Aus dem Max von Pettenkofer-Institut

Lehrstuhl für Virologie

Institut der Ludwig-Maximilians-Universität München

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LFA1 and ICAM1 are critical factors for fusion and spread of murine leukemia virus

Dissertation
zum Erwerb des Doktorgrades der Naturwissenschaften
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität München

vorgelegt von

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aus

Laupheim

Jahr

2024

Mit Genehmigung der Medizinischen Fakultät

der Universität München

Betreuerin:	PD Dr. Barbara Adler
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Tag der mündlichen Prüfung:	15. November 2024

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Zusammenfassung

Retroviren können sich durch zwei verschiedene Formen der Zellkontakt-abhängigen Übertragung effizient von Zelle zu Zelle ausbreiten. Zum einen können sich Retroviren von produktiv infizierten Zellen über Zell-Zell Kontakte zu benachbarten Zellen ausbreiten. Zum anderen können Zellen, die nicht infizierbar sind Viruspartikel an ihrer Oberfläche binden und auf empfängliche Zellen übertragen, was als *Trans*-Infektion bezeichnet wird. Makrophagen und dendritische Zellen, die das Sialinsäure-bindende Protein CD169 exprimieren, unterstützen die *Trans*-Infektion von Lymphozyten mit HIV und MLV *in vitro* und *in vivo* durch die Sequestrierung von Viruspartikeln. Kürzlich zeigte man mittels Intravitalmikroskopie in den peripheren Lymphknoten lebender Mäuse *Trans*-Infektion von MLV, indem Viruspartikel von CD169+ Makrophagen über enge Zell-Zell-Kontakte auf Lymphozyten transferiert wurden. Dies weist auf eine entscheidende Rolle von Zelladhäsionsproteinen bei der *Trans*-Infektion hin.

In dieser Arbeit untersuchten wir die Rolle der Zelladhäsionsproteine LFA1 und ICAM1 bei der retroviralen Ausbreitung. Zunächst etablierten wir ein Ko-Kultur Modell für *Trans*-Infektion mit primären Zellen, um die Adhäsionsprotein-abhängige Ausbreitung *in vitro* zu untersuchen. Als Spenderzellen fungierten hierbei CD169+ Makrophagen, die in der Lage sind MLV-Partikel an ihrer Zelloberfläche zu binden. Als Zielzellen dienten mit MLV infizierbare FoxP3+ CD4+ T-Zellen. Mit diesem Assay konnten wir eine entscheidende Funktion von LFA1 auf Empfängerzellen und seines Liganden ICAM1 auf Trägerzellen bei der interzellulären Übertragung von MLV nachweisen. Um diese Ergebnisse *in vivo* zu verifizieren, führten wir adoptive Tansferexperimente in lebenden Mäusen durch. Dabei konnten wir nachweisen, dass die Oberflächenexpression von LFA1 auf den Empfängerzellen für die Ausbreitung von MLV *in vivo* entscheidend ist. Interessanterweise zeigte sich keine Rolle von ICAM1 *in vivo*, eine Beteiligung ist daher weiterhin unklar.

Um ein breiteres Spektrum an Experimenten für die Erforschung von zellulären Oberflächenproteinen im Kontext der Virusübertragung zur Verfügung zu stellen, haben wir einen Fusionstest für MLV entwickelt, um die Fusion von MLV im Rahmen der *Trans*-Infektion *in vitro* und *in vivo* zu quantifizieren. Damit konnten wir erstmals zeigen, dass Adhäsionsproteine bereits in diesem ersten Schritt der Infektion eine wichtige Rolle spielen.

Um festzustellen, ob unsere Ergebnisse auch für andere Retroviren gelten, haben wir die Rolle von LFA1 bei der Ausbreitung von HIV in primären menschlichen CD4+ T-Zellen getestet. Durch die Erzeugung LFA1-defizienter T-Zellen durch Knock-out des *ITGAL*-Gens mittels CRISPR/Cas9, konnten wir zeigen, dass eine effiziente Ausbreitung von HIV in einer T-Zell-Population die Oberflächen-Expression von LFA1 erfordert.

Mithilfe der konfokalen Mikroskopie lebender Zellen analysierten wir die dynamische Interaktion zwischen Spender- und Zielzellen während der *Trans*-Infektion und charakterisierten die Rolle von LFA1 und ICAM1 auf Einzelzellebene. Durch die

Visualisierung subzellulärer Strukturen konnten wir die dynamische Interaktion zwischen WT und Adhäsionsprotein-defizienten Zellen *in vitro* quantifizieren und zeigen, dass LFA1 und ICAM1 die Zellkontaktdynamik jeweils in unterschiedlichem Ausmaß beeinflussen.

Zusammenfassend hat diese Studie neue mechanistische Details über die Ausbreitung von Retroviren durch *Trans*-Infektion und die Rolle von zellulären Adhäsionsproteinen in diesem Prozess offenbart. Wir konnten die Bedeutung der LFA1-ICAM1-Interaktion bei der retroviralen Fusion und Ausbreitung *in vitro* und *in vivo* bestätigen und darüber hinaus neue, nützliche Werkzeuge für die weitere Untersuchung der viralen Fusion und Kontaktdynamik *in vivo* zur Verfügung stellen.

Abstract

Retroviruses can efficiently spread from cell to cell by two different modes of cell contact-dependent transmission. In *cis*-infection retroviruses spread from productively infected cells to neighboring lymphocytes via cell-cell contacts. The other mode is called *trans*-infection. Here, non-permissive cells capture and transfer virus particles to susceptible cells. Macrophages and dendritic cells expressing the sialic acid-binding protein CD169 support *trans*-infection of lymphocytes by HIV and MLV *in vitro* and *in vivo* through viral particle sequestration. Recently, intravital imaging of MLV *trans*-infection in peripheral lymph nodes of living mice revealed tight cell-cell contacts for the transfer of viral particles from sinus-lining CD169+ macrophages to target lymphocytes, indicating a critical role of cell adhesion proteins in *trans*-infection.

In this study, we investigated a role of the cell adhesion proteins LFA1 and ICAM1 in retroviral spread. First, we established a *trans*-infection co-culture model with primary cells to study adhesion protein-dependent spread *in vitro*. CD169+ macrophages that are able to capture MLV particles served as donor and MLV permissive FoxP3+ T cells served as target cells. With this assay we could demonstrate a crucial function of LFA1 on target cells and its ligand ICAM1 on donor cells for MLV cell-cell transmission. To confirm these results *in vivo*, we performed adoptive transfer experiments in living mice. Importantly, we could verify that LFA1 expression by target cells is essential for MLV spread *in vivo*. Interestingly, the role of ICAM1 on donor cells remains obscure *in vivo*.

Further, we established a fusion assay for MLV, to quantify MLV fusion in the context of *trans*-infection *in vitro* and *in vivo*. For the first time, we could thereby show that adhesion proteins play an important role already in this very first step of infection.

To see if our results are also true for other retroviruses, we tested the role of LFA1 in HIV spread within primary human T cells. Generating LFA1-deficient primary human T cells by knock-out of the *ITGAL* gene using CRISPR/Cas9, we could show that efficient HIV spread in T cell populations requires surface expression of LFA1.

Using live cell confocal microscopy, we analyzed the dynamic interaction between donor and target cells during *trans*-infection and characterized the role of LFA1 and ICAM1 at the level of individual cells. By visualizing subcellular structures, we could quantify the dynamic interaction between WT and adhesion protein-deficient cells *in vitro* and show that LFA1 and ICAM1 each influence cell contact dynamics to different extents.

In conclusion, this study provides novel mechanistic details about retroviral spread by *trans*-infection and the role of cellular adhesion proteins in this process. We could confirm the importance of the LFA1-ICAM1 interaction in retroviral fusion and spread *in vitro* and *in vivo* and further provide useful new tools for further investigation of *in vivo* viral fusion and contact dynamics.

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List of Abbreviations

BlaM	Beta-lactamase
BSA	Bovine serum albumine
BSL	Bio Safety Level
CD	Cluster of Differentiation
CMV	Cytomegalovirus
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylendiaminetetraacetic acid
FBS	Fetal Bovine Serum
H_2O	Water
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HTLV	Human T cell lymphotropic virus
ICAM1	Intercellular adhesion molecule 1
IFNalpha	Interferon alpha
IFNAR	Interferon alpha/beta receptor subunit 1
IgG	Immunglobulin G
IL	Interleukin
IS	Immunological Synapse
IU	Infectious units
kDa	kilodalton
KO	Knock-out
LFA1	Lymphocyte function-associated antigen 1
LTR	Long Terminal Repeat
MLV	Murine Leukemia Virus
MOI	Multiplicity of infection
MR	Mannose receptor
MTOC	Microtubule-organizing center
MW	Megawatt
PBS	Phosphate-buffered saline
PEI	Polyethylenimine
PFA	Paraformaldehyde
pLN	Popliteal lymph node
PMA	Phorbol 12-myristate 13-acetate
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RT	Room temperature
SMAC	Supramolecular activation cluster
VLP	Virus like particle
VS	Virological synapse
WT	Wild type

1. Introduction

1.1 The HIV pandemic – a persistent thread to health

Isolated and described for the first time in 1983, the human immunodeficiency virus (HIV) is a global health thread and the cause for an estimated number of 40 million deaths to date (Barré-Sinoussi et al., 1983; WHO, 2023). In 2022, over 630,000 people died from HIV-related causes and around 1.3 million people newly acquired HIV, resulting in a total population of 49 million people living with HIV in 2022 (WHO, 2023).

Upon infection, HIV spreads to lymph nodes within 3-6 days, where it rapidly infects large populations of target CD4+ T cells, allowing for a fast following systemic dissemination in under one month time (Cohen et al., 2011; Wong & Yukl, 2016). With no cure available so far, infection with HIV has to be treated life-long with highly active anti-retroviral therapy (HAART), resulting in a reduction of the viral load to undetectable levels and prevention of further transmission between individuals. Although therapy enables a life expectancy comparable with that of healthy individuals, lapses in the daily treatment results in a rapid viral rebound originating from latent viral reservoirs (Calin et al., 2016; Chun et al., 1997; Li et al., 2016; Scholz & Kashuba, 2021; Van Sighem et al., 2010). Moreover, long-term infection with HIV is increasing the risk for cardiovascular diseases, cancer and diabetes mellitus significantly, which can cause serious health complications in affected patients (Cihlar & Fordyce, 2016; Duffau et al., 2018; Guaraldi et al., 2011, 2013).

To finally find a cure for HIV, understanding the underlying mechanisms of viral spread - especially in the crucial lymph node environment - is essential. So far, *in vitro* studies of HIV cell-to-cell spread gave striking evidence that cell contact-dependent transmission is up 10^3 times more efficient than cell-free infection (Chen et al., 2007; Dimitrov et al., 1993) and even appears resistant to certain neutralizing antibodies (Abela et al., 2012; Duncan et al., 2014; Malbec et al., 2013). In this study, we will focus on cellular factors that may favor effective cell-to-cell transmission of HIV.

1.2 Retroviruses and their life cycle

Retroviruses such as the human immunodeficiency virus (HIV) and the murine leukemia virus (MLV) are enveloped RNA viruses that are 80-100 nm in diameter (J. M. Coffin, 1992a; John M. Coffin, 1992b; Coffin J.M., 1996). The linear, single-stranded RNA is about 7-12 kb in size and of positive polarity. All retroviruses have four basic genomic

domains in common: *pro, gag*, *pol* and *env*, encoding information for the production of new virions. *Pro* encodes for the protease and *gag* for the nucleoprotein. *Pol* encodes for the genetic information of the enzyme's reverse transcriptase and integrase. *Env* encodes for the viral surface envelope protein that is essential for the specific binding to the host cell receptor (Coffin et al., 1997).

The first step of retroviral infection is binding of the viral particle to the cell and subsequent entry into the host cell. Binding is thereby initiated by viral surface glycoproteins engaging with specific receptor molecules on the cell surface (Hunter E, 1997). A functional receptor for binding and fusion of the ecotropic MLVs is the amino acid transporter mCAT-1 (mouse cationic amino acid transporter 1), that serves as a receptor independently of its transporter function (Hunter E, 1997; H. Wang et al., 1994). Similarly, the human T cell leukemia virus (HTLV) uses the glucose transporter Glut1 as an entry receptor (Manel et al., 2003; Mueckler, 1994). In contrast to that, for HIV and the simian immunodeficiency viruses (SIV) the CD4 antigen serves as a receptor for viral attachment to a host cell (Dalgleish et al., 1984; Klatzmann et al., 1984). For HIV, in addition to CD4, a co-receptor such as CXCR4 or CCR5 is necessary for HIV in order to fuse with the host cell after attachment (Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996).

The namesake mechanism of the retrovirus family is its replicative strategy, where the virus uses reverse transcriptase to translate the viral genomic RNA into linear double-stranded DNA (Coffin et al., 1997). Reverse transcription of the viral RNA into DNA leads to the formation of long terminal repeats (LTRs) that are formed at both ends of the DNA (Coffin et al., 1997). These LTRs regulate viral gene expression and replication and are necessary for permanent integration of the reverse transcribed virus DNA into the host genome as a so-called pro-virus (Coffin et al., 1997). Integrated pro-virus is henceforth part of the cellular genome and is expressed via the host RNA polymerase II, that binds to the LTR, and further replicated by cellular enzymes (Coffin et al., 1997). When all viral glycoproteins as well as the viral RNA are synthesized, assembly of pre-mature virus particles takes place at the cell membrane. It is mainly driven by the Gag protein (Coffin JM, Hughes SH, Varmus HE, 1997; Göttlinger, 2001; Hunter E, 1997). Subsequent budding and release of the virus particle from the host cell membrane is facilitated by the cellular endosomal sorting complexes required for transport (ESCRT) (Morita & Sundquist, 2004; Peel et al., 2011; Sundquist & Kra, 2012). Finally, maturation of the virus particle is facilitated by the viral protease (PR) during budding by cleaving Gag and Gag-Pro-Pol at several sites, resulting in a mature and infectious virus particle (Coffin JM, Hughes SH, 1997; Erickson-Viitanen et al., 1989; Hatanaka & Nam, 1989; Ledbetter, 1979; Sundquist & Kra, 2012).

1.3 Cell contact-dependent retrovirus spread

1.3.1 Modes of retrovirus spread

Besides cell-free virus spread, retroviruses, such as HIV, HTLV, and MLV can spread efficiently between leukocytes in a cell contact-dependent manner (Phillips, 1994; Sattentau, 2008). Cell contact-dependent spread is thereby divided into two categories: First, the so-called *cis*-infection describes spread with cell-cell contacts formed between a productively infected donor cell and a permissive target cell (Hübner et al., 2009; Igakura et al., 2003; Jin et al., 2009; Jolly et al., 2004; Sherer et al., 2007; Yamamoto et al., 1982) (Figure 1). The established cell contact for virus transfer is termed virological synapse (VS) and shares several features with the immunological synapse (IS) between immune cells during T cell priming (Igakura et al., 2003; Vasiliver-Shamis et al., 2009, 2010). Second, retroviruses can spread between cells by a mechanism called *trans*-infection. This process is characterized by a non-infected cell, which is able to bind virus particles to its surface and present them to permissive target cells for infection. The established contact site between cells is designated as an infectious synapse (Cameron et al., 1992; Geijtenbeek, Kwon, Torensma, van Vliet, et al., 2000; Jin et al., 2009; McDonald et al., 2003). In *trans*-infection, binding of virus particles to the surface of donor cells is mediated by lectin-binding proteins such as CD209b (DC-SIGN), CD169 (Siglec-1) or the mannose receptor (MR), that can be expressed on the surface of DCs and macrophages (Erikson et al., 2015; Geijtenbeek, Kwon, Torensma, van Vliet, et al., 2000; Izquierdo-Useros, Lorizate, Contreras, et al., 2012; Puryear et al., 2013; Sallusto et al., 1995; Taylor et al., 1992; Torow et al., 2015; Turville et al., 2002). DC-SIGN and the MR recognize specific glycosylation patterns within the Env glycoprotein of the retrovirus envelope. In contrast, Siglec-1 (CD169) is binding the ganglioside GM3 which is highly enriched in the membrane of the retroviruses HIV and MLV (Chan et al., 2008; Geijtenbeek, Kwon, Torensma, Van Vliet, et al., 2000; Geyer et al., 1988; Lai et al., 2009; Puryear et al., 2012, 2013).

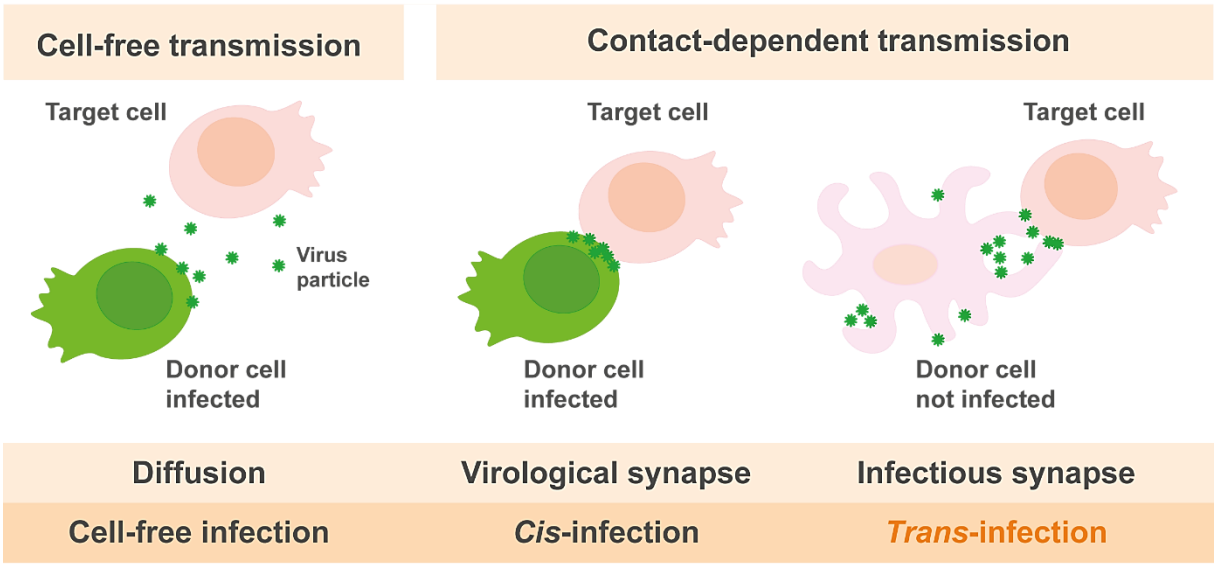

Figure 1: Schematic illustration of retroviral modes of transmission.

Retroviruses can either spread cell-free via diffusion in the extracellular space or via a cell contact dependent manner (Phillips, 1994; Sattentau, 2008). Cell contact-dependent spread is thereby divided into two categories: *Cis*-infection in which the contact is designated as a virological synapse, and *trans*-infection in which the contact side is denoted as an infectious synapse (Hübner et al., 2009; Igakura et al., 2003; Jin et al., 2009; Jolly et al., 2004; Sherer et al., 2007; Vasiliver-Shamis et al., 2010; Yamamoto et al., 1982). While *cis*-infection describes the contact between a permissive target cell and a productively infected donor cell (Jolly et al., 2004; Piguet & Sattentau, 2004), *trans*-infection describes the contact between a permissive target cell and a non-infected donor cell, that is able to bind virus particles at its surface (Geijtenbeek, Kwon, Torensma, Van Vliet, et al., 2000; McDonald, 2010; Piguet & Sattentau, 2004). Illustration was adapted from (Piguet & Sattentau, 2004).

1.3.2 Contact-dependent retroviral transmission is highly efficient

Cell contact-dependent spread is efficient and influences the pathogenesis of retroviruses (Chen et al., 2007; Dimitrov et al., 1993; Vasiliver-Shamis et al., 2010).

In HIV *cis*-infection, virus assembly and budding from the infected donor cell is localized at the cell-cell contact side. This results in a high density of virus particles at the synaptic cleft between donor and the permissive target cell, thereby favoring infection of the latter (Fais et al., 1995; Jolly et al., 2004; Jolly & Sattentau, 2004; Perotti et al., 1996). Remarkably, this mechanism was shown to result in an increase of infectivity by up to 10^3 compared to cell-free spread (Dimitrov et al., 1993). Further, investigation on HIV spread between T cells (*cis*-infection) revealed that upon contact a rapid recruitment of cellular and viral proteins takes place. While on the donor cell side, an enrichment of viral proteins Env and Gag can be observed, recruitment of talin and LFA1 as well as HIV receptors CD4 and CXCR4 on the target cell is observed (Jolly & Sattentau, 2004). The rapid reorganization of cellular components in both cases is facilitated by the actin

cytoskeleton, which seems to be further induced by Env in case of the donor cell (Jolly et al., 2004; Jolly & Sattentau, 2004).

In HIV *trans*-infection, between mature dendritic cells and T cells, similar enrichment of virus particles at the side of cell contact could be observed. Here, high viral titers are accumulated through binding of HIV particles to C-type lectins, resulting in efficient viral transfer upon contact with a target cell (Arrighi et al., 2004; Kwon et al., 2002). Moreover, it was shown that *in vitro* captured HIV particles remain infectious for a longer timespan than cell-free virus (Geijtenbeek, Torensma, et al., 2000; Kwon et al., 2002). In addition, similar to cis-infection, recruitment of cellular adhesion proteins LFA1 and ICAM1 to the side of cell-contact were shown, stabilizing the contact side and thereby potentially prolonging cell-contact (Rodriguez-Plata et al., 2013).

Due to tight cell-cell contacts mediated by adhesion proteins in both, *cis*- and *trans*-infection, cell contact-dependent transmission is likely to be more resistant to certain neutralizing antibodies (Abela et al., 2012; Chen et al., 2007; Duncan et al., 2013; Jolly et al., 2007; Jolly & Sattentau, 2004; Malbec et al., 2013; Reh et al., 2015). This may allow retroviruses to evade the humoral immune response as well as treatment attempts with broadly neutralizing antibodies (Abela et al., 2012; Ganesh et al., 2004; Martin & Sattentau, 2009).

Moreover, *in vitro* and *in vivo* studies of HIV revealed that virus transmission through tight cell-cell contacts can lead to integration of multiple proviruses (Del Portillo et al., 2011; Law et al., 2016; Russell et al., 2013). This might result in a high chance of diversification of HIV sequence variations (Del Portillo et al., 2011; Law et al., 2016) and increasing the chance of developing resistance to current treatments (Carvajal-Rodriguez et al., 2007).

Given these features and the striking *in vitro* evidence that cell-to-cell spread is considered to be highly more efficient than cell-free spread (Chen et al., 2007; Dimitrov et al., 1993), further investigation of cell contact-dependent spread is essential to gain better insight into retroviral transmission and spread and to further improve treatment of associated diseases.

1.3.3 LFA1 and ICAM1 in cell contact-dependent retroviral spread

The lymphocyte function-associated antigen 1 (LFA1) belongs to the family of integrins, which are cellular adhesion receptors, initially discovered to mediate connection of the extracellular environment with the cytoskeleton (Dustin & Springer, 1989, 1991; Walling

& Kim, 2018). One of its main interaction partners is the intercellular adhesion molecule 1 (ICAM1), belonging to the immunoglobulin superfamily (Dustin & Springer, 1989; Makgoba et al., 1988; Williams & Barclay, 1988). Mediating lymphocyte adhesion and migration, LFA1-ICAM1 interaction is critical for adaptive and innate immune responses (Anderson & Springer, 1987; Dustin & Springer, 1989, 1991; Patarroyo & Makgoba, 1989).

