Molecular Therapy
Oncolytics
Original Article

Efficacy of coxsackievirus A21 against drug-resistant neoplastic B cells

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Primary drug resistance and minimal residual disease are major challenges in the treatment of B cell neoplasms. Therefore, this study aimed to identify a novel treatment capable of eradicating malignant B cells and drug-resistant disease. Oncolytic viruses eradicate malignant cells by direct oncolysis and activation of anti-tumor immunity, have proven anti-cancer efficacy, and are safe and well tolerated in clinical use. Here, we demonstrate that the oncolytic virus coxsackievirus A21 can kill a range of B cell neoplasms, irrespective of an anti-viral interferon response. Moreover, CVA21 retained its capacity to kill drug-resistant B cell neoplasms, where drug resistance was induced by co-culture with tumor microenvironment support. In some cases, CVA21 efficacy was actually enhanced, in accordance with increased expression of the viral entry receptor ICAM-1. Importantly, the data confirmed preferential killing of malignant B cells and CVA21 dependence on oncogenic B cell signaling pathways. Significantly, CVA21 also activated natural killer (NK) cells to kill neoplastic B cells and drug-resistant B cells remained susceptible to NK cell-mediated lysis. Overall, these data reveal a dual mode of action of CVA21 against drug-resistant B cells and support the development of CVA21 for the treatment of B cell neoplasms.

INTRODUCTION

B cell malignancies comprise a diverse group of diseases resulting from malignant transformation of B lymphocytes at different stages of differentiation.[1] However, while there is an array of novel therapeutic agents showing promising results in clinical trials,[2] reciprocal cell-to-cell interactions that occur within the tumor microenvironment (TME) can induce a drug-resistant phenotype.[3–6] Primary drug resistance is responsible for the maintenance of low-level residual disease, which is often below the sensitivity of detection by routine clinically employed methods including cross-sectional imaging or flow cytometry; this minimal residual disease (MRD) is a major driver of disease relapse.[7,8] As such, identification of novel treatment strategies that are effective against drug-resistant B cells remains a high priority.[8]

Oncolytic viruses (OVs) offer an attractive addition to current clinical practice as they utilize a range of killing mechanisms to eradicate disease, including (1) oncolysis, through tumor cell infection and viral replication, and (2) development of both innate and adaptive anti-tumor immunity.[9–11] Furthermore, OVs display distinct cellular tropisms and have the capacity to highjack oncogenic signaling pathways to support viral replication.[12,13] To date, OVs have been safe and well tolerated in clinical trials, even in immunocompromised patients,[14–16] and they have induced durable clinical responses in both solid and hematological malignancies.[15,17,18] An OV capable of eradicating drug-resistant disease could revolutionize current treatment practices.

Resistance to drug-induced apoptosis can occur via up-regulation of anti-apoptotic proteins (e.g., Bcl-2, Mcl-1, and IAPs).[19] Therefore, OVs that act independently of apoptosis have the potential to target drug-resistant cells. Previous literature has reported that several OVs, including reovirus,[20] herpes simplex virus type 1 (HSV-1),[21,22] and vesicular stomatitis virus (VSV)[23] depend on apoptotic signaling for direct oncolysis; however, the cellular mechanisms required for coxsackievirus A21 (CVA21)-induced cell death remain undefined. CVA21 is a member of the picornavirus family of viruses and requires intracellular adhesion molecule 1 (ICAM-1) for viral entry and decay-accelerating factor (DAF) for virus sequestration.[24] Clinically, CVA21 has been safe and well tolerated (with no grade 3 or 4 adverse events) and has demonstrated promising results (increased progression-free survival) in advanced melanoma.[25,26] Furthermore, as less than 10% of the population has been exposed to CVA21,[27] pre-existing neutralizing antibodies (NAbs) would not present a barrier for initial CVA21 delivery. However, the consequence of anti-viral immunity remains controversial, and clinical responses have been reported in the presence of NAbs.[28]

Received 19 October 2022; accepted 21 March 2023;
https://doi.org/10.1016/j.omto.2023.03.002.

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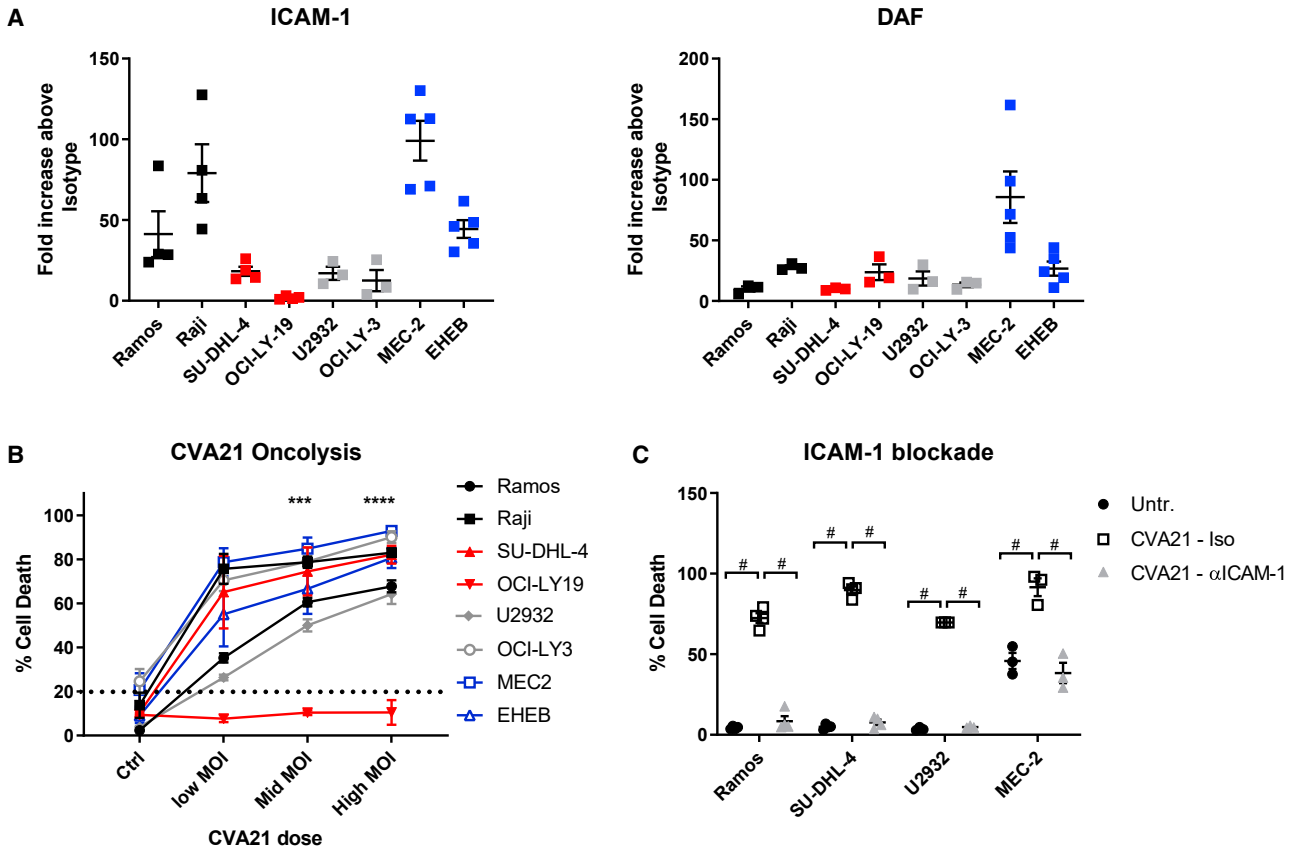


