CHARACTERIZATION OF A NOVEL *MYCOBACTERIUM TUBERCULOSIS*-SIMIAN IMMUNODEFICIENCY VIRUS VACCINE TO PREVENT ORAL PEDIATRIC HIV TRANSMISSION

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ABSTRACT

Kara Lindsey Jensen: CHARACTERIZATION OF A NOVEL *MYCOBACTERIUM TUBERCULOSIS*-SIMIAN IMMUNODEFICIENCY VIRUS VACCINE TO PREVENT ORAL PEDIATRIC HIV TRANSMISSION
(Under the direction of Kristina Abel)

Over 3.3 million children are living with HIV, infected primarily by mother-to-child transmission (MTCT). Breast milk exposure of HIV accounts for up to 44% of MTCT events. Despite serious efforts to prevent vertical HIV transmission, infant testing is often delayed and access to antiretroviral therapies is still limited. Compared to adults, HIV-infected infants experience enhanced disease progression and more severe co-morbidities with pathogens like *Mycobacterium tuberculosis* (*Mtb*). The live attenuated BCG vaccine is the only licensed tuberculosis vaccine but BCG can disseminate in immunosuppressed, HIV-infected infants. Considering the significant geographical overlap of the HIV and TB epidemics and the high vulnerability of infants to both pathogens, a pediatric vaccine to safely protect against HIV and *Mtb* is urgently needed.

We hypothesized that human-adapted attenuated *Mtb* strains engineered to co-express HIV genes (rA*Mtb*-HIV) would safely induce the development of dually-immunogenic HIV- and *Mtb*-specific cellular and humoral responses. Three distinct attenuated *Mtb*-SIV strains were evaluated in the neonatal rhesus macaque model. Oral priming with strain $mc^2$6435 plus systemic MVA-SIV boosts successfully induced the development of *Mtb*- and SIV-specific cellular and humoral responses and was identified to be safe in healthy and immunosuppressed, SIV-infected neonatal macaques.

However, despite vaccine-induced immunity, vaccination did not protect infants against low-dose oral SIV challenges designed to mimic oral MTCT during breastfeeding. Two important results emerged from the challenge study. First, higher Env-specific mucosal IgA activities and plasma IgG avidities positively correlated with controlled viremia in a subset of vaccinated animals. These animals also maintained peripheral $CD4^+$ T cell populations and IL-17-expressing lymphocytes in the intestinal mucosa. However, the majority of vaccinated animals required fewer low-dose SIV exposures to become infected than unvaccinated animals. Enhanced viral acquisition was associated with vaccine-induced persistent immune activation. At the time of challenge, CCR5-expressing $CD4^+$ T cells were observed with greater frequencies in blood, oral and intestinal tissues in vaccinated animals only, providing increased frequencies of SIV target cells. Due to the potential impact of these data on BCG vaccine safety and pediatric HIV and TB vaccine development, additional studies are required to confirm these complex and intriguing results.

To my loved ones:

Away from campus, you provided me friendship, humor and adventure. An unplanned cross-country move offered challenges I wasn't particularly prepared for, but your support and encouragement inspired me to stick with it.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS

Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
BSA	Bovine serum albumin
CCR5	C-C chemokine receptor 5
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope protein
FBS	Fetal bovine serum
FSC	Forward scatter
Gag	Gag protein
GM-CSF	Granulocyte macrophage colony stimulating factor
H&E	Hematoxylin and eosin
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
Iono	Ionomycin
$mc^2$5157	Attenuated *Mtb* expressing SIV *Gag*: Δ*nuoG*Δ*panCD*Δ*RD1*:pSIV GAG
$mc^2$6020	Attenuated *Mtb* strain: Δ*lysA*Δ*panCD* (no SIV insert)

$mc^2$6208	Attenuated *Mtb* expressing SIV *Env*: Δ*leuCD*Δ*panCD*Δ*SecA2*:pSIV ENV
$mc^2$6434	Attenuated *Mtb* parental strain: Δ*leuCD*Δ*panCD*Δ*SecA2* (no SIV insert)
$mc^2$6435	Attenuated *Mtb* expressing SIV *Gag*: Δ*leuCD*Δ*panCD*Δ*SecA2*:pSIV GAG
$mc^2$6440	Attenuated *Mtb* expressing SIV *Pol*: Δ*leuCD*Δ*panCD*Δ*SecA2*:pSIV POL
mDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
mg	Milligram
ml	Milliliter
Mtb	*Mycobacterium tuberculosis* (etiological agent of TB)
MTCT	Mother-to-child transmission
ng	Nanogram
ND	Not detected
NS	Not significant
NT	Not tested
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
R848	Resiquimod
RM	Rhesus macaque
RT-PCR	Reverse transcriptase PCR

SD	Standard deviation
SEM	Standard error of the mean
SIV	Simian Immunodeficiency Virus
SSC	Side scatter
TB	Tuberculosis
$TCID_{50}$	Tissue culture infectious dose, 50%
T_{CM}	Central memory T cell
$T_{E/EM}$	Effector/effector memory T cell
TLR	Toll-like receptor
T_N	Naïve T cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell
μg	Microgram
μl	Microliter

CHAPTER 1: INTRODUCTION

THE PEDIATRIC HIV EPIDEMIC

Multiple distinct zoonotic transmission events of simian immunodeficiency viruses (SIV) in western Africa resulted in the first cases of human immunodeficiency virus (HIV) sometime in the 1930s (62, 67, 101, 102, 114, 161, 170, 234, 306). Although HIV had not yet been identified through the 1970s, symptoms such as Kaposi's sarcoma, wasting disease, opportunistic infections and unusual pneumonias became hallmarks of a fatal infection that appeared to disproportionately afflict African sex and migrant workers and western homosexual men and injection drug users (143, 199). Retrovirologists Françoise Barré-Sinoussi and Luc Montagnier performed fundamental experiments identifying HIV as the etiological agent of AIDS in 1983 (17). An estimated 34 million people currently live with HIV, with 2.5 million new infections annually. Over 350,000 of the new annual infections occur in infants (384).

Mother-to-child transmission (MTCT) events are accountable for over one new pediatric HIV infection per minute, with >90% occurring in sub-Saharan Africa (338, 382, 383). MTCT of HIV occurs via multiple mechanisms including *in utero*, intrapartum or following oral exposure to virus in breast milk (185) and up to 44% of MTCT infections occur following oral breast milk exposure in the absence of preventative interventions (237). The risk of vertical transmission positively correlates with maternal viremia in breast milk and, even with maternal HAART, oral transmission events are only reduced but not

eliminated (280, 338, 384).

Viral transmission during breastfeeding is poorly understood. Due to the immunosuppressive state induced by pregnancy, viral replication, and thus virus levels in breast milk, increase during pregnancy and the postpartum period, translating to increased risk of HIV transmission during breastfeeding (97, 153, 168, 171, 233, 300, 343). Pregnancy-induced immunosuppression accounts for increased levels in both cell-free virus particles and cell-associated virus, both of which are potentially infectious following oral exposure although the relative contributions of each route remain controversial. Recent findings in humans implicate cell-associated virus as a correlate of early MTCT oral transmission (0-6 weeks) (233), while studies in humanized mice identify transmission of cell-associated virus at both oral and intestinal mucosal surfaces and *only* cell-free virus as infectious at mucosal sites distal to the esophagus (356). Similarly, the specific location(s) of viral entry following oral exposure is/are unknown. The palatine tonsil, salivary glands and other organized oral lymphoid tissues, along with upper gastrointestinal mucosal surfaces have been implicated as likely sites of entry due to the presence of HIV-infected cells in these tissues.

Considering the frequency of exposure to virus during the breastfeeding period, HIV infection following oral exposure is inefficient. *In vitro* experiments have demonstrated that breast milk can inhibit HIV infectivity, has explicit antiviral properties and contains many innate factors that exhibit immunoprotective effects (e.g. antibodies, cytokines, lactoferrin, mucins, defensins, tenascin-C, secretory leukocyte protease inhibitor (SLPI), bile-stimulated lipase, lipids, long chain polyunsaturated fatty acids and β-globins)(88, 94, 112, 113, 120, 185, 215, 231, 272, 289, 310, 323, 332, 382). In fact, the inhibitory components in HIV-naïve human breast milk appear to render cell-free HIV unable to orally infect humanized

mice (356).

Factors such as human and viral genetics, commensal microbiome and/or localized innate defenses may also contribute to reduced transmission frequencies following oral HIV exposure. There is likely interplay between the virus and those maternal and infant factors operating to protect against transmission. Despite the viral inhibitory properties of breast milk, oral exposure to virus in breast milk remains an important route of pediatric HIV acquisition and protective interventions are urgently needed to curb the pediatric HIV epidemic.

PEDIATRIC HIV PATHOGENESIS

Most of our knowledge about pediatric HIV pathogenesis stems from observational studies of infected infants. We know that the HIV disease course is accelerated in the young and that both morbidity and mortality are exacerbated compared to infection in older individuals (246). In the absence of therapy, neonatal infections progress rapidly to symptoms of AIDS; about 35% of infected individuals will die before their first birthday and 50% will die before their second, often from secondary infections like tuberculosis (26, 228, 237, 302). HIV-infected infants represent just 4% of the HIV-1-infected individuals worldwide but account for over 20% of HIV-associated fatalities (111).

HIV replicates to high viral titers that are roughly equivalent between infants and adults, peaking at about two weeks post-infection (peak viremia). Following peak viremia and dependent on a number of factors such as comorbidities, viral strain and immune function, the amount of virus in the periphery drops and stabilizes at the viral set point (209). While ART-naïve adults are usually able to reduce plasma viremia significantly between peak viremia and set point, infants are typically much less able to resolve viremia, resulting

in a phenotypic age-associated difference in control of viral replication (Figure 1.1). High viremia along with weak antiviral immune responses in infants accelerates the elimination of T cells, destroys the integrity of the intestinal mucosa, increases systemic bacterial translocation and leads to sustained inflammation (29, 81, 248, 249, 263, 284, 287).

Additionally, pediatric HIV infections can manifest as central nervous system (CNS) disease following viral transfer across the blood-brain barrier (BBB)(20, 52, 98, 164, 220, 222, 253). CNS disease is associated with pediatric AIDS and high levels of compartmentalized virus within the CNS have been associated with accelerated disease (98, 269, 329, 345). Because the BBB requires several years to fully mature, infants are at higher risk of HIV-associated CNS disease compared to older individuals (254, 269). Importantly, CNS disease can prevent normal brain development, resulting in delays in motor skills, speech and other neurocognitive functions, as well as growth and physical development.

Further, the immaturity of the immune response at birth predisposes infants, and particularly infants further immunosuppressed by HIV infection, to comorbidities like tuberculosis (TB). Sub-Saharan Africa is heavily burdened by both the HIV and TB epidemics, and the significant geographic overlap results in the high prevalence of comorbidities (Figure 1.2). Due to HIV-induced immunosuppression, species of mycobacteria that were not pathogenic in healthy individuals (e.g. *Mycobacterium avium*) now have a susceptible host population in which to propagate (39, 106, 131, 163, 242). TB disease is the leading killer of HIV-infected individuals of any age and the WHO predicts that HIV-infected individuals have up to 50% lifetime risk of TB (371). TB is responsible for 21-52% of deaths in HIV-infected individuals (384) and infants co-infected with HIV and *M. tuberculosis* are more likely to die from TB (228, 318).

A synergistic relationship exists between HIV and *Mtb* that serves to exacerbate disease morbidity (257). HIV infection reduces immune system function, allowing *Mtb* to freely replicate/disseminate and *Mtb* infection activates immune cells that are the preferential infection target of HIV. Co-morbidity appears to occur in a similar manner independent of the order of infection, with HIV infections well documented to reactivate bacterial replication in individuals latently infected with *Mtb* (257). In fact, infants exposed to HIV through breastfeeding are more likely to become co-infected with TB than infants not exposed to the virus, suggesting again that co-infection is advantageous for these two pathogens, especially in infants (380).

Decades of global antibiotic use have caused the emergence of multi-drug (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* that are undeterred with most first-line antibiotics, significantly boosting the financial and economic costs associated with treatment and care of individuals infected with these strains. The severity of MDR strains is illustrated in the tragic outbreak in KwaZulu-Natal, South Africa, killing 52 of 53 patients co-infected with HIV+ (99). Independent of HIV status, there are an estimated 1 million *new* pediatric TB infections annually (375). Historically endemic alongside conditions of poverty, recent TB outbreaks even in the developed world have prompted new focus on vaccine and therapeutic development, but as TB therapies become less effective, it is essential to consider dual HIV-TB immunogen development as a possible and efficient approach. Here, I describe my research towards the goal of developing a novel pediatric HIV-TB vaccine using the rhesus macaque model.

EFFORTS TO PREVENT PEDIATRIC HIV

Most vertical HIV transmissions occur in resource-limited regions with low testing

rates, restricted access to antiretroviral drugs and/or where the benefits of breastfeeding outweigh potential transmission risk (383). While abstinence from breastfeeding can reduce the risk of pediatric HIV transmission, it does not reduce infant morbidity or mortality because the presence of immunologically protective factors in breast milk can be so effective at reducing other pediatric health concerns and infections (e.g. nutritional deficiencies, diarrheal disorders, sepsis and pneumonia). In the developing world, breastfed children have at least a six-times greater chance of survival in the early months than non-breastfed children (14, 25).

Previously, public health advisories recommended early and abrupt weaning to avoid MTCT. However, it is now clear that mixed feeding (formula, water, juice and/or solid foods in addition to breast milk) drastically *enhances* the risk of infection, suggesting that long-term exclusive breastfeeding may be more protective (61, 64, 177, 286, 339). Studies concluded that reductions in breastfeeding frequency due to alternate calorie sources caused breast milk viral loads to increase and that enhanced transmission resulted from periodic breastfeedings after premature weaning (128). Now mothers are recommended to exclusively breastfeed (>1year), followed by slow weaning with infant ART prophylaxis treatment for the full duration of the breastfeeding period.

Antiretroviral therapies (ART) have drastically improved the longevity and quality of life for those living with HIV (344), but these therapies are not readily available to all infected individuals. Limited access to therapy has aggravated the HIV epidemic disproportionately in resource-constricted regions. In addition, fewer resources are specifically allocated to HIV-infected children. In 2012, access to ART for adults was predicted to be 65% but estimated to be 34% for infants or lower, depending on the region

(384) (Table 1.1). Pediatric ART access was also improving more slowly (up just 11% between 2011-12) compared to access for adults (up 21% between 2011-12) (384). The WHO Global Plan 2015 target of providing ART to all ART-eligible children will require a "huge effort" to reach its goal (384). Because pediatric ART is not accessible to all children in need and efforts to improve ART dispersal are failing to accommodate all those in need, alternative approaches to limit HIV MTCT are urgently needed. Our goal of testing new pediatric HIV vaccines for immunogenicity and efficacy in the infant macaque model of SIV infection are part of the preclinical development of such alternative interventions.

HIV VACCINE DESIGN

There are several types of vaccine strategies that could reduce the prevalence of HIV, and each approach provides a unique mechanism. For example, an effective oral vaccination regimen capable of eliciting broadly neutralizing antibodies at the oral and intestinal mucosa but not at the vaginal mucosa could result in sterilizing immunity from MTCT in breast milk but provide little benefit following vaginal exposure. Alternatively, a vaccine that induces the development of robust cytotoxic lymphocyte populations could work to reduce viremia in an HIV-infected individual sufficiently to prevent subsequent transmission events but could unlikely prevent the initial transmission event. It is important to apply our understanding of the mechanisms of HIV pathogenesis and transmission to vaccine design in a rational way such that even without sterilizing immunity, the appropriate responses are elicited at the site(s) of exposure to stifle replication and limit transmission.

HIV has a complex array of phenotypes that make vaccine design particularly challenging. HIV has a RNA genome and a high rate of mutation leading to patterns of rapid viral escape following immune pressure; high mutation rates have produced multiple strains

and numerous viral clades, further expanding the variant repertoire for the immune cells to recognize. Rapid viral evolution can also lead to drug resistance (216, 226, 238, 250, 260). The HIV Envelope protein is comprised of variable loops, dense glycosylation patterns, inaccessible conserved regions and a trimeric configuration, which makes viral neutralization difficult (16, 115, 158, 169, 186, 357, 358). HIV preferentially targets the $CD4^+$ T helper cells and long-lived memory T cells, providing opportunities for infection latency, genome integration and the establishment of a viral reservoir (18, 134, 240). HIV-infected adults who maintain higher levels of $CD4^+$ T cells experience reduced viremia. While data are scarce for children, long-term non-progressor (LTNP) children similarly have higher HIV-specific $CD4^+$ T cell responses than children with poorer prognoses (44). Host genetic factors such as HLA and TRIM5α genotypes have been identified to affect virus transmission or control, with specific HLA types having either positive or negative associations with infection rates (232, 291, 315, 316). Despite what we have learned, there is still much unknown about the correlates of immune protection against HIV, which offers an additional challenge for effective HIV vaccine design.

HIV vaccine candidates have consisted of an impressive variety of platforms, antigens and regimens including recombinant proteins, inactivated viruses, live attenuated recombinant viral vectors and even antigen-pulsed autologous dendritic cells. Although much time and effort has been dedicated to its pursuit, the HIV vaccine human phase II/III clinical trials list is short. Just three vaccine candidates have progressed through the clinical trials process to reach large-scale testing in human subjects. First in 1998, the VaxGen Corporation initiated trials in the U.S. and Thailand using an HIV Envelope (Env) gp120 subunit platform mixed with alum adjuvant; vaccinated subjects produced limited

neutralizing antibodies and vaccination had no protective efficacy (55). A monovalent Env subunit vaccine was unlikely to be effective for a number of reasons. Subunit vaccines offer limited immunogenic breadth because they contain only a portion of one viral variant. Viral quasi-species diversity- estimated at up to 1 billion *distinct* HIV variants produced each day- defines the need for a highly diverse repertoire of neutralizing antibodies- a nearly impossible task (38, 186). HIV envelope contains multiple epitopes with particularly high variability, termed 'variable loops'. The variability and flexibility of these loops account for the restricted development of robust neutralizing antibodies that can require years of HIV infection to develop. Substantial efforts have focused on mapping and mechanisms of eliciting Env-specific broadly neutralizing antibodies (bNAbs) to simultaneously target multiple variants of the Env protein (115, 158, 169, 358). However, despite studying bans isolated from LTNPs, it is difficult to assess just how these antibodies are generated in the context of a rapidly evolving pathogen and as a result, it remains unclear exactly how to elicit potent and broadly neutralizing antibodies using vaccination.

The second clinical trial, the STEP or Merck trial, began in 2005 (37, 50, 78, 85, 146, 167, 187, 212). STEP subjects were vaccinated with an immunogenic adenovirus 5 (Ad5) recombinant vector expressing HIV-1 Gag, Pol and Nef. Adenoviruses work well to induce the production of vaccine-specific T cell responses, which was observed in male (MSM) subjects participating in this trial. Despite robust T cell responses, no protection, delayed transmission or viremia control after HIV acquisition were noted in vaccinees. Further, additional analyses revealed a number of positive associations, such as the ability to produce HIV Gag- and Nef-specific $CD8^+$ T cells and the subject's HLA genoytype, along with negative associations like preexisting Ad5 immunity and lack of circumcision with delayed

transmission or reduced viremia. The STEP trial was terminated prematurely at 18 months because vaccinees experienced no protective efficacy but instead Ad5 seropositive, uncircumcised men exhibited *enhanced* transmission. Despite other adenoviral vectors without robust seroprevalence concerns that could be used instead of Ad5 (259, 267), the failure of this trial significantly halted adenovirus-based approaches in HIV vaccine design.

The RV144 trial, or Thai trial, began in 2003 and used a combination vaccine approach of the attenuated canarypox ALVAC prime (HIV Env, Gag and Pol)/HIV Env protein boost regimen in serodiscordant couples (8, 28, 70, 110, 124, 157, 188, 193, 223, 251, 278, 333, 398). This combination of immunogens offered 31% protection compared to mock-vaccinated couples, and represents the best HIV vaccine result from human trials to date. While 31% protection is relatively low, the trial offered conceptual proof that HIV infection is preventable following vaccination. An additional finding from the Thai trial was a positive correlation between protection and HIV Env-specific IgG antibodies in plasma, although these antibodies were determined to be working by antibody-dependent cell-mediated cytotoxicity (ADCC) and not neutralization (110, 193). Alternatively, there was an inverse relationship with plasma IgA anti-Env antibodies to protection, where high concentrations of plasma IgA had an interference effect on IgG binding function (333). Future studies should include the measurement of mucosally secreted pathogen-specific IgA because this antibody, working at the site of viral exposure, is more likely to affect transmission events.

One feature of HIV that has led to incredible challenges for eradication is its ability to integrate into host DNA. While integration events may not always occur (65, 336), integrated HIV plus HIV infection of latent memory cells are responsible for establishing the

viral reservoir (65). The reservoir is capable of providing new infectious particles in the absence of HAART or reactivation events, leading to patient life-long dependency on ART drug cocktails. A vaccine that induces potent immunity prior to reservoir establishment has the potential to prevent the development of these 'sleeper' cells could make great strides towards HIV eradication.

Despite failure in clinical trials, we have learned much about what components are essential for an effective HIV vaccine (38). We understand that the antibodies, in particular broadly neutralizing antibodies at the mucosal surface of exposure, play an integral role in preventing initial transmission events and limiting dissemination (125, 130, 210, 224, 258). Conversely, the CTL response likely is more important early after transmission to eliminate infected cells, control viral replication and reduce genomic integration events (194, 305). For example, Hansen et al. have recently observed effector cell-mediated 50% protection against SIV challenge in macaques vaccinated with persistent rhesus cytomegalovirus vectors expressing SIV proteins (RhCMV-SIV), followed remarkably by seemingly complete viral clearance *after* documented systemic SIV infection (117, 118). And, although RhCMV-SIV vaccination only prevented SIV infection by 50%, viral clearance was reported even in the absence of vaccine-induced antibody responses. So, while we understand that immune correlates like neutralizing antibodies and CTLs are important, a successful vaccine may not have to elicit both before the vaccine can be considered effective.

SPECIFIC CHALLENGES FOR PEDIATRIC HIV VACCINES

Some infants will acquire HIV *in utero* or peripartum and, for these infants, vaccination at birth would not be protective. However, the primary objective of this discussion aims to detail the specific challenges of a vaccine designed to protect against oral

MTCT in breast milk. A protective neonatal vaccine against a pathogen with immediate and repeated exposures, such as HIV in breast milk, must exhibit all features important in an adult vaccine with the additional requirements of i) safety in healthy and immunosuppressed neonates, ii) an accelerated regimen starting at birth capable of rapidly inducing immune development and iii) potent immunogenicity in the context of the developing infant immune system.