In addition, by supporting formation of the IS (Grakoui et al., 1999; Krummel & Davis, 2002; Sims & Dustin, 2002), LFA1 and ICAM1 are essential for immune cell priming (Campi et al., 2005; Choudhuri et al., 2014; Monks et al., 1998; Scholer et al., 2008) and cell-mediated killing (Jenkins & Griffiths, 2010; Walling & Kim, 2018). The IS is initiated when the major histocompatibility molecule peptide complex (MHC-peptide) of an antigen presenting cell is recognized by the T cell receptor (TCR) of a T cell (Unanue, 1984). During formation of the IS, LFA1 and ICAM1 are part of an adhesive ring structure at the cell-cell contact side, called the peripheral supramolecular activation cluster (pSMAC). This ring structure surrounds a cluster of T cell receptors, a structure called central supramolecular activation cluster (cSMAC) (Monks et al., 1998). Engagement of LFA1 was shown to be linked with multiple signaling cascades. Inside-out signaling allows for the conformational extension of LFA1, enabling intermediate affinity binding to interaction partners like ICAM1. Initiated by that binding, outside-in signaling subsequently induces a ZAP-70 kinase dependent cascade, resulting in a cytoskeleton remodeling and the final stage of integrin activation. This leads to a strengthening of the integrin interaction and finally the cell-cell interaction itself (Evans et al., 2011; Hogg et al., 2011; Salas et al., 2006; Shamri et al., 2005). Interaction of LFA1 and ICAM thereby allows for stable, highly effective T cell stimulation as it slows down cell motility by generating a strong adhesive surface and furthermore shielding the MHC-peptide-TCR interaction in the cSMAC center (Dustin, 2009; Dustin et al., 1997; Dustin & Springer, 1989; Hogg et al., 2011; Monks et al., 1998; Scholer et al., 2008). T cell activation then results in activation of numerous genes and polarization of cell organelles and vesicle release to the side of cell-cell contact (Huppa & Davis, 2003; A. Kupfer et al., 1983; A. Kupfer & Dennert, 1984).

Similar to their function within the IS, LFA1-ICAM1-interaction was shown to be critical for the formation of the VS and infectious synapses during *cis*- and *trans*-infection, respectively, and thereby supporting the highly efficient contact-dependent spread of HIV and other retroviruses (Jolly et al., 2004, 2007; Rodriguez-Plata et al., 2013; Starling & Jolly, 2016; Vasiliver-Shamis et al., 2008, 2010). In the following two paragraphs, formation of the VS and infectious synapse as well as the role of LFA1 and ICAM1 will be explained in more detail.

The Virological Synapse

The virological synapse is a tight adhesive junction that is formed between an infected donor cell and a permissive target cell during *cis*-infection (Jolly & Sattentau, 2004; Vasiliver-Shamis et al., 2010). To date, the virological synapse formation is best described between an HIV-infected donor CD4+ T cell and target CD4+ T cell (Figure 2). It was shown that, in this case, the VS is initiated by binding of the HIV envelope glycoprotein gp120 on the donor cell to CD4 on the target cell (Chen et al., 2007; Vasiliver-Shamis et al., 2008).

Similar to the MHC-peptide-TCR complex in the IS, gp120-CD4 interaction initiates recruitment of cell surface proteins and formation of a stable, adhesive interphase in an actin-dependent manner (Jolly & Sattentau, 2004; Vasiliver-Shamis et al., 2010). HIV co-receptors CCR5 and CXCR4 were shown to accumulate together with the gp120-CD4 complex at the VS center, and are critical for efficient infection of the target cell (Chen et al., 2007; Hübner et al., 2009; Vasiliver-Shamis et al., 2008). Like in the IS, adhesion molecules LFA1 and ICAM1 on the donor and the target cell side were described to cluster in a ring-like structure around this center (Jolly et al., 2004, 2007; Piguet & Sattentau, 2004; Starling & Jolly, 2016; Vasiliver-Shamis et al., 2010). The LFA1-ICAM1 interaction thus supports spread of HIV-1 by stabilizing and shielding the gp120-CD4 interaction (Jolly et al., 2004, 2007; Vasiliver-Shamis et al., 2008).

Besides recruitment of surface proteins, binding of gp120 to CD4 also initiates rearrangement of the actin cytoskeleton and microtubule polarization to the contact side (Jolly et al., 2007). Further the presence of LFA1 at the VS interface is triggering cellular rearrangement via ZAP-70 kinase pathways and translocation of the MTOC and the Golgi to the VS (A. Kupfer et al., 1983; B. A. Kupfer et al., 1987; Starling & Jolly, 2016). These modifications result in polarization of the HIV Gag precursor and envelope glycoproteins to the cell-cell interface (Rudnicka et al., 2009; Vasiliver-Shamis et al., 2010). With that, polarized viral assembly at the contact side is facilitated, resulting in efficient cell-to-cell spread (Starling & Jolly, 2016).

The Infectious Synapse

The infectious synapse is a tight adhesive junction, formed between an uninfected donor and a permissive target cell during *trans*-infection (Figure 2) (McDonald, 2010; Rodriguez-Plata et al., 2013). To date, the infectious synapse is not studied as extensively as the virological synapse but there is evidence that the infectious synapse formation is mainly a hijacking of normal cellular processes like the interaction between DCs and T

cells (Arrighi et al., 2004; Bracq et al., 2018). For example, it was shown that recognition of the MHC-peptide complex by the TCR actively enhances productive HIV transmission to the target cell, but in contrast to the IS is not necessarily involved in the formation of the infectious synapse (Bracq et al., 2018; Garcia et al., 2005; Rodriguez-Plata et al., 2013). Unlike in the VS, the HIV infectious synapse is also not dependent on the gp120-CD4 interaction. Interestingly, the formation of the infectious synapse seems to be mainly dependent on an interaction between the cellular adhesion proteins LFA1 and ICAM1, since disruption of this interaction resulted in a decrease of synapse formation by 60% (Rodriguez-Plata et al., 2013). After initiation of the contact via LFA1 and ICAM, reorganization of cell surface proteins as well as structural organization of the actin cytoskeleton like in the IS and VS was shown (McDonald et al., 2003; Ménager & Littman, 2016). Thereby C-type lectins like DC-SIGN and CD169 on the donor cell as well as CD4 and its co-receptors CXCR4/CCR5 on the target cell are accumulating at the cell-cell interface and allow for efficient virus transfer (Dimitrov et al., 1993; McDonald et al., 2003).

In addition to the central role of LFA1 and ICAM1 during initiation of the infectious synapse, intravital imaging of MLV *trans*-infection in peripheral lymph nodes of living mice indicated a critical role of cell adhesion proteins in *trans*-infection by revealing tight cell-cell contacts for transfer of viral particles from sinus-lining CD169+ macrophages to target lymphocytes (Sewald et al., 2012, 2015).

With the knowledge of LFA1 and ICAM1 essentially supporting contact-dependent retroviral transmission *in vitro*, the actual contribution of these adhesion proteins *in vivo* could add to understanding the pathogenic features of retroviral spread.

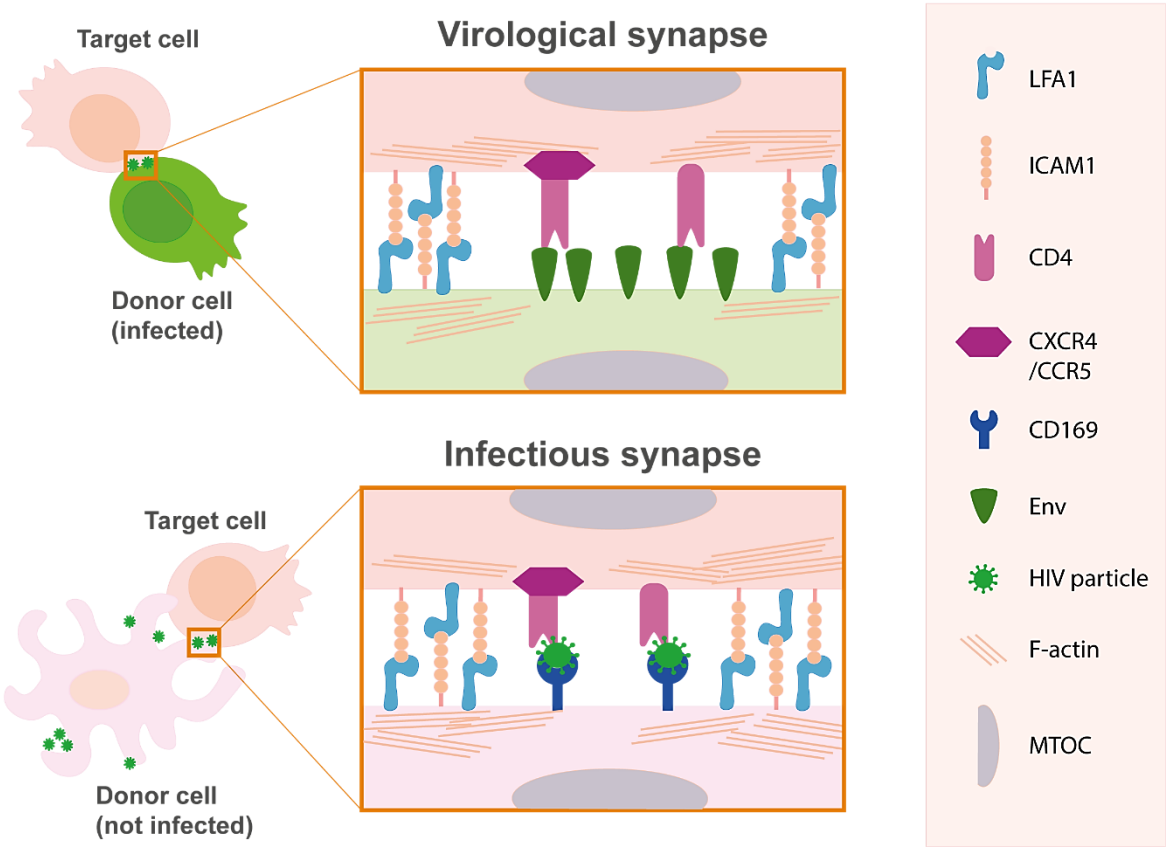

Figure 2: Schematic Illustration of the HIV virological synapse and infectious synapse during *cis*- and *trans*-infection, respectively.

The virological synapse is initiated by binding of the gp120 subunit of the HIV glycoprotein Env on the donor cell to the HIV receptor CD4 on the target cell (Chen et al., 2007; Vasiliver-Shamis et al., 2008). Subsequently HIV co-receptors CXCR4 and/or CCR5 are recruited to the contact side (Chen et al., 2007; Hübner et al., 2009; Vasiliver-Shamis et al., 2008). In addition, cell adhesion molecules LFA1 and ICAM1 are recruited and accumulate in a ring like structure around the core of viral proteins and receptors (Jolly et al., 2004, 2007; Vasiliver-Shamis et al., 2008). Further synapse formation triggers reorganization of the cytoskeleton and cellular organs towards the side of cell-cell contact, allowing for polarized virus assembly and release at the cell interface (Jolly et al., 2007, 2011; A. Kupfer et al., 1983; B. A. Kupfer et al., 1987). The infectious synapse is initiated by binding of LFA1 to ICAM1 (Rodriguez-Plata et al., 2013). Subsequently, other surface receptors like CD4 and co-receptors CXCR4/CCR5 on the target cell and for example C-type lectins on the donor cell are recruited to the cell-cell interface (McDonald et al., 2003). Hence, viral particles bound to C-type lectins like DC-SIGN or CD169 on the donor cell can bind to CD4 on the target cell. Like in the virological synapse, rearrangement of cytoskeleton and cellular organs likely to contributes to efficient infection (Ménager & Littman, 2016). Illustration was adapted from Bracq et al., 2018, Rodriguez-Plata et al., 2013 and Sattentau, 2008.

1.4 Investigating retrovirus fusion

Cell-to-cell spread of retroviruses is often studied by examining cell infection. However, besides the necessity of an entry receptor, additional factors can influence the productive infection of a cell. In MLV for example, cell division is crucial for integration of the viral DNA into the host genome (Roe et al., 1993). Moreover, several retroviruses are blocked before productive infection of the cell by cellular restriction factors such as Trim5α which interferes with viral uncoating or APOBEC3, which is able to mutagenize the viral retrotranscribed DNA (Reuben S Harris *et al.*, 2003a; Reuben S. Harris *et al.*, 2003b; Zhang *et al.*, 2003; Goff, 2004; Mangeat *et al.*, 2004). Therefore, the read-out of infection alone might not be sufficient to study the true effect of LFA1 and ICAM1 on retroviral spread. To circumvent this problem, we decided to additionally investigate an earlier step in the infection cycle, retrovirus fusion.

To allow for the analysis of fusion *in vitro* and *in vivo*, an adaption of the BlaM fusion assay was used. Originally discovered in penicillin resistant bacteria, beta-lactamases are an enzyme family that can cleave the beta-lactam ring structure that is found in all penicillins and cephalosporins (Abraham & Chain, 1940; Christensen et al., 1990). In 1998, Zlokarnik et al. designed the beta-lactamase reporter substrate CCF2, which, as its analog CCF4, is able to perform a rapid shift in the wavelength of fluorescent emission upon cleavage based on fluorescent resonance energy transfer (FRET) (Förster, 1948; Zlokarnik et al., 1998). In its esterified form (CCF2-AM), CCF2 is able to efficiently cross cell membranes due to its nonpolar feature (Zlokarnik et al., 1998). Located in the cytosol of the cell, the four ester groups of CCF2-AM get hydrolyzed, trapping CCF2 in the cell and allowing to perform analysis in single living cells (Zlokarnik et al., 1998).

Using this system, it was possible for the first time to detect fusion events of HIV-1 virions with their target cells by fusing beta-lactamase to the HIV-1 accessory protein Vpr (BlaM-Vpr) while leaving the activity of both proteins intact (Bukrinsky & Adzhubei, 1999; Cavrois et al., 2002). Upon fusion of viral particles with target cells and uncoating of the HIV capsid BlaM-Vpr gets released into the cytoplasm of the cell, able to cleave loaded CCF2 substrate, leading to a detectable shift of emission from 520 nm to 447 nm (Cavrois et al., 2002). Unfortunately, this system is restricted to quantify fusion of HIV-1 due to linking of BlaM to the HIV accessory protein Vpr and further requires uncoating of the retroviral capsid.

In 2021, Albanese et al. modified the BlaM-based fusion assay by linking BlaM to a membrane protein of the host cells that is known to be part of the envelope of the Epstein-Barr Virus and retroviruses such as HIV and MLV. In particular, BlaM is linked to the carboxy terminus of CD63 (CD63-BlaM) a member of the tetraspanin family

(Albanese et al., 2021; Andreu & Yáñez-Mó, 2014; Escola et al., 1998). Using modified HEK293T cells that constitutively express CD63-BlaM, viruses and virus-like particles (VLPs) that incorporate CD63-BlaM in their membrane can be produced. Upon fusion with a permissive cell, BlaM is located in the cytoplasm of the cell and is able to cut present substrate. With that, this assay is versatile and is, compared to the Vpr-BlaM approach, independent of the uncoating of the retroviral capsid.

1.5 Aims of the thesis

Cell-to cell spread in retroviral transmission was shown to be much more effective than cell-free infection of cells, yet the mechanisms behind that phenomenon are not fully understood. In this study, we aim to reveal the role of cellular adhesion proteins ICAM1 and LFA1 and their importance in retroviral *trans*-infection *in vitro* and *in vivo.*

In particular, we wanted to shed more light on cell contact-dependent spread in retroviral transmission and provide tools for further investigation with the following objectives:

- Establishment of an *in vitro* assay to study MLV *trans*-infection
- Investigating the role of ICAM1 and LFA1 during MLV *trans*-infection of primary cells *in vitro* and *in vivo*
- Adapting a BlaM-based fusion assay for the use in MLV research and to assess the role of ICAM1 and LFA1 in virus particle fusion
- Examine the role of LFA1 in HIV cell-to-cell transmission of primary cells
- Study cell contact dynamics by labelling cellular organs to visualize the formation of stable cell contacts.

2. Materials & Methods

2.1 Reagents and overview of key resources

Table 1: Overview of cells

Designation	Species	Source	Details
HEK293T	human	ATCC	Human Embryonic Kidney cells, Cat #CRL-1573
S49.1	mouse (BALB/c)	ATCC	T lymphoid cell line, Cat #TIB-28
Primary macrophages	mouse	isolated from mice	Isolated by negative selection from peritoneal cells
Primary B-1 cells	mouse	isolated from mice	Isolated by negative selection from peritoneal cells
Primary naive CD4+ T cells	mouse	isolated from mice	Isolated by negative selection from splenocytes
Primary CD4+ T cells	human	isolated from leukocyte enriched blood of healthy donors	Isolated by negative selection from leukocyte-enriched blood samples
HEK293T-BlaM	human	Gift from the Wolfgang Hammerschmidt lab	Human Embryonic Kidney cells stably expressing beta-lactamase (BlaM) fused to the transmembrane protein CD63
MAX Eff Stbl2	*E.coli*	Thermo Fisher Scientific	Chemically competent *E. coli,* Cat #10268019

Table 2: Mouse strains

Designation	Strain	Source	Details
WT	C57BL/6J	Charles River	Strain Code: 632
CD11a-KO/ LFA1-deficient	B6.129S7-Itgaltm1Bll/J	Jackson Laboratory	Stock No: 005257
ICAM1-KO	B6.129S4-Icam1tm1Jcgr/J	Jackson Laboratory	Stock No: 002867
RFP	Tg(CAG-DsRed*MST)1Nagy/J	Jackson Laboratory	Stock No: 006051

Table 3: Consumables

Description	Company	Product Number
2-Mercaptoethanol	Gibco™	21985023
Albumin Fraction V, endotoxin-tested	Carl Roth	CP84.2
Alt-R® S.p. Cas9 Nuclease V3	IDT	10000735
Aspiration pipette, 2 ml	Greiner	07-000-175
BD Cytofix/Cytoperm™ kit	BD	554714
Cell strainers; 70 µm	Corning	352350
Cellstar® serological pipettes; 5 ml, 10 ml, 25 ml	Greiner	P7615, P7740, P7865
CellTrace™ Far Red Cell Proliferation Kit, for flow cytometry	Invitrogen™	C34572
CO2-independent medium	Gibco™	18045054
Corning® HTS Transwell® 96 well permeable supports; 3 µm	Corning	3386
Counting chamber C-Chip Neubauer improved	Carl Roth	PK36.1
DNase I	Roche	10104159001
DPBS, ohne Calcium, ohne Magnesium	Gibco™	12037539
DYNAL™ Dynabeads™ humaner T-Aktivator CD3/CD28	Gibco™	11161D
EasySep™ Human CD4+ T Cell Enrichment Kit	Stemcell Technologies	19052
ECM Select® Array Kit Ultra-36	Advanced BioMatrix	5170
EDTA 0.5 M	Invitrogen™	15575020
Eppendorf safe-lock tubes; 1.5 ml, 2 ml, 5 ml	Eppendorf	0030120086, 0030120094, 0030119401
Falcon tubes; 15 ml, 50 ml	Corning	-
FalconTM standard tissue culture dishes; 3.5 cm, 10 cm	FalconTM	150460
Fetal Bovine Serum, qualified, One Shot™ format	Gibco™	A3160501
Fibronectin, Solution (human)	Advanced BioMatrix	5050
Glass bottom 35 mm Dish, No. 1.5 Coverslip, 14 mm Glass Diameter, Uncoated	MatTek	P35G-1.5-14-C
Glycin CELLPURE® ≥99 %	Carl Roth	HN07.1
Ionomycin calcium salt	Sigma	I3909-1ML
Liberase™ TL Research Grade Roche)	Roche	5401020001
LiveBLAzer™ FRET-B/G Loading Kit with CCF4-AM	Thermo Scientific	K1095
LPS-B5	InvivoGen	tlrl-pb5lps
MEM NEAA (100X)	Gibco™	11140035
MojoSort™ Streptavidin Nanobeads	Biolegend	480016
Naive CD4+ T Cell Isolation Kit, mouse	Miltenyi Biotech	130-104-453
Normal rat serum	Jackson Immuno Research Laboratories, Inc.	012-000-120
NucleoBond Xtra Midi (100)	Macherey Nagel	740.410.100

NucleoSpin Plasmid (NoLid) (250)	Macherey Nagel	740.499.250
Opti-MEM™ I Serumreduced Medium	Gibco™	11524456
P3 Primary Cell 4D-NucleofectorTM X Kit S	Lonza	V4XP-3032
Paraformaldehyde, 10% w/v aq. soln., methanol free	Thermo Scientific	047317.9M
Pipette filter tips; 10 µl, 20 µl, 200 µl, 1000 µl	Starlab	-
PMA (phorbol 12-myristate-13-acetate)	Sigma	19-144
Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI 25K™)	Polysciences	23966
Polystyrene tubes	Applied Biosystems	112101
Probenecid, water-soluble	Invitrogen™	P36400
ProLong™ Diamond Antifade Mountant	Invitrogen™	P36965
Recombinant Human IL-15 (carrier-free)	Biolegend	570304
Recombinant Human IL-2 (carrier-free)	Biolegend	589104
Recombinant Human IL-7 (carrier-free)	Biolegend	581904
Recombinant Human TGF-β1 (carrier-free)	Biolegend	781804
Recombinant Mouse IL-4 (carrier-free)	BioLegend	574304
Recombinant Mouse IL-5 (carrier-free)	BioLegend	581504
Recombinant Mouse IL-6 (carrier-free)	BioLegend	575704
Retinoic acid	Sigma-Aldrich	R2625
Roti®-CELL HEPES-Lösung, pH 6,98-7,3	Carl Roth	9157.1
RPMI 1640 Medium, GlutaMAX™ Supplement	Gibco™	61870044
Sodium Pyruvate (100 mM) 100X	Gibco™	11360070
Sterican needles; 21 gauge, 26 gauge, 27 gauge	B. Braun	4657527, 4657683, 4657705
Sterile syringes; 10 ml	Fisher Scientific	14-955-459
Tissue culture plates; 48-well, 24-well, 12-well, 6-well	Sarstedt	3923, 3922, 3921, 3920
Tissue culture plates; 96-well flat bottom, round bottom	Sarstedt	3924, 3925
Tissue culture plates; 96-well, V bottom	Corning	3894
Trypan blue solution 0.4 %	Carl Roth	1680.1

Table 4: Antibodies

Designation	Source	Clone	Reference number	Purpose
Alexa Fluor® 647 anti-mouse CD11a Antibody	BioLegend	M17/4	101113	staining
Alexa Fluor® 647 anti-mouse CD169 (Siglec-1) Antibody	BioLegend	3D6.112	142407	staining
Alexa Fluor® 647 anti-mouse CD54 Antibody	BioLegend	YN1/1.7.4	116114	staining
APC/Fire™ 750 anti-human CD11a Antibody	BioLegend	HI111	301225	staining
Biotin anti-mouse CD11c Antibody	BioLegend	N418	117303	biotin labelling
Biotin anti-mouse CD19 Antibody	BioLegend	6D5	115503	biotin labelling
Biotin anti-mouse CD4 Antibody	BioLegend	RM4-5	100507	biotin labelling
Biotin anti-mouse CD8a Antibody	BioLegend	53-6.7	100704	biotin labelling
Biotin anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody	BioLegend	RB6-8C5	108403	biotin labelling
Biotin anti-mouse TER-119/Erythroid Cells Antibody	BioLegend	TER-119	116204	biotin labelling
Brilliant Violet 421™ anti-human CD184 (CXCR4) Antibody	BioLegend	12G5	306517	staining
Brilliant Violet 605™ anti-human CD4 Antibody	BioLegend	RPA-T4	300555	staining
LEAF™ Purified anti-mouse CD11a Antibody	BioLegend	M17/4	101109	blocking
LEAF™ Purified anti-mouse CD54 Antibody	BioLegend	YN1/1.7.4	116109	blocking
LEAF™ Purified anti-mouse IFNAR-1 Antibody	BioLegend	MAR1-5A3	127303	blocking
LEAF™ Purified Rat IgG2a, κ Isotype Ctrl Antibody	BioLegend	RTK2758	400516	isotype control
LEAF™ Purified Rat IgG2b, κ Isotype Ctrl Antibody	BioLegend	RTK4530	400622	isotype control
PE anti-mouse F4/80 Antibody	BioLegend	BM8	123110	staining
Purified anti mouse CD16/32	BioLegend	93	101302	blocking
Purified anti-mouse CD169 (Siglec-1) Antibody	BioLegend	3D6.112	142402	blocking
Purified anti-mouse FOXP3 Antibody	BioLegend	MF-14	126402	staining
Ultra-LEAF™ Purified anti-mouse CD28 Antibody	BioLegend	37.51	102116	coating
Ultra-LEAF™ Purified anti-mouse CD3ε Antibody	BioLegend	145-2C11	100340	coating
Ultra-LEAF™ Purified Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend	MOPC-21	400165	isotype control

Table 5: Viruses

Designation	Plasmids used for transfection	Source
MLV LTR-GFP	Full Length MLV Friend57	Sewald Laboratory
	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	MLV Env	Sewald Laboratory
MLV Gag-GFP	Full Length MLV Gag-GFP (delta Pol)	Sewald Laboratory
	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	MLV Env	Sewald Laboratory
MLV BlaM LTR-GFP ecoEnvVLP	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	MLV LTR-GFP	Sewald Laboratory
	MLV Env	Sewald Laboratory
MLV BlaM LTR-GFP ΔEnvVLP	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	MLV LTR-GFP	Sewald Laboratory
MLV BlaM LTR-GFP VSV-G-VLP	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	MLV LTR-GFP	Sewald Laboratory
	VSV-G	Sewald Laboratory
MLV LTR-lifeact-GFP	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	Lifeact-GFP	Sewald Laboratory
	MLV Env	Sewald Laboratory
MLV LTR-lifeact-mScarlet_i	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	Lifeact-mScarlet_i	Sewald Laboratory
	MLV Env	Sewald Laboratory
MLV LTR-giantin-mScarlet_i	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	Giantin-mScarlet_i	Sewald Laboratory
	MLV Env	Sewald Laboratory
MLV LTR-tubulin-GFP	MLV Gag-Pol (CMV promotor)	Sewald Laboratory
	Tubulin-GFP	Sewald Laboratory
	MLV Env	Sewald Laboratory
HIV NLENG1-IRES (X4 tropic, GFP+)	-	Keppler Laboratory

2.2 Experimental procedures

2.2.1 Mice

For mouse experiments C57BL/6 mice (WT) were obtained from Charles River. Icam1tm1Jcgr/J (ICAM-KO), B6.129S7-Itgaltm1Bll/J (CD11a-KO) and B6.129S4- Tg (CAG-DsRed*MST)1Nagy/J (RFP) were obtained from Jackson Laboratory. All mouse experiments were approved by local authorities. Six- to 12-week-old male and female mice were used for all experiments.