Figure 1. CVA21 induces direct oncolysis in a range of B cell malignancies

(A) ICAM-1 and DAF expression in a range of neoplastic B cell lines (black: BL; red: DLBCL-GCB; gray: DLBCL-ABC; blue: CLL) was determined by flow cytometry. The fold increase in mean fluorescence intensity (MFI) above the isotype control is shown (± standard error of the mean [SEM]) for a minimum of n = 3 independent experiments. (B) CVA21-induced cytotoxicity was examined by LIVE/DEAD after 48 h. Ctrl, untreated; low MOI, 0.01 PFU/cell for NHL cells and 0.005 PFU/cell for CLL cells; mid MOI, 0.1 PFU/cell (NHL) and 0.05 PFU/cell (CLL); and high MOI, 1 PFU/cell (NHL) and 0.5 PFU/cell (CLL). Statistical comparisons confirmed significant cell killing over Ctrl for all cell lines except OCI-LY19 at the mid-high MOI (mean of at least n = 3 ± SEM). (C) B cell lines were treated with 1 PFU/cell CVA21 alone or in the presence or absence (±) of either an isotype control or an anti-ICAM-1 antibody. After 48 h, CVA21-induced cytotoxicity was determined using LIVE/DEAD (n = 3, ±SEM). *** = $p<0.001$, #/**** = $p<0.0001$.

To date, the efficacy of CVA21 against B cell neoplasms, with the exception of multiple myeloma (MM),[10] has not been reported, though multiple factors suggest that CVA21 could be efficacious against malignant B cells. For example, (1) neoplastic B cells express the CVA21 entry receptor ICAM-1 (CD54)[29,30]; (ii) CD40/CD40L-interactions, reflective of B cell:T cell interactions within the TME, can enhance ICAM-1 expression[31]; (3) alternative coxsackievirus strains act independently of apoptosis, and therefore CVA21 oncolysis may persist despite resistance to apoptosis[32,33]; and (iv) coxsackievirus replication can be dependent on nuclear factor κB (NF-κB) and/or PI3K/AKT/mTOR signaling,[34,35] pathways that are commonly up-regulated in B cell neoplasms.[36,37]

This study aimed to investigate the efficacy of CVA21 against a range of B cell neoplasms, including Burkitt lymphoma (BL), chronic lymphocytic leukemia (CLL), and diffuse large B cell lymphoma (DLBCL); both germinal center B cell (GCB) and activated B cell (ABC) subtypes. This is the first study to investigate the efficacy of CVA21 against a diverse range of B cell malignancies and to investigate the efficacy of CVA21 against drug-resistant disease.

RESULTS

Initial studies aimed to test the direct cytotoxic capacity of CVA21 against a range of B cell neoplasms. Firstly, cell surface expression of ICAM-1 and DAF was examined by flow cytometry, confirming the expression of both receptors on all malignant B cell lines tested except OCI-LY-19, which did not express ICAM-1 (Figure 1A). Next, the cytotoxic effect of CVA21 was determined; all cell lines were highly susceptible to CVA21-induced cell death except OCI-LY-19 cells, as expected, as these lacked the CVA21 entry receptor ICAM-1 (Figure 1B). Following this initial screening, one representative cell line for each disease subtype was selected for further experimentation: Ramos (BL), SU-DHL-4 (GCB-DLBCL), U2932 (ABC-DLBCL), and MEC-2 (CLL). Using these selected cell lines, the importance of ICAM-1 for CVA21 cytotoxicity was examined; blockade of ICAM-1 significantly abrogated CVA21-induced cell death, demonstrating a dependence on

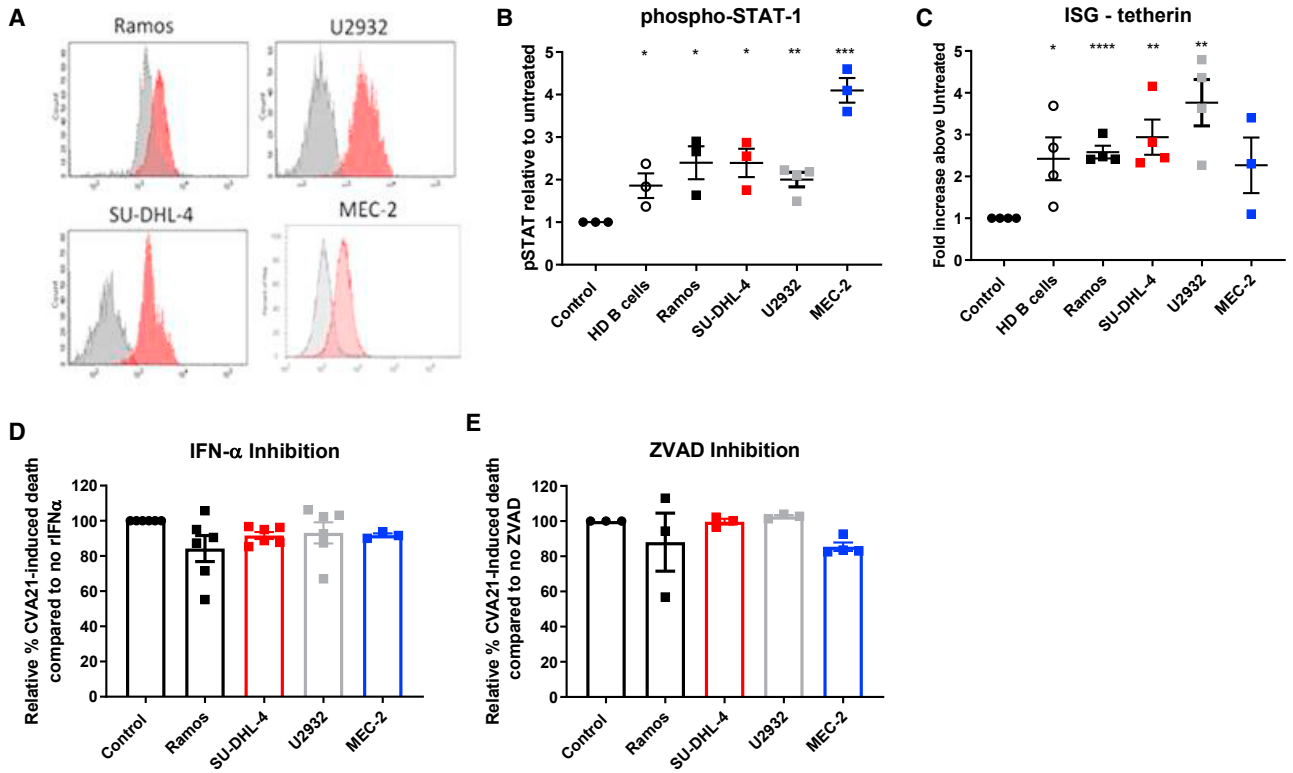


Figure 2. B cell neoplasms have a functional type I IFN response

(A) Expression of IFNAR on B cell lines; a representative histogram plot overlaying IFNAR (red) and isotype control (gray) (representative of n = 2). (B) Neoplastic B cell lines and HD B cell controls were treated with 5,000 pg/mL rIFN-α for 30 min, and phosphorylation of STAT-1 was determined. Results are normalized to treatment-matched isotype controls and show the relative phosphorylation of STAT1 compared with untreated samples (±SEM). (C) Cells were treated with 5,000 pg/mL rIFN-α for 72 h, and surface expression of tetherin was examined. Results are normalized to treatment-matched isotype controls and expressed relative to untreated samples (±SEM). Statistical significance compared with untreated controls is shown. (D) Cells were treated with CVA21 for 48 h, ±5,000 pg/mL rIFN-α, and CVA21-induced cell death was determined by LIVE/DEAD. Relative cell death compared with CVA21 treatment alone is shown for at least n = 3, ±SEM. (E) Cells were treated with CVA21 for 48 h, ±50 μM ZVAD, and CVA21-induced cell death was determined by LIVE/DEAD. Relative cell death compared with CVA21 treatment alone is shown for at least n = 3, ±SEM. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

