Title

Envelope protein-specific B cell receptors direct lentiviral vector tropism in vivo

Journal

Molecular Therapy, 32(5)

ISSN

1525-0016

Authors

Takano, Kari-Ann
Wong, Anita AL
Brown, Rebecca
et al.

Publication Date

2024-05-01

DOI

10.1016/j.ymthe.2024.03.002

Peer reviewed

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

Molecular Therapy
Original Article

Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*

Kari-Ann Takano,[1,2] Anita A.L. Wong,[1,2] Rebecca Brown,[3] Kathy Situ,[1,2] Bernadette Anne Chua,[1,2] Angel Elma Abu,[4] Truc T. Pham,[3] Glania Carel Reyes,[1,2] Sangeetha Ramachandran,[3] Masakazu Kamata,[5] Melody M.H. Li,[2,3,6] Ting-Ting Wu,[2,6,7,9] Dinesh S. Rao,[6,7,8] Vaithilingaraja Arumugaswami,[6,9,10] Kenneth Dorshkind,[8] Steve Cole,[11] and Kouki Morizono[1,2]

[1]Division of Hematology and Oncology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA; [2]UCLA AIDS Institute, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA; [3]Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095, USA; [4]Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, CA 90095, USA; [5]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35233, USA; [6]Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, Los Angeles, CA 90095, USA; [7]Jonsson Comprehensive Cancer Center (JCCC), University of California, Los Angeles, Los Angeles, CA 90095, USA; [8]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA; [9]Department of Molecular and Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA 90095, USA; [10]California NanoSystems Institute, University of California, Los Angeles, Los Angeles, CA 90095, USA; [11]Departments of Psychiatry & Biobehavioral Sciences and Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA

While studying transgene expression after systemic administration of lentiviral vectors, we found that splenic B cells are robustly transduced, regardless of the types of pseudotyped envelope proteins. However, the administration of two different pseudotypes resulted in transduction of two distinct B cell populations, suggesting that each pseudotype uses unique and specific receptors for its attachment and entry into splenic B cells. Single-cell RNA sequencing analysis of the transduced cells demonstrated that different pseudotypes transduce distinct B cell subpopulations characterized by specific B cell receptor (BCR) genotypes. Functional analysis of the BCRs of the transduced cells demonstrated that BCRs specific to the pseudotyping envelope proteins mediate viral entry, enabling the vectors to selectively transduce the B cell populations that are capable of producing antibodies specific to their envelope proteins. Lentiviral vector entry via the BCR activated the transduced B cells and induced proliferation and differentiation into mature effectors, such as memory B and plasma cells. BCR-mediated viral entry into clonally specific B cell subpopulations raises new concepts for understanding the biodistribution of transgene expression after systemic administration of lentiviral vectors and offers new opportunities for BCR-targeted gene delivery by pseudotyped lentiviral vectors.

INTRODUCTION

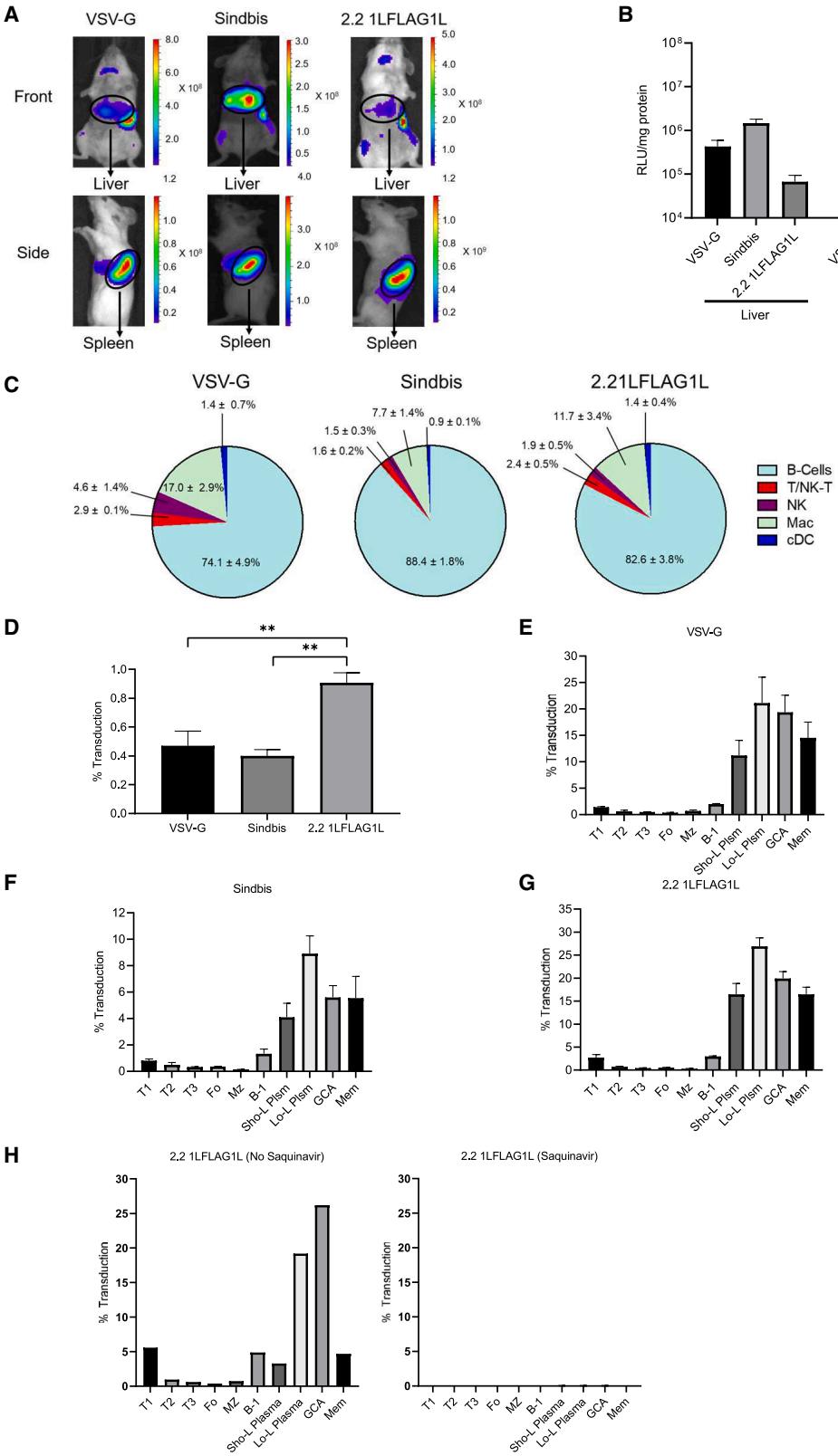
The host range of lentiviral vectors is defined by the tropisms of the envelope proteins pseudotyping the vectors.[1] The tropisms of the envelope proteins are usually defined by two factors. One is the cognate receptor(s) to which the envelope proteins bind, and the other factor is the types of cells, tissues, organs, and species that express these cognate receptor(s). Thus, when we need to transduce broad types of cells, tissues, and organs, we pseudotype lentiviral vectors with envelope proteins, whose cognate receptors are broadly expressed in a wide variety of cells, tissues, and organs.[2] On the contrary, when we need to selectively transduce only desired cell types, we must first eliminate the native binding activity of the pseudotyped envelope proteins to their cognate receptors by mutating their receptor-binding domain and then conjugating the vectors with the targeting ligands that can specifically bind target cells.[3–11]

Based on this principle, we developed lentiviral vectors that selectively transduce only desired cell types.[12–17] We achieved this by pseudotyping lentiviral vectors with modified Sindbis virus envelope proteins. The Sindbis virus envelope protein consists of two types of envelope proteins: E1 and E2. E1 mediates the fusion with the target cell's membrane, and E2 mediates the binding to the target cells. To redirect the tropism of the Sindbis virus envelope protein, we mutated the receptor-binding domains of E2 to eliminate its binding to its cognate receptors (laminin receptor and heparin sulfate) and then conjugated it with targeting ligands that specifically bind to desired cell types.[18,19] When we administered the lentiviral vector pseudotyped with the modified Sindbis virus envelope protein, which contains mutations in its receptor-binding domains, into immunodeficient mice, we observed a decrease of systemic transgene expression (especially from the liver and spleen).[15,16] These results demonstrated that the interaction of the pseudotyped envelope protein with its cognate

Received 19 October 2023; accepted 4 March 2024;
https://doi.org/10.1016/j.ymthe.2024.03.002.

Correspondence: Kouki Morizono, Division of Hematology and Oncology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA.
E-mail: koukimo@ucla.edu

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002



(legend on next page)

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

receptors defines the transduction efficiency of cells, tissues, and organs *in vivo* and the biodistribution of lentiviral transgenes. However, these results were obtained using immunodeficient mice that lack T and B cells. Therefore, the lentiviral transduction of these immune cell populations was not examined in our previous experimental settings. Thus, we investigated how the abrogation of the receptor binding domains affects the biodistribution of lentiviral transgene expression after systemic administration of the vectors into immunocompetent mice.

In the study described here, we found that systemic administration of lentiviral vectors into immunocompetent mice results in robust transduction of splenic B cells, regardless of the types of pseudotyping envelope proteins used. Envelope protein 2.2 1LFLAG1L, derived from Sindbis virus, maintains efficient transduction of splenic B cells despite the loss of its original receptor-binding activity, suggesting that transduction of splenic B cells involves (a) novel mechanism(s) of virus binding. Here, we report that B cell receptors (BCRs) specific to pseudotyped envelope proteins mediate binding to pseudotyped lentiviral vectors and facilitate lentiviral transduction. This vector entry mechanism supports not only viral entry of the 2.2 1LFLAG1L pseudotype but also the vesicular stomatitis virus G protein (VSV-G) pseudotype, which has a broad host range and is widely used in gene therapy. We also report that viral entry via BCRs leads to the activation of BCR-mediated signaling pathways that promote cell proliferation and the differentiation of transduced B cells into mature effectors that include memory B and plasma cells.

RESULTS

Spleen cells are robustly transduced *in vivo* using lentiviral vectors pseudotyped with modified Sindbis virus envelope proteins

We have previously altered the tropism of lentiviral vectors by pseudotyping them with modified Sindbis virus envelope proteins containing mutations in multiple receptor-binding domains; these mutations reduced systemic transduction in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice.[15] However, these studies used immunodeficient mice lacking T and B cells, which potentially under-represented the tropism for these cell types. To address this issue, we injected immunocompetent C57BL6 mice with lentiviral vectors pseudotyped with either the 2.2 1LFLAG1L envelope protein (Sindbis virus envelope protein with mutations to abrogate its receptor-binding activity; Figure S1), the wild-type Sindbis virus envelope (Sindbis), or VSV-G envelope protein. Transduction was assessed by detection of bioluminescence and/or fluorescence from the LumiScarlet transgene.[20] Consistent with previous results from immunodeficient mice, the receptor-binding domain abrogation in 2.2 1LFLAG1L substantially reduced liver transduction compared with the Sindbis or the VSV-G pseudotypes (Figure 1A). However, we unexpectedly observed similar or increased transduction of the spleen by 2.2 1LFLAG1L compared with the VSV-G and Sindbis pseudotypes. We confirmed these results by isolating each organ and measuring luciferase activity per milligram of extracted protein (Figure 1B). These results suggest that splenocyte transduction by the 2.2 1LFLAG1L-pseudotyped vector is mediated by molecular mechanisms independent of interactions with Sindbis virus cognate receptors.

Flow cytometry analysis found that B cells are the major splenocyte subpopulation transduced by all pseudotypes (Figure 1C). Within the total B cell population, all pseudotypes showed more efficient transduction of mature or differentiated cell subpopulations (i.e., short- and long-lived plasma cells, germinal center-activated cells, and memory B cells) relative to less differentiated subpopulations (i.e., transitional B cells; Figures 1D–1G and S2A and S2B).[21,22] All three pseudotypes, particularly 2.2 1LFLAG1L, also increased the absolute cell numbers of later B cell populations while reducing the prevalence of relatively immature populations, such as T1 B cells (Figures S2C–S2M). Although these shared patterns of transduction with Sindbis and 2.2 1LFLAG1L pseudotypes might suggest binding to common Sindbis virus cognate receptors.[18,19] it would not explain the additional enhancement of spleen B cell transduction by the (receptor-binding site-mutated) 2.2 1LFLAG1L pseudotype compared with the Sindbis pseudotype for most B cell subsets.

We next investigated whether the fluorescent signals observed in B cells were caused by pseudotransduction mediated by the binding of fluorescent proteins contaminated in lentiviral vector virions. To eliminate this possibility, we injected the 2.2 1LFLAG1L pseudotype produced in the presence of an HIV-1 protease inhibitor, saquinavir, which does not block production and budding of lentiviral vectors but inhibits post-binding steps of lentiviral transduction.[23–25] The saquinavir-treated vectors did not show any EGFP expression in B cells (Figure 1H), demonstrating that the fluorescent signals observed in our prior experiments are the results of *bona fide* viral transduction but not pseudotransduction.

