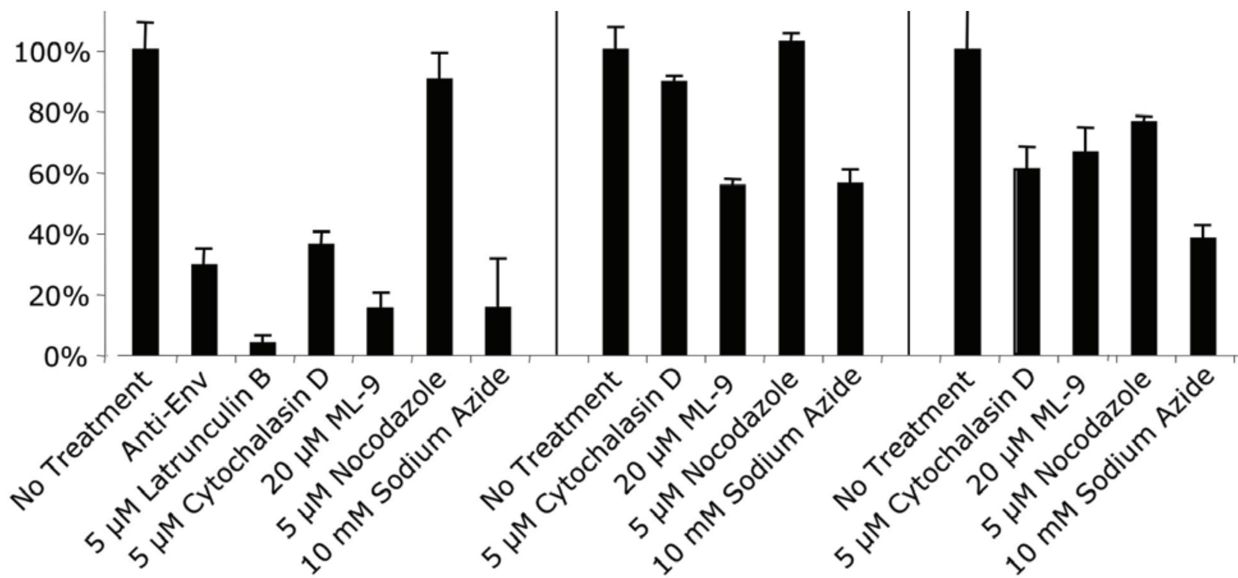

Figure S1 | Virus spreading is sensitive to neutralizing antibodies and reagents affecting the actin/myosin machinery. To measure the effects of neutralizing antibodies and disruption of the actin cytoskeleton on cell-to-cell transmission of MLV, we performed co-culture assays and monitored the spread of MLV infection. HEK 293 cells were transiently transfected to generate a virus encoding GFP (MLV-GFP) and co-cultured with target XC cells in the presence of neutralizing antibodies or various inhibitors as indicated. After 4 hrs of co-cultivation, cell-cell contacts were disrupted by treatment with trypsin and cells were re-plated at a 25-fold dilution to reduce the incidence of cell-cell contact during continuous culture. The spread of MLV-GFP throughout the culture was measured 24 h later by fluorescence-activated cell sorting. The effects of these drugs on MLV budding and entry were measured in parallel as described in Methods.

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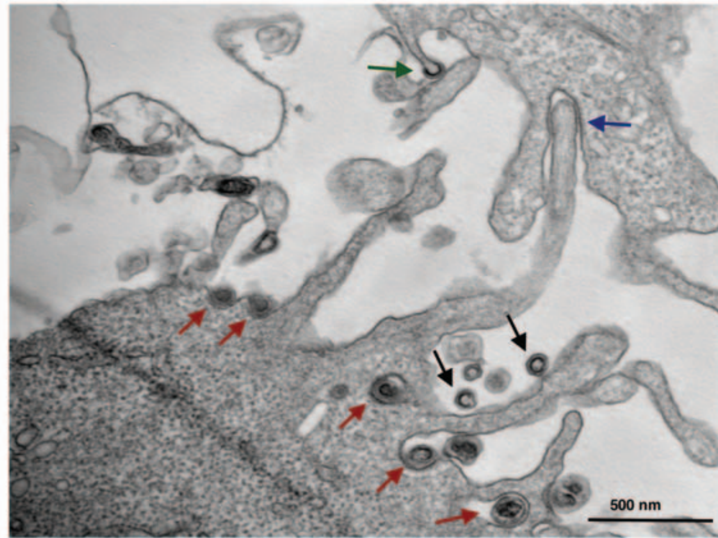

