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The mechanism of TLR4 dependent binding of commensal LPS on retroviral surface and its role in infection

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„Die bloße Empirie erschöpft und wiederholt sich, die Wissenschaft führt zu einem stetigen Fortschritt, und ihr gehört die Zukunft.“

Max von Pettenkofer

Agenda

I. Introduction

I.1 The Murine Leukemia Virus as a model for studies on retroviral infection

As a gamma retrovirus, the Murine Leukemia Virus (MLV) causes rapidly progressive hematopoietic diseases, including leukemia or lymphoma in mice, by infecting lymphocytes and other cell types expressing the mouse cationic amino acid transporter 1 (mCat-1) receptor.[1-3]

In the early phase of MLV infection, viral envelope molecules bind to the surface of target cells expressing mCat-1. The following structural changes of viral envelope proteins will lead to the entry of the viral core, containing the MLV genome, into the intracellular space by fusion of the viral and cellular lipid membranes. [4] One of MLV's essential components to establishing infection is the enzyme reverse transcriptase, an RNA dependent DNA polymerase. It is required to transcribe viral single-stranded RNA into proviral double-stranded DNA. [5] However, integration of the proviral MLV DNA into the host's genome is dependent on the breakdown of the nuclear envelope during mitosis since MLV lacks active nuclear import elements. [6] Therefore, successful infection of MLV target cells is only possible when they get stimulated and are dividing. [7]

The genome of MLV is simple and consists of three groups of genes framed by two long terminal repeats (LTRs). The LTRs are necessary for the initiation of transcription of proviral DNA. Group associated antigens (GAG), such as matrix and capsid domains, and polymerase (POL) proteins are encoded in the same open reading frame (ORF). However, the sequences for envelope proteins are located on a different ORF. [8,4] Understanding the structure of the MLV genome allows researchers to design a genetically modified and replication-deficient MLV, label single viruses, or track MLV infection. For example, after inserting the sequence for a bioluminescent protein into the viral genome, visualization of infected host cells or infection events at all with fluorescent microscopes is possible. Even the detection and quantification of infected host cells via flow cytometric analysis is realizable. [7,9]

MLV shares many features with other retroviruses and is frequently used as a model to study virus and host interactions. For example, retroviral dissemination in secondary lymphatic organs was recently studied with MLV and could be transferred to Human Immunodeficiency Virus (HIV). [9] Furthermore, retroviral interaction with the

immune surveillance function of residential macrophages were shown in several experiments. [9,10] Apart from that, studies on MLV provided early insights into the pathogenesis and treatment of leukemia. [11,12] Although many retroviruses share analogous strategies for host-to-host transmission, there are also differences between simple gamma retroviruses and other more complex retroviral infections. While complex viruses infect host cells independently from their metabolic status, simple retroviruses such as MLV are only infective during the mitosis of host cells. [6,13] Therefore, findings on MLV infection should never simply be transferred to another retroviral genus. [8]

I.2 Horizontal and vertical transmission of retroviruses

Retroviruses such as HIV, Human T-Lymphotropic Virus type 1 (HTLV-1) or MLV can be transmitted horizontally or vertically between individuals. Horizontal transmission of HIV and HTLV-1 occurs during sexual intercourse or intravenously through needle-stick injections by intravenous drug abuse or transfusion of blood products in a medical setting. In contrast vertical transmission occurs by ingesting contaminated maternal milk during breastfeeding. In many transmission settings, the retroviral particles are considered to cross the host's mucosal barriers, leading to the infection of tissue-specific lymphocyte subpopulations. [14-16]

Horizontal MLV transmission is happening during sexual intercourse or fighting behavior. There was a model recently established to investigate early events of horizontal transmission. Sewald et al. found that after simulating horizontal virus transmission with a subcutaneous injection of MLV into the hind footpads of mice, viruses were captured by tissue resting $CD169^+$ macrophages and transported to the subcapsular sinus floor of the draining lymph node and were presented to B cells. This process leads to a subsequent transfer of viral particles and infection of target cells. During this process, retroviruses could infect host cells, such as B1 cells, a rare B cell subpopulation, and CD4+ T cells in peripheral lymph nodes.[7,9]

In addition, retroviruses, especially HIV-1, can be transmitted vertically from the mother to the child. Three major ways of transmission at different child development stages have been described: *in utero*, at birth, or during breastfeeding. [17,18] While the placenta's trophoblasts are progressively infected, mainly caused by a high virus load during pregnancy of women poorly adjusted to their antiretroviral medication, and viral particles make their way through the placental barrier towards the fetal blood

circulation. [19,20] Furthermore, small ruptures in the placenta allow viral transmission of endogenous and exogenous retroviruses *in utero*. Viral infection during delivery occurs when the child gets in contact with infected maternal secretions while passing the birth canal. [21] After birth, uninfected children are still at risk of being infected by their mothers because breastfeeding is an essential source of acquiring infective material. [22] As of late 2020, UNAIDS estimated that about 150.000 children (0-14 years) per year are newly infected with HIV, while 1.7 million children live with HIV infection. Over three-quarters of the cases are detected in sub-Saharan and Western Africa. [23] According to WHO data, the risk of HIV mother to child transmission ranges between 15% to 45% as long as no sufficient antiretroviral therapy (ART) is administered to HIV positive mothers during pregnancy and breastfeeding. [24]

Epidemiological studies in Japan revealed prolonged breastfeeding as a significant route for Mother to child transmission of HTLV-1. [25,26] Interestingly, the source of infection are HTLV-1 infected cells, especially lymphocytes secreted into the milk. [16] Successful prevention of viral spread was achieved by switching from breastfeeding to bottle-feeding in cases with a high proviral load in the mother. This measure will reduce the risk of developing a virus-associated adult T cell leukemia in some endemic regions. [27] Similar to HTLV-1 transmission, understanding the vertical transmission of other human retroviruses such as HIV would help to reduce the incidence of children living with HIV.

In analogy to HIV, murine retroviruses are transferred vertically by mother to child transmission via breastfeeding after virus particles infected mammary gland tissue. [10] Especially the mouse mammary tumor virus (MMTV), a retrovirus that can be orally transferred as a provirus stably integrated into epithelial and lymphatic cells secreted into the breast milk or exogenous particles that are also secreted and get ingested by suckling pups. [28] Once it passes the stomach, the virus is transferred in endosomal vesicles across the endothelial barrier by so-called M cells and infects the underlying lymphoid cells, like T and B cells located in intestinal Peyer's patches. [10,29] Interestingly, MMTV exploits virion-associated LPS acquired from the commensal microbiome to establish infection. How far this mechanism correlates to MLV vertical transmission still has to be elucidated.[30]

I.3 Direct and indirect stimulation of retroviral infection

Since the gut is one of the most extensive interfaces between mammals and their environment, it inhabits the highest density of microorganisms on its surface. As a consequence, there is a vast amount of specially adapted immune cells located in the gastrointestinal mucosa, also called gut-associated lymphatic tissue (GALT), in order to maintain the homeostasis of the host and microbiome as well as the protection of the organism from opportunistic pathogens invading the body via the intestine. [31,32]

Moreover, the pathogens must compete with the commensal microbiome to occupy nutrient niches within the gut. However, there is not only competition between the diversity of microorganisms. As shown recently, pathogens, i.e. viruses, take advantage of the microbiome by interacting together. [33] There are two different ways of interaction between viruses and the microbiome existing. The microbiota inhibits virus infection by stimulating the host's immune system, which leads to an increased migration of antigen-presenting cells (APCs) to the mucosa and consequently blocks virus invasion. On the other hand, the microbiome can promote infectivity of viruses directly or indirectly, mainly with the help of lipopolysaccharides (LPS) and enables successful viral infection of the host. For example, stimulation of lymphatic-cell division induced by microbiota will cause an increase of MLV replication and a higher occurrence of leukemia in specific pathogen-free (SPF) mice than in germ-free mice. [34,35]

Especially two different orally transmitted virus classes depend on commensal microbiome or at least remaining LPS to successfully induce infection and replication. [36,37] The first study describes a lower poliovirus associated mortality of mice treated with antibiotics to eliminate the gut microbiome than mice with a healthy microbiome. Interestingly, the secretion of infectious viruses was also lower in antibiotic-treated or germ-free mice. The decrease of mortality and virus shedding leads to the conclusion that enteric viruses' replication and pathogenesis are associated with microbiota. [36] Retroviruses such as MMTV use LPS of commensal microbiota to successfully establish infection by triggering interleukin 6 (IL-6) and IL-10 production. MMTV associated IL-10 production is significantly lower in LPS free experiments, and vertical transmission of MMTV infected germ-free mice is not detectable since MMTV

exploits features of commensal microbiota to induce immunotolerance towards itself. [37]

In LPS dependent promotion of viral infection, a difference between direct and indirect stimulation is made (**Figure 01**). Speaking of indirect stimulation means that the microbiome creates an optimal environment for viral infection by presenting LPS which leads to an enhancement or suppression of the immune system. Contrary to that, the direct stimulation postulates that viruses can actively bind components of the bacterial membrane, especially LPS, to their surface, which leads to ideal conditions for their infection by deceiving the host's immune system comparable to molecular mimicry. [33]

While budding from their host cells, viruses randomly gather membrane components located on some regions of the host cell's membrane. One can be pattern recognition receptors such as the Toll-like-receptor 4 (TLR4), mainly associated with murine and human leukocytes, which are also popular targets of retroviral infection. Although the signaling function of the receptors might be inactive once fused into the viral membrane, they are still able to bind metabolites such as LPS and attach them to the virus.[30]

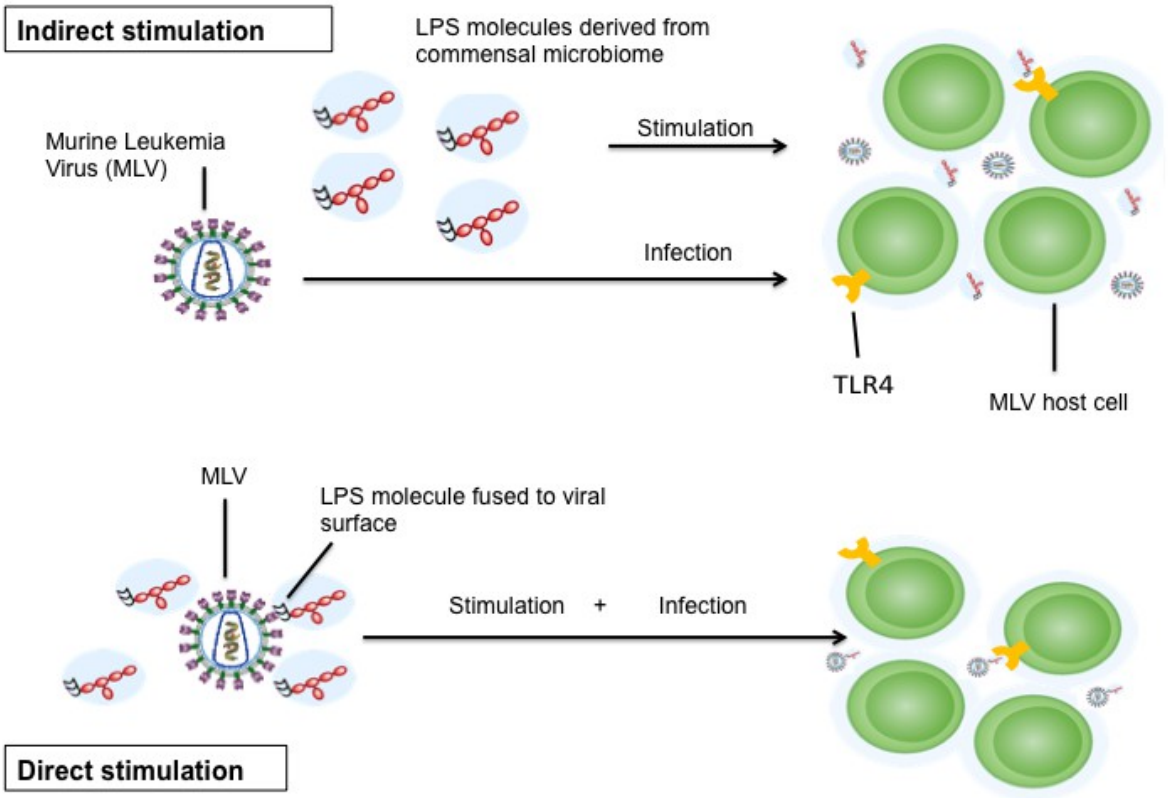


Figure 01: Schematic demonstration of indirect enhancement and direct enhancement of stimulation in retroviral host cells with LPS derived from commensal microbiome. MLV host cells are recognizing LPS with TLR4. Adapted from Wilks et al. (2012)

I.4 Pattern recognition receptors and their role in immunity

Pattern recognition receptors (PRRs) are part of the innate immune system. They recognize pathogen-associated molecular patterns (PAMPs) mainly descended from microorganisms or damage-associated molecular patterns (DAMPs) from damaged tissue.[38] There are 13 different so-called Toll-like-receptors (TLRs) that function as PRRs expressed in different cellular compartments of mammalian cells. Most of them are located on the cell surface as transmembrane proteins, while others are found in endosomes or the cytoplasm. [39]

Endotoxins such as LPS are located to the outer membrane of Gram-negative bacteria and lead to massive immunostimulation once they get into the circulation. Consequently, a massive release of cytokines can be expected, which will end in a septic shock, as already shown by Medzhitov et al. in 1997. [40] Responsible for LPS detection is the complex of the molecules TLR4, CD14, MD-2. While TLR4 is the activator of the intracellular pathway of LPS signaling, CD14 and MD-2 interact with the extracellular domain of TLR4. CD14 as a glycosylphosphatidylinositol (GPI)-anchored molecule into the cellular membrane and transfers LPS to TLR4-MD-2 complex to induce an immune response. MD-2 is responsible for the surface dimerization of two TLR4 molecules and subsequent activation of downstream signaling processes once it gets in contact with LPS transferred by CD14 or other LPS binding proteins. [41]

After dimerization of two TLR4 molecules on the extracellular surface, activation of the intracellular adaptor molecules MyD88 and TRIF induced by the intracellular domains of the Toll-IL1-resistance complex (TIR) follows. Both adaptor molecules play a crucial role in influencing the direction of further activation steps in the cascade. [42,43]

In case of MyD88 activation, several kinases such as IRAK1, IRAK2 and IRAK 4 get recruited, leading to the ubiquitination of TAK1 and activation of the transcription factor NF-κB. Activation of TRIF will also lead to the recruitment of the kinases TBK1 and IKKi, which will lead to the activation of the transcription factor IRF3. Consequently, activation of two nuclear transcription factors as a consequence of TLR4 stimulation will lead to a secretion of pro-inflammatory substances by the cell, i.e. cytokines such as IL-6, IL-1β, TNF-α or interferon-α/-β. Usually, the amount and class of secreted cytokines depend on the stimulating pathogen and the intracellular

activation route described above. [42,44] This knowledge is necessary, especially when it comes to LPS dependent vertical transmission of viruses, and it depends if viruses require immunosuppression or -stimulation to establish infection successfully. [33,45]