ICAM-1 cell surface expression (Figure 1C).[24] CVA21-induced cell death also required viral replication as (1) viral replication was confirmed in malignant B cells (Figure S1A); (2) UV inactivation of CVA21 inhibited cell death (Figure S1B); (3) CVA21-induced cell death was inhibited by 2-methylycytodine—an inhibitor of protein translation and viral replication (Figure S1C); and (4) significant cell death occurred at an extremely low multiplicity of infection (MOI; 0.005 or 0.01 plaque-forming unit [PFU]/cell; Figure 1C). Collectively, these data demonstrate the potential of CVA21 for the treatment of B cell neoplasms, provided ICAM-1 expression is maintained.

While the ability of CVA21 to induce direct oncolysis was promising, *in vivo* administration of OV stimulates an anti-viral immune response mediated by type I interferon (IFN), in particular IFN-α.[10,38] Moreover, we have previously shown that IFN-α can inhibit CVA21 direct oncolysis in acute myeloid leukemia (AML).[10] Encouragingly, neoplastic B cells did not secrete IFN-α in response to CVA21 treatment (data not shown); however, it was possible that IFN-α secretion by peripheral blood mononuclear cells (PBMCs) could limit CVA21 replication. To test this hypothesis, we first examined the expression of the IFN-α receptor (IFNAR) on malignant B cell lines and confirmed its expression (Figure 2A). Functional IFN signaling was also detected in response to recombinant (r) IFN-α through increased STAT-1 phosphorylation (Figure 2B) and the induction of tetherin, an IFN-stimulated gene (Figure 2C). However, despite the IFN-α responsiveness of malignant B cell lines, the addition of rIFN-α did not significantly abrogate CVA21-induced cell death (Figure 2D), demonstrating the ability of CVA21 to kill neoplastic B cells despite the induction of an anti-viral type I IFN-α response. Importantly, we also confirmed that CVA21-induced cell death occurred independently of apoptotic mechanisms, as ZVAD (a PAN-caspase inhibitor, which can inhibit reovirus-induced apoptosis)[20] did not abrogate CVA21-induced cell death (Figure 2E); ZVAD function was confirmed using reovirus-treated Mel-888 cells as a positive control[20] (Figure S2). These findings highlight the potential of CVA21 to eradicate malignant B cells in the context of a host anti-viral immune response. Moreover, they also suggest that CVA21 could be effective against drug-resistant cells that have developed strategies to evade caspase-mediated apoptosis. Consistent with this hypothesis,

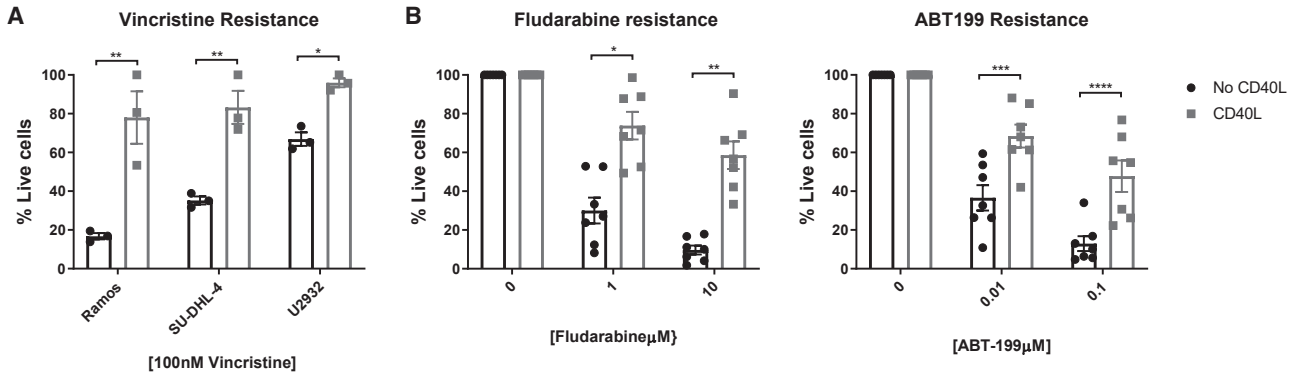


Figure 3. CD40L-stimulated B cell neoplasms are resistant to chemotherapeutic drugs

(A) NHL B cell lines were either cultured alone or co-cultured with CD40L-L929 cells for 24 h prior to treatment with 100 nM vincristine for 48 h; cell death was determined using LIVE/DEAD. Data show mean cell death following subtraction of untreated control values (n = 3, +SEM). (B) Primary CLL samples were either cultured alone or co-cultured with CD40L-L929 cells for 48 h and treated with fludarabine (n = 8, +SEM) or ABT-199 (n = 7, + SEM) at indicated doses for 7 days; cell death was determined using LIVE/DEAD. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

MEC-2 CLL cells, which are inherently resistant to both fludarabine (a chemotherapy drug commonly used in the treatment of CLL) and ABT-199 (a BH3 mimetic that targets the anti-apoptotic protein BCL-2; Figure S3) were highly susceptible to CVA21 oncolysis (Figure 1C).

To further explore the potential of CVA21 to eradicate drug-resistant B cells, we developed a human *in vitro* model system to induce a drug-resistant phenotype. Specifically, neoplastic non-Hodgkin's lymphoma (NHL) cell lines (Ramos, SU-DHL-4, and U2932) and primary CLL cells, which were usually sensitive to the cytotoxic effects of chemotherapy (vincristine and fludarabine, respectively), were co-cultured with CD40L-L929 cells to recapitulate B cell (CD40):T cell (CD40L) interactions within the lymph node (LN).[3,39] Importantly, CD40 ligation of NHL cell lines induced resistance to vincristine (Figure 3A), and primary CLL cells became resistant to fludarabine and ABT-199 (Figure 3B). Co-culture with CD40L-L929 cells was associated with increased expression of the anti-apoptotic protein Bcl-xL (Figure S4), suggesting that modulation of pro- vs. anti-apoptotic machinery may be responsible for the drug-resistant phenotype observed, in accordance with previously published work.[4,40]

Critically, despite B cell lines and primary CLL cells displaying resistance to standard chemotherapeutic agents and the BH3 mimetic ABT-199, CD40L-stimulated B cells remained susceptible to CVA21 direct oncolysis (Figures 4A and 4B). Moreover, CVA21-induced cell death was enhanced upon co-culture with CD40L-L929 cells in Ramos and U2932 cell lines (Figure 4A) and primary CLL cells (Figure 4B) in association with increased ICAM-1 expression (Figures 4C and 4D, respectively). Encouragingly, B cell lines co-cultured with bone marrow stromal cells (BMSCs), to mimic a protective bone marrow niche, also remained susceptible to CVA21-induced oncolysis (Figure S5). Upon assessment of primary NHL cells isolated from LN biopsies at diagnosis, we observed elevated ICAM-1 expression compared with healthy donor (HD) B cells (Figure 4E), which could reflect CD40L stimulation *in vivo*; moreover, primary NHL cells were also susceptible to CVA21-induced cell death (Figure 4F). Notably, not all OVs have the capacity to kill drug-resistant B cells. For example, similar studies carried out using an alternative OV (reovirus) demonstrated that primary CLL cells became more resistant to reovirus-induced cell death following co-culture with CD40L-L929 cells (Figure S6).