2.2.2 Primary cell isolation

Murine primary cells were isolated from WT, ICAM-KO, CD11a-KO or RFP mice. Macrophages were isolated by wash-out of the peritoneal cavity and subsequent isolation by negative selection using biotinylated antibodies against CD19, CD8, CD4, Gr-1, TER119 and CD11c (see Table 4: Antibodies) and magnetic separation with MojoSort™ Streptavidin Nanobeads (Biolegend) (Figure 3A). Naïve CD4+ T cells were isolated from spleen, using the Naïve CD4+ T Cell Isolation Kit (mouse, Miltenyi Biotec) according to manufacturer's protocol (Figure 3B).

Human primary CD4+ T cells were isolated from blood cones (Terumo BCT leukocyte reduction system) of healthy, anonymized donors with ethical approval by the Ethics Committee of the LMU München (project no. 17-202-UE). Isolation of CD4+ T cells was performed by negative selection with the EasySep™ Human CD4+ T Cell Enrichment Kit (Stemcell Technologies) according to the manufacturer protocol.

2.2.3 Cell cultivation, activation, and differentiation

All eukaryotic cells were cultured at 37°C and 5 % CO_2. HEK293T cells were cultured in RPMI 1640 Medium (Gibco, GlutaMAX™) supplemented with 10 % Fetal Bovine Serum, (Gibco). S49.1 cells and primary murine macrophages were cultured in RPMI 1640 Medium (Gibco, GlutaMAX™) medium supplemented with 10 % Fetal Bovine Serum (Gibco), 1 % MEM Non-Essential Amino Acids (100X, Gibco), 10 mM Sodium-Pyruvate (100X, Gibco), 55 µM 2-Mercaptoethanol (Gibco) and 10 mM Roti®-CELL HEPES (Carl Roth) further referred to as 'primary cell medium'.

Naïve CD4+ T cells were cultured in primary cell medium supplemented with mouse IL-7 and mouse IL-15, both 100 ng/ml (Biolegend). T cells were activated either with 1 µM Ionomycin (Life technologies) and 10 ng/ml PMA (Sigma Aldrich) or by incubation with surface-bound antibodies against CD3ε and CD28 (Biolegend). For CD3ε and CD28 surface-coating, 96-well flat bottom plates were coated with 100 µl antibody mix containing 1 µg CD3 and 1 µg CD28 in PBS, incubated for 2-3 h at 37°C and washed once

with 200 µl PBS. For differentiation into FoxP3+ T cells, cells were cultured on CD3/CD28-coated surfaces in primary cell medium supplemented with mouse IL-7 and IL-15 (100 ng/ml each), 20 ng/ml of human IL-2 (Biolegend), 5 ng/ml human TGF-beta1 (Biolegend) and 10 nM retinoic acid (Sigma-Aldrich) for 48 h. FoxP3-expression was confirmed by flow cytometry with intracellular staining using the BD Cytofix/Cytoperm Kit and anti-FoxP3 antibody (clone MF-14, Biolegend) (Figure 3C).

Human primary CD4+ T cells were cultured in RPMI 1640 Medium (Gibco, GlutaMAX™) supplemented with 10 % Fetal Bovine Serum (Gibco), human IL-2 (50 IU/ml, Biolegend), human IL-7 and human IL-15 (both 100ng/ml, Biolegend). For activation, Dynabeads™ Human T-Activator CD3/CD28 (Gibco) were added to the cells every two weeks at a ratio of 1:10 (beads to cells).

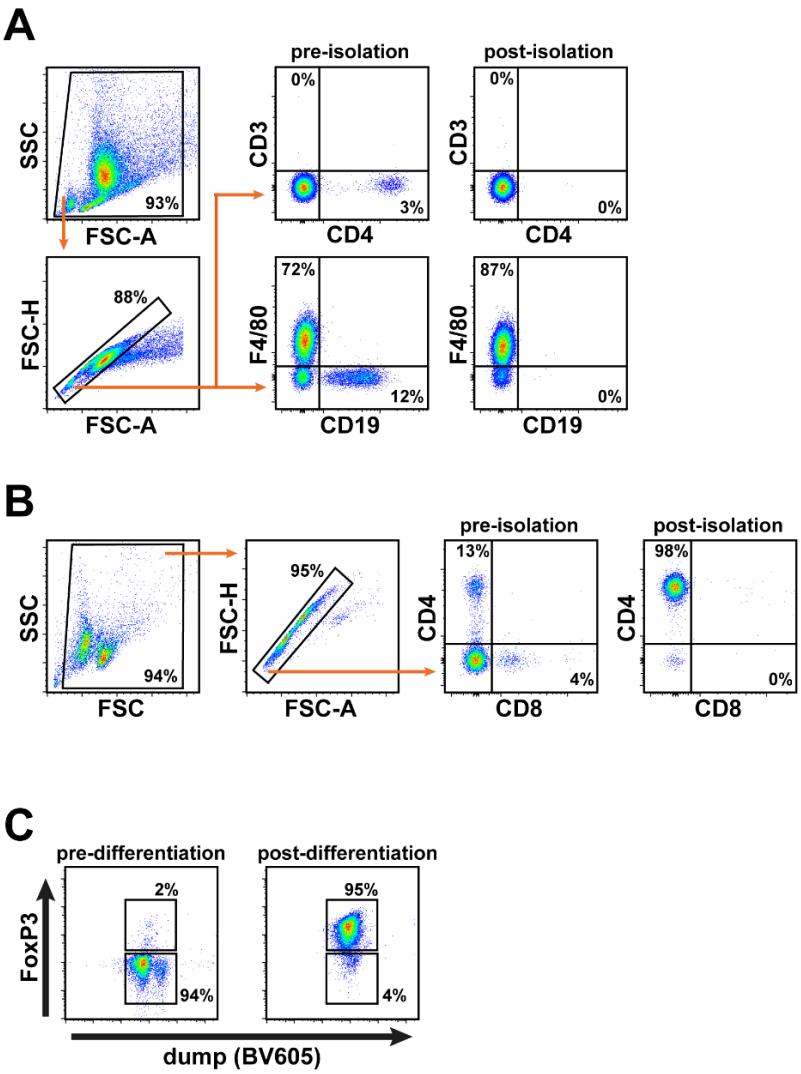

Figure 3: Primary cells can be isolated and differentiated with high purity from mice

(A) Flow cytometric analysis of a cell suspension from the peritoneal cavity, before and after negative selection of macrophages. Washed-out cells were co-stained with anti-F4/80, anti-CD19, anti-CD3 and anti-CD4 antibodies. **(B)** Flow cytometric analysis of cells isolated from spleen, before and after negative isolation of naïve CD4+ T cells. Cells were co-stained with anti-CD4 and anti-CD8 antibodies. **(C)** Flow cytometric analysis of naïve CD4+ T cells before and after differentiation into FoxP3-expressing CD4+ T cells. CD4+ T cells were activated by surface-bound CD3/CD28 and differentiated by addition of cytokines to support FoxP3 expression. Differentiation control are CD3/CD28-activated CD4+ T cells without cytokine differentiation.

2.2.4 Virus production, concentration, and titer determination

MLV viruses and VLPs were generated by co-transfection of HEK293T cells with the plasmid combinations stated in Table 5: Viruses. For that, HEK293T cells were seeded at 70 % confluency in a 10 cm tissue culture plate. For transfection, a total of 12 µg plasmid mix was combined with 36 µl of PEI (Polysciences) in Opti-MEM (Gibco). Transfection mix was incubated for 30 min and distributed dropwise on the cells. After 24 and 48 h, the supernatant was collected, filtered with a 0.45-µm nylon membrane filter, aliquoted, and stored at –80°C. MLV Viruses and VLPs were concentrated by sedimentation with a PBS/15 % sucrose cushion at 20.000 x g for 2 h at 4°C. Viral titers were determined by titrating concentrated virus on S49.1 cells and analyzing fluorescence reporter expression rates after 24 h by flow cytometry using a BD FACSLyric.

Concentrated HIV NLENG1 IRES (X4) was provided by the Keppler laboratory.

2.2.5 PFA fixation

Fixation of cell lines and primary cells (BSL-1 and -2)

For fixation and subsequent flow cytometric analysis, adherent macrophages were detached by 10 min incubation with PBS/2mM EDTA at 4°C and subsequent gentle pipetting and before transfer into reaction tubes. Suspension cells were mixed thoroughly and transferred into reaction tubes. In both cases cells were pelleted, washed once in PBS/1 % BSA and then resuspended in PBS/1 % BSA and fixed with 4 % PFA for 1 h at RT. Cells were washed afterwards once with PBS/1 mM glycine to stop the fixation reaction and were subsequently resuspended in PBS/1 % BSA.

Fixation of adherent macrophages for imaging

Macrophages were washed two times with pre-warmed PBS and fixed with 4 % for 1 h at RT. Cells were washed afterwards once with PBS/1 mM glycine to stop the fixation reaction and subsequently mounted with ProLong™ Diamond Antifade reagent.

Fixation of primary T cells infected with HIV (BSL-3)

Suspension cells were pelleted and resuspended in PBS/4 % PFA. After 90 min of incubation at RT, cells were pelleted and resuspended in PBS for further analysis.

2.2.6 Antibody staining

To analyze surface protein expression, cells were blocked and stained with fluorescent dye-coupled antibodies. In case of fixed cells, blocking and staining was performed at RT. Non-fixed cells were kept at 4 °C throughout staining. First, 1 x 10^6 cells were pelleted and resuspended in 50 µl blocking buffer (PBS/1 % BSA/10 % rat serum) and 10 µg/ml purified anti-mouse CD16/32 antibody (Biolegend). After incubation for 30 min, staining antibodies (see Table 1) were diluted in PBS/1 % BSA, added to the cells in a 1:1 volume ratio and incubated for 1 h. After that, cells were washed two times with 1 ml PBS/1 % BSA and resuspended in PBS/1 % BSA for fixation with PFA (as described in 2.2.5) or short time storage at 4 °C prior to flow cytometric analysis on a FACSLyric™ (BD Bioscience).

2.2.7 IFNAR-1 dependent CD169 expression by peritoneal cavity-derived macrophages

To analyze the contribution of interferon (IFN) to CD169 expression on murine peritoneal macrophages *in vitro*, blocking of IFNAR-1 was performed. For that, macrophages were seeded at a density of 2 x 10^5 cells/well in a 96-well format and anti-mouse IFNAR-1 antibody was added at a concentration of either 1 or 5 µg/well in 200 µl volume per well. For isotype control, 5 µg of mouse IgG1 were added per well in 200 µl medium. Cells were incubated for 24 h at 37 °C. For staining of CD169, macrophages were detached and fixed as described in 2.2.5 and stained with PE-conjugated anti-mouse F4/80 antibody (Biolegend) and Alexa Fluor® 647-conjugated anti-mouse CD169 (Sigec-1) antibody (Biolegend) as described in 2.2.6. Expression levels were analyzed via flow cytometry on a FACSLyric™ (BD Bioscience).

2.2.8 Binding of MLV Gag-GFP virus particles by CD169+ macrophages

To analyze CD169-dependend binding of MLV particles by murine peritoneal cavity-derived macrophages, antibody-blocking of CD169 was performed. For that, macrophages were isolated as described in 2.2.2 and seeded at a density of 2 x 10^5 cells/well in a 96-well flat bottom plate and incubated for 24 h to allow for CD169 surface expression. Cells were washed once with primary medium followed by incubation with purified anti-mouse CD169 (Siglec-1) antibody (Biolegend) at a concentration of 1.7 µg/well in 100 µl primary medium for 20 min at RT. After washing once with 200 µl primary medium, cells were incubated with 100 µl primary medium containing MLV Gag-GFP virus particles at a MOI = 1.5 for 30 min at RT. Unbound virus was removed by extensive washing and macrophages were detached and fixed as described in 2.2.5. MLV binding was quantified measuring GFP intensity by flow cytometry on a FACSLyrik (BD

Bioscience), comparing the mean GFP intensity of cells with and without antibody blocking.

2.2.9 Transduction of CD169+ macrophages with MLV VLPs

To investigate if murine peritoneal cavity-derived macrophages are permissive for MLV infection *in vitro*, cells were transduced with MLV VLPs. This enabled us to have a direct infection read-out of a single-round infection, without virus spreading which would distort the results. Further we had the possibility to use VSV-G-pseudotyped viruses as a direct comparison.

For the assay, macrophages were seeded in a 96-well flat-bottom plate at a density of 2 x 10^5 cells per well in 100 µl volume. After 24 h, VLPs pseudotyped with glycoproteins from ecotropic MLV (ecoEnv) or VSV (VSV-G) containing the reporter genome LTR-GFP were added to the cells at a MOI = 1. Cells were incubated at 37°C for 24 h to allow infection. Next, macrophages were detached and fixed as described in 2.2.5 and analyzed for GFP expression by flow cytometry on a BD FACSLyric (BD Bioscience).

2.2.10 *In vitro trans*-infection assay

To study MLV *trans*-infection *in vitro*, co-culture assays with murine donor and target cells were performed. Peritoneal cavity-derived macrophages were used as donor cells and S49.1 cells, primary PMA/ionomycin-activated CD4+ T cells or FoxP3+ CD4+ T cells as target cells.

Macrophages were seeded at a concentration of 2 x 10^5 cells per well in a 96-well format 24 h prior to co-culture to allow for CD169 surface expression. Macrophages were then loaded with either MLV LTR-GFP or MLV LTR-GFP BlaM VLPs at an MOI = 0.8 and incubated for 30 min at room temperature (RT) to allow virus binding. After extensive washing, target cells were added to macrophages in a 1:2 ratio (target to donor) and incubated for 24 h at 37°C.

For *in vitro trans*-infection assays in a transwell, macrophages were seeded in a 96-well transwell insert (3 µm pore size, Corning) and loaded with virus as described above. After extensive washing, target cells were seeded in the lower transwell compartment at a 1:1 ratio (target to donor). Transwell compartments were combined and incubated for 24 h at 37°C. In both cases, target cells were collected after 24 h of co-culture and fixed with 4 % PFA (as described in 2.2.5). Infection of target cells was analyzed based on GFP expression by flow cytometry on a FACSLyric (BD Biosciences).

2.2.11 *In vitro trans*-infection assays with antibody blocking

Blocking of CD169

To investigate the contribution of CD169-mediated virus binding on macrophages to *trans*-infection, blocking with purified anti-mouse CD169 (Siglec-1) antibody (Biolegend) was performed. Prior to incubation with virus particles, macrophages were incubated with 1.7 µg/well anti-CD169 antibody in a volume of 100 µl for 20 min at RT and washed afterwards once with fresh medium. *Trans*-infection assay was then performed as described in 2.2.10.

Blocking of ICAM1 and LFA1

To investigate the contribution of ICAM1 and LFA1 to *trans*-infection *in vitro*, *trans*-infection assay as described in 2.2.10 was combined with functional blocking of LFA1 and ICAM1 by antibodies. For blocking, purified mouse anti-CD11a antibody (clone M17/4, Biolegend) and purified mouse anti-CD54 antibody (clone YN1/1.7.4, Biolegend), as well as corresponding isotype controls IgG2b and IgG2a (Biolegend), were used. Antibodies were added to the target cells at a concentration of 2 µg/well in 200 µl medium prior to the start of the co-culture with virus-laden macrophages.

2.2.12 MLV BlaM-VLP fusion assay *in vitro*

To detect fusion of virus particles with primary cells *in vitro*, VLPs that stably overexpress a codon-optimized β-lactamase enzyme (BlaM) were produced in HEK293T cells (as described in 2.2.4). BlaM is in this case fused to the carboxy-terminus of the cell-membrane tetraspanin CD63 (CD63-BlaM) (Albanese et al., 2021).

To quantify fusion of MLV BlaM VLPs *in vitro*, *trans*-infection assay was performed as described in 2.2.10 using MLV BlaM VLPs with (ecoEnv) or without ecotropic glycoprotein (ΔEnv) and BlaM VLPs pseudotyped with VSV-G. After 4 h of co-culture, target cells were removed from macrophages (donor cells) and washed once with primary medium to remove unbound virus. Next, target cells were resuspended in 100 µl CCF4-AM staining solution consisting of CO_2-independent medium, 10 % FBS, 2.5 mM probenecid (Invitrogen), 2 µl/ml CCF4-AM, 8 µl/ml Solution B (both from LiveBLAzer™ FRET B/G Loading Kit, Invitrogen) and incubated over night at RT to allow uptake of CCF4-AM into the cytoplasm. The next day, cells were washed two times with PBS / 1 % BSA and fixed with 4 % PFA (as described in 2.2.5). BlaM-VLPs fusion with cells will result in cleavage of the CCF4 substrate, resulting in an emission wavelength shift from 520 nm to 447 nm. Fusion was analyzed by calculating the ratio of fused to unfused cells via flow cytometry on a BD FACSLyric (BD Bioscience).

2.2.13 Retrovirus capture *in vivo*

For virus capture experiments *in vivo*, MLV Gag-GFP was concentrated as described in 2.2.4 and injected subcutaneously into the footpads of WT, ICAM-KO and CD11a-KO mice at a concentration of 8 x 10^4 IU. Mice were euthanized after 1 h and draining pLNs were isolated. Each pLN was incubated in 125 µl RPMI Medium 1640 (Gibco) with DNase I (20 µg/ml, Roche) and Liberase™ TL (0.2 mg/ml, Roche) for 30 min at 37°C. After incubation, 1 ml of RPMI Medium 1640 (Gibco)/10 % FBS was added to stop the enzyme reaction. Tissue suspension was passed through a 70 µm cell strainer (Corning) and washed once with PBS/1 % BSA/0.5 mM EDTA. Binding of MLV was analyzed by flow cytometry on a BD FACSLyrik (BD Bioscience) by mean GFP intensity.

2.2.14 Adoptive transfer experiments to study *in vivo trans*-infection and fusion

To study *trans*-infection and fusion *in vivo*, naïve CD4+ T cells were isolated from spleens of WT, ICAM-KO and CD11a-KO mice as described in 2.2.2 and differentiated into FoxP3+ CD4+ T cells as described in 2.2.3. To be able to discriminate adoptively transferred cells from cells of the acceptor mouse, FoxP3+ CD4+ T cells were stained with 1 µM CellTrace™ Far Red dye (Thermo Fisher Scientific) in Opti-MEM (Gibco) according to manufacturer´s instructions. A total number of 1 x 10^6 cells per acceptor mouse (WT, ICAM-KO, CD11a-KO) were subsequently injected into the hind hock. After 24 h, mice were infected with 1.5 x 10^5 IU of MLV LTR-GFP (for infection analysis) or 8 x 10^4 MLV BlaM VLPs (for fusion analysis) by subcutaneous injection into the footpads. Mice were euthanized 2 h (fusion) or 40 h (infection) post injection and draining pLNs were isolated. To prepare single cell solutions each draining pLN was incubated in 125 µl RPMI Medium 1640 (Gibco) with DNase I (20 µg/ml, Roche) and Liberase™ TL (0.2 mg/ml, Roche) for 30 min at 37°C. After incubation, 1 ml of RPMI Medium 1640 (Gibco)/10 % FBS was added to stop the enzyme reaction. Tissue suspension was passed through a 70 µm cell strainer (Corning) and washed once with PBS/1 % BSA/0.5 mM EDTA.

Fusion of BlaM-VLPs with pLN cells was analyzed via flow cytometry on a BD FACSLyric (BD Bioscience) after over night treatment with CCF4-AM (as described in 2.2.12). Fused cells could be detected by a $CCF4_{cleaved}$ signal, and the difference between transferred cells and host cells could be further distinguished by a Far Red+ signal of the transferred cells and the Far Red- signal of host cells. To analyse for infection (MLV LTR-GFP) of transferred (Far Red+) and host (Far Red-) cells, ratios of infected (GFP+) to uninfected (GFP-) cells for the respective cell types were calculated.

2.2.15 Generation of CD11a-KO in primary human T cells

To induce a stable CD11a-KO in primary human T cells, nucleofection of isolated cells with Cas9 - guide RNA (gRNA) - complexes targeting Exon 2 of the human CD11a gene (*ITGAL*) was performed. For that, Cas9/gRNA complexes were formed by mixing 20 µl of single gRNAs (100 µM each, ACCCUUGCCUUCCUCAGCGC-Modified, UCCAGGUUGUAGCUCGAGGC-Modified, designed and ordered at Synthego) with 14 µl Alt-R® S.p. Cas9 Nuclease V3 (60µM, IDT) and 66 µl sterile filtered PBS. The mixture was then incubated for 15 min at RT. Afterwards, complexes were aliquoted and frozen at -80°C. Human T cells were isolated as described in 2.2.2. After 24 h of cultivation, 2 x 10^6 cells were transferred in a reaction tube and washed once with PBS/1 % BSA. Cells were resuspended in 20 µl P3 Primary Cell Nucleofector™ Solution (Lonza) freshly mixed with Supplement 1 (Lonza) and 5 µl of die Cas9/gRNA complexes (100 pmol of gRNA and 40 pmol of Cas9 are used per reaction). Per reaction 20 µl cell suspension were transferred into a Nucleocuvette™ (16-well format, Lonza). Electroporation was performed in a 4D-Nucleofector™ Core and X Unit (Lonza), using the EH100 manufacturer program. Following electroporation, cells are transferred with 100 µl RPMI Medium 1640 (Gibco, GlutaMAX™) into a 48-well plate. After 15 min of incubation at 37°C, 500 µl of RPMI 1640 Medium (Gibco, GlutaMAX™) supplemented with 10 % FBS (Gibco), human IL-2 (50 IU/ml, Biolegend), human IL-7 and human IL-15 (both 100ng/ml, Biolegend) were added and cells were incubated over night before activation with Dynabeads™ Human T-Activator CD3/CD28 (Gibco) as described in 2.2.3.