Local effects of IL-6 secretion are lymphocyte activation, especially B-lymphocytes and increased antibody production. Furthermore, it has several systemic effects, such as fever, inducing the production of acute-phase proteins and attracting immune cells of adaptive immunity to the infection site or secondary lymphatic tissue. [46]

I.5 Structural analysis of Lipopolysaccharides

In the mid of the 20th century, when the term 'lipopolysaccharide' (LPS) arose, it was already known that they consisted of lipids and carbohydrates. [47] LPS is one of the main components of the outer leaflet of the outer bacterial membrane in Gram-negative bacteria. Structural analyses showed that those molecules consist of three covalently attached regions: a very hydrophobic domain known as Lipid A or endotoxin, a nonrepeating oligosaccharide in the center and a distal polysaccharide or O-antigen. [48,49]

Since Lipid A is crucial for TLR 4 activation, LPS molecules from different bacterial serotypes mainly differ in their composition of the O-antigen. At the same time, Lipid A is very conserved in its structure with a narrow diversity. Even at low concentrations, Lipid A is a very potent stimulator of the immune system and is a leading force speaking of LPS toxicity. Therefore, variations in Lipid A and other ligands of innate immunity might play a role in escaping immune detection of the host and lead to a selective advantage for specific pathogens. However, the options of modifying the structure of Lipid A without losing its stimulatory potency are minimal. Altering the number, the order, the length, or the saturation of the fatty acids might be one option, while additional terminal phosphate residues and other inorganic residues be another option.[50]

I.6 The gastrointestinal immunity

One of the most prominent interfaces between the environment and the human body is the gastrointestinal tract. Due to those circumstances, good protection of this large surface by immunologically active structures and cells is essential. Epithelial cells and lymphatic cells, which are located in the submucosal layer, contribute to gastrointestinal immunity by producing cytokines and vary between a pro and an anti-inflammatory role during inflammatory or infectious processes in the gut.[51]

There is a single sheet of different intestinal epithelial cells connected via tight junctions makes up the barrier to the lumen of the gut. The different epithelial cells consist of cells with specialized functions and are constantly renewed by stem cells found near the bottom of the intestinal crypts. Enterocytes and Paneth cells can produce antimicrobial peptides such as alpha defensins, lysozyme C, phospholipases and C-type lectin. Goblet cells are mucin-secreting cells that protect and lubricate the intestinal epithelial surface. The tight junction complexes establish a seal towards the environment. However, persistent inflammation or infection results in an imbalance of the epithelial integrity and a barrier breach and the invasion of microbes. [52]

First-line defenders of the intestinal tract are the so-called intraepithelial lymphocytes (IELs). Those T cells can be divided into two groups and reside between the epithelial cells. One type is carries a conventional α:β T-cell receptor and a CD4 or CD8 receptor. It is responsible for producing IFN-γ during infection to activate other lymphocytes or directly eliminate infected cells. The other types are T cells with unconventional phenotypes such as the γ:δ T-cell receptor. They distinguish from the other T cells by not undergoing positive or negative selection in the thymus. Their primary function is recognizing and killing injured or stressed intestinal epithelial cells. [43,53]

However, most lymphocytes are located in anatomically defined microcompartments throughout the gut, which are Peyer's patches in the small intestine and solitary lymphoid follicles in the large intestine. Histomorphological, they differ from the normal endothelium in that they form dome-like structures that protrude into the intestinal lumen. They are coated by microfold endothelial cells on their surface, which have neither secretory nor absorptive function. Their primary function is the transcytosis of antigens for antigen presentation by antigen-presenting cells. The

development of these structures is relevant for shuttling viruses across the mucosa since experiments showed that a lack of M cells results in decreased MMTV infectivity of mice. [29,43]

When looking at antibodies in the gut lumen, the majority is immunoglobulin A (IgA). They are produced by plasma cells located in the lamina propria as dimeric IgA and secreted via transcytosis to their destination, which is the luminal surface of the gut, by immature epithelial cells at the base of the intestinal crypts. Once they reach the mucus layer overlying the epithelium, they can bind to and neutralize gut pathogens and toxic products. [43,54]

I.7 Composition of human breast milk

In the first months of a newborn, breastfeeding provides essential nutrients to the growing organism and contributes to developing a sound immune system. Therefore, some components in this highly variable body fluid are obligatory to fulfill those demands. Next to proteins, lipids, carbohydrates and other smaller molecules, there is also a heterogeneous mix of cells and specific bacteria located in the breast milk. [55,56]

Focusing on the immunological role of human breast milk, breastfed children have a reduced incidence of respiratory and gastrointestinal infections and less inflammatory diseases such as autoimmune diseases of the lung, the skin and the gastrointestinal tract. There is evidence that human breast milk continues the development of the infant's immune system, which already has started *in utero*. The passive protection of the organism by IgA- antibodies and the active protection with personalized microbial and immune factors play an essential role in this development. [57]

There are several hundreds of different bacterial species located in human breast milk, which reduce the number of bacterial infections in infants. Firstly, they express antimicrobial properties and bactericides against pathogenic bacteria. Secondly, they are activators of the child's innate and acquired immune system, especially natural killer cells, CD4+ and CD8+ T cells and regulatory T cells. [57]

Moreover, extracellular vesicles (EV) secreted into human breast milk are involved in both stimulation and suppression of the immune system. EVs are packed with heterogeneous cargo. All kinds of RNA, especially miRNA and cytosolic and membrane proteins, are transported via those small (50nm-1µm) particles.

Subsequently, they can communicate with other cells and influence their metabolism.[57]

However, there are also some contraindications for breastfeeding on both the children's and the maternal sides. Children with metabolic disorders such as galactosemia or phenylketonuria should be excluded from breastfeeding because of the complications. Also, mothers who are positive for infectious diseases such as seropositivity for HTLV or HIV and untreated tuberculosis should not breastfeed their child because there is evidence for transmitting those pathogens via breastmilk.[58]

I.8 Methods to visualize retroviral transmission

Many different methods exist to visualize specific details of viral replication and metabolic cycles.[59] Especially bioluminescent imaging and fluorescence microscopy played an essential role in revealing retroviral dissemination and infection mechanisms.[9]

In vivo bioluminescent imaging (BLI) is a non-invasive method to detect molecular pathways in living organisms and track virus-infected cells over a long time and distance in the body by administering modified pathogens with genetic information for luciferases.[60] Based on a chemiluminescent protein of the luciferase family, which is naturally expressed in a wide range of different life forms, it is possible to create a bioluminescent output via oxidation of a substrate that penetrates the organism and is detected by an externally applied camera. However, to enable deep-tissue imaging, a wavelength of >600nm is obligatory to penetrate viable tissue because of its high absorbance rates.[59,61] In some organisms, the luciferase is coupled to a fluorescent protein to redshift the emission, defined by the term Bioluminescence resonance energy transfer (BRET).[60]

Contrary to chemiluminescent proteins, a fluorescent protein requires light excitation to produce a signal. Reporter viruses tagged with a fluorescent protein fused to their capsid proteins or carrying genetic information to express fluorescent proteins in the cytoplasm of infected cells correspondingly are used to detect and analyze retroviral host cells and modelling host-pathogen interactions.[7,59] Despite *in vitro* imaging studies in artificial cell lines, fluorescence microscopy can also be deployed in intravital microscopy experiments under physiological conditions. While BLI resolution is often limited to the macroscopic scale of the organs of the organisms,

fluorescence microscopy can visualize single cellular processes at nanometer resolution in living animals.[59]

I.9 Aim of this study

Retroviruses can be transmitted between hosts via oral infection. [10,62] Recent studies demonstrate in how far MLV particles exploit intestinal Peyer's patches to access the host and establish infection. [10] However, spatial details about how retroviruses especially MLV use LPS derived from commensal microbiome to promote infection are unknown. Therefore, first line lymphocytes such as Th17, Treg and B-1, that are part of the intestinal immune system might represent susceptible cell populations that allow vertical transmitted retroviruses to establish an infection. [53,63] The questions will be experimentally addressed as following:

o To reveal mechanistic details for retrovirus capture and infection at mucosal surfaces, modified virus strains with surface proteins (TLR4) in the envelope will be analyzed regarding their infectivity and their ability to bind LPS successfully on their surface.

o To identify retrovirus entry tissue, a new luciferase/GFP- encoding MLV reporter virus will be designed that fits our demands to detect virus-infected tissue by bioluminescence *in vivo* imaging.

o To characterize the first infected cells, retrovirus-infected and target organs are harvested and prepared for multicolor flow cytometry of single cells. Reporter virus-infected cells are identified based on cytoplasmic GFP expression and analyzed for surface marker expression.

II. Material and Methods

II.1 Cell lines and culture

Infectivity of the Murine Leukemia Virus (MLV) preparations was tested by titrating them on the S49.1 T lymphoma cell line (ATCC Tib-28). The same S49.1 cell line was used to measure luciferase activity *in vitro*. HEK293 or HEK293/mTLR4-MD2-CD14 cell lines (Invivogen) were used for production of MLV. HEK293/mTLR4-MD2-CD14 cell line express murine TLR4-MD2-CD14 receptor complex through stably transfection with the pUno-mTLR4 plasmid and the pDUO-mMD2-mCD14 plasmid containing the murine TLR4 and the murine MD2-CD14 gene as well as an antibiotic resistance gene against Blasticidin and Hygromycin B, respectively.

HEK 293 cells were cultured in RPMI (GIBCO) plus 10% fetal calf serum (FCS) (Sigma-Aldrich). S49.1 cells, primary lymphocytes, primary macrophages and HEK293/mTLR4-MD2-CD14 were cultured in RPMI medium (GIBCO) supplemented with 10% FCS (Thermo Fisher), 10mM HEPES (Carl Roth), 1% non-essential-amino-acids (GIBCO), 1% sodium pyruvate (GIBCO) and 55 µM ß-Mercaptoethanol (Carl Roth). For longer culture of HEK293/mTLR4-MD2-CD14 cells medium was supplemented with 1 µg/ml Blasticidin (Invivogen) as well as 0.5 µg/ml Hygromycin B (Invivogen). Primary lymphocytes were cultured in the presence of 200 ng/ml of Interleukin-4 (IL-4) and 200 ng/ml of Interleukin-6 (IL-6) (both BioLegend). All Cells were maintained at 37°C with 5% CO_2.

II.2 Plasmids

All plasmids (**Tab. 01**) are provided with a long terminal repeat (LTR) sequence.
Genes encoding for GFP and nLuc separated by P2a were synthesized by GeneArt (Thermo Fisher) and cloned by enzymatic restriction into a plasmid containing a LTR sequence. The same procedure was done with genes encoding for GFP and AkaLuc. All plasmids additionally encode for an ampicillin resistance.

Tab. 01: Plasmid types used in the study

Plasmid type	Name	Insert characteristics	*E.coli* strain	Restriction Sites for gene insert
Viral DNA	Friend 57 MLV WT, full length genome [64]	Friend MLV	MAX Efficiency Stbl. 2 (Thermo Fisher)	NO
	Ecotropic MLV Env	MLV envelope protein, not part of viral genome		
	Friend 57 MLV Gag-GFP Δ Pol, full length	GFP fused to Gag, replication insufficient, Pol deletion		
	MLV Gag Pol	Gag protein, Pol proteins		
	MLV Gag-Scarlet-I Δ Pol, full length	Scarlet_I fused to Gag, replication insufficient, Pol deleted		
Reporter plasmids	LTR-Luc2	luciferase	MAX Efficiency Stbl. 2 (Thermo Fisher)	Nco1, BamH1
	LTR-nanoLuc [65]	luciferase		
	LTR-Antares [66]	luciferase, fluorophore		
	LTR-GFP	fluorophore		
	LTR-AkaLuc [67]	luciferase		
	LTR-GFP-P2A-nanoLuc	fluorophore, luciferase		Nco1, BamH1
	LTR-GFP-P2A-AkaLuc	fluorophore, luciferase		Nco1, BamH1
	LTR-AkaLuc-P2A-GFP	luciferase, fluorophore		Nco1, Not1, Xho1, BamH1

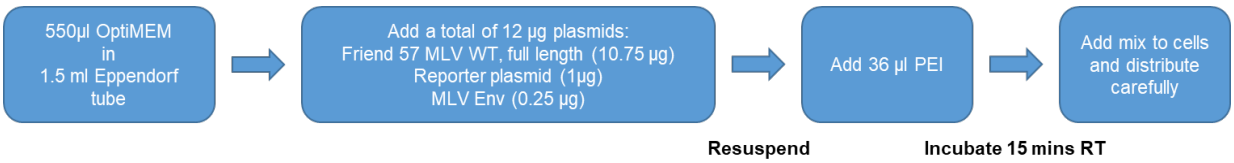
II.3 Virus production

MLV WT was produced by transfection of HEK293 cells at a confluency of 70-80% cultured in a 10 cm tissue culture dish (Falcon) containing 8.5 ml fresh RPMI/10% FCS medium supplemented with 10mM HEPES.

In order to produce MLV co-packed with a reporter plasmid for single-round infection experiments on primary lymphocytes and S49.1 cells, 10.75 µg of Friend 57 MLV full length DNA, 1 µg of a reporter plasmid and 0.25 µg of ecotropic MLV Env DNA was added to 550 µl OptiMEM transfection medium in a sterile 1.5 ml Eppendorf tube. After careful resuspension and application of 36 µg Polyethylenimine (Polysciences, Inc.) as transfection reagent, the mixture was incubated for 15 min at room temperature (RT). Finally, the mixture was carefully applied to the cells (**Tab. 02**).

In order to produce infectious MLV tagged with the red fluorophore Scarlet-I to the Gag protein to visualize MLV particles binding on primary macrophages, 7.75 µg of MLV Gag-Scarlet-I DNA and 4 µg of MLV Gag-Pol DNA was added in analogy to previous description to the transfection medium instead of Friend 57 MLV full length DNA and a reporter plasmid.

Tab. 02: Composition of total DNA used for Friend 57 MLV production



After 24 h supernatant containing viral particles was filtered using a 0.45 µm sterile filter (Pall Acrodisc, 0.45 µm HTTuffryn Membrane) and 8 ml of fresh RPMI/10% FCS medium supplemented with 10mM HEPES is applied to the cells. Supernatant was stored in 2 ml aliquots at -80°C labeled as early harvest. After 48 h again supernatant containing viral particles was filtered using a 0.45 µm sterile filter, aliquoted in 2 ml Eppendorf tubes and stored at -80°C labeled as late harvest.

