DISSERTATION

HIV PROPHYLAXIS: AN ESSENTIAL ROLE FOR T CELLS AND ADJUVANTS IN RECOMBINANT MUCOSAL *LACTOBACILLUS ACIDOPHILUS* VACCINES

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ABSTRACT

HIV PROPHYLAXIS: AN ESSENTIAL ROLE FOR T CELLS AND ADJUVANTS IN RECOMBINANT MUCOSAL *LACTOBACILLUS ACIDOPHILUS* VACCINES

Current HIV vaccines have poor efficacy, with inconsistent levels of protection following mucosal HIV exposure. Lactic acid bacteria offer an alternative vaccine vector targeting the primary site of HIV infection, the mucosa. In these studies we evaluated the immunogenicity of several strains of *Lactobacillus acidophilus* expressing HIV membrane proximal external region (MPER), a portion of HIV envelope that contains broadly neutralizing antibody binding sites. We evaluated MPER-only expressing strains along with strains expressing adjuvants (interleukin-1β or flagellin) to improve immunogenicity against the HIV MPER. We compared the adjuvant strains to the MPER-only strain by oral administration in BALB/c mice to observe these improvements, and in CD40L-/- mice to observe if T cell help was necessary. Some BALB/c animals were also placed on a modified diet supplemented with prebiotic rice bran to observe any influence on vaccine immunogenicity. Resulting antibody responses and interleukin-17 levels were measured by ELISA, and T and B cell levels were measured by flow cytometry.

Here we show that the addition of adjuvants, including dietary rice bran, to *L. acidophilus* vaccine strains improves their immunogenicity against HIV MPER. Our results indicate that anti-MPER IgG and IgA levels, as well as the number of anti-MPER antibody secreting cells, are improved with adjuvants, and that T cell help is required for an effective immune response. These results, combined with the many advantages offered by this lactic acid bacteria vaccine system make *L. acidophilus* an attractive vaccine vector for primate and human trials.

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1.1 Mucosal Immunity

Mucosal membranes provide required functions for the host, particularly nutrient absorption and as barriers of entry against foreign objects, both living and non-living. The cells that comprise these ectodermal membranes provide a barrier of entry against foreign organisms, and are thus priority targets for pathogen entry. Numerous pathogens target mucosae directly or exploit mucosal membranes to gain access to other host tissues. Co-evolution of pathogens and their hosts has resulted in a myriad of immune responses to eliminate pathogens and prevent infection.

1.1a Cell Junctions and Mucus

All mucosae rely on intercellular junctions between epithelial cells as a basic physical barrier against microbial invasion. In the respiratory, intestinal and sexual mucosae, **adherens** and **tight junctions** provide strong interactions between adjacent cells (1-4). Adherens junctions function to maintain cell-to-cell contact and barrier integrity, while tight junctions are typically associated with the movement of ions and other material between cells (5). These junctions are targeted by several mucosal pathogens, including *Listeria monocytogenes*, *Salmonella* species, and enteropathogenic *E. coli,* implying their importance for barrier integrity (4).

The secretion of mucus by mucosal epithelial cells also provides a strong physical barrier and a scaffold for antimicrobial peptides and antibodies. Mucus itself is comprised of multiple heavily glycosylated mucin proteins that are either secreted to form outer and inner luminal layers, or associated with the epithelial cell surface (4). Mucins can have direct antimicrobial

effects against certain pathogens, such as *Helicobacter pylori*, but typically serve to nonspecifically bind and aggregate bacteria, facilitating their removal (6-8). Mucus layers also exhibit physical properties that add more barriers for pathogens, including variations in pH (9), carbohydrates (10) and oxygen concentration (11), and have been associated with anti-inflammatory tolerance in the gut (12, 13).

1.1b Antimicrobial Peptides

A large number of antimicrobial peptides (AMPs) are secreted by mucosal cells on mucosal tissue surfaces and within the matrix of mucus proteins (14, 15). **Lysozymes** are able to cleave the peptidoglycan linkages of gram-positive bacterial cell walls (16). **α- and β-defensins** are AMPs that embed within bacterial membranes, forming destructive pores when enough defensins are present (17). **LL-37**, a human cathelicidin, is able to destroy both gram positive and gram negative bacteria by cell wall destruction (18). **Histones** and their fragments can function as AMPs (19, 20), as well as **Ubiquitin**, which has demonstrated antifungal properties (21).

1.1c Innate Lymphoid Cells

Mucosae rely on various cell types to maintain homeostasis and avoid destructive inflammation. Among these cell types are Innate Lymphoid Cells (ILCs), a relatively recent classification of innate immune cells. Three groups of ILCs have been established (22): **Group 1** ILCs include natural killer (NK) cells as well as non-cytotoxic cells ($Tbet^+$) capable of enhancing Th1 responses via IFN-γ and TNF-α secretion, and are associated with defense against intracellular bacteria and protozoan parasites. **Group 2** ILCs ($GATA3^+$) typically secrete Th2

cytokines and are thus associated with allergies and helminthic parasites. **Group 3** ILCs (RORγt^{+}) can secrete IL-17, as well as similar cytokine profiles as Group 1 ILCs, and predominantly target extracellular bacteria. All ILCs are associated with specific pathogen defenses, and groups 2 and 3 have been shown to induce mucosal tissue repair and stimulate mucus and AMP secretion by goblet and Paneth cells (23). ILC functions are often similar to T helper cells, but ILCs do not require antigen stimulation, instead becoming activated via cytokine stimulation from other resident cell, typically APCs (24).

1.1d Sampling of Mucosal Lumen

The uptake of antigen for the adaptive immune response can vary based on the mucosa. The respiratory tract for example is primarily served by a variety of sentinel dendritic cells (CD103+, CD11b+, plasmacytoid, monocyte-derived) and pulmonary macrophages (alveolar, bronchial, interstitial) (25, 26). Each subset is capable of different effects that can vary based on pathogen or disease context, but all are able to influence the adaptive immune response by variations in cytokine secretion and antigen presentation (25-28). These antigen presenting cells usually initiate adaptive immune responses at lymphoid cell foci known as bronchus associated lymphoid tissue (BALT) (29).

Antigen uptake within the intestinal tract employs several cell types not found in the respiratory tract. These cells are associated with gut associated lymphoid tissue (GALT), the intestinal BALT counterpart. The intestinal mucosa and its associated immune cells vary with each major anatomical gut segment. Within the small intestine are Peyer's patches (PP), lymph node-like structures that increase in number from the duodenum to the ileum. At the luminal surface of PP are microfold (M) cells, epithelial cells that constantly sample lumen contents and

transport those contents to underlying immune cells (30, 31). Underlying DCs have been shown to sample luminal contents on their own by extending dendrites through the M cell or between epithelial cells (32) (33, 34). Goblet cells, which produce mucins, have also been associated with DC antigen uptake via goblet cell associated-antigen pathways (GAPs) (35). The colon also contains colonic patches, similar to Peyer's patches, with overlying M cells for lumen sampling (36). Smaller lymphoid aggregates called solitary isolated lymphoid tissues (SILT) are found throughout the intestinal tract and increase in number as intestinal bacteria numbers increase (31). There are associations between the mechanism of antigen uptake (M cell, goblet cell, dendrite, paracellular leakage) and the resulting immune response, be it inflammation, tolerance, or IgA secretion (37).

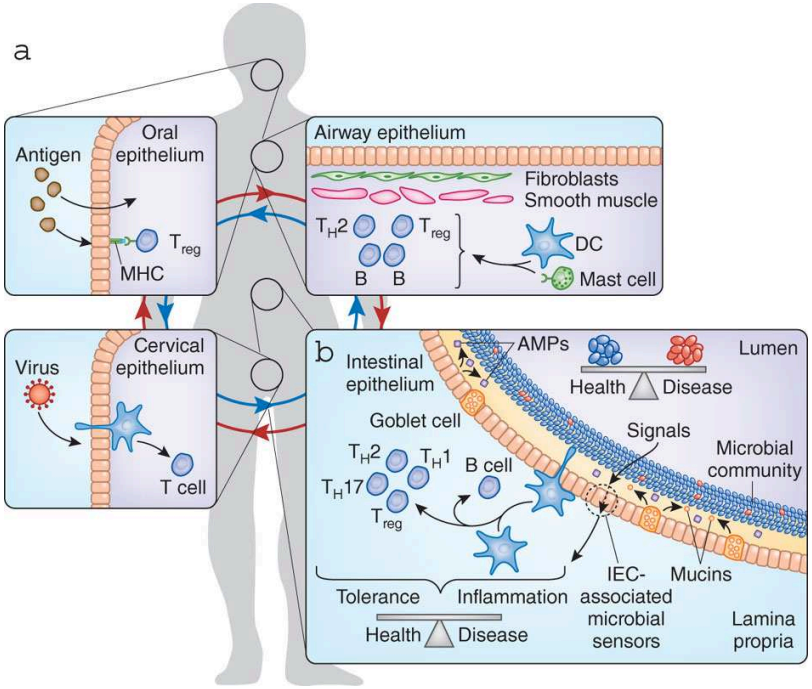

Figure 1.1 Differences in antigen uptake and immunity at mucosal sites. M cells are not shown but provide luminal sampling for the intestine alongside the pictured DCs (b). IEC-associated microbial sensors include Toll-like receptors and other pattern recognition receptors described in the following section. Courtesy Gill et al. (38).

1.1e Pattern Recognition Receptors

The sentinel cells of mucosae express surface pattern recognition receptors (PRR) to detect common pathogen structures. There are several types of PRRs. **Toll-like receptors** (TLRs), responsible for recognizing a diverse set of pathogen motifs, are expressed on virtually all APCs and mucosal epithelia and upon stimulation initiate cytokine and AMP secretion. Some TLRs cause different effects based on their mucosal tissue location. Oral and respiratory mucosa tend to drive AMP secretion, while the intestinal mucosa tends to drive inflammation and antibody class switching (39, 40). The potential for overstimulation of TLRs in the intestine leads to negative-TLR regulation to promote homeostasis and prevent destructive inflammation (41). The intestines also have reduced apical expression of TLR4 and TLR5, sequestering these receptors to intracellular compartments to prevent over-stimulation by commensal bacteria (42, 43).

Nucleotide-binding oligomerization domain-containing proteins (NOD1, NOD2), detect gram-negative and gram-negative/positive bacterial cell wall motifs, respectively (44, 45). Similar PRRs called **NOD-like receptors** (NLR) recognize specific bacteria or toxin motifs, such as anthrax or gout crystals. Both NOD and NOD-like receptors activate inflammatory pathways in the host cell (44). **C-type lectin** PRRs detect sugar-based pathogen motifs, resulting in Th1 responses, inflammation and antigen presentation (46). **N-formyl methionine** receptors may be responsible for the tolerance of commensal bacteria detected through non-inflammatory pathways (47).

1.1f B cells, class switching, and IgA

B cells, responsible for the secretion of antibodies, traffic from the blood and are distributed throughout the intestinal tract. Dimeric IgA is a class of antibody secreted by mature B cells that passes to the mucosal surface via transcytosis (48). Unlike other antibody classes IgA is typically secreted locally, as opposed to systemically, and resists mucosal protease digestion (49, 50). In secondary lymphoid organs, Th2-type cytokines combined with B-cell receptor (BCR) and CD40L binding induces IgA class switching (51). This process is referred to as T-dependent IgA class switching since CD40L-CD40 binding requires a T helper cell and results in the formation of memory B cells. However, within inductive sites like Peyer's patches, mesenteric lymph nodes, colonic patches and SILT's, B cells can receive signaling through TLRs and cytokines APRIL and BAFF causing class switching independent of T cell help (52). IgA induced by this T-independent mechanism do not undergo significant affinity maturation and are thus low-affinity, but still prevent bacteria from binding intestinal epithelia. This prevents overgrowth of commensal bacteria and avoids damaging inflammation. Recently established subsets of B cells (B-1a B-1b) can also become activated without T cell help and may contribute to commensal bacteria control (53).

1.1g T cells

Progress in identification of cellular markers has led to a dramatic increase in the number of T cell subsets. While many T cells can be classified within typical groups (CD4, CD8, Treg), new subsets of these cells have complicated the definitions of mucosal T lymphocytes. T helper cells still serve as key mediators for strong adaptive immune responses, either inflammatory or tolerance. **Tolerance** typically arises from T regulatory (Treg) cells, which are stimulated by DC

and mucosal epithelial cells (via REG3γ or TLR2) to secrete IL-10 and create a more homeostatic, tolerogenic mucosa (54). Under the right circumstances γδ T cells may also exert an anti-inflammatory response (55). **Inflammation** arises from APC detection of pathogens and drives multiple subsets of T cells, often via IL-23 stimulation (56). These include: **γδ T cells**, which use alternative non-MHC antigen detection via multiple receptors (57). **NKT cells,** which bind glycolipids (rather than proteins) presented via MHC-like receptors like CD1d (58). **ILC3** cells (See section 1.1c), and **Th17 cells**, a distinct pro-inflammatory lineage (59). Both Th1 and Th2 subtypes can drive inflammation, with Th1 cytokines (IFN-γ and TNF-α) associated with gut inflammation, and Th2 cytokines (IL-4, IL-13) associated with airway inflammation (60). CD8+ T cells also contribute to mucosal immunity, migrating to mucosae following activation and establishing resident memory cells for rapid response to viral mucosal infections (61).

1.2 Human Microbiome

Bacteria of various genera are associated with all human mucosal surfaces (62). Microbial diversity of these local microbiomes can vary drastically based on host hygiene, health and diet (62). By mapping out the species within these microflora we can better understand the symbiotic relationship humans and other creatures have with the organisms that live on and within them. This mapping is typically performed by amplicon sequencing of a highly conserved gene segment in prokaryotes, usually a region within the 16S portion of ribosomal ribonucleic acid (rRNA), though other portions have been used in the past (63). Ribosomes consist of RNA and protein complexed together to form two subunits, the large (LSU) and small (SSU). Functionally these subunits catalyze the translation process from RNA to amino acid chain via tRNA (64). The SSU in prokaryotes contains the 16S subunit, which is relatively conserved

genetically in all bacteria, leading to its common use for phylogenetic analysis, despite some bias (65, 66). Both the 16S rRNA, and its counterpart rDNA, can be used for sequencing, with rDNA allowing for identification of quiescent cells with lower numbers of active ribosomes (67, 68).

1.3 Microbiome and Disease

As analytical techniques have improved specific associations between microbiomes and health have been identified (69). Bacteria of the gut can have a huge effect on host metabolism and nutrient availability, resulting in correlations between the gut microbiome and obesity, and thus diabetes and other diseases associated with obesity (70). Studies of germ-free mice show severe losses in gut-associated immunity, such as reduced IgA production, the loss of CD4+ and CD8+ T cells, and reduced expression of pattern recognition receptors, resulting in susceptibility to pathogen infection (71). Destruction/dysbiosis of the gut microbiome has been associated with inflammatory compounds, leading to potential associations with inflammatory bowel diseases including cancer (72), Crohn's disease (73)) and arthritis (74). Psoriasis and acne of the skin have been associated with specific bacterial species (75, 76), and peptic ulcers are famously associated with *Helicobacter pylori* colonization (77). Interestingly, the microbes acquired during birth have been associated with a number of diseases, usually correlated with vaginal versus cesarean birth (78). Clearly the gut microbiome is a key component of the overall health of individuals. However, expectations of associations between the microbiome and disease can lead to bias, which can only be mitigated by increasing the number of cause-and-effect studies (79).

1.4 Lactic Acid Bacteria and Probiotics

Lactic acid bacteria (LAB) are an extremely large and diverse clade of gram-positive bacteria. Because of their close associations with human food they have been extensively studied for over a century. Subdivisions within the clade are very complex, compounded by periodic reclassifications within families and genera, as well as the persistence of 'classical' genera from work in the early 1900's by Dutch biochemist Sigurd Orla-Jensen (80). These classical genera were established by studying the various attributes of bacteria under different growth conditions, but plasticity within LAB regarding their typical abilities has necessitated the use of modern genome sequencing to establish sub-genera relationships (81). While large differences exist within genera and species, major commonalities exist in LAB. All LAB utilize fermentation to generate energy and lactic acid. Some LAB homoferment, converting simple sugars to lactic acid or lactate, its conjugate base. Other LAB are heterofermenters, utilizing a more complex metabolic pathway to generate lactic acid, acetic acid, ethanol and carbon dioxide. See Fig. 1.2 (82).

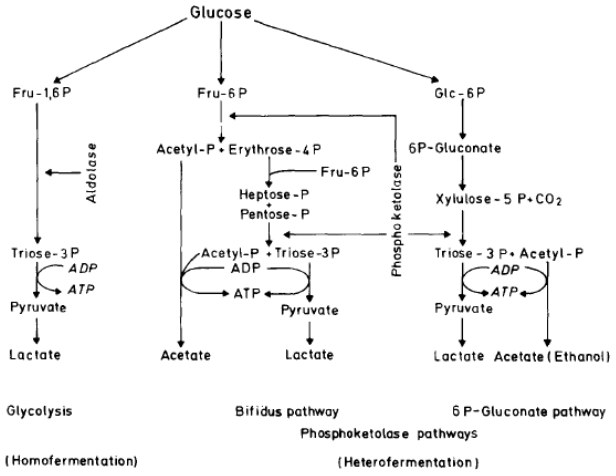

Figure 1.2 Homo- and Heterofermentation pathways. Lactic acid bacteria breakdown glucose through one of several potential pathways, resulting in lactate, or a combination of lactate and other byproducts. Courtesy Kandler et al. (82).

These compounds and their concentrations can be altered by variations in growth conditions, leading to the extremely diverse uses LAB provide to humans (83). These include a millennial-long association with milk and bread products, from cheese and yogurt in ancient Iraq to sourdough bread in ancient Switzerland (84). In addition to enhanced flavor, LAB provide excellent natural means of food preservation, preventing spoilage by acidification (lactic and acetic acids) and secretion of antibacterial proteins targeting competing bacteria (85). Of increasing modern relevance is the characterization of LAB (usually *Lactobacillus* or *Bifidobacterium* spp.) as probiotics: bacteria that provide specific benefits when administered to mucosa. There is evidence of strain-specific probiotic effects on obesity and psychological disorders in animal models, and intestinal and psychological disorders in humans (86-88)). More stringent clinical studies and strain-specific analysis is required to isolate specific probiotic effects (87-89).

1.5 Lactobacilli

Over 80 species of the genus *Lactobacillus* have been classified (80). Some have long been associated with human foods, especially cheese, yogurt and a variety of fermented products (90). This strong safety record means many lactobacilli are Generally Recognized As Safe (GRAS) by the FDA (91). Other lactobacilli are associated with mammalian gastrointestinal, oral and vaginal mucosa (90, 92, 93). Lactobacilli are located throughout the human gut, increasing in number from stomach to colon (31). The actual diversity *Lactobacillus* species that colonize the gut is likely much smaller than previously thought (90). Lactobacilli are able to survive and thrive in the mammalian gut: They are intrinsically bile and acid tolerant due to bile salt pumps and proton pumps (94), they thrive in the low oxygen environment of gut mucus, and they

possess surface proteins to allow binding to mucus (31, 95). Lactobacilli have been studied for their anti-inflammatory and anti-pathogen properties, as well as their ability to adjuvant vaccine responses (see section 1.6).

1.5a Anti-Pathogen Effects

There have been multiple experiments showing that *Lactobacillus* can confer protection against pathogens and their symptoms. *L. acidophilus* reduced cold and flu-like symptoms in children and decreased the number of *Clostridium difficile* bacteria in elderly GI tracts (96, 97). *L. plantarum* increased mouse survival from *Pseudomonas aeruginosa* and *Escherichia coli* challenge and reduced bacterial translocation in rats (98, 99). *L. rhamnosus* reduced the duration of rotavirus diarrhea in children and increased the number of children with rotavirus-specific IgA from ~50% to nearly 100% (100). *L casei* reduced complications in patients with systemic inflammatory response syndrome, and lowered the number of preterm infants with gastrointestinal *Candida albicans* (101, 102). *L. reuteri* showed *in vitro* antibiotic effects against several enteric bacteria, as well as antiviral activity against Coxsackievirus A and Enterovirus 71, the viruses responsible for hand, foot and mouth disease (103, 104). The mechanisms behind these effects are not fully known, but suppression of pathogens can involve competition with pathogens for nutrients or space, acidification (via lactic acid) that inhibits growth, enhancement of the mucosal immune response, or the production of bacteriocins/antimicrobials (105).

1.5b Anti-Inflammatory Effects

Many strains of probiotics exhibit anti-inflammatory effects in the host. *L. rhamnosus* lowered TNF-α levels in healthy adults and lowered inflammatory cytokines in an *in vitro* cigarette smoke inflammation assay (106, 107). Inactivated *L. crispatus*, common in vaginal mucosa, caused DC secretion of IL-10 and Treg polarization *in vitro* (108). *L. acidophilus* counteracted the inflammatory effects of chemotherapy on the intestinal mucosa in mice (109). *L. casei* reduced colitis in mice and inhibited IL-6 production by blocking NF-κB nuclear trafficking (110, 111). The body's downregulation of inflammatory components may simply be a mechanism to ensure beneficial bacteria (nutrient digestion help, pathogen protection) are allowed to thrive.

1.5c Adjuvant/Immune Effects

Many studies have shown that lactobacilli can influence co-administered vaccines. *L. acidophilus* alone was able to reduce tumor growth via Th1 enhancement (112-114), with similar results observed using *L. casei* (115). *L acidophilus* and *L. rhamnosus* both improved T cell proliferation, IFN-γ secretion, and serum antibody responses to both oral cholera toxin or subcutaneous tetanus toxoid in mice, with no changes in T cell % or mucosal antibodies (116). These same strains showed increased neutralization, IgG and IgA levels in conjunction with oral polio vaccine in humans (117). *L. rhamnosus* by itself enhanced specific IgA responses in humans exposed to rotavirus (100), and coadministration with an influenza vaccine improved protection (118). *L. fermentum* with an inactivated influenza vaccine increased TNF-α and IFN-γ and boosted serum anti-influenza IgA and total IgM, though significance was somewhat questionable (119). This strain also generated a balanced Th1/Th2 humoral response in mice

administered either Salmonella Typhimurium or ovalbumin, with a strong Th1 cellular immune response (120). Oral delivery of an inactivated SIV alongside *L. rhamnosus* or *L. plantarum* induced a protective CD8+ Treg cell response, protecting macaques from SIV challenge by preventing CD4+ T cell activation in the mucosa (121). Surgical patients treated with *L. plantarum* did not show the increased total mucosal IgA levels measured in animal studies (122). A theme of enhanced Th1 cellular responses emerges from many studies, while a more balanced Th1/Th2 humoral immune cytokine profile has also been observed. Recent work has shown that this *Lactobacillus* inflammatory balance is complex and likely a balance between Tregs and Th17 cells (123, 124).

1.6 Lactic Acid Bacteria Vaccines

The use of a live bacterial vaccine vector was described in 1981, when Formal et al. expressed *Shigella* antigens on the surface of *Salmonella* Typhi (125). Other bacterial vectors have been attempted, often using attenuated, non-pathogenic versions of common pathogens like *Listeria* and *Salmonella enterica* serotype Typhimurium (126). These vectors pose a serious safety risk, and the host will often develop antibodies against the vector and not the antigen of interest (127). The idea of using safe commensal bacteria as vectors was fielded in the 1990's and required a number of proof-of-concept experiments to demonstrate that non-pathogens could generate an adequate vaccine immune response. Lactic acid bacteria (LAB), with their strong safety record, relatively simple genetic manipulation and association with mucosa led to their candidacy as vaccine vectors (127). Early attempts with LAB included *Streptococcus gordonii, Streptococcus lactis*, *Lactococcus lactis*, and various lactobacilli (128).

All of these strains are still studied as vector platforms (129), but the majority of research has moved towards *Lactococcus* or *Lactobacillus* because of inherent strain-specific probiotic and antigenic properties (130), particularly for lactobacilli (131, 132).

1.6a Antigen Expression

LAB vaccine vectors generally function as delivery vehicles for heterologous antigens expressed either intracellularly, embedded within the surface membrane, or secreted. This expression is typically controlled by plasmids encoding for fusion domains of the antigen of interest with trafficking domains from other bacteria (133). For example, fusion of an antigen with a peptidoglycan-binding domain or a surface-embedded enzyme can allow for surface expression of antigen motifs (134, 135). Other systems embed the antigen of interest within surface layer proteins (136, 137). Intracellular expression requires typical plasmid promoter expression (138), while extracellular secretion of antigen can be accomplished by adding secretion signals to antigens (139). Other variables can affect the immune response to LAB vaccines, including the route of administration (oral, intranasal, vaginal) and the addition of adjuvants either as fusion peptides with the antigen (140, 141) or as secreted (136, 142) or surface proteins (143).

1.6b Immune Response

Immune responses against LAB heterologous antigens can vary based on expression system, route of administration, strain, and adjuvants. Vaccination can induce both Th1 and Th2 cytokines (with a tendency towards Th1) as well as antibodies associated with either T helper response (144-147). An interesting push-and-pull between inflammatory (Th17/IL-17) and

tolerant (Treg) cell types and cytokines has also been shown (146, 148-150). Overall, LAB vaccination is able to induce both systemic and mucosal antibodies and can protect hosts from disease morbidities and mortality (See Appendix A). These results indicate that mucosal immunity is the result of a complex interaction between powerful arms of the immune system that must maintain a delicate homeostasis, preventing autoimmune damage via inflammation while also targeting pathogens.

1.7 Brief History and Epidemiology of HIV

In 1981 a large number of homosexual patients with opportunistic diseases typically associated with immune suppression (Kaposi's sarcoma, Pneumocystis pneumonia) began appearing at California and New York health clinics (151). Over the next five years this severe immunosuppression, coined Acquired Immune Deficiency Syndrome (AIDS) by the CDC in 1982, continued to expand throughout the world (152). Of the many proposed causes for AIDS, several retroviruses targeting CD4+ T cells were identified by multiple research groups (153). Initially separate viruses (Lymphadenopathy Associated Virus, Human T Lymphotropic Virus-III), these were shown to be the same virus in 1984 (154), and renamed Human Immunodeficiency Virus type 1 (distinct but related to HIV type 2) in 1986 (155). Since its identification HIV has become one of the most prevalent and deadly modern infectious diseases, estimated to have killed over 35 million people (156). Treatments and prevention tactics have drastically reduced mortality and virus acquisition, but the rate of new infections has alarmingly stabilized both in the United States (157) and globally (158).

1.8 HIV Genome Structure and Encoded Proteins

The HIV genome follows the typical organization of a retrovirus, possessing three major genes (*gag, pol*, *env*) that encode the major structural proteins and essential enzymes of the virus. These large genes are cleaved by either viral or cellular proteases to yield smaller gene segments for translation.

1.8a Gag proteins

Short for group-specific antigen, *gag* encodes four proteins located within the envelope of the virus particle. These proteins are generated from the cleavage of a larger polyprotein (Gag precursor, pr55) by HIV protease following budding from the host cell (159). They include (abbreviation, kilodaltons):

1. Matrix Antigen (MA, p17): Functions as an assembly scaffold between capsid proteins and the inside of the viral envelope (160).
2. Capsid Antigen (CA, p24): Forms a conical-shaped structural shell to contain the viral genome and enzymes (161).
3. Nucleocapsid (NC, p9): Binds to the unspliced HIV RNA genome and assists reverse transcription and integration steps by stabilizing interactions between the genome and HIV enzymes (162).
4. Proline-rich (p6): Recruits cellular factors associated with vesicle transport to allow for viral budding (163).

These gag proteins often have additional functions as well. Capsid binding of Cyclophilin A has been shown to protect against host restriction factors (164). Soluble matrix proteins stimulate host inflammatory cytokine release (165), and p6 can initiate recruitment of Vpr into the nucleocapsid (166).

1.8b Pol proteins

Short for polymerase, the *pol* gene encodes three enzymes required for productive viral replication. These enzymes are generated by the cleavage of a larger polyprotein (Gag-Pol precursor, Pr160) by HIV protease (159). They include (abbreviation, kilodaltons):

1. Protease (PR, p10): Cleaves the Gag and Gag-Pol precursor proteins into smaller functional subunits (167).

2. Reverse Transcriptase (RT, p66, p51): Generates viral double stranded complementary DNA (dscDNA) from viral RNA (168). When bound to integrase, Vpr, MA, and certain host proteins, dscDNA is more stable and can traffic to the nucleus as the Pre-Integration Complex (PIC) (169).