Next, to aid patient stratification and the development of novel combination therapies, we sought to determine whether signaling pathways associated with aberrant B cell receptor (BCR) signaling were utilized by CVA21 to promote oncolysis. Using ibrutinib, a Bruton's tyrosine kinase (BTK) inhibitor that acts downstream of the BCR, we demonstrated that CVA21-induced cell death was partially dependent on BTK in all cell lines, except Ramos (Figures 5 and S7). Moreover, the MEK inhibitor U0126 identified a role for RAS/MEK/ERK signaling in all cell lines, and abrogation of mTORC1 signaling (using rapamycin) suggested that mTOR was also important for CVA21 cytotoxicity in 3 of the 4 cell lines (not U2932 cells) (Figures 5 and S7). Collectively, these data suggest that CVA21 cytotoxicity is partially dependent on oncogenic B cell signaling and highlight the need for careful consideration when designing novel combination approaches. However, while combination with agents that modulate downstream BCR signaling would not be advised, our work has confirmed that CVA21 cytotoxicity was not abrogated when used in combination with fludarabine or ABT-199. Indeed, additive killing was observed in CD40L-L929-stimulated (drug-resistant) primary CLL cells when CVA21 was used in combination with fludarabine or ABT-199 (Figure S8). Importantly, these supplementary data demonstrate that CVA21 can kill CLL cells that remain viable in the presence of drug alone, further supporting its ability to eradicate "drug-resistant" cells.

In view of the highly susceptible nature of malignant B cells and their dependence on ABC signaling, we next examined potential

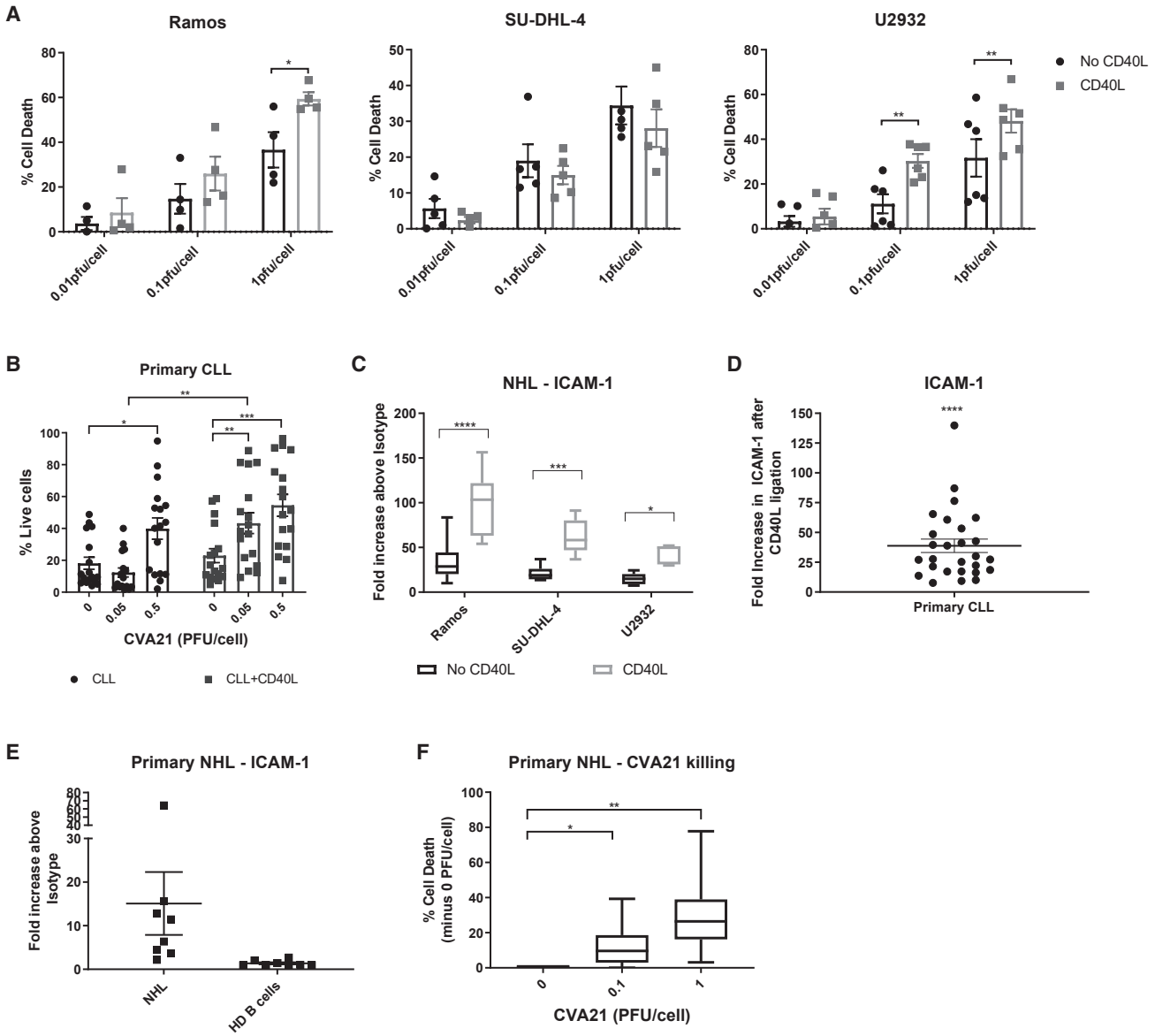


Figure 4. Drug-resistant B cells remain susceptible to CVA21-induced cell death

(A) B cell lines were cultured alone or co-cultured with CD40L-L929 cells for 24 h prior to treatment with CVA21 for a further 24 h; cell death was measured using LIVE/DEAD. Spontaneous cell death observed in untreated controls was subtracted to standardize the data (n = 3, +SEM). (B) Primary CLL cells were cultured alone or co-cultured with CD40L-L929 cells for 48 h and treated with CVA21 for a further 5 days; cell death was measured using LIVE/DEAD (n = 17 samples, ±SEM). (C and D) ICAM-1 expression on NHL cell lines (C) and primary CLL cells (D) when cultured alone or co-cultured with CD40L-L929 cells. (E) PBMCs were isolated from NHL LN samples at diagnosis, and ICAM-1 expression on primary NHL B cells and HD B cells was determined by flow cytometry. (F) PBMCs were isolated from NHL LN samples and treated with CVA21 for 5 days. B cells were identified using $CD19^+$ or $CD20^+$, and CVA21-induced cell death was determined using LIVE/DEAD. The percentage of dead cells in untreated samples was subtracted (n = 10 samples, ±SEM). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

"off-target" cytotoxic effects of CVA21 against non-neoplastic B cells. Previously, we reported that CVA21 does not kill natural killer (NK) cells, $CD4^+$ T cells, $CD8^+$ T cells, or monocytes isolated from HD PBMCs; however, the susceptibility of HD B cells was not reported.[10] Moreover, as CD40 ligation can up-regulate ICAM-1 expression, it was important to examine CVA21-induced cytotoxicity in CD40L-stimulated HD B cells. Indeed, while CD40 ligation did up-regulate ICAM-1 expression on HD B cells (Figure 6A), no significant increase in CVA21-induced cell death was observed (Figure 6B). These data validate the reported safety profile of CVA21 in the clinic[25,41] and support the requirement for aberrant cellular signaling pathways, such as those explored in Figure 5.