MLV TLR4+ was produced in HEK293/mTLR4-MD2-CD14 cells with 12 µg DNA and 36 µl Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. 48 and 72 h post transfection supernatant of HEK293/ mTLR4-MD2-CD14 cells was

filtered using a 0.45 µm sterile filter and was frozen in 2 ml aliquots at -80°C accordingly to MLV WT production.
Infectious titer of produced virus was determined by infection of S49.1 cells and quantification of GFP expressing cells by flow cytometry (see **II.4**)

II.4 *In vitro* inoculation with MLV WT and MLV TLR

Equal amounts of virus containing supernatant from both harvest timepoints (see **II.3**) were thawed for 2 minutes at 37°C and kept on ice the whole time. Then the 2 ml aliquots were underlaid with 130 µl of 15% sucrose/ PBS in a 2 ml Eppendorf tube. After 2 h of centrifugation at 20,000 x g and 4°C the supernatant was carefully removed and the virus pellet was resuspended in 50 µl RPMI / 10% FCS / 10mM HEPES for 30-45 min on ice. To remove aggregates, samples were sedimented by another centrifugation step of 5 min at 5,000 x g at 4°C and supernatants were collected and pooled from early harvest and late harvest for infection experiments.
Finally, different amounts of concentrated virus were added to $2x10^5$ S49.1 cells or $1x10^5$ primary lymphocytes in 50 µl primary cell medium and incubated for 24 or 48 h at 37°C in a flat bottom 96-well plate. After 6 to 18 h of incubation, 100 µl of primary cell medium was added.

II.5 Primary cell isolation from C57/BL6 mice

To isolate primary cells for the experiments C57/BL6 mice (provided by the Max von Pettenkofer animal facility, Ludwig-Maximilians-Universität, Munich, Germany) were sacrificed with an overdose of carbon dioxide. B1 cells, macrophages (M□) and naïve T cells were enriched via negative selection, to avoid cell activation through antibody binding during isolation. Biotinylated antibodies (BioLegend) against cell surface proteins are used to remove cells by streptavidin magnetic beads (BioLegend). B1 cells and macrophages are enriched from the cell suspension created by washout of the peritoneal cavity with 5ml of phosphate-buffered saline / 1% bovine serum albumine / 2mM ethylenediaminetetraacetate (EDTA) (MACS buffer). After centrifugation for 10 min at 500xg and 12°C, up to 10^7 cells were resuspended in 400 µl MACS buffer and the following biotinylated (Bio) antibodies are added for negative selection of B1 cells (Tab. 03) and M□ (Tab. 04).

Tab. 03: Antibody cocktail for magnetic negative separation of CD19+ cells

Antibody (clone)	1 purification [uL]
F4/80-Bio (BM8)	3.5
CD8-Bio	1
CD4-Bio	0.75
Gr-1-Bio (RB6-8C5)	0.75
TER119-Bio (TER-119)	1
CD11c-Bio (N418)	2
NK1.1-Bio (PK136)	2
CD23-Bio	0.5
Fcε-Bio	1
CD88-Bio	1
CD117-Bio	1
CD115-Bio	2

Tab. 04: Antibody cocktail for magnetic negative separation of peritoneal macrophages

Antibody	1 purification [uL]
CD19-Bio	3
CD8-Bio	0.75
CD4-Bio	0.75
Gr-1-Bio	0.75
TER119-Bio	1
CD11c-Bio	2
NK1.1-Bio	0.75

After incubation for 15 min at 4°C, 25 µl of magnetic streptavidin beads were added to the suspension. Cells were negatively isolated in 4 ml MACS buffer using a MojoSort magnet (BioLegend) for 5 min on 4°C. Cell suspension was decanted and contained the cells of interest. To increase the amount of total enriched cells, the positive fraction was resuspended again in 4 ml MACS buffer and magnetic separation was repeated for a second time. Untouched cells of interest were

subsequently cultivated in primary cell medium in a flat bottom 96-well plate (Sarstedt) for further experiments.

For naïve CD4+ T cell isolation, spleens from mice were removed and minced through a 70 µm cell strainer (Corning) in serum-free RPMI. Homogenized splenocytes were enriched by negative selection with a murine naïve CD4+ T cell isolation kit (Miltenyi) according to manufacturer's protocol.

II.6 Regulatory T cell (Treg) and Interleukin 17 secreting T helper cell (Th17) differentiation

To differentiate freshly acquired naïve CD4+ T cells into Treg and Th17 cells, the surface of a flat bottom 96-well plate (Sarstedt) was coated with antibodies (BioLegend) against CD3 (clone 17A2) and CD28 (clone 37.51). A total concentration of 1µg/100 µl of each antibody diluted in PBS was added to each well. After 3 hours of incubation at 37°C, the wells were washed with 200 µl of PBS to remove unbound antibodies. Around $1.5x10^5$ cells in 200 µl primary cell medium were added to each well and celltype specific factors for Treg differentiation (see **Tab. 05**) and for Th17 differentiation were supplemented. Subsequently, cells were incubated for 4 days at 37°C to allow differentiation. T cell differentiation was confirmed by flow cytometry after intracellular staining against transcription factors FoxP3 (Treg) and RORγ-t (Th17) (see **II.11**).

Tab. 05: Substrates needed to differentiate naïve CD4+ cells to Treg cells

Concentration	Substrate
5 ng/ml	Recombinant human TGF-beta
20 ng/ml	Recombinant human IL-2
10nM	Retinoic acid
100 ng/ml	IL-7
100 ng/ml	IL-15

Tab. 06: Substrates needed to differentiate naïve CD4+ cells to Th17 cells

Concentration	Substrate
5 ng/ml	Recombinant human TGF-beta
20 ng/ml	Recombinant mouse IL-2
10 ng/ml	IL-1beta
100 ng/ml	IL-7
100 ng/ml	IL-15

II.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotanalysis

To analyze murine TLR4 expression on HEK293 cells as well as murine TLR4 MLV incorporation by western blot analysis, proteins were separated under denaturized conditions by SDS-PAGE (Thermo Fisher). HEK293 and HEK293/mTLR4-MD2-CD14 cells both at a confluency of 70-80% without supernatant were lysed with 500 µl of Radioimmunoprecipitation assay (RIPA) buffer in a 10 cm tissue culture dish to extract intracellular as well as transmembrane proteins for 10 min on ice. Similarly, 10 µl concentrated virus preparation of MLV WT and MLV TLR+ were lysed in 50 µl RIPA buffer to extract viral proteins. Next the (10X) NuPAGE sample reducing agent (Thermo Fisher) and the (4X) NuPAGE LDS sample buffer (Thermo Fisher) were added accordingly to each sample. After a 10 min incubation at 70°C, samples were chilled on ice and a total volume of 20 µl was loaded on a NuPAGE 10% Bis-Tris Gel (Invitrogen). The gel run took 90 minutes at 120 volts in NuPAGE MOPS SDS running buffer and the transfer on the nitrocellulose membrane (GE healthcare) was performed at 10 volt for 60 minutes in NuPAGE Transfer Buffer (Thermo Fisher) supplemented with 10% methanol.

For immunoblot analysis, the membrane was incubated in Tris-buffered saline (TBS) / 0.05% Tween / 5% milk (Carl Roth) (TBST 5% milk) for 1 hour at room temperature (RT). Afterwards the primary monoclonal antibodies murine Toll-like Receptor 4 (D8L5W) Rabbit mAb (Mouse Specific) (CST), β-Actin (13E5) Rabbit mAb (CST) and p30 Rat mAb (R187) (ATCC CRL-1912) were added in a 1:1000 ratio for mTLR4 and β-Actin and 1:500 ratio for p30. All antibodies were diluted in 5 ml TBS / 0.05% Tween / 5% BSA (Carl Roth) (TBST 5% BSA) and incubated overnight at 4°C.

Nitrocellulose membranes with HEK293 lysate were incubated with mTLR4 mAb and β-Actin mAb, while virus lysate was incubated with mTLR4 mAb and p30 mAb. On the next day membranes were washed 3-times with 15 ml of TBST for 5 min each. The corresponding secondary polyclonal antibodies Anti-rabbit IgG linked with Horseradish Peroxidase (HRP) (CST) and the Anti rat IgG linked with HRP (Jackson ImmunoResearch) were added in a 1:2500 dilution and in a 1:5000 dilution, respectively, in TBST 5% milk for 1 hour at RT. After another three washing steps as described above, the HRP substrate SuperSignal West Femto (Thermo Fisher) was used and chemiluminescence was detected by a Vilber Fusion VX imaging system according to manufacturer's instructions.

II.8 Limulus amebocyte lysate (LAL) assay

Endotoxin quantification was performed with the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo scientific). Before starting with LPS detection MLV WT preparation and MLV TLR preparation were titrated on S49.1 cells (see **II.4**) and normalized to the same titer in RPMI plus 10% FCS. In the following step one ml of both samples was incubated with 50 ng LPS derived from *E. coli* strain K12 (Invivogen) for 1 h at room temperature (RT). After that a first centrifugation of 1 h at 20,000 x g and 4°C on a 30% sucrose cushion was performed, which was adapted from a previous protocol established by Wilks et. al. [30] To further remove unbound LPS the pellet was resuspended in 50 µl PBS and the virus suspension was transferred into a fresh 2 ml centrifugation tube (Eppendorf) containing 1 ml RPMI on a 30% sucrose cushion. Then a second centrifugation of 2 h at 20,000 x g and 4°C was done. That pellet was resuspended in 50 µl PBS for 30 min on ice. The LAL assay was performed with 10 µl, 5 µl and 2.5 µl of concentrated virus according to manufacturer's instruction using the high standard stock solution.

II.9 Luciferase Assay

S94.1 cells were infected with luciferase-reporter virus as described above (see **II.4**) and bioluminescence from *in vitro* infected cells was measured 24 h post infection with the help of the plate reader CLARIOstar (BMG Labtech). For bioluminescent quantification, S49.1 cells were transferred to a white 96 well assay plate (Corning #CLS3600) and the medium was replaced with 100 µl RPMI containing 10% FCS to reduce background. Immediately after the corresponding substrate (**Tab. 07**) was

added, bioluminescent live cell emission was measured in top reading mode in three different defined spectral windows such as λ=430-510 nm, λ=500-600 nm and λ=610-700 nm. To optimize the readout, emission of every well was captured in 21 spots with a settling time of 0.1 s. Finally, a mean relative light unit (RLU) was calculated for each well.

Tab. 07: Luciferases with corresponding substrates

Luciferase	Substrate	concentration
Antares	Furimazine (Promega)	10-50 µM
AkaLuc	AkaLumine-HCl (Sigma-Aldrich)	100 µM
Luc2	D-luciferin (Sigma-Aldrich)	250 µM
nanoLuc	Furimazine (Promega)	10-50 µM

II.10 *In vivo* imaging studies

For *in vivo* infection experiments using bioluminescence imaging C57/BL6 mice were anaesthetized with 2% isoflurane. Luciferase-reporter MLV was prepared as described in **II.4**, but was resuspended in 50 µl PBS / 2% BSA. Mice were shaved at their legs to reduce background caused by hair and a total of $2x10^5$ infectious units of luciferase-reporter viruses were injected into their right footpad.

For quantification of infection with AkaLuc-reporter MLV after 48h, 200 µl of the luciferase substrate AkaLumine-HCl (final concentration 7.5 mM) was intraperitoneally injected into the mice. Infection with Antares-reporter and nLuc-reporter MLV was quantified with 200 µl Furimazine (Promega) (final concentration 13 mM) by intraperitoneal injection.

Whole body images were acquired with 3 min exposure time by the IVIS Lumina (Xenogen Corp.) to track virus infection in the mouse.

II.11 Flow cytometric analysis

Cells from murine tissues or cell culture were fixed in 1-4% paraformaldehyde (PFA) for 2 min. After fixation, cells were washed with 1 ml PBS and resuspended in 400 µl PBS / 1% BSA. 1-5 x 10^5 cells were blocked for 20 min in 50 µl PBS containing 1% BSA, 10% rat serum and 1 µg Fc-blocking antibody against CD16/CD32 (BioLegend) at RT for PFA-fixed cells and on 4°C for living cells. For surface marker staining, antibodies (BioLegend) listed in **Tab. 08** were diluted in 50 µl PBS / 1% BSA and

were added to the cell suspension and incubated in the dark for 30-45 min at RT for PFA-fixed cells and on 4°C for living cells. After another washing step with 1 ml PBS / 1% BSA cells were resuspended in 300 µl PBS / 0.5% BSA for flow cytometric analysis using a BD FACSLyric (BD Biosciences). For intracellular marker (**Tab. 08**) staining the BD Cytofix/Cytoperm (BD Biosciences) was used according to manufacturer's instructions. After intracellular staining cells were resuspended in 300 µl PBS / 0.5% BSA for flow cytometry analysis using a BD FACSLyric. At least 30,000 viable cells were acquired for each sample. Flow cytometric data was analyzed using FlowJo v10 (Treestar).

Tab. 08: Antibodies used for flow cytometric staining

Antibodies	Clone	Identifier	Dilution
Brilliant Violet 605™ anti-mouse CD3ε Antibody	145-2C11	# 100351	1:1000
PE/Cy7 anti-mouse NK-1.1 Antibody	PK136	# 108713	1:1000
APC Anti-mouse TCR γ/δ Antibody	GL3	# 118115	1:1000
APC/Fire™ 750 anti-mouse CD4 Antibody	GK1.5	# 100459	1:1000
PE anti-mouse CD8a Antibody	53-6.7	# 100707	1:8000
Brilliant Violet 421™ anti-mouse CD19 Antibody	6D5	# 115537	1:500
APC/Fire™ 750 anti-mouse CD45 Antibody	30-F11	# 103153	1:1000
Alexa Fluor® 647 anti-mouse CD326 (Ep-CAM) Antibody	G8.8	# 118211	1:1000
PE anti-mouse CD14 Antibody	Sa14-2	# 123309	1:8000
PE/Cy7 anti-mouse TLR4 (CD284)/MD2 Complex Antibody	MTS510	# 117609	1:1000
APC anti-mouse CD284 (TLR4) Antibody	SA15-21	# 145405	1:1000
APC anti-mouse Slc7a1 (Cat-1, ERR) Antibody	SA191A10	# 150505	1:1000
APC/Fire™ 750 anti-mouse CD19 Antibody	6D5	# 115557	1:1000

II.12 Microscopy

To investigate LPS binding to MLV particles $5x10^4$ peritoneal cavity-derived macrophages in 100µl primary cell medium were seeded in the center of an uncoated glass bottom microwell dish (MatTek #P35G-1.5-10-C). After 24 h incubation to allow adhesion, MLV WT GAG Scarlet_I or MLV TLR GAG Scarlet_I were incubated for 1 h on 4°C with 500 ng/ml fluorescent LPS (Invitrogen # L23351), concentrated to 100 µl as described above (see **II.4**) and incubated with the macrophages. After incubation for 30 min at room temperature followed by 30 min incubation at 37°C microscopic

analysis on a confocal microscope eclipse Ti2 (Nikon) connected to a confocal scanning unit w1 (yokogawa) was performed.

