In vivo imaging of retrovirus infection reveals a role for Siglec-1/CD169 in multiple routes of transmission

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Abstract

Early events in retrovirus transmission are determined by interactions between incoming viruses and frontline cells near entry sites. Despite their importance for retroviral pathogenesis, very little is known about these events. We developed a bioluminescence imaging (BLI)-guided multiscale imaging approach to study these events in vivo. Engineered murine leukemia reporter viruses allowed us to monitor individual stages of the retrovirus life cycle including virus particle flow, virus entry into cells, infection and spread for retroorbital, subcutaneous and oral routes. BLI permitted temporal tracking of orally administered retroviruses along the gastrointestinal tract as they traversed the lumen through Peyer’s Patch to reach the draining mesenteric sac. Importantly, capture and acquisition of lymph-, blood- and milk-borne retroviruses spanning three routes, was promoted by a common host factor, the I-type lectin CD169, expressed on sentinel macrophages. These results highlight how retroviruses co-opt the immune surveillance function of tissue resident sentinel macrophages for establishing infection.

Keywords: Retrovirus transmission, oral route, bioluminescence imaging, CD169
Introduction

Retroviruses cause cancer and immunodeficiencies [1]. Once retroviruses establish viral reservoirs, it is difficult to eliminate infection as retroviral genomes are permanently integrated into host DNA. Despite the clinical relevance of early processes by which incoming retroviruses establish infection by navigating complex host tissue architecture en route to their first targets, little is known about these events [2-4]. Retroviruses like the human immunodeficiency virus (HIV-1) can enter through the vaginal and rectal mucosa during sexual transmission, orally via milk during mother-to-child transmission, subcutaneously and intravenously through needle stick injections during drug use and blood transfusions [2, 5]. The majority of the Murine leukemia viruses (MLV) transmission in mice occur vertically from dam-to-pup via ingestion of virus-containing milk through the gastrointestinal tract. MLV transmission can also occur parenterally between male mice during infighting and via the venereal route between infected male and female mice [6, 7]. Entry via different routes requires retroviruses to navigate diverse host tissue architecture and overcome barriers for successful infection [8-10]. Whether retroviruses exploit common host factors across these transmission routes remain to be clarified.

We have previously used MLV as a model retrovirus to understand how retroviruses establish infection in mice through the lymph or blood following subcutaneous and intravenous delivery respectively [11, 12]. We found that sentinel macrophages lining blood/lymph-tissue interfaces such as the subcapsular sinus in lymph nodes or the marginal zones in the spleen function to filter out incoming retroviruses from circulation [11, 12]. The “fly-paper” like activity of sentinel macrophages have been observed for various incoming viruses and pathogens [13-15]. The frontline position of sentinel macrophages allows them to orchestrate downstream innate, cell-mediated, and humoral immune responses to incoming pathogens in the lymph and blood [8, 12, 16-18]. These macrophages naturally express the I-type lectin Siglec-1/CD169 that specifically interact with sialosides present on retroviral envelopes [11, 19, 20]. CD169 expression allows sentinel macrophages to capture retroviruses and limits their dissemination [12]. However,
retroviruses like MLV and HIV-1 exploit their CD169 to promote infection of target lymphocytes that sample antigens captured by sentinel macrophages [11, 12, 21]. Whether the observed roles for CD169+ macrophages following subcutaneous and intravenous delivery, are of any relevance for natural mother-to-offspring transmission when viruses enter via the gastrointestinal (GI) tract, has remained unknown.

Given the complexity, functional diversity and length of the entire GI tract that can measure over >7 cm even in neonatal mice, identifying portals of entry such as Peyer’s Patch (PP) in underdeveloped intestines can be very challenging [22]. Here, we developed a whole-body bioluminescence imaging (BLI)-based approach to illuminate areas where MLV concentrates to traverse into the gut tissue from the lumen. We implemented BLI by developing a series of MLV-based reporter viruses to enable observation of specific stages of the retrovirus lifecycle in vivo, including viral particle flow, entry into cytoplasm, first infection events, and spread. We first validated this system by testing its ability to uncover new insights into previously studied subcutaneous and intravenous transmission routes. Second, BLI-imaging permitted temporal tracking of various steps of virus infection for orally administered retroviruses along the gastrointestinal tract as they traversed the lumen through the PP to reach the draining mesenteric sac. Finally, we show that capture and acquisition of lymph-, blood- and milk-borne retroviruses spanning three routes, was promoted by a common host factor, CD169 expressed on sentinel macrophages. Our results highlight how retroviruses co-opt the immune surveillance function of tissue resident sentinel macrophages for establishing infection. Understanding these events will inform design improved prophylactic strategies that target prevention of virus acquisition and establishment of infection.

Results
Generation of reporter viruses to enable visualization of individual stages of retrovirus infection in vivo

We established a BLI-directed approach for studying individual stages of retroviral infection in vivo by strategically inserting reporters into unique genomic loci of MLV (Figure 1A). To track virus particle flow, we generated bioluminescent virus particles by introducing nanoluciferase (Nluc) into the proline-rich region (PRR) of the MLV envelope (Env). Nluc-Env-tagged virus particles produced using a tripartite plasmid system (encoding Nluc-tagged MLV Env, MLV Gag-Pol and MLV-LTR) exhibited 200 times more luciferase activity per virus particle (0.2 RLU/virion) compared to viruses generated using the full-length MLV genome (0.001 RLU/virion) (Figure 1B).

To monitor virus fusion and entry into the cytoplasm of cells, we packaged firefly luciferase (Fluc) within the virus capsid as a C-terminus fusion of MLV Gag (MLV Gag-Fluc Env\textsubscript{wt}) and exploited the ATP-dependence of Fluc for its activity that is restricted to the host cell cytoplasm in vivo. This strategy ensured that Fluc activity was exhibited when both detergent (Triton X-100) and ATP were present (Figure 1C). A Gag-Fluc labeled virus in which MLV envelope was replaced by the fusion-defective SFFV gp55 envelope (MLVGag-Fluc Env\textsubscript{FD}) served as a negative control.

To monitor single-round virus transduction, we utilized a replication-defective virus generated by co-transfecting a dual BLI and GFP reporter (pMIG-Nluc-IRES-GFP) in conjunction with MLV Gag-Pol and Env [23]. In addition, we generated red-shifted reporter viruses encoding Antares, which is a bioluminescence resonance energy transfer (BRET) reporter that enables superior deep-tissue sensitivity over Nluc in vivo [24]. Finally, to permit longitudinal monitoring of progressing infection, we generated replication competent MLV reporter viruses by introducing a shortened internal ribosome entry site (IRES), 6ATRI, to drive Nluc expression downstream of viral Env [23, 25, 26]. This strategy enabled bi-cistronic expression of Env and Nluc in infected cells. Infectivity measurements revealed that MLV 6ATRI Nluc was ~ 60 % as infectious as wild-type MLV (Figure 1D).
BLI-driven characterization of blood-borne retrovirus infection

We revisited the previously studied intravenous (i.v.) route of MLV infection at a whole-body level by applying these new reporter viruses. We challenged mice retroorbitally (r.o.) with MLV Env-Nluc reporter viruses. BLI-driven virus tracking immediately after challenge showed that MLV rapidly reached both liver and spleen within 30 s after administration and accumulated predominantly at the spleen after passing through the liver (2-3 min) (Figure 2A, B; Video 1). This was consistent with the spleen being the main blood-filtering organ in mice. Interestingly, the decaying luminescence over time (>30 min) in the spleen was revived when Nluc substrate (furimazine) was re-administered (Figure 2-figure supplement 1). This indicated that viruses remain captured at the spleen and the exhaustion of substrate contributed to the decay in the signal. We next investigated virus entry into the host cell cytoplasm by utilizing MLV Gag-Fluc-tagged viruses with wild-type (EnvWT) or fusion-defective Env (EnvFD). Inocula were equalized by measuring the luciferase activity (relative light units; RLU) in detergent-lysed viral preparations (Figure 2C). Mice challenged with MLV (r.o.) were monitored at 3 min, 40 min, and 1 h post-infection (hpi) using BLI. In contrast to animals infected with fusion defective MLV, we observed Fluc signal emerging at the spleen (1 hpi) in animals infected with reporter viruses carrying wild type MLV envelope (Figure 2D, E; p=0.0061). Taken together, our data indicated that blood borne MLV was filtered rapidly at the spleen within 2-3 minutes and entered the cytoplasm of cells by 60 minutes after capture.