3. Integrase (IN, p32): Integrates the dscDNA viral genome into the host cell genome, resulting in integrated provirus (170).

As with the gag proteins, pol proteins often have additional functions. Protease has been shown to cleave host cell proteins, leading to CD4+ T cell death and depletion (171). Integrase may be required for proper capsid core assembly during virion maturation (172), and reverse transcriptase may prevent nucleocapsids from aggregating with viral dsDNA, allowing space for protein binding and thus PIC assembly (173).

1.8c Env glycoproteins

The *env* gene, short for envelope, has fewer proteins than *gag* or *pol*, encoding a large, heavily glycosylated precursor protein (gp160) that is cleaved into two subunits, gp120 and gp41. Both proteins remain non-covalently bound to each other in a trimeric form and are transported to the host cell surface for expression on what will become the virion envelope (159). The gp120-gp41 complex, known as a spike, is responsible for binding host cell surface receptors and initiating envelope fusion. Gp41 anchors the spike within the viral envelope and is responsible for viral and host membrane fusion, while gp120 is responsible for binding to host surface proteins, including CD4 and a variety of chemokine receptors (see section 1.6 for more detail on binding and fusion). Both proteins can have effects beyond their typical functions. Soluble gp120 has been shown to increase particle production in infected cells (174) and can impair B cell function by initiating abortive signaling in naïve B cells, causing secretion of TGF-β1 (generally suppressive) and increasing surface expression of the inhibitory receptor FcRL4 (175). Gp41 has been shown to modulate a large number of cytokines associated with the immune response (IL-6, IL-10, IL-22) (176).

1.8d Regulatory and Accessory Proteins

In addition to the three major HIV genes, several auxiliary proteins are also encoded in the HIV genome. These can be organized into regulatory proteins (responsible for interactions with viral RNA) and accessory proteins (perform a variety of functions, usually stopping host factors) (159).

Regulatory proteins:

1. Transactivator of Transcription (Tat): A transcription factor that binds the Tat Responsive Element (TAR) portion of HIV RNA, indirectly phosphorylating RNA polymerase II (via host transcription factor P-TEFb) and thus drastically increasing the rate of HIV RNA transcription (177).
2. Regulator for Expression of Viral Proteins (Rev): Binds to the Rev Response Element (RRE) portion of unspliced Env and allows for nuclear export (178).

Accessory proteins:

1. Viral protein R (Vpr): This protein serves many functions, from cell cycle arrest to proper reverse transcriptase function to import of HIV DNA into the nucleus (179).

2. Viral Infectivity Factor (Vif): Initiates degradation of the host cell innate antiviral enzymes APOBEC3G and 3F, cytidine deaminases that converts cytidine to uridine. When packaged within HIV virions both APOBECs render HIV RNA indirectly useless by hypermutation or directly by blocking reverse transcriptase binding (180).

3. Viral protein U (Vpu): Degrades CD4, thus preventing T cell activation, infection with other HIV virions, and interference with gp120 on newly budding virions (181). Vpu also enhances virion budding (182).

4. Negative Factor (Nef): Downregulates many surface receptors, including CD4 and MHCI, and blocks antiviral protein incorporation into virions (183).

1.8e Other Genome Regions

The HIV genome possesses several important non-coding regions (159). Each end of the genome possesses a long terminal repeat (LTR) region, which primarily acts as a promoter for

polymerase binding. Within the LTR is the target sequence for viral transactivation (TAR or Tat responsive element). This region allows for polymerase and Tat binding, increasing the rate of transcription (177). Unspliced HIV RNA is transported out of the nucleus by the binding of Rev to the Rev response element (RRE), located within the *env* gene (178). Interestingly, the secondary structures of HIV RNA elements are complex and required for proper function (184).

1.9 HIV Transmission

HIV is typically transmitted from one individual's infected fluid (blood, semen, pre-ejaculate, vaginal fluid, rectal fluid, breast milk) to a recipient mucosal surface (80% of transmissions), excepting direct injection into the blood via needle (20% of transmissions) (185, 186). Rates of transmission vary based on activity (sexual or otherwise), number of exposures, and the economic status of the subject's country of residence (187). The risk-per-exposure rate can be mitigated by condom use, dropping from 0.82% to 0.18% in receptive men who have sex with men (MSM) (188). Antiretroviral therapy to prevent mother-to-child transmission can provide a helpful barrier to child seroconversion, especially when multiple drugs are used for the mother's treatment (189). This same trend follows in discordant couples, who have a drastically decreased risk of partner transmission when the infected partner takes antiretrovirals (190). Blood transfusions posed risks early on for transmitting new infections, and despite monitoring of the blood supply recent incidents in India highlight the need for careful surveillance of transfusion blood as a potential route of transmission (191).

1.10 HIV Life Cycle

The life cycle of human immunodeficiency virus type 1 is similar to other retroviruses. It is an enveloped virus, possessing a phospholipid bilayer obtained during budding from a previous host cell. This envelope contains the viral capsid in which HIV RNA and various enzymes are safely stored. The complexities of each step of the HIV life cycle are still being elucidated, but most steps have been analyzed enough to offer a relatively detailed breakdown.

1.10a Attachment and Fusion

HIV entry begins when the surface trimer of gp120 binds to host cell surface CD4. Binding initiates a conformational change, shifting gp120 from a closed, antibody-resistant form to an open conformation (192). Shifting exposes new binding sites within the gp120 variable region loops (V1-V5) for host cell co-receptors. These host co-receptors are chemokine receptors, typically CCR5 or CXCR4, though a number of other co-receptors have been identified (193). Different strains of HIV tend to utilize different co-receptors, with macrophage/DC/T tropic strains using CCR5 and T cell-only strains typically using CXCR4, though exceptions exist (194). Only strains expressing CCR5 or CCR5 and CXCR4 are usually transmitted between humans (195). More conformational changes accompany co-receptor binding, inserting gp41 into the host cell membrane. Gp41 then folds on a hinge region, pulling the viral and cellular membranes together (196). Whether this process occurs within an endosome or at the cell surface has been debated, though it is likely endocytic (197, 198).

1.10b Uncoating, Reverse Transcription and Nuclear Import

Once inside the host cell the HIV capsid must breakdown, or uncoat, to release viral enzymes and RNA. This uncoating cannot occur too early or late following entry (199). Once released from the capsid, HIV reverse transcriptase (RT) begins converting viral RNA to DNA (Fig. 1.3). Synthesis of the minus (-) DNA strand is from 5' to 3' and is primed by host tRNA binding to the primer binding site of viral RNA. This primer binding site is relatively close to the 5' end of the viral RNA, thus generating a relatively small strand of DNA. This small portion of minus DNA is then transferred to the 3' end of the viral RNA for completion. As the RT builds the minus DNA strand it degrades the complementary RNA via ribonuclease H cleavage, except the primer binding site. This leftover primer binding site region is then used as the primer for the plus (+) DNA strand synthesis. This final plus strand synthesis is continuous due to circular formation of the minus strand (200, 201). The now double-stranded viral DNA is then transported into the nucleus with the help of a number of proteins (Vpr, capsid, matrix, host cofactors) that are still being identified (202, 203).

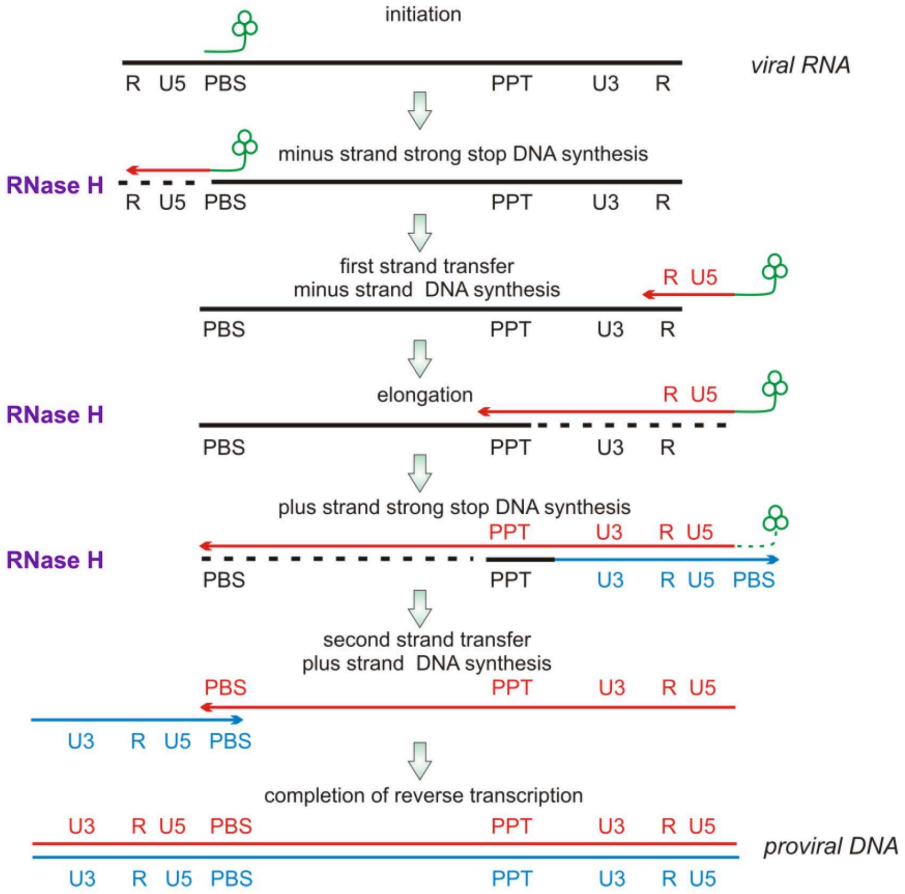

Figure 1.3 Steps in HIV reverse transcription. RNA is black, minus strand DNA is red, and plus strand DNA is blue. tRNA is represented by green drawings. The final completion step uses a circular template strand. Courtesy Ilina et al. (201).

1.10c Integration and Latency

Once within the nucleus the viral genome is incorporated into the host genome by viral integrase. Integrase cleaves host DNA, inserts the viral genome, and ligates the ends in the HIV genome (170, 204). When integrated into non-resting cells, active transcription of the HIV genome (provirus) can begin following recruitment of RNA polymerase II to the LTR region of HIV (205). However, in quiescent or resting T helper cells, the HIV provirus may lie dormant (latent) until the cell becomes activated. These latently infected cells are known as the latent

reservoir (206). This reservoir is estimated to survive for the lifetime of the host, though attempts to target latent provirus are underway (207, 208).

1.10d Transcription and Nuclear Export

Once embedded within the host genome, the HIV provirus can begin transcription by recruiting cellular RNA polymerase II. Transcription is later enhanced by the viral transcription factor Tat binding to the proviral TAR region (177). Early viral RNA exiting the host nucleus is fully spliced, allowing normal host exportins to traffic them out of the nucleus (209). Nuclear export of unspliced RNA is accomplished by the *pol* export protein Rev, which binds to the Rev Response Element (RRE) region of unspliced viral RNA and facilitates their movement through nuclear pore complexes (210). These HIV transcripts are either translated into HIV structural proteins and enzymes for virion formation and assembly, or remain as RNA genomes for later virion inclusion.

1.10e Translation, Packaging and Budding

Translation is controlled by several mechanisms that rely on secondary RNA structure (211). HIV is capable of using both typical mRNA cap-dependent (5' CAP) translation, as well as cap-independent internal ribosomal entry sites (IRES) (211, 212). Translated polyproteins are cleaved by viral and host proteases, resulting in regulatory proteins (Tat, Rev) that affect further transcription, as well as structural proteins and enzymes for virion production. Formation of the virion is initiated by the Env, Gag and Gag-Pol polyproteins associating with the host plasma membrane, with Gag polyprotein also associating with two viral RNA strands (213, 214). As budding of the immature virion begins, the Gag polyprotein forms a lattice structure, associating

(from membrane inward) as matrix, capsid, and nucleocapsid. Following successful budding these proteins will be cleaved to confer full functionality. Budding is achieved by hijacking the host endosomal sorting complexes required for transport (ESCRT), the cell enzymes responsible for endosome budding (215). Final virion maturation involves cleavage of the Gag and Gag-Pol polyproteins by protease, with subsequent conformational changes altering internal virion structure to form a conical capsid that contains the RNA genome, integrase, protease, and reverse transcriptase, stabilized with nucleocapsid (213).

1.11 HIV Vaccine History

Early optimism and a lack of understanding of HIV led to an under-estimation of how long it would take to generate an effective HIV vaccine (216). Many vaccines have been designed, but only a select few have been deemed safe and potentially efficacious enough for human efficacy trials (217). The first efficacy trial relied on recombinant gp120 with alum, a commonly used aluminum salt adjuvant, and failed to elicit any protection (217). New trials using adenoviral (STEP, Phambili, HVTN 071) and canarypox (Thai/RV144) vectors were attempted. The canarypox trial is the only HIV vaccine trial to show any efficacy, with a slightly significant protection of 31% (218). The adenoviral trials were halted early because of a potential enhancement of HIV acquisition, likely due to increased numbers of mucosal T cells targeting the vector and thus creating a strong founder population for virus infection (219). DNA vaccines have also been attempted in combination with adenoviral vectors (HVTN 505), but were stopped early due to a lack of efficacy (220). The seventh and most recent efficacy trial (HVTN 702) is set to begin in 2016, utilizing a modified canarypox with a recombinant boost of gp120 and squalene (a naturally occurring 30-carbon oil) as an adjuvant (221, 222). While many vaccine

strategies have been proposed, safety, a lack of translational immune responses from animals to humans, and a basic lack of data, have prevented their use in extensive human trials (223, 224).

1.12 HIV Broadly Neutralizing Antibodies

Surface expression of gp120 and gp41 make them the most readily available HIV proteins for immune system targeting. In response these proteins have extreme sequence variability, which combined with glycosylation and structural conformation changes results in less than 2% of the Env protein surface available for antibody binding (225). Among these protein segments is a relatively conserved region of gp41 known as the membrane proximal external region (MPER). This ~22 amino acid region contains several binding sites for broadly neutralizing antibodies (bNAbs, see Fig. 1.4) (226, 227).

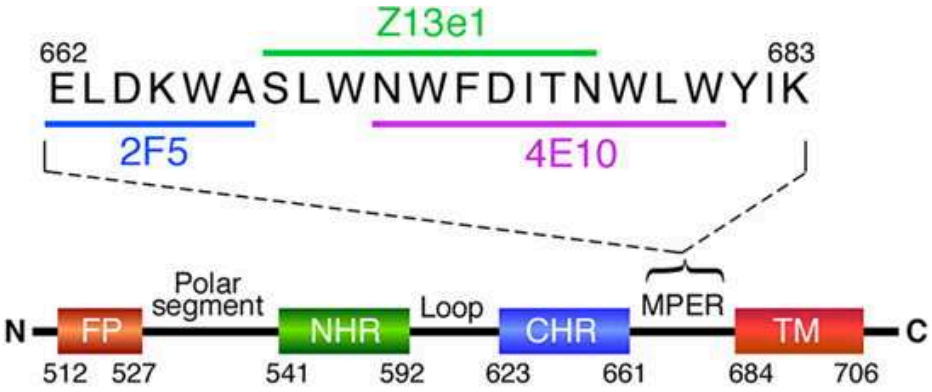

Figure 1.4 Broadly neutralizing binding sites within HIV MPER. The MPER region contains multiple binding sites for neutralizing human anti-HIV antibodies, including the 2F5 and overlapping Z13e1/4E10 regions. Courtesy Song et al (228).

These antibody-binding regions have been extensively studied for use as epitopes for neutralizing antibodies, resulting in dozens of potential therapeutic targets for vaccine and therapeutic antibody development (229-231). However, it is very difficult to generate neutralizing antibodies in the context of a vaccination. Most bNAbs require extensive somatic

hypermutation and have odd heavy chain structure (232). Interestingly, neutralization of HIV may not be required for adequate defense, especially in mucosal tissues. Antibody-dependent cellular cytotoxicity (ADCC) against HIV-infected cells only requires non-neutralizing antibody binding to viral proteins, and the importance of IgA for HIV neutralization at mucosal surfaces is poorly defined (232, 233). Recent experiments have shown serum IgA may interfere with serum IgG HIV neutralization, but mucosal IgA may be the key to preventing viral infection (234, 235).

1.13 HIV Treatment

Anti-HIV drugs are classified by their functionality. **Entry and fusion inhibitors** prevent receptor binding and membrane fusion, respectively, and target structural components of the virus or host cell (236, 237). **Reverse transcriptase inhibitors** fall into two categories: nucleoside/nucleotide RT inhibitors, which compete with viral RNA for binding to RT functional sites, and non-nucleoside RT inhibitors, which disable RT via non-competitive inhibition (238). **Protease inhibitors** bind to the active sites of protease and disable the enzyme via competitive inhibition with HIV proteins (239). **Integrase inhibitors** bind HIV integrase active sites and prevent HIV DNA strand integration into the host genome (240).

Most patients receive a combination of drugs, a cocktail referred to as highly active antiretroviral therapy (HAART). This requires any HIV escape mutants to develop resistance to multiple drugs, which is unlikely (241). The introduction of HAART drastically lowered morbidity and mortality rates (242). However, patients must remain on drugs indefinitely due to latent infection.

Without an efficacious prophylactic HIV vaccine it was proposed that high-risk groups be administered anti-HIV drugs before they acquire the virus. This pre-exposure prophylaxis (PrEP) can be topical or ingested, and promising results were found in some clinical trials (243, 244).

1.14 Humanized Mice

Infectious diseases that only target humans require alternative model systems. Non-human primates are cost prohibitive and require viral tropism modifications. Mouse models with recapitulated human cells are much less expensive and eliminate the need for host tropism alterations. These humanized mouse strains rely on immunodeficiencies in the mouse immune system to prevent graft rejection. Initial models relied on adoptive transfer of adult human peripheral blood, but did not allow for proper immune cell maturation and antigen training and would often suffer from graft-vs-host disease (245). In the late 1980's the implantation of fetal thymus (for T cell maturation) and fetal liver (location of fetal hematopoietic cells) resulted in the generation of human immune cells for an extended period of time (246, 247). The isolation and injection of fetal hematopoietic cells reduced the costly need for surgical implantation and resulted in circulating lymphocytes. These animals could recapitulate certain aspects of HIV infection, including T cell depletion, and could be infected via mucosal challenge (248). Despite these positive results these mice still suffered from poor secondary lymphoid structure formation, inconsistent engraftment, and an inconsistent primary B cell infection/vaccination response (249). This was primarily caused by a lack of human leukocyte antigen (HLA) molecules in the mouse thymus, which was remedied by creating HLA-expressing knock-in mice (250). Other models attempted to remedy lymphocyte maturation problems by combining fetal thymus and liver transplants with an infusion of hematopoietic cells. This did allow for the generation of

human T cells educated in a human thymus, but also suffered from graft-vs-host disease (251). Humanized mouse models continue to evolve, with new xenografts, such as hepatocytes or lung, allowing for interesting new pathogen research that can be rapidly implemented (252, 253) (254).

1.15 Rice Bran

When rice undergoes processing for food consumption the inner kernel is separated from outer components (hull, bran, germ) to prevent spoilage (255). The vast majority of nutrients, including complex carbohydrates, fatty acids, antioxidants, phytochemicals and proteins, are stored within these discarded outer layers (256-258). These layers, colloquially known as rice bran (RB), have been shown to have interesting health effects. RB components possess antibiotic (*Salmonella, Vibrio, Shigella, Escherichia)* and antiviral effects (cytomegalovirus, HIV) (255), can increase neutrophil phagocytosis (259), and increase IgG secretion (260). RB can also cause a down-regulation of inflammatory cytokines (261), the sequestration of IgE (262), and some anticancer effects (255). RB also influences probiotic intestinal bacteria, particularly lactobacilli (263).

Chapter 2: The Influence of Adjuvants on Immunogenicity and Gut Microbiota Following Oral Lactobacillus Vaccination

2.1 Overview

The use of lactobacilli as vaccine vectors has steadily grown in popularity since the 1990's. The natural adjuvant effects of certain species of *Lactobacillus*, particularly *L. acidophilus and L. rhamnosus*, can enhance host immune responses (264-267).

This enhancement is the result of innate immune recognition of several cell wall components, including lipoteichoic acid (LTA) and peptidoglycan (PG) (268). LTA (or live lactobacilli stimulation) of Toll-like Receptor (TLR) 2/6 can trigger induction of IL-12, TNF-α, IL-1β, and IFN-γ, as well as several chemokine ligands (CCL2, CCL20) (267, 269, 270). Bacterial binding of nucleotide-binding oligomerization domain-containing 2 (NOD2), an intracellular pattern recognition receptor that binds muramyl dipeptide (a component of peptidoglycan), was able to induce IL-12 and TNF-α and boost Th17 responses (270, 271). Lactobacilli also possess surface layer proteins (Slp) which interact with dendritic cells via DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN; a C-type lectin receptor (272)) and can drive both inflammatory (IL-12p70, TNF-α, and IL-1β) and anti-inflammatory (IL-10) responses depending on the type of Slp (273, 274). This surface diversity is responsible for the variations in immune responses to different strains of lactobacilli (268, 275, 276).

Lactobacillus vaccine systems typically utilize plasmids for heterologous antigen expression. Antigens are targeted to specific subcellular locations by fusion with trafficking domains from lactobacilli or other bacteria (133). Intracellular expression usually only requires an adequate promoter (139), and extracellular secretion can be accomplished by fusion of antigen

to an antigen-secretion signal (138). Surface expression can be accomplished by fusion with a PG or other cell wall domain, or fusion with a surface-expressed enzyme (134, 135). However, plasmids possess inherent problems, including spontaneous loss and the potential for unintended antibiotic gene spread (277). By integrating the heterologous antigen into the bacterial genome, these plasmid problems can be eliminated. Surface layer proteins are advantageous chromosomal integration sites that provide highly expressed platforms for the antigen and, as previously mentioned, are responsible for DC interactions (136, 137).

The human immunodeficiency virus possesses a highly conserved region within its gp41 envelope protein called the membrane proximal external region (MPER). There have been several human broadly neutralizing antibodies (BnAbs) mapped to specific regions of MPER (229). Thus the MPER region is an enticing region to exploit with vaccine strategies. We have previously shown that embedding a 16 residue peptide portion containing the 2F5 binding site of MPER is weakly immunogenic (136). Other HIV vaccines have suffered similar problems, the result of clonal deletion of anti-Env B cells due to auto- and polyreactivity with host and intestinal bacteria proteins (278-280).

In an attempt to improve the immunogenicity of MPER several adjuvants were identified for co-expression in the *Lactobacillus* vector. IL-1β, a key inflammasome cytokine (281), has been shown as an effective mucosal vaccine adjuvant (282-284) and is required for proper cellular immune responses (285). Flagellin subunits, which signal through TLR5, have also been shown to be effective mucosal adjuvants, with strong inflammasome-dependent and independent responses allowing for redundancy in antigenicity (143, 286, 287).

We have previously shown that recombinant *L. acidophilus* expressing both MPER and secreted IL-1β showed improved immune responses versus MPER alone (136). Our primary goal

was to validate these responses and compare them to MPER+FliC immunogenicity by orally dosing BALB/c mice. Previous experiments with MPER+IL-1β vaccination had increased IgG2b, an antibody associated with T-independent B cell activation (288), and there is evidence that neutralizing anti-MPER antibodies can be elicited via T-independent pathways (278). By circumventing T-dependent activation, a large founder population of T helper cells could potentially be avoided, thus reducing the likelihood of enhancing HIV infection (289). Our secondary goal was thus to measure T cell and B cell responses in mice lacking traditional T-dependent activation. This was accomplished by orally dosing CD40L-deficient mice ($CD40L^{-/-}$), which were incapable of traditional B-cell activation via CD40-CD40L binding (290).

2.2 Materials and Methods

2.2a Bacterial Strains and Culture Conditions

All *Lactobacillus acidophilus* strains used in this study were grown from cultures generated as previously described (291). All strains were grown statically overnight in MRS broth supplemented with 5 μg/ml of erythromycin (Ery) in sealed 50mL or 250mL vials at 37˚C. The strains used in this study are listed in Table 1. Erythromycin resistance was confirmed by growth in Ery supplemented MRS broth and on Ery supplemented MRS agar plate. MPER surface expression was confirmed by flow cytometry (Gallios, Beckman Coulter) after staining cells first with human-anti-HIV (2F5, NIH AIDS Reagent Program) IgG, followed by mouse-anti-human IgG Alexa488 (Molecular Probes).

FliC surface expression was confirmed by flow cytometry after staining cells first with rabbit-anti-FLIC IgG (BioLegend), followed by goat-anti-rabbit IgG PE (Invitrogen). Mouse IL-1β secretion was confirmed by ELISA (Ready-SET-Go!, eBioscience).

2.2b Mice and Immunization Schedule

For study A (Fig. 2.1A), 6-8 week old BALB/c and CD40L-/- mice were obtained from The Jackson Laboratory. CD40L knockout was confirmed by PCR on tail snips using CD40L primers. Animal gut microbiomes were normalized by cage swap with the other mouse type and oral gavage of cecum contents from other mouse type one week prior to first vaccination. Bacterial cells were prepared from overnight culture and suspended in dosing buffer containing $NaHCO_3$ (8.4 mg/mL) and soybean trypsin inhibitor (20 mg/mL, T9128, SIGMA) in ultrapure water. Three daily doses of $2x10^9$ CFU in 200 μl of dosing buffer were administered by oral gavage to each group of mice (6 mice/group) at weeks 0, 2, 4, 6, 8, and 10 (6 doses total). Colony forming units (CFU) were calculated by optical density measurements correlated with plated CFU. Each bacterial strain was administered to matching groups of BALB/c and $CD40L^{-/-}$ mice at the same time.

2.2c Sample Collections and Tissue Processing

Prior to each immunization fluids from each animal were obtained. Blood was collected via tail vein bleed prior to dosing, or by cardiac puncture at termination and centrifuged to isolate serum. Vaginal lavage was performed prior to dosing by washing the vagina 3-4 times with the same 100uL of PBS via pipette. Insoluble debris was removed by centrifugation. Feces were collected prior to dosing by placing mice into clean holding cups for 5 minutes and collecting

feces in pre-weighed tubes. 2x Protease Inhibitor in PBS was added at 10 µL/mg of feces. Contents were then homogenized and centrifuged to isolate supernatant. Two weeks after the final dose mice were euthanized (CO_2 and cervical dislocation as per protocol). Vaginal lavage and feces were collected prior to sacrifice. Blood was collected by cardiac puncture, and cecum contents were transferred to pre-weighed tubes and resuspended like fecal pellets. Spleen, mesenteric lymph node, Peyer's patches (PPs), large intestine (LI; without cecum), and female reproductive tract (FRT) were collected for preparation of cell suspensions in collection medium (RPMI w/o L-glutamine, supplemented with HEPES, pen/strep, and Gentamycin).

For study A (Fig. 1A) single cell suspensions of spleen and PP cells were prepared by mashing tissues in GentleMACS dissociator (Miltenyi Biotec, Auburn, CA) and filtering through cell strainers. Isolation of lymphocytes from the colon and FRT were performed as previously described with some modifications (292). Briefly, mucus and epithelium were gently removed by massaging dissected tissues in PBS supplemented with 1 mM dithiothreitol (DTT) and 5 mM ethylenediaminetetraacetic acid (EDTA). The tissues were then cut into small pieces (~1mm^2), suspended in digestion medium (collection medium with Liberase (125 µg/ml) and DNAse I (100 µg/ml) (Roche)), and applied to GentleMACS dissociator (Miltenyi Biotech). After 30 minutes (LI) or 60 minutes (FRT) incubation at 37˚C cell suspensions were transferred to new tubes through cell strainers. Lymphocytes were isolated by Percoll gradient and suspended in culture medium (RPMI w/o L-glutamine, supplemented with L-glutamine, HEPES, pen/strep, fetal bovine serum, sodium hydroxide, sodium pyruvate, β-mercaptoethanol, non-essential and essential amino acids).