To confirm the successful knock-out of *ITGAL*, tide analysis (Brinkman et al., 2014) was performed. In addition, surface marker expression of CD11a was tested every two days post nucleofection with anti-human CD11a antibody (Biolegend). As a standard control, surface expression of CD4 and CXCR4 was tested with anti-human CD184 (CXCR4) antibody and anti-human CD4 antibody (Biolegend), respectively.

2.2.16 *In vitro cis*-infection assay with primary human T cells

To perform a human *cis*-infection assay with primary human T cells (WT, LFA1-deficient), T cells were isolated from blood and CD11a-KO cells were generated using Cas9-gRNA targeted knock-out of *ITGAL* as described in 2.2.15. For the assay, donor T cells (WT or CD11a-KO) were seeded at a density of 2 x 10^5 cells per well in a 96-well conical bottom plate (Corning). For initial infection, cells were mixed with concentrated HIV NLENG1 IRES (X4) virus at an MOI = 0.6 in 100 µl volume and spin-inoculated at 650 x g for 2.5 h at 37°C. Cells were resuspended afterwards and incubated for three days at 37°C to establish infection. At day three, half of the donor cells were taken out and fixed (as described in 2.2.5) and analyzed via flow cytometry to determine initial infection rates. For co-culture, uninfected human T cells were stained with 1 µM CellTrace™ Far Red dye (Thermo Fisher Scientific) in Opti-MEM (Gibco) according to manufacturer's instructions and used as target cells. Donor and target cells were mixed at a 1:1 ratio and co-cultured

for six days. At day 3 and day 6 of co-culture, cells were fixed (as described in 2.2.5) and analyzed for infection via flow cytometry on a BD FACSLyric (BD Bioscience). Experiments were performed in a BSL-3 facility by Manuel Albanese.

2.2.17 Selection of surface coating for *in vitro* study of cell contact dynamics

To find a suitable surface coating to study cell-cell interactions of primary cells *in vitro*, the ECM Select® Array Kit Ultra-36 (Advanced BioMatrix) was used to test attachment of primary macrophages to different surface coatings. Primary peritoneal cavity-derived macrophages were isolated from RFP+ mice as described in 2.2.2 and 2 x 10^6 cells were seeded on an ECM Select® Array, which was washed once with PBS and once with primary cell medium in prior. Macrophages were incubated over night on the array slide to allow attachment to suitable surface coatings. Visual evaluation of macrophage attachment was performed with a spinning disk confocal microscope (Nikon).

To test if activated T cells can migrate on fibronectin, a glass dish was coated with 2 µg/cm² fibronectin (Advanced Biomatrix) in PBS. PBS/fibronectin solution was incubated for one hour at RT and washed twice with PBS and once with primary medium before use. T cells were seeded in primary cell medium supplemented with IL-7 and IL-15 (100 ng/ml each) on coated and uncoated glass dishes and incubated for 2 h at 37°C. Visual evaluation of T cell migration was performed on a spinning disk confocal microscope (Nikon).

To test virus particle binding to the fibronectin-coated glass surface, glass-bottom dishes were coated with either 2 µg/cm² fibronectin or vitronectin (Advanced Biomatrix) in PBS, for 1 h at RT and washed as described above. Next, MLV reporter virus (LTR-GFP) was incubated on the coated surface for 30 min at 37°C. After extensive washing to remove unbound virus, S49.1 cells were cultivated for 24 h on the virus-laden surfaces and analyzed afterwards for infection (GFP expression) using flow cytometry.

2.2.18 Transduction of FoxP3+ T cells with MLV VLPs to mark cellular compartments

To visualize intracellular compartments of primary FoxP3+ T cells, MLV VLPs with genomes encoding for LTR-driven expression of fluorescent proteins coupled to cellular markers were used. For efficient transduction of the cells spin-infection was performed. For that, cells were seeded in a 96-well flat-bottom plate at a density of 4 x 10^5 cells per well in 100 µl volume. VLPs for fluorescent labelling of compartment markers (see Table 5) were added to the cells at a MOI = 2. Spin-infection was performed at 1000 x g for 90 min at 37°C. Afterwards 100 µl of medium was added to each well and cells were incubated for 24 h at 37°C prior to start of co-culture.

2.2.19 Imaging of primary cell *in vitro* co-culture and contact quantification

For quantification of cellular dynamics during co-culture of experiments, glass bottom dishes (1.5 mm, MatTek) were coated with human fibronectin in PBS (2 µg/cm^2, Advanced BioMatrix) for 1 h at RT, washed twice with PBS and once with primary cell medium. Macrophages were isolated as described in 2.2.2 and seeded at a density of 1 x 10^5 cells on the fibronectin-coated dishes.

FoxP3+ T cells transduced with VLPs to mark cellular compartments (see 2.2.18) were added at a concentration of 1:1 and co-cultured for 24 h at 37°C. Live cell imaging was performed on a spinning disk confocal microscope (Nikon) for 4 h at 37°C and 5 % CO_2. Image acquisition was performed every 30 sec with a 12 µm z-stack in 1 µm steps for every position.

2.3 Flow cytometry

Data were acquired on a BD FACSLyric flow cytometer (BD Biosciences) and were analyzed with the FlowJo software (Version 10, Treestar).

2.4 Figures

Figures were edited and illustrated using Adobe Illustrator 2021 and BioRender.com.

2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.4.3 software. Statistical tests used and the numbers of independent replicates (n) are reported in the figure legends. P values are included in the figures. A difference was interpreted as statistically significant if $P < 0.05$ (two-tailed).

3. Results

3.1 Characterization of peritoneal cavity-derived macrophages as donor cells for *in vitro trans*-infection assays

To investigate the role of cell adhesion proteins in retroviral spread, an *in vitro* co-culture assay was established.

First, primary cells were isolated from mice and characterized for their suitability to study cell contact-dependent transmission of MLV by *trans*-infection. MLV presenting donor cells should be relevant *in vivo* and available in sufficient quantities. In addition, they should fulfill the functional characteristic to bind virus particles on their surface while not being susceptible to infection. A promising cell type are CD169+ macrophages that have been shown to efficiently capture retroviruses *in vivo* and present viral particles for *trans*-infection to lymphocytes without getting infected (Haugh et al., 2021; Sewald et al., 2015). An easily accessible source of macrophages is the peritoneal cavity of mice (X. Zhang et al., 2008) allowing fast isolation of a sufficient number of cells. Around 70 % of the peritoneal wash-out of WT mice are F4/80+ CD11b+ macrophages that can be enriched to a purity of 87 % by negative selection (Figure 3A).

To address their functional characteristics, peritoneal macrophages were analyzed for the expression of CD169, an I-type lectin that was shown to bind GM3 of the retroviral envelope (Izquierdo-Useros, Lorizate, Puertas, et al., 2012; Puryear et al., 2013). Therefore, macrophages were enriched by negative selection and fixed directly or after 3 h, 6 h, 24 h, 48 h and 72 h of *in vitro* cultivation (see 2.2.2, 2.2.3 and 2.2.5). Macrophages were stained with an anti-CD169 antibody and analyzed by flow cytometry (see 2.2.6). Although freshly isolated macrophages did not express CD169 on their surface, 86 % of the cells express CD169 after 24 h in culture and expression slightly increases over the observed period of 72 h (Figure 4A). Similar to human myeloid dendritic cells (Puryear et al., 2013), expression of CD169 by murine macrophages depends on IFNalpha (Figure 4B). Antibody-blocking of the type I IFN receptor subunit 1 (see 2.2.7) reduced surface expression of CD169 compared to untreated samples or IgG1 control antibody treatment (Figure 4B).

In a next step, CD169-dependent binding of MLV virions to macrophages was investigated (see 2.2.8). CD169-expressing macrophages were incubated for 30 min with fluorescent MLV particles (MLV Gag-GFP) followed by extensive washing to remove unbound virus. Analysis of the cells for MLV binding was performed via flow cytometry. About 83 % of CD169+ macrophages were able to bind MLV particles, a process that can be blocked significantly by adding anti-CD169 antibody prior to incubation of the cells with virus (Figure 4C).

Next, susceptibility of macrophages for infection with MLV was examined (see 2.2.9). Freshly isolated (CD169-) and *in vitro*-cultured (for 24 h, CD169+) macrophages were

incubated with MLV reporter virus (MLV LTR-GFP VLPs) pseudotyped with different glycoproteins from ecotropic MLV (ecoEnv) or VSV (VSV-G) and analyzed after 24 h for infection. As a result, neither VLPs pseudotyped with ecotropic Env, nor with VSV-G could infect CD169- or CD169+ macrophages (Figure 4D). In contrast, infection of MLV-permissive S49.1 cells with ecoEnv-VLPs and VSV-G-VLPs was detectable (Figure 4D).

Taken together, primary CD169+ macrophages isolated from the peritoneal cavity can bind MLV particles on their surface by IFN-α inducible expression of CD169. In addition, infection with retrovirus-based VLPs containing different glycoproteins reveal that CD169+ macrophages are not permissive for retrovirus infection.

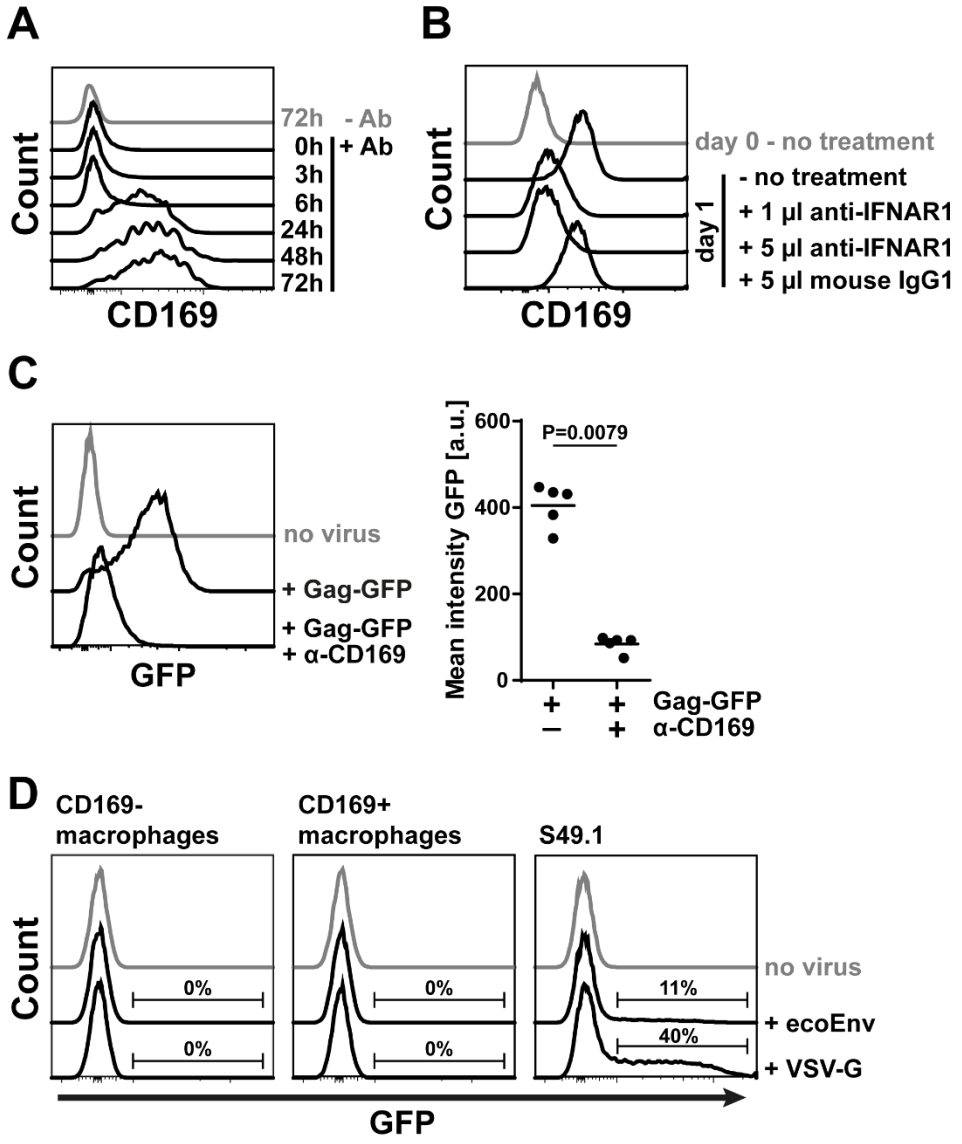

Figure 4: Characterization of peritoneal cavity-derived macrophages

Flow cytometric analysis of WT peritoneal cavity-derived macrophages. **(A)** Surface CD169 expression at different time points after *in vitro* culture. Time points indicate hours in culture at 37°C post isolation. Representative data of three independent experiments are shown. **(B)** Surface CD169 expression after 24 h culture in the presence of IFNAR1-blocking antibodies or isotype control. Representative data of three independent experiments are shown. **(C)** Quantification of MLV Gag-GFP binding to CD169+ macrophages with or without addition of CD169-blocking antibodies. Representative data of five independent experiments are shown. The P value was determined using the Mann-Whitney test. *Figure was partially published before on Poster #117 at Retroviruses CSHL 2021.* **(D)** Analysis of CD169+ macrophages (WT) 24 h after *in vitro* transduction with MLV LTR-GFP VLPs pseudotyped with glycoproteins from ecotropic MLV (ecoEnv) or VSV (VSV-G). Infection of S49.1 cells with the same amount of VLPs serves as positive control. Representative data of three independent experiments are shown. *Figures **A** and **C** were similarly published before in Engels et al. 2022. Figure **D** was similarly published before on Poster #117 at Retroviruses CSHL 2021.*

3.2 *Trans*-infection of target cells requires CD169 on macrophages and cell-cell contact

Peritoneal cavity-derived macrophages bind MLV particles via CD169 on their surface and are not permissive for infection. Whether they are able to support infection of lymphocytes in *trans* in a cell contact-dependent manner was investigated in the following assays.

First, CD169-mediated *trans*-infection of an MLV-permissive target cell line was tested (see 2.2.10). Therefore, CD169+ macrophages were incubated with CD169-blocking or isotype control antibodies and were loaded with MLV LTR-GFP reporter virus. After extensive washing to remove unbound virus, macrophages were co-cultured with MLV-permissive S49.1 cells for 24 h. As a result, 20 % of S49.1 target cells were infected after co-culture with MLV-laden macrophages without antibody treatment (Figure 5A). In the presence of anti-CD169 antibodies almost no infection of target cells was detectable, while infection rates in the presence of isotype control antibodies were comparable to the untreated co-cultures (Figure 5A).

As a next step, *trans*-infection of primary target cells was tested. CD4+ T cells were previously identified as target cells for MLV infection *in vivo* (Sewald et al., 2012). Further marker analysis of our lab revealed that the majority of MLV-infected CD4+ T cells express the transcription factor FoxP3 (Engels et al., 2022). Therefore, naïve CD4+ T cells activated with PMA and ionomycin or differentiated into FoxP3+ T cells (see 2.2.3) were tested as primary target cells for *trans*-infection *in vitro*. As a result, about 3 % of activated T cells could be infected during co-culture with MLV-loaded macrophages and around 8 % of FoxP3+ T cells (Figure 5B). Infection of both cell types could be significantly reduced in the presence of anti-CD169 antibodies during incubation of macrophages with MLV (Figure 5B). To investigate if the observed infection of CD4+ T cells with MLV is cell contact-dependent, co-cultures were performed in a transwell plate to physically separate donor and target cells (see 2.2.10). As a result, neither S49.1 target cells (F4/80-) (Figure 5C) nor primary activated CD4+ T target cells (Figure 5D) were infected when separated from MLV-laden CD169+ macrophages by the transwell insert.

In summary, *in vitro trans*-infection of primary CD4+ T cells is cell contact-dependent and requires CD169 on macrophages.

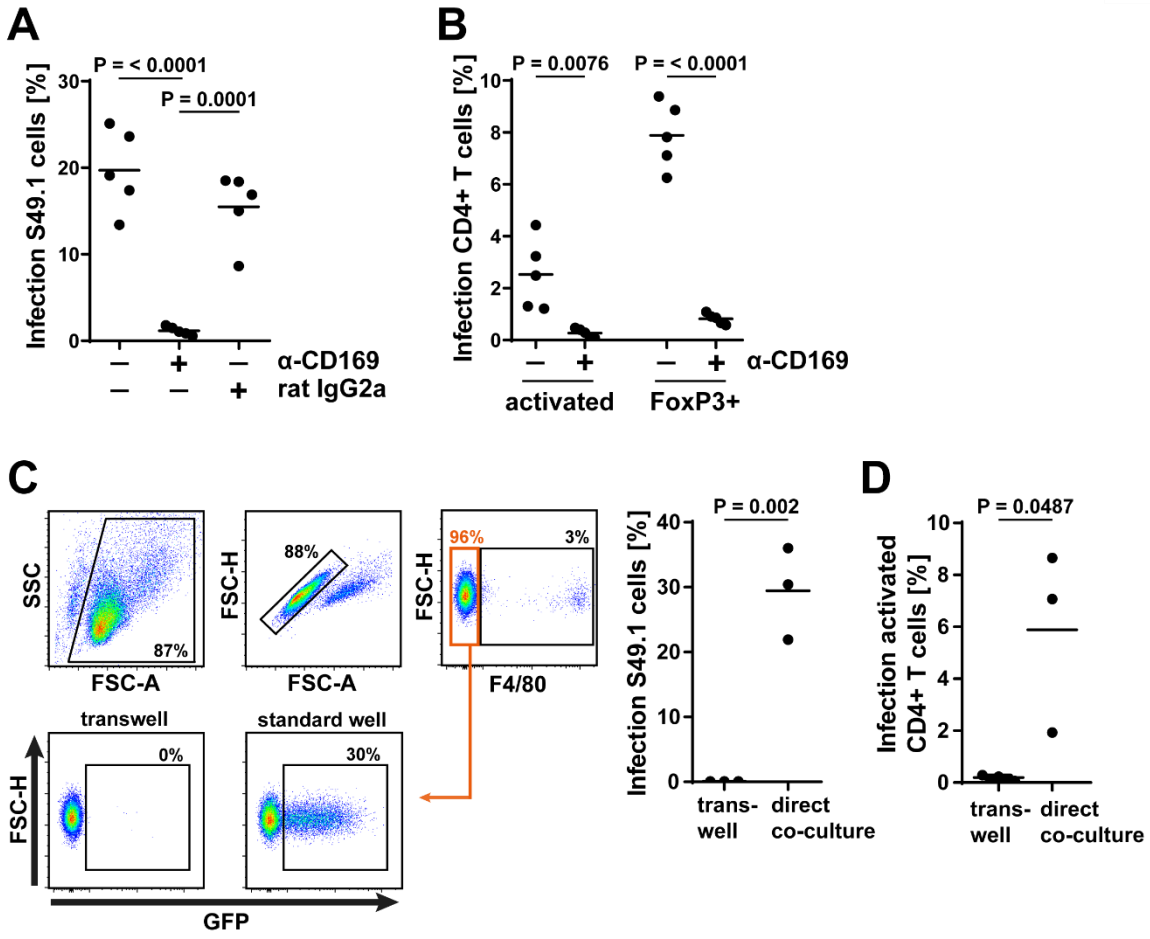

Figure 5: CD169-mediated *trans*-infection of permissive target cells is contact dependent

(A) Quantification of MLV-infected S49.1 cells after 24 h of co-culture with MLV LTR-GFP-laden CD169+ macrophages. Macrophages were pre-treated with antibodies against CD169, the corresponding isotype control (IgG2a) or left untreated before addition of the virus. Representative data of five independent experiments are shown. The P values were determined using an ordinary one-way ANOVA and Tukey's multiple comparison test, not significant comparisons are excluded from the graph. **(B)** Quantification of MLV-infected CD4+ T cells (activated, FoxP3+) after 24 h of co-culture with MLV LTR-GFP-laden CD169+ macrophages. Macrophages were pre-treated with antibodies against CD169 or left untreated before addition of virus. Representative data of five independent experiments are shown. The P values were determined using an ordinary one-way ANOVA. **(C)** Gating strategy and quantification of MLV-infected S49.1 cells. S49.1 were co-cultured directly or in transwell with MLV LTR-GFP-laden macrophages for 24 h. Representative data of three independent experiments are shown. The P value was determined using a Student´s t-test. **(D)** Quantification of MLV-infected PMA/ionomycin-activated CD4+ T cells. T cells were co-cultured directly or in a transwell with MLV LTR-GFP-laden macrophages for 24 h. Representative data of three independent experiments are shown. The P value was determined using a Student´s t-test. *Figures **A-C** were similarly published before in Engels et al. 2022.*

3.3 LFA1 and ICAM1 are expressed on primary donor and target cells and contribute to MLV *trans*-infection *in vitro*

Trans-infection of primary CD4+ T cells with MLV by virus-loaded CD169+ macrophages is cell contact-dependent (see 3.2). Whether the cell adhesion protein LFA1 (CD18/CD11a heterodimer) and its interaction partner ICAM1 (CD54) play a role in this process remains elusive. Therefore, we decided first, to investigate whether both adhesion proteins are expressed on CD169+ macrophages (donor) and CD4+ T cells (target) and second, to perform an initial testing whether their presence has an influence on *trans*-infection by an antibody blocking experiment.

Antibody staining for LFA1 and ICAM1 revealed that both adhesion proteins are expressed on the surface of CD169+ macrophages and CD4+ cells (naïve, activated, differentiated) (Figure 6A).

To test if functional blocking of LFA1 and ICAM1 by antibodies influences *trans*-infection, co-culture experiments in the presence of either anti-CD11a-, anti-ICAM1- or isotype control antibodies were performed (see 2.2.11). Blocking of LFA1 function leads to a reduction of infected target cells by ~ 75% compared to the untreated co-culture or isotype control (rat IgG2a) (Figure 6B). In the presence of anti-ICAM1 antibodies, *trans*-infection of target cells was slightly reduced by ~ 15 % as compared to the untreated cells or isotype control (rat IgG2b) (Figure 6B). These results indicate a role of LFA1 and ICAM1 during *trans*-infection of primary cells with MLV *in vitro*.