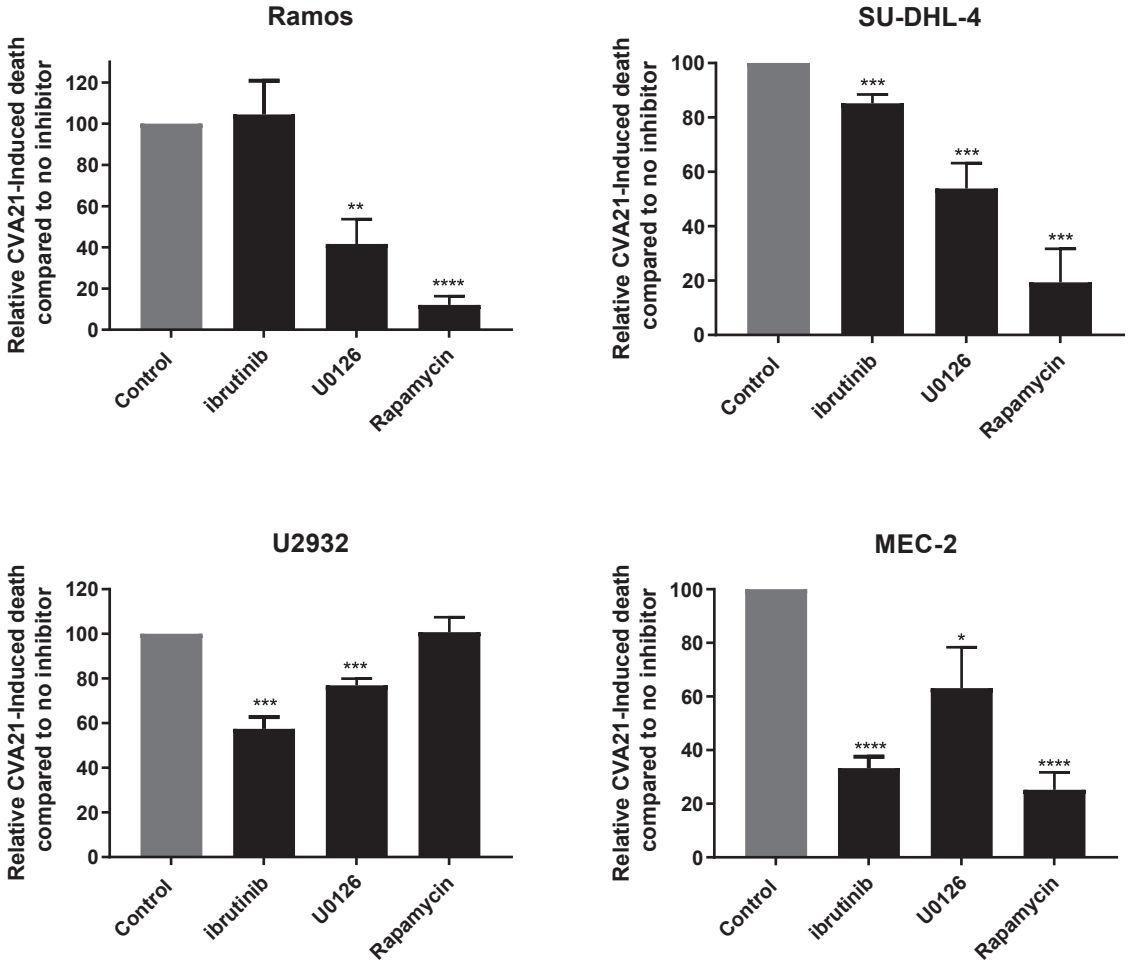


Figure 5. CVA21-induced cell death is dependent on oncogenic B cell signaling pathways

B cell lines were treated with 0.05PFU/cell CVA21 ± sub-lethal doses of ibrutinib, U0126 or rapamycin. For ibrutinib, cells were treated with 1μM (Ramos and SU-DHL-4) and 10μM (U2932 and MEC-2) for 24 (MEC-2) or 48hrs (Ramos, SU-DHL-4 and U2932), to reflect susceptibility to CVA21 infection. For U0126, cells were treated with 1 μM (Ramos) and 10 μM (SU-DHL-4, U2932, and MEC-2) for 24hrs (MEC-2, SU-DHL-4 and U2932) or 48hrs (Ramos). Cells were treated with 50nM rapamycin for 24hrs (MEC-2, SU-DHL-4 and U2932) or 48hrs (Ramos). After treatment, cell death was quantified using LIVE/DEAD® and cell death in the absence of CVA21 was subtracted; relative CVA21-induced cell death compared to no drug is shown. All data shows the mean +SEM for at least n=3 independent experiments (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

Having recently reported that CVA21 can exert its anti-cancer effects by both direct oncolysis and activation of innate anti-tumor immunity,[10] the ability of CVA21-activated NK cells to recognize and kill malignant B cells, including those with a drug-resistant phenotype, was examined. Figure 7 demonstrates that treatment of PBMCs with CVA21 can (1) increase NK cell degranulation against malignant B cells (Figure 7A) and (2) promote NK cell-mediated killing (Figure 7B). Next, to examine the effectiveness of CVA21-activated NK cells against drug-resistant cancer cells (i.e., those co-cultured with CD40L-L929 cells), we used an autologous *in vitro* system consisting of primary CLL cells and non-malignant PBMCs, which were obtained from patients with CLL with a low disease burden. PBMCs isolated from low-count CLL samples were separated and either cultured alone or co-cultured with CD40L-L929 cells to model drug-sensitive and drug-resistant CLL, respectively. In parallel, additional PBMCs were either left untreated or were treated with CVA21 to activate immune cell components, including patient NK cells. PBMCs (±CVA21 treatment) were then co-cultured with autologous CLL cells (±CD40L-L929 stimulation, e.g., conditions that cause resistance to fludarabine/ABT199 [Figure 3B] and/or reovirus [Figure S6]), and CLL cell death was determined. Critically, we observed that CVA21-activated NK cells could kill autologous CLL cells (Figure 8A) and that NK cell-mediated killing was not abrogated upon co-culture with CD40L-L929 cells (Figures 8A and 8B). Interestingly, levels of NK cell killing after CVA21 treatment were similar to those observed with GA101, a next-generation CD20 antibody developed for enhanced NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC; Figure S9) in all cases except for MEC-2 cell targets, which express low levels of NK cell activatory ligands and

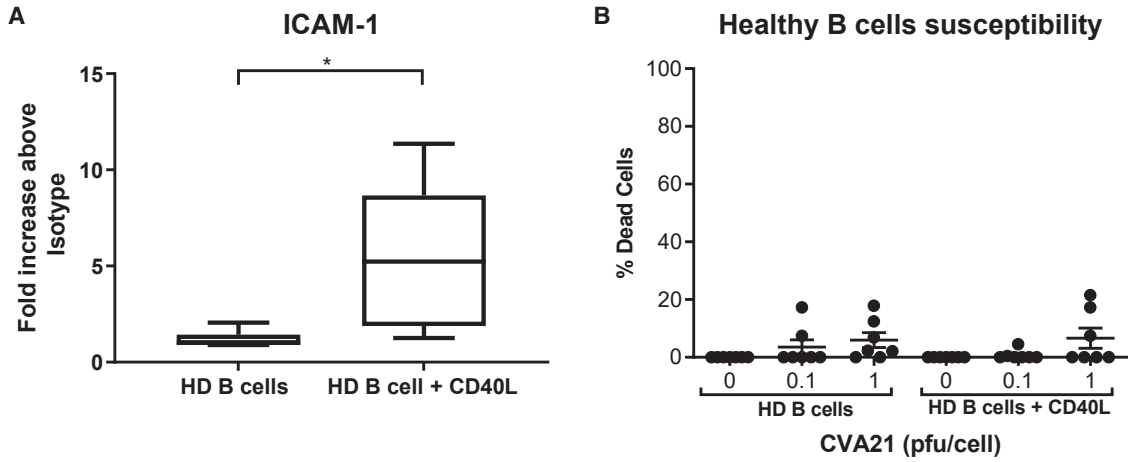


Figure 6. Healthy B cells are resistant to CVA21 oncolysis

(A) ICAM-1 expression on $CD19^+$ or $CD20^+$ HD B cells cultured alone or co-cultured with CD40L-L929 cells was determined by flow cytometry. Results are expressed as the fold increase in MFI over the isotype control (n = 7, ±SEM). (B) HD B cells (±CD40L-L929) were treated with CVA21 for 5 days, and death of $CD19^+$ or $CD20^+$ B cells was quantified using LIVE/DEAD (n = 7, ±SEM). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