For study B (Fig. 1B), 6-8 week old BALB/c mice were immunized as in study A. Mice were euthanized 12 hours after their final immunization. Serum, vaginal lavage, and cecum

contents were removed and frozen at -80°C. Tissues were removed and processed as previously described with modifications (293). Briefly, tissues were cut into small pieces and suspended in PBS supplemented with 5mL of DTT and EDTA in GentleMACS dissociator tubes. Tubes were sealed and placed sideways on ice and gently rocked for 15 minutes. Tubes were spun at 1500 xg for 1 minute, supernatant poured off, and tissues resuspended in another 5 mL of supplemented PBS. Rocking and supernatant removal were repeated and tissues were resuspended in 10mL of collection medium containing 450 U/mL of Collagenase VIII (Sigma-Aldrich). Vaginal samples were incubated at 37˚C for 60 minutes with one GentleMACS spin at 30 minutes and another at the end of incubation. Intestinal samples were incubated for 30 minutes at 37˚C and spun as the vaginal samples at the end of incubation. Resulting suspensions were washed with culture medium and processed as in study A. All single cell suspensions from experiment A were enumerated using Countess automated cell counter (Invitrogen) while suspensions from study B were enumerated using Cellometer Auto 2000 (Nexcelom).

The care and use of experimental animals complied with the guidelines of Colorado State University (IACUC 14-5332A).

2.2d MPER and SlpA specific ELISAs

Maxisorp high-binding 96-well plates (Nunc) were coated overnight at 4˚C with 1 μg/ml of synthetic 17-mer MPER peptide (GNEQELLELDKWASLWN, Bio-Synthesis Inc.) or SlpA isolated from LaWT as previously described (294), suspended in carbonate coating buffer (Na_2CO_3 15Mm, $NaHCO_3$ 35mM in ultrapure water). Wells were blocked with 1% BSA in PBS for 1 hour at room temperature. After washing in 0.05%Tween/1% BSA/PBS, serum, vaginal lavage, fecal pellets and cecum contents were serially diluted 1:10 in PBS, added to wells, and

incubated for 2 hours at room temperature. Following washing, HRP-anti-IgG (serum) or HRP-anti-IgA (other fluids) were added and incubated for 1 hour at room temperature. Color development with 3,3',5,5'-tetramethylbenzidine (TMB) was terminated with sulfuric acid and absorbance (570-450 nm) was measured. To determine endpoint titer, fluids from each vaccination group prior to vaccination were included in the assay. The cutoff was calculated as the mean value of all negative controls for each vaccination group + 3.365 standard deviations, based on the 99% confidence interval standard deviation cutoff multiplier for an n of 6 (295).

2.2e MPER-specific and total IgA ELISPOTs

One day before cell isolation two sterile ELISPOT plates (MAIPSWU, EMD Millipore) were primed with sterile 70% ethanol, washed with water, coated with anti-IgA coating antibody at 10 μg /mL (ELISpot^{Plus} Kit, Mabtech), and sealed and incubated overnight at 4˚C. On the day of tissue isolation, two hours before cells were added the coating antibody was discarded and wells blocked with 200 μL/well of growth media. After incubation one plate (T) received 10,000 cells/well in 100 μL of growth media, in duplicate. The other plate (S) received 250,000 cells/well in 100 μL of growth media, in duplicate if possible. If enough cells could not be obtained from tissue then tissues were combined (typically only LI). Cells were incubated at 37˚C for 20 hours. After incubation cells were discarded and wells washed with PBS-Tween (0.05%). T plates received 100 μL/well of anti-IgA-biotin (1 μg/mL), while S plates received 100 μL/well of MPER-biotin (1 μg/mL, GNEQELLELDKWASLWN-biotin, Bio-Synthesis Inc.) and both were incubated for 2 hours at room temperature. After discard and washing 100 μL/well of Streptavidin-HRP was added and incubated for 1 hour at room temperature. Wells were then washed 3x with PBS-Tween, 3x with PBS, and 100 μL/well of 0.44 μm fresh filtered

TMB was added and incubated for 10 minutes (S plates) and 2 minutes (T plates). Reaction was stopped by washing 9x with distilled water. Plates were dried and spots counted using an Immunospot analyzer (Cellular Technology Limited). The B cell percentage of total cells plated was determined by flow cytometry. The number of MPER-specific antibody-secreting cells (ASC) per 250,000 B-cells was calvulated according to the following formula: (ASC counted) x (250,000/(250,000 x %B cells plated)).

2.2f Flow Cytometry

For B cell staining, single cell suspensions were washed and blocked with purified CD16/CD32 (BD). All antibodies used were anti-mouse unless stated otherwise. Cell suspensions were stained with anti-CD45-FITC, anti-CD38-PeCy7, and anti-CD19 Pacific Blue. For Treg staining, single cell suspensions were stained with anti-CD3-FITC, anti-CD4-PECy7, and anti-CD25-APC. Cells were then fixed and permeabilized according to the FOXP3 Fix/Perm Buffer Set protocol (eBioscience) and stained with anti-FoxP3-PE. All antibodies are from BioLegend unless specified. Cell markers were observed using a Gallios flow cytometer (Beckman Coulter).

2.2g Fecal and cecum content DNA extraction, sequencing, and data processing

Fecal and cecum DNA was extracted using the PowerFecal DNA isolation kit (MoBio). The hyper variable region 4 (V4) of 16s ribosomal RNA was amplified by PCR and resulting amplicon libraries quantified and normalized. Samples were fragmented and sequenced using MiSeq (Illumina) at the Colorado State University Nextgen Sequencing Core, with paired 250-nucleotide reads (296). Resulting overlapping fragment reads were recombined to form contigs

and trimmed to eliminate low quality contig ends. These sequenced reads are filtered and duplicates collapsed, resulting in final sequences. These final sequences were matched by 97% similarity to matching operational taxonomic units (OTUs). Each OTU represents a taxonomic rank, typically down to the bacterial species, with the resulting group of OTUs per sample representing the microbiome of that sample. Processing was performed using mothur software (Dr. Patrick Schloss (297)) and principle component analysis generated using the vegan plugin for R software (R development team).

2.2i Statistics

Groups were compared for significant differences by analysis of variance using the non-parametric Mann-Whitney-Wilcoxon test using Prism 6.0h software (GraphPad). Significance between serum and vaginal antibody levels over time were calculated by comparison of area under independent receiver operating characteristic (ROC) curves, also using Prism 6.0h. Differences between SlpA ELISA groups were compared by Dunn's multiple comparisons test following Kruskall-Wallace H test.

Table 2.1 Mutant *L. acidophilus* strains used in this study. All strains were previously derived from *Lactobacillus acidophilus* substrain NCFM.

Strain	Erythromycin resistance	Surface MPER SlpA Fusion Protein	Adjuvant (via plasmid)	Reference
LaWT	+	−	−	(298)
MPER	+	+	−	(136)
MPER+IL-1β	+	+	+ (Secreted mouse IL-1β)	(136)
MPER+FliC	+	+	+ (Surface-displayed, ionic-bound *Salmonella* flagellin protein C)	(291)

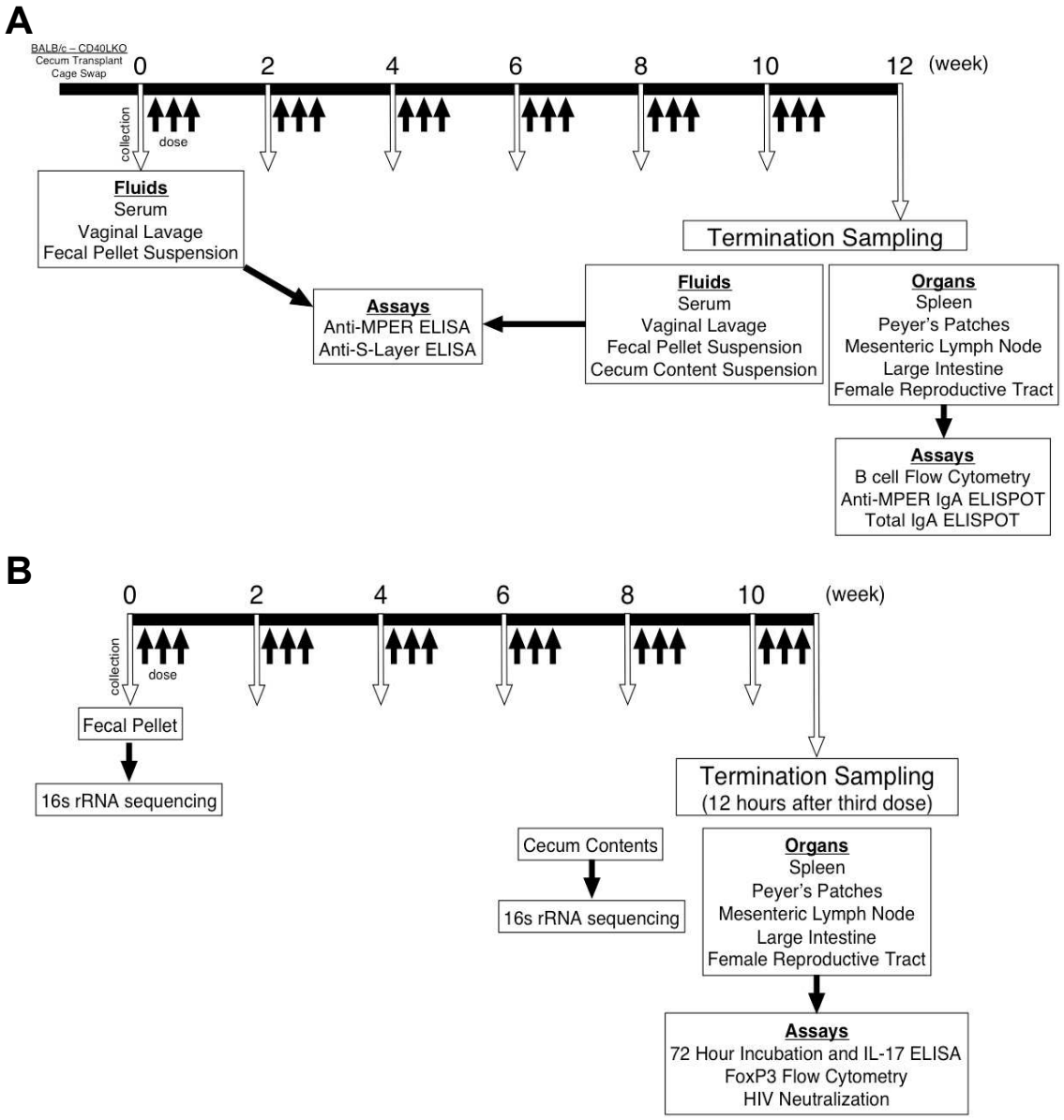

Figure 2.1 Study design. Two study designs were used for these experiments. Both studies have identical vaccination schedules. Animals in study B were terminated 60 hours after the first final immunization (12 hours after third final immunization).

2.3 Results

2.3a Oral administration of adjuvanted MPER-expressing *L. acidophilus* produces significant levels of anti-MPER serum IgG

We have previously shown that oral administration of strains expressing only MPER or MPER+IL-1β can induce anti-MPER antibodies (136). Here we used these two strains, along with a MPER+FliC strain (See Table 2.1), to determine if the addition of FliC would enhance MPER immunogenicity in BALB/c mice. Groups of mice were administered 5×10^9 CFU of each strain for three days every two weeks, for a total of 6 administrations. Two weeks after the final dose animals were sacrificed and serum, vaginal lavage, fecal pellets, and cecum contents were collected. Anti-MPER antibody levels were determined by endpoint dilution in an isotype-specific ELISA. Our results show that when MPER-expressing *L. acidophilus* is administered in conjunction with constitutively expressed secreted IL-1β or surface FliC, anti-MPER antibody levels are significantly higher in serum (Figure 2.2). Anti-MPER serum IgG levels were not significantly different between adjuvant groups. In contrast, no significant difference was observed for anti-MPER IgA levels in vaginal lavage between adjuvant strains or compared to the MPER-only strain. Levels of anti-MPER IgA in fecal pellets and cecum contents were typically too low to be detected, thus no statistical differences were observed between treatment groups.

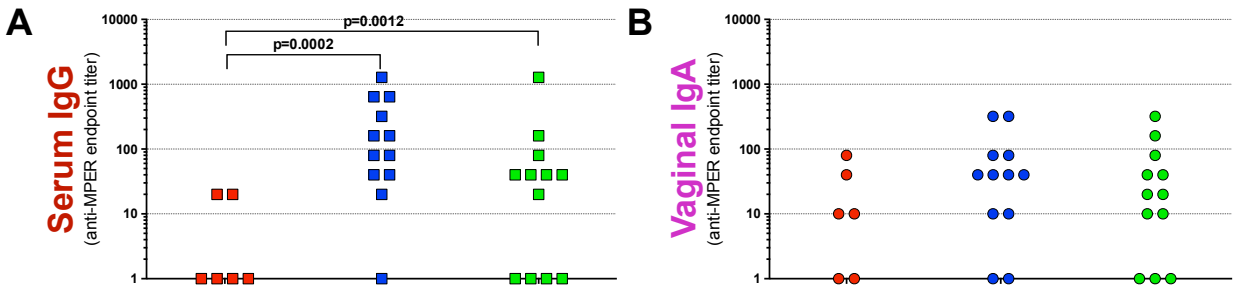

Figure 2.2 Anti-MPER antibody endpoint titers are significantly higher in adjuvant mice. BALB/c mice were treated orally with *L. acidophilus* strains. Serum (A) and vaginal lavage (B) were collected and processed as described in Methods. Anti-MPER IgG was measured in serum, anti-MPER IgA was measured in vaginal lavage. Groups (MPER N=6, MPER+IL-1β and MPER+FliC N=12) were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. If significant, exact two-tailed P-value is displayed.

2.3b Significant antigen-specific serum IgG was induced by *L. acidophilus* strains expressing adjuvant.

We have previously shown that anti-MPER antibody levels in the serum increase over time when mice are vaccinated every two weeks (136). Our goal was to similarly assess immunogenicity of MPER+FliC as measured by serum IgG and vaginal IgA. To determine anti-MPER antibody levels over time, serum and vaginal lavage were collected for each animal prior to each vaccine administration. Serum anti-MPER IgG or vaginal anti-MPER IgA titers were determined by ELISA. Our results showed that *L. acidophilus* combined with either secreted IL-1β or surface FliC induced significantly higher levels of serum IgG over the vaccination course (Fig. 2.3A). MPER-specific vaginal IgA was not significantly different between vaccine groups (Fig. 2.3B).

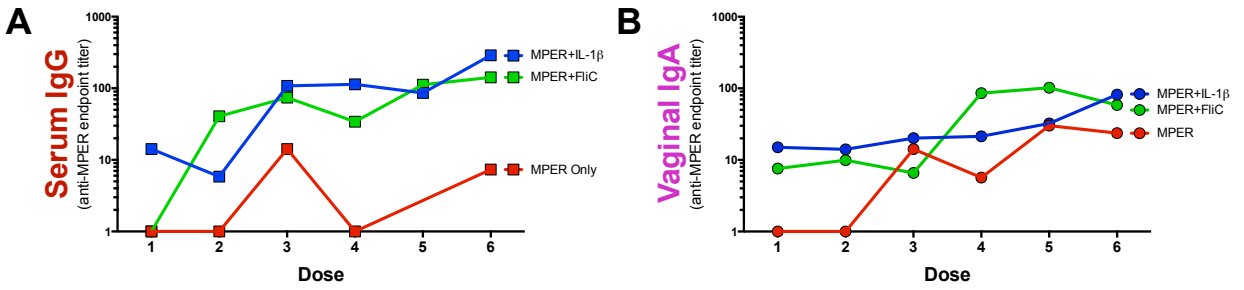

Figure 2.3 Anti-MPER antibody endpoint titers are elevated in mice over time. BALB/c mice were treated orally with *L. acidophilus* strains. Serum and vaginal lavage were collected every two weeks prior to each vaccine administration as described in Methods. Anti-MPER IgG was measured in serum (A), anti-MPER IgA was measured in vaginal lavage (B). The mean (MPER *N*=6, MPER+IL-1β and MPER+FliC *N*=12) for each timepoint is plotted. Significance between vaccinations was calculated by comparison of areas under independent receiver operating characteristic (ROC) curves. Only MPER vs. MPER+IL-1β (IgG; p=0.0225) and MPER vs. MPER+FliC (IgG; p=0.0358) were significantly different.

2.3c Oral *L. acidophilus* stimulated anti-S-layer IgG and IgA in all vaccine groups.

We have previously shown immunization with *L. acidophilus* consistently induces anti-S-layer antibodies (136). To determine whether the IL-1β or FliC adjuvants influenced anti-S-layer antibody titers, mouse fluids were endpoint diluted and measured by anti-S-layer ELISA. Our results show that anti-S-layer antibody titers were not significantly higher in any vaccinated group (Figure 2.4).

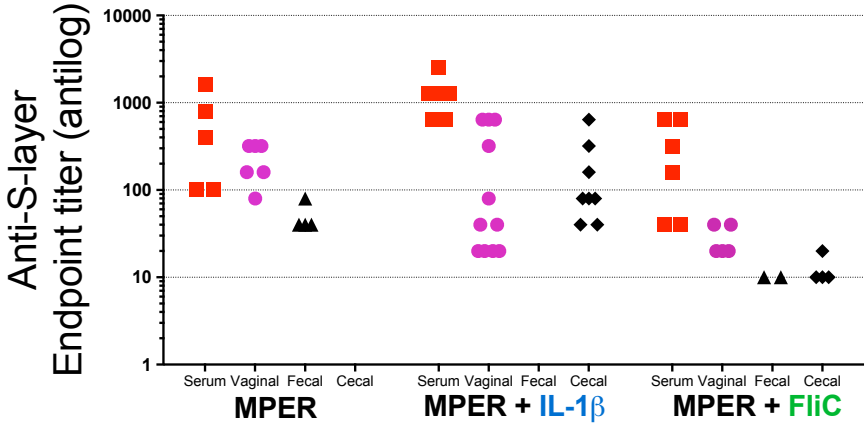

Figure 2.4 Anti-S-Layer antibody endpoint titers are the same regardless of adjuvant strain. BALB/c mice were treated orally with *L. acidophilus* strains. Serum, vaginal lavage, fecal pellets, and cecum contents were collected as described in Methods. Anti-S-layer IgG was measured in serum, and anti-S-layer IgA was measured in other fluids. Groups were compared by Dunn's multiple comparisons test. MPER and MPER+FliC *N*=6, MPER+IL-1β *N*=12. No significance differences between vaccine group fluids were observed.

2.3d MPER-specific IgA-secreting cells are increased following oral vaccination with *L. acidophilus* with adjuvants.

In order to identify actual numbers of antibody-secreting cells and their tissue locations, single-cell suspensions of various tissues were analyzed by anti-MPER ELISPOT. At the time of termination, mouse spleen, mesenteric lymph nodes (MLN; 1-2 per mouse), Peyer's patches (PP; 5-6 per mouse small intestine), female reproductive tract (FRT; vagina to ovaries), and large intestine (LI; from end of cecum to rectum) were removed and digested to single-cell suspensions. 250,000 cells were plated in duplicate and the number of spots after 18 hours of 37C incubation were analyzed by ELISPOT reader. A portion of each cell suspension was analyzed by flow cytometry to determine the percentage of CD45+ CD19+ cells. Our results indicate that MPER-specific B cells following *L. acidophilus* vaccination typically reside within mucosal tissues. Only *L. acidophilus* secreting IL-1β showed a significant increase in antibody-secreting cells within the FRT (Fig. 2.5A). However, when all MPER-specific IgA-secreting cells are pooled (total MPER-specific IgA-secreting cells/animal) both IL-1β and FliC adjuvanted *L. acidophilus* showed significantly higher percentages of secreting cells (Fig. 2.5B).

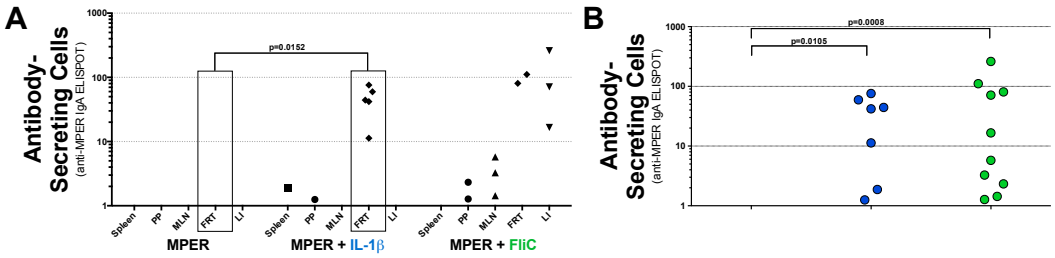

Figure 2.5 Anti-MPER IgA-secreting cells are elevated in adjuvant strain mice. BALB/c mice were treated orally with *L. acidophilus* strains. Spleen, Peyer's patches, mesenteric lymph node, female reproductive tract, and large intestine (sans cecum) were single-cell digested as described in Methods. 250,000 cells/well were plated and resulting spots measured by ELISPOT reader. Number of anti-MPER IgA-secreting cells were extrapolated based on flow cytometry % of CD45+ CD19+ cells (A). MPER *N*=6, MPER+IL-1β and MPER+FliC *N*=12. FRT were pooled in groups of two, LI were typically pooled in groups of two or more. For graph (B) all tissues were pooled, comparing the frequency of cells within each vaccine group. Groups were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. Significant exact two-tailed p-values are displayed. ASC = antibody secreting cells.

2.3e Vaccine strain did not affect total number of IgA-secreting cells.

In order to identify the total number of IgA-secreting cells in each tissue compartment, single-cell suspensions of various tissues were measured by anti-IgA ELISPOT. Our results show that there was no difference between vaccine groups regarding total IgA-producing cells, and that large intestines possessed the most IgA-secreting cells of the isolated tissues (Fig. 2.6).

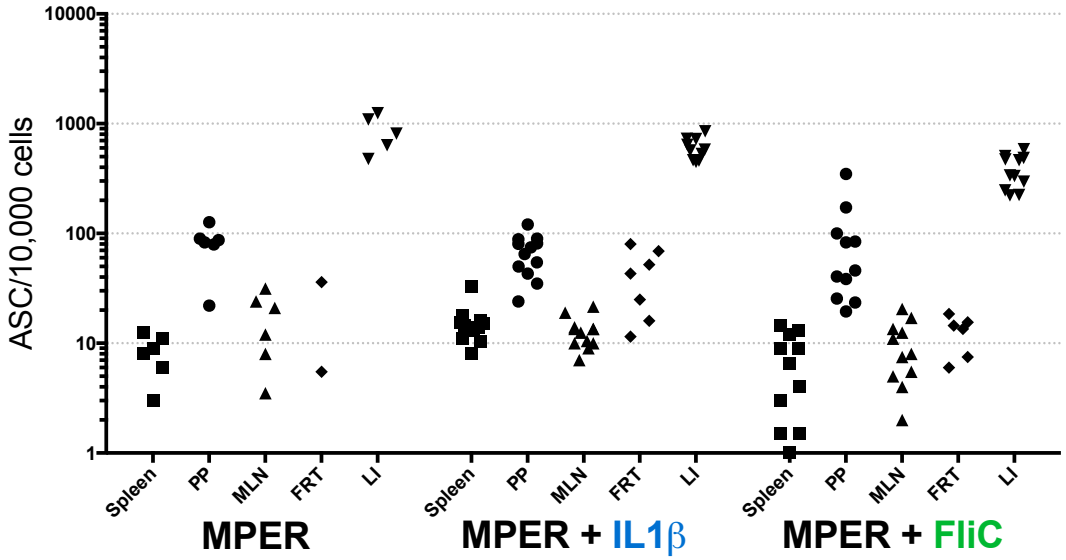

Figure 2.6 BALB/c IgA-secreting cells are elevated in large intestines. BALB/c mice were treated orally with *L. acidophilus* strains. Spleen, Peyer's patches, mesenteric lymph node, female reproductive tract, and large intestine (sans cecum) were single-cell digested as described in Methods. 10,000 cells were plated and resulting spots measured by ELISPOT reader. MPER N=6, MPER+IL-1β and MPER+FliC N=12. FRT were pooled in groups of two, LI were typically pooled in groups of two or more. Groups were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. Significant exact two-tailed p-values are displayed. ASC = antibody secreting cells.

2.3f CD40L$^{-/-}$ animals have lower levels of MPER and S-layer-specific antibodies in serum and vaginal lavage fluid.

We have previously shown that oral administration of *L. acidophilus* expressing MPER+IL-1β resulted in IgG3b antibody secretion (136). Because this antibody subtype is associated with T-independent antibody production, we orally dosed CD40LKO mice with *L.*

acidophilus vaccine strains to identify if the vaccine immune response requires CD40L binding to CD40 (classical T-dependent activation). Our results indicate that anti-MPER antibody levels were significantly lower in CD40L$^{-/-}$ mice versus BALB/c wild type mice, except serum IgG in the MPER only vaccination groups (Fig. 2.7A). Anti-S-Layer antibody levels were significantly higher in all BALB/c groups versus CD40L$^{-/-}$ (Fig. 2.7B).

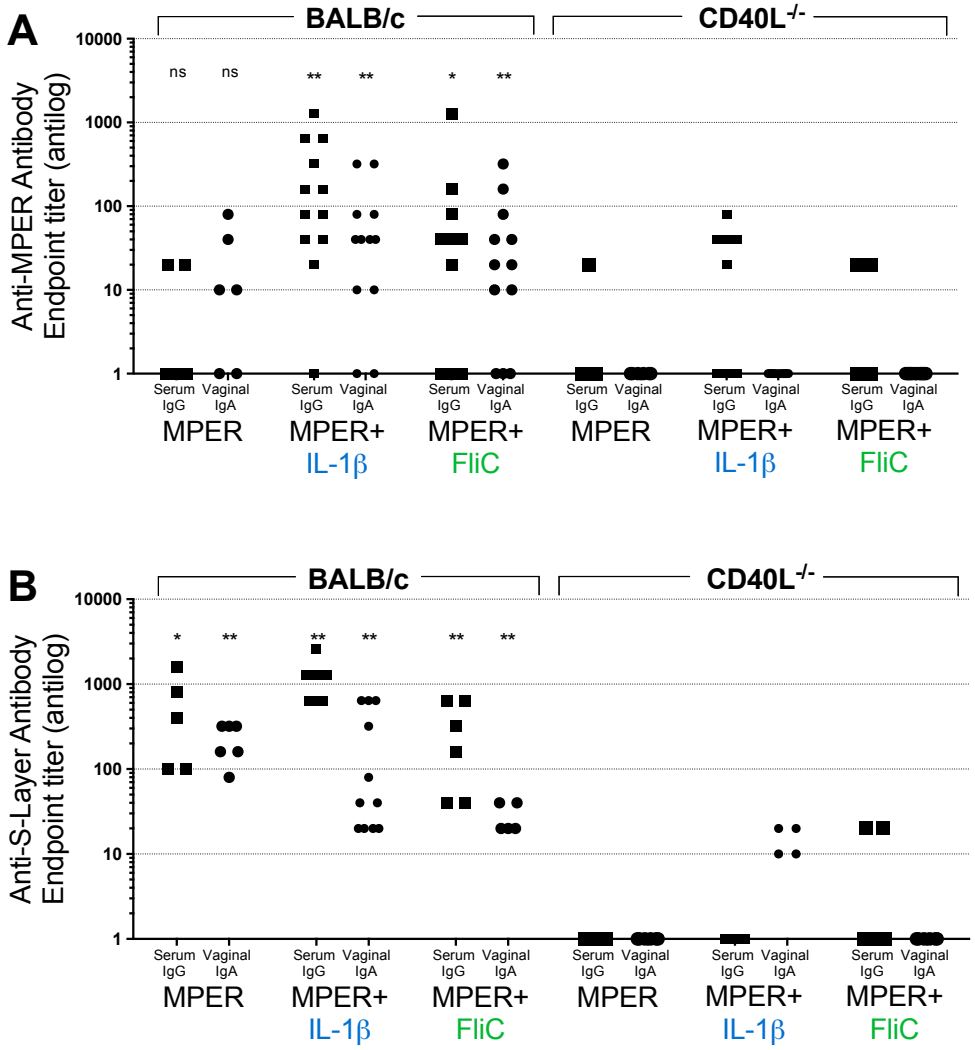

Figure 2.7 Anti-MPER and anti-S-layer antibody endpoint titers are much lower in CD40LKO mice than BALB/c. BALB/c and CD40L$^{-/-}$ mice were treated orally with *L. acidophilus* strains. Serum and vaginal lavage were collected as described in Methods. Anti-MPER serum IgG and vaginal IgA (A), and anti-S-Layer serum IgG and vaginal IgA (B), were measured by endpoint titer of terminal fluids. BALB/c and CD40L$^{-/-}$ had the same number of mice, MPER *N*=6, MPER+IL-1β and MPER+FliC *N*=12. The non-parametric Mann-Whitney-Wilcoxon test was used to tesy for significant differences between mouse strain/fluid/vaccination (ex. BALB/c Serum MPER vs. CD40L$^{-/-}$ Serum MPER). *p<0.05, **p<0.01.