II.13 Statistical analysis

Statistical analysis and data analysis were performed with GraphPad Prism v6 software. For two-group comparisons the non-parametric Mann-Whitney U test (two-tailed) was used under the assumption that the samples did not follow a Gaussian distribution. Exact p-values and the numbers of independent replicates (n) are included in the figures. A difference between groups was considered significant if $p<0.05$. No statistical tests were performed if one of the compared groups contained three or less values (e.g., **Figure 05**: n=3).

III. Results

III.1 *In vitro* infection of primary cells with MLV

III.1.1 Validation of *in vitro* differentiated primary cells for further experiments

First infection experiments in peripheral lymph nodes suggest that MLV specifically infects T and B cell subsets to establish infection *in vivo*. [7] Further investigations revealed a tropism of MLV for CD4+ Treg, CD4+ Th17 and a rare population of CD19+ B1 cells. [9, 68] While intestinal Treg cells are known to fulfill an important role in perpetuating immune tolerance by secretion of IL10, intestinal Th17 cells tend to stimulate the secretion of IgA with their cytokine IL17. [69] Both subpopulations might influence intestinal viral infectivity passively by serving as target cells or actively by secretion of cytokines. [30]

To study the role of indirect and direct stimulation of MLV infection in these primary cells *in vitro*, target cell subsets were either enriched from mouse tissue or in vitro differentiated from naïve precursors. B1 cells are directly isolated from the peritoneal cavity of mice by negative selection using an antibody cocktail (BioLegend) to eliminate other cell types (Tab. 03). Treg and Th17 cells are differentiated from naïve CD4+ T cells isolated from the spleen of C57BL/6 mice by negative selection according to the manual (Miltenyi Biotech). CD4+ T cells are *in vitro* differentiated according to published protocols. [70,71,72] Cell culture purity after the differentiation procedure was checked with an antibody panel for flow cytometry analysis. Treg are detected with an antibody against the intracellular nuclear transcription factor Forkhead-Box-Protein P3 (FOXP3), while antibodies against the RAR-related orphan receptor gamma (RORγt) detect Th17. Flow cytometric analysis reveals that 92.0 % CD4+ T cells had differentiated into FOXP3-positive Tregs and more than 82.0 % CD4+ T cells had differentiated into RORγt positive Th17 cells (**Figure 02**). Those results are firstly validating our differentiation procedure and enable a primary target cell line to conduct further *in vitro* experiments.

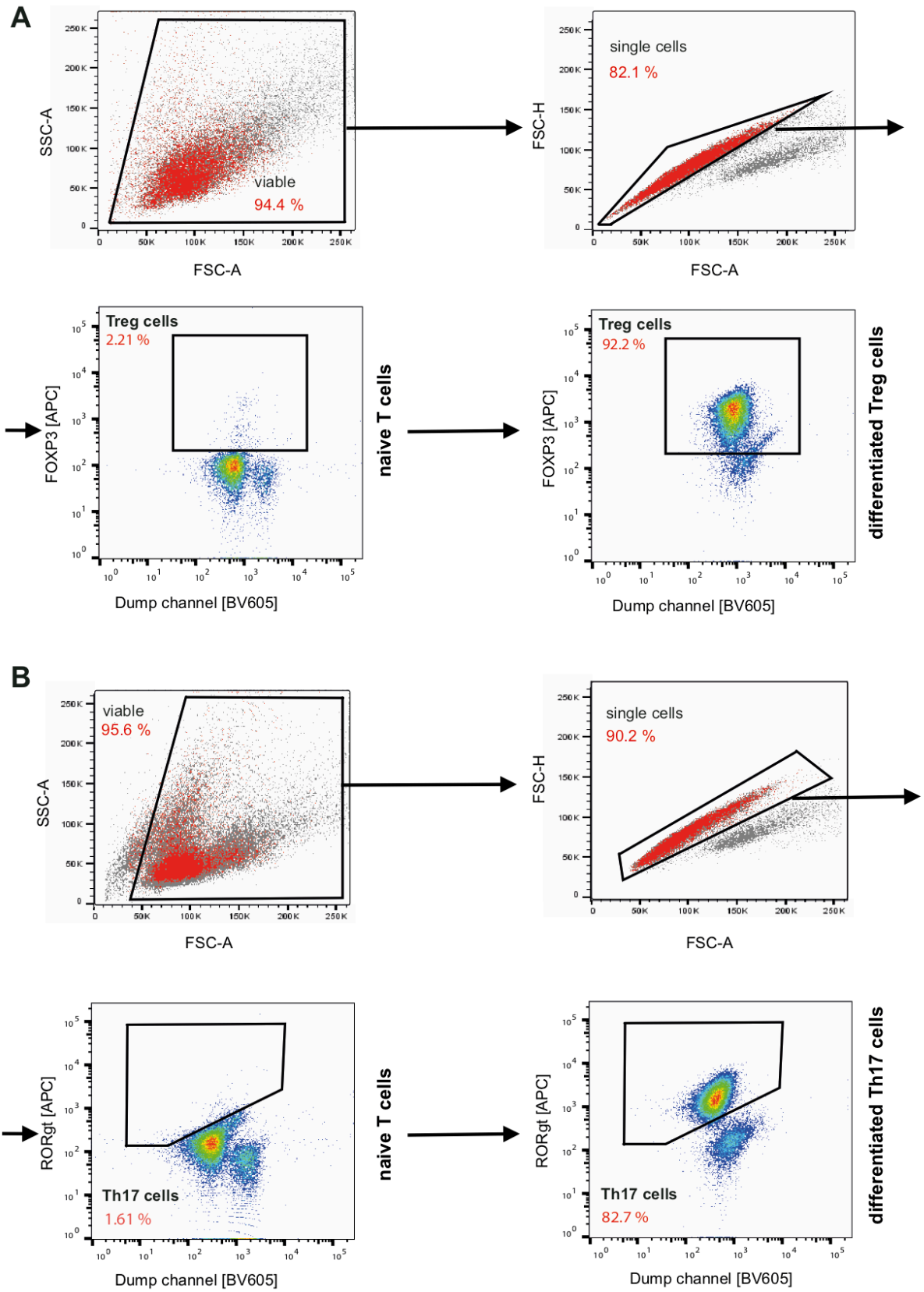


Figure 02: Naïve CD4+ T cells were differentiated *in vitro* into Treg or Th17 cells. Cells after 4 days of differentiation were analyzed by antibody staining of the intracellular antigens FoxP3 **(A)** and RORγt **(B)** for Treg and Th17 cells, respectively. Starting population of naïve CD4+ T cells was used as negative control. Samples were analyzed by flow cytometry and cell populations were gated for viable single cells.

III.1.2 Stable expression of murine TLR4 in HEK293 cells is needed to produce MLV TLR4

Previous studies have revealed that the mTLR4 complex (murine TLR4/MD2/CD14) is incorporated into the envelope of MMTV *in vivo* to support mother to child transmission by LPS accumulation on its surface. [30] To investigate the role of LPS in retrovirus infection at the cellular level, we established a protocol to design MLV particles with LPS receptors incorporated in their envelope analogue to the model that was demonstrated for MMTV by Wilks et al. [30] First to create differently equipped virus particles for further experiments, *in vitro* production of MLV was performed either in HEK293 cells (MLV WT) or HEK293 cells that have been stably co-transfected with the two plasmids pUNO-mTLR4 and pDUO-mMD2-mCD14 to express murine TLR4 (mTLR4)[73], MD2 (mMD2) and CD14 (mCD14) as complex. This reporter system has been used successfully by other groups, who found out that not even human TLR4 is naturally expressed on our HEK293 cells. [74] In order to ensure that producer cells as well as viral particles incorporate the murine TLR4-MD2-CD14 receptor complex we performed flow cytometric analyses and a western blot analysis. We compared TLR4 (**Figure 03 A**) and CD14 surface expression (**Figure 03 B**) between naïve HEK293 and HEK293/mTLR4-MD2-CD14 cells. We were able to prove surface expression of the mTLR4 receptor as well as of mCD14 in the HEK293/mTLR4-MD2-CD14 cells compared to naïve HEK293 cells that serve as negative control. In the western blot analysis (**Figure 03 C**) we observed no expression of mTLR4 in wild type HEK293 cells, which means that proteins of the murine TLR4 complex are neither translated nor assembled in wild type HEK293 cells. Consequently, MLV produced in HEK293 cell line will unlikely incorporate the mTLR4 complex in its surface, while MLV produced in HEK293/mTLR4-MD2-CD14 cell line shows a positive band for mTLR4 in the western blot (**Figure 03 C**).

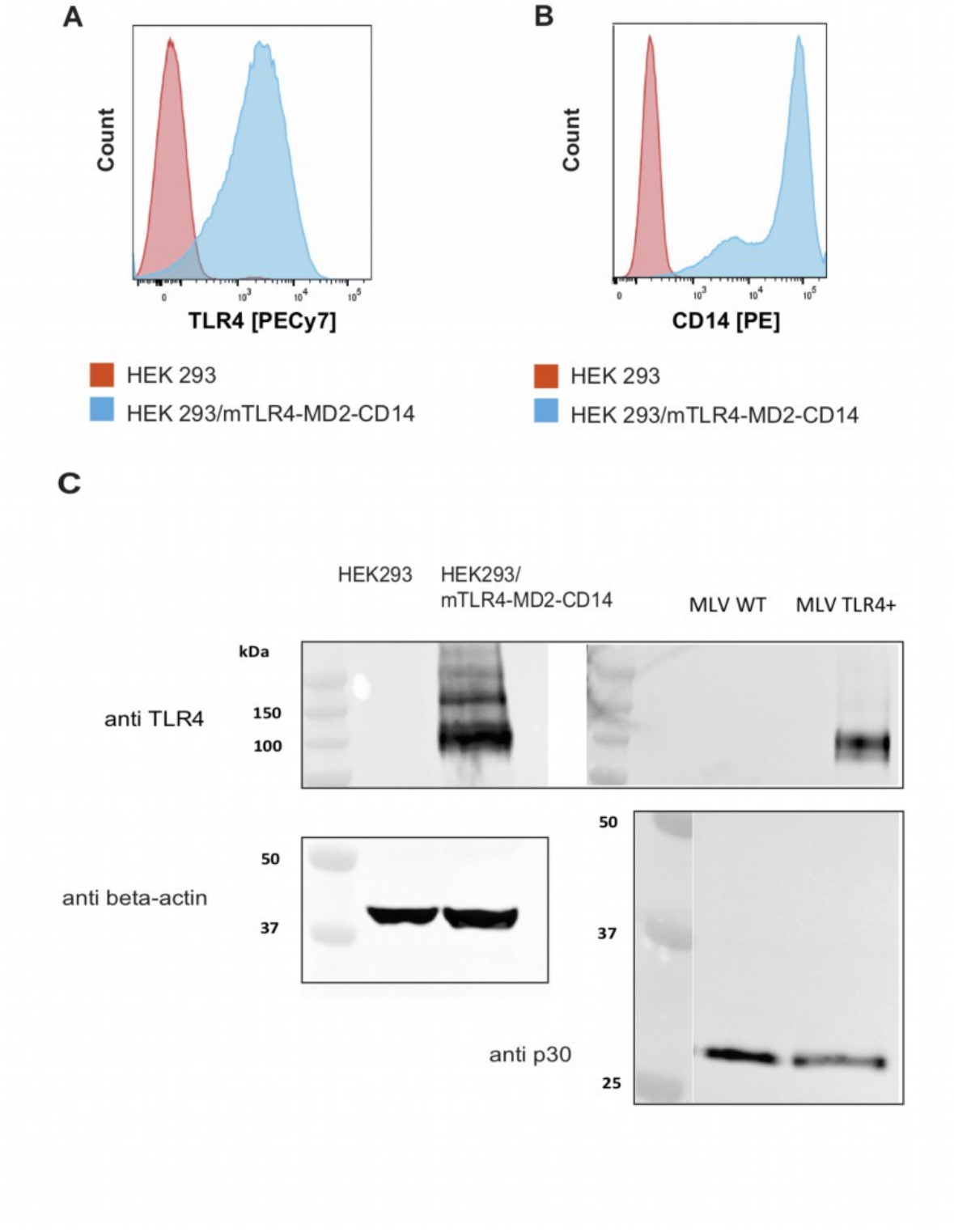


Figure 03: HEK293 cells were used for MLV production. After successful MLV production cells and viral particles were analyzed for mTLR4 incorporation. HEK293 cells and HEK293/mTLR4-MD2-CD14 cells were lysed, stained with extracellular antibodies against mTLR4 and CD14 and compared in their (**A**) mTLR4 and (**B**) CD14 surface expression via flow cytometry analysis. In the same way cells were analyzed for (**C**) mTLR4 expression with western blot analysis. HEK293 blots were normalized to beta-actin expression, which served as a loading control.

MLV or MLV TLR4+ in supernatant of both cell lines was concentrated according to protocol, lysed and analyzed for incorporation of (**C**) mTLR4 protein by western blot analysis. MLV blots were normalized to the presence of p30 capsid protein.

III.1.3 First LPS quantification tests show tendencies of MLV particles to bind LPS

Successful transmission and enhancement of MMTV and enteroviral infectivity is dependent on the transfer of LPS from bacterial cell walls to the viral envelope with the help of LPS binding proteins (LBP) and TLR4 by modulating gastrointestinal immunity. [37] To test, if MLV TLR4+ binds functional LPS with the help of incorporated TLR4 on its membrane, we performed a limulus amebocyte lysate (LAL) assay to quantify the amount of LPS bound to MLV particles. Concentrated MLV WT or MLV TLR4+ particles were incubated with 50 ng/ml Standard LPS, *E. coli* strain K12 and extensively washed before LPS was quantified. Remaining but functional LPS will activate a cascade of proteases in the reagent which leads to the activation of a pro-clotting enzyme. [75] After adding a yellow chromogenic substrate which is metabolized by the reagent the optical density at 405nm (OD405) can be measured. [76] The OD405 of the MLV TLR4+ group is 1.65 (mean, SD 0.10) and of the MLV group is 1.55 (mean, SD 0.04) (**Figure 04**). According to these results a difference in LPS binding ability of the two viruses can be suggested. However, speaking of 0.06 ng/ml (mean, SD 0.002 ng/ml) final LPS concentration in the MLV group vs. 0.07 ng/ml (mean, SD 0.005 ng/ml) LPS concentration in the MLV TLR4+ group calculated in a linear regression model has not a significant outcome. Due to a low specifity of the LAL assay in the OD range above 1.0, these test results must be taken into careful consideration. In addition, the LAL assay showed inconsistent results although specific procedures recommended for work with LPS have been applied.