Safety is an integral component of any vaccine, but safety is of utmost importance in vaccine candidates with potential applications in immunosuppressed neonates. Some infants born to HIV-infected mothers will be infected with HIV *in utero* and some will be infected peripartum, inducing a state of immunosuppression. Despite efforts to improve neonatal HIV testing, only 28% of infants born to HIV-infected mothers are tested by 6 weeks of age (377). Further, more than one infant sample may be required to correctly confirm HIV status, inducing inherent delays in diagnoses for infants that do get tested (378). Therefore, vaccination regimens that begin at birth could include both HIV-naïve and HIV-infected groups, requiring any vaccine candidate to have an outstanding safety profile in healthy and immunosuppressed infants. Although worldwide we have a broad array of effective pediatric vaccines against numerous infectious diseases, some vaccines, e.g. BCG, while safe in healthy infants can be pathogenic in infants immunosuppressed by HIV infection (40, 69, 77, 288).

Abstinence from breastfeeding effectively negates the risk of oral MTCT by breastfeeding but many HIV-infected mothers breastfeed by choice or necessity. Low-level but frequent exposure to virus in breast milk results in low risk of transmission at any one exposure, but cases of nonexclusive breast milk diets, preexisting maternal and/or infant

morbidities, viral concentration in breast milk and damage to the infant oral mucosal surfaces can increase the transmission rate per exposure (61, 64, 75, 147, 172, 175, 176, 344, 382, 388). A breastfed infant born to an HIV-infected mother will be exposed to virus within hours after birth, and therefore, any vaccine regimen aimed at reducing oral HIV transmission events must be administered soon after birth and work quickly.

With the exception of BCG, currently licensed vaccines for neonates rely on antibody-mediated mechanisms of pathogen clearance. Antibody-mediated protection developed in response to vaccination is highly effective in infants against certain pathogens like Hepatitis B, tetanus and pertussis, although protection against these pathogens requires multiple vaccinations and is mediated by serum IgG antibodies only (Table 1.2) (312). There is evidence of cross-placental and breast milk transfer of maternal antibodies that could also provide protective effects to neonates prior to the development of vaccine-induced immunity, although the beneficial effects of oral IgG exposure is debated (49, 63, 138, 189, 236, 362). Early passive maternal antibody transfer is particularly important since infants are unable to produce antibodies for several weeks after birth. In addition, there is evidence that even more time is required for neonates to develop pathogen-specific effective antibodies since their early humoral responses are highly restricted in VDJ arrangement breadth (84, 105, 227, 264, 277, 322, 392). The frequencies of B cells in PBMC are also slow to establish, constituting less than 5-10% of the total PBMC population at birth and requiring many months to stabilize at the 10-20% frequencies observed in adults (139, 225).

In addition to delayed development of the antibody repertoire, infants also encounter difficulties clearing pathogens that require cell-mediated immune responses. To protect the infant against hyperimmune activation following the transition from a sterile environment to

one teeming with pathogens, several age-dependent mechanisms evolved to delay the development of robust cellular immunity; these mechanisms account for the immaturity of the infant immune system. In particular, infants exhibit immune limitations within several key components necessary for the development of the T_h1 lineage that drives pathogen-specific cellular immunity (Figure 1.3) (198, 342).

There are multiple characterized mechanisms of infant immune restrictions, including i) epigenetic age-associated differences like the reduced capacity of infants to produce the proinflammatory cytokine interferon gamma (IFN-γ) due to promoter hypermethylation (367, 368), ii) less efficient antigen processing/presentation capacity of infant antigen presenting cells (APCs) (53), iii) reduced expression of MHC class II and costimulatory molecules by APCs (203, 261), iv) reduced ability of APCs to produce the heterodimeric IL-12 cytokine important for driving the T helper 1 (T_h1) response (108, 109, 173, 229), and v) a decreased ability to respond to Toll-like receptor ligation compared to older individuals (204, 205). All of these mechanisms specifically dampen the quality of the $CD4^+$ T cell response, and subsequent immune development requiring T cell help.

In addition, the impaired infant immune response is dampened by greater frequencies of regulatory T cells (Treg; $CD4^+CD25^+FoxP3^+$) present in both blood and tissues (122). Tregs, important for shutting off the immune response following pathogen clearance, play a major role in restricting the activation of APCs and the development and activation of antigen-specific T cell responses by producing suppressive mediators IL-10 and TGF-β. Tregs also interfere with the induction and function of virus-specific $CD4^+$ T cells in infants leading to reduced $CD8^+$ CTLs and neutralizing antibody development (19, 122). Infants, however, also have significantly fewer $CD8^+$ T cells and, therefore, less $CD8^+$ effector T

cells, at birth compared to adults (139). In addition, the cytolytic ability of CTLs to degranulate upon stimulation develops gradually in infants (22, 271). The reduced frequency and function of infant CTLs contribute to their inability to substantially reduce viral replication following peak viremia (154).

As a result, infants are characterized as having instead a T helper 2 (T_h2)-driven immune response, which typifies the development of humoral responses and anti-parasitic immunity (198, 342). The T_h2 immune skew importantly illustrates again that the infant immune response performs in a muted or altered way compared to responses generated by adults. One way to illustrate this phenomenon is to quantify the response of infant and adult cells to a non-specific immune activator and measure the ensuing immune response (Figure 1.4). Of note, the ability of infant PBMC to produce IL-2 following stimulation mirrors or exceeds the magnitude of this response in adult PBMC, but induction of other cytokines, particularly IFN-γ, is robustly muted in the infant samples. The polyfunctional T cell response (T cells producing $\geq$ two cytokines) is robustly effective at combating pathogens and individuals producing greater frequencies of pathogen-specific polyfunctional cells typically experience better prognoses. However, there is an age-associated delay also in the production of dual and triple polyfunctional T cells in infants, which are clearly ablated compared to adults (Figure 1.4).

The culmination of multiple inefficient immune processes in the infant results in a less robust response to foreign pathogen invasion compared to adults and further validates the need for protective vaccinations to complement known immune immaturities (265). In the context of infection, the mechanisms of how HIV eventually monopolizes host immunity are far beyond the scope of this work, but it is important to note that infants exhibit altered

immune responses compared to adolescents and adults. Although the correlates of protection and controlled infection are not fully understood, the ongoing immune development of the infant makes it particularly more susceptible to HIV infection.

One approach to improve pediatric immune responses to vaccination is to include an adjuvant. Adjuvants can help overcome known immunological delays in infants, particularly acting on the antigen-presenting myeloid dendritic cell (mDC) populations. Adjuvants can increase antigen presenting cell (APC) activation, uptake and processing of antigen, migration to organized lymphoid tissues and presentation of antigen to cognate T cells, as well as upregulate expression of co-stimulatory and adhesion molecules important for the formation of the immunological synapse. For example, CD40 ligand (CD40L) can bypass the requirement for T cell help prior to B cell activation and improve antibody responses, providing a surrogate for functional T cell help in the immature immune system of the infant (6, 123, 190, 311, 328). Other adjuvants, such as known TLR agonists, GM-CSF and cytokines can serve as APC activators (31, 74, 144, 155, 173, 181, 182, 204, 274, 311, 394). While other cells can serve as APC, mDC are the most efficient, with important roles in both innate and adaptive immune maturation. T cell priming by mDCs and the ensuing cytokine milieu determines the lineage fate of the T cell and instructs the type of immune response that will be produced upon presentation of cognate antigen. The immune maturation events that delineate the T cell lineages in the context of immune ontogeny are not fully elucidated, but proper adjuvant use could work to preferentially drive the infant T_h1 lineage towards effector and memory T cell responses.

The gestational age at birth, infant and maternal morbidities, exposure to breast milk and bacterial colonization, vaccination record, exposure to environmental and pathogenic

agents and genetic factors all contribute to the rate of immune maturation with age. And, while many phenotypic immune distinctions between infants and adults are described, what we lack is a mechanistic understanding of how immunity develops with age. Ongoing research in our lab and others seeks to characterize age-associated immune maturation events using longitudinal and cross-sectional sampling approaches in human and macaque infants to elucidate the mechanistic changes responsible for age-specific immune phenotypes. A comprehensive understanding of immune maturation will improve the efficacy of pediatric interventions.

DEVELOPMENT OF A PEDIATRIC HIV-TB VACCINE

One hallmark benefit of the live attenuated mycobacterial vaccine BCG is its ability to drive the development of robust and persistent cellular immunity, despite the immaturity of the infant immune system. In fact, BCG itself has intrinsic adjuvant activity and can be administered in tandem with other infant vaccines to enhance dual immunogenicity (255, 270, 314). Early acceptance of BCG vaccination was slow following disastrous attempts to prove its safety and efficacy but despite early setbacks, BCG vaccination was adopted by the WHO predecessor, the League of Nations, in 1928, implementing large-scale infant vaccination efforts. By 1980, global BCG vaccination hovered around 20% and has jumped to 90% coverage in the past 30 years, making it the most widely administered immunogen (Figure 1.5) (126, 140, 183, 196). The BCG vaccine remains largely unchanged since its first use in humans in 1921 and remains the only live attenuated vaccine licensed for use at birth (87). BCG vaccination dramatically reduces the incidence of miliary and meningeal TB disease in infants (5, 140, 325). Further, BCG can effectively induce the development of cellular immunity via a T_h1 lineage, which is a significant accomplishment considering the immature

immune system of infants (159, 202, 206, 353). The live attenuated nature of BCG ensures that the immune response is continually primed with antigen throughout childhood, eliminating the need for boosts.

There is much we have learned after nearly a century of BCG use. Perhaps most notably, BCG vaccination has a variable 0-80% efficacy rate. Studies conducted in the U.K., India and South Africa report substantial variation in the protective efficacy of BCG vaccination, although uncontrollable variables like BCG strain genetics (e.g. virulence factors and biochemical properties (218)), population genetics and environmental mycobacteria make vaccine efficacy results difficult to interpret (1, 319, 335). In addition, BCG protection wanes significantly by adolescence and offers little to no protection against the pulmonary disease phenotype that most commonly afflicts older individuals (276). Although elements of multiple mechanisms are likely responsible for the observed variation in BCG efficacy, a retrospective analysis of studies conducted across multiple sites using different vaccine strains in diverse population cohorts suggest the following common themes: i) vaccinating older children with more developed immune systems (4 months to 15 years) does not improve rates of protection (15, 35, 57, 59, 326), ii) boosting with BCG provides little benefit, iii) BCG-induced protection wanes within about 15 years and iv) adult BCG vaccination does not protect against pulmonary TB infections.

The specific attenuations that render BCG less pathogenic include deletions in regions of known immunodominance, like the *RD1* locus harboring the *ESAT-6* and *CFP-10* genes, the loss of which occurred during the initial attenuation of virulent *M. bovis*. In fact, when the ESAT-6 gene was reintroduced into a BCG backbone, the altered vaccine was more immunogenic (206). Other important deletions in BCG include mutations/deletions in

known *Mtb* virulence factors ESX-1, PDIM/PGL and PhoP (1, 100, 142, 192). Polymorphisms present in a subset of BCG strains have also been implicated as virulence factors but are not conserved across standard vaccine strains. The loss of virulence factors was important for BCG safety but came at the cost of immunodominant factors.

In the context of the HIV epidemic, the live attenuated nature of the BCG vaccine relies on a minimum level of infant immune capacity to maintain low levels of replication and restrict bacterial dissemination. HIV-infected infants, however, experience variable levels of virus-induced immunosuppression compounded by age-specific immune immaturity and BCG vaccination in immunosuppressed infants can result in BCG-osis, a disease resulting from failure of the immune system to adequately regulate the bacterial replication (40, 69, 77, 126, 201, 288). BCG-osis results in lymphadenopathy and TB-like symptoms including granuloma formation in the axillary lymph nodes that drain the dermal inoculation site (126, 364). To limit BCG-osis, the WHO advises against BCG vaccination for infants infected with HIV or at high risk of becoming HIV-infected (379) but the high prevalence of TB has prompted some countries to vaccinate all infants with BCG (e.g. South Africa) with the intent to treat BCG-osis if it presents rationalizing that accurate neonatal HIV testing typically requires several weeks and the risk of TB infection far outweighs that of BCG complications (126, 241, 400). In light of the safety and immunological concerns surrounding use of the BCG vaccine, we proposed vaccinating with attenuated *Mycobacterium tuberculosis* strains instead of *M. bovis*, hypothesizing that a human-adapted attenuated *Mycobacterium tuberculosis* backbone may provide enhanced protection and greater immune persistence and improved safety compared to BCG.

To engineer a safe vaccine strain, genes important for bacterial replication and host

immune evasion were deleted in H37Rv *M. tuberculosis*. Specifically, the *panCD* and *leuCD* loci, which encode for pantothenate and leucine, respectively, were deleted to generate auxotroph strains (45, 184). Pantothenate and leucine are essential nutrients for mycobacterial growth and their deletion severely ablates bacterial replication in unsupplemented cultures. Deletion of the *lysA* gene also reduces replication (132, 256). Further, the *SecA2* gene locus was removed to limit host immune cell evasion and *nuoG* was deleted to enhance immunogenicity (33, 179, 330, 355). However, in contrast to BCG, the *RD1* operon was maintained in two of three strains evaluated here because of its immunodominant properties. Table 2.1 lists the attenuations that have been tested in neonatal macaques as potential vaccine candidates.

BCG was first administered orally but was transitioned to the intradermal route following reports of cervical lymphadenopathy (196, 365). Intradermal vaccination, done using a scarification needle or syringe at birth on the upper arm, delivers live attenuated bacteria to dermal-resident Langerhans cells. However, *Mtb* can selectively bind mucosal M cells, a primary cell type at the tonsil mucosa that passes antigen basally to dendritic cells (32, 235). Therefore, attenuated *Mtb* with an improved safety profile and reduced replication compared to BCG could be administered effectively by the oral route without risk of adverse pathology while inducing the potent T_h1 response important for neonatal cellular immune development (90, 239, 243, 353).

With the overlap of the HIV and TB epidemics and the emergence *Mtb* antibiotic resistance, efforts have resurged to improve the current BCG vaccine (21, 36, 135, 141, 200, 244, 283, 317). One formulation, the lysine auxotroph BCG.HIVA (HIVA consists of the HIV *Gag* consensus sequence and HIV-specific $CD8^+$ T cell epitope peptides) followed by

MVA.HIVA boosting, is currently undergoing phase IIb clinical trials in African children (7, 116, 137, 281, 282, 298, 299). However, even auxotroph BCG strains may present some safety risk to immunosuppressed neonates. Instead, we have applied lessons learned from BCG vaccination to hypothesize that a molecularly attenuated *Mtb* strain recombinantly expressing HIV genes and administered orally could safely result in persistent immunity against both HIV and *Mtb*. We predict that this approach provides greater advantages compared to BCG because:

i) specific bacterial loci important for replication, virulence and immune evasion in *Mtb* were eliminated to produce a safer vaccine candidate while maintaining the *RD1* immunodominant domain

ii) the live attenuated human-adapted *Mtb* pathogen will retain the ability to potently induce cellular immune activation but offer greater immunogenicity against *Mtb*

iii) oral vaccination will specifically target the M cell-rich palatine tonsil and oral lymphoid tissues, shown to readily take up vaccines (174, 235) and induce a robust immunological response at the oral mucosa to protect against oral MTCT of HIV in breast milk

iv) using bacterial strains expressing a variety of HIV antigens will induce both broad and persistent cellular and humoral responses

v) vaccinees may maintain specific cellular responses into adulthood with conferred protection against pulmonary TB.

Although direct comparisons to BCG were not within the scope of these studies, in the chapters to follow the predicted advantages of attenuated *Mtb* strain vaccine candidates are

explored using an infant macaque model.

NONHUMAN PRIMATE MODEL OF PEDIATRIC SIV

Because we aimed to evaluate specific immune development in response to vaccination in neonates, it was important that we identified an animal model able to best recapitulate the HIV disease that occurs in human infants. The development of HIV vaccine candidates has relied heavily on preclinical research in the rhesus macaque model of SIV infection, particularly because macaques are susceptible to simian immunodeficiency virus (SIV), which behaves in macaques analogously to HIV-1 infection in humans (166, 221, 346, 396). In addition, macaques are also highly susceptible to the same *Mycobacterium tuberculosis* strains as humans, experience similar disease pathology and are a widely validated animal model for TB research (89, 160). Dual immunogenicity studies could best be completed in the nonhuman primate model.

Humanized mice have become an important small animal model for HIV research (71), but the oral anatomy of the mouse lacks potential HIV target tissues in humans (e.g. tonsils), solely relying instead on a patch of nasal associated lymphoid tissue (NALT) for oral antigen sampling, inaccurately modeling oral vaccination and MTCT in humans. We postulated that using a nonhuman primate infant model was superior to the humanized mouse because infant macaques are highly immunologically, developmentally and anatomically similar to human infants. Suckling macaque infants closely mimics human infant nursing, and the macaque oral cavity contains human-like organized lymphoid tissues (including palatine tonsil, submandibular and retropharyngeal lymph nodes) that could be important for oral transmission events following HIV exposure in breast milk. Oral exposure of SIV in infant rhesus macaques mimics viral acquisition via breast milk in humans (9) and the model

has been developed to study oral infection and pathogenesis of HIV (27, 207, 209, 350). Orally infected infant macaques rapidly progress to simian AIDS (SAIDS) following viral dissemination within one week (4, 219) and experience a dramatic and irreversible loss of intestinal $CD4^+$ T cells (360), accurately mimicking disease progression in human infants.

Despite many benefits, there are some limitations of the macaque model. Macaques are exclusively outbred population, which is more representative of human populations but can provide substantial individual variation. In addition, studies requiring the use of neonatal macaque studies are restricted to a single breeding season without the benefit of staggered experiments. Like human infants, neonatal macaques are small, provide limited blood sample volumes and can only be sampled sparingly from the periphery without cull experiments. Due to the high expense to purchase and house infant macaques, group sizes are highly restricted, which can limit power and statistical calculations. Despite the limitations of our chosen model, we propose that neonatal macaques provided the best available animal model in which to evaluate the safety (Chapter 2), immunogenicity (Chapter 3) and protective capacity (Chapter 4) of pediatric HIV-TB vaccine candidates.

TABLES

	A. Number of children living with HIV (2011)		B. Pediatric access to ART in resource-limited countries (2012)	
Region	**Infected Children**	**Burden (%)**	**On ART**	**ART Coverage (%)**
Sub-Saharan Africa	3100000	92.31	544,000	17.55
South/East Asia	166000	4.94	12,000	7.23
Latin America	60000	1.79	19,100	31.83
North Africa/Middle East	15000	0.45	1,100	7.33
Europe/Central Asia	12600	0.38	8,500	67.46
Totals	**~3.3 million**	**100**	**584,700**	**17.72**

Table 1.1 Pediatric HIV infection and access to ART. A. The prevalence of HIV infection in children aged 0-14 years, estimated to total 3.3 million, and the burden value indicates the percentage of the pediatric epidemic in each region (2011). B. Frequencies of HIV-infected children accessing ART in resource-limited countries with the percentage of ART coverage reported in the right column (2012). Data in A and B are adapted from (376, 384); North America and Australia were omitted for simplicity.

Global Routine Childhood Immunization Schedule				U.S. Routine Childhood Immunization Schedule			
Antigen	**Etiological Target**	**Age at First Dose**	**No. of Doses in Primary Series**	**Antigen**	**Etiological Target**	**Age at First Dose**	**No. of Doses in Primary Series**
BCG[a]	*M. tuberculosis*	birth	1				
HepB	Hepatitis B	birth	3-4	HepB	Hepatitis B	birth	3
RV	Rotavirus	≥ 6 weeks	3	RV	Rotavirus	2 months	3
DTP	Diptheria Tetnus Pertussis	≥ 6 weeks	3	DTaP	Diptheria Tetnus Pertussis	2 months	4
Hib	*H. influenzae*	≥ 6 weeks	3	Hib	*H. influenzae*	2 months	4
Pneumococcal conjugate	Pneumococcal	≥ 6 weeks	3	PCV	Pneumococcal	2 months	4
OPV or IPV	Polio	1-2 months	3	IPV	Polio	2 months	3
Influenza	Influenza	6 months	annually	Influenza	Influenza	6 months	annually
HepA	Hepatitis A	12 months	2	HepA	Hepatitis A	12 months	2
Measles	Measles	6-12 months	2	MMR	Measles Mumps Rubella	12 months	2
Mumps	Mumps	≥ 6 months	2				
Rubella	Rubella	9-12 months	1				
				Varicella	V zoster	12 months	2

Table 1.2 Childhood vaccine schedules. Recommended immunization schedules for infants from birth to one year. The vaccination schedule issued from the World Health Organization, Geneva, Switzerland (left; adapted from (372)) and Centers for Disease Control, Atlanta, GA, U.S. (right; adapted from (41)) were designed for global or domestic populations, respectively. BCG vaccination for infants born in non-western countries is the primary difference between the advised pediatric immunization schedules. [a] BCG vaccination is advised for all infants unless contraindicated due to HIV infection. The WHO vaccination schedule advises immunizations in addition to those listed here for infants less than 12 months of age on a region- and population risk-specific basis, but were omitted here for simplicity. The WHO guidelines were last updated August 1, 2013 and those from the CDC on March 20, 2013.

FIGURES

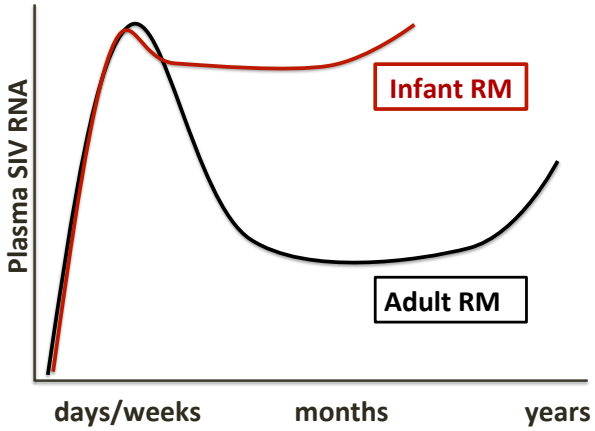


Figure 1.1 Schematic age-associated differences in HIV plasma viremia. HIV/SIV infection in the young is often less controlled due to immature immune function, resulting in little-to-no decrease in plasma viremia at the viral set point compared to recently infected adults. RM= rhesus macaques.