2.3g CD40L MPER and Total IgA ELISPOT levels

MPER-specific responses in CD40L$^{-/-}$ mice would indicate the potential for a T-independent B cell activation pathway. Cell suspensions from all CD40L-/- tissues were subjected to MPER-specific IgA ELISPOT and total IgA ELISPOT, as with BALB/c mice. No MPER-specific IgA secreting cells were detected in any vaccination group (Fig 2.8A). Total IgA secreting cells were detected in most tissues (Fig. 2.8B), but had ~2-4 fold less cells than their wild-type counterparts (Table 2.2).

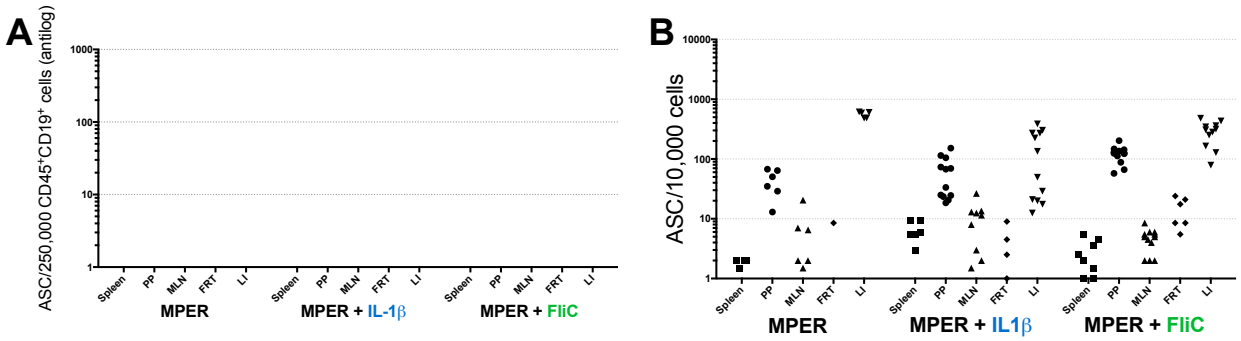

Figure 2.8 No MPER-specific antibody secreting cells were detected in CD40LKO tissues. CD40L$^{-/-}$ mice were treated orally with *L. acidophilus* strains. Spleen, Peyer's patches, mesenteric lymph node, female reproductive tract, and large intestine (sans cecum) were single-cell digested as described in Methods. 10,000 cells were plated and resulting spots measured by ELISPOT reader. MPER *N*=6, MPER+IL-1β and MPER+FliC *N*=12. FRT were pooled in groups of two, LI were typically pooled in groups of two animals or more. Groups were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. Significant exact two-tailed p-values are displayed. ASC = antibody secreting cells.

Table 2.2 Total IgA is elevated in BALB/c mice versus CD40LKO. Total numbers of spots for each tissue and vaccination were averaged for BALB/c mice. These averages were then divided by the corresponding organ/vaccination in CD40L$^{-/-}$ mice. A ratio greater than 1 indicates a higher number of ASC in BALB/c mice.

BALBc/CD40L$^{-/-}$	MPER	MPER+ IL-1β	MPER+FliC	Average
Spleen	6.2	4.5	3.6	4.8
Peyer's Patch	1.9	1.1	0.7	1.2
Mesenteric Lymph Node	2.5	1.7	2.1	2.1
Female Reproductive Tract	4.9	0.9	0.9	2.2
Large Intestine	1.8	4.1	1.3	2.4

2.3h Foxp3 and CD25 expression by T helper cells is stable regardless of vaccination.

The lack of specific immune responses in CD40L$^{-/-}$ mice implies that *L. acidophilus* vaccination requires T cell help. To better characterize the T cell responses, a second set of experiments using only BALB/c mice was initiated (Fig. 2.1C). Mice received the same *L. acidophilus* and dosing conditions as in the initial study but were sacrificed 12 hours after the final dose (60 hours after the first of the final three doses). The resulting single cell suspensions were stained for CD3, CD4, CD25, and FoxP3 and analyzed via flow cytometry. CD3+CD4+ cells typically fall under the category of T helper cells. CD25 is one of several markers of activation, and FoxP3 (required for T regulatory function) expression in the presence of CD25 expression generally marks T regulatory cells. Initially, the CD25-FoxP3+ T helper cell thought to be a minor population of inactive but potential Tregs (299). Recently, these cells have been shown to be the primary subset of functional Tregs within mucosa (300). In this study activated T helper cells (FoxP3-CD25+) in the spleen remained stable regardless of vaccination. The percentage of traditional Tregs was reduced in MPER+IL-1β mice and trended towards an increase in MPER+FliC mice, while the percentage of non-traditional Tregs was increased in the MPER+FliC mice (Fig. 2.9A). Peyer's patches from MPER+FliC immunized mice also exhibited an increase in both subsets of Tregs (Fig. 2.9B). In the mesenteric lymph node this trend continued in MPER+FliC versus MPER+IL-1β animals regarding non-traditional Tregs, but LaWT animals had significantly higher levels of traditional Tregs and activated T helper cells (Fig. 2.9C).

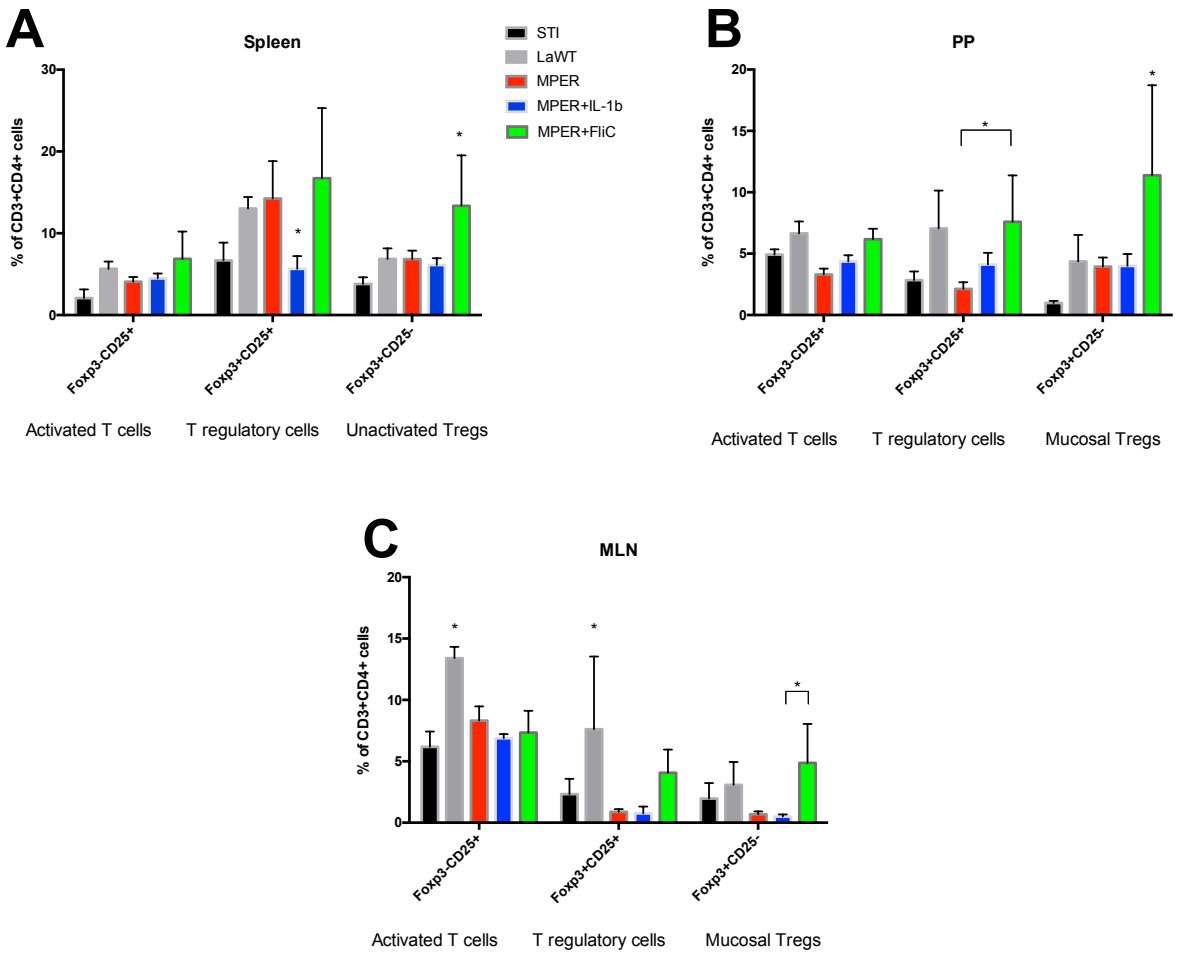

Figure 2.9 Treg marker expression is elevated in CD3+CD4+ T cells of Peyer's patches. Single cell suspensions of each organ were stained with antibodies against CD3, CD4, CD25 and intracellular FoxP3. Significance was calculated by Tukey's multiple comparisons test following two-way ANOVA.

2.3i IL-17 ELISA in 72 hour supernatants are slightly elevated in Peyer's patches.

In order to measure IL-17 production single cell suspensions of 1 million cells in 200 L of growth medium were incubated for 72 hours. Supernatants were removed and run in duplicate on IL-17 ELISA. IL-17 expression was low or undetectable in spleen and MLN regardless of vaccination. However, cells from Peyer's patches in MPER-only and MPER+FliC vaccinated mice had significantly higher levels of IL-17 than either control vaccination (Fig. 2.10). IL-17 levels after MPER+IL-1β vaccination was significantly lower versus both MPER-alone and

MPER+FliC, and MPER+FliC was significantly lower than MPER-alone (Fig. 2.10, bottom two significance lines). These results indicate that vaccination does induce elevated levels of IL-17 that might raise safety concerns for inflammation in the mucosa.

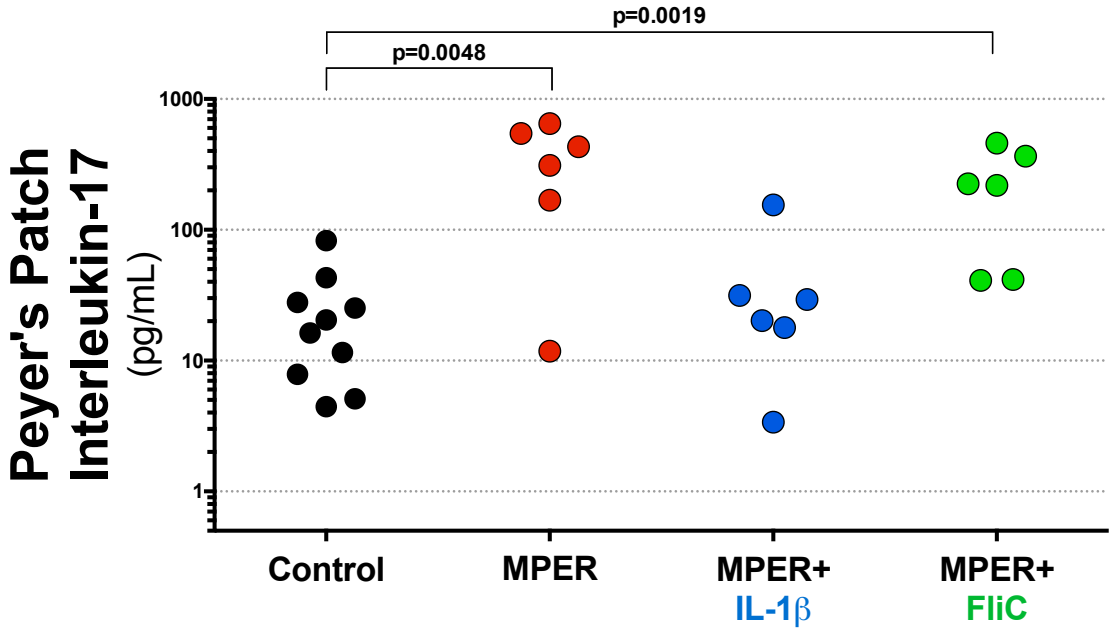

Figure 2.10 IL-17 is elevated in MPER and MPER+FliC incubated cell supernatant. $1x10^6$ cells were isolated from Peyer's patches and incubated for 72 hours in 200uL of growth media. Values below limit of detection are not displayed. Groups were compared by analysis of variance using Holm-Sidak's multiple comparisons test following a normal one-way ANOVA (**p<0.01, *p<0.05).

2.3j *L. acidophilus* vaccine strains induce changes in intestinal microbial community structure.

The gut microbiome is a powerful organ, protecting against invading pathogens and helping maintain a homeostatic mucosal environment (71, 301). The influence of the microbiome and probiotics on vaccine efficacy has been tested in a number of studies, with some showing improved vaccine efficacy and others showing no difference or even negative effects (302, 303). However, the effects of mucosal vaccine vectors on the gut microbiome are relatively unexplored (304).

Research on mucosal bacterial influence is often hampered by expensive sequencing assays, daunting data analysis and confounding variables such as diet, genetics and the number of study participants. Of the few studies conducted all utilized live attenuated versions of pathogens, with two studies showing no effect on the microbiome (305, 306), and one showing variations in diversity that correlated with non-vaccine variables such as animal handling and host geographic origin (307). These results point to mucosal vaccination as a relatively minor perturbation for the host microbiome, often with no evident consequences. This has not been tested with non-pathogenic mucosal vaccines, such as lactobacilli vectors. These vectors provide an interesting window into the potential influence dual-function bacteria (probiotic and vaccine) may serve in the host. Any potential alterations could impact, for better or worse, the delicate symbiosis between host mucosal immune system and commensal bacteria. To this end the fecal microbiome of BALB/c mice was analyzed to determine if any changes in microbiome species structure occurred in conjunction with vaccination.

The fecal microbiome of three mice per group at three timepoints for each immunization group were analyzed by sequencing of bacterial 16s rRNA to detect changes in microbial community structure. Differences between timepoints within each treatment group were measured by principle components analysis (PCA). Untreated animal microbiome clustering had little variation over time, resulting in overlapping of PCA ovals (Fig. 2.11A). When vaccine strains were introduced significant microbial community structure changes were observed. MPER-only vaccination caused considerable changes after 2 weeks, with a slight restoration towards pre-vaccine species at 6 weeks (Fig. 2.11B). MPER+IL-1β vaccination revealed changes as well, with the microbiome shifting further over time (Fig. 2.11C).

MPER+FliC vaccination caused an initial shift, which changed slightly over time but still possessed similarities versus timepoint 0 (Fig. 2.11D).

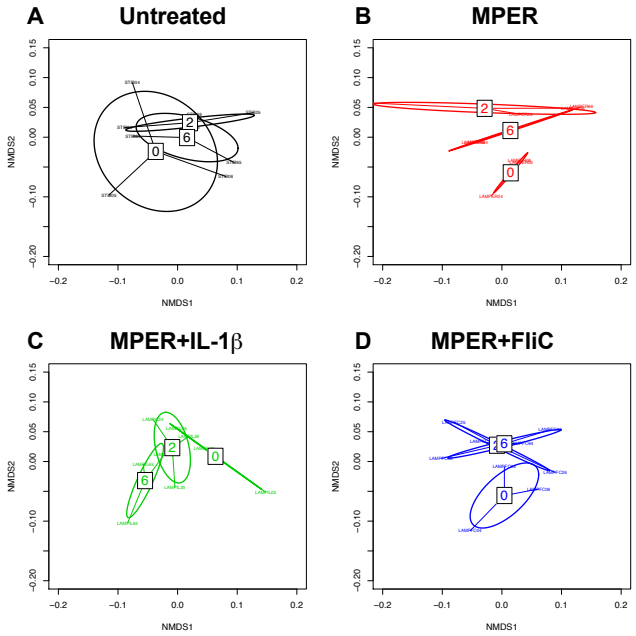

Figure 2.11 Variations in microbial community structure over time following vaccination. Following sequencing and contig cleanup OTUs for each vaccination and timepoint were compared via nonmetric multidimensional scaling principle components analysis (NMDS). Timepoints 0 (pre-vaccination), 2 (2 weeks after first vaccination), and 6 (6 weeks after first vaccination) are shown for untreated (A), MPER-only (B), MPER+IL-1β (C) and MPER+FliC (D).

2.4 Discussion

An effective HIV vaccine is desperately needed. Even the most promising vaccination strategies have had limited success (217). Alternative HIV vaccination strategies offer advantages over traditional parenteral (non-mucosal) vaccination. One of these alternative strategies is the lactic acid bacteria (LAB) vector. The past two decades have seen a drastic increase in the number of studies utilizing LAB as vector systems, particularly of the genus *Lactobacillus* (130, 133).

Lactobacilli offer a number of advantages over other vaccine methods, including safety, straightforward non-parenteral administration, and relatively simple mass production (127). Most importantly lactobacilli readily accept heterologous antigens and possess intrinsic adjuvanticity that can be augmented with heterologous adjuvants. However, before lactobacilli can be used clinically their ability to target notoriously non-immunogenic HIV antigens must be better understood (308).

To that end we constructed several *Lactobacillus acidophilus* strains expressing the highly conserved MPER portion of HIV Env gp41 (136). Previous vaccines have encountered problems with immunogenicity of this region (136, 278-280). The addition of adjuvants could enhance the anti-MPER immune response, with evidence pointing to toll-like receptor signaling as a key mechanism for this enhancement (136, 278, 309). To take advantage of TLR signaling an adjuvant *L. acidophilus* strain targeting TLR5 was constructed (291). Our primary goal was to determine if these adjuvant-expressing lactobacilli could improve immunogenicity against HIV MPER. Our secondary goal was to better elucidate the mechanisms behind enhanced immunogenicity.

Our primary conclusion is that adjuvants significantly improved lactobacilli immunogenicity as measured by both systemic and mucosal antibody immune responses. Our secondary conclusions show T cell help is required for antibody induction, that *Lactobacillus* vaccination modulates IL-17 and Treg responses, and vaccination induces significant changes to the gut microbiome.

A variety of adjuvants to improve lactobacilli vaccine responses have been attempted, with promising results for improved antibody and cell-mediated immunity (143, 144, 310, 311). In this study MPER-lactobacilli expressing either surface FliC or secreted IL-1β significantly

improved systemic IgG versus non-adjuvant MPER-*L. acidophilus*. Levels of vaginal anti-MPER IgA were not significantly increased with adjuvant strains, but the presence of anti-HIV antibodies in the vaginal mucosa is likely key for protection against HIV challenge (312, 313). The number of anti-MPER IgA secreting cells was significantly higher with adjuvant lactobacilli, indicating that these adjuvants may circumvent clonal deletion of MPER-specific B cells (278).

Previous experiments by our lab and others indicated the potential for a T independent B cell response, which would be advantageous against HIV (39, 136, 278). We addressed this question by vaccinating CD40L knockout mice, a strain incapable of traditional CD40-CD40L T-cell dependent B cell activation. Anti-MPER ELISA and ELISPOT had virtually no anti-MPER antibody or anti-MPER antibody secreting cells. This indicates that T cell help through traditional CD40-CD40L binding is required for proper anti-MPER antibody production in this model system.

The oral route of delivery, as opposed to parenteral routes, is a key factor in understanding resulting immune responses. The intestinal immune environment is complex, having to strike a delicate homeostatic balance between tolerance of commensal bacteria and inflammation against potential pathogens (314). This has been demonstrated by lactobacilli vaccine studies that have variations in Th17 (inflammatory) and Treg (tolerance) cytokines and cell levels (146, 148-150, 315, 316). In this study we made similar observations of this push-and-pull between inflammation and tolerance. Proinflammatory IL-17 levels were elevated in the Peyer's patches of vaccinated animals, while mucosal Treg levels were also elevated.

These variations were not consistent across all vaccinations and more data are needed, but evidence in our study and others points to lactobacilli vaccines as potent influencers of intestinal homeostasis.

The homeostatic balance of the gut is influenced not only by the host but the commensal bacteria within. Dysbiosis of the gut microbiome can have severe consequences (317). Therefore the effects of oral vaccines on the microbiome should be considered. Only a few studies have attempted to identify any changes in the gut microbiome following mucosal vaccination, with most evidence pointing to vaccines having little effect (305-307). In order to observe the effects of our lactobacilli strains on the gut microbiome we measured fecal 16s rRNA for each vaccine group over time. To our surprise there were significant differences in microbiome composition within each vaccination group over time. These data suggest that mucosal vaccination with probiotic bacteria can alter the gut microbiome. Such alterations may be beneficial, detrimental or inconsequential to the host, but regardless future vaccine studies must take these changes into account.

Although the results and implications of these experiments are exciting and intriguing, there are several weaknesses. By conducting these studies in mice we were limited by immune differences between mice and humans, particularly in the mucosa (318, 319). There is also no standardized measure of anti-MPER antibodies, thus comparisons between endpoint titrations must be used. Other assays suffer from low cell numbers from mucosal tissues, as well as the small volumes of sera and vaginal fluid, limiting the number of experiments that can be performed. Ultimately, challenge studies in non-human primates will be required to determine the efficacy of the *Lactobacillus* vaccine platform.

Despite some limitations in assays and the model system, results from these experiments allow us to make several important conclusions. The adjuvants used to augment MPER-*L. acidophilus* significantly improved anti-MPER antibody responses. These responses required traditional T cell help for antibody production, and vaccination caused variations in markers of inflammation and tolerance, as well as variations in the gut microbiome over time. In conclusion *L. acidophilus* vaccine vectors targeting HIV offer a promising system that demands further research.

Chapter 3: Effects of Rice Bran on *L. acidophilus* Vaccine Strains *In Vitro* and *In Vivo*

3.1 Overview

Prebiotics are dietary fibers and starches that are indigestible by the host but may be used as substrates for bacterial fermentation. Prebiotics have been shown to influence the mucosal immune system by altering the intestinal flora, typically by providing nutrients to selected bacteria (typically probiotics like lactic acid bacteria) (320, 321). This boost of beneficial commensals usually results in a reduction of inflammation and an increase in Tregs and anti-inflammatory cytokines (321). It was proposed that prebiotic supplementation could improve vaccine immune responses, especially in undernourished youth (322). However, studies conducted had mixed results, with animal studies showing some successful use against *Salmonella* and influenza (323, 324), while human trials generally showed no change in vaccine efficacy (325, 326), though interestingly allergic responses tended to be reduced (302, 327).

When rice undergoes processing for food consumption the inner kernel is separated from outer components like the hull, bran and germ preventing spoilage (255). The vast majority of nutrients, including several classified as potential prebiotics, are stored within these discarded outer layers (256-258). These layers, colloquially known as rice bran (RB), have been shown to have interesting health effects outside of improved nutrition. RB components have been shown to possess antibiotic (*Salmonella, Vibrio, Shigella, Escherichia)* and antiviral effects (cytomegalovirus, HIV) (255), and to modulate immune cell activity (neutrophil phagocytosis (259), increased IgG secretion (260)). Down-regulation of inflammatory cytokines (261) and sequestration of IgE (262) can reduce allergy symptoms, and a number of studies both *in vitro* and *in vivo* have demonstrated RB anticancer effects (255). Rice bran has also been shown to

influence probiotic intestinal bacteria (prebiotic effects). Mice on a 10% rice bran diet showed increased total mucosal IgA levels, likely by increasing *Lactobacillus* concentration in the gut (263), and the addition of rice bran to *Lactobacillus*-treated pigs greatly improved pathologies associated with rotavirus infection (328). These results prompted us to test the influence, if any, that rice bran and its extracted nutrients can have on vaccine-strains of *L. acidophilus*. We first measured any variations in growth and antigen expression in vaccine strains grown in rice bran extract supplemented media. We then observed if a diet supplemented with rice bran altered bacterial gut survival and immunogenicity of the MPER-only vaccine strain.

3.2 Materials and Methods

3.2a Rice bran nutrient extraction

4 g of crushed rice bran (Neptune variety) was combined with 42.6 mL of 80% Methanol, vortexed, and incubated overnight at -80°C. The solution was then centrifuged at 1500 xg for 5 minutes. Supernatant was removed and transferred to pre-weighed 1.5 mL tubes and incubated at -80°C. Samples were then dried using a SpeedVac (conditions: 45°C, 5 minute heat time, 8 hour run time, vacuum 7.5). Samples were weighed after each session until liquid loss was undetectable by scale. Samples were then stored at -20°C until used.

3.2b MRS broth with rice bran extract supplement

De Man, Rogosa Sharp (MRS) broth w/o dextrose (US Biological) was supplemented with dextrose (DEX, 20 g/L) and/or rice bran extract (RBE, 100 mL/L) to yield four separate media types (–DEX–RBE, +DEX–RBE, –DEX+RBE, and +DEX+RBE). All broths were

supplemented with 5 µg/ml of erythromycin. All *in vitro* figures use the following color scheme = –DEX–RBE, +DEX–RBE, –DEX+RBE, +DEX+RBE. **Green lines** denote the – control LaWT for FliC and IL-1β expression experiments.

3.2c Measuring growth over time

Each strain was grown in 2 mL/well of the specified media in a 24-well plate. Inoculum of $1x10^6$ bacteria/well was measured by optical density and applied to each well and mixed. 24-well plate was then sealed with Parafilm (Bemis NA) and placed in the sealed EnSpire Multimode Plate Reader chamber (Perkin Elmer). Sample chamber was maintained at 37°C. Samples were mechanically shaken by the plate reader and optical density measured at 600nm every 20 minutes for 20 hours.

3.2d Surface antigen and secreted IL-1β detection

All strains were grown statically in separate tubes in each MRS media. At the designated timepoint post inoculation (0, 4, 8, 15, 19, and 25 hours) bacteria were pelleted, washed twice in PBS, and stained with either human-anti-HIV IgG (2F5, NIH AIDS Reagent Program), followed by mouse-anti-human IgG Alexa488 (Molecular Probes), or for FliC surface expression first with rabbit-anti-FLIC IgG (BioLegend), followed by goat-anti-rabbit IgG PE (Invitrogen). For mouse IL-1β secretion, MPER+IL-1β and LaWT strains were grown in 2 mL/well of a 24-well plate as in the growth study. Bacteria were pelleted and supernatant was removed and frozen at -20°C until all samples were collected. Mouse IL-1β secretion was measured by ELISA (Ready-SET-Go!, eBioscience).

3.2e Fecal collections and plate growth

Feces (at least one pellet) were collected (Fig. 2B) by direct excretion into screw-cap tubes containing 1 mL of sterile PBS held close to the mouse anus. Feces were then homogenized using a Fast Prep-24 (MP). The resulting homogenate was then diluted 1:1000 in sterile PBS. The resulting solution was the plated on Rogosa agar + Erythromycin (where from and concentrations) using an Eddy Jet Spiral Plater (Neutec Group Inc). Plates were placed in sealed anaerobic chambers and incubated at 37°C for 48 hours. CFU/mL was calculated based on fecal weight, pre-plating dilutions, and spiral plating dilution calculations using Flash & Go Analysis software (IUL).

3.2f Mouse treatments, sample collection and sample analysis

BALB/c mice (Jackson Labs) were immunized and samples collected following the previously established regimen (Fig. 2A) with modifications. Mice receiving a 10% rice bran diet (Harlan custom feed) had samples removed for analysis prior to being placed on their custom diet. Animals were on the diet for 1 week prior to the administration of MPER-only *L. acidophilus* strain, and the diet maintained until termination. All sample collections, termination, and single cell suspensions were the same as previously described, and MPER-specific ELISA and MPER-specific IgA ELISPOTS were also as previously described.

3.2g Statistics

ELISA and ELISPOT data were compared among treatment groups for significant differences by analysis of variance using the non-parametric Mann-Whitney-Wilcoxon test using Prism 6.0h software (GraphPad). In order to eliminate any individual timepoint bias, significance between entire

in vitro curves was calculated by comparison of areas under independent receiver operating characteristic (ROC) curves.