Figure 1. Robust transduction of splenic B cells by intravenous administration of lentiviral vectors

(A) Whole-body imaging of the LumiScarlet lentiviral transgene after intravenous injection of lentiviral vectors pseudotyped with either VSV-G, Sindbis, or 2.2 1LFLAG1L. The amounts of the vectors injected were adjusted by the HIV p24 CA protein (3 μg p24/mouse). Representative images of each experimental group are shown. (B) Transgene expression in the liver and spleen. Averages of relative luminescence units per 1 mg of protein are shown. Error bars represent standard deviation (SD). n = 3 for VSV-G and Sindbis, n = 6 for 2.2 1LFLAG1L. (C) Average percentages of cell populations in the Scarlet-expressing cells analyzed by flow cytometry. Numbers are shown as average ± SD. (D–G) Averages of percentages of transduced cells in (D) whole B cell or B cell subpopulations in the spleen after intravenous injection of (E) VSV-G; (F) Sindbis or (G) 2.2 1LFLAG1L pseudotypes. (H) Percentages of transduced cells in whole B cell or B cell subpopulations in the spleen after intravenous injection of 2.2 1LFLAG1L pseudotypes produced in the presence (n = 1) or absence (n = 3) of saquinavir. The transduction data of saquinavir-treated 2.2 1LFLAG1L pseudotype is the average and SD of three mice. (D) Significance was calculated using a two-sample, two-sided, unpaired Student's t test (**$p < 0.01$). Fo, follicular B cells; Mz, marginal zone B cells; Sho-L Plsm, short-lived plasma cells; Lo-L Plsm, long-lived plasma cells; GCA,germinal center activated B cells; Mem, memory B cells. Error bars represent SD.

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

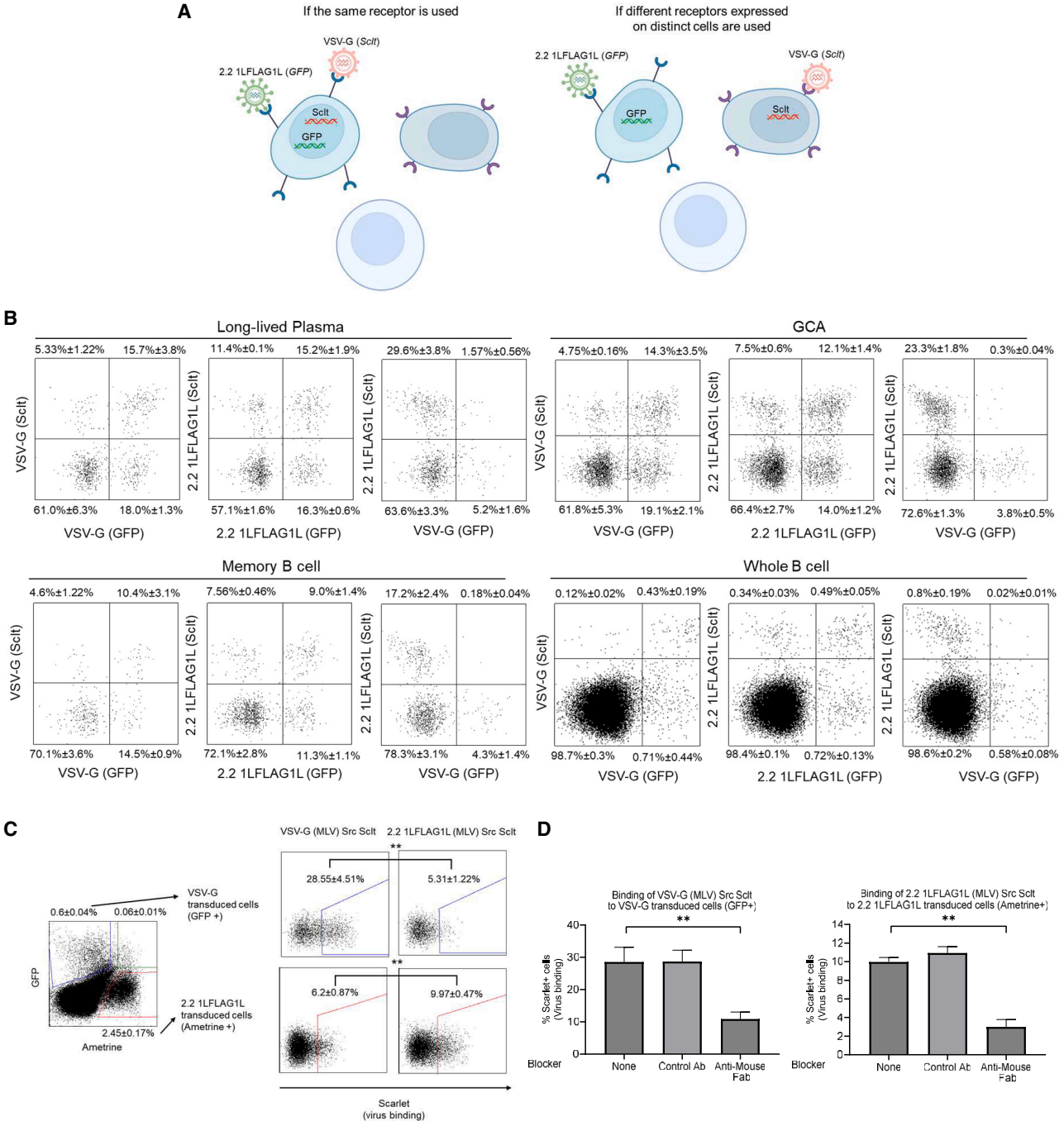


Figure 2. Properties of viral receptors mediating binding of pseudotyped lentiviral vectors

(A) Schematics of the hypothesis of transduction of B cells with lentiviral vectors expressing GFP and Sclt pseudotyped with different types of envelope proteins, respectively, when both envelope proteins use the same receptor (left) or different receptors expressed on different cells (right). (B) Expression of GFP and Scarlet in splenic B cells and their subpopulations when these transgenes are delivered by lentiviral vectors pseudotyped with the same or different envelope proteins. Averages and SDs derived from three replicates as well as representative flow cytometry profiles are shown for each experimental condition. n = 3 for each experimental group. (C) Binding of Scarlet-labeled MLV pseudovirions pseudotyped with VSV-G (VSV-G [MLV] Src Sclt) or 2.2 1LFLAG1L (2.2 1LFLAG1L [MLV] Src Sclt) to splenic B cells (C57BL6) transduced with lentiviral vectors pseudotyped with VSV-G (GFP+) or 2.2 1LFLAG1L (Ametrine^{+}). Averages and SDs derived from 3 replicates as well as representative flow profiles are shown for each experimental condition. Significance was calculated using a two-sample, two-sided, unpaired Student's t test (**$p < 0.01$).

(legend continued on next page)

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

We also conducted histological analysis of the transduced spleens. Morphologically, a small eccentric germinal center is noted within the follicle in VSV-G transduction (Figure S2N), but a larger and more prominent germinal center is seen in the follicle in 2.2 1LFLAG1L transduction (Figure S2O). Transduced cells (GFP^+) are seen in the perifollicular areas in both spleens and within the germinal center (Figure S2O, bottom right and inset). Morphological analysis of GFP-expressing cells suggested that both 2.2 1LFLAG1L and VSV-G pseudotypes also transduce marginal zone macrophages, which is consistent with their known function to trap blood-borne viruses.[26]

We then tested whether these pseudotypes could transduce various murine, rat, and human B cell lines as well as human primary B cells *in vitro*. Unlike the pantropic VSV-G pseudotype, the Sindbis and 2.2 1LFLAG1L pseudotypes failed to efficiently transduce any of the B cell lines or primary B cells (Figures S3A–S3H). Given that the 2.2 1LFLAG1L pseudotype transduced primary mouse splenic B cells more efficiently than the VSV-G pseudotype (Figure 1D), *in vivo* transduction of splenic B cells appears to be mediated by different binding/entry mechanisms than those analyzed in *in vitro* settings.

We observed previously that the viral envelope lipid phosphatidylserine (PtdSer) can mediate virus attachment by binding to host PtdSer-recognizing molecules such as TIM-1 and Axl, regardless of the types of envelope proteins (i.e., independent of envelope protein cognate receptors).[27,28] Thus, we investigated whether B cells transduced with the 2.2 1LFLAG1L-pseudotyped vector express TIM-1 and/or Axl (Figure S3I). However, neither molecule was expressed on transduced B cells, suggesting that other receptor(s) besides PtdSer receptors mediate *in vivo* transduction of splenic B cells.

Receptors specific to 2.2 1LFLAG1L or VSV-G pseudotype facilitate transduction of splenic B cells

To identify the receptor(s) mediating splenic B cell transduction, we first examined whether the transduction of B cells with each pseudotype was mediated by the same receptor(s). Because differentiated B cells, which is the relatively small number in the total B cells, expressed the transgene at a high percentage, we hypothesized that, if two different pseudotypes use the same receptor(s), then the percentage of co-transduced (double-positive) cells would be higher than expected by random distribution (Figure 2A, left). To test this hypothesis, we co-injected the VSV-G (Scarlet-expressing) and 2.2 1LFLAG1L (GFP-expressing) pseudotypes into the same mouse and measured the frequency of co-transduction (percentage GFP^+/$Scarlet^+$). We first analyzed this in the B cell subpopulations with relatively high transduction efficiency (long-lived plasma cells, germinal center activated [GCA]) B cells, and memory B cells). As a positive control, animals were injected with two vectors bearing the same envelope protein and showed a significantly greater percentage of double-positive (co-transduced) cells than expected under independence, demonstrating shared tropism (Figure 2B). When animals were injected with two vectors bearing different envelope proteins, the percentage of double-positive cells was significantly lower than expected under independence (i.e., distinct tropism), suggesting that the different pseudotypes use distinct receptors expressed on different cell populations (Figure 2A, right). Within the total B cell pool, the rate of co-transduction by heterologous envelopes was also significantly lower than that observed for homologous envelopes. These results indicate that the VSV-G and 2.2 1LFLAG1L pseudotypes employ different transduction receptors.

We next examined whether binding of VSV-G and 2.2 1LFLAG1L to splenic B cells is mediated by different receptors specific to each pseudotyping protein. We co-injected a VSV-G-pseudotyped vector expressing GFP and 2.2 1LFLAG1L-pseudotyped vector expressing Ametrine, and we investigated whether GFP-expressing cells preferentially bind VSV-G-pseudotyped MLV (Murine Leukemia Virus) pseudovirions and whether Ametrine-expressing cells preferentially bind 2.2 1LFLAG1L-pseudotyped MLV pseudovirions *in vitro* (with MLV pseudovirions labeled with Scarlet protein in both cases to quantitate its binding to cells; Figure 2C). VSV-G-transduced (GFP^+) cells preferentially bound VSV-G-pseudotyped MLV pseudovirions, while cells transduced with 2.2 1LFLAG1L ($Ametrine^+$) preferentially bound 2.2 1LFLAG1L-pseudotyped MLV pseudovirions.[29] Thus, transduction of splenic B cells with VSV-G or 2.2 1LFLAG1L pseudotypes appears to be mediated by receptors specific to each envelope protein. These results were observed not only in C57BL6 but also BALB/c mice (Figure S3J), suggesting that these results are not specific to any specific genetic background. Similar experiments using a lentiviral vector pseudotyped with the Ebola virus glycoprotein (EboZ) showed that the EboZ-pseudotyped MLV pseudovirion preferentially bound to the splenic B cell population transduced with the EboZ-pseudotyped lentiviral vector (Figure S3K).[30] These results again support the hypothesis that distinct receptors mediate the binding of lentiviral vectors pseudotyped with distinct envelope proteins.

Our data indicated that the same subpopulation (differentiation stage) of B cells has specific virus-binding receptors for VSV-G, 2.2 1LFLAG1L, or EboZ exclusively. Similarly, it is known that B cells express BCRs specific to one antigen, which can be different for each B cell. Because of this similarity, we examined whether BCRs participate in differential tropism of pseudotyped viral particles. We concealed the antigen-binding domain (antibody fragment [Fab] domain) of the BCR using an anti-mouse Fab antibody and found substantial reductions in attachment of MLV pseudovirions to splenic B cells (Figure 2D). To confirm that these effects were mediated by BCRs, we

(D) Binding of MLV pseudovirions pseudotyped with VSV-G to splenic B cells transduced with the VSV-G-pseudotyped lentiviral vector and binding of MLV pseudovirions pseudotyped with 2.2 1LFLAG1L to splenic B cells transduced with the 2.2 1LFLAG1L-pseudotyped lentiviral vector in the absence or presence of goat anti-mouse Ig Fab domain or control goat Ab. Averages and SDs derived from three replicates are shown. Significance was calculated using a two-sample, two-sided, unpaired Student's t test (**$p < 0.01$).