We next infected mice with WT FrMLV or MLV 6ATRi-Nluc reporter virus and compared infection levels at 7 dpi. In vivo infectivity of MLV 6ATRi-Nluc virus was reduced in comparison to WT FrMLV (Figure 2F). This was not unexpected based on the reduced released infectivity of MLV 6ATRi-Nluc in vitro (Figure 1D), and the known effect of genomic reporter insertions on retrovirus fitness (17). To visualize the first round of infected cells and virus spread at the whole animal level, we challenged mice with single-round MLV reporter virus (pMIG-Antares) as well as replication-competent reporter MLV, and monitored replication dynamics using BLI every 2-3 days.
over the course of two weeks (Figure 2G). In contrast to the decline of luminescent signal observed with single-round Antares-encoding MLV, luminescent signal in organs infected by MLV 6ATRi-Nluc increased over time, indicative of fresh rounds of infection (Figure 2G, H). We observed infection in auricular and inguinal LN in addition to the spleen (Figure 2G, H). The gradual decline of luminescent signal in mice infected with MLV 6ATRi-Nluc is consistent with the immune control of MLV infection in C57BL/6J (B6) mice [11, 12, 21, 27, 28]. Taken together, these results demonstrate the utility of our bioluminescent reporter viruses in monitoring particle flow, capture, cytoplasmic entry, transduction, and subsequent virus spread following intravenous infection of mice. The validation of this reporter system also set the stage for applications to other infection routes.

**BLI-driven characterization of lymph-borne retrovirus infection**

Intrafootpad (i.f.p.) infection is widely used to study subcutaneous (s.c.) infection and model antigen trafficking to draining lymph nodes via lymphatics [29, 30]. The draining lymph node for i.f.p. infection is the popliteal lymph node (pLN) [14, 15, 31]. Previous studies using two-photon intravital microscopy indicated that incoming viruses accumulated at the subcapsular sinus of the pLN within a few minutes following viral challenge [11, 14, 15, 32]. We revisited this well-studied route using BLI imaging of incoming virus particle flow from the administration site to the target organ by infecting mice i.f.p. with MLV Env-Nluc. Incoming viruses accumulated rapidly at the pLN, with detectable signal occurring within 1 minute 30 seconds p.i. (Figure 3A, B; Video 2). This was indicative of lymph flow-mediated dissemination of MLV to pLN and was consistent with previous multiphoton microscopy studies [11]. However, we observed that most of the incoming virus particles localized to the injection site at the footpad (Figure 3A, B). Quantification of virus particle accumulation in the footpad and pLN, displayed as photon flux (photons/sec), revealed that virus accumulation in the footpad was over 200-fold higher than that in the pLN. This observation remained constant for the entire imaging window. Even when virus eventually...
accumulated in the pLN, plateauing at ~9 minutes pi., the level of virus in the footpad remained ~150-fold above that of the pLN (Figure 3A, B). Thus, our imaging analyses showed that only a small fraction of incoming viruses surpass tissue barriers including collagen fibrils, muscle tissue, and antigen-capturing cells at the footpad to reach the draining site.

We next explored the location where viruses fuse and gain access to the host cell cytoplasm. We challenged mice with MLV Gag-Fluc EnvWT or MLV Gag-Fluc EnvFD via the i.f.p. route and monitored these events by BLI. Bioluminescent signal in the mouse footpads infected with MLV Gag-Fluc EnvFD did not increase over time (Figure 3C, D). In contrast, luciferase activity in footpads challenged with MLV Gag-Fluc EnvWT increased over time, indicating progressive virus access to cell cytoplasm in vivo (Figure 3C, D). We observed that fusion-competent viruses gained access to the host cell cytoplasm in the footpad as early as 3 minutes post-challenge (Figure 3C, D). Despite accumulation of virions in the pLN within minutes following challenge (Figure 3A, B), we were unable to detect Fluc activity in the first 40 minutes of the continuous imaging time frame (Figure 3C, D). However, viruses had gained cytoplasmic access in the pLN by 6 hpi (Figure 3C, D). The delay was likely due to the mode of infection at the pLN, where MLV was first captured by CD169 macrophages before trans-infection of permissive B-1a cells [21, 27].

Next, we infected mice subcutaneously (s.c.) in the footpads with MLV 6ATRi-Nluc or WT MLV, harvested pLN at 3, 5, 7, 9, or 11 dpi, and assessed the number of infected cells in individual pLN by flow cytometry using antibodies to MLV GlycoGag. WT MLV exhibited an expected infection profile in vivo, peaking at 5 dpi and subsequently decreasing due to immune control (Figure 3F). As in r.o. infection (Figure 2F), MLV 6ATRi-Nluc replicated with slower infection kinetics compared to those of WT MLV and peaked at 7-8 dpi instead of 5 dpi (Figure 3E, F). We also confirmed luciferase activity resulting from single cell suspensions isolated from pLN isolated from mice infected with MLV 6ATRi-Nluc at different time points (Figure 3G). Luciferase activity mirrored infection curves obtained by flow cytometric enumeration of infected cells using
antibodies to MLV GlycoGag. In contrast to r.o. challenge, virus infection during s.c. challenge was restricted to the draining pLN and new infection events were not observed beyond the target organ before elimination by mounting immune responses in resistant B6 mice (Figure 3E and H). These data are consistent with our earlier work describing how capture by CD169+ macrophages at pLN limits systemic spread and initiates effective immune responses [12, 33].

**CD169+ macrophages contribute to virus capture in the footpad**

We next characterized the cell types that capture MLV at the footpad by challenging mice with MLV Gag-GFP particles (i.f.p) (Figure 4A). Surprisingly, immunostaining of footpad cryosections (15 min post challenge) revealed that in addition to CD11c+ DCs, CD169+ macrophages also reside in the footpad and predominantly captured MLV (Figure 4A). In many cases, we observed virus-capturing CD169+ macrophages in close contact with DCs, indicative of synaptic cell-cell contacts (Figure 4A). Electron tomography revealed the presence of virus-laden macrophages with viruses present in membrane invaginations, as well as tethered to plasma membranes, suggestive of CD169-mediated capture (Figure 4A, figure 4-supplement 1, Video 3). We also observed viruses within cell-cell contacts between virus-capturing macrophages and DCs (Figure 4B, C). Quantification of cell-associated viruses in tomographic sections were consistent with immunostaining data (Figure 4A) and revealed that incoming viruses predominantly associated with macrophages (Figure 4- figure 4 supplement 1) and to a lesser extent with DCs.

**BLI-driven characterization of oral route of retrovirus transmission**

We utilized the capacity of BLI to pinpoint events of interest in extensive organs like the gastrointestinal tract, by characterizing individual steps in the less understood oral route of MLV transmission. To study mother-to-offspring transmission, we infected a lactating dam in the mammary glands (s.c.) with WT MLV carrying a co-packaged Fluc reporter driven from the viral LTR and allowed the infection to establish itself for 6 days. BLI confirmed the presence of
luminescent signal in infected teats at 6 dpi (Figure 5A). Subsequent transfer of neonatal mice (1-3 days old) for fostering resulted in successful transmission of MLV as seen by luciferase-positivity in the gastrointestinal regions at 2 days post-transfer (dpt) (Figure 5A). Electron tomography of neonatal stomach contents at 3 dpt, revealed cell-free viruses in milk (Figure 5B), congruent with studies of mother-to-offspring transmission of cell free MMTV to suckling pups [34, 35]. Due to the internal location and convoluted nature of the GI tract, necropsy was required for revealing anatomic details of the infected regions. BLI analyses of GI tracts from neonates fostered by infected dams revealed that viruses had established infection in the PP and mesenteric sacs by 8 dpt (Figure 5C).

To explore the dynamics of virus transit in the intestinal tract, we orally inoculated 3-day old mice with replication-defective MLV Env-Nluc particles and analyzed their distribution at 12, 24, or 48 hpi via BLI. We observed a sequential movement of virus particles from the stomach at 12 hpi to a striking, temporal accumulation in PP and mesenteric sacs of the small intestine (Figure 6A, B). There was a significant and simultaneous increase in particle accumulation between 24 to 48 hpi at both PP and mesenteric sac (Figure 6B). These results indicated that incoming particles from PP can reach the draining mesenteric sac via the lymph drainage without first undergoing replication. There was less frequent accumulation of incoming particles in cecal patches, likely because they are downstream of PP in the direction of intestinal traffic. Our data reveal that PP, followed by mesenteric sacs, were the earliest intestinal structures to accumulate incoming MLV.