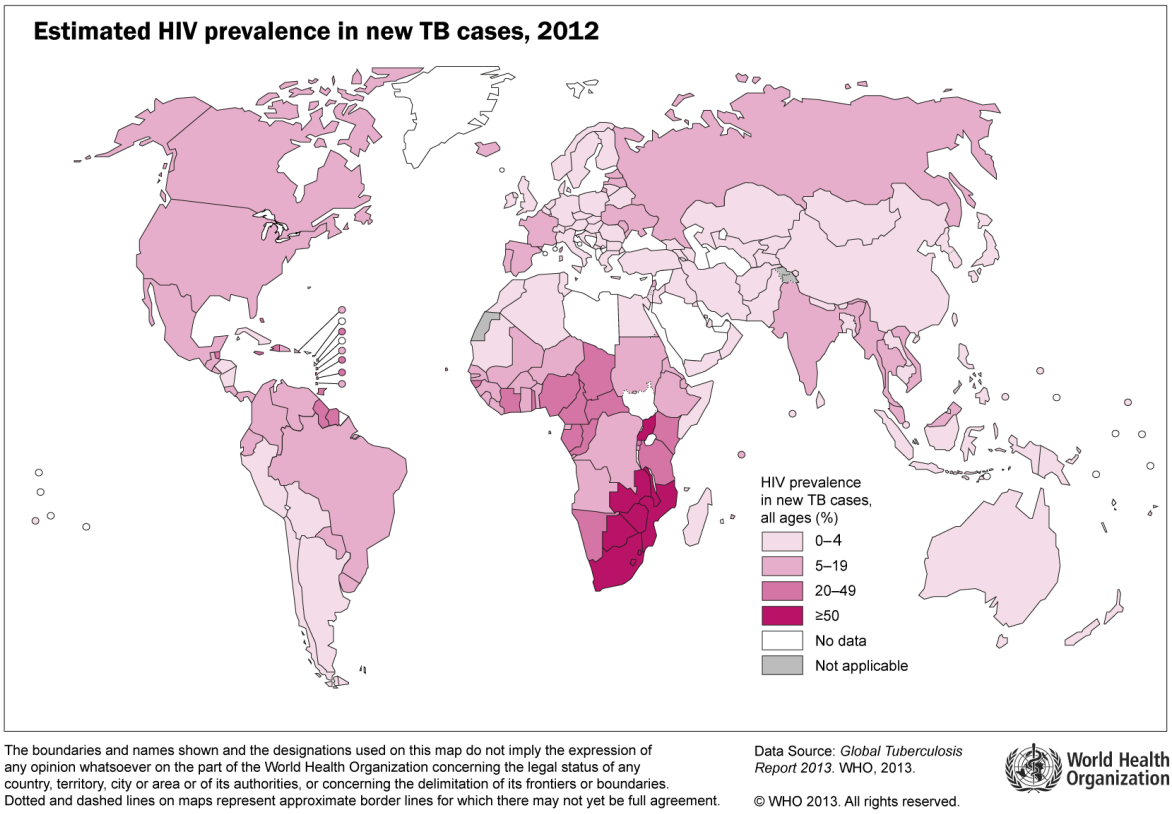


Figure 1.2 Global perspective of HIV prevalence in new TB cases (2012). Geographic representation of the global prevalence of HIV infection in patients reporting *new* TB infections. While co-infections are reported globally, sub-Saharan Africa is burdened with the highest rates of co-infection. Reproduced, with permission from the publisher, from (375).

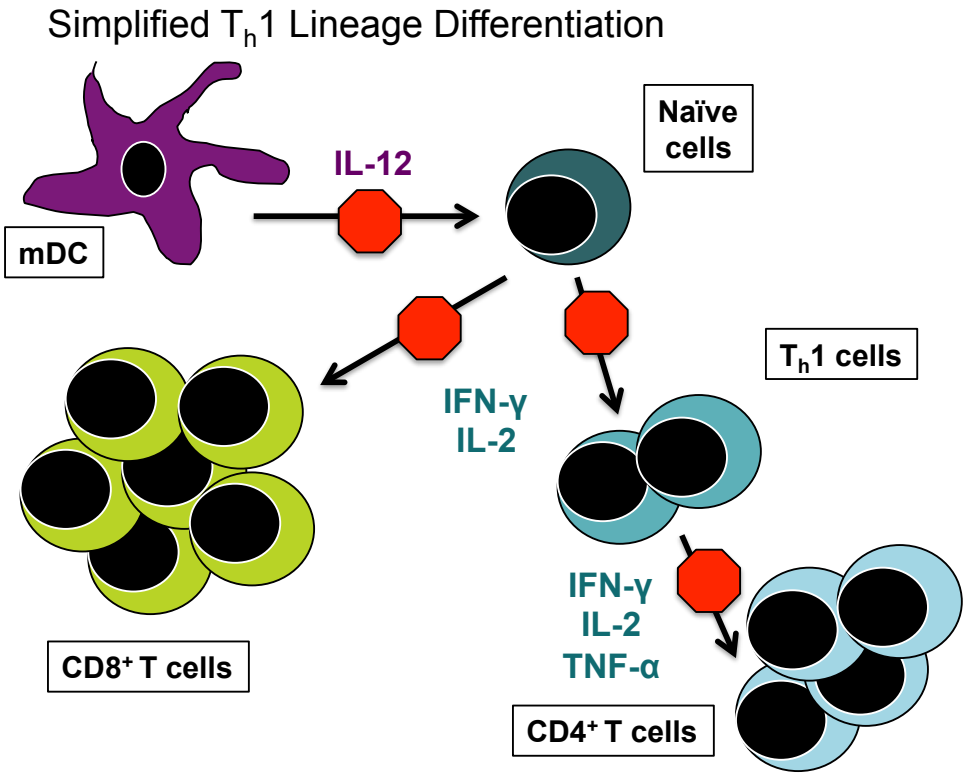


Figure 1.3 Schematic of T_h1 lineage development. A simplified T_h1 lineage development diagram, illustrating steps at which age-dependent limitations have been identified; red octagons indicate known immune immaturities in infants.

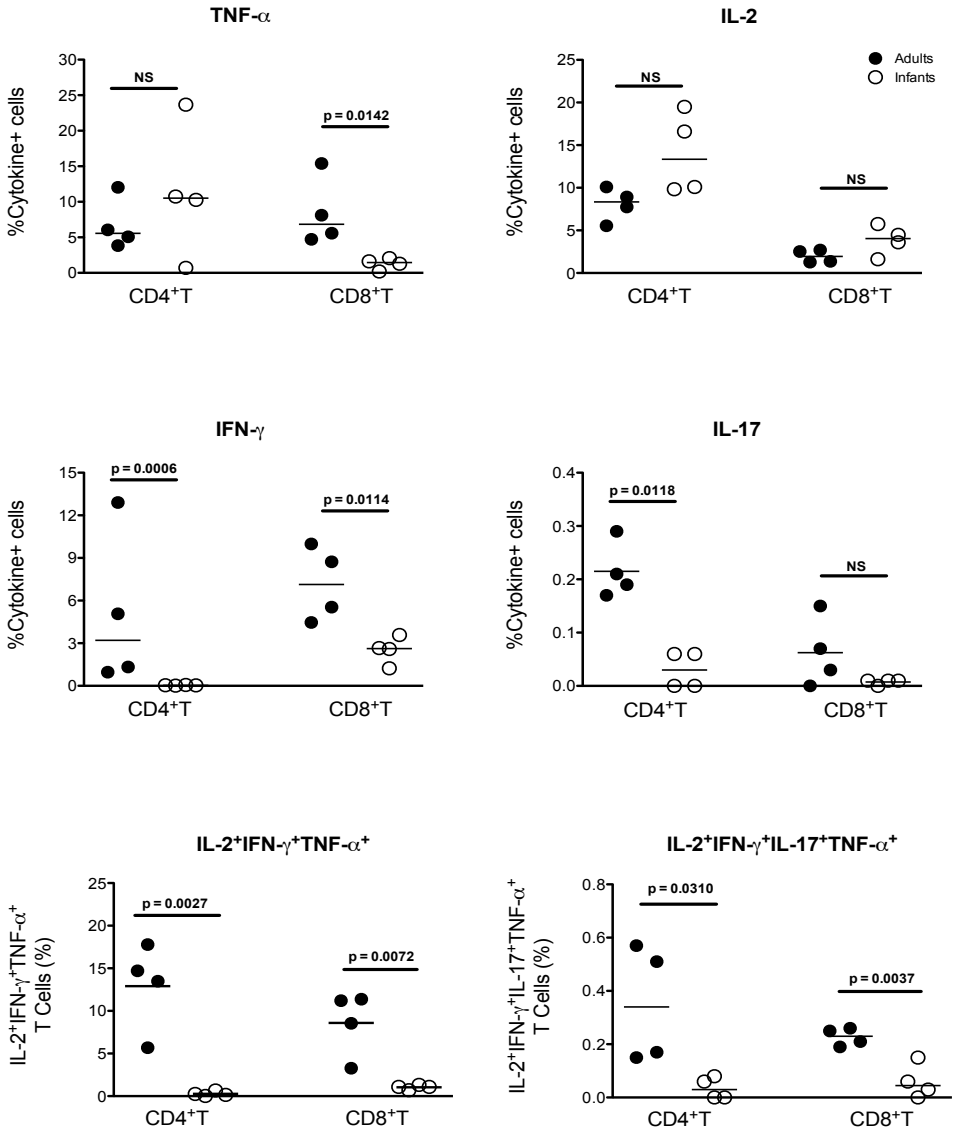


Figure 1.4 Age-dependent differences in T cell responses to nonspecific stimulation. Infant and adult peripheral blood from rhesus macaques was stimulated using a non-specific stimulant PMA and Ionomycin for 6 hours before evaluating the percentages of $CD4^+$ and $CD8^+$ T cells expressing TNF

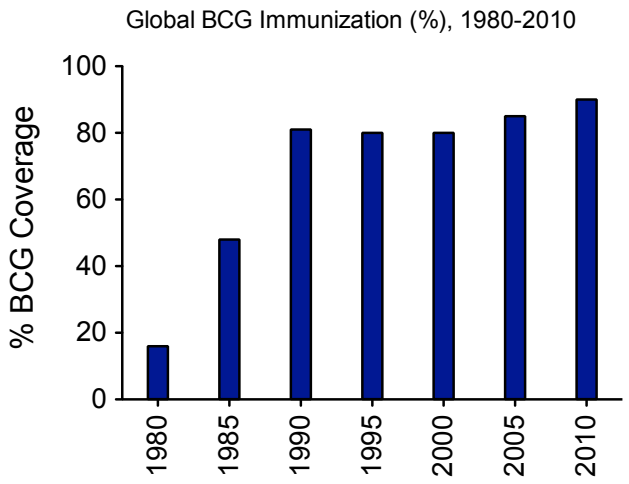


Figure 1.5 Global BCG vaccination rates. The percentage of infants vaccinated with BCG at birth experienced dramatic increases in coverage since the late 20th century. Adapted from (373).

CHAPTER 2: A RECOMBINANT ATTENUATED MYCOBACTERIUM TUBERCULOSIS VACCINE STRAIN IS SAFE IN IMMUNOSUPPRESSED SIMIAN IMMUNODEFICIENCY VIRUS-INFECTED INFANT MACAQUES[1]

OVERVIEW

Many resource-poor countries are faced with concurrent epidemics of AIDS and tuberculosis (TB), caused by HIV and *Mycobacterium tuberculosis (Mtb)*, respectively. Dual infections with HIV and *M. tuberculosis* are especially severe in infants. There is, however, no effective HIV vaccine and the only licensed TB vaccine, the Bacille Calmette-Guérin (BCG) vaccine, can cause disseminated disease in HIV-infected children. Thus, a pediatric vaccine to prevent HIV and TB infections is urgently needed. We hypothesized that a highly attenuated *M. tuberculosis* strain (A*Mtb*) containing HIV antigens could be safely administered at birth and induce mucosal and systemic immune responses to protect against HIV and TB infection, and we rationalized that vaccine safety could be most rigorously assessed in immunocompromised hosts. Among three vaccine candidates tested, the recombinant A*Mtb* strain mc^{2}6435 encoding an SIV Gag expression plasmid and harboring attenuations in genes critical for replication (*panCD* and *leuCD*) and immune evasion (*secA2*), was found to be safe after oral or intradermal administration in SIV-uninfected *and* SIV-infected infant macaques. Safety was defined by absence of clinical symptoms, histopathological changes indicative of TB infection, and lack of mycobacterial dissemination. These data represent an important step in the development of novel TB

[1] This chapter has been reproduced/amended from Jensen *et al*. (152).

vaccines and suggest that a combination rA*Mtb*-HIV vaccine could be a safe alternative to BCG for the pediatric population as a whole, but importantly for the extreme at-risk group of HIV-infected infants.

INTRODUCTION

About one third of the world's population is infected with *Mycobacterium tuberculosis (Mtb)* (374). Every year, 8-10 million new individuals become infected with *M. tuberculosis*, and almost 1.5 million people die of tuberculosis (TB) (374). The recent development of multi-drug and extensively multi-drug resistant strains of circulating *M. tuberculosis* further underscores the need for novel approaches to combat TB infections. The only licensed TB vaccine, the Bacille Calmette Guérin (BCG), is a live attenuated vaccine derived from the bovine *Mycobacterium bovis* strain. It is the oldest and most widely used vaccine worldwide. Although the BCG vaccine can induce potent cellular immune responses in infants and protect against disseminated TB in children (202, 230, 354), the duration of protection is questionable since immunity wanes with time in many vaccinated individuals and the vaccine shows only variable protection in adults (58, 275, 337). In addition, BCG vaccination offers little to no protection against pulmonary TB, cannot eliminate latent *M. tuberculosis* and is ineffective at preventing subsequent TB infections.

TB is the leading cause of death in HIV-infected individuals (369, 374). Given the large geographical overlap between *M. tuberculosis* and HIV infection, BCG vaccination was recommended at birth for all infants because infants with HIV-induced immune suppression have a higher risk than adults of contracting TB (381). Recently, however, it became apparent that the annual risk for disseminated BCG disease in untreated HIV-infected infants (~0.42%) that is associated with a 75% mortality rate (127-129) clearly outweighs the

potential benefits of BCG vaccination in children with HIV (128). Therefore, the WHO now advises against BCG vaccination in any infant infected with HIV or at risk for HIV infection (385). As a result, the number of infants co-infected with HIV and TB in resource-poor countries is expected to remain the same or even rise.

Alternative methods to control TB in infants with HIV are urgently needed. In response to this challenge, we aim to develop a novel infant combination HIV-TB vaccine based upon a safe, orally administrable attenuated *M. tuberculosis* strain expression HIV antigens. Although the rate of in utero and perinatal mother-to-child-transmission (MTCT) of HIV has been significantly reduced with the introduction of antiretroviral therapy (ART) to mother and/or child (369), breast milk transmission of HIV remains a serious problem. Ideally, a vaccine to prevent *oral* HIV acquisition by breast-feeding should be administered orally. BCG-based vaccines are advantageous because they can be administered at birth, are effective orally, and rapidly generate long-lived T cell responses against dually administered mycobacterial and co-expressed non-mycobacterial antigens when administered simultaneously in human infants (245).

To address the safety concern associated with the current BCG vaccine, we hypothesized that a rationally attenuated strain of human *M. tuberculosis* might be a better vaccine platform than the bovine-adapted *M. bovis* BCG. We developed auxotroph mutants of the human *M. tuberculosis* strain H37Rv in which mycobacterial genes important for replication and persistence were deleted or modified to attenuate replication. In addition, in an attempt to increase immunogenicity, several genes important for the evasion of host immune responses were deleted. The construction of these attenuated *M. tuberculosis* (A*Mtb*) strains, their safety and immunogenicity profile in comparison to the licensed BCG vaccine

in SCID mice have been reportedly previously (145, 184, 268, 293-295). Some of these TB vaccine candidates were also characterized in nonhuman primates as an important step towards potential human clinical trials. Vaccine safety, immunogenicity and efficacy data obtained in nonhuman primates would be expected to be highly relevant to humans (184). The A*Mtb* vaccine strains $mc^2$6020 and $mc^2$6030 were safe and well tolerated in *adult* cynomolgus macaques and did not cause TB-disease, but provided only partial protection against an intrabronchial *M. tuberculosis* challenge (184). Based on these data, we developed novel replication-attenuated *M. tuberculosis* vaccine strains with increased immunogenicity.

Due to obvious ethical concerns, *pediatric* HIV-TB vaccine safety assessments and challenge studies for efficacy cannot be performed in HIV-infected human infants. To account for the infant's relatively inexperienced and still developing immune system early after birth, we therefore choose to test vaccine safety in infant macaques that show similar immune system ontogeny after birth compared to human infants. Thus, in a first step towards the generation of a pediatric combination HIV-TB vaccine, we constructed A*Mtb* strains that express the SIV Gag gene. The safety profile of three distinct rA*Mtb*-SIV vaccine candidates with different degrees of attenuation in replication and/or immunogenicity (Table 2.1) was initially tested in healthy, SIV-uninfected infant macaques. None of these vaccine candidates induced clinical symptoms of TB. The vaccine strain ($mc^2$5157) that was predominantly attenuated for immune evasion and less for replication caused *M. tuberculosis* dissemination to multiple tissues and was therefore excluded as a potential pediatric vaccine. The safety of the two other vaccine candidates, rA*Mtb*-SIV $mc^2$6020 and $mc^2$6435, was then evaluated under even more stringent conditions in immunosuppressed SIV-infected infant macaques, analogous to HIV-infection in human infants.

The infant macaque model of SIV infection is a well established animal model of pediatric HIV infection and is suitable for the testing of safety and efficacy of intervention strategies for a wide range of infectious diseases (3, 9, 10, 209, 350). Vaccination of SIV-infected infant macaques with these two rA*Mtb*-SIV strains did not cause TB-like lung pathology. Importantly, in animals vaccinated with $mc^2$6435, local or systemic dissemination of mycobacteria did not occur, and live mycobacteria could not be recovered from any tissues under optimal culture conditions. The data represent an important step in the clinical testing of these novel live-attenuated *M. tuberculosis* vaccine candidates and suggest that a combination rA*Mtb*-HIV vaccine could be a safe alternative to BCG for the pediatric population as a whole, but importantly for the extreme at-risk group of infants infected with HIV.

MATERIALS AND METHODS

Animals.

Newborn rhesus macaques (*Macaca mulatta*) from the SIV negative and type D retrovirus-free colony were hand-reared in a nursery at the California National Primate Research Center (CNPRC, Davis, CA). Animals were housed according to the "Guide for Care and Use of Laboratory Animals" and the standards outlined by the American Association for Accreditation of Laboratory Animal Care; all animal protocols were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee prior to study initiation. Animals were randomly assigned to the various study groups and were between 3 and 7 days of age at the first immunization (see Table 2.2). For vaccinations and blood collections, animals were immobilized by intramuscular injection of 10mg/kg body weight of ketamine-HCl (Parke-Davis, Morris Plains, NC). Trained veterinary staff

monitored the animals daily for clinical symptoms associated with TB (e.g. difficulties in breathing, coughing, mucus secretions, lethargic behavior, weight loss) and/or SIV infection.

Vaccine Strains.

The three rA*Mtb* vaccine strains tested in the current study were rationally attenuated from the wild-type *M. tuberculosis* strain H37Rv. *M. tuberculosis* H37Rv was modified through several deletions in genes that attenuated replication to increase vaccine safety, and in genes important for mycobacterial immune evasion to enhance the immunogenicity (13). As outlined in Table 2.1, strain $mc^2$6020 was predominantly attenuated for replication with deletions in the *lysA* and *panCD* loci (Δ*lysA,* Δ*panCD*), no loci important for immune evasion were deleted. In contrast, strain $mc^2$5157 was designed primarily to test for enhanced immunogenicity (Δ*nuoG,* Δ*RD1*) and contained only the panCD locus deletion to attenuate replication. Strain $mc^2$6435 was developed based on the initial results obtained with strains $mc^2$6020 and $mc^2$5157 to harbor replication (Δ*leuCD,* Δ*panCD)* and immune evasion (Δ*secA2*) attenuations. The construction of the rA*Mtb* vaccine strains has been previously described (184, 268, 294-296, 363, 397). In these prior studies, the introduced deletions were stable and reversions were not observed (184, 268, 294-296, 363, 397). Prior to use in rhesus macaques, the safety and immunogenicity of all three rA*Mtb* strains was confirmed in mice (184, 268, 294-296, 363, 397). The rA*Mtb* $mc^2$5157 and $mc^2$6435 vaccine strains were further manipulated to incorporate a mycobacterial expression plasmid with a full length SIVmac239 Gag insert (268). Expression of SIV Gag in vaccine preparations was confirmed by Western blot immunolabeled with a V5 antibody-HRP, as previously described (Figure 2.1A) (268). The immunogenicity was confirmed in C57BL/6 mice (Figure 2.1B).

Immunization Regimens.

An overview of the vaccination schedule including route and dose is provided in Table 2.2. Briefly, strain $mc^2$6020 was administered both orally (PO) and intradermally (ID) at one week of age, and animals were followed for 6 months (Group A). Animals vaccinated with $mc^2$5157 were primed orally and received a homologous ID booster vaccination at either 2 (Group C) or 3 weeks (Group E). Animals were then euthanized at week 4 or 6, respectively, to test for vaccine-induced immune responses and *M. tuberculosis* dissemination in various tissues. The time intervals were selected based on our previous pathogenesis and pediatric HIV/SIV vaccine studies in the oral SIV infant macaque infection model in which we generally challenge at 4 weeks of age to reflect early breast milk transmission of HIV in humans (4, 208, 347, 350). Finally, we tested two heterologous prime-boost regimens using either recombinant adenovirus 5 expressing SIVmac239 Gag (rAd5-SIVgag; Groups G and H) or recombinant modified vaccinia virus Ankara expressing SIVmac239 Gag, Pol and Env (rMVA-SIVgpe; Group J) that were kindly provided by the International AIDS Vaccine Initiative (IAVI, Brooklyn, NY), and Dr. B. Moss (NIAID, NIH, Bethesda, MD), respectively (208). The heterologous boosts were administered 3 weeks (rAd5-SIVgag), or at 3 and 6 weeks (rMVA-SIV constructs) after the initial $mc^2$6435 vaccination. In the studies described here, the vaccine boosts are reported solely for the purpose of revealing all study variables; vaccine immunogenicity will be reported separately (manuscript in preparation). Note that all experiments were carried out using mock-infected (saline) age-matched infant macaques in parallel (Table 2.2, Groups D, F, I, and K).

SIV Infection.

A subset of animals were infected with 10^3 $TCID_{50}$ of SIVmac251 (stock 6/04; (208) by the intravenous (IV) route within 72 hours of birth, and then immunized one week later with $mc^2$6020 (Group B) or $mc^2$6435 (Group L) (Table 2.2). SIV-infected animals were euthanized when they met criteria established for retrovirus-infected animals (352).