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

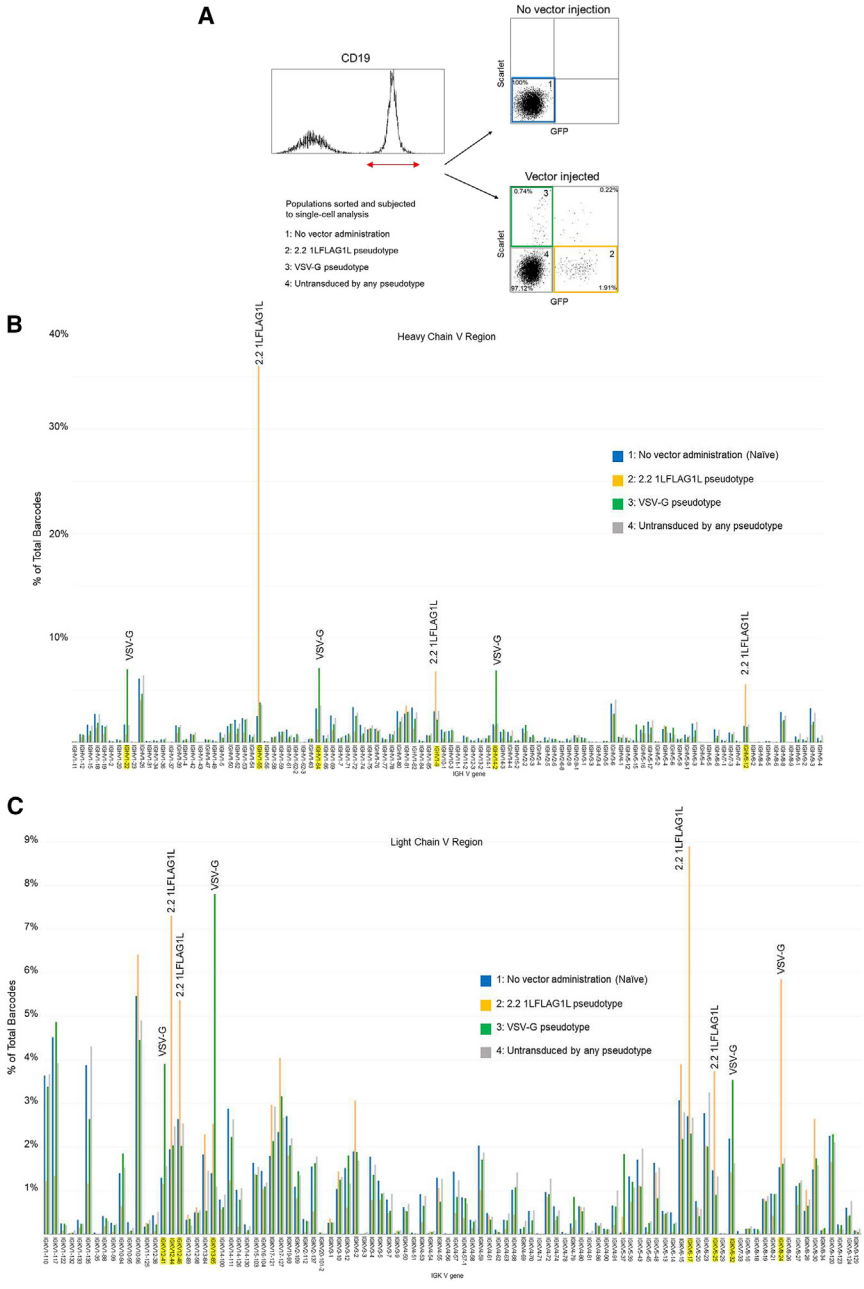


Figure 3. BCR mediates envelope protein-specific transduction of pseudotypes

(A) Sorting strategy for single-cell analysis of a splenic B cell population transduced with VSV-G or 2.2 1LFLAG1L pseudotype and untransduced with any pseudotype and splenic B cells of a mouse with no vector injection. (B) Heavy chain and (C) light chain V region usage of B cells transduced with VSV-G pseudotype, 2.2 1LFLAG1L pseudotype, untransduced splenic B cells, or splenic B cells with no vector injection.

next used single-cell sequencing to isolate the BCR sequences from the transduced cells and examined their binding specificity and ability to mediate lentiviral vector transduction.

Lentivirally transduced B cells express BCRs specific to the envelope protein used for their transduction

To identify the specific BCR sequences of the cells transduced by VSV-G (Scarlet-expressing) or 2.2 1LFLAG1L (GFP-expressing) pseudotypes, we administered both vectors to a C57BL6 mouse and conducted single-cell BCR sequencing of VSV-G-transduced (Scarlet$^+$/GFP$^-$), 2.2 1LFLAG1L-transduced (Scarlet$^-$/GFP$^+$), and untransduced (Scarlet$^-$/GFP$^-$) splenic B cells isolated 4 days post vector administration (Figure 3A). (Co-transduced cells could not be analyzed due to insufficient numbers.) B cells that were exposed to the vector but remained untransduced showed a heavy-chain (Figure 3B) and light-chain (Figure 3C) V-gene distribution similar to splenic B cells from a control naive mouse unexposed to any vector. B cells that were transduced with VSV-G or with 2.2 1LFLAG1L both showed a marked skew in V-gene distribution, but the specific V-gene usage patterns differed markedly by pseudotype. These results suggested BCR genetic sequence as the key determinant of selective viral entry.

To analyze BCR specificity toward pseudotyping envelope proteins, we identified the 10 most frequent BCR sequences of 2.2 1LFLAG1L-transduced cells and the 9 most frequent BCR sequences of VSV-G-transduced cells (Table 1) and tested whether the antibodies based on these BCR sequences specifically bind to human and mouse cells expressing 2.2 1LFLAG1L (Figure S4A) or VSV-G (Figure S4B). Eight of 10 antibodies derived from 2.2 1LFLAG1L-transduced cells specifically recognized 2.2 1LFLAG1L expressed on both the mouse B16F10 cell line and the human HEK293T cell line (Figure S4A). The other two antibodies did not bind to any cells tested (2.2 08 and 10 hIgG1), possibly because recognition epitopes are abundantly present on the virus but not on the envelope-expressing cells.[31] Thus, we analyzed the binding specificity of these two BCRs by ectopically expressing them as the BCR form in CD79 HEK293T cells (HEK293T cells ectopically expressing CD79 as the chaperone for the BCR) and tested binding of Scarlet-labeled lentiviral vectors pseudotyped with either 2.2 1LFLAG1L or VSV-G (Figure S4C).[32] These two BCRs mediated specific binding of the 2.2 1LFLAG1L pseudotype, confirming the

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

Table 1. Most frequent BCRs isolated from transduced splenic B cells

Rank by frequency	No. of cells sharing the same BCR sequence	BCR name	Heavy-chain V region[a]	Light-chain V region[a]	Binding antigen	Pseudotypes supported for transduction	Isotype/subclass
BCRs isolated from splenic B cells transduced with the 2.2 1LFLAG1L pseudotype (total of 5,715 cells)							
1	12	2.2 01	*IGHV1-55*	*IGKV12-46*	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG3
1	12	2.2 02	IGHV1-18	*IGKV6-17*	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG2c
3	10	2.2 03	*IGHV1-55*	IGKV10-96	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG2c
4	9	2.2 04	*IGHV1-55*	IGKV6-15	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG2c
4	9	2.2 05	*IGHV1-9*	IGLV1	2.2 1LFLAG1L	2.2 1LFLAG1L	IgM
6	7	2.2 06	*IGHV1-55*	*IGKV8-24*	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG2c
7	6	2.2 07	*IGHV1-55*	*IGKV8-24*	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG3
7	6	2.2 08	*IGHV8-12*	IGKV17-127	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG2c
7	6	2.2 09	IGHV1-75	IGKV5-48	2.2 1LFLAG1L	2.2 1LFLAG1L	IgG2c
7	6	2.2 10	*IGHV1-9*	*IGKV12-44*	2.2 1LFLAG1L	2.2 1LFLAG1L	IgM
BCRs isolated from splenic B cells transduced with VSV-G pseudotype (total of 5,998 cells)							
1	10	VSV 01	IGHV11-2	IGKV14-126	VSV-G on human cells	VSV-G	IgM
1	10	VSV 02	*IGHV14-2*	IGKV17-121	VSV-G on human cells	VSV-G	IgM
3	6	VSV 03	IGHV1-12	*IGKV6-32*	human antigen	VSV-G and 2.2 1LFLAG1L	IgG2b
4	5	VSV 04	*IGHV14-2*	IGKV4-79	VSV-G	VSV-G	IgM
4	5	VSV 05	*IGHV1-64*	*IGKV13-85*	VSV-G	VSV-G and 2.2 1LFLAG1L[b]	IgG2c
4	5	VSV 06	IGKV13-85	*IGKV13-85*	VSV-G	VSV-G	IgM
4	5	VSV 07	IGHV1-18	IGKV5-37	VSV-G	none	IgM
4	5	VSV 08	IGHV1-80	IGKV2-109	human antigen	VSV-G and 2.2 1LFLAG1L	IgG2c
4	5	VSV 09	*IGHV1-64*	IGKV12-46	VSV-G	VSV-G	IgM

[a]V-regions in italic font are shown to be enriched in each transduced population as shown in Figures 3B and 3C.
[b]Enhancement of 2.2 1LFLAG1L pseudotype transduction was not observed on Ramos cells.

antigenic specificity of all BCRs isolated from 2.2 1LFLAG1L-transduced primary B cells to be specific to 2.2 1LFLAG1L. The antigenic specificity of BCRs derived from VSV-G-transduced cells was distinct from that of 2.2 1LFLAG1L-transduced primary B cells and showed a more complex array of binding targets (Figure S4B). VSV-G-specific BCR sequences 04, 05, 06, 07, and 09 encoded antibodies that specifically bound to VSV-G. VSV-G 03 and 08 sequence antibodies bound to a human antigen present on HEK293T cells but not mouse B16F10 cells, and VSV-G 01 and 02 sequence antibodies bound weakly to untransfected and 2.2 1LFLAG1L-expressing HEK293T cells while binding more strongly to VSV-G-expressing HEK293T cells (but not VSV-G-expressing mouse B16F10 cells). VSV-G 01 and 02 likely recognize human antigens exposed and/or upregulated in response to the cytotoxicity of VSV-G and, thus, were abundantly present on VSV-G-transfected HEK293T cells (and possibly the VSV-G pseudotype).[33,34] In summary, among nine antibodies derived from VSV-G-transduced cells, five specifically recognized VSV-G, two recognized epitopes related to VSV-G expression, and two recognized human antigens that were derived from vector producer HEK293T cells (Table 1). These results encouraged us to test the hypothesis that BCRs mediate viral binding and entry.

Virus-specific BCRs mediate viral transduction

We ectopically expressed each of the isolated BCRs on CD79 HEK293T cells and investigated whether these BCRs mediate pseudotype-specific increases in transduction efficiency (Figure 4A). As expected, all BCR sequences isolated from 2.2 1LFLAG1L-transduced primary B cells selectively enhanced CD79 HEK293T cell transduction by the 2.2 1LFLAG1L pseudotype. These results demonstrate that entry of the 2.2 1LFLAG1L pseudotype is specifically mediated by the BCR. Likewise, three BCRs isolated from VSV-G-transduced cells also specifically enhanced target cell transduction by the VSV-G pseudotype (VSV 04, 06, and 09) (Figure 4B).