To determine the tissues sites where incoming viruses entered the cytoplasm of intestinal target cells, we orally infected neonatal mice MLV Gag-Fluc EnvWT or MLV Gag-Fluc EnvFD, which served as a control. Fluc signal was first observed in PP (Figure 6C). We saw a significant temporal increase in access to the cytoplasm of target cells of PPs by incoming MLV Gag-Fluc EnvWT viruses compared to the fusion-deficient MLV Gag-Fluc EnvFD control viruses (Figure 6D). However, Fluc activity at the mesenteric sac began to increase weakly over control particles only...
by 48 hpi (Figure 6C, E), despite early particle accumulation (Figure 6B). Delayed cytoplasmic access was a recurring theme, suggestive of the antigen presenting cell-mediated trans-infection process, as seen in pLN, also effective in mesenteric LNs. Finally, the use of single round (MLV Nluc-IRES-GFP) and replication competent viruses (MLV 6ATRi-Nluc) confirmed true virus infection events at both PP and mesenteric sacs at 96 hpi by BLI (Figure 6F, G). Infection levels, measured by flux, were expectedly lower in PPs compared to the draining mesenteric sacs, which accumulated more viruses than PP (Figure 6B, G).

Incoming retroviruses enter Peyer’s Patches through M cells

Microfold (M) cells in PP function as portals of entry into underlying lymphoid follicles for particulate antigens, bacteria, and viruses such as MMTV present in the lumen [36-38]. M cells have been previously implicated in MMTV infection during oral transmission using mouse models that have significantly reduced levels of M cells [39]. Thus, we asked whether incoming MLV infiltrates PP through M cells. We infected 3-day-old mice with MLV Env-Nluc, sampled intestines at 48 hpi to identify luciferase-positive PPs, and processed them for electron tomography to delineate possible infiltration mechanisms (Figure 7A). MLV particles were observed within endosomes inside of M cells (Figure 7B). These data supported a contribution of M cells and a transcytosis model for retrovirus infiltration from the intestinal lumen into the follicle region of the PP [40-42].

CD169 contributes to virus particle accumulation and establishment of infection in the GI tract

We next asked if viruses are captured after arriving in the PP. Visualizing virus particles (MLV Gag-GFP) at the single cell level by immunostaining of tissue sections was challenging after particles had traveled for days in the GI tract before arriving in the PP follicle. We therefore resorted to performing retrovirus challenge in adult mice by surgically ligating a region in the small
intestine that contained PP. The ligated loop allowed us to increase the local particle concentration of MLV Gag-GFP under live settings [43]. After allowing the virus to be taken up for 1 h, PP region of the ligated intestine was processed for immunostaining and microscopy (Figure 8). In addition to particles that were free or adhered to the epithelial cells, we observed particles invading the epithelial barrier presumably through M cells and dispersed in the PP follicle (Figure 8A). CD169+ macrophages were not previously reported in the PP. Surprisingly, we observed CD169+ macrophages located in the serosal side of the PP that had captured MLV Gag-GFP (Figure 8B). These data suggested a possible role for CD169+ macrophages in capturing and promoting retrovirus infection in intestinal PPs. We explored this possibility further in neonatal mice. In contrast to their serosal location in adult PPs, CD169 macrophages were more dispersed within developing neonatal PPs (Figure 9A). We next asked whether CD169 plays a functional role in promoting retrovirus acquisition during oral transmission from mother to offspring. We first orally inoculated B6 and CD169−/− mice with MLV Env-Nluc to monitor virus particle transit through the intestine. BLI at 48 hpi revealed a significantly reduced distribution of virus particles throughout the intestines and within PP (~5-fold reduction; p<0.0001) as well as mesenteric sacs (~3-fold reduction; p=0.0082) in CD169−/− mice compared to B6 mice (Figure 9B-D). We then asked whether CD169 promoted infection in the mesenteric sac. We infected neonatal B6 and CD169−/− mice orally with WT FrMLV and assessed infection levels in individual mesenteric sacs via flow cytometry for viral GlycoGag protein expression at 5 dpi. Our analyses showed that CD169−/− mice displayed a 26-fold reduction in establishment of infection in the mesenteric sac compared to B6 mice, (p=0.0011) (Figure 9E). These results revealed that CD169 is a novel host factor that contributes to retroviral acquisition via the oral transmission route.

Discussion

We developed a whole-body imaging-guided, top-down approach to study retrovirus infection in vivo. We engineered a series of reporter viruses to monitor individual steps of virus
infection including virus particle flow, entry into cytoplasm, infection of cells, replication, and spread in the context of living animals. We first validated this system by successfully demonstrating its ability to uncover new insights into well-studied subcutaneous and intravenous transmission routes. Importantly, BLI permitted temporal tracking of orally administered retroviruses along the GI tract as they traversed the lumen through PP to reach the draining mesenteric sac. We have also shown that a common host factor expressed on sentinel macrophages, CD169, promotes retrovirus capture and acquisition throughout three routes. Our results highlighted how retroviruses co-opt the immune surveillance function of tissue resident sentinel macrophages to establish infection.

Our visual approach to virus infection in vivo revealed several novel facets of host-virus interplay.

**Route-specific tissue barriers:** Each route of entry comprises a unique set of tissue-specific barriers that viruses must overcome for successful infection of the host. Our particle flow analyses for the retroorbital route revealed that most viruses reach the spleen, where they establish infection after briefly passing through the heart and liver. In contrast, the i.f.p. route presented a much more challenging barrier for viruses to reach their target organ, the pLN. Virus accumulation in the footpad was 150-200-fold higher than that in the pLN (Figure 3B). We frequently observed clusters of incoming virus particles trapped within the dense, tightly packed matrix of footpad muscle and collagen fibrils. In addition, virus particles were taken up by macrophages and DCs that presumably trap them in a non-productive pathway (Figure 4A-C) and initiate immune responses, in contrast to B and T cells located at the pLN that serve as amplifying hosts for MLV.

Despite the high levels of particle capture and infection seen near the injection site at the footpad, our previous studies showed that the establishment of the infection at the draining pLN is independent of cells migrating from the footpad [12]. During oral transmission, enveloped retroviruses must withstand harsh conditions such as low pH in the stomach, digestive enzymes, and bile salts [9, 44] that can solubilize viral membranes. While retroviruses can be destroyed at
pH as low as 4 [45], MLV is stable until pH 3 [46]. Furthermore, mice are most susceptible to MLV infection at day 3 post-partum [38], when stomach acid production is low [47]. Moreover, milk may also shield retroviruses by neutralizing secreted acid in the neonatal stomach. Our analyses comparing the flux of input MLV Env-Nluc viruses (~1 x 10^7 flux/s) with that of signal seen at 48 h in PP and mSac (~1-2 x 10^6 flux/s), suggest that ~50-100-fold fewer viruses are able to surmount the oral and gastrointestinal barriers. This barrier is expected to increase significantly with age due to the upsurge in production of virus-inactivating factors like bile salts and acid thus rendering adult mice resistant to oral MLV transmission [47].

**Differential kinetics of virus particle arrival and cytoplasmic entry:** Following i.v. infection, viruses accumulated in the spleen within 3 minutes and entered the cytoplasm of host target cells by 60 minutes post-challenge (Figure 2D, E). In footpads, cytoplasmic entry corresponded with virus particle arrival (Figure 3, A-D). In pLN, however, viruses took longer (more than 40 min) to enter host cell cytoplasm after arrival, with pLN luminescence first observed at 6 h post-challenge (Figure 3C, D). Similarly, we saw a weak but delayed cytoplasmic entry of viruses in the mesenteric sac compared to PP despite near simultaneous arrival of virus in both the organs (Figure 6D, E). The delayed cytoplasmic entry in the pLN and mesenteric sacs is likely explained by the mode of infection. We have previously documented that in the spleen and pLN, CD169+ macrophages capture incoming viruses and promote infection of target lymphocytes by a process called trans-infection [11, 12, 21]. Sentinel macrophages are resilient to MLV infection at early time points as viruses are held at a distance of ~41 nm from the cell surface, corresponding to the length of the CD169 ectodomain, a distance too far for the MLV Env to engage the receptor [11]. Following capture, viruses must transit through surface-associated membrane invaginations of CD169+ sentinel macrophages before presentation to target cells for trans-infection, delaying cytoplasmic entry. In contrast, in footpads, incoming virus may directly interact with DCs, in addition to the handoff from virus-capturing macrophages, as observed by our immunohistochemistry and electron tomography studies (Figure 4B, C). Congruent with our
observations of rapid virus entry at the footpad, virus-capturing DCs may internalize virus and become infected to perform their natural role of antigen presentation unlike, CD169+ macrophages, which cross-present viruses [12, 33]. A similar situation may occur within PPs, where viruses, after transiting through M cells, have direct access to target cells in the underlying follicle in addition to availing CD169+ macrophage promoted infection. In contrast, free viruses entering mesenteric LN undergo sentinel macrophage-mediated trans-infection, delaying cytoplasmic access like those seen in the pLN.