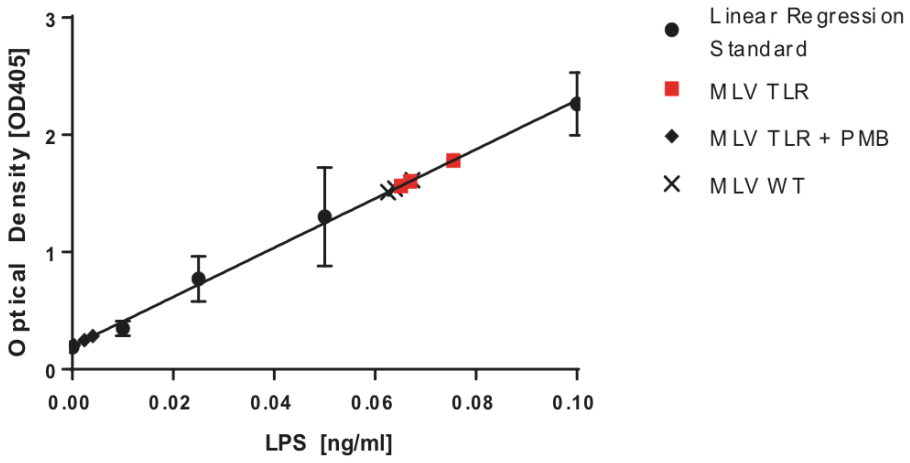


Figure 04: LPS binding and quantification assay of MLV and MLV TLR4+ incubated with 50 ng/ml LPS derived from *E.coli* K12. Samples (n=3) were analyzed with LAL assay by measurement of OD405 while error bars represent SD. All values were aligned to a linear regression model of the standard LPS titration to quantify LPS bound to viral surface. However, linear regression is only validated between OD405 values of 0 and 1. OD405 values above 1 only allow a calculated guess of LPS concentration.

III.1.4 Primary macrophages bind MLV particles fused with LPS

Since the LAL assay is not able to detect TLR4 complex-dependent binding of LPS to virus particles, a more specific approach was established. More sensitive imaging methods such as live cell imaging with a spinning-disk confocal microscope or flow cytometric analysis require fluorescent LPS for quantification. [59] However, single MLV with fluorescent LPS fused to their surface would fall under the resolution threshold of common flow cytometers. [77] Therefore, to enable quantification of viral LPS binding by flow cytometry we used primary CD169 positive murine macrophages to bind MLV on carrier cells. [9] To detect TLR4 complex-dependent binding of fluorescent LPS to the virus envelope, we had to establish a new protocol:

Using MLV with a fusion of the red fluorophore mScarlet-I to the capsid protein Gag allows virus particle detection by flow cytometry and confocal microscopy. Next, we incubate both MLV Gag-Scarlet-I or MLV TLR4+ Gag-Scarlet-I with 500 ng/ml of LPS conjugated with green fluorescent Alexa Fluor 488 (AF488) (Invitrogen) derived from *E. coli* strain 055:B5. Finally, we titrate MLV Gag-Scarlet-I or MLV TLR4+ Gag-Scarlet-I on primary macrophages and analyze both fluorescent emissions with a flow cytometer. Primary macrophages function as carrier cells for MLV and help us to

quantify and visualize LPS MLV interaction, due to their ability to bind MLV on their surface with the help of the molecule CD 169. [9] With the help of the confocal microscope, we can distinguish if the virus-LPS complex binds in total on the cell or if LPS molecules and viruses bind separately, which was not the case.

While in three independently conducted experiments only 0.006% (mean, SD 0.008%) of the macrophages cultured with MLV Gag-Scarlet-I and contrary to that 16.7% (mean, SD 9.2%) of the macrophages cultured with MLV TLR4+ Gag-Scarlet-I show an AF 488 signal representing the LPS load in the flow cytometry analysis, we can conclude a stronger LPS binding capacity of MLV TLR4 positive particles (**Figure 05 A&B**) and therefore prove the functionality of the TLR4 complex in MLV particles.

As a control, we check the total Scarlet-I signal representing the viral load on the macrophages to see if we titrated the similar number of viral particles. In the MLV group a total of 15.4% (mean, SD 23.0%) of the macrophages are binding to the virus and in the MLV TLR4+ group a total of 8.8% (mean, SD 10.0%) of the macrophages are binding to the virus (**Figure 05 A&C**).

Finally, taking both findings together, we can conclude that MLV TLR4+ is able to bind LPS particles to its surface while MLV can’t do so, when incubating the same number of viral particles with the same amount of LPS.

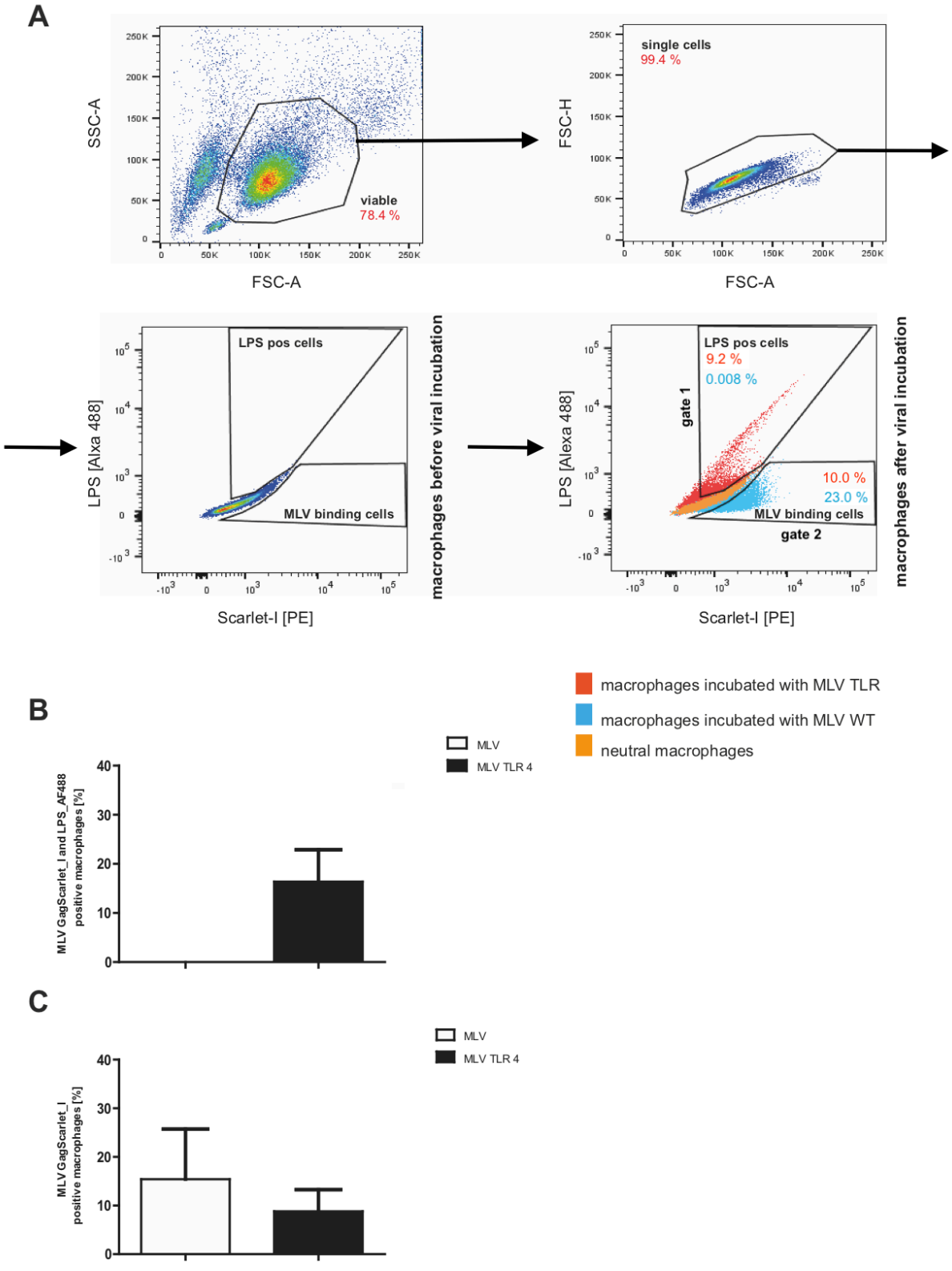


Figure 05: Primary CD169+ macrophages were collected by peritoneal washout. MLV GagScarlet_I or MLV GagScarlet_I TLR4+ were incubated with AF488 tagged LPS molecules. After virus concentration MLV was

incubated with primary murine macrophages. Now macrophages in each experimental group were analyzed by flow cytometry for LPS signal (AF488) and MLV signal (PE). Cell populations were gated as shown in (**A**), while gate 1 represent LPS and MLV positive macrophages and gate 2 represent MLV positive but LPS negative macrophages. MLV negative macrophages were used as negative control. Now, mean of flow cytometric readout in the different experimental groups was graphically depicted, while gate 1 is represented by graph (**B**) and gate 2 by graph (**C**). Error bars represent SD; n=3.

III.1.5 Direct stimulation is significantly increasing MLV infectivity *in vitro*

One way of MMTV increasing its infectivity is binding LPS molecules on its surface and triggering murine T cells to secrete the immunosuppressive cytokine IL-10, which inhibits immunological defense against the virus. [28,37] Especially MMTV-bound LPS leads to a stronger LPS-induced TLR4 activation and subsequently results in a higher IL-10 secretion level than virus-free LPS. [30,37] MLV requires mainly an activating milieu, that stimulates its host cells, to successfully establish infection. [6] To test if LPS-binding to the virus envelope by LBP influences infectivity at the cellular level, we compare direct (virus-bound LPS) and indirect (virus-free LPS) stimulation of MLV infection in the S49.1 T lymphoma cell line and in primary cells (**Figure 06**) *in vitro*. We incubate equal amounts of infectious MLV and MLV TLR4+ for an hour on 4°C with different amounts (50 ng/ml and 500 ng/ml) of LPS, *E. coli* strain K12 for direct enhancement and then purified them (**Figure 01**). To test indirect enhancement, we have to apply a higher concentration (1 µg/ml, 10 µg/ml and 30 µg/ml) of LPS, *E. coli* strain K12 directly to the cells (**Figure 01**), accordingly to already established protocols to achieve B1 cell activation. [7] Around 48h after virus addition under direct enhancement conditions we can show a difference in B1-cell infectivity between the two groups of up to 6.03% (mean, SD 4.73%) in the MLV TLR4+ group vs. 1.06% (mean, SD 0.46%) in the MLV group (**Figure 06**). However, the other tested primary lymphocytes like Th17 and Treg showed no increased infection rates and remain on a low infection level (**Figure 07**). Interestingly, a different TLR4 agonistic LPS isolated from the pathogenic *E. coli* strain 055:B5 showed with a difference in infectivity rate of 1% a less stimulatory potency in direct enhancement compared to LPS isolated from the commensal *E. coli* strain K12 with a difference in infectivity rate of 6% (**Figure 08**).

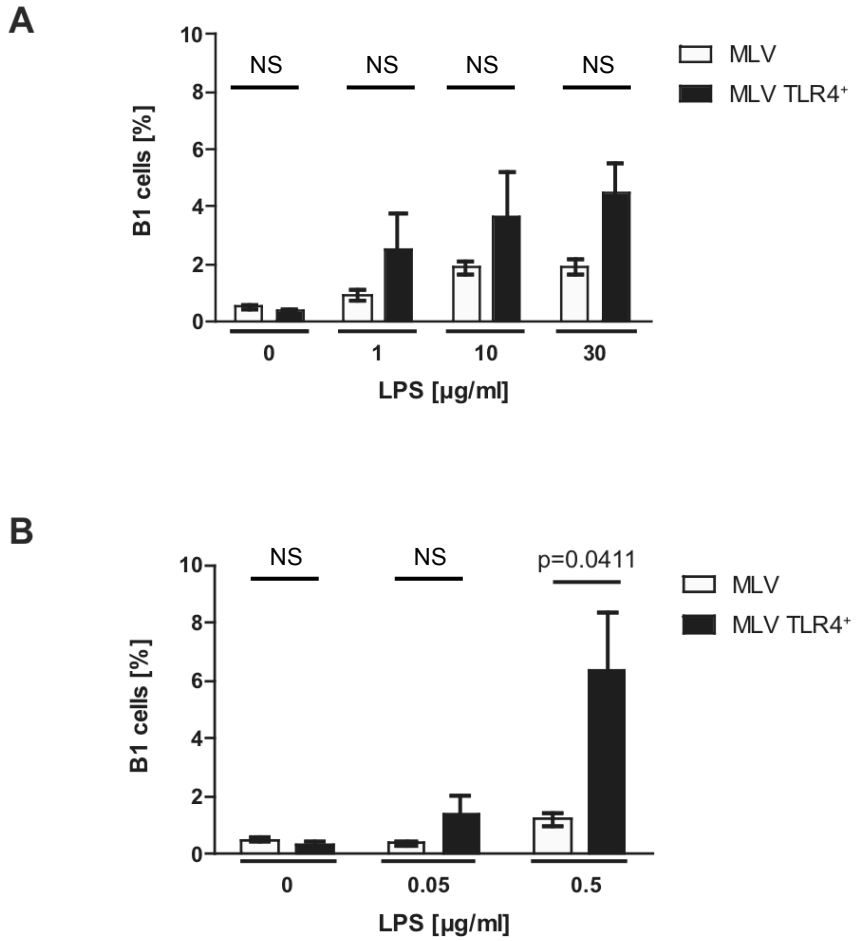


Figure 06: Equal amounts of MLV and MLV TLR4+ were incubated with primary B1 cells. Indirect stimulation (**A**) is simulated with separate titration of LPS to the cell suspension, while direct stimulation (**B**) is simulated via preincubation of viral particles with LPS before suspending them with the cells. Virus titer was normalized to titration on S49.1 cells and diluted for each viral stock, respectively. Samples (n=6) were analyzed for GFP expression in MLV positive B1 cells with the flow cytometer while error bars represent SD of samples. Mann-Whitney U test was used to calculate p-value. NS = Not Significant