It is possible that the roles of BCRs in VSV-G pseudotype transduction cannot be properly quantitated in CD79 HEK293T cells due to lack of signaling from BCRs in CD79 HEK293T cells and/or masking by highly efficient viral entry via the VSV-G cognate receptors. For this reason, we thought to use a human B cell line, Ramos, to express the BCRs and assess transduction. Since Ramos cells have endogenous light and heavy chains, ectopic expression of the BCR can result in heterodimerization between exogenous and endogenous chains, impeding analysis. Thus, we generated single-chain BCRs (ScFv BCRs) of VSV 01–09 and 2.2 1LFLAG1L-associated BCR 2.2 05 (as

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

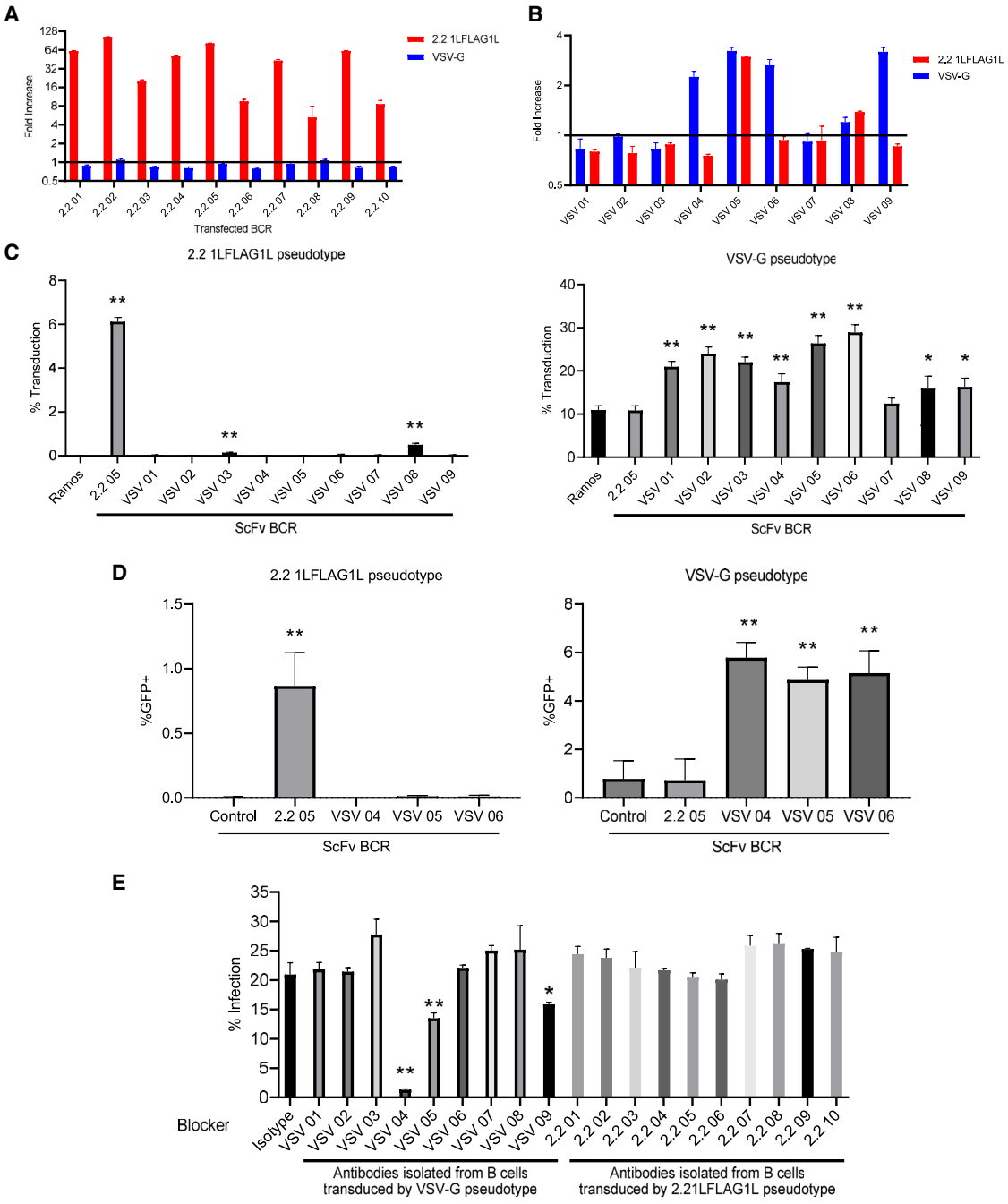


Figure 4. BCRs mediate envelope protein-specific transduction of pseudotypes

(A and B) Enhancement of 2.2 1LFLAG1L or VSV-G pseudotype transduction of CD79 HEK293T cells by ectopic expression of BCRs isolated from splenic B cells transduced with (A) 2.2 1LFLAG1L or (B) VSV-G pseudotype. Averages and SDs derived from three replicates are shown. Transduction efficiencies of untransfected CD79 HEK293T cells with 2.2 1LFLAG1L and VSV-G pseudotypes are 1.32 ± 0.03 and 5.4 ± 0.6%, respectively. (C) Transduction of Ramos cells ectopically expressing ScFv BCRs with the 2.2 1LFLAG1L (left) or VSV-G (right) pseudotype. n = 3, and averages are shown with SD (error bars). Significance was calculated by comparing the transduction efficiency of Ramos cells with that of cells expressing ScFv BCR, using a two-sample, two-sided, unpaired Student's t test (*$p < 0.05$, **$p < 0.01$). (D) Transduction of human primary B cells ectopically expressing ScFv BCRs with the 2.2 1LFLAG1L (left) or VSV-G (right) pseudotype. n = 3, and averages are shown with SD (error bars). Significance was calculated by comparing the transduction efficiency of human primary B cells with that of cells expressing ScFv BCRs, using

(legend continued on next page)

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

a specificity control), to avoid heterodimerization with endogenous BCRs and ectopically expressed them (Figure 4C). The 2.2 1LFLAG1L 05 ScFv BCR specificity control specifically enhanced transduction by the 2.2 1LFLAG1L pseudotype. In this setting, six of the nine ScFv BCRs derived from VSV-G-transduced B cells specifically increased transduction by VSV-G-pseudotyped vectors. Of note, the VSV 03 and 08 ScFv BCRs showed enhancement of both VSV-G and 2.2 1LFLAG1L pseudotype transduction, which is consistent with the observation above that VSV 03 and 08 BCR-derived antibodies bind human antigen(s), which may be commonly present on both pseudotypes. These results confirm that antigen-specific BCRs expressed on target cells play a key role in mediating the transduction of distinct B cell populations by lentiviral vectors pseudotyped with distinct envelope proteins.

We also examined the effects of several anti-VSV-G ScFv BCRs on transduction of primary B cells, which are resistant to transduction by the VSV-G pseudotype. We confirmed that the expression levels of the ScFv BCRs were comparable with that of the endogenous IgG1 BCR (data not shown). As a specificity control, we confirmed that the 2.2 1LFLAG1L 05 ScFv BCR rendered non-permissive primary B cells permissive to transduction by the 2.2 1LFLAG1L pseudotype (Figure 4D). VSV-G-specific ScFv BCRs specifically enhanced the efficiency of primary human B cell transduction by the VSV-G pseudotype 6- to 7-fold. These results confirmed that anti-viral BCRs can facilitate the viral transduction of primary B cells.

Recombinant antibodies derived from lentivirally transduced B cells neutralize viruses containing the same envelope protein as the transducing lentiviral vectors

Because BCRs isolated from the transduced B cells recognized pseudotyped envelope proteins, some of the recombinant antibodies derived from these cells will be able to bind neutralizing epitopes of the envelope proteins, which could lead to a novel approach in isolating neutralizing antibodies against viruses of the origin of the pseudotyping envelope protein. Thus, we tested whether recombinant antibodies derived from B cells transduced by the VSV-G pseudotype could neutralize infection by VSV.[35] Three of nine (VSV 04, 05, and 09) recombinant antibodies derived from VSV-G pseudotype-transduced cells neutralized VSV infection (Figure 4E). Conversely, none of the antibodies derived from B cells transduced by the 2.2 1LFLAG1L pseudotype neutralized VSV infection, confirming the binding specificities of BCR/antibodies of transduced cells and presenting novel approaches for the generation of neutralizing antibodies against viruses.

Viral transduction promotes proliferation and differentiation of B cells

We next examined whether virus binding to BCRs activates BCR signaling pathways in Ramos cells. Binding of the 2.2 1LFLAG1L pseudotype to the 2.2 05 ScFv BCR phosphorylated the BCR signaling molecules ERK and Akt, while binding of VSV-G pseudotype to the VSV 03, 04, 05, and 06 ScFv BCRs induced phosphorylation (Figure S5A).[36] Of note, both the VSV-G and 2.2 1LFLAG1L pseudotypes elicited signaling via the VSV 08 BCRs that recognize human antigens possibly present on the envelope of both pseudotypes. These data demonstrate that vector binding to pseudotype-specific BCRs can induce B cell signaling.

To determine whether BCR signaling plays a functional role in pseudotype-specific vector transduction, we blocked BCR signaling with Ibrutinib and idelalisib (Figure S5B).[37,38] Neither ibrutinib nor idelalisib had any significant effect on transduction (Figure S5C), suggesting that BCR signaling does not play an essential role in the BCR-mediated viral entry step.

However, it is possible that BCR signaling may contribute to proliferation and/or differentiation of the transduced cells *in vivo* even if it does not play an essential role in the viral entry step. To assess this hypothesis, we compared the single-cell transcriptomics profile of VSV-G- or 2.2 1LFLAG1L-transduced splenic B cells with that of untransduced or uninfected mouse B cells (Figure 5A). Consistent with the flow cytometry analysis (Figures 1E and 1G), single-cell transcriptomic phenotyping found that the highly differentiated B cell subpopulations, including germinal center, memory B cells, and plasma cells, were predominately transduced. We observed elevated expression of *Blimp1* and *Xbp1*, which control plasma cell terminal differentiation, and the proliferation markers *Pcna* and *Ki67* in transduced B cells compared with uninfected and untransduced populations (Figure 5B).[39–41] The 2.2 1LFLAG1L pseudotype upregulated proliferation and differentiation indicator genes more strongly than the VSV-G pseudotype.

Transduced B cells also produced higher amounts of antibodies and underwent BCR/antibody class switching from immunoglobulin D (IgD) to IgM, IgG, and IgE (Figure 5B). The 2.2 1LFLAG1L pseudotype induced class switching more efficiently than the VSV-G pseudotype, which is consistent with our data showing that eight of 10 of the most frequent antibodies from the B cells transduced by the 2.2 1LFLAG1L pseudotype are IgG, while only three of nine are IgG for those transduced by the VSV-G pseudotype (Table 1).

These results indicate that BCR-mediated viral entry induces proliferation and differentiation of transduced cells. Given that the absolute cell numbers of the relatively immature B cell subpopulations decreased while those of differentiated mature B cell subpopulations increased in lentivirally transduced spleens (Figures S2D–S2M), it is likely that lentiviral transduction via BCRs induced

a two-sample, two-sided, unpaired Student's t test (*$p < 0.05$, **$p < 0.01$). (E) Recombinant Abs derived from lentivirally transduced B cells neutralize viruses containing the same envelope protein as the transducing lentiviral vectors. Shown is blocking of infection of HEK293T cells with VSV by recombinant Abs. Significance was calculated by comparing the transduction efficiency without blockers with that with the blockers using a two-sample, two-sided, unpaired Student's t test (*$p < 0.05$, **$p < 0.01$).

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

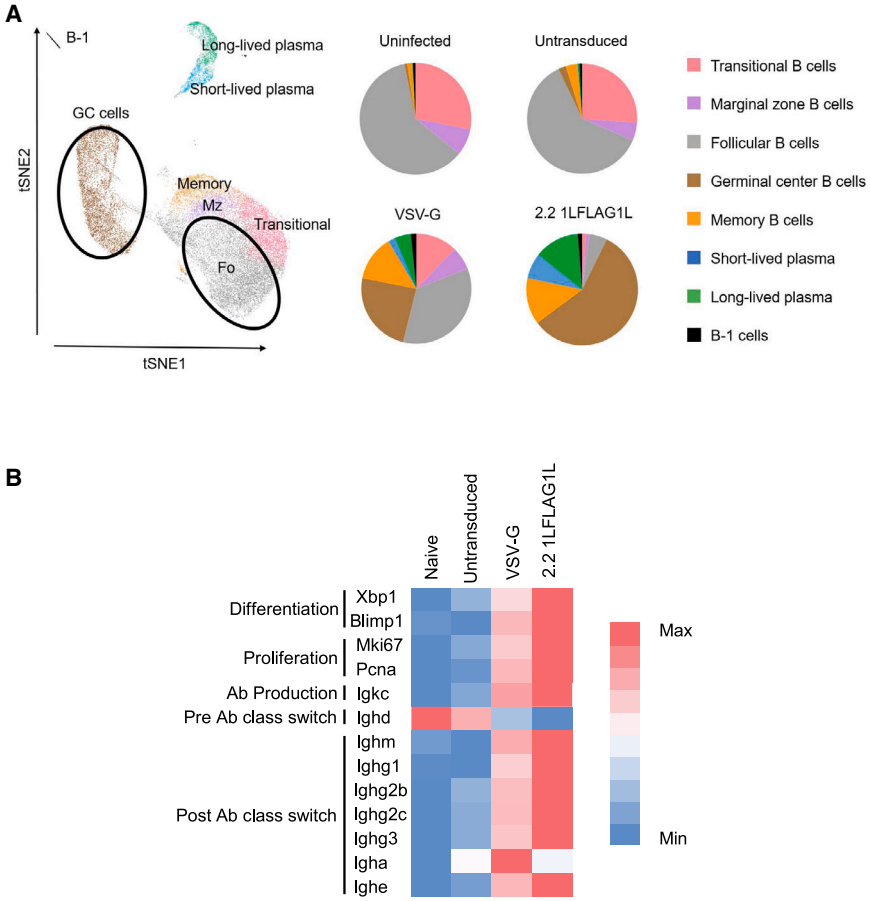


Figure 5. Antiviral BCR-mediated viral entry induces clonal response of infected B cells

(A) Single-cell gene expression analysis to compare subpopulations of splenic B cells of naive, untransduced cells and those transduced with the VSV or 2.2 1LFLAG1L pseudotype. (B) Heatmap of expression of genes mediating various B cell functions in naive, untransduced cells and those transduced with the VSV or 2.2 1LFLAG1L pseudotype.

differentiation of immature transduced B cells into mature effector cells.