Differential capture of retroviruses by CD169 in spleen and liver: We observed that retroorbitally-administered viruses briefly passed through the liver before accumulating in the spleen (Figure 2A, B). The vastly reduced virus-retention in the liver, despite the presence of sinusoidal CD169+ Kupffer cells, was surprising. However, these results could be explained by several-fold lower levels of CD169 expression in Kupffer cells compared to CD169+ SCS macrophages in the LN or marginal zone metallophilic macrophages in the spleen [48]. A similar predominant capture of intravenously delivered exosomes in spleen compared to liver was also observed previously [48]. Alternatively, specific tissue environments may govern the capacity of lectins to bind incoming viruses as the binding capacity of Siglecs is often regulated by endogenous ligands [49, 50].

Novel facets of oral retroviral transmission: While requiring necropsy to increase resolution and sensitivity, BLI assisted the study of long organs such as the GI tract, as it can illuminate areas of interest to guide directed investigations and shed light on the sequential course of infection events. Particle flow analyses with replication-defective reporters demonstrated that it takes 24-48 h for viruses to reach portals of entry (PP) and accumulate in the draining mesenteric sac. Interestingly, prior replication in PP was not required for transiting to the draining LN (Figure 6A-C). Incoming particles were able to access the lymph flow for transit to the mesenteric sac. This contrasts with vaginal infection of SIV, in which local replication was critical for further virus dissemination [2, 3, 51]. The use of Fluc-tagged viruses revealed that entry into the cytoplasm...
occurs at ~24 hpi in the PP and begins at ~48 hpi in the mesenteric sac which houses mesenteric
LNs. This was followed by infection at 96 hpi (Figure 6F, G). As with other infection routes we
tested, CD169 expression on sentinel macrophages was crucial for promoting oral transmission
(Figure 9). Particle retention and subsequent infection at PP and mesenteric sacs were
significantly reduced in the absence of CD169. These data were reminiscent of our previous
study, where we observed a CD169-requirement to promote MLV infection at both pLN and
spleen [11, 12]. Thus, our studies revealed the existence of CD169-mediated capture and
infection promotion as a second crucial step downstream of likely entry through M cells that
augments oral retroviral transmission.

Overall, our BLI-guided analyses have highlighted how retroviruses, during a million years
of co-evolution, have co-opted CD169, primarily used for immune surveillance by tissue resident
sentinel macrophages, as a common host factor for promoting host colonization via various
naturally occurring infection routes. Our study opens avenues for a localized CD169-blockade
based strategy to curb retrovirus acquisition and transmission.
Materials and Methods

Ethics statement

All animal experiments were performed according to protocols approved by the Institutional Review Board and the Institutional Animal Care and Use Committee (IACUC) of Yale University. Yale University is registered as a research facility with the United States Department of Agriculture (USDA), License and Registration number: 16-R-0001. It also is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). An Animal Welfare Assurance (#A3230-01) is on file with OLAW-NIH.

Mice

C57BL/6 (B6) and BALB/cJ, mice were obtained from the Jackson Laboratory (Bar Harbor, ME). CD169−/− mice (B6 background) were from Paul Crocker [52], University of Dundee, UK. Animals were housed under specific pathogen-free conditions in the Yale Animal Resources Center (YARC). The Institutional Animal Care and Use Committees (IACUC) and the Institutional Biosafety Committee of Yale University approved all experiments. 6–8-week-old male and female mice were used for all experiments involving adult mice. Breeder mice acting as foster mothers for litter transfer experiments were 3-6 months of age. Oral inoculation experiments in neonatal mice were performed on 3-day-old mice.

Generation of viral vector plasmids

Virus-encoding plasmids were generated using Gibson Assembly (NEB Gibson Assembly kit, NEB, Ipswich, MA). Insert amplicons containing 25 bp overlaps to target regions were generated using Kapa HiFi Hotstart™ high-fidelity polymerase (Kapa Biosystems/Sigma-Aldrich, St. Louis, MO) using a touchdown PCR protocol. MLV Env-Nluc: For generating full-length MLV Env-Nluc construct, MLV pLRB303 Env-PRR-GFP plasmid encoding a full length MLV genome [53] was
digested with NheI to release the GFP cloned into the Proline Rich Region (PRR) of MLV envelope. Nluc was amplified from the pNL1.1 plasmid (Promega, Madison, WI) with primers to ensure in-frame Nluc expression as well as containing 25 bp overlap to the insertion site to allow cloning by Gibson Assembly. A similar strategy was used to generate MLV envelope expressor plasmid for insertion of Nluc in-frame into PPR region. MLV Gag-GFP: MLV Gag-GFP in the full length viral context was described previously [11]. MLV Gag-Fluc EnvWT: GFP was released from MLV Gag-GFP using EcoRI and HindIII digestion. Fluc was amplified from pGL4.32[luc2P NF-kB-RE] plasmid (Promega, Madison, WI) and inserted into the vector backbone using Gibson Assembly. For MLV Gag-Fluc EnvFD generation, MLV envelope was replaced with SFFV envelope using restriction digestion and ligation. SFFV envelope was amplified from pBR322-SFFV LS, a gift from Leonard Evans and Frank Malik (NIH). Full-length MLV 6ATRi-Nluc: gBlocks™ encoding 6ATRi-Nluc along with flanking region were obtained from Integrated DNA Technologies (Coralville, IA). Amplified gBlocks and Full-length Friend MLV plasmid were digested with ClaI and BlpI and assembled into the FrMLV backbone via Gibson Assembly. pMIG-Nluc-IRES-GFP: pMIG-Nluc-IRES-GFP was described previously [23]. pMIG-Nluc-IRES-GFP which was described previously [23]. pMIG-Fluc-IRES-mCherry was a gift from Xiaoping Sun (Addgene plasmid # 75020; http://n2t.net/addgene:75020; RRID:Addgene_75020). pMIG-Antares: pMIG-Antares was generated by replacing the IRES GFP cassette of pMIG-w with Antares luciferase from the pNCS-Antares. pMIG-w was a gift from Luk Parijs (Addgene plasmid # 12282; http://n2t.net/addgene:12282; RRID:Addgene_12282) [54], and pNCS-Antares was a gift from Michael Lin (Addgene plasmid # 74279; http://n2t.net/addgene:74279; RRID:Addgene_74279) [24]. Plasmids were transformed into DH5α Max Efficiency competent E. coli (ThermoFisher, Waltham, MA). E. coli were grown overnight in 1L cultures of 2X Yeast extract Tryptone (YT) media at 30-37 °C under shaking conditions. Plasmids were isolated using Machery-Nagel DNA preparation kits. Reporter gene expression was tested by transfecting 50 ng of plasmid and 450
ng of pcDNA3.1 into HEK293 cells seeded in 24-well plates. 24 h post-transfection, reporter gene expression was monitored via flow cytometry and/or luciferase assay.

Generation of viral stocks

Plasmids encoding each vector were transfected into HEK293 cells seeded in 10 cm plates using Fugene 6™ (Promega, Madison, WI) or polyethyleneimine. 12 μg of plasmid encoding full-length viral vectors were transfected into each 10 cm plate. Replication-defective viruses were produced via transfection of 12 μg total DNA consisting of a mixture of MLV Gag-Pol, LTR-Reporter plasmid, and Ecotropic MLV Envelope in a 5:5:2 ratio. ViralBoost™ reagent (ALSTEM, Richmond, CA) was added to producer cell plates 12 h following transfection. 48 h following transfection, culture supernatants were collected and filtered through 0.45 μM low protein-binding filters (Pall Corporation, Port Washington, NY). Viral stocks were aliquoted into 2-mL tubes and stored at -80 °C.