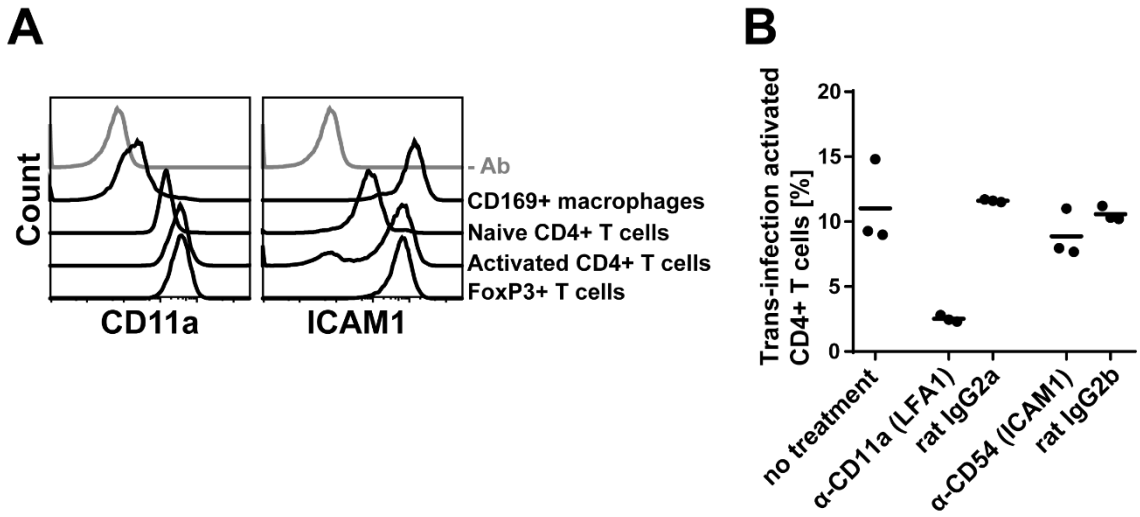

Figure 6: Antibody blocking of the adhesion proteins LFA1 and ICAM1 during *trans*-infection results in reduced infection of target cells

(A) Flow cytometric analysis of CD11a (LFA1) and ICAM1 surface expression in primary cells derived from WT mice. Peritoneal cavity-derived CD169+ macrophages and CD4+ T cells isolated from spleen (naïve, PMA/ionomycin activated, FoxP3+) are shown. Unstained CD169+ macrophages were used as unstained control (-Ab). Representative data of three independent experiments are shown. **(B)** Quantification of MLV-infected, CD4+ T cells (activated with PMA/ionomycin) after 24 h of co-culture with MLV-LTR-GFP-laden CD169+ macrophages. Cells were co-cultured in the presence of blocking antibodies against CD11a, ICAM1 or respective isotype controls. Representative data of three independent experiments are shown.

3.4 LFA1 on target cells and ICAM1 on donor cells support *trans*-infection *in vitro*

Antibody blocking experiments indicate a role of LFA1 and its ligand ICAM1 in *trans*-infection of lymphocytes with MLV by virus-presenting macrophages (see 3.3). Since donor and target cells express both adhesion proteins (Figure 6A), the relevance of the orientation in the receptor-ligand interaction cannot be addressed in this experimental setup. To investigate a donor cell- and target cell-specific function of LFA1 and ICAM1 during *trans*-infection, primary cells from KO mice lacking surface expression of LFA1 or ICAM1 were used.

Before co-culture experiments, primary donor and target cells isolated from CD11a-KO (LFA1-deficient) and ICAM1-KO mice were compared with WT cells for their ability to bind MLV particles and their permissiveness to infection. Macrophages isolated from CD11a- and ICAM1-KO mice were shown to bind MLV to nearly the same extent as WT cells (Figure 7A). In addition, LFA1- and ICAM1-deficient FoxP3+ T target cells could be equally transduced with cell-free MLV reporter virus (LTR-GFP) compared to WT cells (Figure 7B).

WT and KO *trans*-infection co-culture experiments were performed in various combinations (see 2.2.10). In detail, donor CD169+ macrophages from WT or KO mice were loaded with MLV reporter virus (LTR-GFP). Afterwards unbound virus was removed by extensive washing. Next, WT or KO FoxP3+ T target cells were added to the virus loaded macrophages and co-cultured with for 24 h at 37°C. FoxP3+ target T cells were removed and analyzed for infection (GFP expression) by flow cytometry. Co-culture experiments with CD11a-KO and WT cells demonstrate that the infection of target cells lacking LFA1 is significantly reduced by 80 % compared to WT-WT co-cultures (Figure 7C, left panel). Compared to that, deficiency of LFA1 on MLV-presenting donor cells does not affect the rate of *trans*-infection (Figure 7C, left panel). Interestingly, co-culture experiments with ICAM-KO and WT cells show that only ICAM1-deficiency on donor cells leads to an impaired *trans*-infection of T target cells (Figure 7C, right panel). Here, *trans*-infection of WT target cells is reduced by 80 %, while ICAM1-deficiency on target cells has no effect (Figure 7C, right panel).

Taken together, MLV *trans*-infection of lymphocytes by virus-presenting macrophages requires LFA1 on target cells and ICAM1 on donor cells.

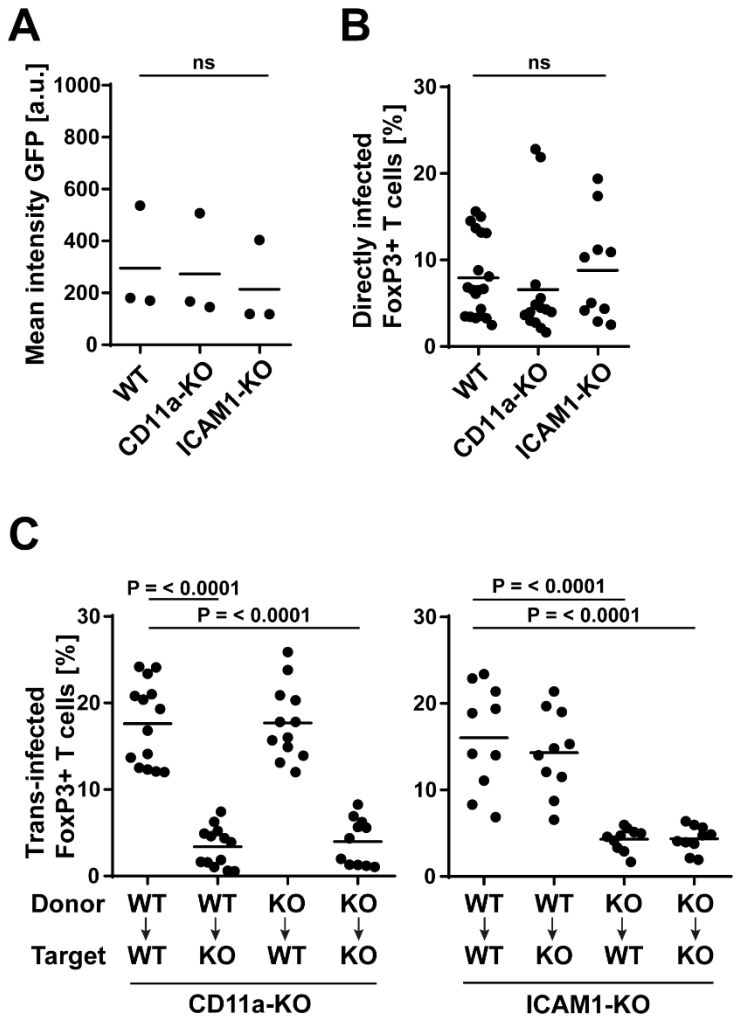

Figure 7: *Trans*-infection of target cells is reduced in co-cultures with LFA1-deficient target cells or ICAM1-deficient donor cells

(A) Quantification of MLV Gag-GFP binding to CD169+ macrophages by GFP mean intensity. Macrophages were isolated from WT, CD11a-KO and ICAM1-KO mice. Representative data of three independent experiments are shown. Statistical analysis was performed using the Kruskal-Wallis test, no significant difference could be determined between the samples (ns). **(B)** Quantification of MLV-infected FoxP3+ T cells 24 h post *in vitro* transduction with MLV LTR-GFP. CD4+ T cells were isolated from WT (n=20), CD11a-KO (n=14) and ICAM1-KO (n=10) mice and *in vitro*-differentiated for 48 h prior to cell-free infection. Representative data of three independent experiments are shown. Statistical analysis was performed using the Kruskal-Wallis test, no significant difference could be determined between the samples (ns). **(C)** Quantification of MLV-infected FoxP3+ T cells (target) after 24 h of co-culture with MLV-laden CD169+ macrophages (donor). Cells were isolated from WT, CD11a-KO and ICAM1-KO mice. Co-cultures of WT with CD11a-KO cells (left, n=11-14) and WT with ICAM1-KO (right, n=10) are shown. The P values were determined using an ordinary one-way ANOVA and Dunnett's multiple comparison test against the WT-WT control, respectively. Indication of not significant comparisons are excluded from the graphs. *Figure **C** was similarly published before on Poster #117 at Retroviruses CSHL 2021 and Engels et al. 2022.*

3.5 MLV CD63-BlaM VLPs allow quantification of retrovirus particle fusion and infection in primary cells

LFA1 on target cells and ICAM1 on donor cells are critical for *trans*-infection of MLV *in vitro* (see 3.4). Whether the LFA1-ICAM1 interaction supports productive infection of lymphocytes directly by mediating cell-cell contacts or indirectly by downstream signaling is unclear (Davis et al., 1990; Guasch et al., 2017; Postigo et al., 1991; Wacholtz et al., 1989). This is of importance, since integrin-induced lymphocyte activation/proliferation has been described before (Davis et al., 1990; Guasch et al., 2017; Postigo et al., 1991; Wacholtz et al., 1989) and, therefore, LFA1-ICAM1 interaction might influence mitosis-dependent MLV infection (Harel et al., 1981; Roe et al., 1993). To address this, a beta-lactamase (BlaM) assay was adjusted to quantify MLV fusion, a very early step of the retroviral infection cycle. With that, it is possible to investigate the impact of LFA1 and ICAM1 directly at the level of cell-cell contact, excluding possible downstream signaling effects and cell cycle dependency.

For the fusion assay, MLV VLPs containing a reporter genome (LTR-GFP) were produced in HEK293T cells that overexpress the CD63-BlaM fusion protein (see 2.2.12). This results in the production of MLV VLPs with CD63-BlaM incorporated into the retroviral envelope and with a packaged reporter genome (LTR-GFP) to quantify fusion and infection, respectively. Upon fusion with a target cell, CD63-BlaM becomes part of the cellular membrane with BlaM facing the cytoplasm (Figure 8). When cells are loaded with CCF4 substrate, BlaM is able to cut the substrate in the cytoplasm of the cell, which results in a blue (450 nm) fluorescent signal, detectable by flow cytometry. In addition to quantification of MLV fusion within the first hours of infection, productive infection can be quantified by flow cytometric analysis of cytoplasmatic GFP expressed from the LTR-GFP reporter about 24 h after virus contact.

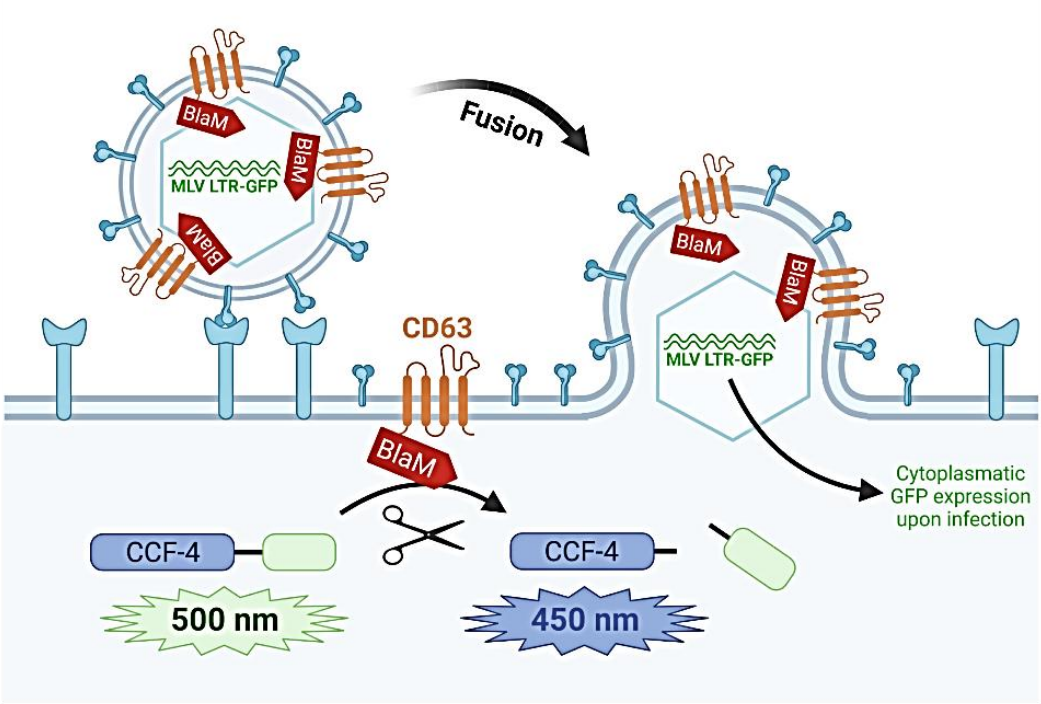

Figure 8: Schematic illustration of MLV CD63-BlaM LTR-GFP VLPs particle fusion

Upon fusion, CD63-BlaM becomes part of the cellular membrane with BlaM facing the cytoplasm and is able to cut the CCF4 substrate (Albanese et al., 2021). Cleaved substrate can be detected by a V450+ signal via flow cytometric analysis. LTR-GFP genome integration allows for detection of infection by GFP signal via flow cytometry. *Figure 6 was similarly published before on Poster #117 at Retroviruses CSHL 2021.*

To test whether the MLV CD63-BlaM VLP based system is functional, S49.1 cells were directly inoculated for 4 h with MLV CD63-BlaM VLPs and then thoroughly washed to remove unbound virus. Subsequently, cells were divided for incubation overnight at RT in CCF4 staining solution to quantify fusion and incubation at 37°C for 24 h to allow infection (GFP expression). MLV CD63-BlaM-VLP particles pseudotyped with glycoproteins from ecotropic MLV (ecoEnv) and VSV (VSV-G) fuse efficiently with MLV permissive S49.1 cells (Figure 9A). Fusion rates of ~ 80 % for ecoEnv and up to 95 % for VSV-G were observed (Figure 9A). MLV CD63-BlaM VLP particles without Env (ΔEnv) result in no detectable V450+ signal by the inoculated cells excluding unspecific fusion events (Figure 9A). To compare rates of MLV CD63-BlaM VLP fusion with productive infection, S49.1 were analyzed 24 h after inoculation for GFP expression by flow cytometry. About 30 % of S49.1 cells were infected when transduced with MLV CD63-BlaM VLPs pseudotyped with ecoEnv and up to 75 % with VSV-G (Figure 9B) indicating that not every fusion event leads to a productive infection of the cell. In a next step, fusion of MLV CD63-BlaM VLPs was tested with peritoneal cavity-derived macrophages, which were earlier shown to be non-permissive for retroviral infection (see 3.1). In brief, CD169+ macrophages were incubated with MLV CD63-BlaM VLPs (ecoEnv, ΔEnv and VSV-G) and thoroughly washed to remove unbound particles. Macrophages were then incubated overnight with CCF4 substrate and fusion was quantified the next day by flow cytometry (see 2.2.12). To our surprise, although VSV-G-pseudotyped MLV CD63-BlaM

VLPs revealed high fusion rates with primary macrophages, fusion of MLV CD63-BlaM ecoEnv-VLPs with CD169+ macrophages was almost absent and reached similar level as the MLV CD63-BlaM ΔEnv-VLP control (Figure 9C).

Consequently, primary macrophages represent optimal donor cells to investigate fusion in the context of *trans*-infection since MLV particles can be bound by surface CD169 without subsequent particle fusion.

To test if fusion can also be detected in primary FoxP3+ target cells, the assay was performed as before with S49.1 cells and evaluated for fusion and infection. Around 17 % of FoxP3+ T cells were able to fuse with MLV CD63-BlaM ecoEnv-VLPs (Figure 9D) and no unspecific fusion was observed after incubation with ΔEnv-VLPs (Figure 9D). When incubated with MLV CD63-BlaM VSV-G-VLPs around 70 % of FoxP3+ T cells had fused with VLPs (Figure 9D). When looking at the corresponding infection rates, only half of the cells (~ 8 %) that fused with MLV CD63-BlaM ecoEnv-VLPs were productively infected and expressed GFP (Figure 9E). As expected, no GFP expression was detectable after incubation with MLV CD63-BlaM ΔEnv-VLPs (Figure 9E). Interestingly, although fusion with MLV CD63-BlaM VSV-G-VLPs was highly efficient, very few GFP-expressing primary FoxP3+ T cells were observed (Figure 9E).

To summarize, MLV CD63-BlaM VLPs allow the quantification of retrovirus fusion and infection in cell lines and primary cells *in vitro*.

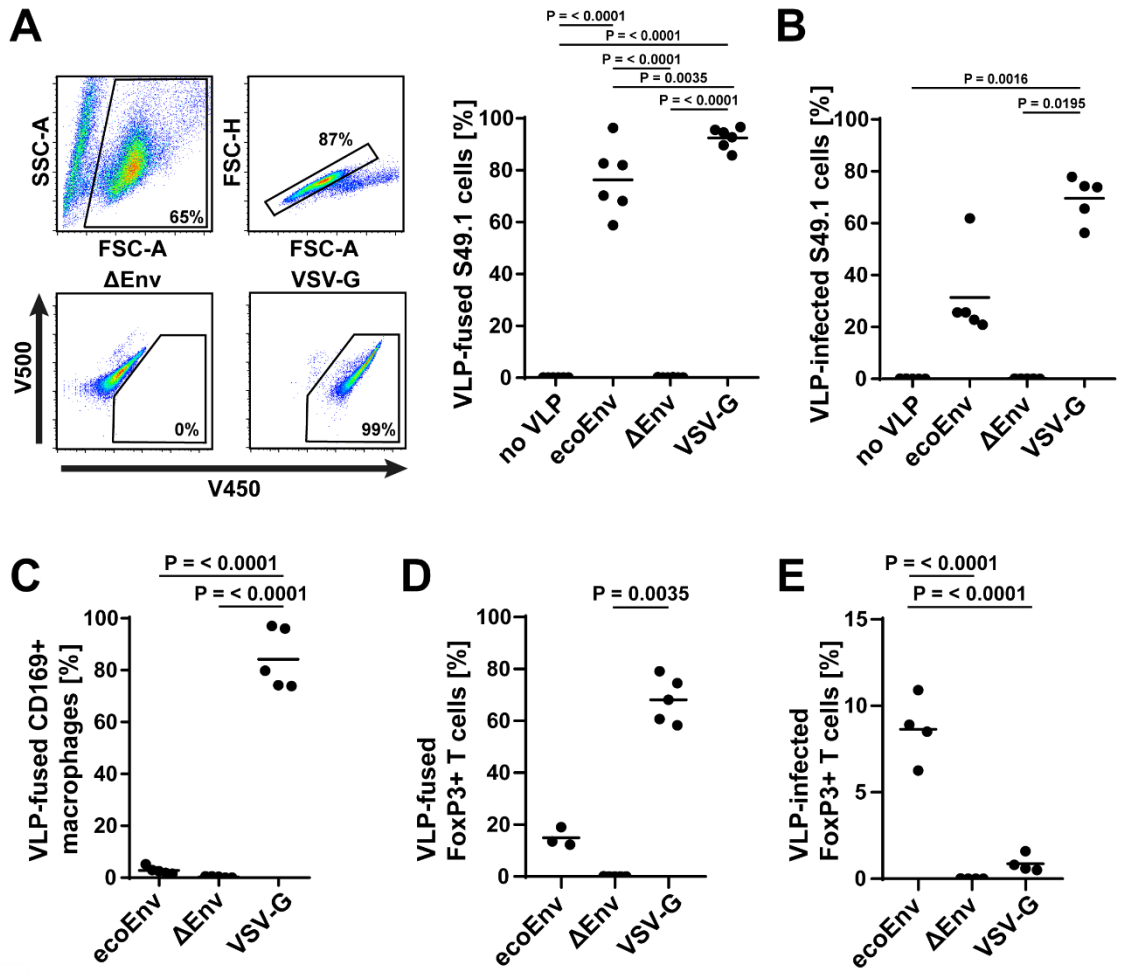

Figure 9: Establishment of a BlaM-based fusion assay for MLV

(A + B) Gating strategy and quantification of MLV LTR-GFP BlaM-VLP fused (**A**, n=6) and infected (**B**, n=5) S49.1 cells after 24 h *of in vitro* transduction. S49.1 were cultured without VLPs or transduced with BlaM-VLPs without glycoprotein from ecoptropic MLV (ΔEnv) and pseudotyped with glycoprotein from ecotropic MLV (ecoEnv) or VSV (VSV-G). The P values for Figure **A** were determined using an ordinary one-way ANOVA and Tukey's multiple comparison test. The P values for Figure **B** were determined using an Kruskal-Wallis and Dunn's multiple comparisons test. Indication of not significant comparisons are excluded from the graph. *Figure* **A** *was similarly published before on Poster #117 at Retroviruses CSHL 2021.* **(C)** Quantification of peritoneal cavity-derived CD169+ macrophages after 24 h of *in vitro* transduction with MLV LTR-GFP BlaM VLPs. VLPs without glycoprotein from ecoptropic MLV (ΔEnv) and pseudotyped with glycoprotein from ecotropic MLV (ecoEnv) or VSV (VSV-G) were used. Representative data of five independent experiments are shown. The P values were determined using an ordinary one-way ANOVA and Tukey's multiple comparison test. Indication of not significant comparisons are excluded from the graph. *Figure* ***C*** *was similarly published before on Poster #117 at Retroviruses CSHL 2021.* **(D + E)** Quantification of MLV LTR-GFP BlaM-VLP fused (**D**, n = 3-5) and infected (**E**, n = 4) FoxP3+ T cells after 24 h of *in vitro* transduction. FoxP3+ T cells were transduced with BlaM-VLPs without glycoprotein from ecoptropic MLV (ΔEnv) and pseudotyped with glycoprotein from ecotropic MLV (ecoEnv) or VSV (VSV-G). The P values for Figure **D** were determined using an ordinary one-way ANOVA and Tukey's multiple comparison test. The P values for Figure **E** were determined using an Kruskal-Wallis and Dunn's multiple comparisons test. Indication of not significant comparisons are excluded from the graph.

3.6 LFA1 and ICAM1 are important for cell contact-dependent fusion and *trans*-infection *in vitro*

Virus particle fusion with and subsequent infection of primary cells could be successfully analyzed using MLV CD63-BlaM VLPs (see 3.5). In a next step, MLV CD63-BlaM VLP particles were used to quantify the role of LFA1 and ICAM1 in fusion during *trans*-infection of WT and KO cells by VLP-presenting macrophages.

Macrophages were loaded with MLV CD63-BlaM ecoEnv-VLPs and co-cultured with FoxP3+ T cells for 4 h at 37°C. FoxP3+ T cells were then split up and one half was incubated with CCF4 substrate overnight, while the other half was kept at 37°C for 24 h. As a result, MLV CD63-BlaM ecoEnv-VLP fusion was significantly reduced by > 50 % in co-cultures with either CD11a-deficient target cells or ICAM1-deficient donor cells as compared to WT-WT control (Figure 10A). As already observed before (3.4), this was also the case for infection of target cells in the respective co-cultures (Figure 7D). Overall, about 50 % of fused cells were infected after 24 h (Figure 10B). To exclude that different fusion efficiency of WT, LFA1- and ICAM1-KO FoxP3+ target T cells lead to the observed differences in *trans*-infection, cells were directly transduced with MLV CD63-BlaM ecoEnv-VLPs. Although CD11a-KO FoxP3+ T cells appear to be slightly more permissive for fusion, no significant differences in fusion rates were observed for all WT and KO FoxP3+ target cells (Figure 10C). Therefore, differences in *trans*-infection experiments with primary WT and KO cells are likely caused by LFA1 and ICAM1, mediating cell-cell contacts, supporting fusion and infection.

In conclusion, LFA1 expression on target cells and ICAM1 surface expression on donor cells is important for retrovirus fusion and *trans*-infection of primary target cells *in vitro*.