Antiviral BCRs mediate transduction of redirected lentiviral vectors

We also investigated whether transduction of lentiviral vectors redirected by conjugation with targeting ligands can be mediated by antiviral BCRs. We used a modified Sindbis virus envelope protein redirected to integrin αV/β3 and αV/β5 by replacing the FLAG tag of 2.2 1LFLAG1L to an integrin-binding peptide (RGD-4C), designated as 4CRGD (Figure S1).[13] BCRs recognizing 2.2 1LFLAG1L enhanced transduction of the 4CRGD pseudotype *in vitro* (Figure 6A). When we co-injected VSV-G pseudotype-expressing EGFP and 4CRGD pseudotype-expressing Ametrine, these two pseudotypes transduced distinct B cells populations (Figure 6B). B cells transduced by the 4CRGD pseudotype specifically bound to Scarlet-labeled MLV pseudovirions pseudotyped with 2.2 1LFLAG1L (Figure 6C). This specific binding is blocked by antibodies against the Fab domain of BCRs (Figure 6D). These results demonstrated that lentiviral vectors redirected by insertion of targeting ligands can still be recognized by BCRs recognizing the backbone viral envelope proteins, resulting in antiviral BCR-mediated lentiviral transduction.

DISCUSSION

These studies demonstrate that BCRs that specifically recognize viral envelope protein epitopes can mediate the binding and cell entry of lentiviral vectors. Viral entry via anti-envelope protein BCRs allowed for transgene expression in B cells with lentiviral vectors pseudotyped with 2.2 1LFLAG1L that bear no receptor-binding domains for their cognate receptors. This suggests that BCRs potentially mediate entry of various types of pseudotyped lentiviral vectors into B cells that lack any expression of cognate viral receptors that would otherwise restrict the vector tropism for other cell types.

Our studies utilized mice with no prior exposure to any forms of the envelope proteins, ensuring that all identified antiviral BCRs existed prior to vector administration, which can be explained through several molecular mechanisms. Natural polyreactive IgM antibodies are produced independent of prior antigenic exposure to specific pathogens and play a critical role in protecting the host from VSV and other viruses.[42,43] Naturally occurring polyreactive BCRs are likely to play a significant role in transduction with VSV-G pseudotypes, which may explain why the majority of the BCR/antibody sequences derived from B cells transduced by VSV-G pseudotyped vectors were IgM isotypes (Table 1). By contrast, the majority of BCRs of 2.2 1LFLAG1L pseudotype-transduced cells were class-switched IgGs, which usually occur in germinal centers following antigen binding to the BCR. One study showed that BALB/c mice have germline BCRs that bind the influenza virus hemagglutinin (HA) protein with high affinity, which induced germinal center formation.[44,45] Our tissue analysis of the spleen from the 2.2 1LFLAG1L pseudotype-injected mouse showed enhanced germinal center formation, which would be consistent with the hypothesis that germline BCRs recognizing 2.2 1LFLAG1L undergo class switching and affinity maturation in germinal centers.

Although lentiviral transduction via this route will occur in small populations in B cells, it is highly selective to the specific B cell subsets capable of generating antibodies against the envelope proteins.

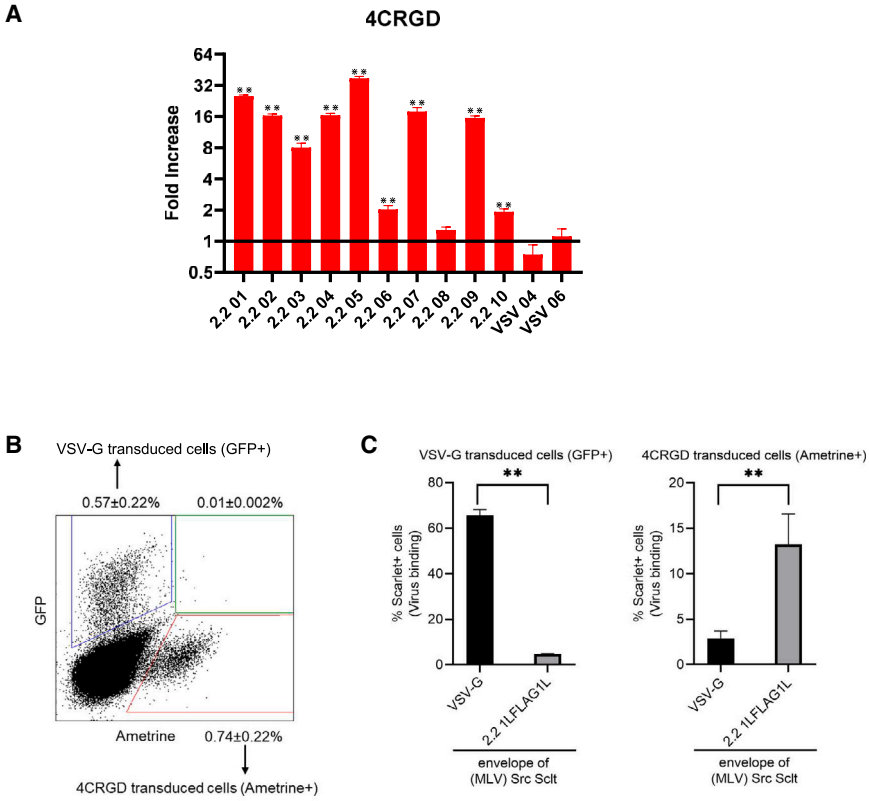


Figure 6. Antiviral BCRs mediate transduction of redirected lentiviral vectors

(A) Enhancement of 4CRGD pseudotype transduction of CD79 HEK293T cells by ectopic expression of BCRs isolated from splenic B cells transduced with the 2.2 1LFLAG1L or VSV-G pseudotype. Averages and SDs derived from three replicates are shown. The transduction efficiency of untransfected CD79 HEK293T cells with 4CRGD pseudotypes is 0.99% ± 0.2%. Significance was calculated by comparison with the transduction efficiency of untransfected CD79 HEK293T cells using a two-sample, two-sided, unpaired Student's t test (**$p < 0.01$). (B) Transduction of splenic B cells (C57BL6) with lentiviral vectors pseudotyped with VSV-G (GFP^+) or 4CRGD ($Ametrine^+$). Averages and SDs of the percentages transduced cells derived from 3 replicates are shown. (C) Binding of Scarlet-labeled MLV pseudovirions pseudotyped with VSV-G (VSV-G [MLV] Src Sclt) or 2.2 1LFLAG1L (2.2 1LFLAG1L [MLV] Src Sclt) to splenic B cells transduced with lentiviral vectors pseudotyped with VSV-G (GFP^+) or 4CRGD ($Ametrine^+$). Averages and SDs were derived from 3 replicates. Significance was calculated using a two-sample, two-sided, unpaired Student's t test (*$p < 0.01$). (D) Binding of MLV pseudovirions pseudotyped with 2.2 1LFLAG1L to splenic B cells transduced with 4CRGD-pseudotyped lentiviral vector in the absence or presence of goat anti-mouse Ig Fab domain or control goat Ab. Averages and SDs derived from three replicates are shown. Significance was calculated using a two-sample, two-sided, unpaired Student's t test ($p < 0.01$).

Our results suggest new opportunities to selectively transduce B cell subpopulations expressing BCRs against desired envelope proteins by pseudotyping the vectors with the envelope proteins of the target virus. For example, lentiviral vectors pseudotyped with EboZ will transduce B cells expressing anti-Ebola virus BCRs *in vivo*, which enables expression of antiviral effector molecules from the cells that can proliferate and differentiate in response to Ebola virus replication. It is also conceivable that epitopes might be engineered into vector particles to direct transduction based on B cell antigen specificity (e.g., conjugating 2.2 1LFLAG1L with target antigens, etc.)[17]

Beyond the general opportunity for BCR-specific vector targeting by engineered envelope proteins, the present results also identify key empirical opportunities for optimizing the biological impacts of this approach through strategic selection of envelope backbones. The 2.2 1LFLAG1L pseudotype transduces splenic B cells more efficiently than the Sindbis pseudotype, likely due to the abrogated binding of 2.2 1LFLAG1L to cognate receptors so that the 2.2 1LFLAG1L pseudotype can reach splenic B cells efficiently without being trapped by the liver and/or other cell types in the blood and spleen. This preferential tropism for splenic B cells may also increase the effective "multiplicity of binding, entry, and transduction" and thereby enhance BCR signaling, proliferation of transduced B cells, and their differentiation into memory B and plasma cells. Because the half-life of plasma cells and memory B cells can be as long as the life of the host, and B cells can induce tolerance to their transgene products, *in vivo* B cell transduction with the 2.2 1LFLAG1L pseudotype may have more durable biological effects than other pseudotypes.[22,46–49]

Our data also proposed a genetic strategy for isolating BCR/antibody sequences that encode neutralizing antibodies. Current protocols for mapping neutralizing antibodies by single-cell sequencing depend on immunization with viral antigens followed

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

by staining of BCRs with fluorescence-conjugated viral antigens and subsequent cell sorting.[50] This method cannot identify high-affinity antibodies from plasma cells due to the downregulation of BCRs on plasma cells. Our method uses integrated lentiviral reporter genes to identify antiviral B cells; therefore, such B cells can be identified after differentiation into plasma cells with antibody affinity maturation.

Our results indicated differentiation of transduced cells into long-lived plasma cells within 4 days of vector administration. It is conceivable that such rapid differentiation into long-lived plasma cells occurs when splenic B cells encounter replication-competent viruses. Because long-lived plasma cells downregulate BCRs, it is conceivable that rapid differentiation into long-lived plasma cells protects B cells capable of producing antiviral antibodies from antiviral BCR-mediated infection with viruses.

Given the vast diversity-generating mechanisms of BCR genomic rearrangement, it is conceivable that BCRs may recognize not only lentiviral vectors but also exterior features of other enveloped and non-enveloped viruses and viral vectors as well as other types of gene delivery vehicles and thereby facilitate their entry into B cells. Future research is needed to establish this potential and to map the breadth of the effects of BCR-mediated gene transduction/viral infection *in vivo* across a wide range of gene delivery vectors and viruses.

MATERIALS AND METHODS

Cells

Ramos, Raji, Daudi, Sp2/0, P3X63, Y3-Ag 1.2.3, HEK293T, and HEK293T-related cells were cultured in IMDM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Cytiva, Marlborough, MA, USA) and 1× pen/strep. CD79 HEK293T and CD79 Sp2/0 cells were generated by co-transduction of HEK293T cells with lentiviral vectors expressing human CD79A with a hygromycin-resistant gene and human CD79B with a puromycin-resistant gene, followed by culture in the presence of hygromycin (800 μg/mL, Thermo Fisher Scientific) and puromycin (2 μg/mL, Thermo Fisher Scientific). Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy normal donors and purchased from the UCLA CFAR Virology Core. PBMCs were cultured in B cell stimulation medium (IMDM supplemented with 10% FBS, 1× pen/strep, 500 IU/mL human interleukin-2 (IL-2; Shenandoah Biotech, Warminster, PA, USA), 50 ng/mL human IL-10 (Shenandoah Biotech), 10 ng/mL human IL-15 (Shenandoah Biotech), 50 nM 2-mercaptoethanol, 1 μg/mL ODN2006 (InvivoGen, San Diego, CA, USA), and 100 ng/mL human soluble CD40 ligand (Shenandoah Biotech).[38]

Plasmid cloning and construction

Primers and synthetic genes were ordered from Integrated DNA Technologies (Coralville, IA, USA). All constructs were made by the In-fusion kit (Takara Bio, San Jose, CA, USA) in general. pLV-EF1a-CD79A-IRES-Hygro was generated from pLV-EF1a-IRES-Hygro (Addgene, Watertown, MA, USA) by inserting human CD79A cDNA between the BamH1 and EcoR1 sites.[51] pLV-EF1a-CD79B-IRES-Puro was generated from pLV-EF1a-IRES-Hygro (Addgene) by inserting human CD79B cDNA between the BamH1 and EcoR1 sites. Zeocin hIgG1-mem was generated from pFUSEss-CHHIh-hG1 (InvivoGen) by inserting a cDNA sequence of the human IgM transmembrane and the cytoplasmic domains between the Nsi1 and Hpa1 sites. The expression vectors of membrane-anchored and soluble forms of immunoglobulin heavy chains were generated by inserting V regions of synthesized heavy-chain DNAs between the EcoR1 and Nhe1 sites of zeocin hIgG1-mem and pFUSEss-CHHIh-hG1, respectively. Expression vectors of immunoglobulin light chains were generated by inserting V regions of synthesized light-chain DNAs between the EcoR1 and BsiW1 sites of pFUSE2ss-CLIg-hk. cDNAs of single-chain antibodies of BCRs were designed by connecting the 3' end of the light-chain variable region sequences to a flexible linker (GGGGSX3), followed by the 5' end of the light-chain variable region sequences. To distinguish ScFv BCRs from endogenous BCRs when expressed on human B cells, we added a FLAG tag sequence at the 5' end of the ScFv sequences. The designed cDNAs were synthesized and inserted between the EcoR1 and Nhe1 sites of zeocin hIgG1-mem to generate ScFv BCRs. The ScFv BCRs sequences were then cloned between the BamH1 and Sal1 sites of cppt2e or the Age1 and Spe1 sites, pRRL MND GFP (Addgene), to generate lentiviral vectors expressing ScFv BCRs. The LumiScarlet, Scarlet, and Ametrine sequences were synthesized and inserted between the BamH1 and EcoR1 sites of FUGW to generate FULumiScltW, FUScltW, and FUAW, respectively.[52] The cDNA of Src Sclt was designed and synthesized by replacing the VPX sequence of Src-Pro-FLAG-VPX with the Scarlet sequence.[53] The Src Sclt expression vector was generated by replacing the VSV-G sequence of pCMV-VSV-G with synthesis of the Src Sclt sequence. 2.2 1LFLAG1L was generated by inserting the cDNA of the FLAG tag between the AVR2 and BstE2 sites of 2.2 1L1L.

