VSVd51ΔG (VSVd51ΔG-iLOV) or UV-inactivated MG1 (UV-MG1-iLOV) (n=5 each group). The unimmunized and iLOV immunized cohorts shown here were performed simultaneously with experiment shown in Figure 3 F. P = 0.0133, MG1-iLOV or VSVd51-iLOV versus UV-inactivated MG1-iLOV; P = 0.32, MG1-iLOV or VSVd51-iLOV versus VSVd51ΔG-iLOV; P = 0.0228, VSVd51ΔG-iLOV versus unimmunized; P = 0.0019, MG1-iLOV or VSVd51-iLOV versus unimmunized. D, Survival following leukemic challenge in DBA/2 mice immunized with PFA-fixed iLOV versus freshly prepared unfixed iLOV, PFA-fixed L1210 cells, or unimmunized (n=5 each group). P = 0.6649 PFA-fixed iLOV vs. iLOV; P = 0.0023 PFA-fixed iLOV vs. PFA-fixed L1210; P = 0.0017 PFA-fixed iLOV vs. unimmunized.

Figure 5. Host or leukemia cell stimulation with TLR agonists, or injection of necrotic or apoptotic leukemia cells are insufficient to produce effective anti-leukemia immunity. A, Survival following leukemic challenge of DBA/2 mice immunized with MG1-iLOV versus immunization with L1210 cells previously cultured for 18 hours in TLR agonists (LPS or poly I:C) or L1210 cells incubated with both TLR agonists and UV-inactivated MG1 (MOI 10) (n=5 each group). P ≤ 0.002, iLOV versus other groups. B, Survival following leukemic challenge of DBA/2 mice immunized with MG1-iLOV versus immunization with L1210 cells injected with poly I:C or LPS (n=5 each group). P = 0.0034, iLOV versus unimmunized; P = 0.0018, iLOV versus poly I:C; P = 0.0128, iLOV versus LPS. C, Flow cytometry dot plots of Annexin V (apoptotic cells) versus viability dye 7-AAD (necrotic cells). MG1-iLOV preparations include substantial proportions of apoptotic and necrotic cells at 18 hours –
shown is a representative analysis. D, E, Survival following leukemic challenge of DBA/2 mice immunized with iLOV versus immunization with apoptotic or necrotic L1210 cells respectively. MG1 was either admixed (apoptotic L1210 + MG1, necrotic L1210 + MG1) or omitted (apoptotic L1210, necrotic L1210) with cells 1 hour prior to injection. (n=5 each group). The unimmunized and iLOV-immunized cohorts shown were performed simultaneously for this experiment and the experiment shown in Figure 4 A. *P* = 0.0133, iLOV versus apoptotic cells + MG1; *P* = 0.002, iLOV versus apoptotic cells; *P* = 0.0018, iLOV versus necrotic cells +/- MG1 and unimmunized.

**Figure 6. Pre-existing anti-viral immunity does not impair the development of anti-leukemia immunity.** A, Survival of iLOV-immunized DBA/2 mice, challenged with 1x10⁶ L1210 cells, previously immunized against MG1 acquiring neutralizing antibodies (MG1/iLOV) versus mice that were not pre-immunized against MG1 (-/iLOV) and unimmunized (-/-) (n=5 each group), *P* = 0.0019. B, Survival of iLOV-immunized DBA/2 mice, challenged with 1x10⁷ L1210 cells, previously immunized against MG1 acquiring neutralizing antibodies (MG1/iLOV) versus mice that were not pre-immunized against MG1 (-/iLOV) and unimmunized (-/-) (n=5 each group). *P* = 0.0486 (MG1/iLOV) vs. (-/iLOV); *P* = 0.0019 (MG1/iLOV) vs. (-/-).
Figure 2

A

B

Days after L1210 challenge

Days after L1210 challenge

Survival (%)

Survival (%)

- iLOV, n=60

- Unimmunized, n=55

- iLOV

- Unimmunized
Figure 5

A. Survival (%) of different treatment groups over days after L1210 challenge.

B. Survival (%) of different treatment groups over days after L1210 challenge.

C. Flow cytometry analysis of non-infected cells and cells incubated with MG1 oncolytic virus (MOI 10) for 18 hours, showing survival rates.

D. Survival (%) of different treatment groups over days after L1210 challenge.

E. Survival (%) of different treatment groups over days after L1210 challenge.
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