Virus Titration

For titration of viral stocks, DFJ8 chicken fibroblasts (50,000 cells/well, 48-well plate) were infected with varying dilutions of viral stocks in the presence of 8 μg/mL polybrene (Sigma Aldrich). 48 h following infection, infected DFJ8 cells were analyzed by flow cytometry using antibodies to MLV GlycoGag. For preparation of single-cell suspensions, infected cells were incubated with Accutase™ (StemCell technologies, Vancouver, Canada) for 5 minutes at 37°C. Accutase was neutralized via addition of RPMI containing 10 % FBS. Cells were centrifuged for 5 minutes at ~110 × g and resuspended in PBS containing 1 % BSA. Cells were fixed with 4 % PFA for 7 minutes at room temperature. PFA was neutralized by addition of 0.1 M glycine in PBS. Cells were washed with PBS-0.1M glycine twice and resuspended in FACS buffer solution (5% FBS, 1 % BSA, 0.2 % Gelatin in PBS) for 15 minutes at room temperature. Cells were incubated with 1:500 anti-GlycoGag antibody conjugated with Alexa 647 at room temperature for 1 h. Cells were
washed twice with and resuspended in MACS Buffer (1X PBS, 2 mM EDTA, 1 % BSA). Infectious units of fluorescent protein-encoding constructs were additionally enumerated by estimating the number of GFP or mCherry-expressing cells. Viral titers were determined by calculating infectious units per mL, based on numbers of infected cells resulting from each volume of virus supernatant used in titration.

**In vitro luciferase assays**

Single cell suspensions obtained from tissues or infected cells were lysed in 1X Passive Lysis Buffer (Promega) for 5 min at 37°C and lysates were added to white-bottom 96-well flat-bottom plates (Costar, Corning, NY). Luciferase activity was measured after adding appropriate substrate (Promega Firefly Luciferase Assay substrate for firefly luciferase, or Promega nanoGlo™ nanoLuciferase substrate for NLuc and Antares, diluted 1:40 in PBS per manufacturer’s instructions) using a Berthold luminometer (Berthold technologies, Bad Wildbad, Germany). Luciferase activity associated with virions were performed on partially purified viruses that were sedimented through a 15 % sucrose cushion in PBS at 25000 x g for 2 h at 4 °C. Sedimented viral pellets were resuspended in 0.1 % BSA/PBS and diluted accordingly for detection within the luminometer linear range. For MLV Gag-Fluc virion luciferase assays, sedimented viral pellets were resuspended in 0.1 %BSA/PBS or Passive Lysis Buffer (Promega). ATP-free or ATP-containing (150 μM ATP) substrate solutions were prepared containing 150 μg/mL D-Luciferin (Goldbio, St. Louis, MO), 100 mM Tris pH 8, and 5 mM MgCl₂. Relative light units (RLU) were determined by taking luciferase readings of lysis buffer or PBS/0.1 % BSA.

**Retrovirus administration**

Virus stocks were stored at -80 °C, thawed at 37 °C, quickly placed on ice, and concentrated by sedimentation through a 15 % sucrose-PBS cushion for 2 h at 4 °C, at 25,000 x g. After sedimentation, virus pellets were resuspended in endotoxin free 0.1 % BSA in PBS at appropriate
luciferase light units or infectious units (IU) for administration. Retroorbital (100 μL) and subcutaneous (intra footpad or intra mammary glands; 25 μL) injections were carried out using an insulin syringe with 31 G needle on anesthetized mice (0.5 - 5 % isoflurane delivered using precision Dräger vaporizer with oxygen flow rate of 1 L/min). During retroorbital and subcutaneous virus inoculations, mice received 1 x 10^5 IU of MLV Env-Nluc for viral particle flow monitoring, 2 x 10^4 IU of MLV Gag-Fluc Env_{WT} or MLV Gag-Fluc Env_{FD}, for monitoring of cytoplasmic entry, or 5 x 10^5 IU of MLV 6ATRi-Nluc, MLV-Antares or WT MLV for monitoring of longitudinal virus spread and infectivity comparison. For mother-to-offspring transmission experiments, dams were inoculated with 1 x 10^7 IU of MLV Fluc-IRES-mCherry subcutaneously distributed into the mammary gland 6 days prior to transfer of neonates for fostering. For oral inoculation of neonatal mice, appropriate amounts of virus were resuspended in 15 μL sterile endotoxin free PBS containing 0.1 % BSA and 5 % sucrose and fed using a p10 pipette tip. Mice were orally inoculated with 1 x 10^6 IU of MLV Env-Nluc for monitoring virus particle flow, 2 x 10^4 RLU of MLV Gag-Fluc for monitoring virus entry into cytoplasm, or 1 x 10^6 IU of MLV 6ATRi-Nluc or MLV pMIG-Nluc for monitoring establishment of infection.

Bioluminescence Imaging (BLI)

Image acquisition: All mice were anesthetized via isoflurane inhalation (3 – 5 % isoflurane, oxygen flow rate of 1.5 L/min) prior and during BLI using the XGI-8 Gas Anesthesia System. Images were acquired with an IVIS Spectrum® (PerkinElmer) and analyzed with the manufacturer’s Living Image v4.7.3 in vivo software package. Image acquisition exposures were set to auto, with imaging parameter preferences set in order of exposure time, binning, and f/stop, respectively. Images were acquired with luminescent f/stop of 1, photographic f/stop of 8. Binning was set to medium.

Short interval imaging for particle flow: Prior to imaging MLV Env-Nluc virion flow, mice received furimazine (Promega) diluted 1:40 in PBS. 100 μL diluted furimazine were administered
retroorbitally. Image sequences were then acquired at 30 sec intervals following administration of
MLV Env-Nluc (r.o or i.f.p). Image sequences were assembled and converted to videos using
Image J.

**Imaging of adult mice:** Mice infected with Antares- or Nluc-encoding viruses received 100 μL
furimazine (NanoGlo™ furimazine, Promega, Madison, WI) diluted 1:40 in sterile endotoxin-free
PBS (r.o.) before imaging. Mice infected with Fluc-carrying viruses received in vivo grade D-
Luciferin (15 mg/mL in sterile endotoxin-free PBS, GoldBio) (r.o.) before imaging.

**Imaging of neonatal mice:**

For non-invasive imaging of neonatal mice that were fed from infected dams, 25 ul of pre-warmed
*in vivo* grade D-luciferin in PBS (15 mg/mL) was injected subcutaneously in the scruff of the neck.
The substrate was allowed to diffuse for 10 min before imaging the mice using IVIS. For oral virus
administration, neonatal mice were orally inoculated with various reporter viruses suspended in
15 μL of 5 % sucrose in PBS. Luciferase-specific substrate was subcutaneously injected as
above, 10 min before euthansia. Infected areas of interest were identified by carrying out whole-
body imaging following necropsy [23]. Infected regions indicated by luminescent signal were
sampled and washed in PBS to remove residual blood and placed onto a glass plate. Additional
droplets of furimazine in PBS (1:40) or D-Luciferin (15 mg/mL) were added to organs and soaked
in substrate for 1-2 min before BLI for quantitation.

**Image Processing:** Comparative images were compiled and batch-processed using the image
browser with collective luminescent scales. Photon flux was measured as luminescent radiance
(p/sec/cm²/sr). During luminescent threshold selection for image display, luminescent signals
were regarded as background when minimum threshold levels resulted in displayed radiance
above non-tissue-containing or known uninfected regions.

**Single cell preparation from mouse tissue**
Popliteal lymph nodes, mesenteric sacs and spleens harvested after necropsy were disrupted in serum free media, treated with Liberase TL (0.2 mg/ml, Sigma-Aldrich) and DNase I (20 mg/ml, Roche) at 37 °C for 20 min and passed through a 70 µm cell strainer (Falcon, Cat # 352350). Splenic cell suspensions were treated additionally with red blood cell lysis buffer at room temperature for 10 min (Sigma-Aldrich BioLegend Inc) for removing RBCs to obtain single cell suspensions. Single cells suspensions from each lymphoid tissue were fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) before processing for flow cytometric analysis.

**Flow cytometry**

Fixed Single-cell suspensions were washed twice with 0.1 M glycine in PBS to neutralize excess PFA. Cells were blocked with CD16/32 antibodies (Biolegend® Inc, San Diego, CA) in FACS staining buffer (5% FBS, 1% BSA, 0.2% Gelatin) for 15 minutes to 1 h. Cells were then stained with Alexa647 conjugated antibodies to Glycogag (mAb34 hybridoma) incubated with antibodies for 1-2h at room temperature. For intracellular staining cells were permeabilized with staining buffer supplemented with 0.2% Triton X-100. Flow cytometry was performed on a Becton Dickinson Accuri™ C6 benchtop cytometer. Data were analyzed with Accuri C6 or FlowJo v10 software (Treestar, Ashland OR). 200,000 – 500,000 viable cells were acquired for each sample.