Transfection and lentiviral and retroviral production

To generate lentiviral vectors, we performed transient transfection of HEK293T cells, using TransIT LT1 (MirusBio, Madison, WI, USA) according to the manufacturer's protocol. To generate regular lentiviral vectors, HEK293T cells (1.4×10^7) were transfected with one type of envelope protein expression vector (6–7 μg), packaging plasmid ps PAX2 (12–13 μg), and one type of lentiviral vector plasmid expressing the transgene of interest (12–13 μg). To generate lentiviral vectors treated with saquinavir, HEK293T cells were cultured in the presence of 3 μM saquinavir (NIH HIV Reagent Program, Manassas, VA, USA) after transfection. To generate lentiviral pseudovirions labeled with Scarlet, HEK293T cells (1.4×10^7) were transfected with one type of envelope protein expression vector (6–7 μg), packaging plasmid ps PAX2 (12–13 μg), and Intron2 Src Scarlet (0.5 μg). To generate the S protein-pseudotyped lentiviral vector containing β-lactamase and Src Scarlet, HEK293T cells (1.4×10^7) were transfected with HDM-SARS2-Spike-Δ21-D614G (8 μg), ps PAX2 (12–13 μg), FUGW (6–7 μg), pCMV4-BlaM-Vpr (3 μg), and Intron2 Src Scarlet (0.5 μg).[54] To generate MLV pseudovirions labeled with Scarlet, B16F10 cells (9×10^6) were transfected with one type of envelope

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

protein expression vector (6–7 μg), packaging plasmid pUMVC (12–13 μg), and Intron2 Src Scarlet (0.5 μg).[55] Three days post transfection, the supernatant was subjected to ultracentrifugation (20,000 rpm, 4°C, 2 h) with the SW32 rotor (Beckman Coulter Genomics, Brea, CA, USA), using PBS containing 20% sucrose as a cushion. The pellet containing the virus was resuspended in Hanks' buffered saline (100-fold concentration). The amounts of lentiviral vectors and lentiviral pseudotypes were quantitated by HIV p24 ELISA (XpressBio, Frederick, MD, USA), and the amounts of MLV pseudovirions were quantitated by fluorescent western blotting using a rabbit anti-murine leukemia virus p30 gag capsid polypeptide (Absolute Antibody, Wilton, UK) and anti-rabbit IgG StarBright 700 antibody (Bio-Rad, Hercules, CA, USA).

Generation of replication-deficient VSV

Replication-deficient VSV seed particles (VSVΔG-luc/GFP) pseudotyped with VSV-G were generated as described previously and generously provided by Dr. Michael Letko (Washington State University) and Dr. Vincent Munster (NIH).[35] To make subsequent seed particle stocks, HEK293T cells were transfected with a plasmid encoding VSV-G and infected 24 h later with replication-deficient VSV seed particles pseudotyped with VSV-G. Supernatant containing newly made seed particles was collected 24 h post infection, centrifuged to remove cell debris, and aliquoted and stored at −80°C until use.

Production of recombinant antibodies

Recombinant antibodies were produced by transfecting HEK293T cells with their expression vector plasmids using TransIT LT1. HEK293T cells (1.4×10^7) were transfected with one type of heavy-chain expression vector (12 μg) and one type of corresponding light-chain expression vector (18 μg). One day post transfection, cells were cultured with AIM-V medium supplemented with 1× pen/strep. The supernatants were collected and filtered 5 days post transfection. The supernatants were incubated with MabCapture A (Thermo Fisher Scientific) beads for 1 h at room temperature (RT). The beads were pelleted by centrifugation then washed in columns, and recombinant antibodies were eluted according to the manufacturer's protocol. The buffers of all purified proteins were changed to Tris-buffered solution (Thermo Fisher Scientific) using Zeba spin columns (Thermo Fisher Scientific).

In vivo transduction of splenic B cells by intravenous injection of lentiviral vectors

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was approved by the institutional Animal Care and Use Committees of the University of California, Los Angeles (protocol ARC-2022-033). For bioluminescence imaging and flow cytometry analysis of transduced splenic cells, lentiviral vectors expressing LumiScarlet (FUFlagLumiScltW) pseudotyped with the VSV-G, Sindbis, or 2.2 1LFLAG1L envelope protein were retro-orbitally injected into C57BL6 albino mice (female, 6 weeks old; The Jackson Laboratory, Bar Harbor, ME, USA). The virus amount was adjusted by HIV p24 amount (3 μg HIV p24 in 150 μL PBS). Four days post vector administration, we anesthetized the mice and intraperitoneally injected them with fluorofurimazine (8.8 mM 100 μL/mouse; Promega, Madison, WI, USA). CCCD images were obtained using a cooled IVIS charge-coupled device (CCD) camera (Xenogen, Canbury, NJ, USA) 0, 5, or 10 min after substrate injection. The data were analyzed with Living Image software (PerkinElmer) at the time point showing the maximum bioluminescence signals (predominantly 5–10 min post injection). After imaging, the mice were sacrificed, and the livers and spleens were isolated. The spleen cell suspensions were prepared by passing spleen tissue through a cell strainer (40 μm), followed by red blood cell lysis with LCK red blood cell lysis buffer, and cell numbers were counted by Logos LunaII. The liver and half of the spleen cells were lysed by tissue cell lysis buffer (Goldbio, St. Louis, MO, USA) containing a protease inhibitor cocktail (Thermo Fisher Scientific), using MACSdissociator (Miltenyi Biotec, Gaithersburg, MD, USA), followed by removal of cell debris by centrifugation. Bioluminescence activities in the supernatants were measured by an *in vitro* luciferase assay, and protein concentrations were measured using detergent-compatible Bradford assay (Thermo Fisher Scientific). An *in vitro* bioluminescence assay was conducted by mixing the same volume of 17.6 nM fluorofurimazine in NanoGlo buffer (Promega), using a GloMax 20/20 luminometer (Promega).[56] For flow cytometry analyses, cells were incubated with anti-CD16/CD32 (FcgRII-III, clone 2.4G2; eBioscience, San Diego, CA, USA) to reduce nonspecific staining. Transitional T1 and T2/T3 cells, follicular B cells, marginal zone B cells, and B-1 cells were resolved by flow cytometry after incubation with the appropriate combinations of the following antibodies: BV421-conjugated anti-mouse CD21, BV650-conjugated anti-mouse CD23, APC-conjugated anti-mouse CD19, and SuperBright 780-conjugated anti-mouse IgM antibodies.[22] Short- and long-lived plasma cells, GCA B cells, and memory B cells were resolved by flow cytometry after incubation with the appropriate combinations of the following antibodies: APC-conjugated anti-mouse B220, BV711-conjugated anti-mouse CD138, SuperBright 780-conjugated anti-mouse CD19, APC-conjugated anti-mouse GL7, BV421-conjugated anti-mouse CD38, BV650-conjugated anti-mouse IgD, and BV605-conjugated anti-mouse CD95 antibodies.[21] B, T, and natural killer (NK) cells as well as macrophages and conventional and plasmacytoid dendritic cells were resolved by flow cytometry after incubation with the appropriate combinations of the following antibodies (Abs): BV421-conjugated anti-mouse CD11c, SuperBright 780-conjugated anti-mouse F4/80, BV605-conjugated anti-mouse CD19, BV711-conjugated anti-mouse CD4, APC H7-conjugated anti-mouse B220, and APC-conjugated anti-mouse CD49b Abs. Flow cytometry analyses were performed on an Attune NxT flow cytometer (Thermo Fisher Scientific), and data were analyzed by FCS Express 5 (De Novo Software, Pasadena, CA, USA). Expression levels of Axl and TIM-1 in transduced splenic B cells were also investigated by staining splenocytes transduced with Scarlet-expressing the 2.2 1LFLAG1L pseudotype with BV711-conjugated anti-mouse Axl, Alexa 488-conjugated anti-mouse TIM-1, and APC-conjugated anti-mouse CD19 Abs. For histological analyses, GFP-expressing lentiviral vectors pseudotyped with either 2.2 1LFLAG1L or VSV-G (2 μg HIV p24 in 150 μL PBS) were

Please cite this article in press as: Takano et al., Envelope protein-specific B cell receptors direct lentiviral vector tropism *in vivo*, Molecular Therapy (2024), https://doi.org/10.1016/j.ymthe.2024.03.002

retro-orbitally injected into C57B6 mice (female, 6 weeks old; The Jackson Laboratory). Four days post injection, the mice were sacrificed, and their spleens were isolated and fixed in 10% formalin overnight. This was followed by rinsing with water, storage in 70% ethanol, and submission to the UCLA TPCL for making slides, H&E staining, and staining GFP, mouse CD3, and mouse B220. For double infection of GFP- and Scarlet-expressing lentiviral vectors, each type of lentiviral vector expressing LumiScarlet (FUFlagLumiScltW) and GFP (FUGW) pseudotyped with the VSV-G, 2.2 1LFLAG1L, EboZ envelope protein was retro-orbitally injected into C57BL6 mice (female, 6 weeks old). Both types of vectors were adjusted to 3 μg HIV p24 in 150 μL PBS. Four days post injection, the mice were sacrificed, and the splenic cells were stained and analyzed by flow cytometry as described above. For the virus binding assay, Ametrine-expressing lentiviral vectors (FUAW) pseudotyped with either 2.2 1LFLAG1L or 4CRGD and FUGW pseudotyped with either VSV-G or EboZ were co-injected into C57BL6 and BALB/c mice. Both types of vectors were adjusted to 3 μg HIV p24 in 150 μL PBS. Four days post-injection, the splenocytes were isolated as described above and stained with anti-CD3 APC, anti-CD19 BV650, and Scarlet-labeled MLV pseudovirions pseudotyped with either VSV-G, 2.2 1LFLAG1L, or EboZ in the presence or absence of either Fab'_2 goat anti-mouse IgG Fab'_2 or control goat IgG Fab'_2 (20 μg/mL) at RT for 1 h. Virus binding to B cells was analyzed by measuring $Scarlet^+$ populations in the $CD19^+CD3^-$ population.

Transduction of various types of B cells

Sp2/0, Y3-Ag 1.2.3, Ramos, Raji, and Daudi cells (1×10^5) were incubated with 200 μL of GFP-expressing lentiviral vector (FUGW) pseudotyped with VSV-G, Sindbis, or 2.2 1LFLAG1L at the virus concentrations shown in Figures 1A–1D and S3A–S3D (1,000, 100, or 10 ng HIV p24/mL). Three days post transduction, GFP expression was analyzed by flow cytometry. PBMCs from healthy donors were cultured for 3 days in B cell stimulation medium, and then the cells were transduced with 200 μL of various pseudotypes (FUGW) at 1,000 or 100 ng HIV p24/mL. Three days post transduction, the transduced cells were stained by BV421 conjugated anti-mouse CD19 Ab, and percentages of GFP-expressing cells in the CD19 populations were analyzed by flow cytometry.