Figure S2 | Electron microscopy of cytonemal cell-cell contacts reveals individual particles at various stages of viral replication. Electron micrograph of a cytonemal contact (blue arrow) between Cos-1 cells producing virions carrying ALV Env (green arrow) and receptor-expressing XC cells. ALV virions are observed at the outer surface of filopodia (black arrows) and in coated pits at the base of each filopodium (red arrows).

SUPPLEMENTARY MOVIES

Movie S1 | **Cell-to-cell transmission of MLV via long-range and long-lived filopodia.** A Cos-1 cell generating MLV labeled with Gag-CFP (green) and Env-YFP (red) in contact with filopodia of a non-infected target XC cell expressing mCAT1-CFP (green). Several virus particles positive for both Gag-CFP and Env-YFP undergo directional movement toward the infected cell. Images were taken every ~60 s over a period of 44 min. The video is played at a speed of 10 frames/sec. Size bar represents 10 μm. Particles A-E correspond to the analysis presented in Fig. 1b.

Movie S2 | **Cell-to-cell transmission of MLV via filopodial bridges.** A time-lapse movie showing an XC cell chronically infected with MLV and stably expressing Gag-CFP (green) and YFP-actin (red) in co-culture with a non-infected target XC cell expressing mCAT1-YFP (red). Several virus particles move along filopodia and continue along the lamellum toward the cell body. Images were taken every ~90 s over a period of 40 min. The video is played at a speed of 5 frames/sec. Size bar represents 10 μm.

Movie S3 | **Filopodia lengthen after contact with infected cells.** A filopodium from a non-infected target XC cell expressing mCAT1-YFP (red) lengthens as it is pulled toward the cell body of an XC cell generating infectious MLV labeled with Gag-CFP (green). Images were taken every ~45 s over a period of 26 min. The video is played at a speed of 5 frames/sec. Size bar represents 10 μm.

Movie S4 | Tips of cytonemes are internalized into infected cells. Cytonemes originating from XC cells expressing mCAT1-YFP (red) were monitored in contact with an infected Cos-1 cells generating infectious MLV particles (green=MLV Gag-GFP). Images were taken every ~60 s over a period of 30 min. The video is played at a speed of 5 frames/sec.

Movie S5 | Mutant virus unable to bud is compromised in its ability to be transferred from cell to cell. A Cos-1 cell generating mutant MLV impaired in its ability to be released from cells in contact with filopodia of a non-infected target XC cell expressing mCAT1-YFP (red). The mutant virus is labeled with mutant Gag-CFP (green) and Env-YFP (red). Several virus particles (white dashes) are seen in contact with cytonemes, but fail to initiate movement. One particle engages one cytoneme, but then stalls. Images were taken every ~20 s over a period of 18 min. The video is played at a speed of 5 frames/sec.

Movie S6 | Viruses move in the opposite direction of cytonemes extension. An experiment as in Movie 3 was performed. The red and green dashes indicate the opposite movement of filopodia and viruses in contact zones. Images were taken every ~45 sec over a period of 15 min. The last 6 frames were recorded after a gap of 15 min to illustrate the lengthening and internalization of the filopodium. The video is played at a speed of 5 frames/sec.

Movie S7 | **HIV-1 transmission via cytonemes.** A time-lapse movie showing a Cos-1 cell generating infectious HIV-1, labeled by co-expression of HIV-1 Gag-CFP (green), in co-culture with a non-infected target XC cell expressing CXCR4 and CD4-YFP (red). Several HIV particles are transferred along filopodia from infected toward non-infected cells. Images were taken every ~60 s over a period of 41 min. The video is played at a speed of 10 frames/sec. Size bar represents 10 μm.

Movie S8 | **Hypothetical model for the polarized spread of viruses in tissues via cytonemes.** The expression of Env in an infected cell would lead to the establishment of cytonemes allowing for the transmission of viruses to neighboring cells. Viral cytonemes would be transient due to the downregulation of receptor following viral infection. Repeated rounds of polarized contacts would provide a model for viral spread in tissues.

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