3.3 Results

3.3a *In vitro* growth is stable regardless of supplementation

Several strains of lactobacilli have been cultured with nutrients from rice bran (263, 329, 330). However, the effects of rice bran extract on *L. acidophilus* vaccine strain growth has been unexplored. In order to measure any influence of rice bran supplementation on modified *L. acidophilus* strain growth, each of the four strains (WT, MPER, MPER+ IL-1β, and MPER+FliC) were grown in MRS medium with or without dextrose (DEX), and with or without rice bran extract (RBE). Optical density over time indicates that *L. acidophilus* fails to grow in the absence of a carbohydrate source like glucose or RBE (Fig. 4.1, black lines). The growth of *L. acidophilus* with supplemented broth (either DEX, RBE, or both) was statistically different from the non-supplemented media, but not different from one another in the LaWT and MPER-only strains. MPER+ IL-1β and MPER+FliC exhibited a significant preference for RBE alone (Fig. 4.1C and D, red lines).

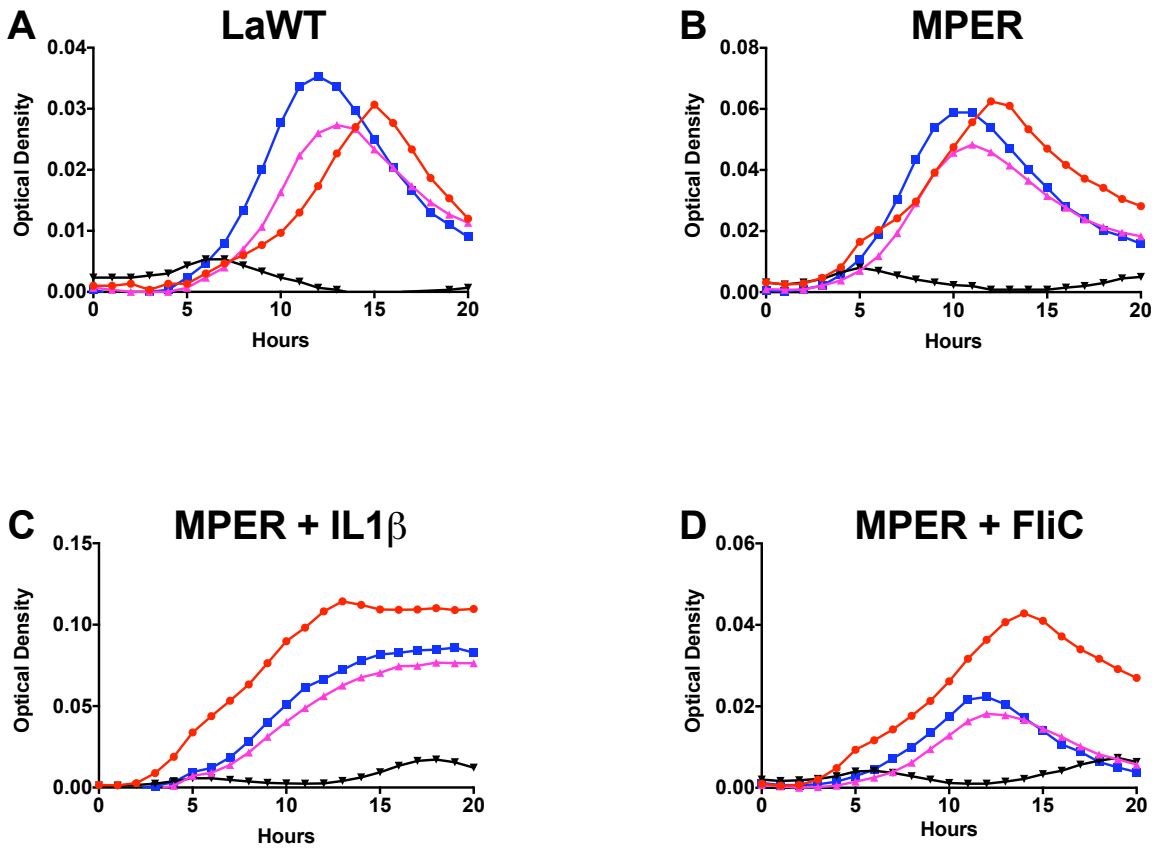

Figure 3.1 *L. acidophilus* growth varies over time under different nutrient conditions. All strains were grown in triplicate under identical conditions (sealed, 37°C) in MRS medium with different supplements (–DEX–RBE, +DEX–RBE, –DEX+RBE, +DEX+RBE). Growth at each timepoint was measured by optical density following machine stirring. Growth differences between media conditions was calculated by comparison of areas under independent receiver operating characteristic (ROC) curves. All supplemented media were significantly different ($p<0.05$) versus non-supplemented medium. –DEX+RBE was significantly different from +DEX–RBE and +DEX+RBE for MPER+IL-1β ($p=0.0044$, 0.0022) and MPER+FliC ($p=0.005$, 0.0009) strains.

3.3b Surface MPER expression remained unchanged in different growth media.

To ensure that the presence of rice bran does not alter the expression of surface MPER, each strain's MPER expression was measured over time under different media conditions for each strain. LaWT does not possess surface MPER, and thus serves as a negative control (Fig. 4.2A). All three vaccine strains showed high levels of MPER expression under all media conditions, with the exception of MPER alone when grown in non-supplemented MRS broth, likely indicating a nutrient-limiting effect of glucose-free medium (Fig. 3.2B).

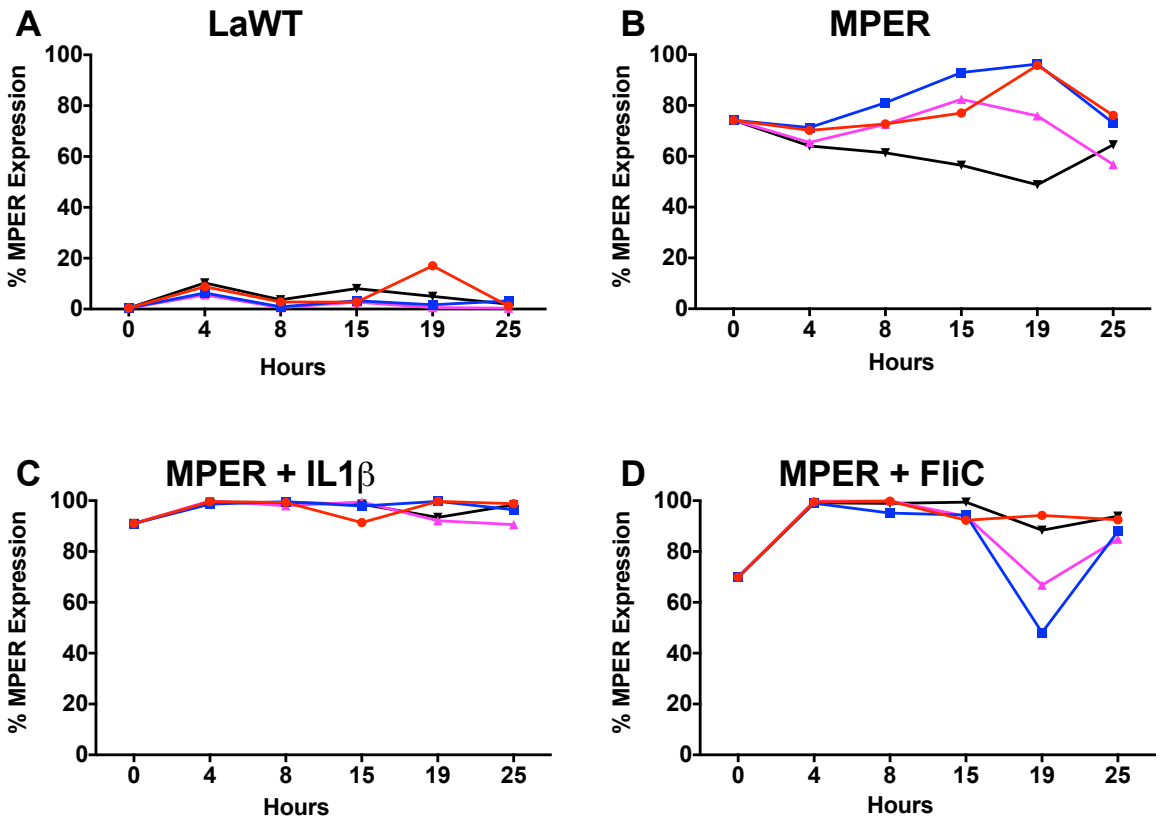

Figure 3.2 Effect of media conditions on MPER surface expression. All strains were grown under identical conditions (sealed, 37°C) in MRS medium with different supplements (–DEX–RBE, +DEX–RBE, –DEX+RBE, +DEX+RBE). MPER expression at each timepoint was measured by indirect flow cytometry after direct staining of MPER. No significant difference in MPER expression was observed between media conditions as calculated by comparison of areas under independent receiver operating characteristic (ROC) curves.

3.3c IL-1 β secretion remains the same regardless of media.

We next measured IL-1β secretion over time in MPER+ IL-1β and LaWT strains. IL-1β secretion requires time to accumulate to measurable levels, thus initial timepoints (0, 2, 4, and 6) are relatively low. By 10 hours and beyond IL-1β levels in the supernatant were significantly higher versus LaWT, regardless of media (Fig. 3.3).

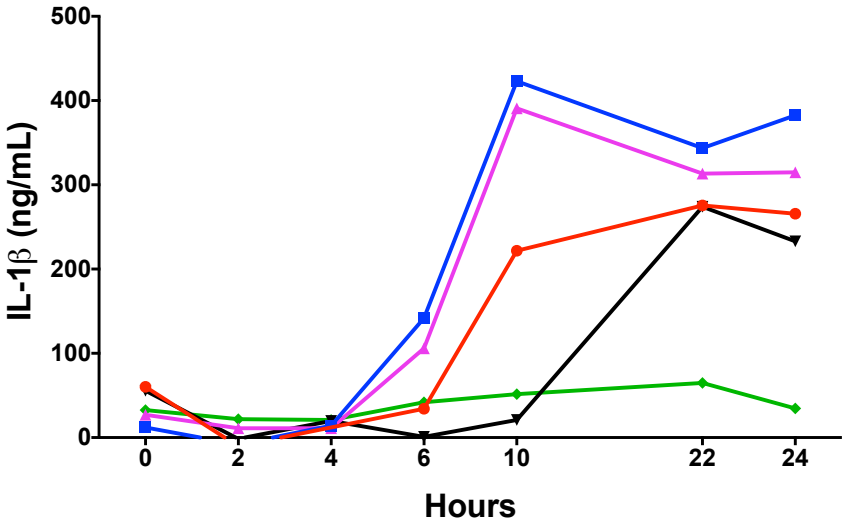

Figure 3.3 Supernatant IL-1β is elevated under all growth conditions. All strains were grown under identical conditions (sealed, 37°C) in MRS medium with different supplements (–DEX–RBE, +DEX–RBE, –DEX+RBE, +DEX+RBE, LaWT+DEX+RBE). Each timepoint is a separate tube to prevent oxygen exposure to future samples. Supernatant from each strain, in duplicate, was analyzed by mouse IL-1β ELISA. Significance between media was calculated by comparison of areas under independent receiver operating characteristic (ROC) curves. When timepoints 0, 2, 4 and 6 are excluded from analysis all strains (except –DEX–RBE) are significantly different from LaWT ($p<0.05$) and not different from each other.

3.3d FliC expression remains the same regardless of media.

To ensure that the presence of rice bran extract does not alter the expression of surface FliC, MPER+FliC and LaWT FliC expression was measured over time under different media conditions. LaWT, which does not possess surface FliC, and thus served as a negative control (Fig. 3.4, green line). All media of MPER+FliC showed significantly higher levels of FliC versus LaWT, and were not significantly different from each other.

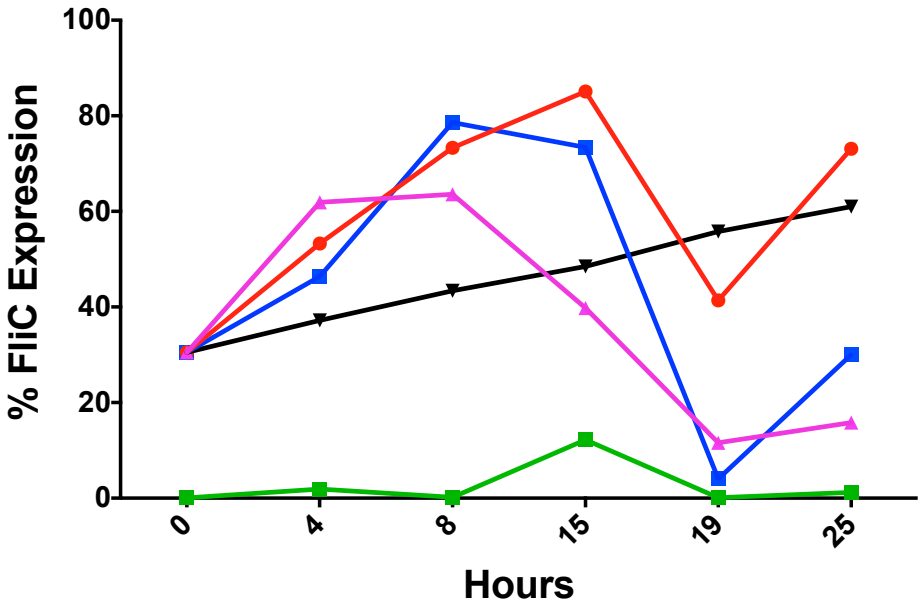

Figure 3.3 Surface FliC is elevated in all growth conditions. All strains were grown under identical conditions (sealed, 37°C) in MRS medium with different supplements (–DEX–RBE, +DEX–RBE, –DEX+RBE, +DEX+RBE, LaWT+DEX+RBE). Each timepoint is a separate tube to prevent oxygen exposure to future samples. FliC expression at each timepoint was measured by indirect flow cytometry staining of FliC. Significance between media was calculated by comparison of areas under independent receiver operating characteristic (ROC) curves. All strains were significantly different from LaWT ($p<0.01$) and not different from one another.

3.3e No difference in *L. acidophilus* vaccine strain survival over time in mice supplemented with rice bran.

Lactobacilli have been shown to utilize rice bran nutrients and are present in higher numbers in feces of animals fed a rice bran diet (263, 329, 331). We hypothesized that higher numbers of vaccine *L. acidophilus* might similarly be increased in mouse feces of animals receiving a rice bran diet. Vaccine strains were followed over the course of a typical dosing regimen (three days of consecutive dosing) and for four days after. No significant differences were observed in the frequency of any strains at any timepoint, with the exception of MPER-only versus the other strains at 24 (except MPER+IL-1β) and 48 hours after the 3rd dose. Lactobacilli levels peaked between 10^3 and 10^4 bacteria/mg of feces, which progressively decreased

following the 3rd dose (Fig. 3.4). By 72 hours after the third dose all animals had 10^2 bacteria/fecal pellet or less, and no vaccine-strain lactobacilli were detectible by 96 hours.

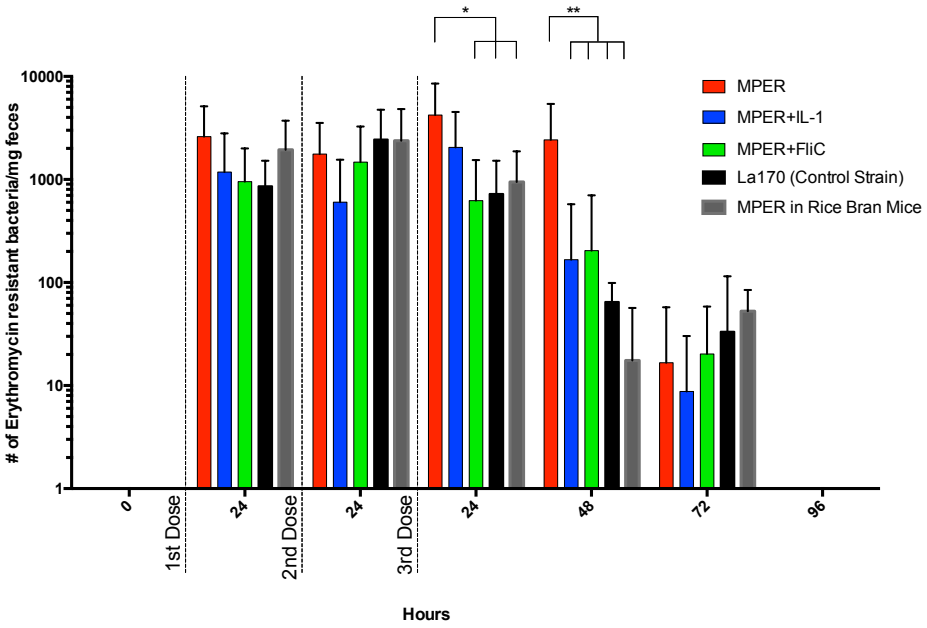

Figure 3.4 Modified *L. acidophilus* is undetectable after 96 hours post dose. Fecal pellets were collected 24 hours prior to the first vaccine dose, then every subsequent 24 hours for 6 days. Fecal pellets were weighed, homogenated in 1mL of PBS, and the resulting suspension diluted 1:1000 in PBS. The diluted suspension was plated using a spiral plater on erythromycin-infused Rogosa agar plates and allowed to grow under oxygen-deprived conditions for 48 hours. MPER *N*=6, MPER+ IL-1β *N*=6, MPER+FliC *N*=6, MPER+RB *N*=5. Colonies were counted and spiral plate dilution calculations used to extrapolate the number of colonies per milligram of feces. Mean and standard deviation are shown. Statistical significance was calculated by Tukey multiple comparison test (*p<0.05, **p<0.01).

3.3f Rice bran supplemented mice show some increased immunogenicity when vaccinated with MPER-only lactobacilli.

We next observed antibody levels and antibody secreting cell frequency in mice fed diet supplemented with 10% rice bran. No significant differences in antibody levels in fluids (Fig. 3.5A) or antibody secreting cells, MPER-specific or nonspecific, were observed versus non-rice bran vaccinations (Fig. 3.5C and D).

When antibody-secreting cells were pooled from multiple tissues (Peyer's patches, female reproductive tract, spleen, mesenteric lymph node, large intestine), rice bran supplemented mice show a significant increase in anti-MPER-secreting cells (Fig. 3.5B).

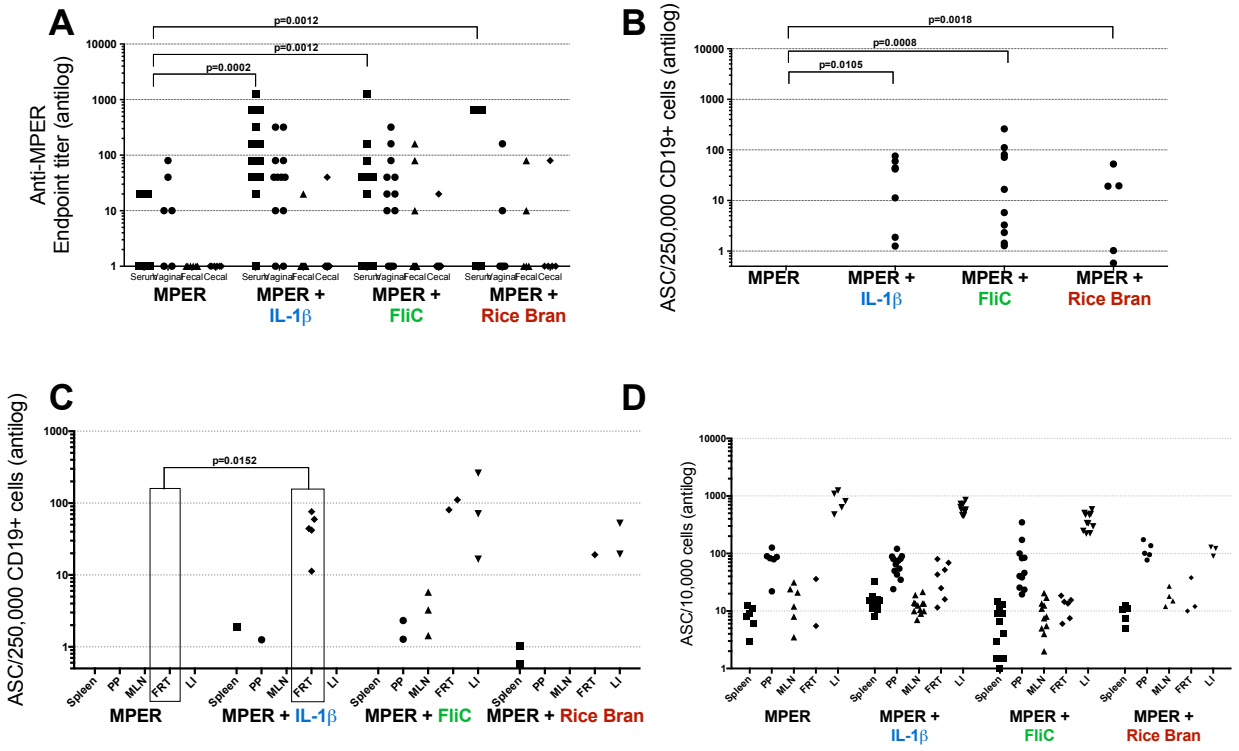

Figure 3.5 Mice supplemented with rice bran have elevated serum IgG and antibody-secreting cells. ELISAs (A) and MPER-specific ELISPOTs (B, C) and total IgA (D) were performed as previously described. MPER *N*=6, MPER+ IL-1β *N*=12, MPER+FliC *N*=12, MPER+RB *N*=5. Groups in panels A, B and C were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. Significance for panel D was performed using Dunn's multiple comparisons test after Kruskal-Wallace H.

3.4 Discussion

Probiotic bacteria can be influenced by prebiotic supplementation (320, 321). Previously, this influence was hypothesized to improve probiotic vaccination, but results were inconclusive (302, 322). However, new data revealed effects of prebiotic rice bran on intestinal lactobacilli (263, 328). Rice bran diet augmentation of mucosal vaccines could offer an alternative strategy

for improved immunogenicity that also provides intriguing prebiotic benefits to the host (255, 259-261). However, the effects of rice bran on vaccine strain lactobacilli have not been studied.

To that end we performed *in vitro* and *in vivo* experiments to determine whether rice bran influences growth and immunogenicity of *Lactobacillus*. Our goal was to measure the effects rice bran nutrients on vaccine strain growth and heterologous antigen presentation *in vitro*. We also sought to measure the effects of a rice bran supplemented diet on MPER immunogenicity in mice. We concluded that rice bran nutrients allow for growth and antigen expression of MPER-vaccine bacteria, and improve anti-MPER immunogenicity *in vivo*.

Rice bran and its nutrient extracts are known to support *Lactobacillus* growth and survival (263, 330). In order to observe the effect of rice bran on *L. acidophilus* vaccine strains we measured the growth and heterologous antigen presentation of bacteria grown with or without rice bran extract. Results show that rice bran supplementation supported growth and exogenous protein expression similar to optimized medium, indicating rice bran may be a useful prebiotic *in vivo*.

As previously mentioned, *in vivo* adjuvant effects of prebiotics on vaccination have been inconclusive (302, 322). However, rice bran has shown promise as a potential adjuvant by influencing lactobacilli growth (263). In order to measure any adjuvant effects of a rice bran diet, mice were fed a diet with 10% rice bran and orally dosed with MPER-*L. acidophilus*. Anti-MPER serum IgG levels were significantly increased versus non-supplemented mice. As with other adjuvants, the number of MPER-IgA antibody secreting cells was significantly higher with rice bran supplementation. These results indicate that a prebiotic rice bran diet can effectively adjuvant *L. acidophilus* vaccination, and provides support for rice bran as a potential adjuvant for mucosal vaccines. Hundreds of compounds have been identified within rice bran, including

squalene, a natural adjuvant used in other vaccine studies. Squalene, and/or other compounds, may be responsible for the observed enhanced immunogenicity. However, the mechanism behind this enhancement requires more experiments.

While promising, these experiments suffered from several weaknesses. A low number of mice were used (*N*=5), limiting statistical power. This also limited the number of assays that could be carried out. As with most model systems, mice do not identically recapitulate higher mammalian immune systems, and the influence of rice bran in the mouse diet may be different in humans or non-human primates.

Overall our purpose was to identify if rice bran could improve the immunogenicity of our *L. acidophilus* vaccine. We were able to conclude that not only was rice bran not detrimental to the growth or antigen expression of vaccine strains, but a mouse diet supplemented with rice bran improved the immunogenicity of anti-MPER responses versus a non-supplemented diet. Future work will help elucidate the mechanisms behind this improvement.

Chapter 4: Summary and Future Considerations

4.1 Summary

HIV vaccines have a history of poor efficacy, with inconsistent levels of protection against mucosal HIV exposure (217). Lactic acid bacteria offer an alternative vaccine platform for delivery of antigen to the primary site of HIV infection, the mucosa. Current understanding of the immunogenicity of this vaccine system is incomplete. In this regard here we present several adjuvant strategies to improve immunogenicity.

We evaluated the mucosal and systemic immune responses of several strains of *Lactobacillus acidophilus* expressing the HIV membrane proximal external region (MPER). The addition of interleukin-1β or flagellin increased systemic MPER-IgG and showed high levels of vaginal IgA, with increased MPER-specific B-cell numbers in mucosal tissues. Importantly, we have demonstrated that *L. acidophilus* oral vaccination, when combined with adjuvants, is effective at generating mucosal and systemic antibodies. This is significant for future vaccine studies by providing a method to target antigens regardless of their immunogenicity, in the context of a safe and simple to use vector.

Understanding of T cell functionality in lactic acid bacteria vaccines is incomplete. Our results indicate T cell help, in the form of CD40L-CD40 binding, is required for efficient B cell responses against *L. acidophilus* expressed antigens. Importantly, we observed similar results to other experiments regarding T cell tolerance and inflammation markers. Many experiments have noted both regulatory and inflammatory cells and cytokines following *Lactobacillus* vaccination, and we observed both elevated Tregs and IL-17 levels. This is unsurprising since the gut immune environment is a delicate balance between immune recognition and tolerance. This balance poses

an interesting conundrum for probiotic vaccination: immunogenicity typically requires an adequate inflammatory response for proper function, however, inflammation of the gut can be damaging, as observed in Crohn's disease and other autoimmune conditions. Striking a balance between these two arms of immune function is key.

The effects of vaccination on the gut microbiome is understudied (304). We present preliminary data showing differences in microbiome community structure over time following vaccination. These results were unexpected and raise the question of interactions between the commensal vaccine vector, mucosal immune system, and intestinal microbiome. A more detailed study of bacterial community shifts at the taxon, genus, and species level is required before conclusions can be made.

We have also shown that an alteration of diet can improve the immunogenicity against MPER. Rice bran has been shown to be a powerful dietary tool in the past, and its influence on lactobacilli in particular prompted us to examine its effects both *in vitro* and *in vivo*. In chapter 3 we have shown that mice consuming a 10% rice bran diet had increased antibody and antibody secreting cell levels. The mechanism behind this increase is not clear but may involve increased bacteria uptake, a nutrient effect on the mucosa itself, or the presence of adjuvant compounds in rice bran.

Collectively our experiments demonstrate that mutant *L. acidophilus* can be an effective vaccine vector. Direct targeting of mucosa is a logical step towards prophylaxis against mucosal pathogens, and our results have identified new aspects of adjuvants, T cells, and microbiota that will aid in future studies. The ease of modifications, inexpensive growth, and excellent safety profile all point to *L. acidophilus* as an important vector for future vaccine studies.

4.2 Future Considerations

Future studies using this vaccine system must expand into new antigenic HIV epitopes, and new antigens in general. There have been many studies of other pathogen antigens, and the inclusion of other HIV epitopes could allow for multiple broadly neutralizing antibodies to be generated from a single vaccine. The use of other adjuvants is also a logical step. The adjuvants in our experiments have proven effective both here and in other studies, but new adjuvants could allow for more tailored immune responses, or may provide a synergistic effect when combined.

More detailed microbiome analysis must be performed to identify species alterations and strain-specific effects on the microbiome, and to that end more data is currently being generated. Deeper understanding of the T cell response is also needed, including a more expansive look at the subsets involved, particularly Th17 and CD8s. Antibody subtypes would be a valuable addition to the adjuvant strain responses, as would other diets for comparison to rice bran and typical mouse feed. The complexity and infancy of the mucosal vaccine field indicates no shortage of future studies.