Single-cell gene expression and BCR sequence analysis

The 2.2 1LFLAG1L-pseudotyped GFP-expressing lentiviral vector (FUGW) and VSV-G pseudotyped Scarlet-expressing lentiviral vector (FUScltW) were retro-orbitally co-injected into C57BL6 mice (female, 6 weeks old, 4 μg p24 for both vectors in 150 μL PBS). Four days post injection, we isolated splenocytes as described above and stained the cells with anti-CD19 APC Ab. B cells that were single positive for GFP or Scarlet or were untransduced B cells were sorted by the UCLA JCCC/CFAR Flow Cytometry Core. We also isolated splenic B cells from a mouse that did not receive any vector administration. The sorted cells were subjected to BCR sequencing using the Chromium Single Cell Mouse BCR amplification kit and gene expression sequencing using Chromium Next GEM Single Cell 5 reagent kit v.2 (10× Genomics, Pleasanton, CA, USA). The next-generation sequencing was done using NovaSeq (paired end 2 × 100 bp; Illumina, San Diego, CA, USA). 6,000 cells were subjected to sequencing. 200,000 and 5,000 reads per cells were analyzed for gene expression and BCR sequencing, respectively. Gene expression was analyzed by Loupe browser v.6.00, and the BCR sequence was analyzed by Loupe VDJ browser v.4.0.0 (10× Genomics).[57,58]

Functional analyses of BCRs as virus receptors

CD79 HEK293T cells (2×10^6) were transfected with light-chain (3 μg) and heavy-chain (2 μg) expression vectors of BCRs. One day post transfection, transfected cells (1×10^5 cells/well) were seeded into 48-well plates. Two days post transfection, the transfected cells were transduced with the 2.2 1LFLAG1L-pseudotyped GFP-expressing lentiviral vector (FUGW) (10 ng HIV p24/mL), VSV-G-pseudotyped Scarlet-expressing lentiviral vector (FUScltW) (0.25 ng HIV p24/mL), or 4CRGD-pseudotyped Ametrine-expressing lentiviral vector (FUAW) (75 ng HIV p24/mL). Two-days post virus transduction, cells were harvested and stained with Alexa 647 goat Fab anti-human IgG (Fc). GFP^+, $Scarlet^+$, or $Ametrine^+$ populations among Alexa 647^+ populations were analyzed by flow cytometry.

Binding of recombinant Abs to VSV-G and 2.2 1LFLAG1L

B16F10 (9×10^6) and HEK293T (1.4×10^7) cells were transfected with 24 μg of either expression vector of VSV-G, 2.2 1LFLAG1L, or control empty vector, together with FUAW, using TransIT LT1. Two days post transfection, cells were stained with recombinant Abs or human IgG1 isotype control Ab, followed by PE-conjugated Fab goat anti-human IgG Ab. The Ab binding to $Ametrine^+$ populations was analyzed by flow cytometry. To analyze binding of Scarlet-labeled HIV pseudovirions pseudotyped with VSV-G (40 ng HIV p24/mL) or 2.2 1LFLAG1L (400 ng HIV p24/mL), CD79 HEK293T cells were transfected with expression vectors of light and heavy chains of the negative control BCR, VSV 04 BCR, 2.2 08 BCR, or 2.2 10 BCR, as described above. Two days post transfection, cells were stained with either VSV-G- or 2.2 1LFLAG1L-pseudotyped pseudovirions together with Alexa 647 goat Fab anti-human IgG (Fc). $Scarlet^+$ populations among Alexa 647^+ populations were analyzed as virus binding by flow cytometry.

Transduction and signaling of ScFv BCR-expressing Ramos cells

Ramos cells were transduced with various ScFv BCR-expressing lentiviral vectors. The transduced cells were stained with APC-conjugated anti-FLAG Ab and sorted by the UCLA JCCC/CFAR Flow Cytometry Core. Ramos cells and ScFv BCR-expressing Ramos cells (1×10^5) were transduced with 200 μL of GFP-expressing lentiviral vector (FUGW) pseudotyped with VSV-G (5 ng HIV p24/mL) or 2.2 1LFLAG1L (1 μg HIV p24/mL). Two days post transduction, GFP expression was analyzed by flow cytometry. When investigating signaling upon virus binding, cells were incubated with control medium or lentiviral vectors pseudotyped with VSV-G or 2.2 1LFLAG1L (5 μg HIV p24/mL) for 1 h at 37°C. The cells were then fixed with 4% paraformaldehyde for 20 min at RT. The fixed cells were then permeabilized by 100% methanol (Thermo Fisher Scientific) on ice for

30 min, followed by staining with Alexa 647 mouse anti-Akt (pS473; BD Biosciences, Franklin Lakes, NJ, USA) or mouse anti-ERK1/2 (pT202/pY204, BD Biosciences) Abs.[36] When investigating the effects of BCR signaling inhibitors, Ramos cells or ScFv BCR-expressing Ramos cells were incubated with 100 nM ibrutinib or idelalisib for 30 min before and during incubation with virus.

Ectopic expression of human ScFv BCR on human PBMCs and transduction

Human PBMCs were cultured in B cell stimulation medium 3 days before transduction of ScFv BCR-expressing lentiviral vectors. Human PBMCs (4×10^6) were incubated with GALV-pseudotyped ScFv BCR-expressing lentiviral vectors (5 μg HIV p24 in 1.5 mL DMEM + 3% BSA) in 6-well plates. The plates were spun at 1,500 rpm for 30 min at RT and then incubated for 6 h. The cells were then cultured in B cell stimulation medium for 3 days. BCR expression was confirmed by flow cytometry after staining the cells with human TruStain FcX, BV421 anti-human IgD, KIRAVIA Blue 520 anti-human CD19, and mouse anti-FLAG IgG PE. The cells were then transduced with the GFP-expressing lentiviral vector (FUGW) pseudotyped with 2.2 1LFLAG1L and Scarlet-expressing lentiviral vector (FUScltW) pseudotyped with VSV-G for 2 h at 37°C. Cells were cultured for 2 days, harvested, and stained with human TruStain FcX, BV421 anti-human CD19, and anti-Flag APC Abs. GFP and Scarlet expression in $CD19^+$ and $FLAG^+$ populations was analyzed by flow cytometry.

Blocking vesicular stomatitis virus infection by recombinant Abs

HEK293T cells (1×10^5 cells/well) were seeded in a 24-well plate 1 day before infection. VSV expressing GFP and luciferase reporter genes (VSV-G/VSVΔG-luc/GFP) was incubated with 10, 1, or 0.1 μg/mL of recombinant Abs (2.2 05 and VSV 04 hIgG1), isotype control human IgG1 Abs, or control medium for 30 min at RT before transduction. HEK293T cells were incubated with VSV-G/VSVΔG-luc/GFP (250 μL/well, MOI 0.15) or an equivalent amount of the virus preincubated with Abs for 2 h at 37°C. One day post transduction, GFP expression was analyzed by flow cytometry.

DATA AND CODE AVAILABILITY

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2024.03.002.

ACKNOWLEDGMENTS

The luciferase and GFP reporter VSV was a kind gift from Dr. Michael Letko (Washington State University) and Dr. Vincent Munster (NIH). We thank the UCLA Neuroscience Genomics Core, UCLA AIDS Institute, and the James B. Pendleton Trust and McCarthy Foundation for support. We thank Drs. Benhur Lee, Sam Kung, Stephen Smale, Gene-Errol Ringpis, Joe DeYoung, and Enca Montecino for discussions and Ms. Wendy Aft for proofreading the manuscript. This work was supported by the UCLA AIDS Institute and the UCLA Center for AIDS Research, the James B. Pendleton Trust and McCarthy Foundation, and NIH grants R21AI095004 (to K.M.), R01AI108400 (to K.M.), R01AI145044 (to K.M.), U19AI149504 (to K.M.), 1R01EY032149-01 (to V.A.), 5U19AI125357-08 (to V.A.), 5R01AI163216-02 (to V.A.), and 1R01DK132735-01 (to V.A.).

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, T.-T.W., D.S.R., V.A., K.D., S.C., and K.M.; investigation, K.-A.T., A.A.L.W., R.B., K.S., B.A.C., A.E.A., T.T.P., D.S.R., S.C., and G.C.R.; resources, M.K., T.-T.W., V.A., S.C., and K.M.; writing, K.-A.T., A.A.L.W., R.B., K.S., B.A.C., G.C.R., T.-T.W., V.A., D.S.R., K.D., S.C., and K.M.; funding acquisition, T.-W.W., D.S.R., V.A., K.D., S.C., and K.M.

DECLARATION OF INTERESTS

K.M. holds 2 US patents (US patent numbers 8449875 and 9163248) related to this work.

REFERENCES

1. Morizono, K., and Chen, I.S.Y. (2011). Receptors and tropisms of envelope viruses. Curr. Opin. Virol. *1*, 13–18. https://doi.org/10.1016/j.coviro.2011.05.001.
2. Finkelshtein, D., Werman, A., Novick, D., Barak, S., and Rubinstein, M. (2013). LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA *110*, 7306–7311. https://doi.org/10.1073/pnas.1214441110.
3. Sandrin, V., Russell, S.J., and Cosset, F.L. (2003). Targeting retroviral and lentiviral vectors. Curr. Top. Microbiol. Immunol. *281*, 137–178.
4. Waehler, R., Russell, S.J., and Curiel, D.T. (2007). Engineering targeted viral vectors for gene therapy. Nat. Rev. Genet. *8*, 573–587.
5. Lévy, C., Verhoeyen, E., and Cosset, F.L. (2015). Surface engineering of lentiviral vectors for gene transfer into gene therapy target cells. Curr. Opin. Pharmacol. *24*, 79–85. https://doi.org/10.1016/j.coph.2015.08.003.
6. Bupp, K., and Roth, M.J. (2003). Targeting a retroviral vector in the absence of a known cell-targeting ligand. Hum. Gene Ther. *14*, 1557–1564.
7. Buchholz, C.J., Schneider, U., Devaux, P., Gerlier, D., and Cattaneo, R. (1996). Cell entry by measles virus: long hybrid receptors uncouple binding from membrane fusion. J. Virol. *70*, 3716–3723.
8. Nakamura, T., Peng, K.W., Harvey, M., Greiner, S., Lorimer, I.A.J., James, C.D., and Russell, S.J. (2005). Rescue and propagation of fully retargeted oncolytic measles viruses. Nat. Biotechnol. *23*, 209–214.
9. Kasahara, N., Dozy, A.M., and Kan, Y.W. (1994). Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. Science *266*, 1373–1376.
10. Strebinger, D., Frangieh, C.J., Friedrich, M.J., Faure, G., Macrae, R.K., and Zhang, F. (2023). Cell type-specific delivery by modular envelope design. Nat. Commun. *14*, 5141. https://doi.org/10.1038/s41467-023-40788-8.
11. Yu, B., Shi, Q., Belk, J.A., Yost, K.E., Parker, K.R., Li, R., Liu, B.B., Huang, H., Lingwood, D., Greenleaf, W.J., et al. (2022). Engineered cell entry links receptor biology with single-cell genomics. Cell *185*, 4904–4920.e22. https://doi.org/10.1016/j.cell.2022.11.016.
12. Morizono, K., Bristol, G., Xie, Y.M., Kung, S.K., and Chen, I.S. (2001). Antibody-directed targeting of retroviral vectors via cell surface antigens. J. Virol. *75*, 8016–8020.
13. Morizono, K., Pariente, N., Xie, Y., and Chen, I.S.Y. (2009). Redirecting lentiviral vectors by insertion of integrin-tageting peptides into envelope proteins. J. Gene Med. *11*, 549–558.