Sample Collection and Preparation.

EDTA blood samples were collected at week 0 (baseline) and then longitudinally as described in Table 2.2. Plasma was collected after centrifugation and stored in multiple small aliquots at -80^{0}C for virological analysis and antibody testing. PBMC were isolated by gradient centrifugation as described (208). At the time of euthanasia, multiple tissues were collected, including tonsil, lymph nodes (LN: submandibular, retropharyngeal, bronchial, axillary, mesenteric), lung and intestinal tissues (ileum, colon). In addition, from animals that received an ID vaccination, we saved tissue from the dermal inoculation sites (Groups A, B, E, F and I). Corresponding skin tissue from orally vaccinated animals was collected as control tissue. Each tissue was divided and saved for multiple applications by preparing snap frozen (*M. tuberculosis* culture), formalin-fixed / paraffin-embedded (pathology, *M. tuberculosis* staining), and fresh tissue aliquots (immunogenicity). The isolation of cell populations from tonsil, LNs and intestinal tissues was performed as described previously (208).

$CD4^+$ T Cell Measurement.

A Complete Blood Count (CBC) was performed on an ABX Pentra 60+ electronic cell counter (ABX Diagnostics, Irvine, CA) with manual differential counts. Absolute counts and percentages of $CD4^+$T cells in PBMC were determined using antibodies specific for rhesus macaque CD3 and CD4 using flow cytometric analysis and CBC values as described (4).

SIV Replication.

Plasma samples were analyzed for viral RNA by a quantitative reverse transcription-PCR (qRT-PCR) assay as described (54).

***M. tuberculosis*-specific Plasma Antibodies.**

The presence of IgG antibodies against the *M. tuberculosis* PSTS1 antigen was determined in longitudinally collected plasma samples using a recently described multiplex microbead immunoassay based on the Luminex system (Austin, TX) (162). Relative antibody levels are reported as mean fluorescence intensity (MFI) (162). Sera from *M. tuberculosis*-uninfected and *M. tuberculosis*-infected rhesus macaques were used as negative and positive controls, respectively.

Pathology Evaluation.

Gross pathology evaluation was performed at necropsy. Formalin-fixed, paraffin-embedded tissues were cut into 5 micron sections and stained with hematoxylin and eosin (H&E) according to standard protocols. Lung sections from an adult macaque infected

experimentally with virulent *M. tuberculosis* were kindly provided by Dr. P. Luciw for comparison (197). In addition, Ziehl-Neelson stained sections were examined for the presence of acid-fast bacilli (AFB). Tissue section slides were read in their entirety by a nonhuman primate pathologist blinded with respect to treatment groups.

***M. tuberculosis* Isolation.**

Snap frozen tissues, stored at -80°C, were shipped to the National Animal Disease Center (USDA-ARS, Ames, IA) to recover viable *mycobacteria* using three different culture methods: (i) the Fast Indicator Tube test (MGIT), (ii) Middlebrook 7H12 media (BacTec), and (iii) Solid Culture media to determine colony forming units (CFU). As rA*Mtb* auxotrophic mutants cannot grow in standard mycobacterial growth media culture, the media was supplemented with pantothenate with or without lysine ($mc^2$6020) or leucine ($mc^2$6435). A tissue was considered positive if one of three culture methods yielded mycobacterial growth. *M. tuberculosis* positive control samples were run in parallel for quality assurance.

Statistical Analysis.

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Antibody data at specific time points (see text) were compared between two or more groups after $\log_{10}$ transformation using a nonparametric Mann-Whitney test or One Way Anova analysis (Kruskal-Wallis test with Dunn's comparisons), respectively. Area-under-the-curve analysis of antibody levels was also performed on $\log_{10}$-transformed data using GraphPad Prism Software. P values ≤ 0.05 were considered significant.

RESULTS

Confirmation of vaccine exposure by the oral and intradermal route

All three vaccine strains were attenuated for replication. We, therefore, wanted to confirm that they were able to induce seroconversion to validate the biological significance of our safety assessment that includes lack of mycobacterial dissemination in an immunocompromised host. Plasma antibodies to the *M. tuberculosis* specific antigen PSTS1 were measured after oral and intradermal vaccination with the various rA*Mtb* vaccines. Independent of the vaccine strain, all SIV-uninfected vaccinated animals developed *M. tuberculosis*-specific plasma IgG antibodies to the PSTS1 antigen (Figure 2.2), but the magnitude was dependent on the vaccine strain and the route of administration. In fact, animals that received the $mc^2$6020 vaccine at the PO *and* ID route at 1 week of age (Group A) developed relative antibody levels reaching up to 10^4 MFI by 4 weeks (Figure 2.2A).

Similar data were obtained in animals orally primed with $mc^2$5157, and receiving a homologous ID boost (Groups C and E; Figure 2.2B). Consistent with more stringent attenuations in replication (deletions of the *leuCD* and *panCD* loci), oral vaccination with the $mc^2$6435 vaccine strain (Groups G and J) PSTS1 antibodies during weeks 4-6 post immunization were significantly lower levels ($p<0.001$) compared to $mc^2$6020 and $mc^2$5157 vaccinated infant macaques. Furthermore, the route of vaccine administration influenced the magnitude of antibody induction in plasma, with ID $mc^2$6435 vaccinated animals (Group H) developing significantly higher ($p=0.0119$) antibody levels than PO vaccinated animals (Groups G and J). Although there was a trend towards higher PSTS1 antibody levels in PO $mc^2$6435 vaccinated infants compared to mock-immunized animals between weeks 5-8 post vaccination, this difference did not reach statistical significance. PSTS1 antibody levels, however, were significantly higher in $mc^2$6435 vaccinated infants compared to mock-

immunized animals by area-under-the curve-analysis of antibody levels from week 0 to week 16 (p=0.0229). The magnitude of PSTS1 antibodies in animals infected with SIV prior to vaccination with rA*Mtb* $mc^2$6020 (Group B) or $mc^2$6435 (Group L) was reduced compared to SIV-uninfected animals (Figures 2.2A and 2.2C), likely due to SIV-induced immunosuppression.

Safety assessment of rA*Mtb*-SIV vaccine strains

The safety profile of the various rA*Mtb* vaccine strains was evaluated based on the combined assessment of (i) clinical observations (e.g. breathing difficulties), (ii) histopathological evaluation of multiple tissues, (iii) detection of AFB in tissues, and (iv) recovery of viable mycobacterial bacilli from tissues. A vaccine was considered to have no or only a minimal safety risk if AFB could not be detected, mycobacterial bacilli could not be recovered from any tissues, histopathology of lung and other tissues/organs appeared essentially normal, and no clinical symptoms were observed. In contrast, a vaccine strain was considered unsafe if one or more tissues tested positive for any of the following: AFB positive, *M. tuberculosis* culture positive, or granuloma detection during histopathological examination.

We assessed the safety of the rA*Mtb* vaccine strains in a stringent two-step study. First, the rA*Mtb* vaccine strains were administered to immunologically immature infant macaques at one week of age. Next, if no clinical signs of *M. tuberculosis* infection were observed and no viable mycobacteria could be recovered from tissues, we tested the vaccine in infant macaques infected with highly pathogenic SIVmac251 at one week prior to rA*Mtb* vaccination to reflect HIV-induced immunosuppression in HIV-1 infected human infants.

(i) Clinical safety assessment

SIV infection (Groups B and L) was confirmed by measuring plasma viral RNA levels. Consistent with our prior studies (4, 208, 350, 351), all six infant macaques developed high peak viremia (>10^7 copies/ ml of plasma) (Figure 2.3A) and viremia persisted at high levels. The loss of $CD4^+$ T cells is a hallmark of HIV/ SIV disease progression in adults, but not a reliable clinical marker of virus-infected infants (4). Furthermore, as $CD4^+$ T cells represent the vast majority of T cells at birth and $CD8^+$ T cell numbers continue to increase after birth, a decline in the percentage of $CD4^+$ T cells is characteristic of the normal developmental process after birth. In fact, a decline in $CD4^+$ T cells was observed in all experimental groups and was not limited to only SIV-infected infants (4) (Figure 2.3). Compared to age-matched control animals (mock-vaccinated animals), SIV-uninfected vaccinated animals showed similar peripheral blood $CD4^+$ T cell frequencies. In addition, the CBC values in SIV-uninfected rA*Mtb* vaccinated infants remained normal throughout the study period (data not shown).

A more critical factor in the evaluation of infant health is weight gain. All vaccinated SIV-uninfected infants showed weight gain similar to the age-matched control animals (Figure 2.3). In contrast, consistent with previous studies (4, 209), SIV-infected animals showed poor weight gain (Figure 2.3). Although wasting is also a common symptom of HIV/SIV-associated disease in adults (95), the lack of weight gain is much more detrimental in infants. Due to failure to thrive and symptoms associated with rapid progression to simian AIDS (e.g. reoccurring episodes of diarrhea, poor appetite and lethargic behavior), the SIV infected animals were euthanized between 6 and 10 weeks after SIV infection (Figure 2.3).

Despite this apparent immunosuppression as a result of SIV infection, these animals did not show clinical symptoms typically associated with *M. tuberculosis* infection. SIV-uninfected infants that received the $mc^2$6020 or $mc^2$6435 vaccines (Group A or Groups G, H, and J, respectively) showed normal weight gain (Figure 2.3).

Clinically, no symptoms indicative of *M. tuberculosis* infection (e.g. breathing difficulties, coughing) were observed at any time during the study period. Similarly, no adverse signs of vaccination with rA*Mtb*-SIV vaccines were observed, with the exemption of local reactivity in ID vaccinated animals. These animals showed a local inflammatory response following ID inoculations and developed indurations. In $mc^2$6020 vaccinated animals, indurations resolved over time. Due to the short follow-up time in $mc^2$5157 vaccinated animals, it could not be determined whether the local inflammation would have resolved over time.

(ii) Histopathological evaluation

To thoroughly assess the safety of the rA*Mtb* vaccine strains in infants, several tissues collected at necropsy were evaluated for TB pathology: (i) the dermal inoculation site or corresponding skin samples from orally rA*Mtb* or mock vaccinated animals, (ii) axillary LNs that drained the dermal inoculation site, (iii) the lung as the primary site of TB-specific pathology, (iv) the lung-draining bronchial LNs, and (v) the spleen as a more distal lymphoid indicator of *M. tuberculosis* dissemination (Table 2.3).

Consistent with clinical signs of inflammation (see above), histopathological examination of $mc^2$5157 vaccinated animals at 4 or 6 weeks of age (Groups C and E,

respectively) revealed that moderate to severe pyogranulomatous dermatitis had persisted at the ID inoculation sites, whereas dermal tissues from mc^{2}6020 animals appeared essentially normal (Table 2.3). In contrast to mc^{2}6020 and mc^{2}5157 vaccinated animals, ID vaccination with mc^{2}6435 (Group H) did not result in an inflammatory response at the site of inoculation (Table 2.3). The mild dermatitis that was observed in some of the vaccinees independent of the vaccine strain was occasionally noted in skin from orally or mock-vaccinated animals as well and thus was probably not due to vaccination (Table 2.3). Consistent with a potential spread of mycobacteria in mc^{2}5157 vaccinated animals (Groups C and E), the majority of the axillary LNs of mc^{2}5157 vaccinated animals showed moderate hyperplasia and pyogranulomatous inflammation, whereas SIV-uninfected animals vaccinated with mc^{2}6020 or mc^{2}6435 showed only mild histopathological changes (Table 2.3).

Evidence of pulmonary TB lesion or granuloma formation in the lung indicative of *M. tuberculosis* infection were markedly absent from the vaccinated animals. In fact, the lungs of animals vaccinated with mc^{2}6020 (not shown) and mc^{2}6435 (Figure 2.4E) were histologically indistinguishable from lungs of mock-vaccinated infant macaques (Figure 2.4C). Among the 10 animals vaccinated with mc^{2}5157, six developed small granulomas in their lungs (Figure 2.4D). However, these lesions were smaller and less frequent when compared to granulomas induced by virulent *M. tuberculosis* infection (Figures 2.4A, 2.4B).

In infants infected with SIV prior to rA*Mtb* vaccination, histopathological changes typical for SIV infection such as lymphoid depletion and mild hyperplasia were commonly observed in various lymphoid tissues (Table 2.3). Although pneumonitis was detected in some SIV-infected rA*Mtb* vaccinated animals, the histopathology was not typical for *M. tuberculosis*-induced lung pathology. Importantly, SIV-infected animals did not develop

granuloma in the lung or other tissues after vaccination with $mc^2$6020 or $mc^2$6435 (Figure 2.4F). Thus, SIV-induced immunosuppression in infant macaques vaccinated with rA*Mtb* strains did not result in TB-induced disease or pathology.

(iii) Strain-dependent differences in mycobacterial dissemination

Miliary tuberculosis is one of the most severe complications of *M. tuberculosis* infection. Although the rA*Mtb* vaccine strains tested in the current study were replication attenuated, the degree of attenuation in replication and immune evasion differed between the strains (Table 2.1). Therefore, we tested the same tissues that were examined for TB-associated pathology for the presence of mycobacteria. First, tissue sections were stained with Ziehl-Neelson dye to detect AFB. However, even in pathogenic *M. tuberculosis* infection mycobacteria detection by AFB staining can be infrequent. Therefore, different culture methods using optimized growth medium specially supplemented for the auxotrophic strains were used to determine whether live mycobacteria could be recovered from any of the tissues. AFB were only rarely (1-2 bacilli per tissue), if at all, detected at dermal inoculation sites after $mc^2$6020 or $mc^2$6435 vaccination. Importantly, AFB were detected in tissue from only 1 of 3 SIV-infected infant macaques that received the $mc^2$6020 vaccine, and only at one site, the axillary LN that drained the ID inoculation site. AFB were not detected by Ziehl-Neelson staining in any other tissues in this animal. Live mycobacteria could be recovered from the same axillary LN of this animal, but not from any other tissue samples.

In contrast, all infant macaques vaccinated with $mc^2$5157, the rA*Mtb* vaccine strain that contained deletions that may increase its immunogenicity and yet result in higher

replication relative to $mc^2$6020, tested positive for AFB in their dermal tissues, and in 6 of 10 animals AFB were also detected in at least one other tissue (Table 2.4). Furthermore, live mycobacteria could be recovered from several tissues of these animals (Table 2.4). Due to the widespread mycobacterial dissemination and more severe immunopathology in $mc^2$5157 vaccinated infant macaques, strain $mc^2$5157 was considered unsafe and not further pursued as a candidate *M. tuberculosis*-SIV vaccine. Remarkably, all tissues from SIV-uninfected (n=20) and also from SIV-infected (n=3) $mc^2$6435 vaccinated animals were negative for AFB and viable mycobacteria could not be recovered by any of the culture methods applied in any of these tissues.

In summary, the data indicate that rA*Mtb*-SIV vaccine strains can be administered orally or intradermally during the first week of age to infant macaques. Although vaccine strain $mc^2$5157 caused dissemination of mycobacteria, the live attenuated vaccine strains $mc^2$6020 and $mc^2$6435 demonstrated a better safety profile in infant macaques. In particular, strain $mc^2$6435 was safe in SIV-uninfected (Groups I and K) *and* in immunocompromised SIV-infected infant macaques (Groups G, H, and J) by all safety criteria applied in this study.

DISCUSSION

An effective vaccine against HIV is not available, and the only approved TB vaccine, BCG, is not safe in immunosuppressed individuals. This safety concern is of particular importance in resource-poor countries affected by the dual epidemics of TB and HIV. In particular, TB infection rates among children have risen in association with HIV prevalence and about 1% HIV-infected infants develop disseminated BCG after vaccination. Although ART coverage now extends to 42% of HIV-infected mothers, only about 23% of their newborn infants receive ART (369, 381) and remain at risk for HIV acquisition by breast-

feeding. These facts underscore the need for a novel safe vaccine to prevent pediatric HIV and TB infections. The current study represents an important first step towards the development of an orally administered highly attenuated *M. tuberculosis* vaccine expressing HIV antigens as a potential pediatric combination HIV-TB vaccine.

BCG was originally administered orally, but this route was discontinued due to cervical lymphadenitis and parapharyngeal complications. The oral route may be more advantageous for a pediatric HIV-TB vaccine because a mucosally administered vaccine could also induce local immune responses protective against orally transmitted HIV by breast-feeding. In fact, experiments in mice have shown that orally fed BCG can infect the submaxillary peri-glandular lymph nodes and Peyer's patches (180), and that *M. tuberculosis* can bind to tonsillar M cells (32, 235). Thus, we assessed the safety of three distinct recombinant attenuated *M. tuberculosis* H37Rv strains engineered with or without the SIV Gag gene in SIV-uninfected and in SIV-infected, and thus immunosuppressed, infant macaques both by the oral and intradermal route. BCG vaccination of SIV-infected macaques has previously been shown to exacerbate SIV disease progression and to cause dissemination of *M. bovis* bacilli (66, 308, 395). In accordance with a recent proposal to improve the classification of TB disease severity in human children by combining multiple factors ranging from disease pathogenesis criteria to bacteriological evaluation and clinical data (387), vaccine safety in infant macaques was rigorously assessed in the current study by comprehensive clinical examination, histopathological evaluations, and multiple *M. tuberculosis* culture methods.

For safety evaluation, animals were grouped by vaccine strain independent of the route of administration, immunization interval, or prime-boost regimen. The *panCD* deletion

in strain mc^{2}5157 was not sufficient to prevent mycobacterial dissemination in infant macaques, and although granuloma size and frequencies in the lung were limited in comparison to macaques infected with pathogenic TB (197), the strain was deemed unsuitable for use in immunocompromised hosts. In contrast to mc^{2}5157, the safety profile of the dual deletion strain mc^{2}6020 (Δ*panCD*Δ*lysA)* was improved. Only one of three SIV-infected infant rhesus macaques showed evidence of *M. tuberculosis* dissemination, and only in the axillary lymph node that drained the ID inoculation site. Whether or not this limited mycobacterial dissemination in immunosuppressed SIV-infected infant macaques correlates with reduced risk for future dissemination or reactivation at other sites, however, remains to be determined. Although we previously confirmed the safety of this strain, these studies were performed only in SIV-uninfected adult cynomolgus macaques (184). Thus, the current data support our hypothesis that auxotroph mutant *M. tuberculosis* strains can be developed as safe vaccine candidates for use in immunocompromised individuals.

M. tuberculosis is a slowly replicating bacterium and encodes genes that limit the host immune response, permitting bacilli to persist within a host, often in a latent state (103, 191). To be safe and effective, a live attenuated *M. tuberculosis* vaccine requires a balance between replication and immunogenicity. In infants with immature immunity, low-level replication may be required to induce *Mtb*-specific immune responses and promote persistent immune memory responses. However, if replication is too robust, attenuated *M. tuberculosis* strains may result in granuloma formation and bacillary dissemination as was observed with mc^{2}5157. In addition to attenuating replication, deletions in mycobacterial genes encoding immune interference may be necessary to enhance A*Mtb* immunogenicity.

Based on the results with mc^{2}5157 and mc^{2}6020, the strain mc^{2}6435 was designed with deletion in both the *panCD* and the *leuCD* loci *(*Δ*panCD*Δ*leuCD*). An additional deletion was introduced in the *secA2* locus that interferes with apoptosis in *M. tuberculosis*-infected macrophages (13, 133). The deletion of this locus has been associated with both enhanced apoptosis of mycobacteria-infected macrophages in vitro, and with increased antigen-specific CD8$^+$T cell responses in vivo, likely due to enhanced cross-presentation of mycobacterial antigens to dendritic cells (13, 133). A similar A*Mtb* strain (Δ*lysA*Δ*secA2*) has previously been demonstrated to be safe and immunogenic in neonatal SCID mice (100% survival for up to 642 days) in which BCG is generally lethal (268). In fact, our results here show that the rA*Mtb* strain mc^{2}6435 was well tolerated and did not cause any TB-like disease symptoms in 20 of 20 infant macaques vaccinated within one week of age. Importantly, the safety of this strain was confirmed in infant macaques infected with highly pathogenic SIV prior to mc^{2}6435 vaccination. The tissue pathology of these SIV-infected infant macaques was consistent only with SIV-induced pathology, yet no signs of TB pathology were detected. Furthermore, there was no histopathological evidence of rA*Mtb* dissemination and live mycobacteria could not be recovered from any tissues studied, even under optimal supplemented culture conditions for the attenuated *M. tuberculosis* strain.

No other TB vaccine candidate has been tested under such stringent conditions in *infant* macaques, a model for infant TB vaccination. Analogous to our study, the safety of a Δ*leuCD*Δ*panCD* auxotroph *M. tuberculosis* vaccine strain was recently evaluated in SIV-uninfected and SIV-infected *adult* macaques (297). Vaccination of adult macaques with this attenuated *M. tuberculosis* strain did not cause adverse effects, AFB were not detectable, and viable mycobacterial bacilli could not be recovered from longitudinally collected blood

samples or tissues at the time of euthanasia (297). The adult SIV-infected animals were followed for up to 1 year, further supporting the conclusion that auxotroph attenuated *M. tuberculosis* strains do not confer a safety risk in immunosuppressed individuals. The safety profile of the rA*Mtb* strain $mc^2$6435 in SIV-infected infant macaques is consistent with this conclusion.

A potential caveat of our study is the relatively short follow-up time in SIV-infected infants. In HIV-infected children and SIV-infected neonatal macaques, disease is often more severe and progression is accelerated. In the current study, SIV-infected infants were euthanized between 6 and 10 weeks after SIV infection due to failure to thrive. Therefore, vaccine-induced *M. tuberculosis*-associated pathology or dissemination had to manifest within a relatively short time period in SIV-infected animals to be detected, and we cannot draw any conclusions about long-term outcome. It should be emphasized though that *M. tuberculosis* dissemination was observed in multiple infant tissues collected at 4 or 6 weeks after vaccination with $mc^2$5157 by pathology, histology and culture assays. The latter data imply that lung pathology and *M. tuberculosis* dissemination should be detectable within a 10-week time frame, especially in immunosuppressed SIV-infected animals.