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Appendix A: Review of Lactobacillus Vaccines And Their Immune Responses

Introduction

Lactic acid bacteria (LAB), alongside other food-based platforms, have been utilized since the 1990's for therapeutic heterologous gene expression, (332). The ability of LAB to elicit an immune response against expressed foreign antigens has led to their use as potential candidates as mucosal vaccine vectors. As vaccine vectors they offer a number of attractive advantages: simple, non-invasive administration (usually oral or intranasal), the acceptance and maintenance of genetic modifications, relatively low cost, and the highest level of safety possible. LAB tend to elicit minimal immune responses against themselves, instead inducing high levels of systemic and mucosal antibodies against the expressed foreign antigen following uptake via the mucosal immune system (333).

LAB as vaccine vectors are generally derived from *Streptococcus gordonii*, *Lactococcus lactis*, or multiple *Lactobacillus* species. *S. gordonii* has fallen out of use, with a few exceptions (129). *L. lactis* and *Lactobacillus* spp. have continued to grow in use, with the number of publications continuing to increase (See Fig. A1.1). Several excellent reviews of *L. lactis* vaccines have been published (130, 334, 335), as well as how to generate these recombinant bacteria (133). Because of the large number of recent articles detailing lactobacilli as vaccine vectors, this appendix will focus on those publications and their resulting immune responses generated *in vivo*.

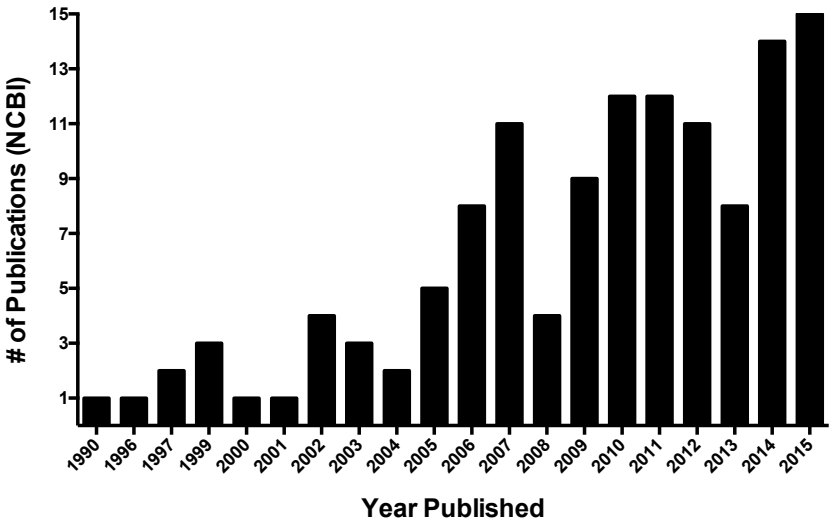

Figure A1.1 Number of primary articles published per year that utilize lactobacilli for heterologous gene expression.

Briefly, this appendix is divided by pathogen/disease of interest (virus, bacteria). Pathogen species or families that have multiple studies (i.e. HIV, *Escherichia coli*) are then highlighted, focusing on the immune responses resulting from *Lactobacillus* vaccination. This appendix only covers research involving *Lactobacillus* strains with heterologous gene expression. Studies conducted with unmodified *Lactobacillus* used either as an adjuvant or for intrinsic antibacterial or antiviral properties are excluded (336, 337). The text of this appendix also only focuses on *in vivo* work and their resulting immune responses, or *in vitro* studies with a significant immune component. When possible the specific animal model will be addressed. Recent evidence has shown that the mucosal responses in C57BL/6 mice can have dramatic problems compared with BALB/c mice (338). Thus results from mucosal vaccine studies in C57BL/6 mice must be taken within this context.

Viruses

Human Immunodeficiency Virus

HIV-positive people are living longer lives, yet infection rates have stabilized (157). An effective HIV vaccine is still elusive via traditional methods, with statistical significance plaguing the only moderately successful clinical trial (218). Utilizing lactobacilli as mucosal vaccine vectors can provide an enhanced immune response at the typical mucosal sites of infection. Several studies have looked at lactobacilli expressing HIV antigens, thus targeting the virus at the most common site of infection, namely the mucosa. Our lab has shown that adjuvanting the bacteria with additional secreted molecules (IL-1β, Salmonella Flagellin C) can drastically improve the mucosal (IgA) and systemic (IgG) immune responses against HIV proteins (MPER, gag) in orally dosed mice (136, 143). We have also shown that these immune responses are T cell-dependent. Kuczkowska et al. have shown *in vitro* evidence of T cell recruitment using a *L. plantarum* expressing a fusion protein of CCL3/HIVgag (339).

An alternative preventative measure against HIV is prophylactic topical microbicides, which have been shown to be effective in high-risk groups (340). By incorporating microbicidal expression into lactobacilli, target mucosal sites can be colonized and continuously protected, reducing cost and required compliance. In two separate studies Lagenauer et al. utilized a vaginal-associated *L. jensenii* secreting cyanovirin-N, a promising microbicide with high affinity for HIV envelope glycoproteins. In Rhesus macaques this application was safe and afforded protection against SHIV challenge (341-343). This group also used lactobacilli to secrete broadly neutralizing antibody fragments to protect the vaginal mucosa, though the work is still *in vitro* (344).

Human Papilloma Virus

The association between human papilloma virus (HPV) and various cancers, particularly cervical cancer, is well known (345). Cervical cancer cells tend to express HPV proteins on their surfaces. This allows for an immune response that not only targets potentially infectious virus, but can also destroy infected, cancerous cells. There are currently two FDA approved vaccines against the most common strains of HPV (Gardasil and Cervarix). Both generate protective immune responses via spontaneous virus-like particle formation of the HPV L1 capsid protein (346). While these vaccines provide excellent protection and potential cancer therapy, they remain prohibitively expensive, particularly in the U.S. (347). Only one research group has utilized *Lactobacillus* to generate VLPs using the L1 protein, resulting in serum IgG following subcutaneuous injection in BALB/c (138). All other research groups utilize surface expression of HPV proteins, either minor capsid protein L2 or the early oncogenes E6 or E7, which are directly responsible for unregulated cellular replication (348). In an extensive set of early experiments, Poo et al. utilized an E7-expressing *L. casei*, observing serum IgG along with intestinal and vaginal IgA in orally immunized C57BL/6 mice. They also observed E7-specific IFN-γ-secreting cells in the vagina and spleen, as well as a therapeutic reduction in tumor size and increased animal survival following TC-1 tumor cell challenge (349). A similar study using E6 had similar results (350). Poo et al. later targeted the L2 protein in BALB/c mice, observing serum IgG, mucosal IgG and IgA, and cross-neutralization with related viruses (351). Using *L. casei* administered to C57BL/6, Adachi et al. observed increased levels of E7-specific T cells in the gut, as well as granzyme-B production. Mucosal lymphocytes were capable of TC-1 cell lysis, which was also repeated by another research group (352, 353). Interestingly, oral administration improved the response versus subcutaneous or intramuscular administration (352). Another

research group utilized *L. plantarum* expressing E7, with similar antibody and anti-tumor results, though they only checked for antibodies in the serum, not the mucosa (142). Because of the observed therapeutic effect of several studies, a human trial using cervical cancer (CIN3) patients was conducted and showed increased numbers of E7-specific cervical lymphocytes, though not PBMC's, with the majority of patient tumor pathologies being downgraded (354). This shows great promise and potential for anti-HPV lactobacilli vaccines to provide a large public health benefit.

Influenza

The unpredictability of future influenza strains, and supply problems stemming from slow growth methods (egg and cell-based), means that anti-influenza *Lactobacillus* vaccines could provide a needed service, particularly for highly pathogenic strains like H5N1. Shi et al. showed that oral administration of a *L. plantarum* expressing H9N2 HA was able to induce fecal IgA, bronchiolar IgA, and serum IgG. B cell levels in secondary lymphoid organs were increased, and CD8+ T cell proliferation and IFN-γ secretion were greatly improved relative to a typical influenza vaccine. Most importantly vaccinated mice survived lethal challenge (355). These results were repeated using the dendritic cell-targeting peptide (DCpep) adjuvant, and included improved immune responses and challenge survival in chickens (356). Similar antibody and T cell results were observed when targeting H5N1 hemagglutanin (HA_1) in BALB/c mice (357) and chickens (358). Other influenza proteins have also been targeted. Chowdhury et al. were able to grant BALB/c mice protection (via oral or intranasal administration) from multiple lethal challenge strains, and showed that inclusion of the cholera toxin subunit 1 (CTA1) significantly improved antibody levels and protection (359). A follow-up study showed that

antibody levels, IFN-γ secretion and proliferation, as well as protection against lethal challenge, lasted 7 months post-vaccination (360).

Coronavirus

Until the recent outbreaks of Severe Acute Respiratory Syndrome (SARS, 2003) and Middle East Respiratory Syndrome (MERS, 2014/2015) coronavirus morbidity and mortality were generally worse for domesticated animals, particularly porcine and poultry farms, rather than humans. Corona viruses usually infect via the gastrointestinal tract in livestock and the respiratory tract in birds and humans, causing drastic economic losses and high morbidities in the young, old and immunocompromised (361). The first corona virus addressed using lactobacilli was Transmissible Gastroenteritis Coronavirus (TGEV), which affects swine, particularly piglets. Several spike protein epitopes have been targeted (S, 6D), resulting in serum IgG and mucosal IgA in mice (362, 363). More recently the muramyl dipeptide (MDP) protein was targeted, utilizing tuftsin, an Fc antibody fragment, as an adjuvant, which showed improved antibody and T cell responses in BALB/c (271). The only human coronavirus addressed was SARS-CoV, with serum IgG and mucosal IgA observed against the spike protein (SA, SB) in C57BL/6 mice (364). Porcine Epidemic Diarrhea Virus (PEDV) is another coronavirus that primarily affects piglets, resulting in large economic losses (365). In a thorough set of experiments Di-qiu et al. showed that by targeting both the spike protein (S1) and nucleocapsid (N) via surface expression (as opposed to secretion), anti-S1 and anti-N antibodies were significantly increased, even in atypically-studied secretions such as ophthalmic and nasal (366). Interestingly they observed a synergy against the spike protein, but not the nucleocapsid, in mice vaccinated against both proteins. To improve the immune response against TGEV's core

neutralizing epitope (COE), Ge et al. fused the COE with *E. coli* enterotoxin B (LTB), which showed some statistical significance, particularly in splenocyte IFN-γ and IL-4 secretion (367). In perhaps the most directly useful study, Hou et al. observed the increased presence of anti-nucleocapsid antibodies in the milk and colostrum of nursing sows, correlating with increased anti-N serum IgG in suckling piglets (368). A recent set of experiments by Jiang et al. delved deeper into the immune response generated by *L. casei*, highlighted by a strong mucosal-dependent protection from infection, a stimulation of the IL-17 pathway, and an imbalance between Th1 and Th2 responses, as indicated by variations in numbers of CD4+ T cells containing either intracellular IFN-γ or IL-4 (369). Interestingly, some Lactobacillus species have been shown to downregulate IL-17 responses (148), but this simply points to the delicate balance Th17 cells must strike between pathogen-stimulated inflammation and the damage errant autoimmune inflammation can cause (149). It is clear that the homeostasis between inflammation, immunity, lactobacilli, and Th17 cells is complex and dependent on a number of factors, including host genetics, pathogen, lactobacilli strain and adjuvants.

Rotavirus

Rotaviruses, from the Reoviridae family, are commonly associated with severe gastroenteritis in children, though not typically mortality outside sub-saharan Africa (370). There are a number of approved oral rotavirus vaccines for humans and animals with excellent mucosal immune responses (371), however, availability and cost in developing countries, especially when competing with other healthcare priorities, can leave many unvaccinated (372). Two main avenues of lactobacilli-based rotavirus protection have been attempted in mice. The first is a typical vaccination style, with oral *L. casei* inducing mucosal IgA and neutralizing serum IgG

against porcine rotavirus major protective antigen VP4 in mice (140). The second avenue uses antibody fragments to confer protection. Alvarez et al. expressed a protective anti-rotavirus llama antibody fragment on the surface of *L. rhamnosus*, protecting against diarrhea in a mouse pup model (373). Another group adapted the use of anti-rotavirus hyperimmune bovine colostrum (HBC) in the same model system, expressing an anti-HBC protein from Streptococcus, which would then bind HBC antibodies, thus conferring protection when orally dosed (374).

Fish-Related

Aquaculture is a serious food supply paradigm, and with it comes the typical pathogen problems that large-scale animal farms encounter. Vaccination against fish pathogens can be performed either IP (which can be cost prohibitive), by immersion or orally via feed, with the latter two options suffering from a lack of vaccine persistence in water and the particularly strong mucosal tolerance observed in fish. For a comprehensive summary of vaccination attempts in fish see Embregts and Forlenza's excellent review (375). Lactobacillus vaccine vectors can provide an effective and easily administered system for pisciculture. The first set of experiments targeting a fish-related virus focused on Infectious pancreatic necrosis virus (IPNV), a Birnavirus that afflicts rainbow trout. Direct oral administration of *L. casei* expressing portions of viral capsid generated significant serum IgM and afforded challenge protection in two studies by the same group (376, 377). Two viruses that primarily affect carp, Koi herpesvirus (KHV) and Spring viremia carp virus (SVCV, a Rhabdovirus), have also been studied. Both antigens, KHV ORF81 and SCVC glycoprotein, were expressed together in *L. plantarum* and dosed orally in carp and koi. The resulting serum IgM and challenge survival were promising, particularly for a vaccine that offers dual protection (378). Further lactobacillus studies must be conducted,

particularly looking at cellular mucosal immunity in fish, as well as the potential for multiple pathogens to be addressed with a single modified lactobacillus.

Other Viruses

Outside of the categories already addressed, a large and diverse number of viruses have been targeted using lactobacillus vector systems. Classical Swine Fever Virus (CSFV), a Flavivirus affecting pigs, has been tested in rabbits, mice and pigs, all resulting in serum and mucosal antibodies (379, 380). Importantly, the addition of Thymosin α-1, a T cell-stimulating peptide, was able to increase IgG, IgA, IFN-γ, IL-2 and TNF-α in pigs (144). Porcine parvovirus has been studied in BALB/c mice and pigs, with excellent IgG and IgA responses, as well as challenge protection and virus neutralization (379, 381, 382). A recent study observed strong protective immune responses in chickens against Newcastle disease virus, a Paramyxovirus primarily afflicting poultry, which were improved by the addition of DCpep, which not only boosted mucosal and serum antibody levels but increased Th cells in the spleen and peripheral blood versus bacteria without DCpep (383). Foot-and-mouth disease virus, a Picornavirus afflicting cloven-hooved animals, had a comprehensive dosing study performed looking at immune responses of anti-capsid *L. acidophilus* administered via intramuscular, intraperitoneal, intranasal, or oral routes. Of note however is the authors' use of the vaccine system as a delivery vehicle for an anti-capsid DNA vaccine plasmid, as opposed to expression of heterologous proteins on the bacterial surface. The resulting antibody responses were thus much higher via IM and IP administration rather than mucosal delivery (384).

As the ease of use and awareness of lactobacillus expression systems and their abilities to induce excellent mucosal and systemic immune responses increases, the number and variety of pathogens addressed will likely increase in the future.

Bacteria

Bacillus anthracis

Though infections are relatively rare, the prevalence of natural *Bacillus anthracis* in soil and its potential as a bioterrorist agent gives anti-Anthrax vaccines some priority. The antigen is also fairly immunogenic and well studied, thus making it useful for model development. All lactobacillus vaccination experiments utilize the *B. anthracis* protective antigen (PA), a highly immunogenic binding protein of Anthrax toxin, which has been tested in other vaccine systems with varying degrees of success (385). One of the earliest proof-of-concept lactobacillus experiments involved dosing BALB/c mice with L. casei either orally or intranasally, importantly showing that antibody responses against heterologous protein exceeded antibody responses against just the bacteria (386). Ten years later Mohamadzadeh et al. combined an *L. acidophilus* or *L. gasseri* with DCpep, resulting in neutralizing antibodies and challenge survival in A/J mice (387, 388). This same group later observed colonic DC activation, both Th17 and Treg up-regulation, and up-regulation of a number of pattern recognition receptor genes, though the vaccine regimen did not consist of any boosts (316).

Escherichia coli

Enteric *Escherichia coli* are a major cause of diarrheal morbidity and mortality, particularly for children in developing countries. The most common antigens targeted for *E. coli* vaccination are fimbrial proteins, bacterial adhesins that aid in host cell binding**.** Most experiments mentioned here, except one, target Enterotoxigenic *E. coli* (ETEC). A prolific group from China utilized several fimbrial protein antigens (F41, K99, K88) over several years and in several models (BALB/c, C57BL/6, BALB/c pups), all in *L. casei*. Of their many findings, an increase in several subclasses of serum IgG (IgG1, IgG2a, IgG2b) followed oral dosing, along with increased IL-4 and slightly less increased IFN-γ by CD4+ T cell ELISPOT. Intestinal and bronchiolar IgA levels were increased, and challenge with standard ETEC (389). These results were repeated using intranasal dosing, which resulted in decreased intestinal IgA with increased bronchiolar IgA versus oral (390). Dosing in C57BL/6 mice induced similar IgG and IgA responses, as well as T cell proliferation and challenge protection (391). Challenge protection was conferred to orally dosed mouse pups (make sure it was direct for pups and not indirect), indicating that this vaccination strategy can be administered early in immune development (392). Wu et al. targeted two enterotoxins (ST and LT-B), rather than fimbrial proteins, with a secreted GFP/enterotoxin fusion protein. Similar increased IgG and IgA were observed as well as challenge protection in a patent mouse gut assay (393). Ferreira et al. were the only group to target Enteropathogenic *E. coli* (EPEC), and attempted the only sublingual dosing regimen. *L. casei* expressing a portion of bacterial β-intimin (a cell surface protein that aids in attachment to the host cell) resulted in serum IgG and fecal IgA, though interestingly oral dosing did not generate an IgG response. Splenocytes also secreted elevated levels of IL-6 and IFN-γ, though only their results from the sublingual vaccination were reported (394). While the authors

performed these studies in C57BL/6 mice, they used C3H/HePas mice as their challenge model, due to this strain's susceptibility to *Citrobacter rodentium*, a commonly used strain that shares some pathology with EPEC (395). They observed at least an increase in survival time, though animals eventually succumbed to disease**.**

Streptococcus pneumonia

One research group, led by Dr. Maria Oliviera of Brazil, has performed most lactobacillus experiments involving *Streptococcus pneumonia*, focused on either pneumococcal surface proteins PspA or PspC, with immunity studies conducted in C57BL/6 mice. Early work noted significant increases in bronchiolar IgA but not IgG following intranasal administration, with some variations due to strain differences (396). Improvements in antigen expression led to increased IgG (IgA was not measured), with a variety of IgG subsets being enhanced (1, 2a, 2b, 3). This study culminated in enhanced challenge survival versus saline alone, though not versus bacteria expressing the empty vector plasmid (147). Further experiments identified a propensity for IgG1 versus IgG2a, which along with increased IFN-γ and low levels of IL-5, indicated a Th1 polarization. IL-17 secretion and neutrophil recruitment in the lungs varied by route of administration, adding to the importance of how vaccines are administered and not just how they express antigen (146). A final set of experiments failed to induce significant levels of IgA prior to challenge, but noted that challenge with *S. pneumoniae* did induce significant IgA, which correlated with reduced bacterial loads (397).

Other bacteria

As with lactobacilli vaccination targeting viruses, a number of pathogenic bacteria have only been targeted in a few research publications. A few will be highlighted here, with the rest addressed in Table 1. *Borrelia burgdorferi*, the causative agent of Lyme disease, was targeted with an *L. plantarum* system. The authors identified what has become an interesting theme with lactobacilli vaccinations, that of a broken Th1/Th2 balance. *In vitro* work with human cells indicates both Th1 and Th2 cytokines, and oral administration in C3H-HeJ mice resulted in both IgG1 (Th2) and IgG2a (Th1) (145). These same authors also targeted *Yersinia pestis* with *L. plantarum*, observing once again both inflammatory (TNF-α, IL-12, IFN-γ and IL-6) and anti-inflammatory (IL-10) cytokines, indicating stimulation of both Th1 and Th2 responses (150). Importantly however, as with the previous experiment, these are human ex vivo cytokine studies not conducted in the study's animal model. A vaccine targeting *Helicobacter pylori*, a common cause of stomach ulcers, would be extremely beneficial. By targeting the *H. pylori* adhesin Hp0410 with an *L. acidophilus*, Hongying et al. generated anti-adhesin serum IgG and intestinal IgA and observed reduced bacterial loads and gastric inflammation following challenge (315). Antibodies against the ε-toxoid of *Clostridium perfringens* were identified in BALB/c mice following oral *L. casei*, and though statistical significance of antibody levels was unclear, animals survived challenge (398).

Other Antigens

Parasite/Fungus

Only a few parasites and fungi have been addressed using lactobacilli expression systems. Malaria was the first, with oral and intranasal dosing of *L. reuteri* and *L. salivarius* strains expressing merozoite surface antigen 2 (MSA2). The authors were only able to observe an increase in mucosal IgA, not serum IgG, though this may have been due to variations in the mouse strain (399). *Cryptosporidium parvum*, another parasite which can infect epithelial cells of the intestines and cause diarrhea, had elevated serum IgG and mucosal IgA, and interestingly elevated mRNA levels of IFN-γ and IL-6, but not IL-4 (400). The only fungus to be addressed was *Candida albicans*, the typical cause of yeast infections. Dosing in C57BL/6 mice induced serum IgG (IgA was not tested) and granted some lethal challenge protection, though not complete (401).

Allergens

A large number of lactobacillus studies have attempted to reduce the severity of allergic pathologies against mostly plant-based allergens, though dust mites have also been addressed. These experiments attempt to either target IgE antibodies, thus preventing subsequent IgE-, or target the allergen itself. Scheppler et al. equipped *L. johnsonii* with either an IgE single chain fragment or an IgE mimotope, both resulting in anti-IgE serum IgG, though intranasal favored the mimotope and subcutaneous favored the IgE fragment (402). In an interesting proof-of-concept, Charng et al. showed that by orally vaccinating the mice with *L. acidophilus* expressing the mite allergen (Der p 5) in BALB/c mice that were sensitized to and then subsequently

challenged with Der p 5, they could reduce the severity of the subsequent allergic reaction, marked by reduced anti-Derp5-IgE and granulocyte infiltration into the airway (403). A similar experiment involving intranasal *L. plantarum* targeting a birch pollen allergen showed that pre-treatment with the bacteria could skew the allergic response towards a typical Th1 response, with reduced airway inflammation, reduced IgE and increased levels of anti-allergen bronchiolar IgA (404). Other oral vaccinations targeting plant allergens observed similar reductions in IgE (405), or increases in anti-allergen IgA (406).

Self-Antigens

Lactobacilli vaccinations targeting self-antigens have been used in several contexts. Two research groups have targeted cancer-associated antigens, observing serum IgG against oncofetal antigen (a common tumor immunogen) (407), and serum IgG (IgG1/Th2 and IgG2a/Th1) and intestinal IgA against cancer testis antigen (408). This latter experiment identified an interesting *in vitro* human dendritic cell response, with only immature DC's, rather than mature DC's, responding to lactobacillus stimulation. They also noted that lactobacillus-stimulated DC's had higher levels of PD-L1 and IDO, both associated with T cell inhibition/regulation, though this is likely just a case of DC's exerting a balanced control of T cell stimulation, be it negative or positive. One research group has attempted to target human chorionic gonadotrophin (hCG-β), a candidate for contraceptive vaccination. In a very thorough set of experiments, Yao et al. compared the serum IgG and vaginal IgA of both BALB/c and C57BL/6 mice, along with route of administration (oral, vaginal, nasal) and number of bacteria dosed (409). In line with other mouse strain research (338) they observed a lower antibody response in the C57BL/6 mice. The

same group observed a significant increase in serum and vaginal antibodies when the adjuvant C3d3 (complement) was included in the inoculated *L. casei* (310).

Conclusions

The number of studies involving lactobacilli has only increased in the last few decades. As more information is available interesting concepts regarding the immune responses elicited are beginning to emerge. There is clearly an interesting balance in the Th1/Th2 paradigm, likely pointing to the plasticity and oversimplicity of driving either one side or the other. The evidence of Th17 involvement as well as the regulation Tregs provide points to a complex T cell response as well. Adding to the mix is the elicitation of multiple IgG subtypes. Clearly more work must be done to identify the players at hand in the mucosa, especially the cells charged with initiating the initial bacterial uptake (M cells, DC's).

As research moves forward, there are several major takeaways when designing new experiments. Boosting is a key component of high antibody levels, as is true with all vaccine strategies. The route of administration, usually oral, can have an effect on the type of response elicited, likely derived from differences in the mucosal tissue where uptake occurs. The actual strain of lactobacilli, with their intrinsic differences (410), as well as the location of antigen expression (surface display, intracellular, secreted), can alter the resulting immune response and therefore must be exploited for specific antigens. As always, the model system used must be taken into consideration. Because of mucosal immune problems the C57BL/6 mouse model should be avoided in favor of BALB/c. Further work in non-human primates should be undertaken to better understand the mucosal immune responses generated by these types of vaccines. Based on animal model safety and efficacy, human trials should be undertaken.

Appendix B: Previous Work For The Akkina Laboratory

B1: HIV Infected Humanized Mice Have Improved T Cell Responses And Lower Viral Loads Following Anti-PD-L1 Antibody Treatment

Palmer BE, Neff CP, LeCureux J, Ehler A, Dsouza M, Remling-Mulder L, Korman AJ, Fontenot AP, Akkina R. (2013) In vivo blockade of the PD-1 receptor suppresses HIV-1 viral loads and improves CD4+ T cell levels in humanized mice. J Immunol. Jan 1;190(1):211-9. doi: 10.4049/jimmunol.1201108.

B1.1 Overview

Immune responses are extremely resource heavy for the host (411, 412). The benefits of this energy expenditure outweigh its loss by eliminating the dangers posed by a pathogen. At the same time it is in the host's best interest to downregulate the immune response as soon as the threat has been neutralized, thus freeing up those resources. To this end the host has several mechanisms for slowing and stopping immune responses, typically by stopping the effector T cell response. T regulatory cells can secrete suppressing cytokines and stimulate apoptosis of APCs, thus blocking APC-T cell stimulation (413). Interleukin-2 (IL-2), a powerful T cell proliferation and survival cytokine, under the right conditions can also drive T cells towards activation-induced cell death, thus causing a contraction in the immune response (414).

Of particular interest is the use of negative surface co-receptors to downregulate the immune response. During a normal adaptive immune response antigen is presented via MHC to the T cell receptor (TCR) by an APC. If secondary signals are received from positive co-receptors (CD40L, CD28), as well as cytokines (IL-2, many others (415)), T cells become

activated, proliferating and carrying out effector functions. As this immune response progresses negative co-receptors on T cells begin to gradually increase and compete with positive co-receptors for control of the T cell response. By the time a pathogen is cleared this negative response typically outweighs the positive, influencing anergy and apoptosis of the cells. This process, along with others previously mentioned, frees up resources for the host.

However, during a chronic infection the pathogen and its antigens are not cleared. T cells continue to receive positive stimulation to carry out effector functions and proliferation, while co-inhibitory receptors begin to wrest control from positive co-receptors. Thus cells are receiving both positive and negative stimulation signals (416). Over time these cells become exhausted, displaying a large number of co-inhibitory receptors (CTLA-4, Tim-3, LAG-3, and PD-1), and possess phenotypes characterized by a loss of proliferative capacity, loss of effector function, and loss of multiple cytokine secretion (a hallmark of healthy effector cells) (416-418). A number of strategies have been proposed to reverse this T cell exhaustion (419). One technique is blocking negative co-receptors with anti-receptor antibodies, thus reinvigorating the exhausted cells by eliminating the co-inhibitory signals (420). Success against certain cancers led to the use of anti-PD-1 antibody for clinical use (421). Blocking the PD-1/PD-L1 pathway was proposed as a treatment for other chronic conditions with exhaustion pathologies, particularly HIV (422-425). To this end anti-PDL-1 antibody blockade was used in a humanized mouse model of HIV infection to better simulate potential human clinical responses.

B1.2 Materials and Methods

B1.2a Generation of humanized $Rag^{-/-}$ $CD132^{-/-}$ mice and measurement of HIV-1 viral load.