are poor NK cell targets.[42] These data demonstrate the ability of CVA21-activated NK cells to kill malignant B cells and support a role for CVA21-activated NK cells in eradicating drug-resistant disease. Therefore, this work supports the development of CVA21 for the treatment of B cell neoplasms, including targeting of drug-resistant disease.

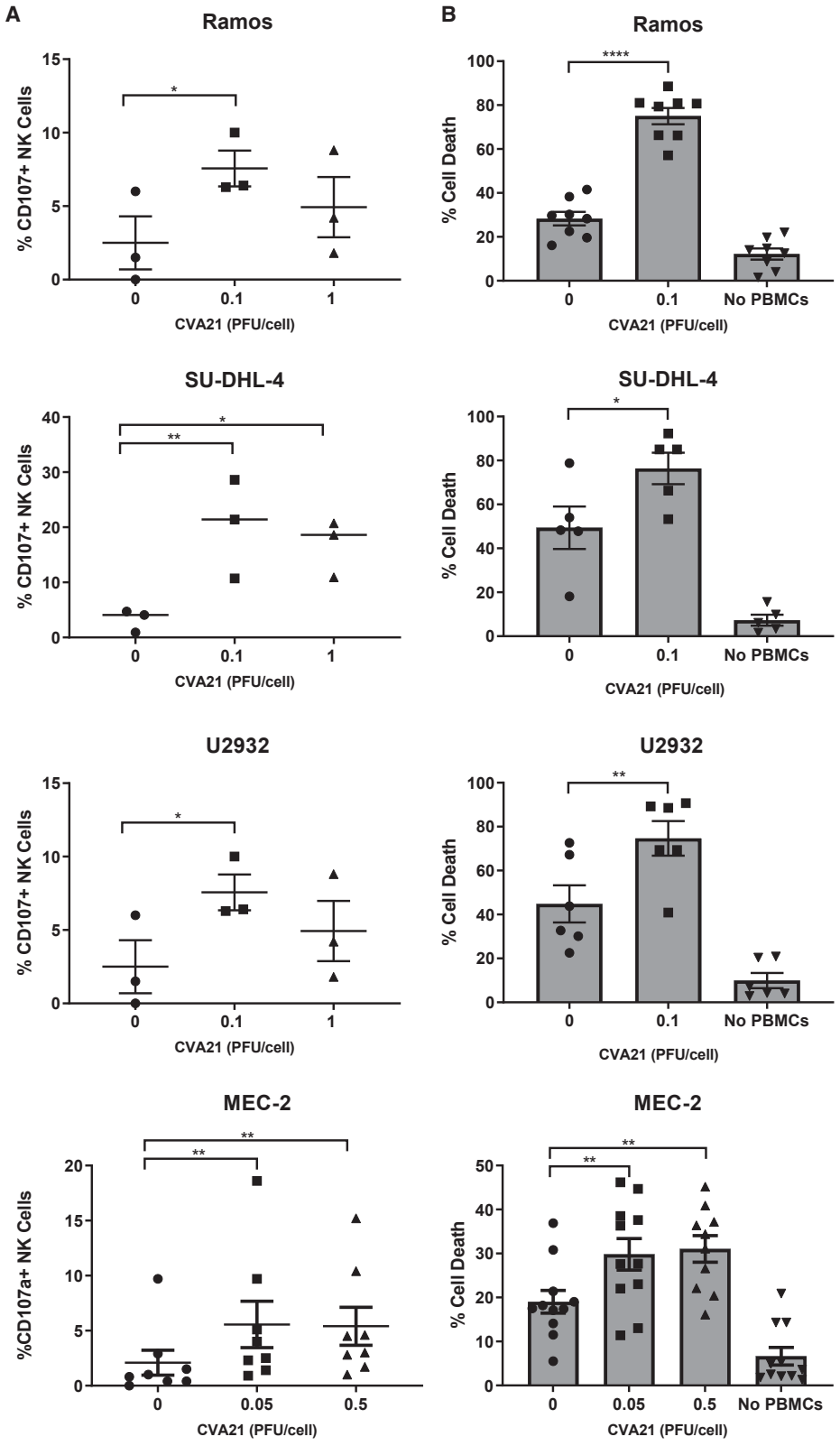
DISCUSSION

Modern therapies for B cell malignancies are often effective at inducing deep clinical remissions, where the disease is no longer detectable by standard radiological or pathological techniques.[43] Unfortunately, a proportion of patients have undetectable disease, which may give rise to clinical relapse; as such, eradication of MRD is the key to curative therapy.[8] Safe and well-tolerated novel treatments to support current therapies and eradicate drug-resistant cells are therefore highly attractive.

Here, we show that CVA21 can kill a range of B cell neoplasms and that oncolysis occurs despite the presence of an anti-viral type I immune response and functional IFN signaling. These results are distinct from those published by Müller et al., who demonstrated that CVA21 oncolysis was abrogated by type I IFN-α in a susceptible AML cell line, KG-1.[10] The susceptibility of B cells, in the face of an anti-viral immune response, could reflect high levels of ICAM-1 on neoplastic B cells and delivery of a greater viral load; the requirement of a threshold level of ICAM-1 has been previously reported.[10,44] Indeed, our work with the MEC-2 CLL cell line also revealed a threshold level of ICAM-1 where a median fluorescence intensity value of ∼5 was required for successful CVA21 infection and oncolysis (Figure S10). Low-level ICAM-1 expression could account for the CVA21 resistance observed in HD B cells; however, an absence of oncogenic signaling[2] is also likely to be important given the established role for MEK and mTOR signaling pathways in CVA21 cytotoxicity. A requirement for BTK signaling was also observed for CLL and ABC-DLBCL, but less so for GCB-DLBCL, which is in accordance with an absence of chronic BCR signaling, and reduced ibrutinib efficacy, in GCB-DLBCL.[2,45] Interestingly, ibrutinib did not abrogate CVA21 cytotoxicity in the BL cell line Ramos despite its ability to inhibit BTK phosphorylation in Ramos cells and its reported pre-clinical efficacy against BL.[46] These results suggest a greater dependence of CVA21 on alternative pathways, such as PI3K/AKT/mTOR, which is constitutively activated in BL.[47] Notably, upon assessment of the importance of BTK, MEK, and mTOR signaling pathways using an alternative cancer model, specifically MEL-624 melanoma cells, these pathways were not critical for CVA21-induced oncolysis. For example, CVA21 cytotoxicity was not affected by BTK inhibition as expected. Moreover, distinct to what we have observed in B cell malignancies, inhibition of mTOR and MEK with rapamycin or U0126 actually enhanced CVA21 cytotoxicity in MEL-624 melanoma cells (Figure S11). Potentiation of coxsackievirus (CV)-induced cell death by rapamycin has been observed using an alternative CV strain, CVB3, in HeLa cells.[48] These results highlight the complex interplay between tumor cells, oncogenic signaling, and OVs as well as the need for careful, disease-dependent consideration of potential combination therapies.