**Retrovirus challenge by ileal ligation**

Ileal ligation was performed on anesthetized mice. Mice were first anesthetized with a ketamine/xylazine cocktail (ketamine 15 mg/mL xylazine 1mg/mL) at 0.01 mL per g of body weight. Mice were then placed on isoflurane inhalation anesthesia using a Dräger vaporizer (1.5-2% isoflurane, flow rate 1L/min). A small, ~5-mm incision was made above the abdominal cavity to expose the peritoneal muscle, in which a small ~3-mm incision was made above the intestine. The small intestine was carefully extracted using forceps. Small intestinal regions of interest were
ligated by tying surgical suture (undyed, Vicryl braided P-3 polyglactin-coated J494G suture, Ethicon, Somerville, NJ) gently around serosal intestinal walls surrounding Peyer's Patches. The suture needle was inserted through the mesenteric membrane, with care to avoid blood vessel obstruction. Knots were gently tied at each end of the Peyer's Patch, spanning a ~1-2-cm length. MLV Gag-GFP viruses corresponding to 1-4 x 10^5 IU based on comparative western blot analyses with antibodies to Gag with equivalent amounts of WT FrMLV. were administered into the intestinal lumen through the intestinal wall using a 31-G needle. Total volume did not exceed 50 μL. Following the intestinal loop, excess suture was trimmed from knots and the intestine was carefully threaded back through the incision. The incision was sealed very gently using a small, low-tension binder clip, while the mouse remained under anesthesia until euthanasia at the end of the terminal surgical procedure. Intestinal loop inoculations did not exceed 1 h. Anesthesia was closely monitored during the duration of the surgery and intestinal loop inoculation. Anesthetic planes were monitored every 15 minutes or more frequently based on heart rate, breathing rate and depth, and noxious stimuli reflexes as recommended by the Yale IACUC. At the end of 1 h, the mouse was sacrificed, and the ligated intestinal region processed for cryosectioning and immunostaining.

Cryo-immunohistology

Non-fluorescent-protein-containing tissue samples were harvested at indicated time points and fixed in 1X PBS containing freshly prepared 4 % PFA for 1 h at 4°C. Fluorescent-protein-containing samples were harvested and fixed in periodate-lysine-paraformaldehyde (PLP) fixative (1X PBS containing 1% PFA, 0.01 M sodium m-periodate and 0.075 M L-Lysine) for 30 min to 1 h to preserve fluorescent protein fluorophores. Tissue samples were washed with PBS, dehydrated in a sucrose gradient consisting of 1h incubation at room temperature in 10, 20, or 30% sucrose in PBS, embedded and snap-frozen in Tissue-Tek® O.C.T.TM compound (Sakura Finetek, Torrance, CA) and stored at –80°C. 15 μm tissue sections were cut on a Leica cryostat
at -20°C and placed onto Superfrost Plus™ slides (Thermo, Waltham, MA). Tissue sections were
dried at 37°C for 15 minutes and stored at -20 for later use or rinsed in PBS for staining. Slides
were washed in PBS (for stains of cell-surface proteins) or permeabilized with PBS containing
0.2% Triton X-100 (for stains of intracellular proteins) and treated with Fc receptor blocker
(Innovex Biosciences), Richmond, CA or Staining solution (5% FBS, 1% BSA, 0.2% Gelatin)
before staining with indicated antibodies in PBS containing 2% BSA. Staining was performed
with following antibodies: CD169-AF647, CD169-AF594, CD11c-eFluor450, CD11c-AF594,
CD11c-AF647, CD68-AF594 were from Biolegend® Inc, San Diego, CA; GlycoGag-CF647
(mAb34 hybridoma, house-conjugated[[12, 55]. Stained sections were washed with PBS and a
final rinse with water to minimize salt precipitation and mounted using ProLong® Glass antifade
reagent (Invitrogen, ThermoFisher) and Fisher Finest thick coverslips (ThermoFisher). Mounted
slides were sealed with clear nail polish and cured for 1 h or overnight at 37°C. Slides were
analyzed by confocal microscopy using a Leica TCS SP8 microscope equipped with a white light
and argon laser, and a Nikon W-1 Spinning Disk microscope. The images were processed using
Volocity™ version 6.3 software (PerkinElmer, Waltham, MA, USA) and Nikon Elements software
(Nikon, Tokyo, Japan). Figures were assembled with Photoshop CC and Illustrator CC (Adobe
Systems, San Jose, CA, USA).

Electron Microscopy and Dual-Axis Tomography Following removal or disarticulation, tissues
were lightly fixed with 3% glutaraldehyde, 1% paraformaldehyde, 5% sucrose in 0.1M sodium
cacodylate trihydrate to render them safe from virus infectivity. Tissues were further dissected in
cacodylate buffer, rinsed with cacodylate containing 10% Ficoll (70kD, Sigma) which served as
an extracellular cryoprotectant, placed in brass planchettes (Tell Pella, Inc, Redding, WA) and
ultra-rapidly frozen with a HPM-010 high pressure freezing machine (BalTec/ABRA, Switzerland).
Samples were then transferred under liquid nitrogen to cryo-tubes (Nunc) containing a frozen
solution of 2% osmium tetroxide, 0.05% uranyl acetate in acetone and placed in an AFS-2 freeze-
substitution machine (Leica Microsystems, Wetzlar, Germany). Tissues were freeze-substituted at -90 °C for 72 h, warmed to -20 °C over 12 h, and held at that temperature for an additional 12 h before warming to room temperature and infiltrating into Epon-Araldite resin (Electron Microscopy Sciences, Port Washington PA). Samples were flat-embedded between two Teflon-coated glass microscope slides and the resin polymerized at 60 °C for 48 h. Embedded tissue blocks were observed by light microscopy to ascertain preservation quality and to select gross regions of interest. Blocks were extracted with a scalpel and glued to plastic sectioning stubs prior to sectioning. Semi-thick (300-400 nm) sections were cut with a UC6 ultramicrotome (Leica Microsystems) using a diamond knife (Diatome, Ltd., Nidau, Switzerland). Sections were placed on formvar-coated copper-rhodium slot grids (Electron Microscopy Sciences) and stained with 3% uranyl acetate and lead citrate. Colloidal gold particles (10 nm) were placed on both surfaces of the grids to serve as fiducial markers for tomographic image alignment. Grids were placed in a dual-axis tomography holder (Model 2010, E.A. Fischione Instruments, Export PA) and imaged with a Tecnai TF30ST-FEG transmission electron microscope (300 KeV; ThermoFisher Scientific). Images were recorded with a 2k x 2k CCD camera (XP1000; Gatan, Pleasonton, CA). Tomographic tilt-series and large-area montages were acquired automatically using the SerialEM software package [56]. For dual-axis tomography, images were collected at 1° intervals as samples were tilted +/- 64°. The grid was then rotated 90° and a second tilt-series was acquired about the orthogonal axis. Tomograms were calculated, analyzed, and modeled using the IMOD software package [57, 58] on MacPro and iMac Pro computers (Apple, Inc, Cupertino, CA). Cell types and frequency within tissue sections were identified using 2D montaged overviews. Virus particles and infected cells were further characterized in 3D by high-resolution electron tomography.

Statistical Analyses
Statistical comparisons were performed using non-parametric Mann-Whitney tests (two-tailed) available in GraphPad Prism software (Graph Pad Software, La Jolla, CA, USA). A difference was considered significant if $P < 0.05$.

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Author Contributions

K.A.H. carried out all the BLI imaging. KA.H and P.D.U. contributed to executing experiments, data analysis, and figure generation. K.A.H. P.D.U., and W.M. shared the conceptualization, experimental design, interpretation, and manuscript preparation. M.S.L. carried out electron tomography of tissue samples. R.P. assisted in carrying out experiments, primer design and maintaining mouse colonies. A.G. assisted with cloning and generation of reporter constructs. P.J.B. and P.K. contributed to interpretation and discussion of the work.

Competing interests

The authors declare no competing interests.
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Figure 1: Construction and characterization of reporter viruses for visualizing individual stages of the retrovirus lifecycle in vivo.

(A) A scheme denoting the location of inserted reporters into unique genomic loci of Friend MLV (FrMLV). 1. To monitor particle flow, Nluc was inserted in-frame into the proline rich region (PRR) of Envelope. 2. To monitor virus entry into the cytoplasm of cells, Gag-Firefly luciferase (MLV Gag-Fluc; ∆Pro-Pol) was employed as Fluc requires both oxygen and ATP in the presence of substrate D-luciferin for its activity. 3. To monitor infection, Nluc was expressed in the cytoplasm using a viral LTR driven construct (single round; ∆gag-pol-env). 4. To monitor spreading infection Nluc was expressed from a short IRES element (6ATRi) downstream of the envelope gene that resulted in replication competent virus.