To our knowledge, this is the first study demonstrating that an attenuated *M. tuberculosis* strain does not cause disease in an infant nonhuman primate model of neonatal TB vaccination and SIV infection that is highly relevant to human infants, including those at risk for perinatal HIV infection. Our data show that all three vaccines were able to induce persistent vaccine-specific antibody responses in infant macaques, albeit the magnitude was dependent on the level of replication-attenuation ($mc^2$6020=$mc^2$5157>$mc^2$6435) and the route of vaccine administration (ID>PO). The persistence of these antibodies suggests that

the rA*Mtb* vaccines primed the infant immune system. Despite the low PSTS1 *M. tuberculosis*-specific plasma antibody responses after oral and intradermal administration in infant rhesus macaques, the rA*Mtb* vaccine strain $mc^2$6435 (that contains a mycobacterial expression plasmid encoding SIV Gag) was effective in inducing both SIV *and* TB-specific $CD4^+$ and $CD8^+$T cell immune responses in systemic and mucosal tissues (manuscript in preparation). Therefore, the rA*Mtb* strain $mc^2$6435 should be further explored and optimized as a valid TB vaccine candidate that could replace BCG and as a combination vaccine to protect against HIV and TB infection in infants. Considering that immune correlates of protection against HIV or TB acquisition are not well defined, these vaccines will ultimately need to be tested for efficacy against an HIV/SIV and *M. tuberculosis* challenge.

TABLES

Table 2.1. rA*Mtb* Vaccine Strains

TABLE 1 Recombinant attenuated *M. tuberculosis* vaccine strains used in this study

Vaccine strain	Groups	Deletion(s) attenuating: Replication	Deletion(s) attenuating: Immune evasion
mc^{2}6020 (Δ*lysA* Δ*panCD*)[a]	A, B	Δ*lysA* Δ*panCD* (low)[d]	None
mc^{2}5157 (Δ*nuoG* Δ*panCD* Δ*RD1* pSIV Gag)[b]	C–F	Δ*panCD* Δ*RD1* (intermediate)	Δ*nuoG*
mc^{2}6435 (Δ*leuCD* Δ*panCD* Δ*secA2* pSIV Gag)[c]	G–L	Δ*leuCD* Δ*panCD* (low)	Δ*secA2*

[a] Δ*lysA* Δ*panCD*, deletions of the *lysA* and the *panCD* loci.
[b] Δ*nuoG* Δ*panCD* Δ*RD1*, deletions of the *nuoG*, *panCD*, and *RD1* loci; pSIV GAG, insertion of a full-length SIVmac239 Gag insert.
[c] Δ*leuCD* Δ*panCD* Δ*secA2*, deletions of the *leuCD*, *panCD*, and *secA2* loci.
[d] Replication levels are shown in parentheses.

Table 2.2. Study Outline

TABLE 2 Study outline

Group	Size[a]	SIV (i.v.)	Vaccination: Prime strain	Vaccination: Dose(s) (CFU)	Vaccination: Route(s)	Vaccination: Age (wk)	Vaccination: Boost strain	Vaccination: Dose	Vaccination: Route(s)	Vaccination: Age (wk)	Sample collection points (wk)	Age at necropsy (wk)
A	3	No	mc^{2}6020	10^9, 10^6	p.o., i.d.	1	None				0, 2, 4, 6, 8, 10, 12, 16, 20, 24	24
B	3	Yes	mc^{2}6020	10^9, 10^6	p.o., i.d.	1	None				0, 2, 4, 6	6
C	5	No	mc^{2}5157	10^9	p.o.	0	mc^{2}5157	10^9 CFU	i.d.	2	0, 2, 4	4
D	2	No	Mock		p.o., i.d.	0	Mock		p.o., i.d.	2	0, 2, 4	4
E	5	No	mc^{2}5157	10^9	p.o.	0	mc^{2}5157	10^9 CFU	i.d.	3	0, 3, 6	6
F	2	No	Mock		p.o., i.d.	0	Mock		p.o., i.d.	3	0, 3, 6	6
G	6	No	mc^{2}6435	10^9	p.o.	0	rAd5SIV	9×10^9 PFU	i.m.[b]	3	0, 3, 6, 9, 12, 16	16
H	6	No	mc^{2}6435	10^6	i.d.	0	rAd5SIV	9×10^9 PFU	i.m.	3		16
I	4	No	Mock		p.o., i.d.	0	Mock		i.m.	3		16
J	8	No	mc^{2}6435	10^9	p.o.	0	rMVASIV	10^8 PFU	i.m.	3, 6	0, 3, 6, 9, 12, 16	18
K	3	No	Mock		p.o.	0	Mock		i.m.	3, 6		16
L	3	Yes	mc^{2}6435	10^9	p.o.	1	None				0, 1, 2, 4, 6, 8, 10	6–10

[a] Number of animals.
[b] i.m., intramuscular.

Table 2.3. Histopathological Evaluations

TABLE 3 Histopathological evaluation

Group(s) (no. of animals)[a]	SIV infection	Vaccine strain (route[s])	Findings on tissue samples[b]					Pathology result[c]	
			Skin	Axillary LN	Lung	Bronchial LN	Spleen	TB	SIV
I, D, F, K (11)	No	Mock (p.o., i.d.)	7/11 normal; 3/11 very mild dermatitis; 1/11 no tissue	4/11 normal; 2/11 mild histiocytosis; 3/11 mild-to-moderate paracortical expansion; 2/11 no tissue	6/11 normal; 3/11 mild bronchiolitis or pneumonia; 2/11 no tissue	4/11 normal; 3/11 mild-to-moderate paracortical expansion; 2/11 mildly reactive; 1/11 mild eosinophilia; 1/11 no tissue	4/11 normal; 3/11 neutrophilia; 3/11 mild hyperplasia; 1/11 no tissue	No	No
A (3)	No	$mc^2$6020 (p.o., i.d.)	2/3 normal; 1/3 very mild dermatitis	1/3 mild hyperplasia; 1/3 mild lymphoid depletion; 1/3 no tissue	1/3 normal; 2/3 subacute mild pneumonitis	1/3 mild lymphoid hyperplasia; 2/3 no tissue	3/3 normal	No	No
B (3)	Yes	$mc^2$6020 (p.o., i.d.)	2/3 normal; 1/3 mild dermatitis	3/3 mild lymphoid depletion	2/3 normal; 1/3 mild pneumonitis	2/3 mild lymphoid depletion; 1/3 normal	3/3 mild-to-moderate lymphoid depletion	No	Yes
C, E (10)	No	$mc^2$5157 (p.o., i.d.)	10/10 moderate-to-severe pyogranulomatous dermatitis	2/10 normal; 3/10 mild-to-moderate hyperplasia; 5/10 pyogranulomatous lymphadenitis	4/10 normal; 6/10 moderate multifocal pneumonitis, some granulomatous inflammation	1/10 normal; 8/10 mild-to-moderate hyperplasia, rare granulomatous lymphadenitis; 1/10 no tissue	10/10 normal	Some	No
G, J (14)	No	$mc^2$6435 (p.o.)	14/14 normal	11/14 normal with mild paracortical expansion; 1/13 mild-to-moderate paracrotical expansion; 2/14 no tissue	8/14 normal; 5/14 mild interstitial pneumonitis, some congestion; 1/14 focal granuloma with foreign material	6/14 normal; 3/14 mildly reactive; 4/14 mild paracortical expansion; 1/14 mild extramedullary hematopoiesis	13/14 normal; 1/14 mild-to-moderate hyperplasia	No	No
H (6)	No	$mc^2$6435(i.d.)	6/6 normal	4/6 normal; 2/6 mild sinus histiocytosis	2/6 normal; 3/6 subacute pneumonitis; 1/6 mild arteriolitis	3/6 normal; 3/6 mildly reactive	4/6 normal; 1/6 increased neutrophils; 1/6 intraparenchymal hemorrhage	No	No
L (3)	Yes	$mc^2$6435(p.o.)	2/3 normal; 1/3 mild dermatitis	2/3 normal; 1/3 sinus histiocytosis	3/3 moderate pneumonia, hemorrhages, pneumocyte hyperplasia	3/3 normal	3/3 lymphoid hyperplasia, neutrophilia; 1/3 focal mineralization	No	Yes

[a] The experimental groups are described in the text and in Table 2.
[b] All of the observed histopathological findings are summarized for each tissue type. The value before the slash is the number of animals within a specific group that showed the pathology described, and the value after the slash is the total number of animals.
[c] The summary conclusion provided by the pathologist is shown as follows: no, negative; yes, positive; some, minor pathology due to SIV or *M. tuberculosis* infection.

Table 2.4. *M. tuberculosis* Detection

TABLE 4 *M. tuberculosis* detection

Group(s) (strain), no. of animals, tissue type	AFB	MGIT[a]				BacTec[b]				Solid culture[c]				Result
		−	+	++	+++	−	+	++	+++	−	+	++	+++	
A (mc^{2}6020), 3														
Skin	0/3	NT[d]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Lung	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Axillary LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Spleen	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
B (SIV + mc^{2}6020), 3														
Skin	0/3	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Lung	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Axillary LN	**1/3**[e]	2/3	**1/3**	0/3	0/3	2/3	**1/3**	0/3	0/3	3/3	0/3	0/3	0/3	**1/3**
Spleen	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
C, E (mc^{2}5157), 10														
Skin	10/10	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	**2/10**	4/10	**1/10**	**3/10**	**2/10**	5/10	0/10	**3/10**	**2/10**	5/10	**1/10**	**3/10**	**1/10**	**5/10**
Lung	**1/10**	5/10	**1/10**	**1/10**	**3/10**	3/10	**5/10**	**1/10**	**1/10**	5/10	**1/10**	**1/10**	**3/10**	**7/10**
Axillary LN	**5/10**	2/10	**1/10**	**3/10**	**4/10**	2/10	**3/10**	**2/10**	**3/10**	3/10	**1/10**	**3/10**	**3/10**	**8/10**
Spleen	0/10	0/10	**5/10**	**5/10**	0/10	0/10	**10/10**	0/10	0/10	0/10	**4/10**	**4/10**	**2/10**	**10/10**
G, H, J (mc^{2}6435), 20														
Skin	0/20	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
Lung	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
Axillary LN	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
Spleen	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	20/20	0/20	0/20	0/20	0/20
L (SIV + mc^{2}6435), 3														
Skin	0/3	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Bronchial LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Lung	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Axillary LN	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3
Spleen	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3

[a] MGIT results: −, no growth; +, ≥24 days; ++, 18 to 24 days; +++, <18 days.
[b] BacTec results: −, no growth; +, ≥24 days; ++, 18 to 24 days; +++, <18 days.
[c] Solid culture results: −, no growth; +, <5 CFU; ++, 5 to 50 CFU; +++, ≥50 CFU.
[d] NT, not tested.
[e] Samples positive for AFB or outgrowth of mycobacteria are in bold.

FIGURES

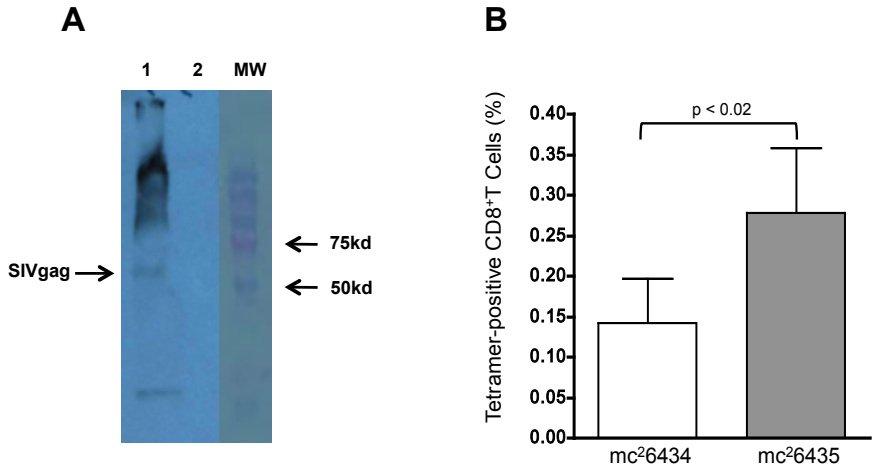


Figure 2.1. Vaccine-induced SIV-specific CD8$^+$ T cell responses in mice. A) Western blot result showing SIVgag protein expression in mc^{2}6435 lysate (Lane 1) and its absence in mc^{2}6434 lysate expressing the control plasmid (Lane 2). Lane 3 represents the molecular weight ladder. rA*Mtb* whole cell lysates were immunoblotted with HRP-conjugated anti-V5 antibodies and developed using chemiluminescence. The principal recombinant SIVgag band migrates at approximately 55 kDa. One lower molecular weight breakdown product is also observed in mc^{2}6435 lysate. B) C57BL/6 mice were immunized subcutaneously with 10^7 CFU of the attenuated *M. tuberculosis* vaccine strain mc^{2}6434 (n=5) or with mc^{2}6434 that contained the SIV Gag expression cassette (named mc^{2}6435, n=5). One week after immunization, splenocytes were isolated and AL11-SIVgag tetramer specific CD8$^+$ T cell responses were determined by flow cytometric analysis. The figure shows the average percentages ± SD of AL11-SIVgag tetramer-specific CD8$^+$ T cells. Note that mc^{2}6435 immunized mice mounted significantly higher AL11-SIVgag-specific CD8$^+$ T cell responses than mc^{2}6434 mice ($p<0.02$), albeit absolute frequencies of tetramer positive CD8$^+$ T cells were low.

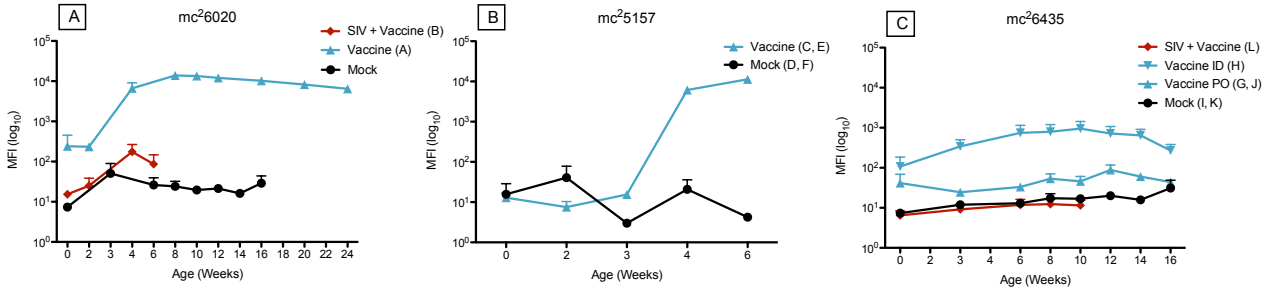


Figure 2.2. *M. tuberculosis*-specific plasma IgG antibody responses. Plasma antibody responses to PSTS1 antigen were measured by multiplex microbead array analysis. Average antibody levels plus standard deviations for mock-vaccinated animals (black lines, closed circles), SIV-infected and vaccinated animals (red lines, diamonds), and non-SIV-infected vaccinated animals (blue lines, triangles) are shown for each vaccine candidate. Data are reported as MFIs. The experimental groups are listed in parentheses in the top right corner of each panel. The mock-vaccinated animals used for groups A and B are the same animals used as controls for groups G, H, J, and L. Note that in panel A, one of three non-SIV-infected vaccinated animals had high PSTS1 antibody levels at day 0 (MFI of 666 compared to MFIs of 40 and 18 in the other remaining animals, and therefore, the average baseline antibody levels at day 0 appear higher than those of groups B, I, and K. Similarly, in panel C, 2 of 6 animals vaccinated i.d. and 2 of 14 animals vaccinated p.o. with mc26435 had PSTS1 MFIs (antibody levels) of 100 at day 0, explaining the higher MFI values at day 0 than those of groups I, K, and L.

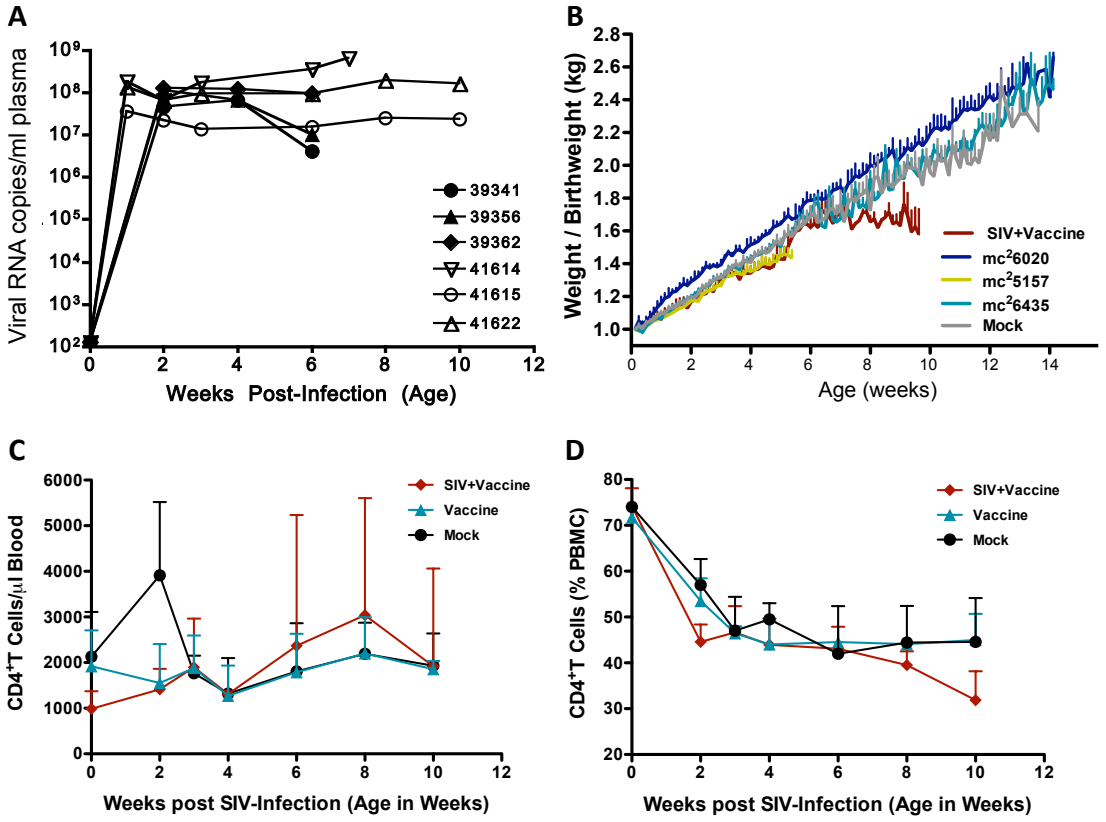


Figure 2.3. Plasma SIV viremia and clinical parameters. A) Numbers of SIV RNA copies per milliliter of plasma in samples longitudinally collected from each animal are shown. Each symbol represents an individual animal. The experimental group of each animal is shown in parentheses. B) The average increase in weight over the birth weight (kilograms) is shown for the animals in each of the three vaccine groups (dark blue, yellow, and light blue lines) and for SIV-infected and subsequently vaccinated animals (red line) in comparison to the weight gain of mock-vaccinated animals (gray line). The averages of absolute numbers ± standard deviations C) and average percentages ± standard deviations of $CD4^+$ T cells D) in peripheral blood are shown for animals vaccinated after prior SIV infection (red lines, diamonds) and for vaccinated non-SIV-infected (blue lines, triangles) animals in comparison to mock-vaccinated animals (black lines, circles). Note that panels C and D include the SIV-infected animals vaccinated with $mc^2$6020 (n = 3) or $mc^2$6435 (n = 3). For this analysis, vaccinated animals (n = 33) included animals from groups A, C, E, G, H, and J and mock-vaccinated animals (n = 11) included animals from groups D, F, I, and K.

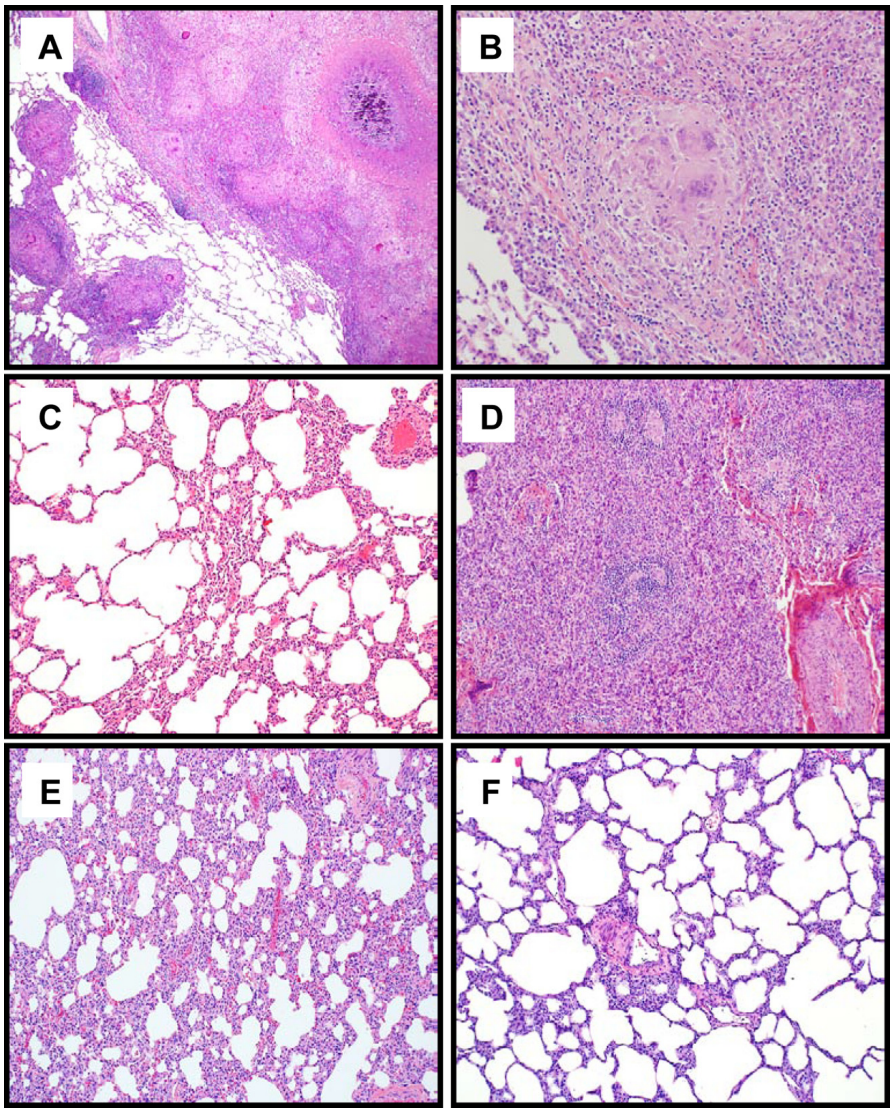


Figure 2.4. Pathology evaluation of lung tissue. Formalin-fixed, paraffin-embedded tissues were stained with H&E and blindly analyzed for histological changes due to vaccination and/or SIV infection. A) Lung tissue (magnification, x100) of an adult rhesus macaque infected with pathogenic *M. tuberculosis* (197). B) The same tissue shown in panel A at x200 magnification. Note the central necrosis, mineralization, and granuloma formation and the presence of multinucleate giant cells. C) Lung section of a mock-immunized infant macaque. D) Representative lung section of an $mc^2$5157-vaccinated infant macaque with granulomatous tissue and signs of extensive atelectasis. Panels E and F show essentially normal lung sections from non-SIV-infected E) or SIV-infected F) infant macaques after $mc^2$6435 vaccination. The sections in panels C to F are at x100 magnification.