Critically, the data presented demonstrated that CVA21 can kill drug-resistant B cells, while resistance to an alternative OV, reovirus, was observed. Moreover, increased ICAM-1 expression, when cells were cultured in a drug-resistant niche, resulted in enhanced CVA21-mediated oncolysis. Reciprocal tumor:stroma (e.g., cancer-associated fibroblasts [CAFs]) interactions have previously been reported to potentiate OV replication in pancreatic cancer cells through mechanisms that were distinct from viral entry receptor expression, including down-regulation of endogenous anti-viral responses.[49]

Throughout this study, we chose to use CD40 ligation to recapitulate survival/proliferation signals received within the LN. This model



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system was selected based on the importance of B cell (CD40):T cell (CD40L) interactions and the reported ability of CD40 ligation to induce a drug-resistant phenotype in NHL cancer models.[3–6,39,50] However, it is well recognized that other LN-derived signals can support neoplastic B cell survival, such as stromal cell stimulation of Hedgehog signaling, chemokine/cytokine interactions, and engagement with immune cell populations (macrophages and neutrophils).[51] To fully recapitulate the TME and the diverse range of cell-cell interactions, *in vivo* models are commonly exploited. CVA21 is strictly dependent on human ICAM-1 for viral entry and immune activation; therefore, *in vivo* assessment of direct oncolysis and NK cell-mediated killing in murine models remains problematic. Humanized mouse models may be applicable but are extremely costly, and technological advances have only recently enabled the development of secondary lymphoid tissue.[52] However, primary samples obtained from LN biopsies and patients with CLL contain a diverse milieu of cells, both malignant and immune, that could provide survival/proliferation signals. Indeed, both NK and T cell populations were identified in primary LN samples (data not shown), and primary CLL cells receive survival signals from monocytes present within isolated PBMC.[42]

We have previously shown that reovirus can enhance NK cell-mediated ADCC[42]; thus, combination of CVA21 with the anti-CD20 antibody obinotuzumab (GA101) was explored. Unfortunately, despite increased NK cell degranulation when CVA21 was used in combination with GA101, a significant increase in NK cell-mediated lysis was only observed for Ramos cells (data not shown). However, as CVA21 was able to activate NK cell anti-tumor immunity to similar levels as GA101, CVA21 could be considered as an alternative to GA101, e.g., following the development of GA101 resistance. Interestingly, intravenous OV administration and/or virus infection is also associated with NK cell recruitment to the LN (Figure S12; Pak-Wittel et al.[53]), suggesting a significant role for NK cell anti-tumor immunity in the eradication of LN disease. Moreover, OV delivery to LN has been reported after *in vivo* administration, further supporting the potential for direct oncolysis within the LN, along with *in situ* NK cell activation.[54]

Here, we demonstrate that in addition to direct oncolysis, CVA21 can induce NK cell anti-tumor immunity and overcome CD40L-induced drug resistance—a key mechanism by which B cell neoplasms escape chemotherapeutic control and give rise to relapse. This modality is likely to be most effective when disease burden is low and immune function is relatively well preserved; therefore, incorporation of CVA21 treatment when patients are in remission holds great therapeutic promise.

MATERIALS AND METHODS

Cell culture

Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C using a CO_2 incubator (Sanyo, Loughborough, UK), and cell culture was performed under aseptic conditions using Nuaire Class II Microbiological Safety Cabinets. Ramos (BL), Raji (BL), SU-DHL-4 (GCB-DLBCL), OCI-LY-19 (GCB-DLBCL), and CD40L-expressing L929 cells (CD40L-L929) were kindly provided by Dr. Tooze and Dr. Doody (LIMR). U2932 (ABC-DLBCL) and OCI-LY-3 (ABC-DLBCL) were purchased from ATCC (Middlesex, UK), and CLL cell lines (EHEB and MEC-2) were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). Genotypic validation of NHL cell lines (Ramos, Raji, OCI-LY-19, and SU-DHL-4) and CLL cell lines (EHEB and MEC-2) was conducted in house using short tandem repeat (STR) profiling and compared with the DSMZ database. MEL-624 cells[55] were obtained from the Cancer Research UK cell bank, and in the absence of a reference profile in the DSMZ database, this cell line was shown to have an original STR profile distinct from other cell lines in the database.

Ramos, Raji, OCI-LY-19, SU-DHL-4, EHEB, and CD40L-L929 cell lines and parental L929 cells were cultured in glutamine-containing Roswell Parks Memorial Institute-1640 medium (RPMI-1640; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% v/v fetal calf serum (FCS; Gibco, Thermo Fisher Scientific, Basingstoke, UK). MEL-624 cells were maintained in glutamine-containing Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% FCS, and MEC-2 cells were grown in glutamine-containing Iscove's modified Dulbecco's medium (Sigma-Aldrich) supplemented with 20% FCS. All cells were routinely tested for mycoplasma and were free from contamination.

Isolation of PBMCs

HD blood was obtained from leukocyte apheresis cones supplied by the National Health Service Blood and Transplant unit (NHSBT). PBMCs were isolated using Lymphoprep (Fresenius-Kabi, Bad Homburg, Germany) density gradient centrifugation and seeded at 2×10^6 cells/mL in glutamine-containing RPMI-1640 containing 10% FCS. LN mononuclear cells (LNMCs) were isolated from LN biopsies of suspected cases of lymphoma; these were supplied by the Haematological Malignancy Diagnostic Service (HMDS, St. James's University Hospital, Leeds, UK); ethical approval was in place (14/WS/0098). LNMCs were maintained in glutamine-containing RPMI-1640 comprising 10% FCS and penicillin (50 units/mL)/streptomycin (50 μg/mL) (Life Technologies, Thermo Fisher Scientific). PBMCs were also isolated from patients with CLL after written informed consent (06/Q1206/106), and freshly isolated PBMCs were cultured at 5×10^6 cells/mL in glutamine-containing RPMI-1640 supplemented with 10% FCS.[42,56]

B cell:CD40L-L929 co-culture

Murine L929 cell lines, stably transfected to express human CD40L (CD40L-L929), were used to recapitulate B cell (CD40):T cell (CD40L) interactions within the LNs. For co-culture experiments,

Figure 7. NK cell-mediated killing of malignant B cells is enhanced by CVA21 treatment

(A) PBMCs were treated with CVA21 for 48 h and co-cultured with malignant B cells at a 10:1 ratio, and NK cell degranulation was determined by quantification of CD107 on $CD3^-CD56^+$ NK cells (±SEM). (B) PBMCs were treated with CVA21 for 48 h and co-cultured with cell Tracker-labeled malignant B cells at a 50:1 ratio. NK cell-mediated killing of $CellTracker^+$ B cells was determined using LIVE/DEAD. All error bars show the mean +SEM and * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