(B) A graph comparing brightness (relative light units, RLU) per infectious unit of full-length FrMLV Env-Nluc viruses with single-round MLV generated using gagpol, env-Nluc and LTR-gfp.
constructs. Infectious units for both viruses were estimated using DFJ8 cells followed by flow cytometry. RLU per infectious virion was determined by measuring Nluc activity in sedimented virus. The error bars denote standard deviations between triplicate samples. p values derived from student’s t-test.

(C) A graph showing detectable Fluc activity in intact (0.1% BSA/PBS) or lysed (0.1% BSA/PBS, Triton X-100) MLV Gag-Fluc virions in the presence or absence of ATP and substrate D-luciferin (15 mg/mL in PBS). The error bars denote standard deviations between triplicate samples. p values derived from student’s t-test; ns, not significant.

(D) A graph comparing released infectivity of replication competent FrMLV Nluc reporter virus (6ATRI-Nluc) and WT FrMLV (WT). Viruses were produced by transfecting equal amounts of virus-encoding plasmids into HEK293 cells in triplicate. 48 h post transfection released infectivity in culture supernatants was determined using DFJ8 target cells followed by flow cytometry with antibodies to MLV GlycoGag to enumerate infected cells. Released infectivity of WT FrMLV was set to 1. The error bars denote standard deviations between triplicate samples. Western blot analyses of sedimented virus from culture supernatants and HEK293 cell lysates for a similar experiment as shown in the graph above using antibodies to MLV Gag.
Figure 2: Real-time visualization of individual steps of retrovirus infection in vivo during retroorbital challenge.

(A) A scheme showing the path of MLV Env-Nluc particles after intravenous retroorbital challenge. Furimazine- (Nluc substrate) administered mice were challenged retroorbitally with 1 x 10^5 IU of MLV Env-Nluc and monitored using IVIS at 30 seconds (s) intervals. Images from one representative experiment from three biological replicates (n = 3) are shown.

(B) Quantification of MLV Env-Nluc bioluminescent signal in the spleen and liver, displayed as photon flux (photons/sec) in each organ following r.o. challenge from the experiment described in (A). Curves represent mean flux over time and error bars denote standard deviation.

(C) A plot showing Fluc activity (RLU) associated with with MLV Gag-Fluc Envwt or MLV Gag-FLuc EnvFD (Fusion defective) inocula after lysis Mice were subsequently inoculated with 2 x 10^4 RLU of indicated unlysed virus preparations. Error bars denote standard deviations from mean.

(D) Representative images of MLV Gag-Fluc Envwt entry into the target cell cytoplasm at the spleen observed via BLI at the indicated time points following r.o. infection. Mice challenged similarly with MLV Gag-Fluc EnvFD served as negative controls for determination of background signals. Images from one representative experiment from 4 to 7 biological replicates are shown.
(E) Quantification of MLV cytoplasmic entry in the spleens (photon flux/s) of mice (n= 4-7) following r.o. infection with MLV Gag-Fluc EnvWT or MLV Gag-Fluc EnvFD at indicated time points from the experiment as in (D). p values derived from non-parametric Mann-Whitney test; ns, not significant; bars represent mean values and error bars denote standard deviations from mean.

(F) A plot showing total number of infected cells per spleen of B6 mice (n =3-4) challenged with 5 x 10^5 IU of FrMLV (WT) and replication competent reporter FrMLV (MLV 6ATRI-Nluc) at 7 dpi (r.o). Infected cells in single-cell suspensions of spleen were determined using flow cytometry with antibodies to MLV GlycoGag. Horizontal lines represent mean values.

(G) Mice were infected r.o. with 5 x 10^5 IU of replication competent reporter FrMLV (MLV 6ATRI-Nluc) or single-round MLV pMIG-Antares as a control for tracking dissemination of transduced infected cells. Dissemination of viral infection was monitored via BLI at the indicated time points. Images from one representative experiment are shown.

(H) Quantification of virus dissemination in indicated organs is displayed as photon flux in mice infected with MLV 6ATRI-Nluc or MLV pMIG-Antares for the experiment described in (G). Antares, n=3; 6ATRI-Nluc, n=8. Images in (G) are from one representative experiment. Symbols represent mean values and error bars denote standard deviations from mean.

Scale bars that accompany the images showing luminescence denote radiance in photons per second per square centimeter per steradian (p/sec/cm²/sr).
Figure 2- figure supplement 1: Stable retention of MLV Env-Nluc in the spleen
demonstrated by re-administration of furimazine substrate after retroorbital challenge.

(A) Timeline of retroorbital (r.o.) substrate administration (red arrows) in mice infected with 1 x 10^5 IU (blue arrow; r.o.) of MLV Env-Nluc.

(B) Images demonstrating waning and resurgence of luminescent signal in mice for an experiment shown in A before and after substrate re-administration at indicated time points.

Scale bars that accompany the images showing luminescence denote radiance in photons per second per square centimeter per steradian (p/sec/cm^2/sr).
Figure 3: Real-time visualization of individual steps of retrovirus infection in vivo during subcutaneous challenge.

(A) A scheme showing the path of MLV Env-Nluc from foot pad to draining popliteal lymph node (pLN) after subcutaneous (intrafootpad; i.f.p.) challenge in mice. Furimazine- (Nluc substrate) administered mice were challenged i.f.p with 1 x 10^5 IU of MLV Env-Nluc and monitored using IVIS at 30 seconds (s) intervals. Images from one representative experiment from three biological replicates (n = 3) are shown.

(B) Quantification of MLV accumulation in footpad and pLN, displayed as photon flux (photons/sec) at each site following i.f.p. inoculation with MLV Env-Nluc (n=3), from the experiment described in (A). Curves represent mean flux over time and error bars denote standard error from mean.

(C) Representative images of MLV Gag-Fluc Env_{wt} entry into the target cell cytoplasm at the footpad and pLN observed by BLI at the indicated time points following i.f.p. infection. Mice challenged similarly with MLV Gag-Fluc Env_{FD} (fusion-defective envelope) served as negative
controls for determination of background signals. Fluc activity were measured for both virus preparations after detergent lysis and equivalent amounts (2 x 10^4 RLU) were delivered i.f.p. before imaging using IVIS. Images from one representative experiment from three to five biological replicates are shown.

(D) Quantification of MLV cytoplasmic entry in footpads and pLN s (photon flux/s) of mice (n= 3-5) following i.f.p. infection with MLV Gag-Fluc EnvWT or MLV Gag-Fluc EnvFD at indicated time points from the experiment as in C. Dotted lines indicate background means. Shaded regions indicate 3 standard deviations above background means. p values derived from non-parametric Mann-Whitney test; ns, not significant; individual data points along with mean values (bars) are shown.

(E) Mice were infected with 4 x 10^5 IUs of replication competent reporter FrMLV (MLV 6ATRi-Nluc (i.f.p.) Dissemination of viral infection was monitored via BLI at the indicated time points days post infection (dpi). Images from one representative experiment are shown.

(F) A plot showing total number of infected cells per pLNs of B6 mice (n = 3 - 4) challenged with 4 x 10^5 IU of FrMLV (WT) (i.f.p.) and replication competent reporter FrMLV (MLV 6ATRi-Nluc) at indicated days post infection (dpi). Infected cells in single-cell suspensions of pLNs were determined using flow cytometry with antibodies to MLV GlycoGag. Connecting lines representing mean values along with individual data points are shown.

(G) A plot showing ex vivo Nluc activity in cells isolated from pLN s of mice infected with MLV 6ATRi-Nluc-infected from the experiment shown in F. Individual data points along with connecting lines (mean values) are shown.

Scale bars that accompany the images denote radiance in photons per second per square centimeter per steradian (p/sec/cm^2/sr)
Figure 4: Sentinel macrophages mediate capture of incoming viruses at the injection site during subcutaneous challenge.

(A) Images of footpad cryosections from B6 mice 15 min after i.f.p. administration of MLV Gag-GFP virus particles (Green). Macrophages and DCs were identified using antibodies to CD169 (blue) and CD11c (red) respectively. Green arrows denote MLV-laden CD169+ macrophages and white arrows denote contacts between CD169+ macrophages and dendritic cells.

(B) Electron tomography of footpads from B6 mice prepared 15 min post-challenge with WT FrMLV. MLV virions can be seen associated with plasma membranes (PM) invaginations of macrophages (mΦ) as well as via a presumed CD169-tether in the magnified inset.

(C) Electron tomography showing synaptic, MLV-containing contacts between macrophages and DCs in footpads of B6 mice at 15 min post-challenge (i.f.p.) WT FrMLV. Green arrow heads indicate macrophage-associated MLV particles; blue arrow heads indicate DC-associated MLV particles.