CHAPTER 3: A NEONATAL ORAL *MYCOBACTERIUM TUBERCULOSIS*-SIV PRIME / INTRAMUSCULAR MVA-SIV BOOST COMBINATION VACCINE INDUCES BOTH SIV- AND *MTB*-SPECIFIC IMMUNE RESPONSES IN INFANT MACAQUES[2]

OVERVIEW

Mother-to-child-transmission of HIV by breast-feeding remains a major obstacle in the eradication of HIV infection. Compared to adults, HIV-infected infants have more rapid disease and show higher susceptibility to co-infections like tuberculosis (TB). Although the Bacille Calmette-Guérin vaccine can be administered at birth to protect against TB, BCG can disseminate in HIV-infected infants and increase mortality. Thus, a pediatric combination vaccine to stop both HIV and TB infection in infants is urgently needed. Towards the goal of developing a pediatric combination HIV-TB vaccine to prevent both oral HIV acquisition by breast-feeding and TB infection, we tested and optimized an immunization regimen using a novel live attenuated *Mycobacterium tuberculosis* vaccine engineered to express simian immunodeficiency (SIV) antigens followed by heterologous MVA-SIV boosting in the infant macaque model.

A single oral dose of the attenuated *Mtb*-SIV vaccine strain $mc^2$6435 during the first week of life was sufficient to induce persistent TB-specific immune responses. SIV-specific immunity was induced at low but comparable magnitudes after oral or intradermal priming,

[2] This chapter has been reproduced/amended from Jensen *et al.* (151).

and was enhanced following MVA-SIV boosts. T cell responses were most pronounced in intestinal tissues and oral lymph nodes. Importantly, in addition to plasma SIV-specific IgG and IgA antibodies, infant macaques developed mucosal SIV-specific IgA in saliva and intestinal IgA and IgG. While future SIV and *Mtb* challenge studies will be needed to determine the protective efficacy of the *Mtb*-SIV / MVA-SIV vaccine, infants at high risk for oral HIV acquisition by breast-feeding *and* TB infection could profoundly benefit from an effective combination vaccine.

INTRODUCTION

The coverage of antiretroviral therapy (ART) for HIV-infected mothers has increased substantially, yet many resource-poor countries are still afflicted by rising rates of HIV mother-to-child-transmission (MTCT) (338). Infant ART coverage remains below 30% and ART prophylaxis does not span the entire breast-feeding period (338). The majority of pediatric HIV infections occur in sub-Saharan Africa where tuberculosis (TB) burden is also high. HIV-infected infants face a higher risk of TB infection (165), and pregnant women co-infected with HIV and TB are more likely to transmit both HIV and TB to their infants (122, 165). The Bacille Calmette-Guérin (BCG) vaccine for prevention of TB infection can disseminate in HIV-infected infants with a case fatality of 75% (128). The high morbidity and mortality associated with HIV and TB disease in infants underscore the urgent need for a safe neonatal vaccine to prevent pediatric HIV and TB infections.

Currently, BCG is the only live attenuated vaccine approved for administration in neonates at birth. BCG-inherent adjuvant properties likely enhance pediatric immune responses because a single BCG dose induces robust cellular immunity comparable to responses in adults. These compelling facts provided the rationale for the development of

combination HIV-TB vaccines. In fact, based on murine TB efficacy data with a recombinant auxothroph BCG vaccine expressing an African consensus HIV-1 clade A Gag immunogen (rBCG.HIVA) (4, 122, 348, 359), phase I clinical trials have been initiated in African neonates. The restriction of preclinical BCG-HIV immunogenicity and safety studies to murine models or adult macaques (4, 27, 122, 136, 281, 348), however, is problematic. Substantial differences in i) infant and adult immune function, ii) immune development between neonatal mice and human newborns, and iii) inherent limitations of BCG-derived vaccines (importantly the safety risk for immunocompromised individuals and lack of relevant protective TB antigens), argue for the tandem pursuit of alternative regimens.

We hypothesized that a live attenuated human-adapted *Mycobacterium tuberculosis (Mtb)* vaccine similar to BCG but with an improved safety profile could be safe and protective, even in immunosuppressed infants. We chose human-adapted *Mtb* H37Rv in lieu of *M. bovis*-derived BCG because *Mtb* contains known immunodominant epitopes for humans that are absent in BCG (46, 73). Secondly, we intentionally deleted specific H37Rv genes important for replication and immune evasion, whereas BCG was attenuated only through serial passaging. Rhesus macaques are an ideal and validated animal model in which to evaluate our combination vaccine due to their extremely sensitivity to *Mtb* and to simian immunodeficiency virus (SIV) (214, 307, 393), and the shared immunological, developmental and physiological similarities between human infants and neonatal macaques (217, 290, 309). The translational potential of our vaccine for application in human infants at risk for HIV is supported by our data demonstrating that neonatal SIV-infected macaques could be safely vaccinated with the live attenuated auxotroph *Mtb* vaccine $mc^2$6435 (152).

Towards the long-term goal of developing a pediatric HIV-TB vaccine, the current

study tested whether this highly attenuated recombinant *Mtb*-SIV vaccine can efficiently prime both *Mtb* and SIV-specific immunity. To establish proof-of concept, we deemed the expression of only a single SIV antigen, SIV Gag, by $mc^2$6435 sufficient. SIV Gag contains numerous immunogenic T cell epitopes, and several vaccine studies support the importance of SIV Gag-specific T cell responses in the control of viral replication (266, 324, 331). Indeed, our data demonstrate that (i) a single dose of $mc^2$6435 induced both *Mtb*- and SIV-specific immune responses in infant macaques, (ii) vaccine-induced SIV immunity was enhanced and broadened by heterologous MVA-SIV Gag, Pol and Env boosts, and (iii) the combined oral *Mtb*-SIV Gag prime/ intramuscular MVA-SIV regimen effectively induced mucosal and systemic immune responses.

MATERIALS AND METHODS

Animals.

Infant rhesus macaques (*Macaca mulatta*), obtained from the SIV-negative and type D retrovirus-free CNPRC colony (UC Davis, CA), were nursery-reared and housed according to the "Guide for Care and Use of Laboratory Animals" and standards by the AAALAC. Animal protocols were approved by the UC Davis Institutional Animal Care and Use Committee. For all interventions, animals were immobilized by 10mg/kg body weight of ketamine-HCl (Parke-Davis, Morris Plains, NC), injected intramuscularly (IM).

Vaccine Strains and Immunization Regimens.

The construction, attenuations and safety profiles of the *Mtb* H37Rv-derived vaccine strains $mc^2$6435 and $mc^2$6020 have been described previously (152, 184, 268, 293, 296, 344, 397). Briefly, in strain $mc^2$6020, the *panCD* and *lysA* loci were deleted to generate a highly

replication-attenuated double-auxotroph strain. The double-auxotroph strain $mc^2$6435 (Δ*panCDΔleuCD*) was further modified by deleting the *secA2* locus, and engineered to co-expresses a mycobacterial codon-usage-optimized full length SIVmac239 Gag cassette. SIV Gag expression from vaccine cultures was confirmed by immunoblot using a V5 antibody-HRP.

Infant macaques (n=8) were orally (PO) vaccinated with $mc^2$6435 at birth (3-7 days old = week 0), IM boosted at 3 and 6 weeks with MVA-SIV expressing SIV Gag, Pol, and Env (kindly provided Dr. B. Moss and Dr. P. Earl, NIAID, NIH, Bethesda, MD) (79, 178, 348), and followed for 18 weeks (Group A). Age-matched control animals (n=3) were mock vaccinated with saline (Group B). To test how replication attenuation affects *Mtb*-specific immune responses, three infants were vaccinated both PO and intradermally (ID) with strain $mc^2$6020 without boosting, and followed for 24 weeks (Group C).

Sample Processing.

Blood and saliva samples were collected prior to all interventions and throughout the study period. Plasma was stored at -80° C for antibody measurement. At the time of euthanasia, tonsils, lymph nodes (LN: axillary, mesenteric, retropharyngeal and submandibular), and intestinal tissues (colon and ileum) were collected, and mononuclear cell suspensions (MNC) were prepared as described (208). Saliva was collected using Weck-Cel sponges (Beaver Visitec), immediately snap frozen, and later eluted as described At the terminal time point, 2-3 grams of fresh stool were added to 0.5% BSA in 5ml sterile PBS supplemented with 1/100 protease inhibitor cocktail (Sigma, P8340) and snap-frozen. Fecal extracts were prepared by vortexing the thawed contents until complete homogenization,

removing debris by ultracentrifugation (4˚C, 10 min), filtration (0.45µm), and concentration to 0.5ml using an Amicon Ultra-4 50K centrifugal filter unit (Millipore).

SIV-specific Antibodies and Immunoglobulin (Ig) Measurements.

Anti-SIV IgG and IgA antibodies in plasma and fecal extracts, and salivary IgA were measured by ELISA as described (208). Briefly, plates were coated overnight with 100ng SIVmac251 Env rgp120 (Immune Technology, New York, NY) or 100µl 1/400 SIVmac239 lysate (Advanced Biotechnologies, Columbia, MD) per well in PBS. Antibodies against the lysate are termed "Gag, Pol"-specific because Env protein is undetectable at the dilution used. Total IgA or IgG in saliva or fecal extracts were measured by capture ELISA (24, 152) using goat anti-monkey IgA (AlphaDiagnostics, San Antonio, TX) or goat anti-monkey IgG (MP Biomedicals), respectively, to capture Ig. The following day, plates were washed, blocked and loaded with diluted samples and a monkey serum standard containing known amounts of Ig, SIV-specific IgA or SIV-specific IgG. After overnight incubation (4˚C), the plates were developed with biotinylated goat anti-monkey IgA or anti-human IgG antibody (SouthernBiotech, Birmingham, AL), and avidin-labeled peroxidase (Sigma). The SIV Env or Gag, Pol-specific IgA or IgG antibody concentrations in each secretion were normalized to total IgA or IgG concentration. The specific activity (ng IgA or IgG antibody per µg total IgA or IgG) was deemed significant if it was greater than the mean + 3SD of negative controls.

Antigen-specific T Cell Responses.

$1x10^6$ blood or tissue MNC were treated with 0.5mg anti-CD28 and anti-CD49d (BD Biosciences, San Jose, CA) in 1ml supplemented RPMI plus i) no stimulant, ii) 5ng/ml PMA /500ng/ml Ionomycin (Sigma, St. Louis, MO) iii) 10 µg SIVmac239 p27 Gag peptide pool, iv) 10 µg SIVmac239 Env peptide pool (both: AIDS Research and Reference Reagent Program), v) 25 µg purified protein derivative (Tuberculin PPD, Statens Serum Institute, Copenhagen, Denmark), vi) 10 µl BCG vaccine (10^5 CFU; Statens Serum Institute), vii) 50 µg *Mtb* H37Rv recombinant culture filtrate protein-10 reference standard (rCFP), viii) 50 µg *Mtb* H37Rv whole strain lysate, or ix) 25 µg Antigen 85b reference standard (Ag85b; all from BEI Resources Manassas, VA). Cells were cultured for 6 hrs (37°C, 5% CO_2) with 1x Brefeldin A (eBioscience, San Diego, CA) treatment after the first hour. Cells were stained using a fixable live/dead discriminator (Invitrogen) and antibodies against rhesus macaque CD3, CD4, IL-2, TNF-α, IFN-γ, (BD Biosciences, San Jose, CA) and IL-17A (eBioscience). When cell counts permitted, unstimulated MNC suspensions were stained for markers of T cell memory (CD45RA, CCR7) and mucosal homing (CD103). All panels included fluorescence-minus-one controls. Paraformaldehyde-fixed samples were acquired on a LSRII instrument and analyzed using FlowJo software with Boolean gating (TreeStar, Ashland, OR). Antigen-specific T cell responses were corrected for background responses in unstimulated controls and are reported as percentage of $CD4^+$ or $CD8^+$ T cells.

Statistical Analysis.

Data were analyzed using Prism (GraphPad, Inc., La Jolla, CA). Correlations between IgA in various compartments (plasma, saliva, intestine) were analyzed using the Spearman

rank test to determine the effect of antibody transudation versus mucosal antibody production. Frequencies of CD103 positive T cell populations were compared by applying a two-tailed Student's t-test with Welch's correction. P values ≤0.05 were considered significant.

RESULTS

TB-specific immunity after a single prime with attenuated *Mtb*

We have previously shown that $mc^2$6435 induces plasma IgG antibodies to the mycobacterial antigen PSTS-1, albeit at significantly lower levels than the more replication-competent strain $mc^2$6020 (152). In contrast, independent of replication capacity, the magnitude and persistence of *Mtb*-specific peripheral blood T cell responses were similar in $mc^2$6020 and $mc^2$6435 vaccinated infant macaques (Figure 3.1A/B, Table 3.1). *Mtb*-specific T cell responses consisted primarily of cells producing IL-2, IFN-g, TNF-α or IL-17 (Figure 3.1C, Table 3.1), but dual and triple polyfunctional T cells were detected at multiple time points (Figure 3.1D). Although only PPD and Ag85b-specific responses were consistently evaluated due to limited infant blood volumes, $mc^2$6435 vaccinated animals also developed rCFP10-specific $CD4^+$ and $CD8^+$ T cell responses, indicating that $mc^2$6435 elicited responses to human immunodominant epitopes that are absent in BCG (Figure 3.1D). Broad polyfunctional *Mtb*-specific T cell responses were also elicited in multiple tissues (Figure 3.1E). Consistent with the oral route of immunization, *Mtb*-specific T cell responses were induced in tonsils and LN draining the oropharynx (Table 3.1), with the highest *Mtb*-specific T cell responses developing in ileum and colon (Table 3.1).

SIV-specific T cell immune responses in peripheral blood and tissues

A single oral immunization with $mc^2$6435 was sufficient to induce SIV Gag-specific T cell responses by 3 weeks of age (Figure 3.2A), prior to MVA-SIV boosting. SIV Gag-specific T cell frequencies did not increase after MVA-SIV boosts (Figure 3.2B), but dual and triple cytokine-producing T cells increased in frequency and diversity (Figure 3.2C), suggesting improved quality of SIV Gag responses. In addition, SIV–specific T cell immunity increased in breadth through induction of Env-specific T cells. SIV Gag and SIV Env-specific T cell responses were observed at multiple time points indicative of induction of SIV-specific T cell memory in blood and tissues (Table 3.2).

Consistent with oral priming, frequencies of CD103 positive central memory ($CD3^+CD4^+CD45RA^-CCR7^+$, T_{CM}) and effector/effector memory ($CD3^+CD4^+CD45RA^{+/-}CCR7^-$, $T_{E/EM}$) $CD4^+$ T cell populations were higher in multiple tissues of vaccinated animals compared to mock animals, including the ileum ($CD4^+T_{E/EM}$: p=0.0326), colon ($CD4^+T_{E/EM}$: p=0.0032), and the retropharyngeal LN ($CD4^+T_{CM}$: p=0.0239) draining the oropharynx (Figure 3.3A). Similarly, a higher percentage of ileal $CD8^+$ T_{CM} expressed CD103 compared to mock animals (p=0.0028). The biological meaning of decreased $CD103^+CD8^+T_{E/EM}$ cells in axillary LN is questionable because CD103 is a mucosal homing marker, and $CD103^+CD8^+T_{E/EM}$ frequencies were similar in all other tissues (Figure 3.3A). Due to limited cell numbers, memory and homing markers were only evaluated on total $CD4^+$ and $CD8^+$ T cell populations. However, tissues with higher $CD103^+$ T cell frequencies generally also had higher numbers of SIV-specific T cells. Thus, SIV-specific T cells were most pronounced in intestinal tissues (Figure 3.3B), but also detectable in at least one other tissue of each vaccinated animal (Figure 3.3C). Responses were directed to SIV Gag and SIV Env and

consisted of single and polyfunctional T cells with $CD8^+$T cells being induced at higher frequencies and in more tissues than SIV-specific $CD4^+$T cells (Figure 3.3C).

Induction of systemic and mucosal SIV-specific antibodies

In contrast to SIV-specific T cell responses, anti-SIV Gag, Pol plasma IgG antibodies were rarely detected prior to boosting, but ultimately developed in all animals after the MVA-SIV boosts. Anti-SIV Gag, Pol- and anti-SIV Env-specific plasma IgG responses peaked at week 8 and were maintained at high levels (Figure 3.4A/B). Plasma SIV Env specific IgA antibodies also peaked by 8 weeks, but subsequently declined, while SIV Gag-specific IgA antibodies showed a more variable pattern (Figure 3.4A/B).

Considering the potential importance of mucosal antibodies in preventing MTCT of HIV by breastfeeding, we measured anti-SIV Gag, Pol and anti-SIV Env-specific IgA in mucosal compartments of vaccinated animals. Even though SIV Gag was a component of both the $mc^2$6435 prime and the MVA-SIV boosts, anti-SIV Gag, Pol-specific IgA was present in saliva of only 1 of 8 vaccinated animals, whereas anti-SIV Env-specific salivary IgA antibodies developed in 4 vaccinees (Figure 3.4C). At euthanasia, we detected anti-SIV Env- or anti-SIV Gag, Pol-specific IgA and IgG intestinal antibodies in 4 of 8 and 6 of 8 animals, respectively. One of 8 animals (#41690) failed to develop SIV-specific intestinal IgG or IgA antibodies (Figure 3.4D), despite high plasma antibodies to SIV Env. In fact, plasma anti-Env IgA levels did not correlate with those in stool ($p = 0.9768$; $R = -0.0238$) or in saliva ($p = 0.3333$; $R= -1.000$), suggesting that anti-Env-specific IgA in the secretions was derived from local mucosal synthesis rather than passive serum transudation (data not shown).

DISCUSSION

Protective immunity against TB is thought to be primarily mediated by T cells (2, 80, 399), while prevention of HIV infection requires broadly-neutralizing antibodies (11) and T cell immunity to control early viral replication should HIV infection occur (117, 324). Towards the long-term goal of developing a pediatric HIV-TB vaccine, the main study objective was to provide proof of concept that a highly attenuated *Mtb* strain expressing SIV antigens could induce both SIV and *Mtb*–specific immune responses in infant macaques. To prevent TB and HIV infections, vaccine-induced memory responses need to be elicited and persist at sites of pathogen entry and replication, i.e. the lung TB, and the oropharynx and intestinal tract, respectively. Thus, a secondary goal was to prove that the oral $mc^2$6435 prime/ IM MVA-SIV boost regimen could effectively induce persistent mucosal and systemic dual immune responses.

Indeed, our results show that a single oral immunization with $mc^2$6435 is sufficient to induce broad and persistent *Mtb and SIV*-specific T cell responses in blood and tissues. *Mtb*-specific T cell responses were elicited to multiple TB antigens, including CFP, a known immunodominant antigen in humans that is absent in BCG (73). Although we did not include a BCG control group in this first study, evidence from mouse studies shows that strain $mc^2$6435 is as immunogenic as BCG (manuscript in preparation, Ranganathan et al.). Furthermore, PPD-specific $CD4^+$ and $CD8^+$T cell frequencies in infant macaques after a single oral $mc^2$6435 immunization are comparable to PPD-specific ELISPOT responses (3,000-5000 SPU/10^6 PBMC or 0.3-0.5% PBMC) elicited by an auxotrophic recombinant BCG-SIV vaccine and by standard BCG in adult macaques (4). Some $mc^2$6435-vaccinated animals had predominantly IL-17A-producing *Mtb*-specific T cells in tissues. The role of IL-17 in TB is still controversial, but some studies suggest a protective role of Th17 cells in the

lung (107, 172, 206, 262, 399). The current study lacked the analysis of *Mtb*-specific lung responses, but future studies will thoroughly assess the quantity, quality and breadth of the lung responses to determine the potential of $mc^2$6435 vaccination to protect against TB infection.

SIV-specific tissue T cell responses were measured in sites draining the oropharynx, in the intestinal tract as an important site for vaccine-induced SIV immunity to prevent $CD4^+$ T cell depletion, and the axillary LN as representative distal site following oral priming. SIV-specific T cell responses were most consistently induced in intestinal tissues and the tonsils. Interestingly, intestinal tissues and lymph nodes draining the oral cavity also contained significantly higher frequencies of $CD103^+$ memory $CD4^+$ and $CD8^+$ T cells compared to mock-immunized infants indicative of mucosal homing.

Support for the successful induction of mucosal immunity was further provided by the presence of SIV-specific IgG and IgA antibodies in intestine and mucosal IgA in saliva. Because the SIV Env immunogen was only present in the MVA-SIV boosts and because the intestinal antibodies had specificity for SIV Env and SIV Gag, Pol antigens, likely the mucosal (PO) prime and systemic (IM) boosts contributed to the development of the intestinal antibody response. The role of IgA in protection against oral SIV infection remains to be demonstrated conclusively. In the HIV Thai vaccine trial, systemic HIV Env-specific IgA antibodies correlated with an increased risk of HIV acquisition (34). However, mucosally-induced SIV Env-induced antibodies persisting at mucosal sites might be important for protection against oral HIV infection in infants. Passive immunization studies demonstrated that systemic administration of antiviral IgG, which transudates into the saliva, could confer protection against oral infection by high in vitro neutralization and/or ADCVI

activity (92, 285, 349). Experiments assessing different vaccine-induced antibody functions will be essential in future studies (91). Oral SIV challenge studies will be needed to determine the relative contribution of SIV-specific T cell responses, SIV-specific salivary and intestinal IgA, and intestinal IgG responses in preventing or controlling infection.

In conclusion, the results of the current study, in combination with our previous safety assessment of $mc^2$6435 in infant macaques, provide the basis for future development of auxotrophic *Mtb* strains candidates as feasible and safe platform for a pediatric combination HIV-TB vaccine. Further optimization of this approach will include the testing a cocktail of attenuated *Mtb* strains expressing SIV Env, Gag and Pol and boosting *Mtb*-specific responses. While an attenuated *Mtb* prime/ heterologous viral boost vaccine strategy may not be sufficient to render sterilizing immunity, this approach may reduce viral transmission rates or viral loads in infants that do become infected. Future SIV and *Mtb* challenge studies will evaluate the efficacy of this or other combination vaccines, but if successful, they could confer protection against both HIV and TB during the early period of life, and potentially through adolescence and into adulthood.