Humanized BALB/c $Rag2^{-/-}$ or $Rag1^{-/-}$ $CD132^{-/-}$ (Rag-hu) mice were prepared as previously described (426). Briefly, newborn mice were irradiated with 350 rad and then injected intrahepatically with 0.5–13 million human CD34+ cells isolated from human fetal liver by magnetic bead separation. Mice were screened for human cell engraftment at 10–12 weeks post-reconstitution. Peripheral blood was collected by tail bleed every week, and RBCs were lysed using the Whole Blood Erythrocyte Lysing Kit (R&D Systems) to isolate white blood cells (WBC). The WBC fraction was stained with anti-human CD45 (Caltag) and analyzed by flow cytometry to determine the levels of human cell engraftment as previously described (426). Only mice with engraftment above 70% were used in the study. Mice were infected by intraperitoneal (i.p.) injection of HIV-1 (strain BaL, $1x10^6$ IU) 12 weeks after engraftment. HIV-1 plasma viral RNA was quantified by qRT-PCR following RNA extraction of plasma using QIAamp Viral RNA kit (QIAGEN). qRT-PCR was performed using LTR-primers and an LTR-specific probe as previously described (426).

B1.2b PD-L1 monoclonal antibody treatment schedule

HIV-1 Bal-1–infected mice were monitored weekly to determine plasma viremia. Consistent viremia was established in all infected mice by week 4 post infection. Seven viremic Rag-hu mice were injected i.p. with 200 mg Bristol-Myers Squibb (BMS) human anti–PD-L1 mAb reconstituted in PBS once every 3 days for 4 weeks (10 total treatments).

Viral loads and CD4+ T cell percentages were determined during the 28-d treatment period, and several weeks after. Six untreated HIV infected mice and four Rag-hu mice that were not infected with HIV-1 were also followed.

B1.2c Flow cytometry

Whole blood was collected, and RBCs were lysed, as reported previously (426, 427). WBCs were stained with anti-human CD45-APC, CD3-PE, CD4-PECy5.5, CD8–Alexa Fluor 405, and PD-1–FITC. Cells were stained with the labeled Abs and analyzed using a Coulter EPICS XL-MCL FACS analyzer (Beckman Coulter,) or BD LSR II (Becton Dickenson). CD4+ T cell levels were calculated as a ratio of the entire human CD45 population (i.e., CD45+ CD4+ CD3+). To establish the baseline CD4+ T cell levels, cells from all mice were analyzed prior to infection. PD-1 expression was analyzed on CD45+ CD3+ CD4+ and CD45+ CD3+ CD8+ T cells and displayed as median fluorescence intensity (MFI) using FlowJo software (TreeStar). Maturation state and PD-1 expression levels were examined before and after treatment with anti–PD-L1. To obtain enough cells,blood from two groups of HLA-matched, HIV-1–infected Rag-hu mice was pooled and stained with anti-human CD45-PE, CD3-BV 605, CD4-V500, CD8–Alexa Fluor 405, CD27–APC–H7, CD45RA-PECy7, and PD-1-FITC and analyzed using a BD LSR II. Fluorescence-minus-one controls were used in all experiments.

B1.2d Cytokine analysis

Plasma samples from Rag-hu mice preinfection, before and after anti– PD-L1 treatment were tested for cytokines using the Human Th1/Th2 Ultra-sensitive Cytokine kit (Meso Scale Development,). TNF-α, IFN-γ, IL-13, IL-10, IL-5, and IL-12 P70 levels in the plasma were

assayed. Plasma samples were stored immediately after collection at -80˚C for this assay. Assays were performed as per manufacturer's instructions and analyzed using a Sector Imager 2400 (Meso Scale Discovery).

B1.2e Statistical analysis

Statistical significances in viral load and CD4+ T cell percentage between mice that were treated or not with PD-L1 mAb were calculated by the Mann–Whitney-Wilcoxen test. Correlations between HIV-1 plasma viral load and the percentage of T cells were assessed by the Spearman test using Prism 3.0 software (GraphPad).

B1.3 Results

B1.3a PD-1 levels are elevated in infected humanized mice

The effect of HIV infection on T cell expression of PD-1 in humanized mice was unknown. In order to measure any differences between control and HIV-infected animals, PD-1 expression was measured by flow cytometry as a percentage of PD-1+CD4+ cells or PD-1+ CD8+ cells. After several months of HIV infection, PD-1 expression was significantly elevated on both CD4+ and CD8+ T cells in HIV infected hu-mice compared with uninfected animals (Fig. B1.1A and B1.1B).

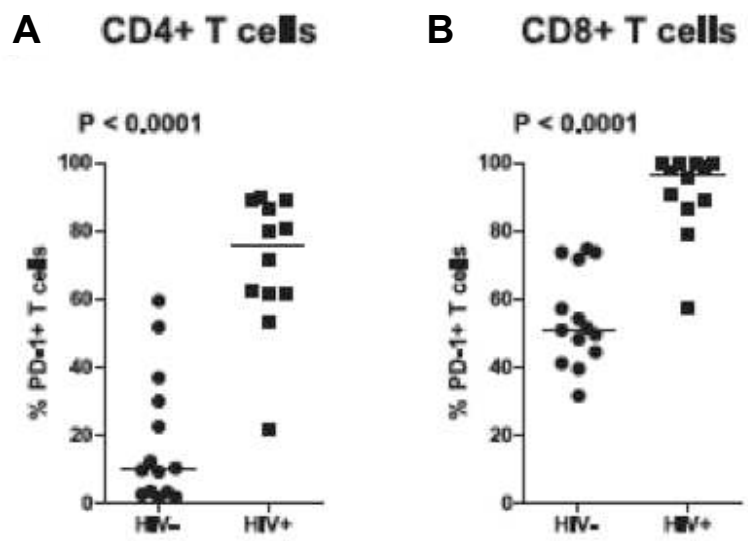

Figure B1.1. PD-1 surface expression on T cells. T cells from peripheral blood via tail vein bleeds of HIV+ and HIV- mice was stained with anti-human CD45, CD3, CD4, CD8, and PD-1 antibodies and assessed by flow cytometry. Groups were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. If significant, two-tailed P-value is displayed.

B1.3b PD-L1 treatment reduces plasma viral load versus untreated HIV infected mice

We next tested the effects of anti-PD-L1 treatment on HIV-infected humanized mice viral loads. HIV+ animals that were untreated maintained relatively stable levels of plasma RNA, averaging approximately $1x10^5$ copies/mL over the 5 weeks they were measured (Fig. B1.2B). Fold-changes for these animals versus day 0 remained relatively small (Fig. B1.2A). HIV+ animals that were treated with anti-PD-L1 showed significant viral load reductions during the first three timepoints after treatment (Fig. B1.2B). Fold-change reductions in these animals at these timepoints were drastically higher than untreated animals (Fig. B2A). Because of reduced viral loads treated animals were followed for an additional four weeks post-treatment, with a progressive restoration of viral loads returning to untreated animal levels (Fig. B1.2B).

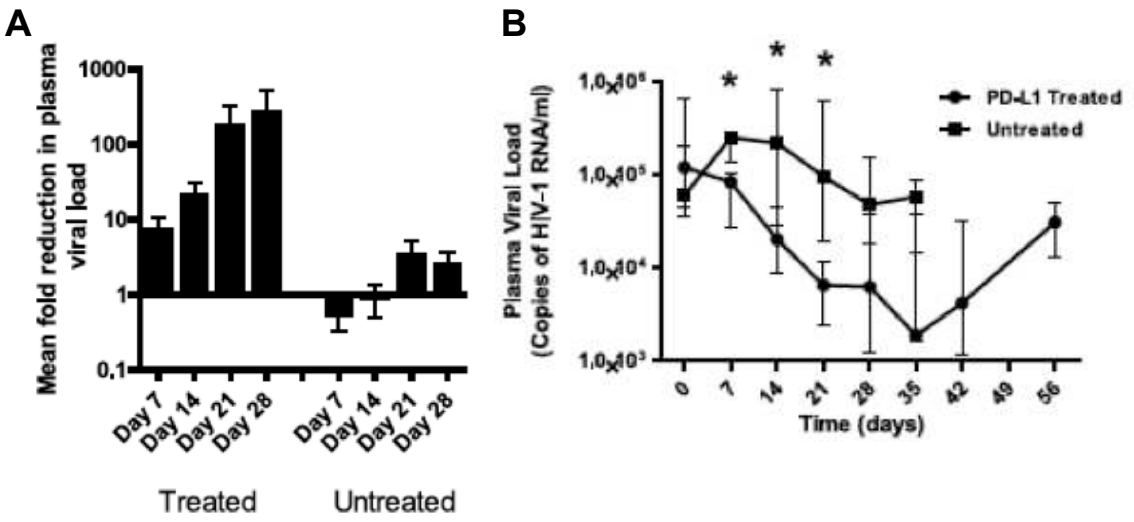

Figure B1.2. Plasma viral loads in HIV+ hu-mice. Animals were HIV positive for one month before undergoing anti-PD-L1 treatment. 200 μg of antibody PD-L1 were administered for 10 treatments over 4 weeks. Timepoints for plasma viral load were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. *$p < 0.05$ (Mann-Whitney-Wilcoxen).

B1.3c PD-L1 treatment increased IFN-γ and IL-12 levels

In order to better understand the mechanism behind anti-PD-L1 treatment down-regulation of HIV viral load, plasma levels of cytokines were measured before, during, and after treatment. Th2 cytokines showed no significant differences between treated and untreated HIV+ animals (Fig. B1.3, bottom panels). However, Th1-related cytokines (IFN-γ and IL-12) were significantly elevated in treated animals, with a trend for significance of elevated TNF-α (Fig. B1.3, top panels).

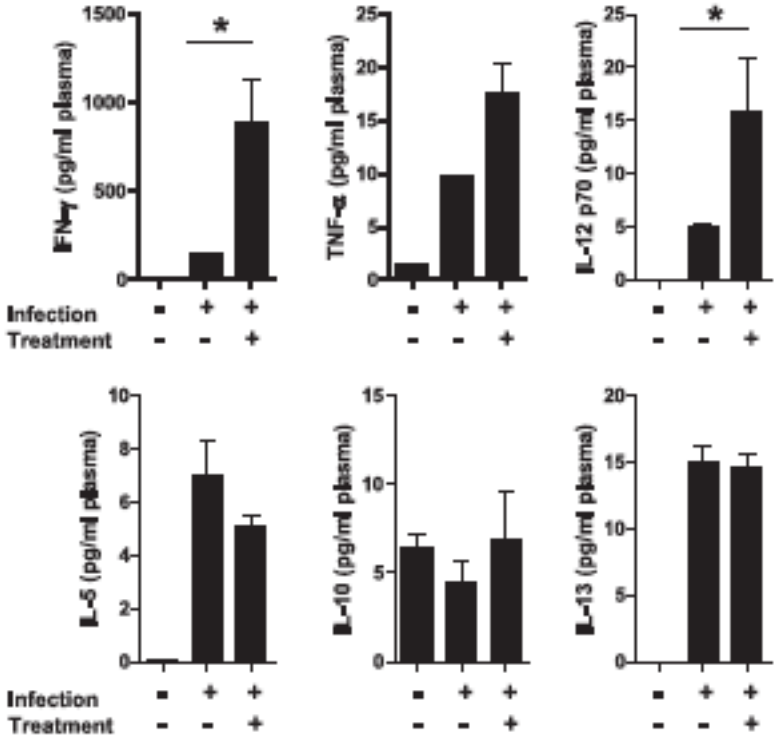

Figure B1.3. Plasma cytokine levels in HIV+ mice before and after infection and treatment. Human plasma cytokines were measured by MESO multiplex cytokine kit. Animals were bled before infection (− −), prior to treatment (+ −), and 7 days after treatment (+ +). Groups were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. *p=0.0002 (Mann-Whitney-Wilcoxen).

B1.3d CD4+ and CD8+ T cell levels were increased in PD-L1 treated mice

In order to address the effect of PD-L1 treatment on T cells, the percentage of CD4+ and CD8+ lymphocytes was measured in treated, untreated, and uninfected animals. Beginning one week after the initiation of treatment (2 treatments in) CD4+ T cell levels were significantly higher than infected, untreated animals, exhibiting a typical CD4 suppression (Fig B1.4A). When these levels continued to rise, the number of sample days was extended for the treated animals, showing that elevated CD4+ T cell levels continued for weeks past the last treatment. CD8 numbers were also significantly increased versus untreated animals, pointing to a possible mechanism of viral suppression (Fig. B1.4B).

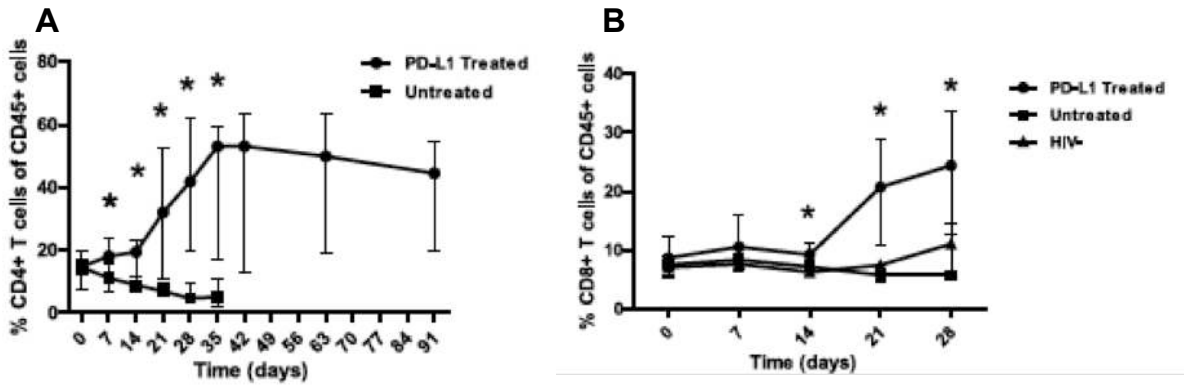

Figure B1.4. CD4+ and CD8+ T cell levels in treated and untreated hu-mice. Beginning at day 0 prior to treatment animals were monitored weekly for CD4+ and CD8+ T cells within the CD45+ lymphocyte population by flow cytometry. CD4 levels were measured beyond the set experimental timepoints in treated animals by 8 weeks. Timepoint comparisons between treated and untreated animals were compared by analysis of variance using non-parametric Mann-Whitney-Wilcoxon test. *$p < 0.05$ (Mann-Whitney-Wilcoxen).

B1.4 Discussion

T cell exhaustion as an HIV-related pathology has only recently been identified as. This exhaustion has been shown to be directly related to inhibitory receptors. Whether the upregulation of these receptors is the cause or symptom of exhaustion is unclear, but their relevance as an avenue for treatment is obvious. We have demonstrated that blocking the interactions between programmed death ligand 1 (PD-L1) on APCs and programmed death 1 (PD-1) on T cells we could restore CD4+ T cells to non-HIV infected levels. The mechanism behind this restoration likely lies in the reduced signaling T cells receive from the PD-1/PD-L1 co-inhibitory pathway, removing the resulting anti-proliferation signals. We have also shown that serum HIV levels are significantly suppressed following PD-L1 treatment. The mechanism behind this reduction is likely due to a combination of CD4 effector and CD8 effector responses against HIV, as demonstrated by improved CD8+ T cell levels and the secretion of IFN-γ and IL-12.

It is important to note that while promising, the HIV levels in treated mice did not remain suppressed. This is despite boosted CD4+ T cell numbers that continued even after the cessation of treatment. It would be expected that after the end of treatment this newly boosted reservoir of

T cells would make an excellent founder population for the virus still present. Further timepoints past those measured may show an eventual CD4 reduction, but if T cell levels continued to maintain perhaps the reinvigoration of T cells by PD-L1 blockade rendered the leftover virus reservoir population weakened. Future work on the effects of PD-L1 blockade on latency, as well as the direct cytoxic improvements of CD8+ T cells, would help elucidate possible mechanisms of control.

Perhaps most important is the demonstration of the hu-mouse as a model system for observing HIV-induced T cell exhaustion. Further studies can improve upon our understanding of the nuances of exhaustion in hu-mice, but the increased levels of PD-1 in infected animals implies an exhausted phenotype. Future studies on other diseases that have associated exhaustion (lymphocytic choriomeningitis virus, cytomegalovirus, hepatitis B, hepatitis C, cancer) could identify if exhaustion is a readily observable phenotype in hu-mice .

B2: Pharmacokinetics And Pharmacodynamics Of Anti-Hiv Drugs In Humanized Mouse Tissues

Veselinovic M, Yang KH, LeCureux J, Sykes C, Remling-Mulder L, Kashuba AD, Akkina R. (2014) HIV pre-exposure prophylaxis: mucosal tissue drug distribution of RT inhibitor Tenofovir and entry inhibitor Maraviroc in a humanized mouse model. Virology. Sep;464-465:253-63. doi: 10.1016/j.virol.2014.07.008.

Veselinovic M, Yang KH, Sykes C, Remling-Mulder L, Kashuba AD, Akkina R. (2016) Mucosal tissue pharmacokinetics of the integrase inhibitor raltegravir in a humanized mouse model: Implications for HIV pre-exposure prophylaxis. Virology. Feb;489:173-8. doi: 10.1016/j.virol.2015.12.014.

B2.1 Overview

The number of deaths from HIV has steadily declined in recent years (156). However the number of new infections has plateaued, tending to affect high-risk groups like minority men who have sex with men (MSM) (157, 158). Once infection is established full eradication becomes impossible by current treatments, thus preventing new infections is key (207). HIV vaccines are still not efficacious enough to afford adequate prophylaxis, thus alternative prevention strategies must be attempted (217). Pre-exposure prophylaxis (PrEP) is one promising strategy that places high-risk patients on anti-HIV drugs before infection (243). Several studies have shown significant levels of protection, while others have had mixed results likely due to problems with adherence (244, 340). In order to properly dose patients with anti-HIV drugs the pharmacokinetics and pharmacodynamics (PK/PD) of said drugs must be understood. Human and non-human primate PK/PD studies are expensive. Mouse models, particularly humanized mice, provide a more cost effective system for PK/PD studies, but drug levels must be measured in various tissue compartments to ensure that future human work can be correlated. To this end we measured the PK/PD of three anti-HIV drugs: raltegravir, an integrase strand transfer inhibitor (428); maraviroc, a CCR5 antagonist fusion inhibitor (429); and tenofovir, a nucleotide analogue reverse transcriptase inhibitor (430).

B2.2 Materials and Methods

B2.2a Generation of humanized mice

Humanized female BALB/c $Rag2^{-/-}$ or $Rag1^{-/-}$ $CD132^{-/-}$ (Rag-hu) mice were prepared as previously described (426). Briefly, newborn mice were irradiated with 350 rad and then injected

intrahepatically with 0.5–13 million human CD34+ cells isolated from human fetal liver by magnetic bead separation. Mice were screened for human cell engraftment at 10–12 weeks post-reconstitution. Peripheral blood was collected by tail bleed every week, and RBCs were lysed using the Whole Blood Erythrocyte Lysing Kit (R&D Systems) to isolate white blood cells (WBC). The WBC fraction was stained with anti-human CD45-PE (Invitrogen) and analyzed by flow cytometry to determine the levels of human cell engraftment as previously described (426). Only mice with engraftment above 50% were used in the study.

B2.2b Administration of antiretroviral drugs and sample collection

Mice were administered tenofovir disoproxil fumarate (TFV, $6.15x10^{-2}$ mg/g, Gilead Sciences), maraviroc (MVC, $6.2x10^{-2}$ mg/g, Selzentry) or raltegravir (RAL, $1.64x10^{-1}$ mg/g, Merck & Co.) by oral gavage for 5 days. Drugs were freshly dissolved in sterile PBS prior to administration. Mouse equivalent drug doses were calculated using an interspecies allometric scaling factor of 12.3 (431, 432). 3-5 mice per timepoint were sacrificed at 2, 8, and 24 hours following the last gavage of tenofovir. 3-5 mice per timepoint were sacrificed at 4, 12, and 24 hours following the last gavage of maraviroc. 2-3 mice per timepoint were sacrificed at 2, 8 and 24 hours following the last gavage of raltegravir. 48-hour plasma samples were also collected for each drug. Plasma samples were obtained by cardiac puncture at termination, except 48 hour tail vein bleeds. At termination each vaginal, rectal and intestinal tissue sample was removed, placed in screw-cap tubes and immediately snap frozen in liquid nitrogen. Untreated animal tissues and plasma were used as negative controls. All samples were stored at −80°C until drug analysis.

B2.2c Plasma and tissue drug concentration measurements

Tissue and plasma TFV and TFV-DP were extracted from tissue homogenate and plasma by protein precipitation with isotopically-labeled internal standards (^{13}C TFV and ^{13}C TFV-DP). TFV was eluted from a Waters Atlantis T3 (100 × 2.1mm, 3µm particle size) analytical column, and TFV-DP was eluted from a Thermo Biobasic AX (50 × 2.1mm, 5µm particle size) analytical column. An API-5000 triple quadrupole mass spectrometer was used to detect all analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1).

Plasma MVC was extracted using solid phase extraction with Varian BondElut C-18, 100 mg, 1CC cartridges. Plasma samples were quantified against the internal standard alprazolam on an Agilent 1200 series HPLC system using a Zorbax Eclipse XDB (50 × 4.6mm, 1.8µm particle size) analytical column. An Agilent 1100 MSD was used to detect the analyte and internal standard.

Tissue MVC was extracted from tissue homogenates using protein precipitation with the internal standard alprazolam. Resulting extract was analyzed on an Agilent 1200 series HPLC system using a Zorbax Eclipse XDB (50 × 4.6mm, 1.8µm particle size) analytical column. An Agilent 1100 MSD was used to detect the analyte and internal standard.

Plasma and tissue RAL was extracted with methanol containing the isotopically-labeled internal standards (^{2}H RAL). RAL was eluted from a Phenomenex Synergi Polar-RP (50×4.6 mm^2, 4 µm particle size) analytical column. An API-5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) was used to detect the analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1).

B2.2d Statistical analysis

Pharmacokinetic data for all drugs were compared using Mann-Whitney two-tailed test using Prism 5 (Graphpad). $P<0.05$ were considered significant. Measures of central tendency were expressed as median and inter-quartile range (IQR 25^{th}, 75^{th}percentile).

B2.3 Results

B2.3a Raltegravir levels in tissue are undetectable by 48 hours.

Levels of raltergravir in vaginal, rectal and intestinal tissue compartments progressively dropped over the course of the experiment. By 24 hours most tissue RAL was barely above the limit of detection, and by 48 hours no RAL was detected in tissue (Fig. B2.1A). Plasma followed a similar trend, though several animals were below the limit of detection by 24 hours (Fig. B2.1A, red line). The ratio of tissue to plasma RAL indicate ~10-fold higher levels of RAL in tissues versus plasma (Fig B2.1B).

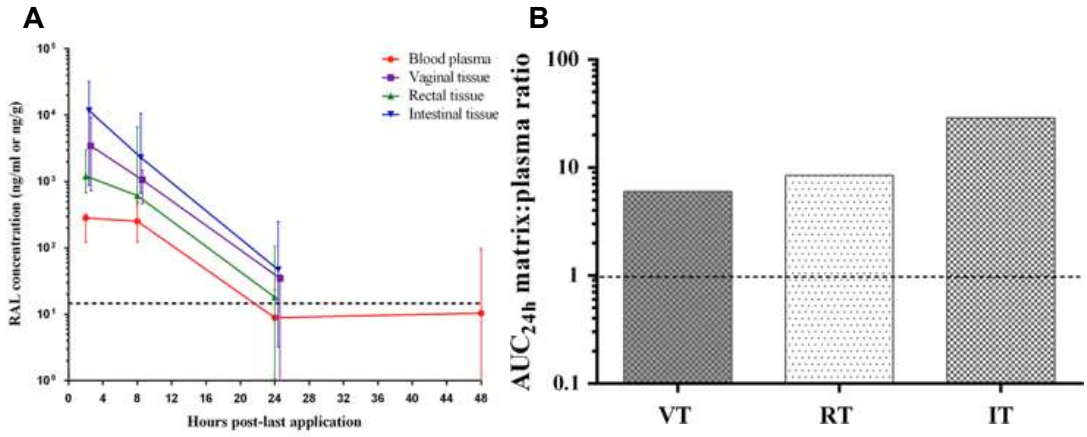

Fig. B2.1 Pharmacokinetic analysis of raltegravir in humanized mouse tissue and plasma. Mice were administered RAL by oral gavage ($1.64x10^{-1}$ mg/g) for 5 days. Plasma and tissue samples were collected at different time points following the last dose and drug concentrations were determined (ng/ml or ng/g). Composite medians and interquartile ranges (IQR) for plasma, vaginal, rectal and intestinal tissue (A). Each tissue to plasma ratio was calculated for the area under the curve (AUC) at 24 hours post dose (B).

B2.3b Maraviroc levels in vaginal tissue are lower than rectal at 24 hours.

Levels of maraviroc in vaginal, rectal and intestinal tissue compartments progressively dropped over the course of the experiment. By 24 hours tissue MVC was around 10ng/g, and by 48 hours no RAL was detected in tissue (Fig. B2.2A). Plasma was basically undetectible by 12 hours (Fig. B2.2A, red line). The ratio of tissue to plasma RAL indicate ~10 to 100-fold higher levels of RAL in rectal and intestinal tissue versus plasma, with about 5-fold higher levels in vaginal tissue (Fig B2.2B).

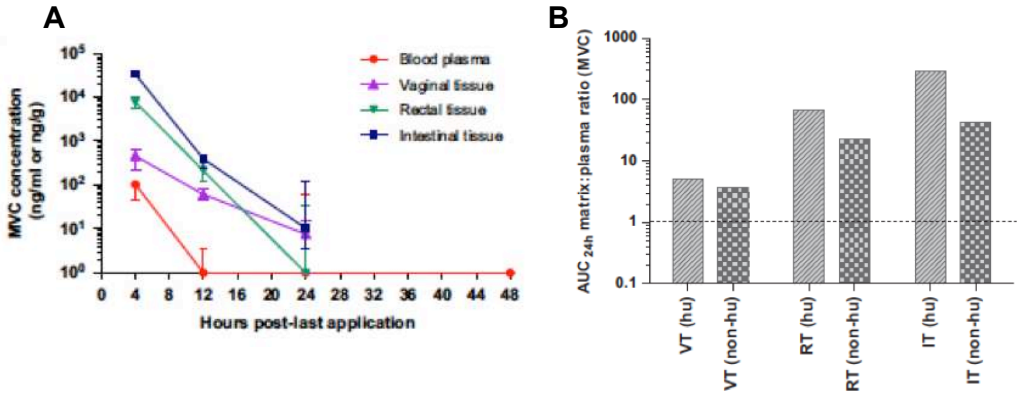

Fig. B2.2 Pharmacokinetic analysis of maraviroc in humanized mouse tissue and plasma. Mice were administered MVC by oral gavage ($6.2x10^{-2}$ mg/g) for 5 days. Plasma and tissue samples were collected at different time points following the last dose and drug concentrations were determined (ng/ml or ng/g). Composite medians and interquartile ranges (IQR) for plasma, vaginal, rectal and intestinal tissue (A). Each tissue to plasma ratio was calculated for the area under the curve (AUC) at 24 hours post dose (B).

B2.3c Tenofovir and Tenofovir-diphosphate are much higher in intestinal tissue than vaginal.

Levels of tenofovir (TFV) in vaginal, rectal and intestinal tissue compartments, and tenfovir-diphosphate (TFV-DP, metabolized active form) dropped more slowly than other drugs, remaining detectible at relatively high levels 24 hours after dose, though becoming undetectable

by 48 hours (Fig. B2.3A, C). Plasma was detectible out to 48 hours (Fig. B2.3A, red line). The ratio of tissue to plasma TFV indicate ~100-fold higher levels of TFV in rectal and intestinal tissue versus plasma, essentially no change in vaginal tissue (Fig B2.3B).