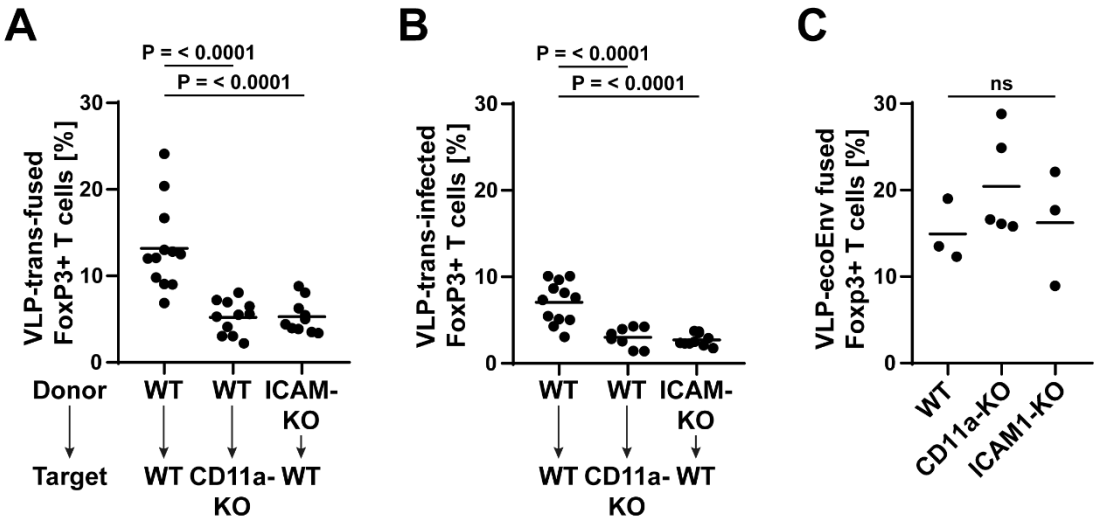

Figure 10: LFA1 and ICAM1 are important for contact-dependent MLV fusion.

(A + B) Quantification of Foxp3+ T cells, *trans*-fused (**A**) or *trans*-infected (**B**) during 24 h of co-culture with MLV LTR-GFP BlaM-VLP-laden, peritoneal cavity-derived CD169+ macrophages. Co-cultures were performed in the following donor-target combinations: WT-WT (n=12 for fusion and infection), ICAM-KO-WT (n=10 for fusion and infection) and WT-CD11a-KO (n=11 for fusion, n=8 for infection). The P values for Figures **A** and **B** were determined using an ordinary one-way ANOVA and Dunnett's multiple comparison test against the WT-WT control, respectively. Indication of not significant comparisons are excluded from the graph. *Figures* **A** and **B** *were similarly published before on Poster #117 at Retroviruses CSHL 2021.* **(C)** Quantification of BlaM-VLP-ecoEnv fused Foxp3+ T cells after 24 h of *in vitro* transduction. FoxP3+ T cells were isolated from WT (n=3), CD11a-KO (n=5) and ICAM1-KO (n=3) mice and *in vitro*-differentiated for 48 h prior to cell-free infection. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey's multiple comparison test, no significant difference could be found between the tested groups (ns).

3.7 Time-resolved, quantitative retrovirus binding and fusion *in vivo*

Virus particle fusion with primary cells could be successfully detected *in vitro* using MLV CD63-BlaM VLPs (see 3.5 and 3.6). To test whether MLV CD63-BlaM VLP fusion can also be detected *in vivo*, the kinetic of MLV fusion with pLN cells was analyzed over time (see 12.2.13). Previous studies have shown that subcutaneously injected (s.c.) retroviral particles are captured by CD169+ macrophages at the subcapsular sinus floor of pLNs and can subsequently infect MLV permissive target cells of draining pLNs (Sewald et al., 2015). Pre-existing data of fluorescent MLV (Gag-GFP) capture by pLN CD169+ macrophages over time (Sewald Laboratory) was used as a temporal reference to analyze fusion kinetics.

For this assay, MLV Gag-GFP particles were s.c. injected into the footpads of WT mice and draining pLNs were isolated at different time points post-injection (0.25 h, 0.5 h, 1 h, 2 h, 4 h and 6 h). Single-cell suspensions of the pLNs were prepared for flow cytometric analysis of GFP-positive cells. To quantify *in vivo* fusion, MLV CD63-BlaM VLPs pseudotyped with MLV ecoEnv or lacking glycoproteins (ΔEnv) were injected s.c. into the footpad of WT mice and pLN were isolated at 0.5 h, 1 h, 2 h, 4 h and 6 h post injection. Isolated pLNs were prepared into single-cell suspensions and total cells incubated overnight in CCF4 staining solution. The next day, pLN cells were analyzed for fusion with MLV VLPs by flow cytometry.

Capture of fluorescent MLV particles (Gag-GFP) by CD169+ macrophages (Sewald et al., 2015) was detectable at 0.25 h post injection, with 0.16 % of total pLN cells being GFP positive. MLV capture at pLNs peaks at 0.5 – 1 h p.i. with 2.4 % of GFP+ cells. Fusion with MLV CD63-BlaM ecoEnv-VLPs is first detected at 0.5 h with a total of ~ 0.3 % of pLN cells fused and peaks at 2 h post injection with 2.8 % of MLV-fused cells (Figure 11A). At 4 h and 6 h post s.c. injection, the amount of fusion-positive cells decreased steadily (Figure 11A). Unspecific fusion is rare since no fusion was detected *in vivo* with MLV CD63-BlaM ΔEnv-VLPs (Figure 11A). Strikingly, these results show that MLV capture by CD169+ macrophages precedes MLV fusion with permissive target cells by around 1 h *in vivo*. By that leaving a plausible amount of time for the MLV CD63-BlaM VLPs to be transferred to and fuse with permissive target cells.

In summary, MLV fusion could be successfully detected *in vivo*, revealing similar kinetic as MLV capture by CD169+ macrophages as recently described (Sewald et al., 2015).

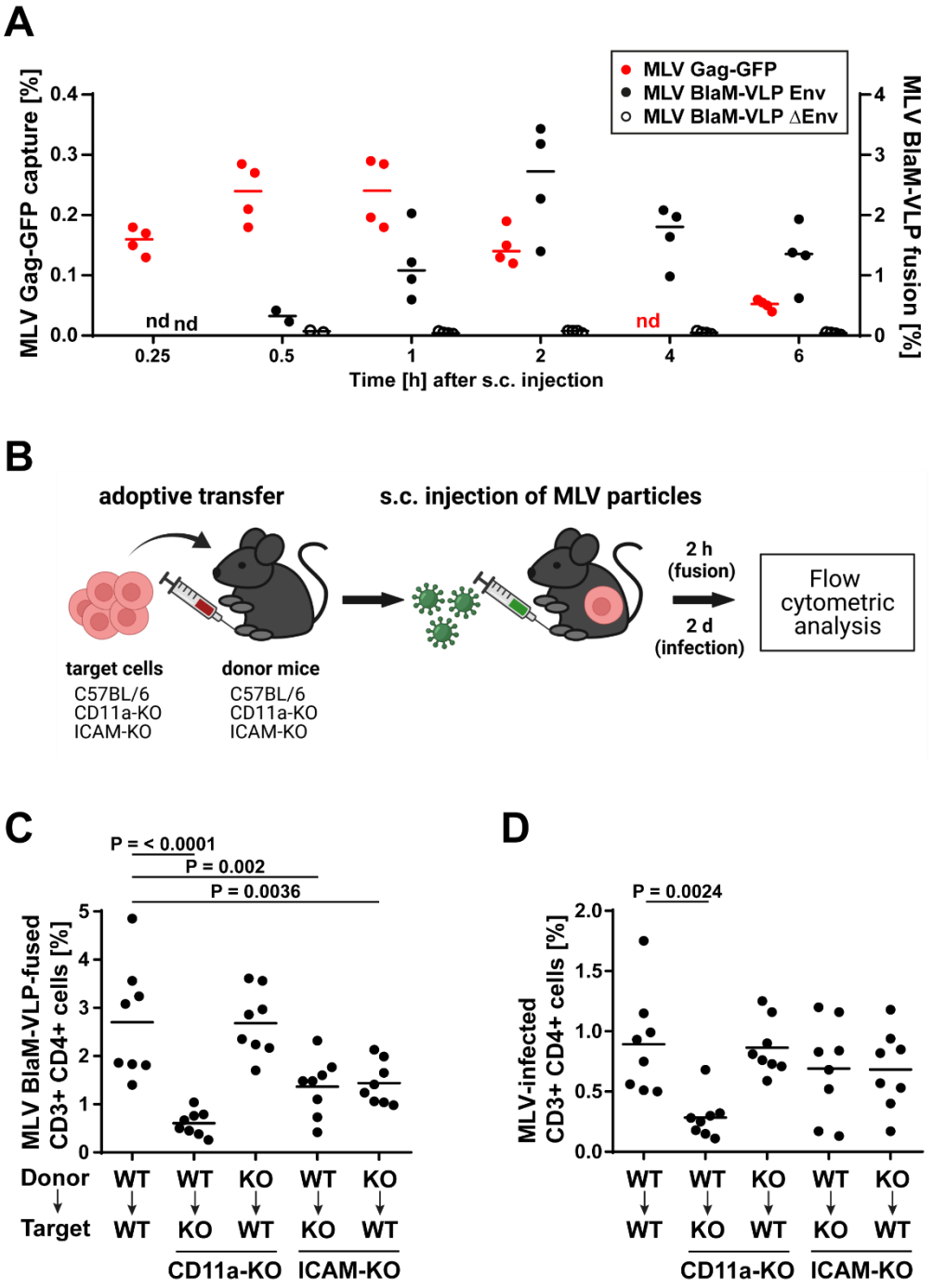

Figure 11: LFA1 is important for MLV fusion and infection *in vivo*.

(A) Quantification and temporal resolution of MLV Gag-GFP capture (n=4, red dots) and MLV CD63-BlaM VLPs fusion *in vivo*. VLPs were pseudotyped with or without ecotropic MLV glycoprotein (ecoEnv (n=2-4, black dots), ΔEnv (n=2-4, open circles)). Each dot represents one WT pLN, isolated at the indicated time point (0.25 h, 0.5 h, 1 h, 2 h, 4 h or 6 h) after s.c. injection of virus. Not determined = nd. *Figure **A** was published before on ePoster at GfV 2021*. **(B)** Schematic illustration of adoptive transfer for *in vivo* MLV *trans*-infection experiments. *In vitro* differentiated and FarRed+ labeled, FoxP3+ target cells were transferred into acceptor mice (donor). After 24 h acceptor mice were infected with MLV VLPs. pLN were isolated after 2 h (fusion) or 2 days (infection) for flow cytometric analysis of transferred FarRed+ target cells. **(C+D)** Quantification of *in vivo* MLV CD63-BlaM ecoEnv-VLPs fused **(C)** and infected **(D)**, adoptively transferred FoxP+ T cells. *In vitro* differentiated, FarRed+ FoxP3+ T cells (target) from WT, CD11a-KO and ICAM1-KO mice were adoptively transferred WT, CD11a-KO and ICAM1-KO acceptor mice (donor). After 24 h, MLV CD63-BlaM ecoEnv-VLPs were s.c. injected. Every dot depicts one pLN, isolated 2 h post injection. Percentage of fused FarRed+ target cells (FarRed+, $CCF4_{cleaved}$) was analyzed via flow cytometry. Representative data of eight independent experiments are shown. The P values were determined using an ordinary one-way ANOVA and Dunnett's multiple comparison test against the WT-WT control, respectively. Indication of not significant comparisons are excluded from the graph. *Figure **C** was published before on ePoster at GfV 2021. Figures **C** and **D** were similarly published before on Poster #117 at Retroviruses CSHL 2021. All figures were similarly published before in Engels et al. 2022.*

3.8 LFA1 is important for MLV fusion *in vivo*

LFA1 on target and ICAM1 on donor cells have an important role in MLV fusion with and *trans*-infection of target cells *in vitro* (3.6, 3.4). Therefore, adoptive transfer experiments (Figure 11B) were performed to analyze LFA1- and ICAM1-dependent fusion with pLN cells *in vivo*.

In brief, target FoxP3+ T cells, isolated from WT, CD11a-KO or ICAM1-KO mice were fluorescently labeled with a cytoplasmatic dye (FarRed+) and transferred into WT, CD11a-KO or ICAM1-KO acceptor mice (donor). After 24 h, acceptor mice were s.c. injected with either MLV CD63-BlaM ecoEnv-VLPs to detect fusion or MLV reporter virus (LTR-GFP) to quantify infection. Since *in vivo* fusion of MLV BlaM-VLP particles peaks at 2 h post infection (Figure 11A), pLNs were isolated 2 h after s.c. injection for analysis. Cell suspensions of draining pLNs were prepared and incubated overnight in CCF4 staining solution to be evaluated the next day for adoptively transferred cells that had fused with MLV VLPs (FarRed+, CCF4-cleaved) by flow cytometry. For infection, pLNs were isolated 2 days after s.c. injection and single-cell suspensions were analyzed for adoptively transferred cells that got productively infected by MLV (FarRed+, GFP+). *In vivo* fusion of CD11a-KO target cells transferred into a WT donor mouse was significantly reduced by ~ 80 % compared to fusion rates of WT target cells transferred into WT donor mice used as control (Figure 11C). In contrast, fusion of WT target cells transferred into CD11a-KO mice was as efficient as fusion of WT target cells transferred into WT mice (Figure 11C). Importantly, *trans*-infection experiments in the same combinations confirm these results. *Trans*-infection of LFA1-deficient target cells transferred in WT mice was significantly reduced by ~80 % when compared to *trans*-infection of WT target cells transferred into WT mice (Figure 11D). In line with that, *trans*-infection of WT target cells transferred into LFA1-deficient mice was as high as the *trans*-infection of WT target cells in a WT/WT constellation (Figure 11D). For ICAM1-KO target cells transferred into WT donor mice, fusion was significantly reduced by 52 % compared to WT and a similar reduction was observed for WT target cells transferred into ICAM-KO mice (Figure 11C). In contrast to that, *in vivo trans*-infection of WT cells transferred in ICAM1-KO donor mice or vice versa only showed a minor reduction by 33 % (Figure 11D).

Taken together, these results confirm a role of LFA1 on target cells during fusion and *trans*-infection *in vivo*, comparable with the results under *in vitro* conditions (Figure 7C and Figure 10A, B).

3.9 HIV spread between primary human CD4+ T cells requires LFA1 on target cells

LFA1 is important for cell contact-dependent *trans*-infection of murine CD4+ T cells with MLV *in vitro* and *in vivo* (Figure 7C, left panel and Figure 11D). Infection experiments with murine lymphocytes deficient in LFA1 and ICAM1 further demonstrate an important role of LFA1 on target cells and ICAM1 on donor cells during MLV *cis*-infection *in vitro* and *in vivo* (data from Lisa Falk, Sewald group). Using *in vitro* cell systems and blocking antibodies against LFA1 and ICAM1 a functional role of these cell adhesion proteins was also described in cell contact-dependent spread of HIV (Arias et al., 2003; Jolly et al., 2007; Rodriguez-Plata et al., 2013; Sanders et al., 2002; J.-H. Wang et al., 2009). Approaches with mutant cell lines lacking LFA1 (Hioe et al., 2001; Jolly et al., 2007) or primary cells carrying a non-functional LFA1 mutant of LAD-I patients (Anderson & Springer, 1987; Groot et al., 2006) strongly support a role for LFA1 in cell contact-dependent spread of HIV *in vitro*. A specific requirement of LFA1 on target cells was, however, not described.

Here the Cas9/RNP technique was used to create LFA1-deficient primary human CD4+ T cells, enabling us to investigate the effect of LFA1 during HIV *cis*-infection between physiologically relevant, primary CD4+ T cells (see 2.2.15, Figure 12).

Complexes of Cas9 and two different guide RNAs, specifically targeting the second exon of *ITGAL* were formed and transferred into primary T cells via nucleofection. Tide analysis (Brinkman et al., 2014) of genomic DNA at the gRNA target site 7 days after nucleofection verified the genomic knock-out of *ITGAL* (data not shown). In addition, every two days post nucleofection, surface marker expression of CD11a was tested by flow cytometry to analyze reduction. After 10 days, surface expression of LFA1 could not be detected in 85 % of the KO T cell population (Figure 13A). Importantly, surface expression of HIV receptor CD4 and its co-receptor CXCR4 was not altered in KO cells as compared to mock-treated WT cells (Figure 13A). Cells were subsequently activated with CD3/CD28-coated beads to enable proliferation and induce migratory behavior to allow for cell contact dynamics (Levine et al., 1996). After 20 days, surface expression of LFA1 was still present in the WT population and absent in the CD11a-KO population (Figure 13B). Surface expression of CD4 and CXCR4 was reduced but still present to similar levels in both, WT and LFA1-deficient cells (Figure 13B).

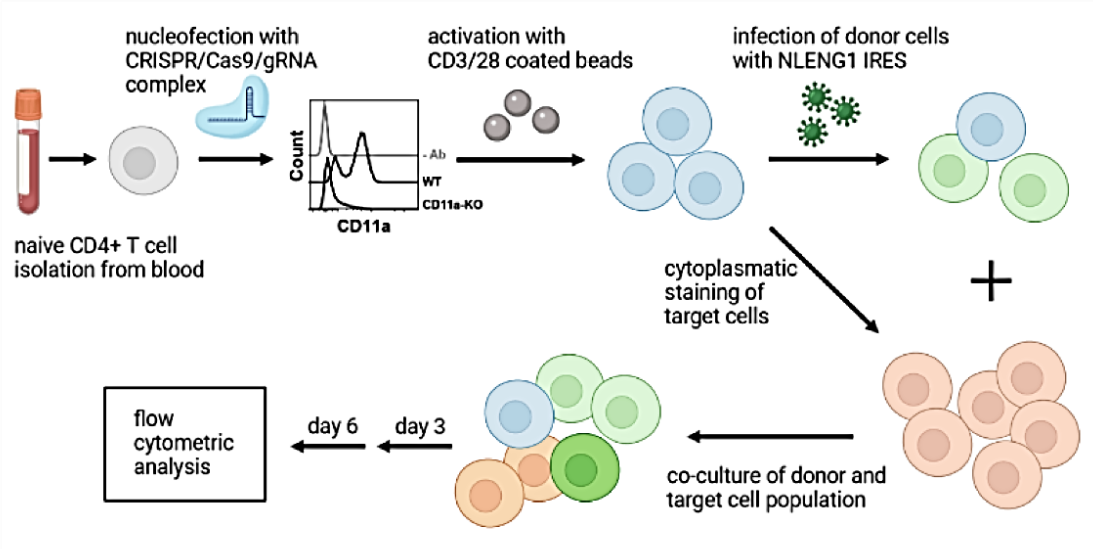

Figure 12: Schematic illustration of CD11a-KO in primary human T cells and subsequent *cis* co-culture assay of donor and target cell populations.

Naïve human CD4+ T cells are nucleofected and subsequently activated with CD3/28 coated beads. Activated cells are divided into two groups, one infected, the other one uninfected and stained. Cells are co-cultured for 3 and 6 days and subsequently analyzed via flow cytometry.

With a stable CD11a-KO produced in human T cells, we were able to perform *cis*-infection assays with co-cultures of human WT and LFA1-deficient cells, to study the influence of LFA1 on cell contact-dependent spread of HIV in human primary cells (see 2.2.16).

Cis-infection assays were performed with WT cells only and in a WT donor and LFA1-deficient target cell combination as well as vice versa. For that, donor human primary CD4+ T cells were spin-infected with HIV NLENG1-IRES (X4, GFP+) and incubated for 3 days to establish infection. Infection rates were measured by infection-induced expression of GFP from the HIV NLENG1-IRES (X4, GFP+). Spin-infected donor cells (WT, CD11a-KO) had comparable infection rates of 20 % on day 3 post infection (Figure 13C). Before the co-culture with infected donor cells was started, target cells were fluorescently labeled with a cytoplasmic dye (“FarRed”, red fluorescent). Co-culture between infected donor cells and fluorescently labeled target cells was started by mixing both populations in a 1:1 ratio. On day 3 and day 6 of co-culture, samples were taken and analyzed for HIV-infected donor (FarRed-) and target cells (FarRed+) by flow cytometry (Figure 12). Importantly, since only 20 % of the donor cell populations were initially infected, HIV was theoretically able to spread (I) from donor to target cells, (II) within the donor cell population itself and (III) from HIV-infected target cells to non-infected donor cells. Therefore, we analyzed the spread of HIV in both, donor and target cell populations over time. In the *cis*-infection assay with WT cells only, infection in the already partially infected donor cell population increased from ~ 10 % on day 3 of co-culture to 15 % on day 6. The corresponding target cell population showed an infection

of 4.5 % on day 3 and ~ 12 % on day 6 (Figure 13D). Notably, the spread of HIV in LFA1-deficient populations, irrespective if donor or target, was shown to be impaired when compared to WT (Figure 13E). In detail, despite equal donor cell infection rates (Figure 13C), only 3.5 % of CD11a-KO target cells were infected on day 3 of culture with WT cells (Figure 13E, left panel), while around 8 % of WT target cells were infected in CD11a-KO/WT co-culture (Figure 13E, right panel). Additionally, WT donor cells had an increase of infection from 11 % to 18 % from day 3 to day 6 and WT target cell populations increased the percentage of infected cells from 8 % to 20 % (Figure 13D). LFA1-deficient target cells also show an increase of infection during this time period from 4 % to 6 %, while LFA1-deficient donor cells show almost no increase in infection (Figure 13E).

These results indicate that transmission of HIV from WT to LFA1-deficient cells is not efficient. We conclude that like in MLV *trans*-infection, LFA1 is important on target cells for HIV cell-to-cell spread by *cis*-infection.

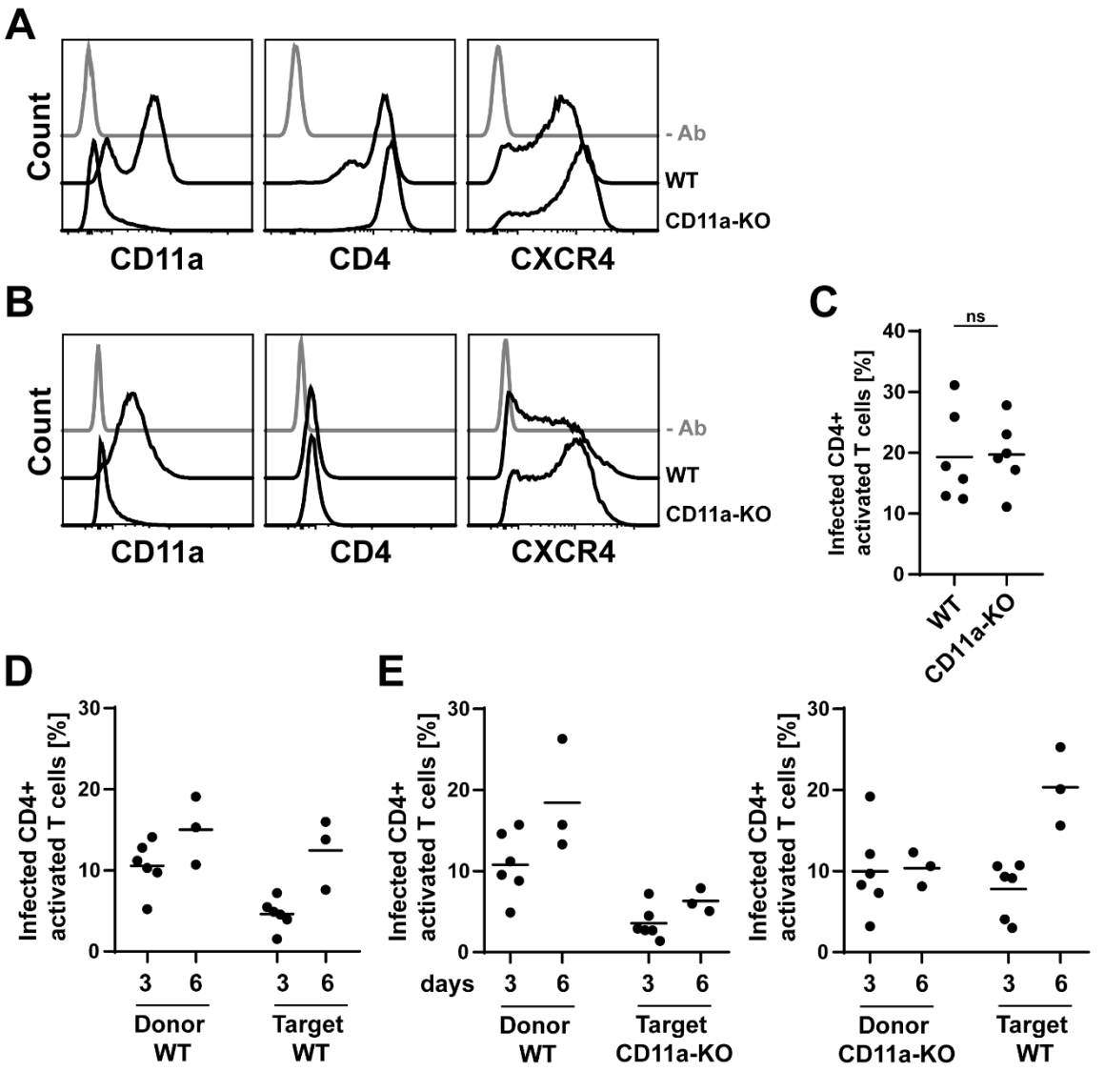

Figure 13: LFA1 is important for HIV spread between primary human T cells *in vitro*.