TABLES

Table 3.1. *Mtb*-specific single cytokine T cell responses.

Table 1
Mtb-Specific single cytokine T cell responses.

Tissue	Time (wks)	PPD					Ag85b				
		IL-2[a]	IFN-γ	IL-17A	TNF-α	Animal no.[b] (%)	IL-2	IFN-γ	IL-17A	TNF-α	Animal no. (%)
PBMC											
$CD4^+T$	3	0.028–0.844	0.014[c]	0.038–0.050	0.016	6/6 (100)	NT[d]	NT	NT	NT	
	6	0.014–0.022	0.013	NS	0.021	6/7 (86)	0.168–16.09	NS[e]	NS	NS	2/2 (100)
	8	0.012–0.084	0.010–0.012	0.039	0.012–0.013	6/8 (75)	0.158–0.239	0.023–0.051	0.037–0.046	1.440–2.220	5/5 (100)
	16–18	0.017–0.130	NS	0.061–0.148	0.013	3/8 (38)	0.148–0.373	NS	0.038–0.191	NS	5/8 (63)
$CD8^+T$	3	0.238–0.911	0.033–0.296	NS	0.047–0.307	6/6 (100)	NT	NT	NT	NT	
	6	NS	0.038–0.139	NS	0.041–0.204	4/7 (57)	0.163–15.19	0.493	NS	NS	2/2 (100)
	8	NS	0.013–0.066	NS	0.048–0.053	6/8 (75)	0.130–0.386	0.121–0.603	0.046–0.137	0.399–1.146	5/5 (100)
	16–18	NS	0.025–0.074	0.141	0.053–0.123	5/8 (63)	0.152–0.212	0.043–0.208	0.093	0.148	3/8 (38)
Tonsil											
$CD4^+T$	16–18	0.011–0.033	NS	0.158	0.013	3/8 (38)	NT	NT	NT	NT	
$CD8^+T$	16–18	0.034–0.131	NS	NS	0.088	3/8 (38)	NT	NT	NT	NT	
Subm. LN											
$CD4^+T$	16–18	0.085–0.968	NS	0.066–0.081	4.258	4/8 (50)	0.041	0.011	NS	0.022	3/7 (43)
$CD8^+T$	16–18	0.440–0.812	NS	0.136	0.040–0.677	4/8 (50)	NS	NS	NS	NS	0/7 (0)
Ileum											
$CD4^+T$	16–18	0.043–0.077	0.039–0.044	0.275–0.800	0.057–0.166	4/6 (67)	0.023–0.062	NS	0.597	0.037–0.633	6/7 (86)
$CD8^+T$	16–18	0.294	0.171	0.305	0.080–1.068	4/6 (67)	0.014–0.097	0.020–0.095	0.239–0.298	0.454–1.328	6/7 (86)
Colon											
$CD4^+T$	16–18	0.068–0.363	0.020–0.450	0.182–0.320	0.055–0.166	6/8 (75)	0.066–0.591	0.019–0.600	NS	0.025–0.156	7/8 (88)
$CD8^+T$	16–18	0.535	1.360	NS	0.055–0.212	4/8 (50)	0.187–0.525	0.023–1.550	0.204–0.360	0.180–0.259	7/8 (88)
Mes. LN											
$CD4^+T$	16–18	NS	NS	0.048	0.011–0.166	4/8 (50)	NS	0.067	NS	NS	1/8 (13)
$CD8^+T$	16–18	NS	NS	0.018	0.024–0.097	6/8 (75)	0.021–0.029	0.033	NS	0.125	7/8 (88)
Ax. LN											
$CD4^+T$	16–18	0.040	NS	NS	0.012	2/8 (25)	0.025	NS	NS	NS	1/8 (13)
$CD8^+T$	16–18	NS	0.049	NS	0.068–0.138	2/8 (25)	NS	NS	0.014	0.036–0.080	5/8 (63)

[a] Shown are the ranges of the percentage of cytokine positive $CD4^+$ or $CD8^+$ T cells.
[b] Only a single animal had a positive response.
[c] NT = not tested.
[d] NS = not significant; responses were not different from media only controls.
[e] Depending on cell numbers, not all animals were tested at all time points for antigen-specific responses. Shown are the number of responding animals/total animals tested. In parentheses we report the percentages of animals with positive responses.

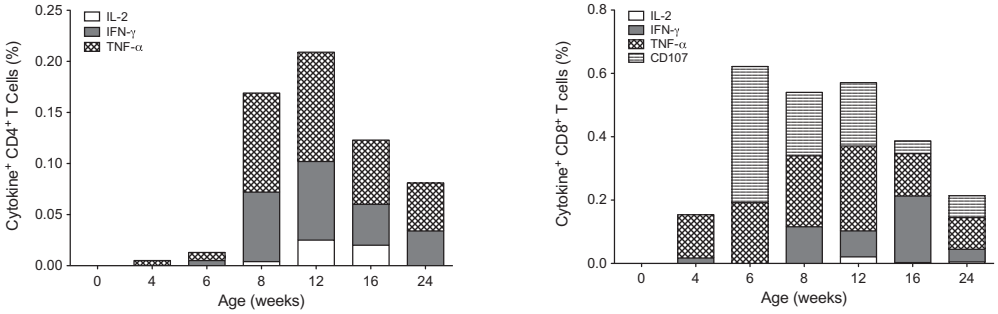
Table 3.2. SIV-specific single cytokine T cell responses

Table 2
SIV-specific single cytokine T cell responses.

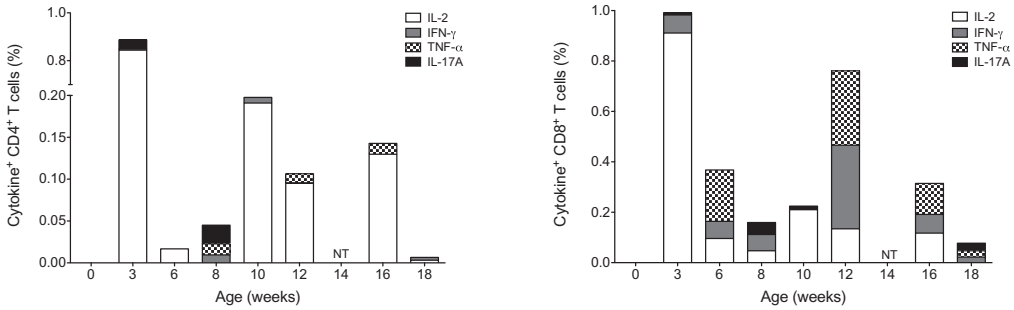
Tissue	Time (weeks)	SIV Gag					SIV Env				
		IL-2	IFN-γ	IL-17A	TNF-α	Animal no. (%)	IL-2	IFN-γ	IL-17A	TNF-α	Animal no. (%)
PBMC											
CD4$^+$T	3	0.068	0.043–0.255	0.061–0.109	0.028–0.207	7/8 (88)	NT	NT	NT	NT	
	6	0.040–0.217	NS	0.056–0.072	0.011–0.016	6/8 (75)	0.030	NS	0.081	0.023	3/4 (75)
	8	0.175	NS	0.113	NS	2/8 (25)	0.038	NS	0.051	0.014	3/6 (50)
	16–18	0.050	NS	0.062–0.136	0.010–0.015	5/8 (63)	0.016–0.096	0.014–0.025	0.045–0.202	0.013–0.019	6/8 (75)
CD8$^+$T	3	0.131–0.181	0.125–1.329	0.077–0.233	0.056–0.281	8/8 (100)	NT	NT	NT	NT	
	6	0.224	0.050–0.100	0.181	0.022–0.096	5/8 (63)	NS	0.063	0.069	0.039–0.044	3/4 (75)
	8	0.219	0.065	0.129	0.022–0.049	5/8 (63)	NS	0.012–0.174	0.041	0.030	4/6 (67)
	16–18	0.048–0.062	0.038–0.063	0.104–0.124	0.080	6/8 (75)	0.139	0.018–0.049	0.089–0.240	0.025–0.120	7/8 (88)
Tonsil											
CD4$^+$T	16–18	0.167	NS	0.196	NS	3/8 (38)	0.015–0.126	NS	0.155	0.019–0.028	5/5 (100)
CD8$^+$T	16–18	0.013–0.213	0.027–0.027	0.176–0.572	0.095–0.105	6/8 (75)	0.042–0.227	NS	0.412	0.053–0.190	5/5 (100)
Subm. LN											
CD4$^+$T	16–18	NS	NS	NS	NS	0/8 (0)	0.011–0.013	NS	NS	0.018	3/8 (38)
CD8$^+$T	16–18	0.010–0.018	0.011–0.016	NS	NS	4/8 (50)	0.035–0.048	0.012	NS	0.010–0.030	7/8 (88)
Ileum											
CD4$^+$T	16–18	0.058–0.174	0.021–0.144	0.165–0.900	0.144–0.396	6/7 (86)	0.0221–0.075	0.024–0.242	0.315–0.395	0.241–0.499	7/7 (100)
CD8$^+$T	16–18	0.023–0.294	0.011–0.077	0.426	0.026–0.878	7/7 (100)	0.064	NS	0.090–1.065	0.032–1.008	7/7 (100)
Colon											
CD4$^+$T	16–18	0.333	0.290	0.118	0.029	3/8 (38)	0.228–0.711	0.033–0.920	0.010–0.130	0.011–0.019	6/8 (75)
CD8$^+$T	16–18	0.143–0.625	0.043–1.130	NS	0.069–0.237	4/8 (50)	0.022–0.505	0.015–0.820	0.050–0.161	0.156–0.193	6/8 (75)
Mes. LN											
CD4$^+$T	16–18	NS	0.010	0.025–0.464	0.016–0.033	6/8 (75)	0.010	0.014	0.101	0.033	5/8 (63)
CD8$^+$T	16–18	0.022–0.029	NS	0.024–0.568	0.062–0.121	7/8 (88)	0.012–0.048	0.027	0.075	0.049–0.073	6/8 (75)
Ax. LN											
CD4$^+$T	16–18	NS	NS	NS	NS	0/8 (0)	NS	NS	NS	0.016	1/8 (13)
CD8$^+$T	16–18	NS	0.052	NS	0.053–0.19	5/5 (100)	0.047–0.062	NS	NS	0.073–0.083	3/8 (38)

FIGURES

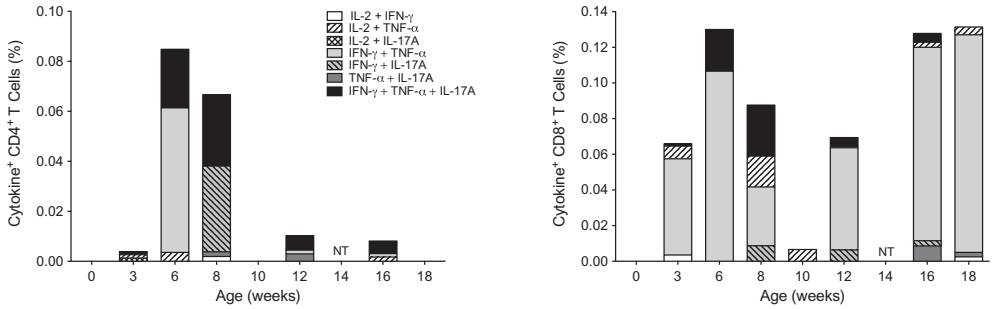
A. *M. tuberculosis*-specific Longitudinal PBMC T Cell Responses- mc^{2}6020



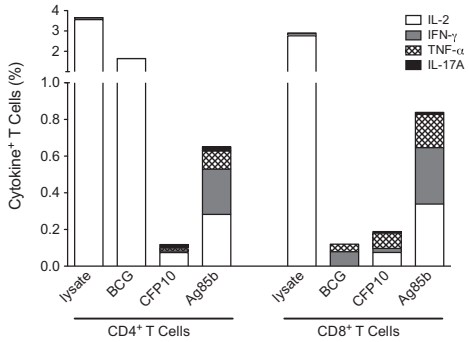
B. *M. tuberculosis*-specific Longitudinal PBMC T Cell Responses- mc^{2}6435



C. *M. tuberculosis*-specific Polyfunctional T Cells in PBMC



D. *M. tuberculosis*-specific T Cell Breadth



E. *M. tuberculosis* Polyfunctional T Cells in Tissues

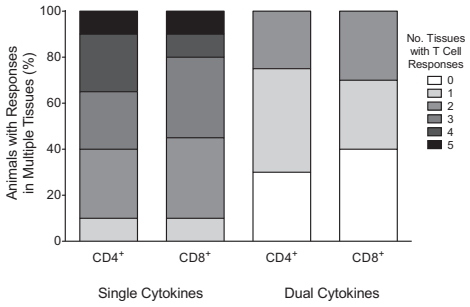
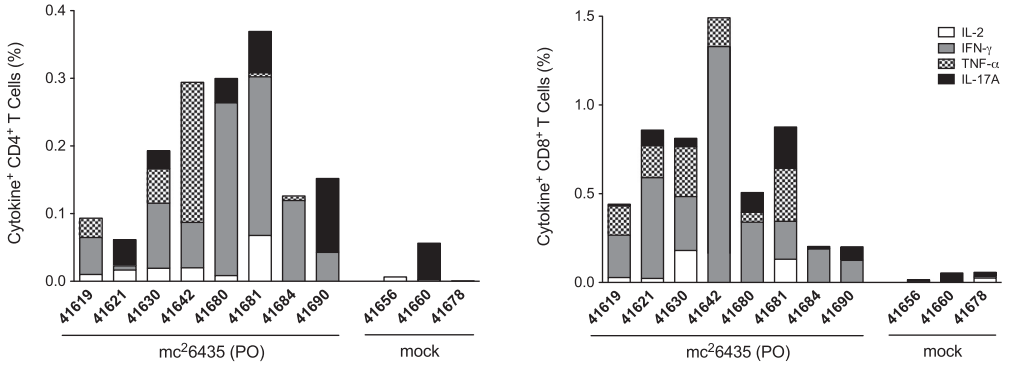
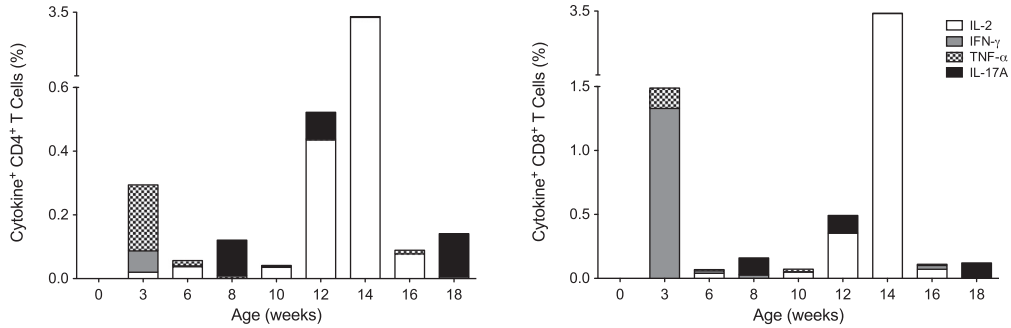


Figure 3.1. *Mtb*-specific peripheral blood T cell responses. Panels A and B: CD4+ and CD8+ T cytokine responses after in vitro BCG or PPD stimulation of longitudinally collected blood samples from a representative animal vaccinated with strain $mc^2$6020 (panel B) or with strain $mc^2$6435 (panel C), respectively. Note, only single-functional responses are shown. Panel C: polyfunctional T cell responses in a representative animal in longitudinally collected blood samples. Panel D: T cell responses are induced against a number of *Mtb*-specific antigens (PBMC data from a representative animal). Panel E: the percentage of animals with vaccine-induced single and polyfunctional PPD-specific T cell responses in tissues. T cells were isolated from selected tissues (tonsil, submandibular LN, ileum, colon and axillary LN) from all animals in group B. Using a gray scale gradient, the percentage of vaccinated animals that have PPD-specific cytokine producing T cells in all five tissues (indicated as '5') is indicated in black, whereas animals lacking PPD-specific responses in all five tissues (indicated as '0') are shown in white. Data are stratified by CD4+ and CD8+ T cells. CFP, *Mtb* culture filtrate protein; lysate, *Mtb* whole cell lysate; BCG, Bacille Calmette–Guérin vaccine strain; Ag85b, antigen 85b.

A. SIV Gag-specific PBMC T Cell Responses at Week 3



B. SIV Gag-specific Longitudinal PBMC T Cell Responses



C. SIV Gag-specific Polyfunctional T Cells in PBMC

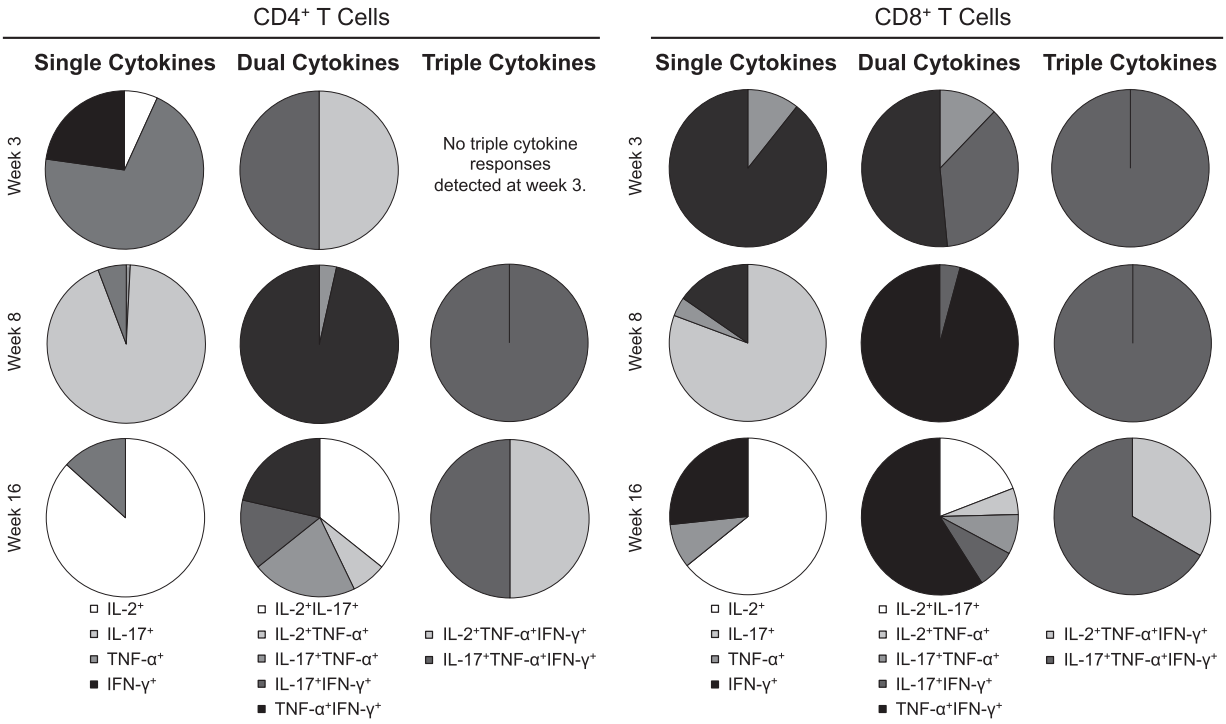
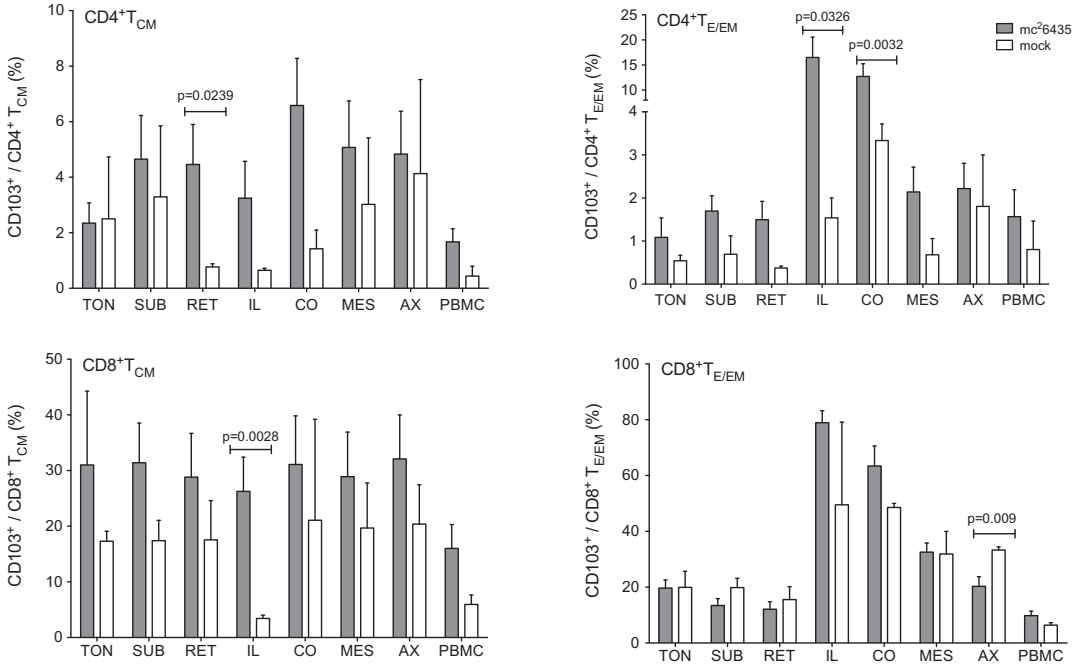
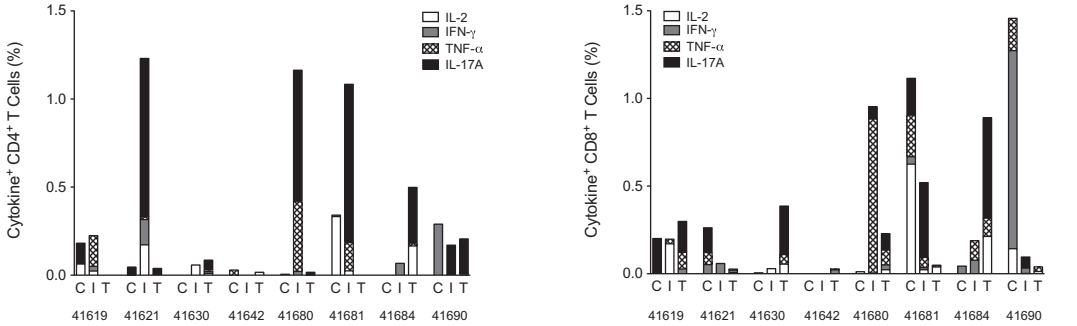


Figure 3.2. SIV-specific peripheral blood T cell responses. Panel A: Cytokine production following SIV Gag stimulation in peripheral T cells at week 3 for all $mc^2$6435-primed animals in Group B. Panel B: $CD4^+$ (left panel) and $CD8^+$ (right panel) T cell cytokine production in response to *in vitro* SIV Gag stimulation in longitudinally collected blood samples of a representative animal after vaccination. Panel C: SIV-specific single, dual and triple positive cytokine responses in peripheral blood $CD4^+$ (left panel) and $CD8^+$ (right panel) T cells at weeks 3 (post-prime, pre-boost), 8 (post-boosts) and 16. Distinct cytokines or combinations thereof are indicated by different colors in the graph legend.