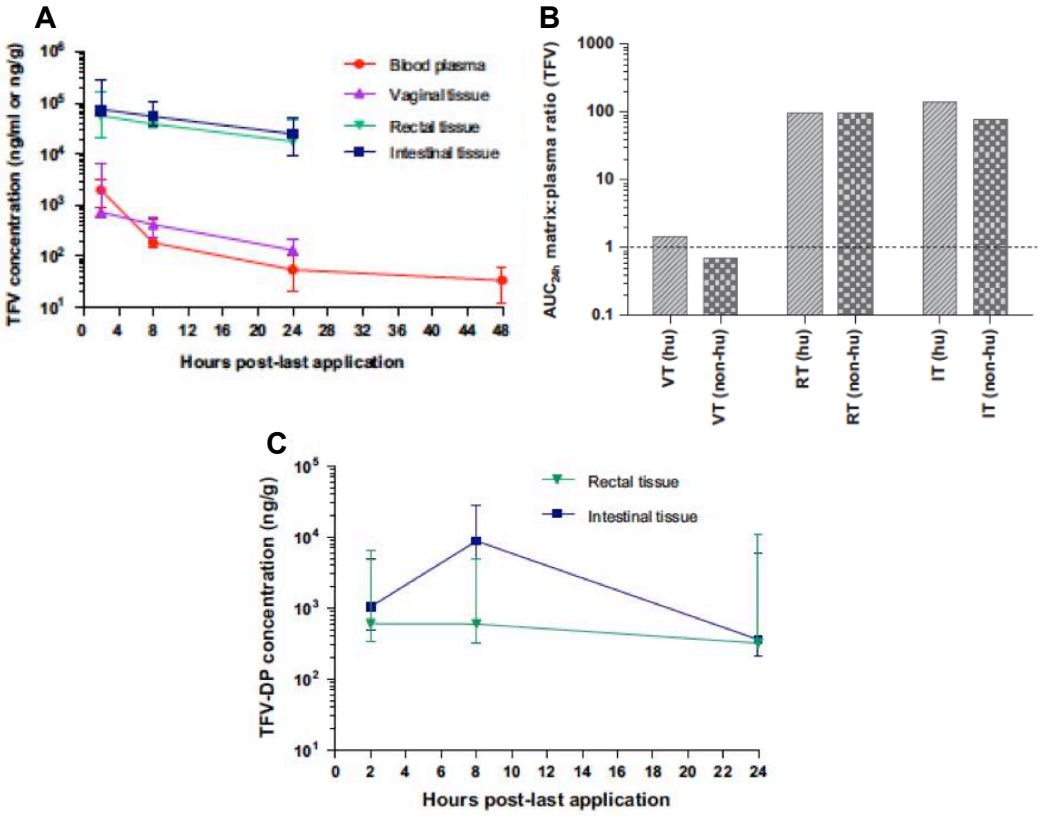

Fig. B2.3 Pharmacokinetic analysis of tenfovir and tenofovir-diphosphate in humanized mouse tissue and plasma. Mice were administered TFV by oral gavage ($6.15x10^{-2}$ mg/g) for 5 days. Plasma and tissue samples were collected at different time points following the last dose and drug concentrations were determined (ng/ml or ng/g). Composite medians and interquartile ranges (IQR) for plasma, vaginal, rectal and intestinal tissue (A, C). Each TFV tissue to plasma ratio was calculated for the area under the curve (AUC) at 24 hours post dose (B).

B2.4 Discussion

All drugs were detectible in humanized mouse tissues for at least the first 24 hours following the end of dosing. Levels of RAL and MVC were much lower at 24 hours, indicating a shorter tissue half-life than TFV. TFV appeared to have a relatively weaker ability to enter vaginal tissue than intestinal tissue. These vaginal levels correlated with plasma TFV levels,

implying a close relationship between blood and vaginal levels. As expected, drug levels in plasma were more rapidly cleared, implying a larger number of variables to remove drugs from circulating plasma (filtering, movement into tissues). All three drugs were detectible for at least a day following dosing, making them excellent candidates for PreP. However, this turnover, especially in MVC and RAL, is relatively quick and may be a difficult hurdle to overcome in populations with poor adherence. Other strategies to extend the release of drugs and thus maintain tissue levels over an extended period of time would be helpful. Future work will observe the ability of varying drug concentrations to protect against HIV challenge (433), as well as the presence of drugs secreted into the vaginal or intestinal lumens. Humanized mice provide an excellent model system for observing PK/PD levels and are now a platform for new anti-HIV drugs to be tested, such as Vorinistat, an anti-latency histone deacetylase inhibitor (434), or Truvada, a combined TFV and emtricitibine cocktail (435).

B3: CD34 Infections *In Vitro* and *In Vivo*

B3.1 Overview

The hope that HIV-1 infections could be cured with long term highly active antiretroviral therapy (HAART) was shattered by the discovery of latently infected resting memory CD4+ T cells (436). While HAART can substantially reduce the numbers of cells actively producing virus, these same drugs have little efficacy against latently infected cells which may later become reactivated (437), potentially initiating new rounds of viral replication should the patient stop taking HAART (438). This latent viral reservoir is a massive barrier to a cure for HIV-1, and new drugs are being investigated for their ability to activate latent provirus (439). While resting

memory CD4+ T cells are likely the primary latent reservoir, evidence has been mounting that other cell subsets may also harbor latent provirus (440).

One such subset may be bone marrow hematopoietic stem cells (HSCs) (441). These cells are CD34+, and serve as precursors to multiple cells types involved in innate and adaptive immunity, such as T cells, B cells, monocytes, dendritic cells, macrophages, neutrophils, and NK cells (442). *In vitro* work has previously demonstrated that CD34+ HSCs isolated from fetal cord blood are capable of becoming latently infected with HIV-1 (443, 444). Other studies, however, have failed to demonstrate HIV-1 infection of these cells, and the topic remains controversial (445, 446).

Our study addresses this issue of *in vivo* reactivation of HIV-1 infected CD34+ HSCs through the use of a humanized mouse model. $Rag^{-/-}$ $CD132^{-/-}$ newborn mice, when engrafted with human fetal liver derived CD34+ HSCs, develop an intact immune system with multiple lineages of human immune cells present, including human CD4+ T cells, rendering these mice susceptible to HIV-1 infection (427). We examined whether HIV-1 infection of CD34+ HSCs, followed by their engraftment into these mice, could provide the spark necessary to initiate sustained viremia following development of a human immune system in these mice. As the isolated fetal liver derived CD34+ HSCs are unlikely to contain CD4+ T cells, the initiation of infection in these mice would be due to CD34+ HSCs which serve as an *in vivo* source of new virus. We also wished to examine previous findings that CD34+ HSCs can be infected with CXCR4 tropic, but not CCR5 tropic, strains of HIV-1 (447).

Our study demonstrates that CD34+ HSCs can be infected with both CCR5 and CXCR4 tropic strains of HIV-1, and shows for the first time that these cells are capable of serving as a viral reservoir in vivo to establish infection. By showing the capacity of these cells to serve as

reservoirs for new viral infection *in vivo*, a strong argument is made that all potential latent viral reservoirs, not simply latently infected CD4 T cells, must be targeted in order to fully eliminate the virus and potentiate a cure for HIV through the use of ART.

B3.2 Materials and Methods

B3.2a Fetal CD34+ stem cell extraction, growth and infection.

Human fetal liver-derived CD34+ cells were prepared as previously described (426). Briefly, fetal liver was enzymatically digested with DNase, hyaluronidase and collagenase to obtain a single cell suspension, then incubated with anti-human CD34 magnetic bead antibodies and concentrated through two Miltenyi magnetic columns. Isolated CD34+ cells were grown in cytokine-supplemented medium (SCF, 50ng/ml TPO, 100ng/ml IGFBP-2, and 50ng/ml Flt3-L (447)). Once large colonies were grown, approximately 0.5-1*10^6 cells were infected with HIV-1 strains BaL or NL4-3 at an approximate MOI of 5. The CD34s were incubated for 4 hours then washed and plated in stem cell media. Supernatant was collected by spinning down CD34+ cells, collecting supernatant, and resuspending CD34s in fresh media.

B3.2b Generation of HIV-1 engraftment-infected Rag-hu mice.

Humanized BALB/c $Rag2^{-/-}CD136^{-/-}$ mice were prepared as previously described using infected or uninfected human fetal liver-derived CD34+ cells (448). Briefly, neonatal mice were conditioned by irradiating at 350 rad then injected intrahepatically with 0.5–1x10^6 human CD34+ cells (either uninfected or infected). 8 weeks after reconstitution mice were screened for human lymphocyte engraftment. Blood was collected by tail bleed and red blood cells were lysed

using the Whole Blood Erythrocyte Lysing Kit (R&D Systems). The white blood cell fraction was stained with anti-CD45-PE (Invitrogen) and analyzed using an Accuri C6 flow cytometer (BD) (426).

B3.3c Real time PCR to measure HIV viral load.

Plasma HIV-1 was detected by qRT-PCR. RNA was extracted from 25–50 ul of EDTA-treated plasma using the QIAamp Viral RNA kit (Qiagen). QRT-PCR was performed using a primer set specific for the HIV-1 LTR sequence and One-Step SYBR Green qPCR kit (Bio-Rad) (426).

B3.3a Infected CD34s release higher levels of BaL HIV than NL4-3

To assess the ability of two common laboratory strains of HIV-1 to infect fetal liver derived CD34+ stem cells, extracted CD34+ cells were subjected to HIV-1 Bal or NL4-3 infection *in vitro*. Supernatants were collected over the course of 3 weeks and viral RNA was quantified by RT-qPCR. Both viral infections had initial viral RNA levels around $10^{4.5}$ copies/mL (Fig. B3.1). Bal-infected CD34s began to release progressively higher levels of viral RNA, exceeding 10^6 copies/mL at 23 days post infection. NL4-3-infected CD34+ cells showed a progressive but still detectable decline over the same time period.

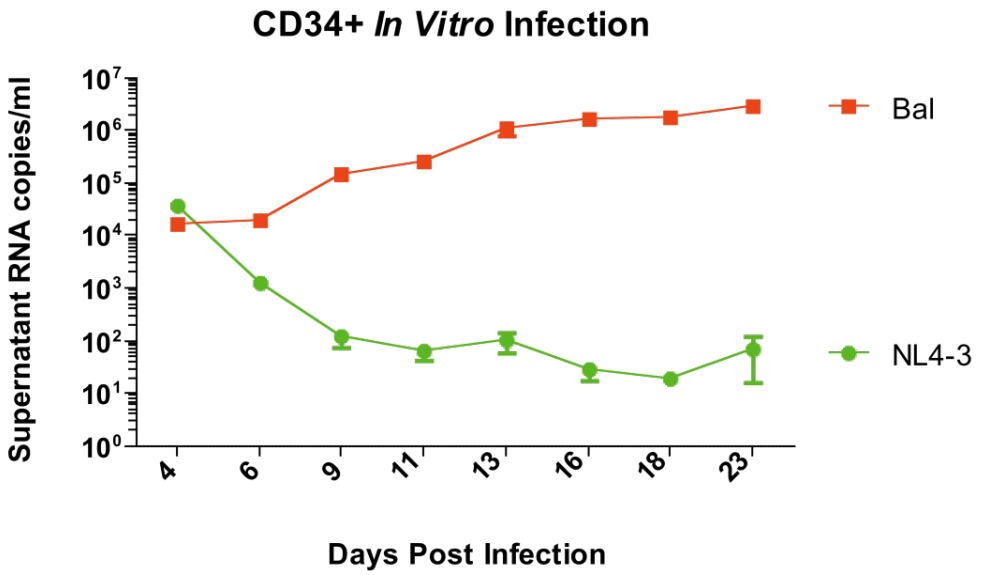

Figure B3.1 Viral supernatant RNA over time following *in vitro* CD34+ HSC infection: After infection (MOI 5), cells were pelleted and supernatant removed by pelleting cells and pulling off supernatant. Cells were then washed twice in PBS and resuspended in fresh media. Each time point represents only the virus accumulated after the previous collection.

B3.3b Viral RNA in vivo

For *in vivo* experiments, isolated CD34+ cells were infected with either BaL or NL4-3 strains of HIV-1 and engrafted into neonatal RAG$^{-/-}$ CD136$^{-/-}$ mice 24 hours later. Serum viral RNA levels were measured over time, with multiple sets of animals being observed following engraftment (Figure B3.2). 3 weeks after engraftment most animals were HIV positive, with the exception of 3 NL4-3 and 1 BaL. By 8 and 9 weeks post engraftment all animals had detectible viral loads between 10^3 and 10^4 copies/mL. As time progressed some BaL and NL4-3 animals had undetectable viral loads (BaL after 25 weeks and NL4-3 after 30).

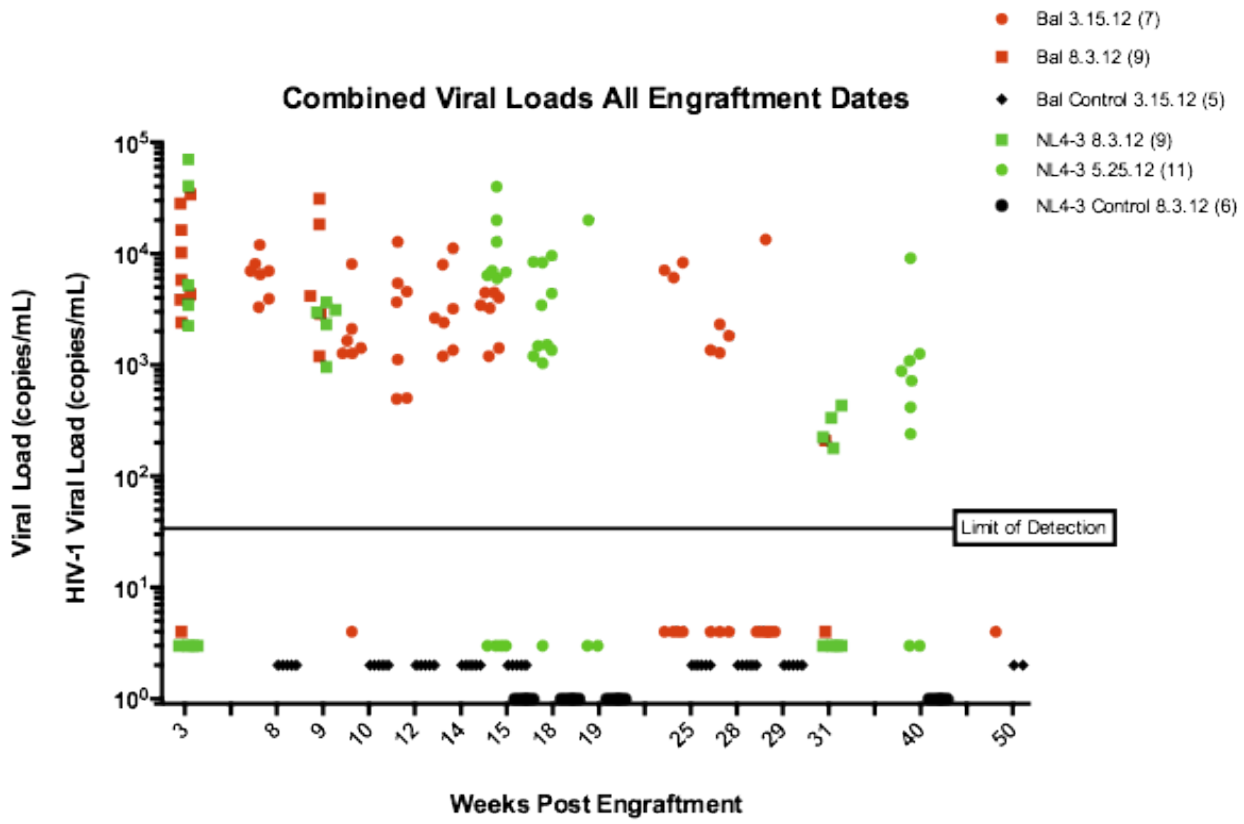

Figure B3.2 *In vivo* viral loads of HIV infected mice. Humanized mice were engrafted with CD34 HSCs that had been infected at an MOI of 5 with either BaL or NL4-3 strains of HIV-1. Viral RNA loads in engrafted mice were followed for a period of 40-50 weeks. Two sets of mice for each HIV strain were initiated at different timepoints based on availability of pups. 16 BaL mice, 20 NL4-3 mice, and 11 uninfected controls were observed.

B3.3c Control and BaL engrafted animals showed typical CD4 loss but not NL4-3 animals

Next, we assessed the ratio of CD4/CD3 in HIV-1 infected mice. In BaL engrafted mice, there was a typical reduction in the number of CD4 T cells (Fig. B3.3, red line). This was confounded by very low human cell numbers at later timepoints. Control animals did not exhibit this CD4 loss (Fig. B3.3, black and blue lines). Interestingly, NL4-3 engrafted animals did not show the typical loss of CD4 cells (Fig. B3.3, green line).

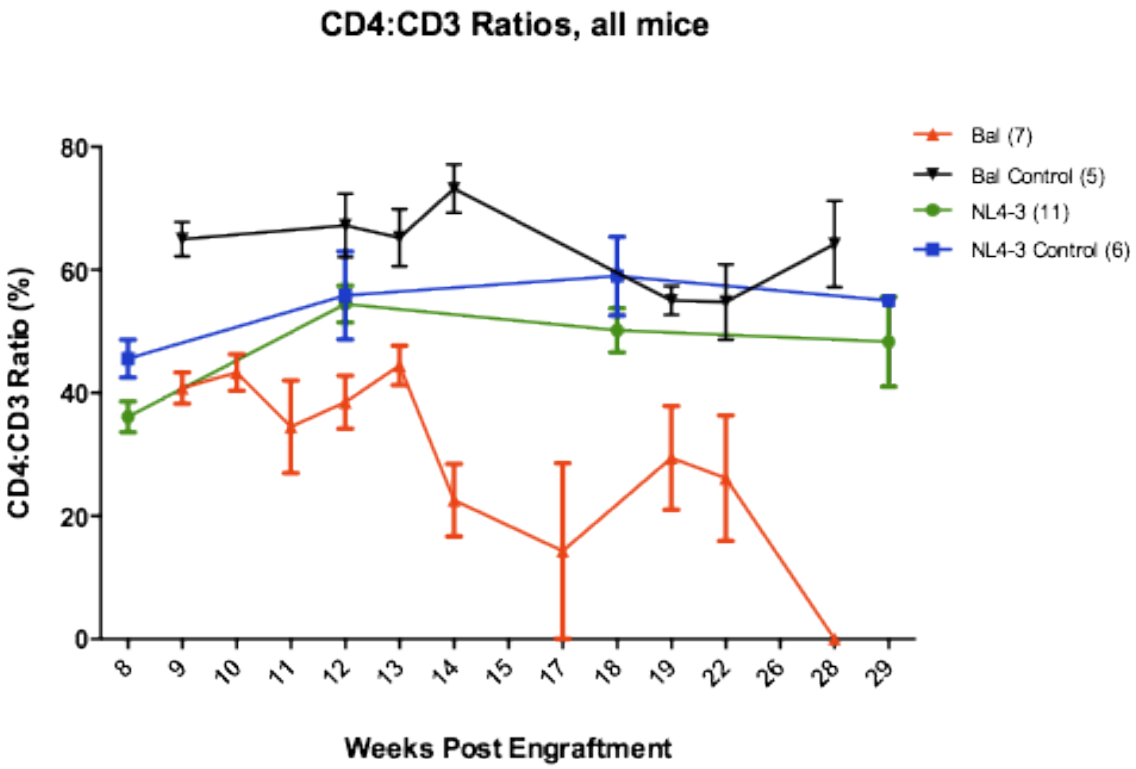

Figure B3.3 percentage of CD4 to CD3 cells in infected and uninfected mice. Frequencies of CD3+ and CD4+ T cells in mouse peripheral blood (tail bleed) were assessed by flow cytometry every one or two weeks following engraftment.

B3.3d Human cell engraftment loss in humanized mice over time.

We next assessed levels of engraftment in mice initially engrafted with HIV-1 infected CD34 HSCs (Fig. B3.4). Through week 13 levels of engraftment between control mice and HIV infected mice were similar. By week 17 BaL infected mice had extremely low engraftment, while other groups showed more progressive engraftment loss.

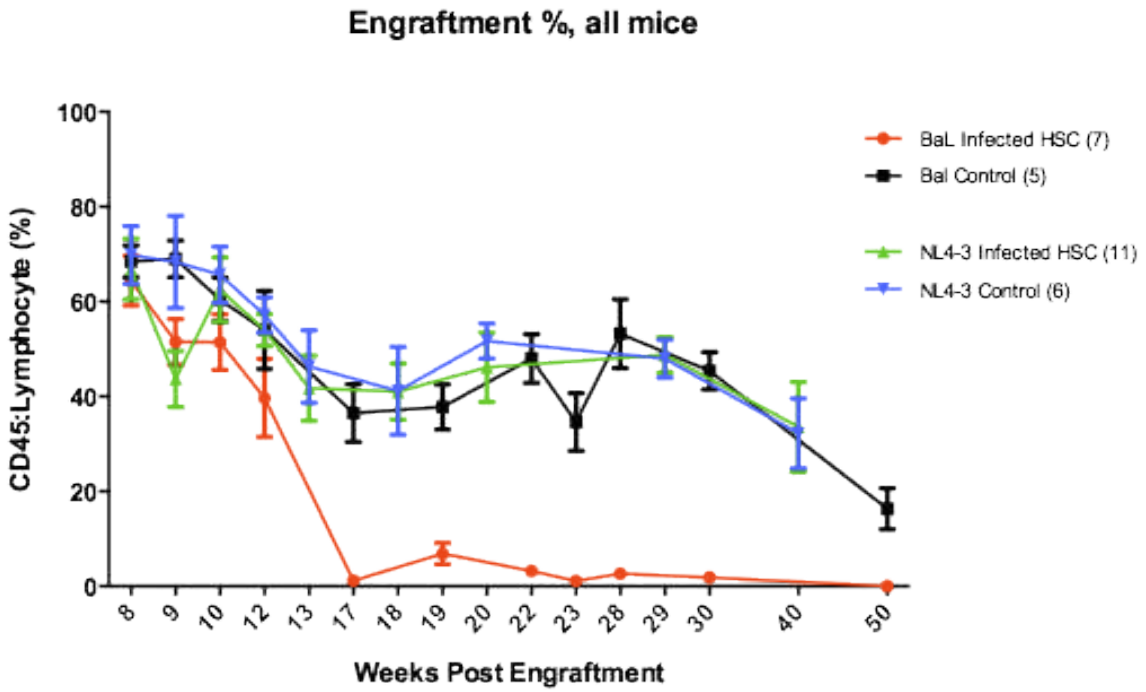

Figure B3.4 Levels of engraftment in mice engrafted with HIV infected or uninfected CD34 HSCs: Overall levels of engraftment are assessed by the frequency of human CD45+ cells present in peripheral blood (tail bleed) of engrafted mice taken at various time points. Cells were gated on lymphocyte subpopulations based on forward scatter and side scatter profile.

B3.4 Discussion

Whether CD34+ HSCs are capable of being infected by HIV and become latently infected is a matter of debate. Previous studies have shown that CD34+ CD133+ cells isolated from bone marrow, and CD34+ HSCs from fetal cord blood are capable of being infected by HIV *in vitro* (443, 444). However conflicting studies have failed to infect HSCs (445, 446). In this study, we demonstrate for the first time that in a humanized mouse model that CD34+ HSC derived from human fetal liver are capable of being infected with both CCR5 and CXCR4 tropic strains of HIV, and that upon engraftment of these infected cells into $Rag^{-/-}$ $CD136^{-/-}$ mice, a productive infection is established.

Our *in vitro* data show that CD34+ HSCs are capable of becoming infected with a CCR5 tropic strain of HIV-1 (BaL). This is at odds with previous published reports showing HSCs being infected with only CXCR4 tropic HIV-1 (447). This may be explained by differences in isolation techniques and markers used to identify HSCs in this study compared to others, as well as potentially the source of HSCs. Previous studies examining this issue used either bone marrow derived CD34+ CD133+ cells, or fetal cord blood derived CD34 HSCs. In our study, we examined bulk CD34+ HSCs derived from human fetal liver. As CD34+ HSCs are not a homogenous population it is possible that certain cell types present in our study were excluded from other studies examining this issue (449). We observed that among our CD34+ HSC from human fetal liver that a significant proportion were CCR5 positive, which may help explain the CCR5 tropism (data not shown). Of the few CD4+ cells among the isolated CD34+ cells, most were CCR5 positive (data not shown).

While CD4 levels did appear to decline for BaL infected mice, the lack of significant CD4 decline in NL4-3 infected mice is puzzling. Viral replication of NL4-3 was possibly deficient in these mice, as seen in Fig. B3.2, where NL4-3 viral loads became undetectable for several mice by week 15. It is important to note that for BaL infected mice, frequencies of CD4 T cells of total CD3+ T cells are difficult to assess accurately, as overall levels of engraftment for BaL infected mice are significantly lower than controls after week 17. This dramatic loss of engraftment in the BaL infected mice is possibly due to HIV-1 mediated killing of CD34+ HSCs in the mouse bone marrow, and is an issue which warrants further investigation. It is possible that some of the decline in immunity as a result of HIV-1 infection may be due to loss of this reservoir of progenitor cells due to their susceptibility to HIV-1 infection.

One potential limitation of this study is that CD34 HSCs isolated from human fetal liver, rather than HSCs from adult human bone marrow. There may be important differences between CD34s isolated from human fetal liver versus CD34s isolated from human bone marrow. However, contamination with CD4+ T cells in adult bone marrow would make such a study difficult. We conclude that CD34+ fetal liver cells can be infected with both BaL and NL4-3 virus *in vitro* and these cells can be engrafted and maintained in mice for an extended period. Infected CD34+ cells are enough to establish an infection in humanized mice and indicate the potential for HSC as a reservoir for HIV. Future work will include looking for latently infected CD34's in the mice and testing anti-latency drugs for their effect on the HSC reservoir.

Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity
AMP: antimicrobial peptides
APC: antigen-presenting cell
APRIL: a proliferation inducing ligand
ART: antiretroviral therapy
BAFF: B-cell activating factor
BaL: HIV-1 isolate
BALB/c: inbred mouse strain
BALT: bronchus-associated lymphoid tissue
bNAbs: broadly-neutralizing antibodies
C57BL/6: inbred mouse strain
CD40L-/-: inbred mouse strain without CD40L
CTLA-4: cytotoxic T-lymphocyte-associated protein 4
DC: dendritic cell
DEX: dextrose
ELISA: enzyme-linked immunosorbent assay
ELISPOT: enzyme-linked immunospot
ESCRT: endosomal sorting complexes required for transport
FliC: *Salmonella* flagellin subunit C
FRT: female reproductive tract
GALT: gut-associated lymphoid tissue
GAPs: goblet cell associated-antigen pathways
GRAS: Generally Regarded As Safe
HAART: highly active antiretroviral therapy
HIV: human immunodeficiency virus
HLA: human leukocyte antigen
HSC: hematopoietic stem cell
hu-Mice: humanized mice
IFN-γ: interferon-gamma
IgA: immunoglobulin class A
IgG: immunoglobulin class G
IgM: immunoglobulin class M
IL-*X*: interleukin-*X*
ILC: innate lymphoid cell
LAB: lactic acid bacteria
LAG-3: lymphocyte-activation gene 3
LPS: lipopolysaccharide
LTA: lipoteichoic acid
MAIT: mucosal-associated invariant T cell

MALT: mucosa-associated lymphoid tissue
MHC: major histocompatibility complex
MLN: mesenteric lymph node
MPER: membrane-proximal external region
MRS: deMann-Rogosa-Sharpe medium
MVC: maraviroc
NK: natural killer cell
NL4-3: HIV-1 isolate
NLR: NOD-like receptor
NOD: nucleotide-binding oligomerization domain-containing protein
PCA: principle components analysis
PD-1: programmed death-1
PK/PD: pharmacokinetics/pharmacodynamics
PP: Peyer's patch
PrEP: pre-exposure prophylaxis
PRR: pattern recognition receptor
qRT-PCR: quantitative reverse transcriptase polymerase chain reaction
RAG: recombination-activating gene
RAL: raltegravir
RB: rice bran
RBE: rice bran extract
ROC: receiver operating characteristic
SILT: solitary isolated lymphoid tissues
SPL: spleen
TED: transepithelial dendrites
TFV: tenofovir
Th1: T helper type 1
Th17: T helper 17 cell
Th2: T helper type 2
Tim-3: T-cell immunoglobulin and mucin-domain containing-3
TLR: toll-like receptor
TNF-α: tumor necrosis factor-alpha
Treg: T regulatory cell