Scale bars as indicated.
Figure 4- figure supplement 1: MLV primarily associates with sentinel macrophages in the footpad

(A) Montaged EM overview of mouse footpad tissue in cross-section with resident structures and cell types labeled.

(B) Overview image showing a macrophage (Mφ) and Dendritic Cell (DC) in synaptic contact in footpads subcutaneously challenged with WT FrMLV (15 min post-challenge; intrafootpad, i.f.p.). Inset: Tomographic detail of the synapse zone between Mφ and DC. Red arrows indicate three MLV particles associated with the macrophage cell surface.

(C) Tomogram and quantification of MLV particles associated with each cell type in a synaptic contact 15 min post-challenge. Green dots indicate virus particles on the macrophage surface, yellow dots indicate virus particles present in macrophage endosomes, and blue dots indicate DC-associated viruses. The majority (287) of virus particles present in the volume are associated with the macrophage while few (24) are associated with the DC.

(D) A graph showing quantification of FrMLV associated with macrophages and DCs for an experiment shown in C. All cell-associated viruses within a 400-nm thick section of footpad tissue were quantified.

Scale bars as indicated.
Figure 5: Visualization of MLV transmission from mother to offspring.

(A) A scheme showing MLV-infected dam fostering pups. \(1 \times 10^7\) IU of WT FrMLV carrying MLV LTR-Fluc were inoculated by distribution in mammary glands of a lactating dam. At 6 days post infection (dpi), virus replication was in the dam was confirmed using non-invasive BLI (NBLI). Neonatal mice from a separate litter were provided to foster and successful MLV transmission from dam to pups was visualized 2 days post-transfer (dpt) by NBLI.

(B) Electron tomography of stomach contents at 3 dpt from an infected neonate for an experiment as in A, revealing free viruses in the stomach. Inset: Details of a single cell-free MLV particle within the sample volume. Scale bars as indicated.

(C) Merged luminescence and photographs gastrointestinal (GI) tract from an uninfected neonatal mouse or from a neonatal mouse that was allowed to feed for 8 days (8 dpt) from infected dam for an experiment as in A showing luminescent signal in Peyer’s Patches (PP) and mesenteric LN (mLN).

Scale bars shown for BLI denote radiance in photons per second per square centimeter per steradian (p/sec/cm²/sr)
Figure 6: Real-time visualization of individual steps of retrovirus infection in vivo during oral challenge.

(A) Overlaid bioluminescence images of gastrointestinal tract from neonatal B6 mice orally challenged with 1 x 10^6 IU of replication-defective, luciferase-labeled Env-Nluc MLV particles at 3 days of age. Mice were sacrificed at the indicated time points and their gastrointestinal tracts were subjected to BLI for monitoring the sequential flow of incoming virus particles through stomach, and developing Peyer’s patches, mesenteric sac (mSac) and cecal patch.

(B) Temporal accumulation of orally challenged MLV Env-Nluc displayed as Flux (photons/sec) for an experiment as in A, in Peyer’s Patch (PP, n = 25) and mesenteric sacs (mSacs, n = 4). Error bars denote standard deviation.

(C) Overlaid bioluminescence images of gastrointestinal tract from neonatal B6 mice orally challenged with 2 x 10^4 RLU of MLV Gag-Fluc EnvWT at 3 days of age. Mice were sacrificed at the indicated time points and their gastrointestinal tracts were subjected to BLI for monitoring virus entry and access to target cell cytoplasm.
(D and E) Quantification of MLV cytoplasmic entry at indicated times in PP (D; n= 6 - 19) and mSacs (F; n= 1 - 8) displayed as Flux (photons/sec) from the experiment described in C. Neonatal B6 mice orally challenged with 2 x 10^4 RLU of MLV Gag-Fluc EnvFD were used as control. Dotted lines indicate 3 standard deviations above background (uninfected mice) means at each location.

(F and G) Overlaid bioluminescence images of gastrointestinal tract from neonatal B6 mice orally challenged with 1 x 10^6 IU of single-round MLV pMIG-Nluc-IRES-GFP (G) or full-length replication competent MLV 6ATRI-Nluc at 3 days of age. Mice were sacrificed at 96 hpi and their gastrointestinal tracts were subjected to BLI for visualizing infection in PP and mSacs. The plot shows quantification of signal at PP and mSac for an experiment as in accompanying image. p values were derived from non-parametric Mann-Whitney test; mean values denoted by horizontal line.

Scale bars shown for BLI denote radiance in photons per second per square centimeter per steradian (p/sec/cm²/sr)
Figure 7: MLV infiltrates intestinal Peyer's Patches and can be found in endosomes of M cells after oral challenge.

(A) BLI of gastrointestinal tract isolated from a neonatal B6 mice 48 h after oral challenge with 1x $10^6$ IU of MLV Env-Nluc at 3 days of age, showing luciferase-positive Peyer's Patch (PP) and mesenteric sac (mSac). Scale bars shown for BLI denote radiance in photons per second per square centimeter per steradian (p/sec/cm²/sr)

(B) EM tomogram and magnified insets of BLI-identified PP as in A, showing MLV within endosomes of Microfold (M) cells. Scale bars as indicated.
Figure 8: Gut-infiltrating MLV is captured by Peyer’s Patch-resident CD169+ macrophages.

(A) Images of cryosections from ligated gut tissue containing a Peyer’s Patch from B6 mice that was challenged for 1 h with MLV Gag-GFP (green) corresponding to 1-4 x 10⁵ IU based on comparative western blot analyses with antibodies to Gag with equivalent amounts of WT FrMLV. Tissue sections were stained with phalloidin (red) to visualize actin in the PP tissue structure. Dotted lines demarcate the epithelium from intestinal lumen. Free, adhered, and infiltrating MLV Gag-GFP particles are as indicated with arrows.

(B) Images of cryosections from PP for an experiment as in A. Macrophages identified using antibodies to CD169 (magenta) were predominantly located at the serosal side of PP. MLV Gag-
GFP (green) viruses within marked insets of PP were identified and modeled into spheres (modeled virions) which were then merged to depict close association with CD169+ macrophages. Scale bars as indicated.
Figure 9: CD169 contributes to retrovirus particle acquisition and establishment of infection during oral challenge

(A) Image of a Peyer’s Patch cryosection from a 3-day old neonatal B6 mouse. Macrophages in the developing follicle were identified using antibodies to surface marker CD169 (red) and dotted lines demarcate the epithelium from intestinal lumen.

(B) Representative images of gastrointestinal tracts isolated from a neonatal B6 and CD169−/− mice 48 h after oral challenge with 1x 10^6 IU of MLV Env-Nluc at 3 days of age to show comparative accumulation of bioluminescent viruses in Peyer’s Patch (PP) and mesenteric sac (mSac). Scale bars shown for BLI denote radiance in photons per second per square centimeter per steradian (p/sec/cm²/sr).

(C-D) Quantification of virus transit to PP (C) and mSacs (D) at 48 hpi in B6 and CD169−/− mice from the experiment described in (B). Virus transit was quantified as Nluc photon flux (photons/sec).
FrMLV-infected cells 5 dpi (oral., 1 × 10⁶ IU) in mesenteric sacs (mSac; n = 6-8) from neonatal B6 and CD169−/− mice challenged at 3 days of age. Single cell suspensions of cells from individual mSacs were obtained at 5 dpi and processed for flow cytometry. Infection levels were determined using antibodies to FrMLV GlycoGag. p values derived from non-parametric Mann-Whitney test; mean values denoted by horizontal line.
Rich Media Files:

Video 1: MLV particle flow through the liver and spleen in the retroorbital infection route
Virus particle flow was monitored via BLI immediately following retroorbital infection with $1 \times 10^5$ IU of MLV Env-Nluc. Mice were continuously imaged at 1-minute intervals starting at 30 s post-challenge.

Video 2: MLV particle flow to the pLN following intrafootpad inoculation
Mice were infected via the intrafootpad (i.f.p.) route with $1 \times 10^5$ IU MLV Env-Nluc. MLV particle flow was monitored by BLI starting at 30 s post-challenge. Images were acquired at 30-second intervals for 16 minutes. Mice were subsequently imaged at 60 min post-challenge.

Video 3: MLV is captured by footpad macrophages following subcutaneous virus challenge
Mice were infected via the intrafootpad (i.f.p.) route with WT FrMLV. Footpads were processed for electron tomography at 15 min pi. Tomograms show MLV-capturing footpad macrophages. Captured, tethered virus particles are present in macrophage membrane invaginations.