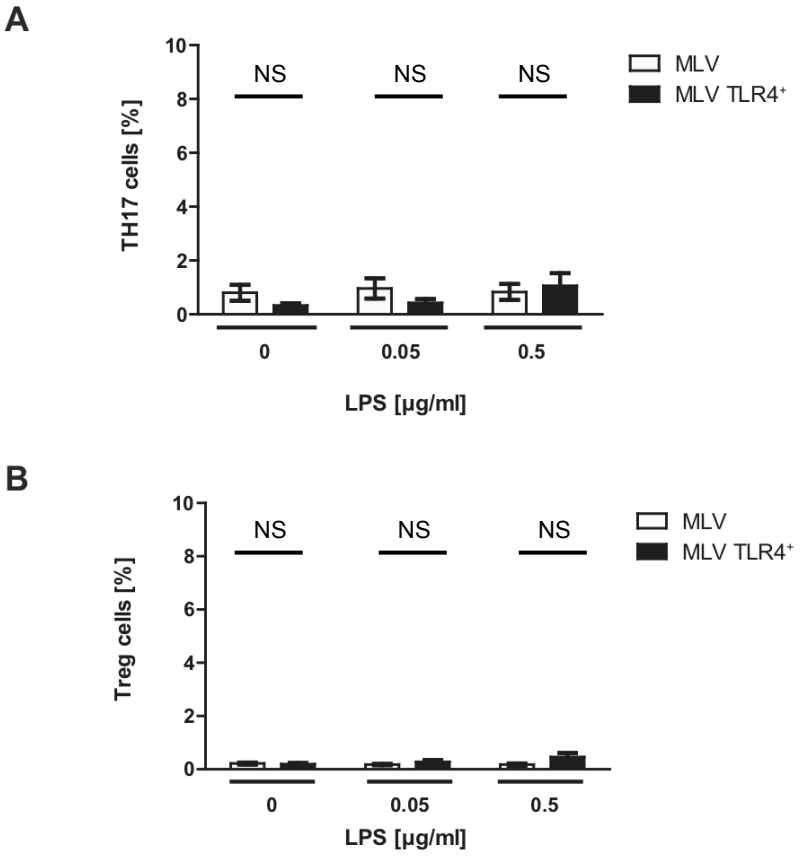


Figure 07: Equal amounts of MLV and MLV TLR4+ were incubated with primary Th17 (**A**) and Treg (**B**) cells. Both graphs represent direct stimulation experiments which were implemented by preincubation of viral particles with LPS before titrating them to the cells. Virus titer was normalized to titration on S49.1 cells and diluted for each viral stock, respectively. Samples (n=5) were analyzed for GFP expression in MLV positive lymphocytes with the flow cytometer while error bars represent SD of samples. NS = Not Significant

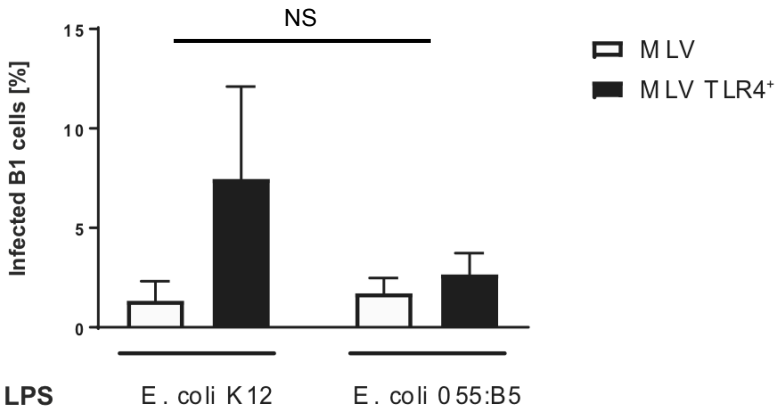


Figure 08: Equal amounts of MLV and MLV TLR4+ were incubated with primary B1 cells. Direct stimulation of 0.5 µg/ml LPS derived from *E. coli* K12 was compared to direct stimulation of 0.5 µg/ml LPS derived from *E. coli* 055:B5. Virus titer was normalized to titration on S49.1 cells and diluted for each viral stock, respectively.

Samples (n=3-5) were analyzed for GFP expression in MLV positive B1 cells with the flow cytometer while error bars represent SD of samples. NS = Not Significant

III.1.6 For successful direct enhancement of infection an TLR4 expression in host cells is obligatory

Since we showed that murine CD4+ T cells are less sensitive to direct and indirect LPS stimulation of MLV infection (see **III.1.5**), we analyzed and compared host cells especially B1, Th17 and Treg cells for the surface expression of LBP.

In this experiment we want to find out if the expression of TLR4 on MLV host cells correlates with successful direct and indirect LPS stimulation in these cells. We can observe that MLV TLR+ infection correlates with TLR4 expression in host cells. While B1 cells show a TLR4 positive signal (**Figure 09 A**), the other cell types such as Th17 (**Figure 09 B**) and Tregs (**Figure 09 C**) do not. Those results lead to a different response on LPS stimuli, which can be seen indirectly in our titration experiments testing MLV infectivity in primary lymphocytes (**Figure 06** and **Figure 07**). While B1 cells strongly react to an increasing concentration of LPS, Th17 cells and Treg cells have almost no response to different amounts of LPS.

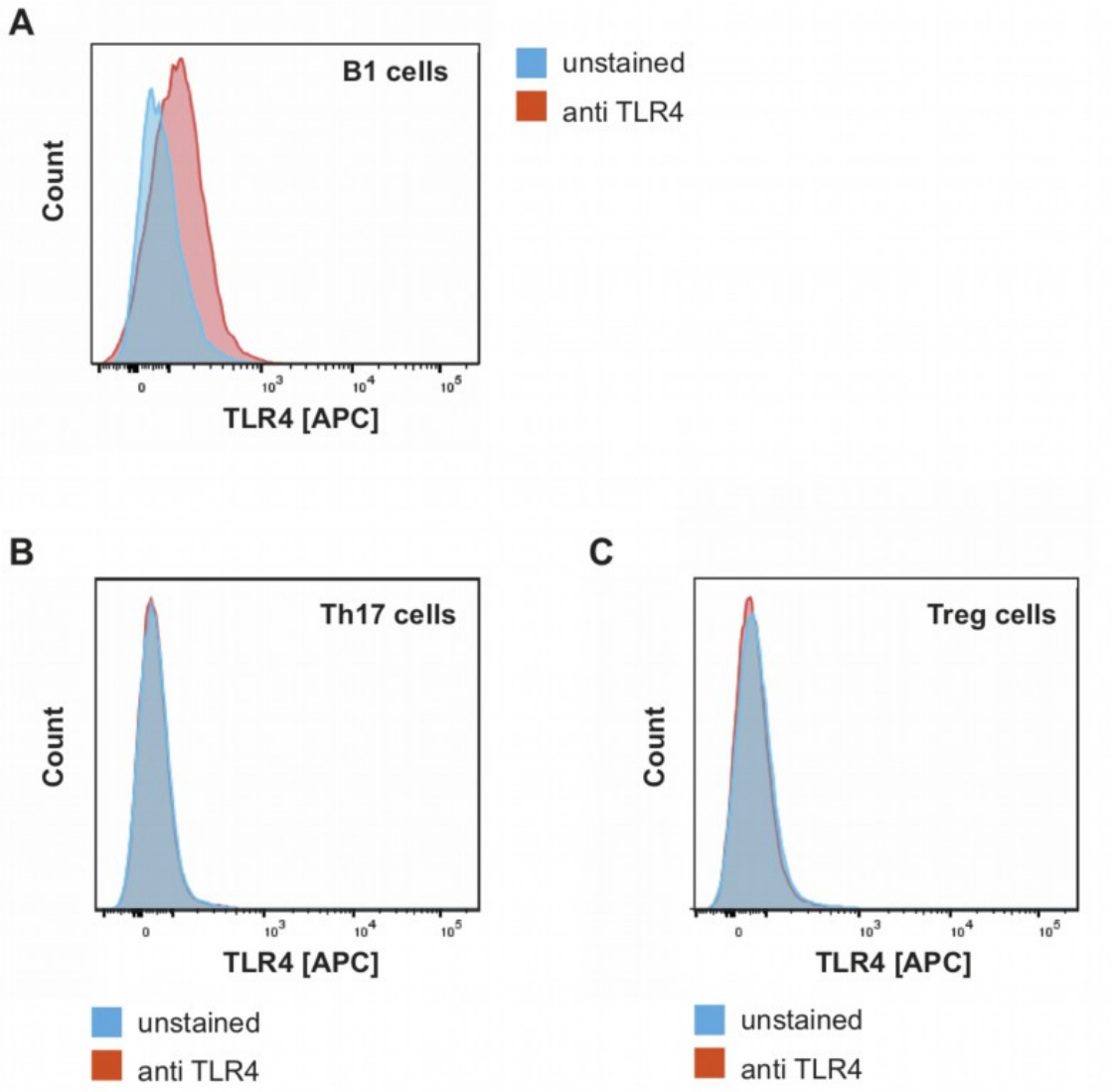


Figure 09: Surface TLR4 expression was analyzed on primary isolated B1 cells (**A**) and on *in vitro* differentiated Th17 (**B**) and Treg cells (**C**). Unstained population of the corresponding cell type was used as negative control. Samples (n=1) were analyzed by flow cytometry.

III.1.7 TLR4 and LPS interaction is obligatory for successful infection

Our previous experiments indicate an LPS-dependent stimulation of MLV infection in murine CD19+ and CD4+ lymphocytes which express TLR4 on their surface. However, to test if MLV infectivity is dependent on the stimulatory potency of functional LPS bound to the viral surface, we had to find an LPS antagonist and add it to the cells before incubating them with the virus. Polymyxin B (PMB) is a cationic,

polypeptide antibiotic drug that directly inhibits various biological effects of LPS by blocking the lipid A region of LPS molecules and subsequently neutralizing the interaction of LPS with TLR4. [78] We use a non-toxic concentration of 10 µg/ml of PMB for our experiments. By blocking the interaction of virus-bound LPS to B1 cells during incubation, we can observe a stable cell viability but a decrease of infectivity in the MLV TLR4+ group from 4.7% (mean, SD 3.0%) to 1.0% (mean, SD 0.3%) while the MLV group stays nearly the same with a decrease from 1.3% (mean, SD 0.8%) to 0.5% (mean, SD 0.4%) (**Figure 10**).

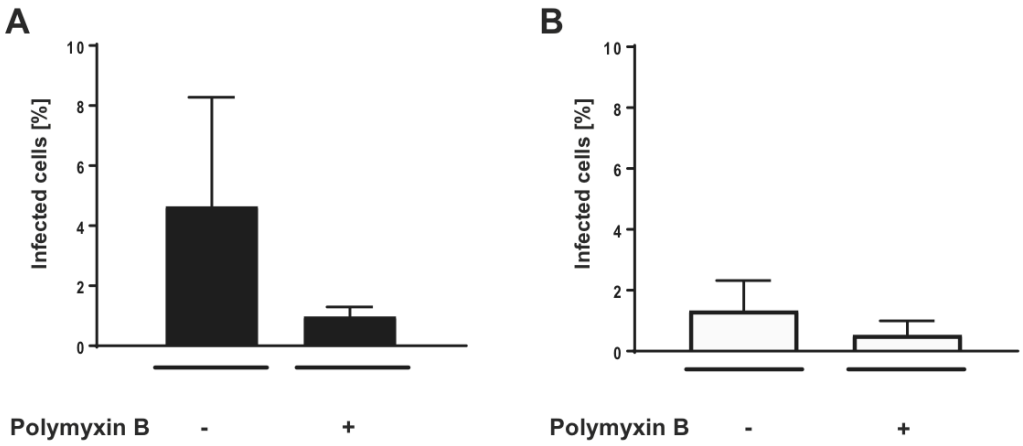


Figure 10: Equal amounts of MLV TLR4+ (**A**) and MLV (**B**) were titrated on primary B1 cells. Direct stimulation of 0.5 µg/ml LPS derived from *E. coli* K12 was blocked with 10µg/ml PMB directly titrated to the cell suspension right after virus titration. Virus titer was normalized to titration on S49.1 cells and diluted for each viral stock, respectively. Samples (n=3) were analyzed for mean GFP expression in MLV positive B1 cells with the flow cytometer while error bars represent the SD of samples.

III.2 Bioluminescence *in vivo* imaging

To gain more information about MLV infectivity *in vivo* and specially to compare MLV with MLV TLR+, we have to apply an already established technique for *in vivo* imaging in our experiments. Bioluminescence *in vivo* imaging (BLI) allows us to detect real time cellular processes, such as retroviral infection, in the living animal based on luciferase gene reporters interacting with their substrates, which leads to photon emission. [79]

However, deep tissue imaging is limited due to high light absorption within the tissue while photons penetrating the body and the lack of reporter enzymes with near-infrared emitting systems, which are known to have the optimal wave lengths for deep tissue imaging. [61,79]

III.2.1 Comparing near-infrared bioluminescence reporters and their substrates for deep tissue imaging

While visible light that penetrates living organisms and tissues is mainly absorbed by hemoglobin (λ=415-577nm) and melanin (λ<600nm), red-shifted (λ>600nm) bioluminescence imaging systems, like AkaLuc and Antares with their substrates Aka Lumine HCl and Furimazine, have an improved detection sensitivity of targets in deep tissue. [80,66,67] In order to be able to visualize infected tissue and to study virus dissemination within the host, the MLV genome was modified with different gene sequences coding for luciferases to detect MLV infected cells. All infected cells showed a luminescent signal in a different light spectrum 24 h after infection. To find out which of our luciferases has the best light emission in MLV infected cells especially in the far-red spectrum (λ>600nm), we titrate MLV *in vitro* on S49.1 T lymphoma cells and measure light emission in relative light units (RLU) 24 h post infection (**Figure 11**). By comparing the emission of the infected cells in the different spectral frames, we are able to analyze the power of intracellular luciferase reactions. Extremely important are proteins, which have a increasement of luminescent intensity such as AkaLuc with a measured RLU of 91.4 (mean, SD 30) in the beginning at 470nm (**Figure 11 A**) towards a RLU of 2643 (mean, SD 674) at 660nm (**Figure 11 C**) in their plateauing phase at 10µl conc. MLV, which equals 10^5 infected cells. Other proteins, however, are decreasing in intensity such as nano Luc (nLuc) with a RLU of 75604 (mean, SD 14780) at 470 nm (**Figure 11 A**) and a RLU of only 404 (mean, SD 72) at 660 nm (**Figure 11 C**) in their plateauing phase at 10µl conc. MLV. Moreover, a strong representative of the 550nm group (**Figure 11 B**) is the molecule Antares

with a RLU of 48806 (mean, SD 9424). Therefore, it is also taken into further *in vivo* investigations together with AkaLuc and GFP-P2A-nLuc.