14. Morizono, K., Xie, Y., Helguera, G., Daniels, T.R., Lane, T.F., Penichet, M.L., and Chen, I.S.Y. (2009). A versatile targeting system with lentiviral vectors bearing the biotin-adaptor peptide. J. Gene Med. *11*, 655–663.
15. Morizono, K., Xie, Y., Ringpis, G.E., Johnson, M., Nassanian, H., Lee, B., Wu, L., and Chen, I.S.Y. (2005). Lentiviral vector retargeting to P-glycoprotein on metastatic melanoma through intravenous injection. Nat. Med. *11*, 346–352.
16. Pariente, N., Morizono, K., Virk, M.S., Petrigliano, F.A., Reiter, R.E., Lieberman, J.R., and Chen, I.S.Y. (2007). A novel dual-targeted lentiviral vector leads to specific transduction of prostate cancer bone metastases in vivo after systemic administration. Mol. Ther. *15*, 1973–1981.
17. Situ, K., Chua, B.A., Bae, S.Y., Meyer, A.S., and Morizono, K. (2018). Versatile targeting system for lentiviral vectors involving biotinylated targeting molecules. Virology *525*, 170–181. https://doi.org/10.1016/j.virol.2018.09.017.
18. Wang, K.S., Kuhn, R.J., Strauss, E.G., Ou, S., and Strauss, J.H. (1992). High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. J. Virol. *66*, 4992–5001.
19. Byrnes, A.P., and Griffin, D.E. (1998). Binding of Sindbis virus to cell surface heparan sulfate. J. Virol. *72*, 7349–7356.
20. Yeh, H.W., Xiong, Y., Wu, T., Chen, M., Ji, A., Li, X., and Ai, H.W. (2019). ATP-Independent Bioluminescent Reporter Variants To Improve in Vivo Imaging. ACS Chem. Biol. *14*, 959–965. https://doi.org/10.1021/acschembio.9b00150.
21. Barber, C.L., Montecino-Rodriguez, E., and Dorshkind, K. (2011). Developmental relationships between B-1 and B-2 progenitors. Cell Cycle *10*, 3810–3811. https://doi.org/10.4161/cc.10.22.18190.18190.
22. Jones, D.D., Wilmore, J.R., and Allman, D. (2015). Cellular Dynamics of Memory B Cell Populations: IgM+ and IgG+ Memory B Cells Persist Indefinitely as Quiescent Cells. J. Immunol. *195*, 4753–4759. https://doi.org/10.4049/jimmunol.1501365.jimmunol.1501365.
23. Kaplan, A.H., Krogstad, P., Kempf, D.J., Norbeck, D.W., and Swanstrom, R. (1994). Human immunodeficiency virus type 1 virions composed of unprocessed Gag and Gag-Pol precursors are capable of reverse transcribing viral genomic RNA. Antimicrob. Agents Chemother. *38*, 2929–2933. https://doi.org/10.1128/AAC.38.12.2929.
24. Humphrey, R.W., Ohagen, A., Davis, D.A., Fukazawa, T., Hayashi, H., Höglund, S., Mitsuya, H., and Yarchoan, R. (1997). Removal of human immunodeficiency virus type 1 (HIV-1) protease inhibitors from preparations of immature HIV-1 virions does not result in an increase in infectivity or the appearance of mature morphology. Antimicrob. Agents Chemother. *41*, 1017–1023. https://doi.org/10.1128/AAC.41.5.1017.
25. Rabi, S.A., Laird, G.M., Durand, C.M., Laskey, S., Shan, L., Bailey, J.R., Chioma, S., Moore, R.D., and Siliciano, R.F. (2013). Multi-step inhibition explains HIV-1 protease inhibitor pharmacodynamics and resistance. J. Clin. Invest. *123*, 3848–3860. https://doi.org/10.1172/JCI67399.
26. Borges da Silva, H., Fonseca, R., Pereira, R.M., Cassado, A.D.A., Álvarez, J.M., and D'Império Lima, M.R. (2015). Splenic Macrophage Subsets and Their Function during Blood-Borne Infections. Front. Immunol. *6*, 480. https://doi.org/10.3389/fimmu.2015.00480.
27. Morizono, K., and Chen, I.S.Y. (2014). Role of phosphatidylserine receptors in enveloped virus infection. J. Virol. *88*, 4275–4290. https://doi.org/10.1128/JVI.03287-13.
28. Morizono, K., Xie, Y., Olafsen, T., Lee, B., Dasgupta, A., Wu, A.M., and Chen, I.S.Y. (2011). The Soluble Serum Protein Gas6 Bridges Virion Envelope Phosphatidylserine to the TAM Receptor Tyrosine Kinase Axl to Mediate Viral Entry. Cell Host Microbe *9*, 286–298. https://doi.org/10.1016/j.chom.2011.03.012.
29. Ai, H.W., Hazelwood, K.L., Davidson, M.W., and Campbell, R.E. (2008). Fluorescent protein FRET pairs for ratiometric imaging of dual biosensors. Nat. Methods *5*, 401–403. https://doi.org/10.1038/nmeth.1207.
30. Diehl, W.E., Lin, A.E., Grubaugh, N.D., Carvalho, L.M., Kim, K., Kyawe, P.P., McCauley, S.M., Donnard, E., Kucukural, A., McDonel, P., et al. (2016). Ebola Virus Glycoprotein with Increased Infectivity Dominated the 2013-2016 Epidemic. Cell *167*, 1088–1098.e6. https://doi.org/10.1016/j.cell.2016.10.014.
31. Li, L., Jose, J., Xiang, Y., Kuhn, R.J., and Rossmann, M.G. (2010). Structural changes of envelope proteins during alphavirus fusion. Nature *468*, 705–708. https://doi.org/10.1038/nature09546.
32. Lin, J., and Justement, L.B. (1992). The MB-1/B29 heterodimer couples the B cell antigen receptor to multiple src family protein tyrosine kinases. J. Immunol. *149*, 1548–1555.
33. Hoffmann, D., Bayer, W., and Wildner, O. (2007). Therapeutic immune response induced by intratumoral expression of the fusogenic membrane protein of vesicular stomatitis virus and cytokines encoded by adenoviral vectors. Int. J. Mol. Med. *20*, 673–681.
34. Fu, M., Fan, P.S., Li, W., Li, C.X., Xing, Y., An, J.G., Wang, G., Fan, X.L., Gao, T.W., Liu, Y.F., and Ikeda, S. (2007). Identification of poly-reactive natural IgM antibody that recognizes late apoptotic cells and promotes phagocytosis of the cells. Apoptosis. *12*, 355–362. https://doi.org/10.1007/s10495-006-0581-z.
35. Letko, M., Marzi, A., and Munster, V. (2020). Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nat. Microbiol. *5*, 562–569. https://doi.org/10.1038/s41564-020-0688-y.
36. Wolf, C., Maus, C., Persicke, M.R.O., Filarsky, K., Tausch, E., Schneider, C., Döhner, H., Stilgenbauer, S., Lichter, P., Höfer, T., and Mertens, D. (2022). Modeling the B-cell receptor signaling on single cell level reveals a stable network circuit topology between nonmalignant B cells and chronic lymphocytic leukemia cells and between untreated cells and cells treated with kinase inhibitors. Int. J. Cancer *151*, 783–796. https://doi.org/10.1002/ijc.34112.
37. Honigberg, L.A., Smith, A.M., Sirisawad, M., Verner, E., Loury, D., Chang, B., Li, S., Pan, Z., Thamm, D.H., Miller, R.A., and Buggy, J.J. (2010). The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. Proc. Natl. Acad. Sci. USA *107*, 13075–13080. https://doi.org/10.1073/pnas.1004594107.
38. Ikeda, H., Hideshima, T., Fulciniti, M., Perrone, G., Miura, N., Yasui, H., Okawa, Y., Kiziltepe, T., Santo, L., Vallet, S., et al. (2010). PI3K/p110delta is a novel therapeutic target in multiple myeloma. Blood *116*, 1460–1468. https://doi.org/10.1182/blood-2009-06-222943.
39. Schebesta, M., Heavey, B., and Busslinger, M. (2002). Transcriptional control of B-cell development. Curr. Opin. Immunol. *14*, 216–223. https://doi.org/10.1016/s0952-7915(02)00324-2.
40. Gerdes, J., Schwab, U., Lemke, H., and Stein, H. (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer *31*, 13–20. https://doi.org/10.1002/ijc.2910310104.
41. Celis, J.E., Bravo, R., Larsen, P.M., and Fey, S.J. (1984). Cyclin: a nuclear protein whose level correlates directly with the proliferative state of normal as well as transformed cells. Leuk. Res. *8*, 143–157. https://doi.org/10.1016/0145-2126(84)90135-8.
42. Roost, H.P., Bachmann, M.F., Haag, A., Kalinke, U., Pliska, V., Hengartner, H., and Zinkernagel, R.M. (1995). Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. Proc. Natl. Acad. Sci. USA *92*, 1257–1261. https://doi.org/10.1073/pnas.92.5.1257.
43. Ochsenbein, A.F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., and Zinkernagel, R.M. (1999). Control of early viral and bacterial distribution and disease by natural antibodies. Science *286*, 2156–2159. https://doi.org/10.1126/science.286.5447.2156.
44. Rothaeusler, K., and Baumgarth, N. (2010). B-cell fate decisions following influenza virus infection. Eur. J. Immunol. *40*, 366–377. https://doi.org/10.1002/eji.200939798.
45. Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L., Herzenberg, L.A., and Herzenberg, L.A. (1999). Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. Proc. Natl. Acad. Sci. USA *96*, 2250–2255. https://doi.org/10.1073/pnas.96.5.2250.
46. Andraud, M., Lejeune, O., Musoro, J.Z., Ogunjimi, B., Beutels, P., and Hens, N. (2012). Living on three time scales: the dynamics of plasma cell and antibody populations illustrated for hepatitis a virus. Plos Comput. Biol. *8*, e1002418. https://doi.org/10.1371/journal.pcbi.1002418.PCOMPBIOL-D-11-01013 [pii].
47. Wang, X., Herzog, R.W., Byrne, B.J., Kumar, S.R., Zhou, Q., Buchholz, C.J., and Biswas, M. (2017). Immune Modulatory Cell Therapy for Hemophilia B Based on CD20-Targeted Lentiviral Gene Transfer to Primary B Cells. Mol. Ther. Methods Clin. Dev. *5*, 76–82. https://doi.org/10.1016/j.omtm.2017.03.005.S2329-0501(17)30050-5.
48. Skupsky, J., Zhang, A.H., Su, Y., and Scott, D.W. (2010). B-cell-delivered gene therapy induces functional T regulatory cells and leads to a loss of antigen-specific effector

cells. Mol. Ther. *18*, 1527–1535. https://doi.org/10.1038/mt.2010.95.S1525-0016(16)31104-2.

49. Wang, X., Moghimi, B., Zolotukhin, I., Morel, L.M., Cao, O., and Herzog, R.W. (2014). Immune tolerance induction to factor IX through B cell gene transfer: TLR9 signaling delineates between tolerogenic and immunogenic B cells. Mol. Ther. *22*, 1139–1150. https://doi.org/10.1038/mt.2014.43.

50. Scheid, J.F., Barnes, C.O., Eraslan, B., Hudak, A., Keeffe, J.R., Cosimi, L.A., Brown, E.M., Muecksch, F., Weisblum, Y., Zhang, S., et al. (2021). B cell genomics behind cross-neutralization of SARS-CoV-2 variants and SARS-CoV. Cell *184*, 3205–3221.e24. https://doi.org/10.1016/j.cell.2021.04.032.

51. Hayer, A., Shao, L., Chung, M., Joubert, L.M., Yang, H.W., Tsai, F.C., Bisaria, A., Betzig, E., and Meyer, T. (2016). Engulfed cadherin fingers are polarized junctional structures between collectively migrating endothelial cells. Nat. Cell Biol. *18*, 1311–1323. https://doi.org/10.1038/ncb3438.

52. Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868–872.

53. Durand, S., Nguyen, X.N., Turpin, J., Cordeil, S., Nazaret, N., Croze, S., Mahieux, R., Lachuer, J., Legras-Lachuer, C., and Cimarelli, A. (2013). Tailored HIV-1 vectors for genetic modification of primary human dendritic cells and monocytes. J. Virol. *87*, 234–242. https://doi.org/10.1128/JVI.01459-12.

54. Crawford, K.H.D., Eguia, R., Dingens, A.S., Loes, A.N., Malone, K.D., Wolf, C.R., Chu, H.Y., Tortorici, M.A., Veesler, D., Murphy, M., et al. (2020). Protocol and Reagents for Pseudotyping Lentiviral Particles with SARS-CoV-2 Spike Protein for Neutralization Assays. Viruses *12*, 513. https://doi.org/10.3390/v12050513.

55. Stewart, S.A., Dykxhoorn, D.M., Palliser, D., Mizuno, H., Yu, E.Y., An, D.S., Sabatini, D.M., Chen, I.S.Y., Hahn, W.C., Sharp, P.A., et al. (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA *9*, 493–501. https://doi.org/10.1261/rna.2192803.

56. Su, Y., Walker, J.R., Park, Y., Smith, T.P., Liu, L.X., Hall, M.P., Labanieh, L., Hurst, R., Wang, D.C., Encell, L.P., et al. (2020). Novel NanoLuc substrates enable bright two-population bioluminescence imaging in animals. Nat. Methods *17*, 852–860. https://doi.org/10.1038/s41592-020-0889-6.

57. Laidlaw, B.J., and Cyster, J.G. (2021). Transcriptional regulation of memory B cell differentiation. Nat. Rev. Immunol. *21*, 209–220. https://doi.org/10.1038/s41577-020-00446-2.

58. Kennedy, D.E., Okoreeh, M.K., Maienschein-Cline, M., Ai, J., Veselits, M., McLean, K.C., Dhungana, Y., Wang, H., Peng, J., Chi, H., et al. (2020). Novel specialized cell state and spatial compartments within the germinal center. Nat. Immunol. *21*, 660–670. https://doi.org/10.1038/s41590-020-0660-2.