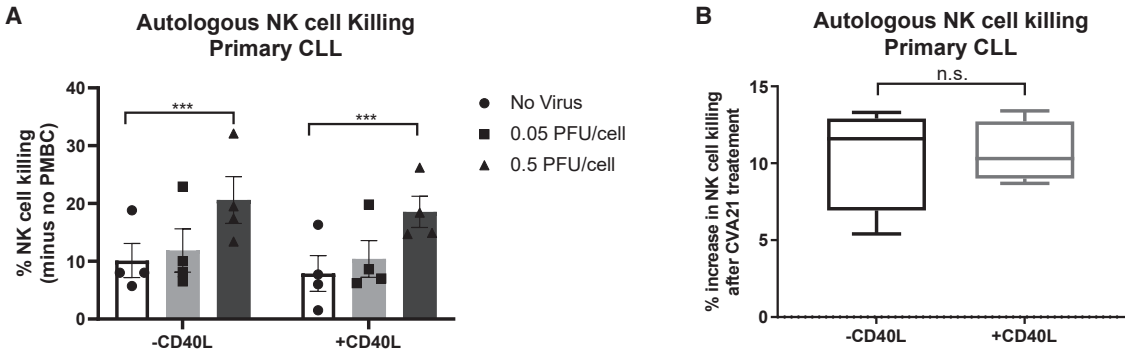

Figure 8. CVA21-activated NK cells can kill autologous primary CLL cells

Primary CLL cells were either cultured alone or co-cultured with CD40L-L929 cells to induce a drug-resistant phenotype. CLL cells (±CD40L-L929) were labeled with CellTracker and co-cultured with CVA21-treated autologous patient PBMCs at a 100:1 ratio for 5 h. NK cell killing of CellTracker-labeled CLL cells was determined using LIVE/DEAD. (A) The percentage of NK cell killing in the presence or absence of CD40L-L929 stimulation. (B) The percentage increase in NK cell killing after CVA21 treatment ± CD40L-L929 co-culture. Error bars show the mean +SEM and *** = $p<0.001$.

CD40L-L929 cells were irradiated with 50 Gy to prevent proliferation and left to adhere to culture vessels overnight. B cell lines, HD-PBMCs, or patient CLL cells were added to CD40L-L929 cells in an equal volume of fresh medium. The cells were cultured on CD40L-L929 feeder layers for at least 24 h before use in downstream experiments.

CellTracker staining

A 5 mM stock solution of CellTracker Green (CTG) CMFDA fluorescent dye (Invitrogen, Thermo Fisher Scientific) was prepared in DMSO, and a 2.5 μM working dilution was prepared in serum-free RPMI-1640. Cells were stained at 10^6/mL for 30 min at 37°C and washed three times in complete RPMI-1640 before use.

ICAM-1 blockade

A recombinant human anti-ICAM-1 (rh-α-ICAM-1, Biolegend, London, UK) antibody was used to block ICAM-1. Recombinant anti-ICAM-1 or immunoglobulin G1 (IgG1) isotype control antibody (R&D Systems, Abingdon, UK) was added for 30 min prior to treatment with CVA21.

Cell treatment

Wild-type CVA21, Kuykendall strain, was either provided by Viralytics (Sydney, NSW, Australia) or propagated in house from wild-type CVA21 obtained from ATCC (ATCCVR-850) as described by Müller et al.[10] Viral titer was determined by plaque assay on MEL-624 cells, and CVA21 concentrations were quantified as PFU/cell. Stock solutions of 100 μM rapamycin (Selleckchem, Houston, TX, USA), 10 mM Ibrutinib (Selleckchem), and 10 mM U0126 (Selleckchem) were prepared by reconstitution in DMSO. rIFN-α (R&D Systems) was used at 5,000 pg/mL, consistent with concentrations reached in tissue culture supernatant after OV treatment of PBMCs.[10,42,57] ZVAD (Sigma-Aldrich) was used at 50 μM, as previously described.[20]

Flow cytometry

All flow cytometry was performed on either a 2-laser Attune Acoustic Focusing Cytometer (Applied Biosystems, Thermo Fisher Scientific) or a 4-laser CytoFLEX S (Beckman Coulter, High Wycombe, UK). For analysis, Attune software and CytExpert software were used, respectively. Details of antibodies used are provided in Table S1.

LIVE/DEAD assay

Samples were harvested, washed in 1 mL PBS, and stained with LIVE/DEAD Fixable Yellow Dead Cell Stain (Invitrogen; 500 μL diluted 1:1,000 in PBS) for 30 min in the dark. Samples were washed with 2 mL PBS and fixed with 300 μL 1% PFA in PBS, or combined with antibodies, for exclusion of dead cells.

Intracellular Phosflow staining

Treated cells were harvested and washed with fluorescence-activated cell sorting (FACS) buffer (PBS, 1% FCS, and 0.1% sodium azide) and stained to identify immune cell populations (Table S1). Cells were subsequently resuspended in a 1:1 mix of growth medium and Cytofix (BD Biosciences, San Jose, CA, USA), incubated at 37°C for 12 min, and permeabilized using Perm Buffer III (BD Biosciences) for 30 min (in the dark). Then, cells were washed twice with FACS buffer and stained with appropriate intracellular antibodies (Table S1) for 60 min; cell acquisition was performed within 60 min.

NK cell CD107 degranulation assays

PBMCs were incubated alone or with target tumor cells at a 10:1 ratio. After 1 h at 37°C, anti-CD107a, anti-CD107b, and anti-CD3/CD56 were added to identify NK cells, along with 10 μg/mL Brefeldin A (BioLegend). Samples were then incubated for a further 4 h at 37°C and subsequently washed with FACS buffer and fixed in 1% PFA until acquisition.

Flow cytometry-based killing assay

Target cells were either stained with CTG immediately prior to the assay or co-cultured with CD40L-L929 cells for 48 h prior to CTG staining. Target B cells were harvested by washing with PBS and co-cultured with PBMCs at a 50:1 effector:target ratio (100:1 was used for autologous CLL assays) for 5 h at 37°C.

Cells were subsequently stained with LIVE/DEAD and fixed in 1% PFA.

Statistical analysis

All statistical analysis was performed using Graphpad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). p values were calculated using either Student's t test with two-tailed distribution or one-way or two-way analysis of variance (ANOVA) with post-hoc tests and correction for multiple testing. Results were considered significantly different from the null hypothesis if $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, #/****$p < 0.0001$).

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and supplemental information. Computational datasets were not generated or used in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.03.002.

ACKNOWLEDGMENTS

The authors would like to thank the Tony Bramall Charitable Trust for financial support and acknowledge Cancer Research UK (CRUK: C16708/A21855), Yorkshire Cancer Research (YCR; L374RA), and Kay Kendall Leukaemia Fund (KKLF; KKL1071) for additional financial support. We also thank the National Health Service Blood and Transplant (NHSBT) unit for provision of leukocyte apheresis cones and NIHR Leeds Clinical Research Facility and Leeds ECMC for facilitating sample collection. Additionally, we would like to thank Prof. Mark Cragg (University of Southampton) for providing GA101, Dr. G. Doody and Dr. R. Tooze (University of Leeds) for providing some of the cell lines used in this work, and Dr. Darren Shafren for supplying CVA21 during the early stages of this project. Finally, the authors thank all patients and HDs who volunteered to participate in this study.

AUTHOR CONTRIBUTIONS

M.H. and G.B.S. performed the majority of the experimental work, including experimental design and data acquisition. S.H., T.B., B.A., L.M.E.M., and V.A.J. contributed significantly to experimental work. C.R., C.B., A.M., P.H., and C.P. all provided intellectual input and reviewed the manuscript. C.R., C.B., P.H., and C.P. contributed to patient consent and sample collection. F.E.-M., M.H., and G.B.S. were responsible for experimental design. F.E.-M. prepared the figures and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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