(A + B) Flow cytometric analysis of primary human CD4+ T cells (WT and CD11a-KO) for surface expression of CD11a, CD4 and CXCR4. Analysis was performed 10 days post nucleofection **(A)** and at the start of co-culture (20 days post nucleofection) **(B)**. Unstained WT T cells serve as representative unstained control (- Ab). Representative data of three independent experiments. **(C)** Quantification of HIV NLENG1 IRES infected, primary CD4+ human T cells (WT and CD11a-KO) after 72 h of *in vitro* transduction. Representative data of six independent experiments are shown. Statistical analysis was performed using an ordinary one-way ANOVA, no significant difference could be found between the tested groups (ns). **(D)** Quantification of HIV NLENG1 IRES infected, activated primary CD4+ human T cells at day 3 (n=6) and 6 (n=3) of *cis* co-culture. Co-culture was performed with WT donor and target cells. **(E)** Quantification of HIV NLENG1 IRES infected, activated primary human CD4+ T cells at day 3 (n=6) and 6 (n=3) of *cis* co-culture. Co-culture was performed with WT donor and CD11a-KO target cells (left) and CD11a-KO donor and WT target cells (right).

3.10 Establishment of an *in vitro* co-culture set up with fluorescent marker proteins for the analysis of cell dynamics using live-cell microscopy

Although LFA1 and ICAM1 were shown to be important for cell contact-dependent spread by *cis*- and *trans*-infection of HIV and MLV (Arias et al., 2003; Engels et al., 2022; Jolly et al., 2007; Rodriguez-Plata et al., 2013; Sanders et al., 2002; J.-H. Wang et al., 2009), the underlying mechanism remains elusive. A newly modified fusion assay using MLV CD63-BlaM VLPs (Figure 8) revealed that LFA1 and ICAM1 also support fusion during *trans*-infection, indicating that they support MLV spread by mediating or even stabilizing cell-cell contacts during *trans*-infection. To analyze the function of LFA1 and ICAM1 in cell-cell contact dynamics during retrovirus spread, a murine primary cell co-culture assay for live-cell imaging was established.

First, surface coating of glass bottom imaging dishes was tested that allows adhesion and migration of primary cells during co-culture. Importantly, a suitable surface coating should not interfere with the LFA1/ICAM1-mediated interaction of cells during *trans*-infection and not cause unspecific virus particle binding to the surface. Therefore, different extracellular matrix (ECMs) proteins were tested for adhesion and migration of primary cells using the ECM array slide (Advanced Biomatrix) (see 2.2.17). First, fluorescent peritoneal cavity-derived macrophages (RFP expressing) were cultured on the ECM array slide for 4 h at 37 °C. Afterwards, adhesion was visually analyzed by fluorescent microscopy. Macrophages were able to attach efficiently to the ECM proteins collagen VI, fibronectin, laminin and vitronectin (Figure 14A). Since fibronectin was the most cost-efficient, it was chosen to be further investigated. Next, to test the ability of primary target cells to migrate on fibronectin, FoxP3+ T cells were seeded on either untreated glass or glass coated with fibronectin (2 µg/cm²) and incubated for 2 h at 37°C. Migratory behavior of FoxP3+ T cells was followed over time by live-cell microscopy and migration speed was analyzed. While not able to migrate properly on glass, T cells were able to migrate on fibronectin-coated surface (Figure 14B). Tracking of T cells on fibronectin revealed an average velocity of 12 µm/min (data not shown) that is comparable to *in vivo* and *in vitro* migration velocity in other studies (Bousso & Robey, 2003; Hons et al., 2018; Lopez et al., 2019; Miller et al., 2002, 2003).

Finally, virus particle binding to the fibronectin-coated glass surface was investigated. MLV reporter virus (LTR-GFP) was incubated on glass dishes coated with either fibronectin or vitronectin. After extensive washing to remove unbound virus, S49.1 cells were cultivated for 24 h on the virus-laden surfaces and analyzed afterwards for infection (GFP expression) using flow cytometry. S49.1 cells incubated on reporter virus-loaded fibronectin coating showed lower infection rates (~ 1 %) than cells incubated on vitronectin (2,7 %) (Figure 14C). In conclusion, fibronectin is a suitable surface coating to investigate cell dynamics during *trans*-infection of MLV, since macrophages and T cells

are able to adhere and to migrate on fibronectin and its retrovirus binding capacity is low.

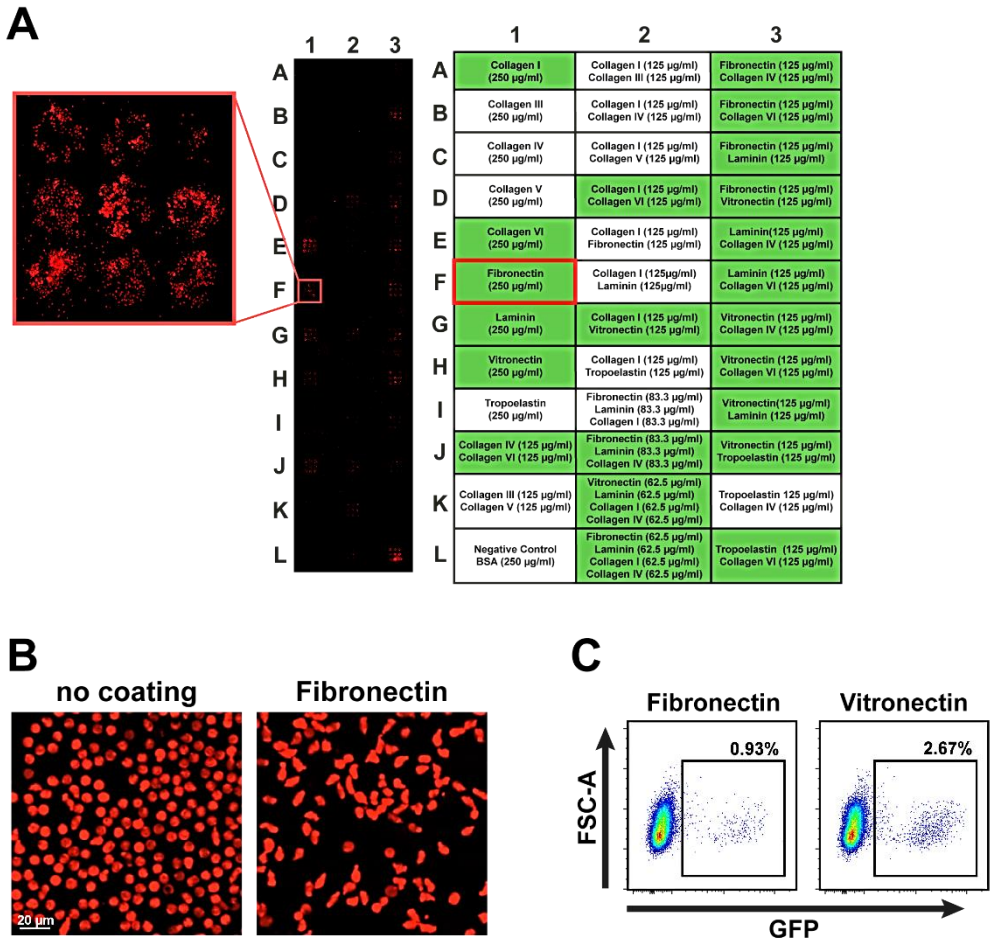

Figure 14: Fibronectin as a suitable surface coating for adhesion if CD169+ macrophages and migration of activated primary CD4+ T cells

(A) Visualization of RFP macrophages attached to variable surface coatings on an ECM Assay slide (Advanced Biometrix). Enlargement of fibronectin dotted coating area with attached RFP macrophages. **(B)** Visualization of PMA/ionomycin activated RFP CD4+ T cells on glass culture area without coating (left) and with fibronectin coating (right). **(C)** Flow cytometric analysis of MLV infected S49.1 cells (GFP+) after overnight incubation on Fibronectin (left) and Vitronectin (right) coated culture areas, previously incubated with MLV LTR-GFP reporter virus.

For the retroviruses HIV and HTLV it is reported that immunological and virological synapses share the characteristic accumulation of actin at the cell-cell contact site (Jolly et al., 2004; Vasiliver-Shamis et al., 2010) and the translocation of intracellular compartments such as the MTOC and the Golgi towards the side of cell-cell contact (Igakura et al., 2003; A. Kupfer et al., 1983; B. A. Kupfer et al., 1987; Starling & Jolly, 2016).

To be able to investigate and visualize this process in the case of contact-dependent MLV infection, FoxP3+ T cells were *in vitro* transduced with VLPs to stably express

fluorescent marker proteins to image cellular compartments (see 2.2.18). To visualize the actin cytoskeleton, the peptide lifeact (staining F-actin) fused to EGFP or mScarlet_i was used. For the microtubule-organizing center (MTOC) and the Golgi apparatus, the fusion proteins EB3-EGFP and Giantin-mScarlet_i, respectively, were expressed. Transduced FoxP3+ T cells were cultured for 48 h to allow expression of fluorescently labeled compartment markers prior to visualization by fluorescent life cell microscopy on a Nikon spinning disc confocal microscope. To test if the relocation of the compartment marker proteins is functional during synapse formation, beads coated with antibodies against the T cell receptor (anti-CD3/-CD28) were used to trigger the formation of immunological synapses (Figure 15A). Within 1-2 min after the first contact between cells and beads, the Golgi apparatus as well as the MTOC relocated to the bead-cell contact site and remained stable for several hours (Figure 15A). Additionally, a ring-like accumulation of actin could be observed at the contact sites (Figure 15A). Further, expression of fluorescent marker proteins did not interfere with cell behavior as transduced cells showed identical migration performance and cell division as non-transduced cells (data not shown).

3.11 LFA1 on target cells and ICAM1 on donor cells favor stable synapse formation during *trans*-infection

With a functional system established to visualize stable contact formation in primary FoxP3+ T cells, contact formation during co-culture between fluorescently labeled FoxP3+ T cells and MLV-laden macrophages was analyzed next.

Therefore, peritoneal cavity-derived macrophages were seeded on fibronectin-coated glass-bottom dishes and incubated for 24 h at 37°C to allow for CD169 surface expression. CD169+ macrophages were loaded with fluorescent MLV (MLV Gag-GFP) and afterwards washed extensively to remove unbound virus. FoxP3+ T cells were transduced two days before co-culture with MLV VLPs for expression of compartment markers (see 2.2.18) and were then added to the macrophages to start co-culture. After 24 h of incubation at 37 °C, live-cell imaging of the co-culture was performed (see 2.2.19). Thereby image acquisition was performed every 30 sec with a 12 µm z-stack in 1 µm steps for 4 h, with acquisition of fluorescent channels (488 nm and 561 nm excitation) as well as the differential interference contrast (DIC) channel. A stable contact was defined by a round-shaped contact side and localization of compartment markers at the side of cell-cell contact (Figure 15B, lower panel). In contrast to that, unstable contact was defined by an irregular contact side and altering positions of compartment markers (Figure 15, upper panel). The frequency and duration of stable cell-cell contacts in WT and KO primary cell co-culture was investigated. Due to the strong reduction of target cell *trans*-infection rates that were observed in WT donor/CD11a target and ICAM-KO donor/WT co-cultures in comparison with WT/WT co-culture (see 3.4), these combinations were chosen for the analysis of cell contact dynamics. Co-culture of CD11a-KO target cells with WT donor macrophages showed a 10 % increase of short contacts (< 2.5 min) and a 50 % decrease in the number of stable contacts lasting 2.5-10 min and > 30 min as compared to WT/WT co-culture (Figure 15C). Co-culture of WT FoxP3+ T cells and ICAM1-KO macrophages, resulted in a 25 % increase of short cell-cell contacts (< 2.5 min) compared to WT/WT co-culture. In line with that, stable contacts lasting 2.5-10 min, 10-30 min and > 30 min were decreased by 50 % compared to WT/WT co-culture (Figure 15C).

Taken together, LFA1 and ICAM1 are important for long-lasting and stable cell-cell contacts since KO of either LFA1 or ICAM1 resulted in a reduction of stable contacts during co-culture.

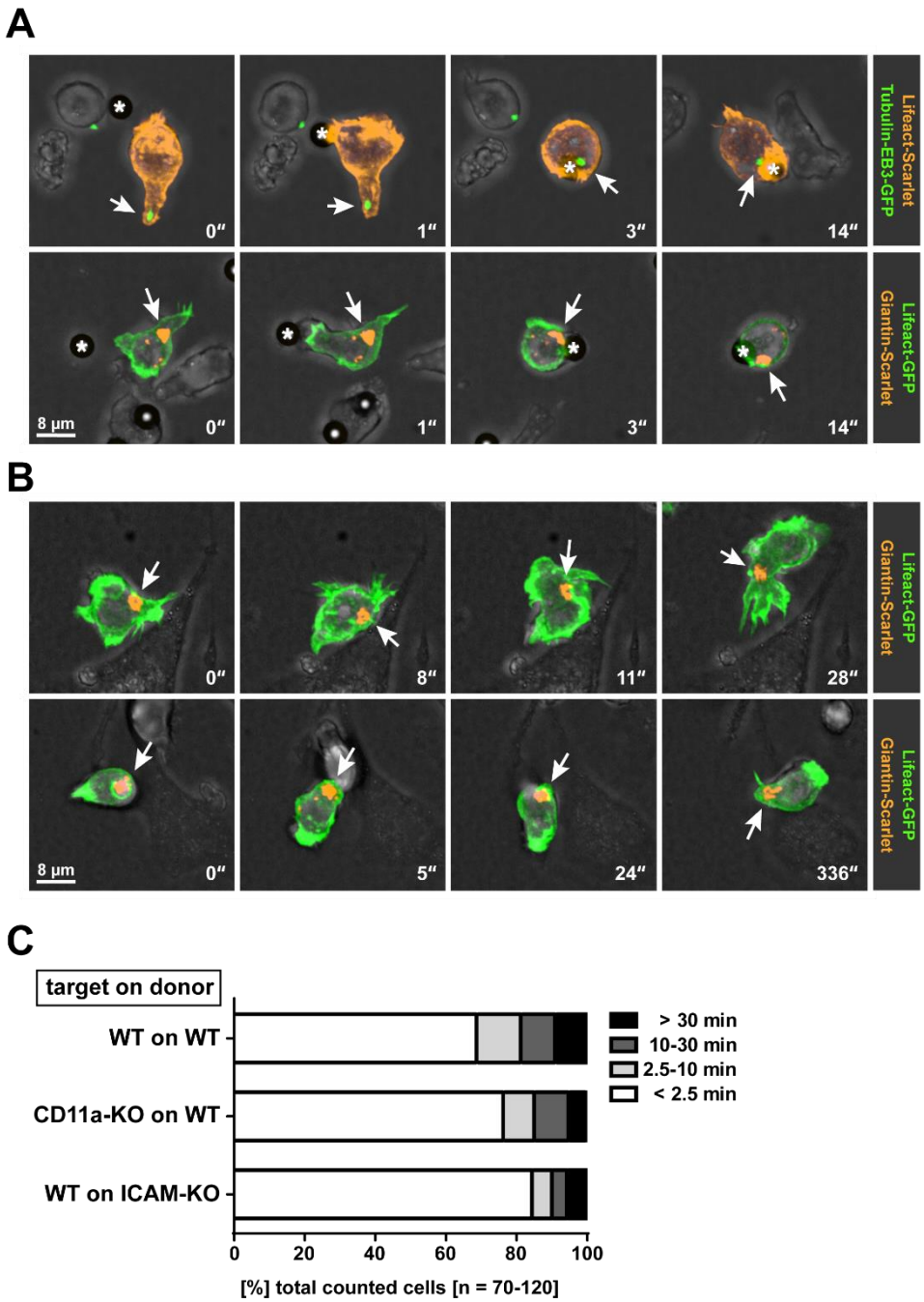

Figure 15: Fluorescently labelled cellular compartments allows the visualization of stable cell contact formation. LFA1-KO on target cells and ICAM1-KO on donor cells result in reduction of long-lasting, stable cell-cell contacts.

(A) Image sequence of *in vitro* differentiated FoxP3+ T cells (WT) with fluorescently labelled cellular compartments. Images were taken at consecutive time points during stable contact formation with CD3/CD28-coated beads. Upper panel: FoxP3+ T cells with LTR-driven expression of GFP coupled to Tubulin-EB3 and mScarlet_i coupled to lifeact (F-actin). Lower panel: FoxP3+ T cells with LTR-driven expression of mScarlet_i coupled to Giantin and GFP coupled to lifeact. Arrows indicate position of intracellular compartments MTOC (upper panel, green) and Golgi (lower panel, orange). Stars indicate position of CD3/CD28 coated beads. Elapsed time in minutes. **(B)** Image sequence of *in vitro* differentiated FoxP3+ T cells (WT) with LTR-driven expression of mScarlet_i coupled to Giantin (Golgi) and GFP coupled to lifeact. Images were taken at consecutive time points during unstable (upper panel) and stable (lower panel) contact formation with primary peritoneal-derived CD169+ macrophages loaded with MLV LTR-GFP. Arrows indicate position of Golgi (orange). Elapsed time in minutes. **(C)** Quantitative analysis of *in vitro* differentiated FoxP3+ T cell contacts with peritoneal cavity-derived macrophages in WT-KO co-culture combinations. Co-cultures were performed with following donor-target combinations: WT-WT, WT-CD11a-KO and ICAM-KO-WT. Bars indicate the relative percentage of contacts (n=70-100) that lasted < 2.5 min (white), 2.5-10 min (light grey), 10-30 min (dark gray) and > 30 min (black). *Figures **A, B** and **C** were similarly published before on Poster #117 at Retroviruses CSHL 2021.*

4. Discussion

In this thesis, we investigated the role of the cell adhesion proteins LFA1 and ICAM1 in contact-dependent spread of retroviruses. For that, we established a co-culture model for *trans*-infection with physiologically relevant primary cells. To reveal a role of the cell adhesion proteins LFA1 and ICAM1 in *trans*-infection, we used donor and target cells isolated from WT and LFA1-KO as well as ICAM1-KO mice. With that, we were able to prove that LFA1 on target cells and ICAM1 on donor cells are critical for *trans*-infection *in vitro*. Adoptive transfer experiments further demonstrate that LFA1 on target cells is also essential for *trans*-infection *in vivo*, while a lack of ICAM1 on either donor or target cell showed little effect. By adjusting a BlaM-based fusion assay for MLV, we could - for the first time - investigate fusion of retrovirus particles *in vivo*. Thereby, we show that the critical involvement of LFA1 and ICAM1 in *trans*-infection occurs already at the very early step of particle fusion.

To investigate if LFA1 and ICAM1 are also critical for the transmission of other retroviruses, we investigated HIV spread *in vitro*. By performing *cis*-co-culture assays with primary human T cells, we showed that viral spread was reduced from LFA1-KO to WT cells, as well as within LFA1-KO populations. These results, obtained for the first time with an LFA1-KO in primary human cells, provide a strong argument that LFA1 is also critical for the spread of retroviruses other than MLV.

Despite that evidence, the actual dynamic behind that phenomenon remains elusive. By establishing a novel visual screening method for stable contacts between cells, we could prove that LFA1 and ICAM1 contribute to cell contact durations and provide a new tool to analyze cell-contact dynamics.

4.1 Establishment of an *in vitro* MLV *trans*-infection assay

In the LN, sinus-lining macrophages provide a physical barrier by capturing pathogens and by retaining molecules bigger than 70 kDa (Farrell et al., 2016; Gretz et al., 2000; Iannacone et al., 2010; Sewald et al., 2015). However, MLV and HIV are able to overcome this barrier and infect permissive cells in the LN interior (Sewald et al., 2015). By surface expression of CD169 on sinus-lining macrophages, virus particles are captured and transferred to permissive cells located in the inner LN. Once few permissive cells are infected, the virus can spread within the permissive cell population via *cis*-infection. Therefore *trans*-infection is considered as an essential step in the systemic infection of mice (Sewald et al., 2015). The same mechanism was previously described *in vitro* for DCs, which are able to capture and transfer MLV and HIV particles via CD169 *in vitro* (Cameron et al., 1992; Geijtenbeek, Kwon, Torensma, Van Vliet, et al., 2000). For HIV, DCs have been shown to be able to efficiently *trans*-infect T cells *in vitro* and thus, they

likely play a critical role in the establishment of HIV infection *in vivo* (Cameron et al., 1992; Geijtenbeek, Kwon, Torensma, Van Vliet, et al., 2000; Hyun et al., 2008).

Although *trans*-infection was shown to be significant for retroviral infection and spread, little is known about the mechanisms behind this process. To gain more knowledge on individual steps of *trans*-infection, the first aim of this thesis was to establish a suitable assay to study *trans*-infection with primary cells *in vitro*.

For that we started by thoroughly characterizing primary isolated mouse cells to use as donor and target cells in our co-culture model. Key features of *trans*-infection are (i) donor cells that are able to capture virus particles on their surface but are non-permissive to infection and (ii) target cells that are permissive to infection (Geijtenbeek, Kwon, Torensma, van Vliet, et al., 2000). As donor cells, macrophages washed out from the peritoneal cavity of mice were tested. The crucial feature of capturing MLV particles by CD169 was confirmed by antibody staining, and an MLV binding assay. Further, CD169+ macrophages were not susceptible to infection as tested with MLV pseudotyped with ecotropic Env or VSV-G. For the target cells, we focused on CD4+ T cells, that were previously identified as target cells for MLV infection *in vivo* (Sewald et al., 2012). In our hands, the subset of CD4+ T cells, expressing the transcription factor FoxP3 (Engels et al., 2022) was highly permissive for MLV infection and thereby best suitable for our approach. In a first *trans*-infection assay using CD169+ macrophages and FoxP3+ T cells, we were able to successfully observe *trans*-infection. Including a transwell set-up, we were able to confirm that infected cells were the product of *trans*-infection and not of cell-free infection.

With the *in vitro trans*-infection assay in our hands, we were now able to investigate further aspects of retroviral *trans*-infection as described in the following chapters.