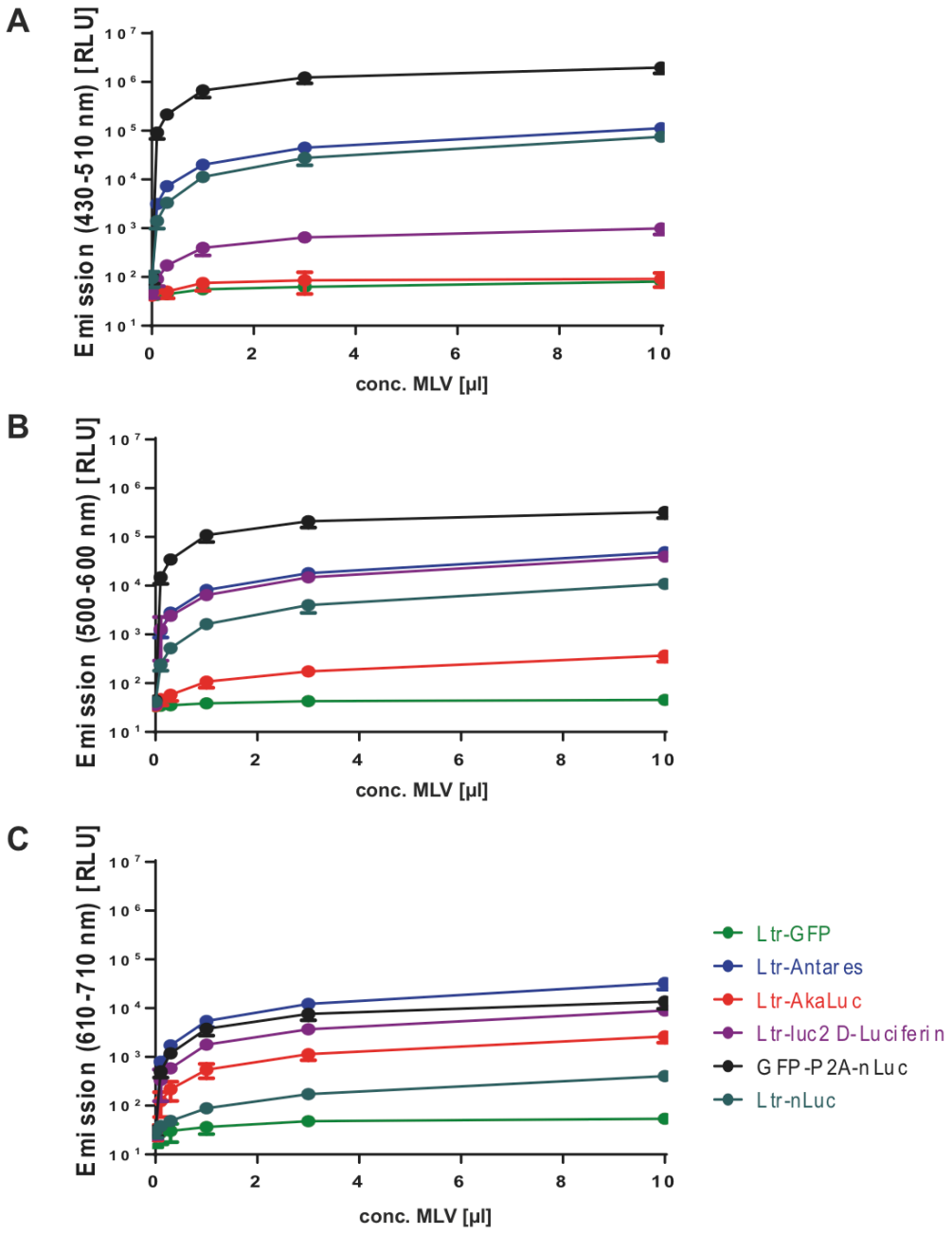


Figure 11: Concentrated luciferase-reporter MLV was titrated on S49.1 cells *in vitro*. Luciferase emission of MLV infected cells was detected in the blue light spectrum (**A**), the green/yellow light spectrum (**B**) and the far-red light spectrum (**C**). Mean RLU of samples (n=5) was measured by a plate reader while error bars represent the SD of samples.

III.2.2 AkaLuc is visualizing MLV infected lymphocytes in living C57/BL6 mice

While our previous *in vitro* experiments showed good results in near-infrared emitting systems, we still have to test our modified luciferase-reporter MLVs in their ability of emitting detectable signals under realistic conditions in living animals. In order to test the previously selected luciferases in their emission intensities *in vivo*, injection of MLV was performed according to already established protocols in the hind footpads of C57/BL6 mice.[9] In this first experiment, the brightest signal for detection of all tested luciferases has the combination of the luciferase AkaLuc together with the substrate Akalumine-HCl represented by region of interest 1 (ROI 1) (**Figure 12**). The other two reporters (ROI 2 and ROI 3) are not able to show a relevant higher signal than the background (ROI 4 and ROI 5). This might be due to a reduced cell permeability of the shared substrate furimazine. Consequently, we decide to modify our AkaLuc reporter plasmid with a green fluorescent protein (GFP) to be able to investigate single cells we have harvested from infected murine tissue. For that, we cloned a self-cleaving peptide (P2A) between the AkaLuc sequence and the GFP sequence to achieve an expression of both, a luciferase and a fluorophore, in infected cells.

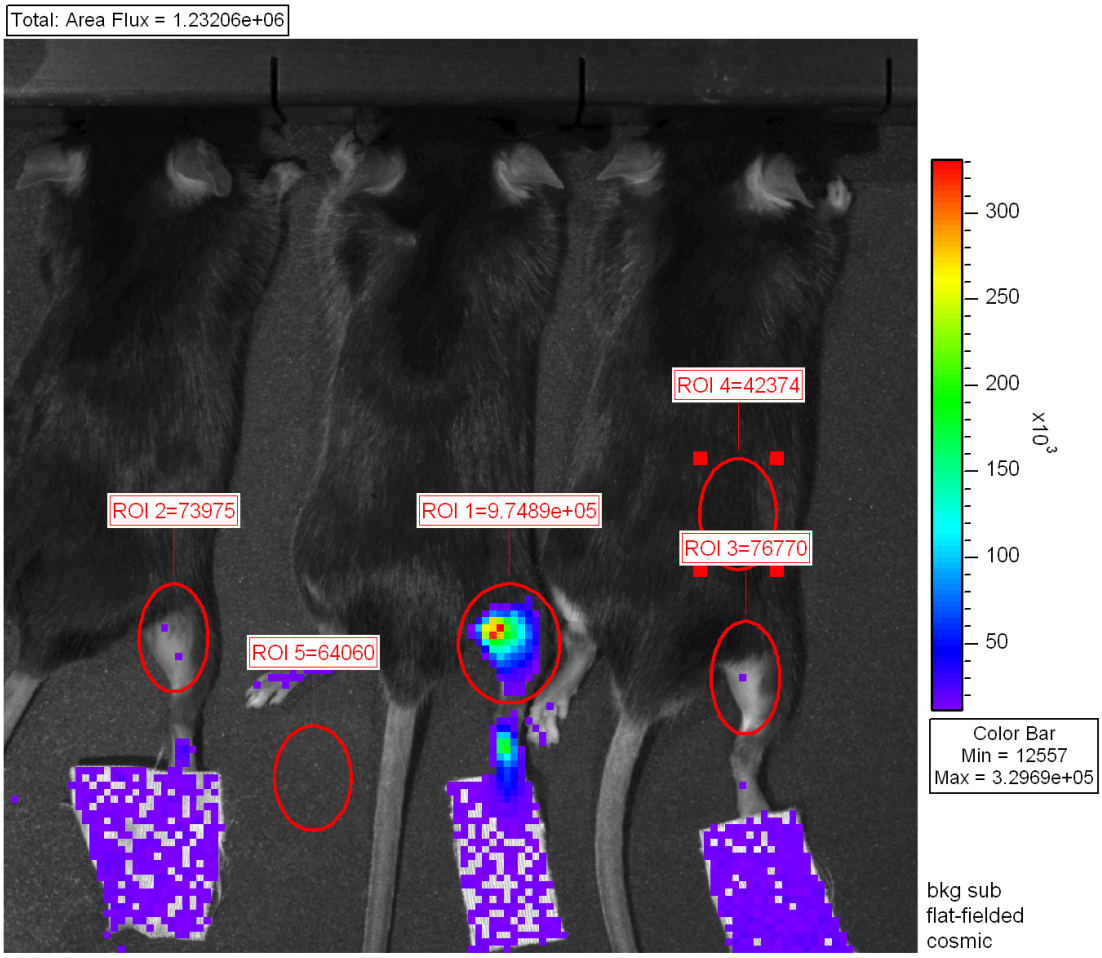


Figure 12: 20µl of concentrated luciferase-reporter MLV was injected into the right dorsal footpads of three C57/BL6 mice. After 48 h and intraperitoneal injection of the luciferin, *in vivo* bioluminescence of infected lymphocytes located in the popliteal lymph node was detected with a camera. Emissions of GFP-P2A-nLuc construct (**ROI 2**), AkaLuc (**ROI 1**) and Antares (**ROI 3**) were specified in RLU and compared to the background signal from murine fur (**ROI 4**) and the chamber (**ROI 5**)

IV Discussion

Recent reports shed some light on the mechanism of how viruses establish infection through oral transmission. [10] Strikingly, microbiota-derived LPS was identified as one of the critical factors for successful vertical viral transmission and establishing infection.[30,33,36,37]

Here, we have investigated LPS dependent stimulation of MLV infection, which might occur in vertical MLV transmission. Compared to the wild type, MLV with LBPs incorporated into its envelope significantly increases infectivity after LPS preincubation. This mechanism occurs especially in TLR4 expressing murine B1 cells, which leads us to conclude that a functional TLR4 dependent pathway in host cells is necessary for direct and indirect stimulation of MLV infection. TLR4 activation in host cells might lead to solid intracellular activation and proliferation signals. [30] LPS acquired from the non-pathogenic commensal *E. coli* K12 can potentiate direct stimulation effects in MLV TLR4+ infection, while LPS from pathogenic *E. coli* 055:B5 showed no difference to wild type infectivity. Moreover, antagonizing LPS by blocking the functional Lipid A region with PMB leads to the same reduction of infection in both cohorts.

By using LPS tagged with fluorescent AF 488, we were able to quantify and visualize LPS binding on the viral surface. Quantification of virus-bound LPS was necessary to compare the LPS binding ability of MLV WT with MLV TLR4+ and confirmed the assumption that the WT virus has no LBPs incorporated in its surface and therefore no ability to bind LPS like MLV with TLR4. Furthermore, this experiment verifies our MLV production protocol by transfecting different HEK293 cells.

For our experiments, we mainly used cell populations that might play a role in vertical transmission. Therefore, we investigated *in vitro* differentiated intestinal Th17, Treg and B1 cells since these lymphocytes are identified as hosts of MLV infection in horizontal routes of infection. [9] Also, vertical transmission experiments of MLV WT revealed that CD19+ B cells and CD4+ T cells, located in the mesenteric sac and Peyer's patches, make up the majority of MLV WT infected cells. [10]

However, the mechanistic details of how the MLV binds LPS and establishes direct stimulation of infection *in vivo* have still to be elucidated. Nevertheless, our microscopic and flow cytometric data suggest that commensal LPS attached to the virus's surface plays a key role in enhancing MLV infectivity.

We also developed and initially tested a luciferase and GFP encoding dual reporter MLV. After subcutaneous injection of reporter virus into C57BL/6 mice, the infection could be detected via bioluminescence at popliteal lymph nodes. Single cells from infected regions can be analyzed for GFP expression in analogy to *in vitro* experiments. Using these dual reporter plasmids for MLV or MLV TLR4+ production for further experiments, we might be able to demonstrate direct and indirect stimulation *in vivo*.

Virus production in artificially modified cell lines ensure stable mTLR4 expression patterns

There is evidence that enveloped viruses are acquiring host proteins during their budding process from the cell. [81] Some retroviruses are able to perform a selection process by degradation or enrichment of host proteins during viral intracellular maturation to incorporate them into their envelope. [82] In how far this incorporation of surface receptors is linked to virus replication, transmission and pathogenesis was shown in studies of HIV-1, that incorporates the major histocompatibility complex (MHC) class II receptor or cyclophilin A in its membrane or capsid to accelerate infection of human T lymphocytes. [83,84] Of course, studying HIV infectivity *in vivo* is limited due to its human-specific tropism, but fundamental principles of retroviral immune recognition can also be transferred on murine models. [85]

Wilks et al. already described the occurrence of viral particles with murine TLR4 in mice. In some MMTV particles isolated from the milk of infected females, they discovered the LPS binding protein TLR4 incorporated into the viral membrane. Virions with TLR4 incorporated into their membrane trigger an increased production of IL-6, which causes Treg cells to secrete immunosuppressive IL-10. High IL-10 levels helped MMTV to evade immunodetection and establish infection. [30] The examined viruses were supposed to gain these receptors during the budding process of their host cells. [30]

In our experimental approach, MLV with mTLR4 incorporated in its envelope hijacks proteins from mammalian MLV producer cell lines. Two different virus types were produced by transfection of HEK293 cell lines positive for the mTLR4 complex and WT HEK293 cell lines with the same transfection protocol. One virus type that is positive for mTLR4 and one that is negative for the receptor. Overall, our results showed that MLV TLR4+ could bind LPS from commensal *E. coli* K12 to its surface

and lead to a 60-fold (0.5 µg/ml vs 30 µg/ml LPS concentration) enhancement of LPS stimulation effects in MLV infection. This enhancement effect might be explained because LPS aggregates more than monomers represent the activating unit of TLR4. TLR4 incorporated into MLV membrane may lead to aggregation and intercalation of LPS on its membrane, and direct stimulation effects are observable. [86]

Fluorescent LPS allows a more specific approach to detect LPS-virus interactions

Since LPS is a highly specific mitogen for murine B cells and an immunostimulatory reagent [87], this molecule's detection and quantitative analysis is necessary for our experiments and allows us to demonstrate its role in infection. However, limitations of the LAL assay are a common phenomenon and occur in different settings. First, the interference of the LAL assay with different substances makes it difficult to distinguish between actual LPS and false-positive results due to other nanomaterials, especially in biological reagents. [88] Secondly, the LAL assay is a qualitative LPS detection tool than a quantitative method. Therefore, it is mainly used in detecting LPS contaminations in different industrial sectors and not for diagnostic purposes. [89]

However, we found a powerful method to detect and quantify MLV-LPS interaction. We used primary macrophages because they were shown to capture and bind MLV particles via the CD169 molecule but do not present LPS on their cellular surface in our experiments. [9] Especially mTLR4 cell surface expression on peritoneal acquired murine macrophages is initially deficient (data not shown) and even declines during maturation of the immune system. [90] With this knowledge, we designed an assay to quantify virally bound LPS without testing unavoidable free LPS in the pulldown. After all, we were able to show that MLV TLR4+ particles could bind functional LPS on their surface to enhance infection. Contrary, the LAL assay might have shown more likely false-positive results in the MLV WT cohort due to free LPS in the pulldown. Our new assay also proved the contamination with free LPS in the LAL assay since the MLV WT cohort showed no LPS signal in flow cytometry.