4.2 The role of LFA1 and ICAM1 in MLV *trans*-infection

LFA1 and ICAM1 are critical components of the immune system, as they mediate lymphocyte adhesion and migration (Anderson & Springer, 1987; Dustin & Springer, 1989, 1991; Patarroyo & Makgoba, 1989). In cell contact-dependent virus spread of HIV, LFA1 and ICAM1 were observed to accumulate along with other cellular and viral proteins at the side of cell-cell contact (Grakoui et al., 1999; Jolly et al., 2004, 2007; Krummel & Davis, 2002; McDonald et al., 2003; Sims & Dustin, 2002; Starling & Jolly, 2016; Vasiliver-Shamis et al., 2008, 2010). Using *in vitro* antibody-blocking experiments, others could demonstrate that LFA1 and especially its interaction partner ICAM1 functionally contribute to efficient HIV cell-cell transmission in *cis* and *trans in vitro* (Arias et al., 2003; Jolly et al., 2007; Mothes et al., 2010; Rodriguez-Plata et al., 2013; Sanders et al., 2002). It was shown that LFA1 and ICAM1 are critical for HIV *trans*-infection between mDCs and primary CD4+ T cells, as well as *cis*-infection between T cells (Jolly et al., 2007; Rodriguez-Plata et al., 2013). Moreover, first *in vitro* co-culture

approaches with cells of immunodeficient patients carrying a non-functional LFA1 mutant, strongly support a role of LFA1 in HIV transmission *in vitro* (Anderson & Springer, 1987; Groot et al., 2006).

By using primary cells isolated from mice, we could prove that these results are also true for *trans*-infection of MLV between CD169+ macrophages and FoxP3+ T cells. And in addition, we were able to demonstrate that in MLV, polarity of the LFA1-ICAM1 interaction is critical for MLV transmission. We could show that for *trans*-infection presence of ICAM1 on donor cells and LFA1 on target cells is critical.

Using a mouse model, we verified these results for *trans*-infection at pLN *in vivo*. In contrast to the *in vitro* experiments though, we could only observe little effect for ICAM1-KO on donor cells. Interestingly, in *cis*-infection, which was investigated by my colleague Lisa Falk, ICAM1 was indeed critical on target cells *in vivo*. Why ICAM1 on donor cells during *trans*-infection *in vivo* only has a marginal effect on virus spread could have several reasons. One possible explanation is that there are interaction partners other than ICAM1 present during *trans*-infection in pLNs *in vivo* (Engels et al., 2022). Human LFA1 has been found to interact beside ICAM1 (Makgoba et al., 1988) also with ICAM2 (de Fougerolles et al., 1991; Staunton et al., 1989), ICAM3 (de Fougerolles & Springer, 1992), ICAM4 (Bailly et al., 1994, 1995), ICAM5 (Mizuno et al., 1997; Tian et al., 1997; Yoshihara et al., 1994) and JAM-A (Ostermann et al., 2002). Regarding our experimental conditions, several of these possible interaction partners can be excluded: First, it was shown that ICAM3 has been lost in the rodent genome (Sugino, 2005). Second, ICAM4 is reported to be mainly expressed by erythroid cells (Bailly et al., 1994) and similar to that, ICAM5 expression is limited to telencephalic neurons of the central nervous system (Yang, 2012; Yoshihara et al., 1994). Therefore, the remaining two ligands of LFA1, ICAM2 and JAM-A, could possibly have an influence on the results by disrupting LFA1-ICAM1 interaction or compensate for the lack of ICAM1 in our KO model system. Therefore, it is necessary to conduct these experiments in the context of ICAM1/2- and JAM-A-deficient mice to clarify the interaction of those surface adhesion proteins during *trans*-infection *in vivo*. Another explanation for the observed results could be the incorporation of host adhesion proteins into virions during budding. It has been demonstrated that among other host proteins LFA1 and ICAM1 can be incorporated into the viral membrane upon budding (Fais et al., 1995). High amounts of ICAM1, incorporated into HIV virions were shown to increase infectivity of HIV virions in a LFA1-ICAM1 dependent manner (J.-F. Fortin et al., 1998; J. F. Fortin et al., 1997; Paquette et al., 1998). Therefore, ICAM1 incorporated into the MLV virions could bind the LFA1 on target cells, leading to efficient infection. In our case it is unlikely that this quite interesting effect could influence our results. Since our MLV particles are produced in HEK293T cells, ICAM1 incorporated into virions would be of human nature. Although cross-species interaction is possible for some proteins, human ICAM1 is not able to bind murine LFA1 (Johnston et al., 1990). In addition, effects caused by the virus itself should have also occurred in *in vitro* experiments and not only *in vivo*. In that matter, a different

ligand composition on LN macrophages is the more probable explanation, not excluding that the results observed *in vitro* might resemble what we would see during systemic infection in other body parts where alternative ligands are not present.

4.3 LFA1 outside-in signaling as a possible explanation for reduced infection rates of LFA1-deficient cells in MLV spread

An explanation for the reduced infection rates of LFA1-deficient cells in our experiments, could be the involvement of LFA1-signaling in viral infection of target cells. Several viruses are known to be dependent on the cell cycle or the activation status to support productive infection. In the case of MLV, virus genome integration into the host genome is only possible during cell division after breakdown of the nuclear envelope (Roe et al., 1993). Therefore the cycling cell status of the cell plays an essential role in virus spread. In contrast, HIV does not depend on an actively cycling cell, as accesory proteins mediate the transport of the HIV genome into the nucleus for integration. However, HIV benefits from an active cell metabolism for productive infection and spread, multiple host proteins and cellular pathways are involved in HIV1 replication (Len et al., 2017; Sedger et al., 2018). As an example, HIV1 is able to activate TCR signalling independent of antigens to drive viral spread, although the exact mechanism behind this observation remains elusive (Len et al., 2017). Other studies show that host adhesion proteins can also be involved in cell activation. In the IS, TCR and LFA1 are organized in distinct areas within the synapse called supra-molecular activation complexes (SMACs) (Monks et al., 1998). Further, after ICAM1 binding LFA1 was shown to provide a co-stimulatory signal that supports TCR-mediated activation of resting CD4+ T cells (Lebedeva et al., 2005; Van Seventer et al., 1990). While additionally crosslinking of TCR/CD3 complex induces LFA1 mediated binding to ICAM1 (Diamond & Springer, 1994).

In addition, it was shown for several viral proteins that they can support cellular activation and infection through interaction with cellular adhesion proteins. In the case of HIV it was shown that gp120 can trigger a co-stimulatory signal in CD4+ T cells by interacting with the integrin α4β7, which results in an increase in productive infection (Goes et al., 2020). For HTLV1, the transcriptional transactivator protein Tax have been shown to upregulate ICAM1 expression, leading to potent polarization of intracellular compartments to the VS and efficient cell contact-dependent spread between lymphocytes (Barnard et al., 2005; Nejmeddine et al., 2009). Further, HTLV1 accesory protein p12, which also enhances T cell activation was shown to promote cell-to-cell spread by inducing LFA1 clustering on infected cells (Kim et al., 2006).

Taking these findings into account, further investigation of possible signaling cascades that are induced by LFA1 could reveal even more cellular mechanisms alienated for efficient viral spread.

4.4 A BlaM-based fusion assay to study fusion of enveloped viruses *in vitro* and *in vivo*

Using viruses incorporating BlaM and a reporter for productive infection of the cell (e.g. GFP), we aimed to address the impact of LFA1 and ICAM1 directly at the level of cell-cell contact to exclude possible integrin-induced downstream effects that may favor infection and influence our results.

In a first attempt we implemented our BlaM-based fusion assay in our *in vitro trans*-infection co-culture experiments. Strikingly, we could obtain similar results for fusion as we saw before for infection *in vitro*. Thereby we could prove that LFA1 and ICAM1 both directly influence contact-dependent viral infection *in vitro* already at the very early level of cell-cell contact.

Moreover, we have shown here for the first time that this system also allows the detection of virus particle fusion *in vivo*. In adoptive transfer experiments, we demonstrated that the presence of LFA1 on target cells is important for fusion *in vivo* to a similar extent as it is important for infection (Figure 11C+D). Thus, we can exclude that the reduced infection numbers in our trans-infection assays are due to impaired downstream signaling caused by the absence of LFA1. However, since we did not observe a significant effect on fusion with an ICAM-KO *in vivo*, we are encouraged by the assumption already discussed in 4.2, that *in vivo* other ligands of LFA1 might abrogate the effect of ICAM1-KO on donor cells that we see *in vitro*.

In summary, we demonstrated here that our MLV-BlaM-based fusion assay is functional and paves the way for in vivo fusion experiments for other enveloped viruses. However, a potential limitation of this assay could be its use in long-term experiments, as it is not yet clear how long BlaM remains in the cell membrane *in vivo* before it is translocated or degraded. Therefore, I would like to emphasize at this point the importance of studying the kinetics of this technique in more detail before performing any long-term experiments.

4.5 The role of LFA1 in retroviral spread

Several studies revealed that cell-to-cell transmission is an important part in HIV-1 spread. Besides evidence for transmission of high-copy numbers of proviruses during contact-dependent spread, close contact can also shield viral transmission against some classes of antiretroviral agents and neutralizing antibodies *in vitro* (Abela et al., 2012; Agosto et al., 2015; Chen et al., 2007; Del Portillo et al., 2011; Duncan et al., 2014; Law et al., 2016; Malbec et al., 2013; Reh et al., 2015; Russell et al., 2013; Sigal et al., 2011; Titanji et al., 2017; Zhong et al., 2013). During contact-dependent spread of HIV-1, the cellular receptor CD4 and co-receptors CXCR4/CCR5, as well as other cellular surface proteins like LFA1 and ICAM1 were shown to accumulate at the side of cell-cell contact,

supporting cell infection (Fais et al., 1995; Jolly et al., 2004, 2007; McDonald et al., 2003). The interaction of LFA1 and ICAM1 at the cell-cell contact side was shown to support contact dependent spread of HIV1 in several studies using *in vitro* antibody blocking (Arias et al., 2003; Jolly et al., 2007; Rodriguez-Plata et al., 2013; Sanders et al., 2002; J.-H. Wang et al., 2009). *In vitro* co-culture approaches with primary cells from immunodeficient patients carrying a non-functional LFA1 mutant, strongly support a role of LFA1 in HIV transmission *in vitro* (Anderson & Springer, 1987; Groot et al., 2006).

In our approach using primary human T cells, carrying a CRISPR/Cas induced KO of itgal, we aimed to investigate the effects of LFA1 in HIV spread without the influence of antibodies and with the option of a wide donor range.
Here we could confirm that LFA1 is critical for efficient spread of HIV by *cis*-infection as the spread of HIV in LFA1-deficient populations, irrespective if donor or target, was shown to be impaired when compared to WT (Figure 13E). Thus, our results support the findings of previous experiments on the importance of LFA1 in HIV spread performed by others, using non-functional LFA1 mutants or antibody blocking of LFA1 (Arias et al., 2003; Groot et al., 2006; Jolly et al., 2007; Rodriguez-Plata et al., 2013; Sanders et al., 2002; J.-H. Wang et al., 2009). Thereby we conclude that our assay is functional and might serve as a basis for further experiments to gain more in-depth knowledge about the role of LFA1 and other cellular adhesion proteins in HIV contact-dependent spread.

However, to have a solid read-out it is essential that these experiments have to be repeated and gradually optimized to produce significantly relevant results. For one, although contact-independent infection cannot be excluded in our experimental setting, we assume only a minor contribution to the overall infection rate. This is based on the observation that the original cell population can hardly be infected and spinoculation with high titer virus is required to reach initial infection rates of 20 %. Nevertheless, a parallel conduction of transwell experiments could help to determine the actual ratio of cell-free infection here and make the read-out more accurate.
Additionally, it would be interesting to establish a *trans*-infection assay with human primary cells. Thereby the role of LFA1 and ICAM1 in HIV in both forms of contact-dependent spread (*cis* and *trans*) could be investigated. Therefore, it would be necessary to determine and thoroughly characterize human primary cells that can be used as donor cells as it was done in this study for the donor cells used in MLV *trans*-infection experiments. Unfortunately, this was not possible in this thesis due to time reasons.

4.6 A new imaging-based tool to investigate cell contact-dynamics in retroviral spread *in vitro* and *in vivo*

One explanation for the critical role of LFA1 in retroviral spread is that its function as cellular adhesion protein influences the contact dynamic between cells and allows more or longer cell-cell time for virus particle transmission and subsequent infection. To gain a better understanding of the dynamic during retrovirus *trans*-infection, we established a co-culture model that allows a quantitative analysis by visualizing cell-cell contacts between virus-presenting cells and target cells.

Assembly of cellular contacts like IS or VS is tightly linked to a reorganisation of the cytoskeleton for site-directed, efficient secretion of virus particles (Jolly et al., 2011; Starling & Jolly, 2016). For the IS and the VS it was shown that in favor of better supply of cellular components at the side of contact, cellular organs are rearranged and both, the microtubule-organizing center (MTOC) and the golgi apparatus translocate to the side of cell-cell contact (Geiger et al., 1982; A. Kupfer & Dennert, 1984). By visualizing these cellular compartments, we were able to differentiate stable from unstable contacts between FoxP3+ T cells and CD169+ macrophages. Unstable contacts were definded by cell membrane contact of the two observed cells without rearrangement of the cellular organs. On the other side, stable contacts showed a translocation of the MTOC and the golgi apparatus to the site of cell-cell contacts within seconds after the contact of the two cell membranes. The observed stable contacts were further defined by us in differnet categories of contact duration. While some stable contacts only lasted for a short amount of time (< 2.5 min), others could be categorized in durations of 2.5 - 10 min, 10 - 30 min and > 30 min. In MLV *trans*-infection co-culture models with CD169+ macrophages and CD4+ T cells, CD11a-KO as well as ICAM1-KO both lead to an increase of short contacts < 2.5 min and an overall decrease of contact duration. With our experimental setting we could show that LFA1-deficient target cells as well as ICAM1-deficient donor cells resulted in a strong decrease of long-lasting (2.5 to > 30 min) stable contacts compared to the WT control. Along with this we could observe an increase of stable contacts that were lasting only less than 2.5 min in both cases. Although the measured effects are not as strong as in the infection assays before, they nevertheless show a clear impact of LFA1- and ICAM1- deficiency on the cell-contact dynamics.

Moreover, with this results, we were able to reproduce similar results as shown before for VS in HIV spread (Jolly et al., 2007). There, engagement of LFA1 was shown to influences conjugate formation and support VS assembly, as well as virus transfer. By antibody-blocking of LFA1 Jolly and colleagues showed that VS formation could be reduced by up to 89 % in Jurkat cells *in vitro* and similar effects were observed with an induced LFA1-KO. Antibody-blocking of ICAM1 resulted in a reduction of VS by 31 % in this setting (Jolly et al., 2007). In addition other studies showed before that LFA1 and ICAM1 can both trigger organelle polarization and reorientation of MTOC in HIV-1-infected primary CD4+ T cells (Barnard et al., 2005; Nejmeddine et al., 2005; Starling &

Jolly, 2016).

This leads us to the conclusion that our approach is a new, useful tool to investigate contact-dynamics in retrovirus spread *in vitro* and *in vivo*, as an important addition to infection assays.

4.7 Summary and future perspectives

In this thesis we highlighted the importance of cellular adhesion proteins LFA1 and ICAM1 during retroviral spread, which confirms the findings of others (Arias et al., 2003; Jolly et al., 2007; Mothes et al., 2010; Rodriguez-Plata et al., 2013; Sanders et al., 2002). In addition, we were able to show for the first time that for MLV *trans*-infection the orientation of LFA1 and ICAM1 is crucial and that expression of LFA1 on target cells is essential *in vitro* and *in vivo* (Engels et al., 2022). This provides the basis for future, more targeted research of retroviral spread, as it likely applies to *trans*-infection in other retrovirus models, too.

HIV research is still limited to *in vitro* approaches in cell culture, in organotypic cultures and using humanized mouse models (Fackler et al., 2014). Continuous improvement of the available tools is essential to gain more insight into the relevant mechanisms of HIV and retroviral spread in general (Fackler et al., 2014). In this thesis we were able to establish and further improve useful instruments to enhance the study of retroviral spread.

In vitro approaches often lack physiological relevance to investigate involvement of cell contact dynamics. For example, previous experimental set-ups to investigate the role of LFA1 in HIV spread *in vitro* used co-cultures of cell lines lacking LFA1 or primary cells carrying a non-functional LFA1 mutant (Groot et al., 2006; Hioe et al., 2001). By implementing the targeted knock-out of ITGAL in human HIV primary cells, we provided a useful tool to investigate HIV spread with a broad donor range and physiologically relevant primary cells. Thus, advancing the current standard enabling to have a closer look at the subtleties of spread in an *in vitro* setting.

Using humanized mouse models allows for observation of cell-cell interactions in a physiologically relevant environment. While intravital imaging revealed close and long-lasting cell-cell contacts, resulting in infection of permissive cells (Law et al., 2016; Sewald et al., 2012; Usmani et al., 2018) and *in vitro* experiments support this observation (Arias et al., 2003; Engels et al., 2022; Jolly et al., 2007; Rodriguez-Plata et al., 2013; Sanders et al., 2002; J.-H. Wang et al., 2009), the cell dynamics that influence spread *in vivo* remain elusive. By providing a tool to discriminate stable from unstable

contacts of cells in close proximity with fluorescent labeling, it will be finally possible to visualize stable contact formation *in vitro* and *in vivo*. This could improve research on the integration of multiple proviruses during cell-to-cell spread (Del Portillo et al., 2011; Law et al., 2016; Russell et al., 2013) and further provide insight in the influence of viral or cellular surface proteins on contact formation, as for example HIV Nef which was shown to disrupt host cell actin dynamics and thereby influence motility in dense environments (Laguette et al., 2010; Stolp et al., 2012).

To date, fusion assays have been established but are often limited in their use (Cavrois et al., 2002; Lineberger et al., 2002; Nussbaum et al., 1994). While for HIV a well working model using BlaM coupled to the viral Vpr is available (Cavrois et al., 2002, 2014), this approach is not suitable for other retroviruses. By adapting a BlaM-based fusion assay for the use in MLV, we could prove its functionality *in vitro* and for the first time visualize fusion *in vivo*. Providing an easy way to screen for permissive cells within an organism during retroviral spread and comparing these permissive cells with the ratio of actual infected cells as done by our group for MLV (Engels et al., 2022). Thereby establishment of retroviral infection in different tissues and environments, which is considered to be a bottleneck can be further investigated (Haase, 2011; Lorenzo-Redondo et al., 2016). Since BlaM is linked to the CD63 incorporated into the cellular membrane (Albanese et al., 2021) this method will be also applicable to other enveloped viruses to investigate their fusion properties *in vivo*.

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Acknowledgements

Not a long time ago, there was a girl living in a small town, taking a trip to a bigger city almost every day to learn more about the wonders of science. After several years doing so, she was advised to go to another place, where she would be able to learn even more. Although the girl was a little bit scared, she started searching for such a place. After some time, she was invited by Xaver Sewald to have a look at his lab in Munich, so the girl got on a train and visited. It didn´t took her long to realize that this opportunity was exactly what she wanted and needed. When she was asked if she would like to join the Sewald group, she said yes, still scared but without any hesitation. Little did she know about the great time she was going to have the next three years.

In Xaver Sewald, she got the best group leader she could have asked for during her time in the lab. Always there, always funny, always with a good suggestion on hand. Little did she know how much she would miss the daily “und?” and the hallway meetings. But after just a short time, she knew how much she appreciated Xaver Sewald's way of teaching. - Xaver, thank you for that and everything else.

After some days in the Sewald lab, when the first crazy time of settling down went by, the little girl became more and more aware of the other colleagues in her lab. One girl, Lin Chen immediately made the impression of an extraordinary scientist and a good soul. Little did she know that Lin Chen will become one of her first friends in this big city and little did she know how much gorgeous food and dance parties and yoga lessons she will enjoy with this girl. - Lin, I´m so glad I got to meet you and call you my friend!

The lab was quickly growing, and more and more scientists joined the different groups. One day Ramya Nair, one of the most thoughtful and nicest persons the girl ever met joined the lab. Little did the girl know how close she would be to Ramya and how much bubble tea they will consume in their lives. Little did she know how much awesome food and city strolls and dance lessons they will share. – Ramya, I am so thankful for having you in my life.

Although Lin and Ramya where soon the two closest friends the girl had in Munich, there were so many more amazing people and great scientists worth to mention in this story. Little did the girl know what a great time she will have with Madeleine and Stephanie, with Manuel and Ernesto, with Florian and all the others that she worked with almost every day. – Guys, I’m glad I got to meet you all!

Time went by and although the Sewald group had a lot of fun in the lab, there was still a piece missing. After the girl was in Munich for exactly one year, Lisa Falk joined the Sewald group and made it kind of complete. Little did the girl know how glad she would be for having Lisa as a colleague, how perfect they will be able to work together and how great their experiments will be. – Lisa, thank you for being my partner in crime!

More time passed and great experiments were done, and the time in the lab was sprinkled with lots of great moments, lots of nice beer garden trips, football games, Jamón and birthday table decorations. There were plenty of clubs (running gang, ice-cream club, coffee club - just to mention a few!) and climbing hall lunchtime meetings. And the little girl lived a happy life.

After three years, it was almost time to say goodbye and the little girl started to write down all she learned, so future generations of scientists may build up on that. – Dear Barbara and Xaver, thank you for your thoughtful advice during the writing of this thesis. Dear correctors, thank you for taking the time to read and review this thesis.

When the little girl was done writing, she didn´t felt so little anymore. After all she managed to grow a lot during the time that has passed since she started this journey. And she is so glad to have all the amazing people in her live that supported her along the way.

She is so happy to have a mother who managed to raise her all on her own, being there for her for over thirty years, every day, every hour, loving her unconditionally.

She will be forever glad to have met a boy, that makes her smile every day, who is kind and calm and (almost) never drives her nuts and introduced her to her favorite Henry in the whole world.

Thank you all for this amazing journey, I can´t wait to share more adventures with you.

Affidavit

Eidesstattliche Versicherung

Engels, Rebecca

_____

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

LFA1 and ICAM1 are critical factors for fusion and spread of murine leukemia virus

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München, 27.11.2024 Rebecca Engels

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Conference contributions and publications

Illustrations and graphics in this paper have already been published in the original or with modifications in the posters and publications mentioned below. The corresponding cases have been marked and referenced to the original publication.

Conferences:

Gesellschaft für Virologie Conference 2021 (virtual)

ePoster:

LFA1 and ICAM1 support fusion and cell-to-cell transmission of murine leukemia virus *in vivo*

Rebecca Engels, Lisa Falk, Manuel Albanese, Oliver T. Keppler, Xaver Sewald*

Retroviruses 2021 – CSHL (virtual)

Poster #117:

LFA1 is important for MLV *trans*-infection *in vivo*

Rebecca Engels*, Lisa Falk, Manuel Albanese, Oliver T. Keppler, Xaver Sewald

Poster #192:

LFA1 and ICAM1 are critical factors for fusion and cell-to-cell transmission of MLV *in vivo*

Rebecca Engels, Lisa Falk, Manuel Albanese, Oliver T. Keppler, Xaver Sewald*

(* Presenting author)

Publication:

Cell Reports

LFA1 and ICAM1 are critical for fusion and spread of murine leukemia virus in vivo

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([1] These authors contributed equally)

Engels et al., 2022, Cell Reports 38, 110279 January 18, 2022 ª 2021

https://doi.org/10.1016/j.celrep.2021.110279

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Dekanat Medizinische Fakultät
Promotionsbüro

Confirmation of congruency between printed and electronic version of the doctoral thesis

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I hereby declare that the electronic version of the submitted thesis, entitled

LFA1 and ICAM1 are critical factors for fusion and spread of murine leukemia virus

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Rebecca Engels — Signature doctoral candidate