Besides quantifying virus-bound LPS, we might also visualize MLV-LPS interaction using high-resolution confocal microscopy. Further experiments will show if fluorescent but functional LPS bound to MLV particles enhances virus attachment to B1 cells and increases MLV stability. The verification of LPS dependent

enhancement of MLV stability would confirm parallels to reoviruses such as Polio which increases host cell association by binding bacterial products. [36]

First experiments with the generated reporter viruses show promising results in deep tissue BLI

To test *in vivo* bioluminescent intensity of our reporter viruses, we administered the viral particles into the footpads of C57/BL6 mice. [9] Although we only monitored the luminescence of infected cells in the popliteal lymph node, the measured intensity should be comparable to the signal emitted from infected cells throughout the body. The emitted light is shifted to wavelengths above 600 nm, which do not get absorbed by melanin or haemoglobin and penetrates most mammalian tissues equally. [61] Especially the Aka luciferase, which is classified on its excellent enzyme kinetic (low K_m value) and the high cell-membrane permeability of the substrate Aka Lumine HCl, has a distinct advantage for BLI over the commonly used firefly luciferase with its substrates D-luciferin and CycLuc1. [80] The superior signal-to-noise ratio due to the absence of intrinsic bioluminescence background and the far-red emission spectrum of AkaLuc will allow us to monitor MLV infection in deep tissues in the same way we monitor the popliteal lymph nodes. [61]

Surface bound LPS has a strong mitogenic signal on MLV host cells which is also dependent of its molecular structure

For sufficient cell stimulation and a MyD88 dependent upregulation of proinflammatory and stimulatory cytokines in target cells the expression of pattern recognition receptors is compulsory. [91] Especially B cells proliferate and increase their surface receptor expression in response to LPS. [92,93]

The observed infection-promoting function of LPS bound by TLR4 integrated into the viral surface was different to that seen in MMTV virions. [37] While MMTV is exploiting the immunosuppressing function of endotoxins derived from commensal microbiome by activation of Treg cells to produce the immunosuppressing IL-10 in the host, MLV may increase the stimulation of target cells, especially B1 cells by surface-bound LPS.[30]

However, LPS derived from the most frequent commensal gram-negative bacteria within the gastrointestinal tract are weak TLR4 agonists. [94] Nevertheless, our indirect and direct stimulation data and recent publications about other enveloped viruses

indicate that virus bound LPS has an increased stimulatory potency than free LPS. [30,86] Since host cells are also equipped with the TLR4 receptor, there are two options to be taken into consideration. One option is that the virus and the stimulatory agent (aggregated endotoxins on the viral surface) might strengthen TLR4 activation, and fusion of virus and host cell happens shortly after stimulation. Especially the presentation of aggregated LPS on the viral surface was shown to have a more substantial activation effect on TLR4 and may result in having a greater effective concentration than the free monomeric version of the endotoxin. [86] The other option is that virus particles might form a prolonged and more stable interaction with TLR4 of the target cell with the help of accumulated LPS on the viral surface and trigger inflammatory endocytosis induced by CD14 and TLR4 like it is already described with other pathogens. [95,96]

Moreover, different stimulation patterns of LPS can be explained by the high structural diversity of this endotoxin. Commensal and pathogenic bacteria are equipped with different LPS molecules and have, therefore, either a stimulatory or a suppressing effect on cells of adaptive immunity. [45] While B1 cells may be stimulated by LPS derived from the commensal bacterium *E. coli* K12 leading to an increased MLV infectivity, Treg cells did not show any increment of infectivity after being exposed to virally bound LPS or free LPS in our study. Hence, it would be interesting to determine if Treg cells will secrete the immunosuppressing cytokine IL-10 when facing LPS molecules from *E. coli* K12 and if Treg cells will suppress stimulation when co-cultivated with B1 cells.

Interestingly, the other tested LPS derived from pathogenic *E. coli* 055:B5 showed a less stimulatory effect on MLV infectivity. At first sight, these results might be confusing because *E. coli* K12 is a non-pathogenic strain with a higher stimulation effect in infectivity than pathogenic strains. However, S. Bereswill et al. investigated the pro-inflammatory potential of *E. coli* K12 in a murine model of chronic bowel disease triggered by TLR4 dependent pathways. [97] Apart from that, both LPS molecules are considered solid TLR4 agonists, suggesting that MLV can selectively intensify the immunostimulatory potencies of commensal LPS as already shown in MMTV models. [30]

Biodistribution of the substrate might play an important role in *in vivo* BLI

Interestingly, the observed *in vivo* signal of the tested luciferases was highly dependent on the substrate and differed from the *in vitro* measured intensity. While not every luciferase was able to metabolize the whole setup of luciferins, the carrier substance and the recommended solvent of the luciferin played an essential role in how efficient the metabolism of the substrate was. We discovered that acidic carrier substances like sialic acid diluted in DMSO were a more potent carrier than neutral or alkaline carrier substances diluted in H2O or PBS. Better carrier effects might be explained by a higher membrane permeability of acidic substances and DMSO in vital cells. Hydrophobic membranes coat the cytoplasm to protect cell organelles and maintain the membrane potential created by the cells to enable the transmembrane transport of different substances. Especially DMSO allows substances to cross this border and strengthens their intracellular bioavailability, which is important for our studies to increase luciferase signal to a maximum without lysing our target cells. [98]

There is also a recent publication in which the importance of the solubility and bioavailability of the used luciferin for *in vivo* imaging studies is emphasized. Especially the luciferase substrate furimazine, which is metabolised by nanoLuc and Antares, is claimed for not fitting the demands of *in vivo* BLI. However, to use those highly delicate luciferases for *in vivo* experiments to track tumors and visualize single lymphocytes, the substrates hydrofurimazine and fluorofurimazine with a better bioavailability were designed. [99]

With those additional substrates, the Antares reporter, besides the GFP-P2A-AkaLuc reporter, would be another potent tool to visualize *in vivo* infection of MLV. To analyse and quantify Antares-MLV infected single cells isolated from the gut with the flow cytometer, the fluorophore CyOFP1, which is part of the Antares molecule, may help since it is excitable by cyan light.[66]

Outlook and transfer on vertical transmission of human pathogenic retroviruses

It is still not recommended for HIV positive women to breastfeed their children since up to 40% of mother-to-child transmissions are related to breastfeeding. [100] However, in high-income countries, the mother to child transfection of HIV decreased from about 25 per cent to 1 to 2 per cent due to reduced free virus load in reasons of

effective antiretroviral therapy during pregnancy, elective caesarean sections and recommended formula feeding. [101] Our study and many more demonstrate that different viruses benefit from the LPS they have acquired from commensal microbiome to promote transfection.

They either activate their host cells to proliferate and create an inflammatory milieu or suppress the host's immune system and achieve an evasion of immunodetection. [33] While HIV envelope proteins are already well described in their role of viral fusion and entry, much less literature analyses the role of foreign proteins acquired from the host cells and displayed on the external viral membrane. Therefore, it makes sense to check HIV transmission for interference with the TLR4 pathway of its target cells in analogy to our experiments with MLV and find out if there are parallels to our findings. [102] Especially the background, that HIV hijacks the protein machinery of host cells to control the expression of transmembrane proteins selectively makes further investigations in this field of retroviral transmission interesting. [82]

Immunomodulatory drugs, therapeutic monoclonal antibodies targeting the TLR4 pathway or antibiotic drugs may play an influential role in preventing vertical viral transmission when a correlation of LPS stimulation and viral infection rates can be assumed. Especially LPS dependent activation of TLR4 pathway and the following cytokine release responses in newborn mammalians result in a more substantial lack of blocking agents like monoclonal antibodies against TLR4 and leads to a higher inflammatory response than the adult TLR4 pathway, which emphasizes the sensitivity of the TLR4 pathway in neonates. [103] However, early therapy for at least the first three months of life would be necessary to reduce the high mortality of HIV infected infants. [100] Thinking of the severe side effects of inconsiderate use of antibiotic therapy, we also disagree with the *in vivo* use of antibiotics only to prevent retroviral disease in humans.

More effective prevention of vertical transmission of HIV might be the suppression of virus secretion in the maternal milk by regular intake of antiretroviral therapy and the strict monitoring of maternal viral load. [104] Also, postnatal antiretroviral prophylaxis of zidovudine, a reverse transcriptase inhibitor, lowers the risk of acquiring latent reservoirs of HIV proviruses in CD4+ peripheral-blood mononuclear cells (PBMCs). [100]

Limitations of the current study

Our study has some limitations within which our findings need to be interpreted carefully. Some limitations of this study should be mentioned.

First, using artificial producer cell lines with stable overexpression of TLR4, a single virus produced in the tissue culture might acquire more likely TLR4 molecules on its surface than viruses assembled in primary host cells with a lower TLR4 density. A better and more specific approach is isolating viral particles from the milk of breastfeeding females or the tummies of suckling pups and using an electron microscope for their surface analysis or using those isolated particles for *in vitro* administration on primary lymphocytes. [30]

Further, the research presented here was limited by only generating *in vitro* results specific for MLV infectivity, which cannot be simply transferred on the living organism and probably distinguish from other retroviruses. However, breaking down the MLV replication cycle on critical steps, very profound mechanistic details may appear, making the transfer on or the investigation of another retroviral genus easier. [8] Furthermore, conceiving a dual reporter virus will allow progress in already conducted *in vivo* experiments of vertical MLV transmission. [10]

Conclusion

Retroviruses such as Human Immunodeficiency Virus (HIV) and Human T-Lymphotropic Virus (HTLV), are major pathogens in mammals that can cause life-threatening diseases, including immunodeficiencies and tumors. Retrovirus infections can be treated with drug combinations against different viral targets. The fact that preventive vaccines are not available, antiviral therapies are often associated with side effects and a cure for retrovirus infection is not within reach emphasizes the importance of infection control through other forms of prevention. In this context, unveiling novel mechanisms by which retroviruses establish infection of the host is fundamental to developing innovative strategies against retroviral infection.

Therefore, this study aimed to examine fundamental principles by which retroviruses establish infection in primary host cells. To this end, we performed functional infection studies with the mouse retrovirus murine leukemia virus (MLV). Surprisingly, we find that MLV infection can be markedly enhanced by the interaction with commensal lipopolysaccharide (LPS). Especially MLV that incorporates the mammalian LPS-binding Toll-like receptor (TLR4) into its envelope during the budding process from the productively infected host cell benefits from virus-bound LPS to increase particle infectivity. We established a powerful assay with primary macrophages to detect and quantify the MLV-LPS interaction and constructed a novel reporter virus for these *ex vivo* studies that may also enable deep tissue imaging *in vivo*. We demonstrate that virus-bound LPS aggregates presented by the virus-exposed TLR4 to LPS-sensitive host cells resulted in higher infection rates than free LPS titrated to the virus. As a control of specificity, we were able to block the LPS-induced enhancement of infection by adding nontoxic concentrations of the antibiotic drug polymyxin B that can directly neutralize LPS. The comparison of two agonistic LPS types, derived from non-pathogenic *E. coli* K12 or pathogenic *E. coli* 055:B5, for their infection-stimulating potency revealed a selective enhancement of MLV infection for the non-pathogenic LPS.

Since commensal and non-pathogenic LPS can be found in the gastrointestinal system, we propose that the MLV-LPS interaction might play a crucial pathomechanistic role during the frequently observed mother-to-child transmission of MLV in mice. Based on the current findings, further studies should investigate the molecular mechanisms of LPS-triggered MLV infection in vivo and explore possible

therapeutic interventions. This insight may also contribute to a better understanding of basic processes in the interaction of HIV and HTLV with human target cells.

Zusammenfassung

Retroviren wie das Humane Immundefizienz-Virus (HIV) und das Humane T-Lymphotrope Virus (HTLV) sind wichtige Krankheitserreger bei Säugetieren, die lebensbedrohliche Krankheiten wie Immunschwächen und Tumore verursachen können. Retrovirale Infektionen können mit Medikamentenkombinationen gegen verschiedene virale Ziele behandelt werden. Die Tatsache, dass es keine präventiven Impfstoffe gibt, antivirale Therapien häufig mit Nebenwirkungen verbunden sind und eine Heilung der Retrovirusinfektion nicht in greifbarer Nähe ist, unterstreicht die Bedeutung der Infektionskontrolle durch andere Formen der Prävention. In diesem Zusammenhang ist die Aufdeckung neuer Mechanismen, durch die Retroviren den Wirt infizieren, von grundlegender Bedeutung für die Entwicklung innovativer Strategien gegen retrovirale Infektionen.

Ziel dieser Studie war es daher, die grundlegenden Prinzipien zu untersuchen, durch die Retroviren eine Infektion in primären Wirtszellen etablieren. Zu diesem Zweck haben wir funktionelle Infektionsstudien mit dem Maus-Retrovirus Murines Leukämie Virus (MLV) durchgeführt. Überraschenderweise fanden wir heraus, dass die MLV-Infektion durch die Interaktion mit kommmensalem Lipopolysaccharid (LPS) deutlich verstärkt werden kann. Insbesondere MLV, das den LPS-bindenden Toll-like-Rezeptor (TLR4) von Säugetieren während des Austritts aus der produktiv infizierten Wirtszelle in seine Hülle einbaut, profitiert von virusgebundenem LPS, um die Partikelinfektiosität zu erhöhen. Wir haben einen leistungsfähigen Test mit primären Makrophagen entwickelt, um die MLV-LPS-Interaktion nachzuweisen und zu quantifizieren, und ein neuartiges Reportervirus für diese *ex vivo* Studien konstruiert, das auch tiefe Gewebeaufnahmen *in vivo* ermöglichen könnte. Wir konnten zeigen, dass virusgebundene LPS-Aggregate, die vom Virus über TLR4 an LPS-empfindliche Wirtszellen präsentiert werden, zu höheren Infektionsraten führen als freies, mit dem Virus titriertes LPS. Zur Kontrolle der Spezifität konnten wir die LPS-induzierte Verstärkung der Infektion blockieren, indem wir unschädliche Konzentrationen des Antibiotikums Polymyxin B zusetzten, das LPS direkt neutralisieren kann. Der Vergleich zweier agonistischer LPS-Typen, die aus dem nicht-pathogenen *E. coli* K12 oder dem pathogenen *E. coli* 055:B5 stammen, hinsichtlich ihrer

infektionsstimulierenden Potenz ergab eine selektive Verstärkung der MLV-Infektion für das nicht-pathogene LPS.

Da auch kommensales und nicht-pathogenes LPS im Magen-Darm-Trakt zu finden sind, vermuten wir, dass die MLV-LPS-Interaktion eine entscheidende pathomechanistische Rolle bei der häufig beobachteten Mutter-Kind-Übertragung von MLV bei Mäusen spielen könnte. Auf der Grundlage der aktuellen Ergebnisse sollten weitere Studien die molekularen Mechanismen der LPS-ausgelösten MLV-Infektion *in vivo* untersuchen und mögliche therapeutische Interventionen erforschen. Diese Erkenntnisse könnten auch zu einem besseren Verständnis der grundlegenden Prozesse bei der Interaktion von HIV und HTLV mit menschlichen Zielzellen beitragen.

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