A. Vaccine-enhanced Expression of CD103 by Memory and Effector T Cell Populations



B. SIV Gag-specific T Cell Responses in Mucosal Tissues



C. SIV-specific Polyfunctional T Cell Responses in Tissues

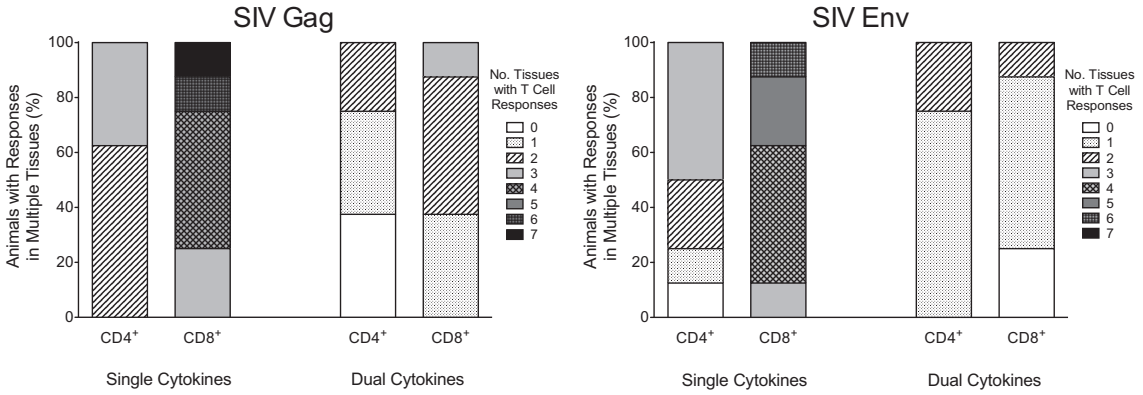
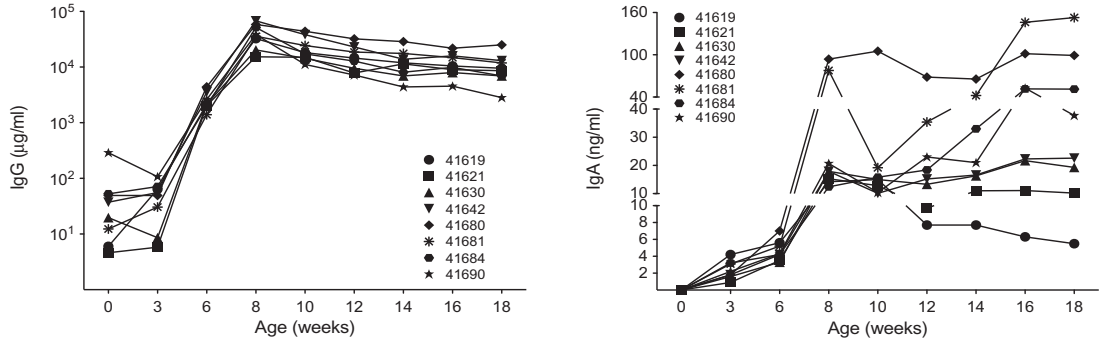
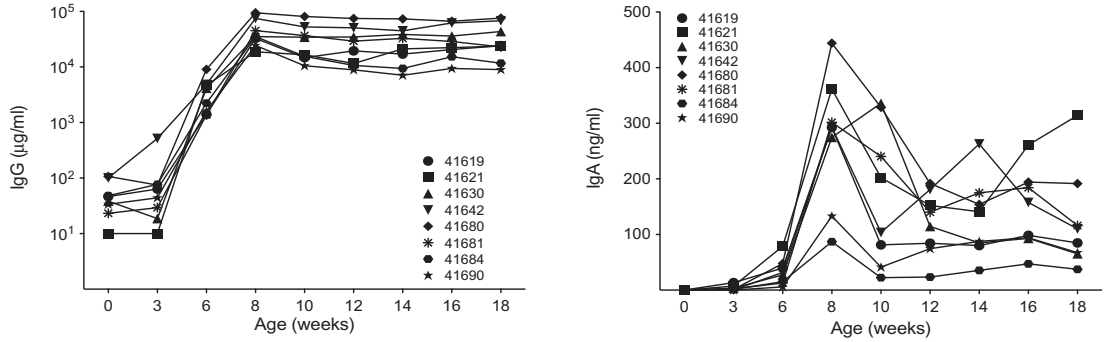


Figure 3.3. Homing and SIV-specific T cell responses in tissues. Panel A: Animals primed with strain $mc^2$6435 and boosted with MVA-SIV (gray bars) have higher percentages of memory and effector T cells expressing CD103 compared to unvaccinated animals (white bars). Memory and effector/effector memory cells were defined within the $CD4^+$ or $CD8^+$ T cell populations as $CD45RA^-CCR7^-$ (T_{CM}) or $CD45RA^{+/-}CCR7^-$ ($T_{E/EM}$), respectively, before measuring CD103 expression. Statistical significance using a one-tailed t-test was calculated using log-transformed values. Shown are mean values ± SEM. Tissues are abbreviated as follows: tonsil (TON), submandibular lymph node (SUB), retropharyngeal lymph node (RET), ileum (ILE), colon (CO), mesenteric lymph node (MES), axillary lymph node (AX), and PBMC. Panel B: $CD4^+$ (left panel) and $CD8^+$ (right panel) T cell single cytokine production in response to *in vitro* SIV Gag stimulation of tissue cell populations. Each bar represents the sum of all single cytokine responses in the relevant tissue (see legend for cytokine color coding). Shown are data for all animals that were vaccinated orally with strain $mc^2$6435 and boosted with MVA-SIV. The three bars for each animal represent from left to right: colon (C), ileum (I), and tonsil (T). Panel C: The percentage of animals with vaccine-induced single and polyfunctional T cell responses to SIV Gag (left panel) or SIV Env (right panel). T cells were isolated from tonsil, submandibular LN, retropharyngeal LN, ileum, colon, mesenteric LN, and axillary LN of all vaccinated animals. Data are stratified for single and dual cytokine producing $CD4^+$ and $CD8^*$ T cells. Using a gray scale gradient, animals with T cells from all seven tissues producing no cytokine(s) in response to SIV are indicated by '0' (white) and animals with all seven tissues producing cytokine(s) are shown as '7' (black).

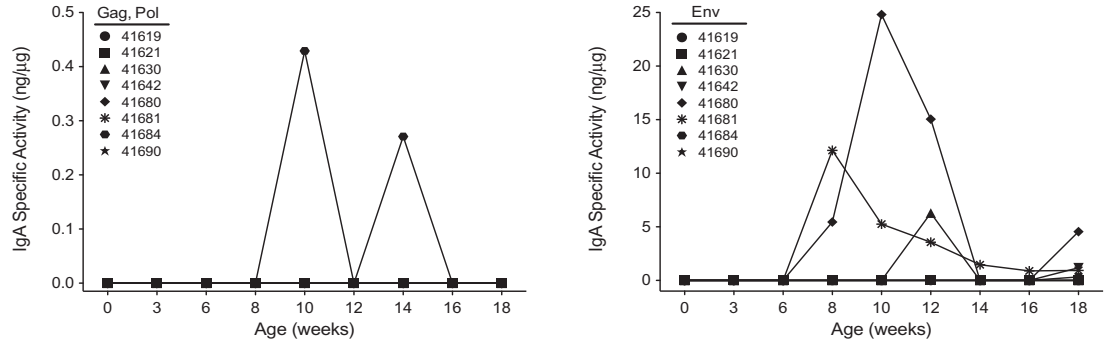
A. Anti-SIV Gag, Pol IgG and IgA (Plasma)



B. Anti-SIV Env IgG and IgA (Plasma)



C. Anti-SIV Gag, Pol and Env IgA (Salivary)



D. Anti-SIV Gag, Pol and Env IgG and IgA (Intestinal)

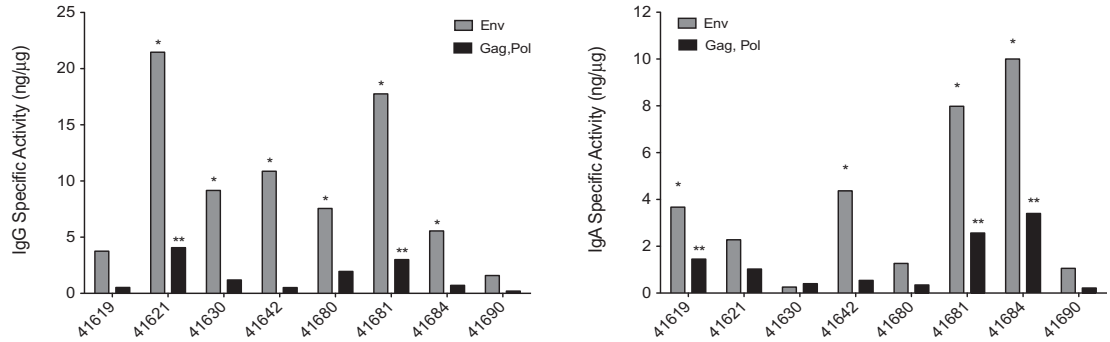


Figure 3.4. SIV-specific plasma and mucosal IgG and IgA antibodies. Panel A and Panel B: Anti-SIV Gag, Pol or anti-SIV Env IgG (left graphs) and IgA (right graphs) antibody development in plasma following oral priming with strain $mc^2$6435 plus MVA-SIV boosts. Note that IgG values are represented on a log scale and IgA values are plotted linearly. Panel C: SIV Gag, Pol (left panel) and Env (right panel) specific activity for IgA in saliva following priming with strain $mc^2$6435 plus MVA-SIV boosts. Panel D: The specific activity for intestinally-secreted anti-SIV Gag, Pol (black) and anti-SIV Env (gray) IgA (left) and IgG (right) antibodies following priming with strain $mc^2$6435 plus MVA-SIV boosts. Intestinal antibodies were only measured at the time of euthanasia. One (*) or two stars (**) above bars indicate that antibody measurements for anti-SIV Env or anti-SIV Gag, Pol, respectively, exceeded mean background levels ± 3SD.

CHAPTER 4: STRIKING A BALANCE: VACCINE-INDUCED IMMUNE ACTIVATION OUTWEIGHS BENFICIAL ANTI-SIV ENV ANTIBODY RESPONSES

OVERVIEW

Over 3.3 million infants and children are infected with HIV. Despite global initiatives to eliminate mother-to-child transmission (MTCT) of HIV by 2015, pediatric ART provision is significantly restricted compared to adults. Oral breast milk exposure to HIV accounts for up to 44% of MTCT but abstinence from breastfeeding is not recommended. We have previously shown that oral vaccination at birth with live attenuated *Mycobacterium tuberculosis* (*Mtb*) strains expressing SIV genes safely induces persistent SIV-specific cellular and humoral immune responses both systemically and at the oral and intestinal mucosa. Here we tested the ability of oral *Mtb* vaccine strains expressing SIV Env and Gag proteins, followed by systemic heterologous (MVA-SIV Env/Gag/Pol) or homologous (*Mtb*-SIV Env/Gag/Pol) boosting, to protect neonatal macaques against oral SIV challenge.

Two important results were observed. First, a subset of heterologously-boosted animals that developed higher specific activities of SIV Env-specific mucosal IgA antibodies and higher avidities of SIV Env-specific plasma IgG antibodies had significantly lower viral titers at peak viremia and were better able to control viral replication. These animals also maintained $CD4^+$ T cell populations better and exhibited higher levels of IL-17-expressing T cells in the colon compared to non-controller animals. Secondly, we observed that vaccination induced persistent immune activation. Vaccine-induced immune activation

increased frequencies of CCR5-expressing $CD4^+$ T cells in blood, oral lymph nodes and intestinal tissues, thus expanding the pool of available SIV target cells and likely enhancing the rate of infection in vaccinated animals. While vaccination is intended to increase immune activation in the immature immune environment of the infant, it will be important to elucidate whether immune activation was the mechanism of enhanced SIV infectivity.

INTRODUCTION

Pediatric HIV infections occur as a result of vertical exposures *in utero*, intrapartum or from oral exposure to virus in breast milk. In the absence of therapy, infant exposure to HIV-1 during breastfeeding causes 39-44% of mother-to-child transmission (MTCT) events (68, 111, 237, 334). Independent of the transmission route, pediatric HIV infections progress at an accelerated rate and with high mortality. Without antiretroviral therapy, fewer than 50% of HIV-infected infants will reach two years of age (237) frequently succumbing to secondary infections like TB. MTCT following oral HIV exposure in breast milk is rare in developed countries with readily available testing, antiretroviral (ART) therapy and safe, nutritional alternatives to breast milk. However, in resource-limited regions where one or more of these interventions are unavailable, there are high rates of MTCT during breastfeeding. Sub-Saharan Africa carries the greatest burden of the pediatric HIV epidemic exacerbated by limited resources with which to prevent new and treat existing infections of HIV (304, 382).

The WHO campaign to eliminate MTCT by 2015 recommends highly active antiretroviral therapy (HAART) for expectant mothers throughout pregnancy, ART to the infant from a minimum 0-6 weeks and standardized HIV testing when needed (30, 370, 384). However, in regions with high rates of pediatric HIV infection, neonates who breastfeed

during their first few months have at least six-times greater survival benefit compared to formula-fed infants (25, 76), indicating that HIV exposure in breast milk may confer fewer overall health risks compared to the nutritional and immune factor deficiencies of breastfeeding alternatives. In response to increased health and survival of breastfed infants, some countries have adopted policies that extend the advised infant ART prophylaxis period until one-week post-breastfeeding cessation to confer added protection (75). Although proven effective in controlled studies, interventions aimed at reducing oral MTCT of HIV have failed in application due to antiretroviral therapy access and infrastructure limitations (178, 252, 340, 344), allowing unnecessary vertical HIV transmission events to occur. Until significant improvements can be made to improve access to anti-HIV interventions during the pre- and antenatal periods, alternative approaches, such as pediatric HIV vaccine development, should be pursued in tandem (148, 149).

A successful vaccine to prevent MTCT of HIV by breastfeeding likely needs to be administered early after birth and with an accelerated immunization regimen. Few pediatric vaccines, however, are approved for use at birth. The live attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine to prevent tuberculosis has been demonstrated to drive the development of persistent and robust cellular immunity after administration at birth, despite the immaturity of the neonate's immune system; BCG is even used to adjuvant immunity to other vaccines administered in tandem (202, 230, 245, 321, 353). BCG vaccination though is not safe for infants that are HIV-infected, immunosuppressed or at high risk of HIV infection due to potential bacterial dissemination (126, 127). We hypothesized that vaccination with human-adapted *Mtb* containing specific deletions in genes necessary for replication, immune evasion and virulence could also induce robust and long-lived cellular

immune responses in infants and at a magnitude comparable to BCG. Further, targeted attenuations and inclusion of immunodominant epitopes such as ESAT-6 and CFP-10 should translate into a safer and more immunogenic vaccine in immunosuppressed infants. Additionally, recombinant plasmids with HIV genes can be co-expressed by attenuated *Mtb* to drive the development of dual immunogenicity specific to both HIV and *Mtb*. In fact, our previous studies in infant rhesus macaques have characterized the ability of attenuated recombinant *Mtb* strains expressing SIV antigens (*Mtb*-SIV) to safely induce persistent cellular and humoral immune responses (151).

Based on these data, the current study tested whether oral priming with recombinant, attenuated *M. tuberculosis* strains $mc^2$6208 and $mc^2$6435 expressing SIV Env and Gag proteins, respectively, followed by MVA-SIV Env/Gag/Pol or homologous systemic boosting could protect infant macaques against oral SIV exposure in breast milk. To more closely recapitulate HIV acquisition by breastfeeding in human infants, we developed and applied a repeated low-dose oral SIVmac251 challenge model. Unexpectedly, however, our results showed that vaccinated infant macaques, independent of boost regimen, had a two-fold higher risk of infection per oral SIVmac251 exposure compared to unvaccinated animals. Subsequent analysis revealed that vaccination was associated with increased systemic immune activation. Importantly, tissues indicated as potential SIV entry sites following oral exposure to virus in breast milk, namely the lymph nodes draining the oropharynx and intestinal tissues, showed elevated frequencies of CCR5-positive $CD4^+$ T cells.

Despite these intriguing data, a subset of vaccinated infants was able to partially control viral replication. At the time of when oral SIV challenges were started, these animals had the highest plasma anti-SIV Env IgG avidities and the highest mucosal anti-SIV Env IgA

specific activities in salivary and intestinal secretions. Thus, plasma IgG and mucosal IgA antibodies directed against SIV envelope appeared to be critical in the early control of SIV infection. In addition, these animals were also able to maintain intestinal IL-17-producing T cells and experienced a less severe loss of $CD4^+$ T cells in blood, mucosal and lymphatic tissues. Combined, these results suggest that our attenuated *Mtb*-SIV vaccination strategy was able to induce strong antibody response in infant macaques, but that vaccine-induced persistent immune activation appeared to promote enhanced infectivity following low-dose oral exposure to SIV.

MATERIALS AND METHODS

Animals.

Infant rhesus macaques (*Macaca mulatta*) were vaginally delivered by colony dams from the SIV-negative and type D retrovirus-free colony at the California National Primate Research Center (CNPRC, Davis, CA) and were nursery-reared. Animals were housed according to the "Guide for Care and Use of Laboratory Animals" and the standards outlined by the American Association for Accreditation of Laboratory Animal Care; all animal protocols were reviewed and approved by the University of California, Davis Institutional Animal Care and Use Committee prior to study initiation. Trained animal technicians monitored animals daily for any clinical symptoms of TB infection. Animals were randomly assigned to groups and were between 3-7 days of age at the first immunization (Table 4.1, "birth/week 0"). For all vaccinations and blood draws animals were immobilized by 10mg/kg body weight of ketamine-HCl (Parke-Davis, Morris Plains, NC), injected intramuscularly. Complete blood counts (CBC) were performed on each peripheral blood sample by the CNPRC Clinical Laboratory.

Vaccine Strains.

The vaccine strains described in this study were derived from the human-adapted *M. tuberculosis* strain H37Rv as described (151, 152, 268). Briefly, the parental strain $mc^2$6434 was engineered with *panCD* and *leuCD* locus deletions to produce a highly attenuated double auxotroph strain unable to express essential nutrients pantothenate and leucine complemented by an additional deletion at the *secA2* locus, a nonessential secretion system important for bacterial growth and host immune response restriction. Strain $mc^2$6434 was further designed to co-express full length SIVmac239 i) *gag* ($mc^2$6435), ii) *env* ($mc^2$6208) or iii) *pol* ($mc^2$6440) multimer cassette that was codon-optimized for expression by mycobacteria. SIV protein expression was confirmed in vaccine cultures by immunoblot using a V5 antibody-HRP (data not shown). Furthermore, we demonstrated recently that strain $mc^2$6435, despite its low replication capacity, could safely induce persistent *M. tuberculosis*- and SIV-specific T cell and antibody responses in neonatal macaques following oral vaccination at birth (151, 152).

Immunizations.

The vaccination regimen is outlined in Table 4.1. All study animals were evaluated in parallel with Group A age-matched mock-vaccinated control animals (n=6). Infant macaques in Group B were orally (p.o.) vaccinated with 10^9 colony forming units (CFU) each of strains $mc^2$6435 and $mc^2$6208 at birth and boosted intradermally (i.d.) at week 3 with a trivalent cocktail of 10^7 CFU each of strains $mc^2$6435, $mc^2$6208 and $mc^2$6440 (n=6). A second vaccine group, Group C (n=8), was also p.o. immunized with 10^9 CFU each of strains

$mc^2$6435 and $mc^2$6208 at birth before receiving systemic boosts intramuscularly (i.m., divided over 4 injection sites) at weeks 3 and 6 with recombinant modified vaccinia virus Ankara expressing SIVmac239 Gag, Pol (MVA vJH4) and MVA-SIV Env (MVA-SIV; each at 10^8 infectious units). MVA-SIV was kindly provided Dr. B. Moss and Dr. P. Earl (NIAID, NIH, Bethesda, MD) (79, 348, 386).

SIV Challenge.

At week 9, weekly oral, low-dose exposures to virulent SIVmac251 were administered under anesthesia to all animals. Each dose contained $5x10^3$ $TCID_{50}$ (6/04 stock grown in rhesus macaque PBMC; previously described (208)) in a volume of 1ml. Animals were challenged weekly until SIV viral RNA levels in plasma indicated persistent infection for a minimum of 3 consecutive timepoints (Table 4.1). Three animals- #42380 (Group A), #42918 (Group B) and #42958 (Group C)- failed to become infected following 10 low-dose exposures. Beginning with the 11th exposure, these three animals received $2x10^4$ $TCID_{50}$ SIVmac251 orally, a dose that resulted in persistent viremia in all three animals. All animals were followed for a minimum of 10 weeks post-SIV infection but were euthanized when they met criteria established for retrovirus-infected animals (352).

SIV RNA Analysis.

Longitudinal plasma samples were used for virological analysis by reverse transcription polymerase chain reaction (RT-PCR) for SIV RNA as described (54) with a limit of detection of about 30 copies/ml. Data are reported as SIV RNA copy equivalents per ml of plasma.

Sample Collection and Processing.

EDTA-anticoagulated blood, saliva and stool were longitudinally collected prior to all interventions throughout the study period. Plasma was isolated from whole blood by centrifugation and stored in multiple small aliquots at -80°C. Saliva was collected using Weck-Cel sponges (Windsor Biomedical, Newton, NH), snap frozen immediately after collection, and later eluted from sponges as described (24). When collectable, 2-3 grams of fresh stool were added to 0.5% BSA in 5ml sterile PBS supplemented with 1/100 protease inhibitor cocktail (Sigma, P8340) and snap-frozen. Fecal extracts were later prepared by vortexing the thawed contents until complete homogenization, debris was removed by ultracentrifugation and filtration and the extracts were concentrated to roughly 0.5ml using an Amicon Ultra-4 50K centrifugal filter unit (Millipore). At the terminal timepoint, bronchoalveolar lavages (BAL) were collected. Recovered BAL fluid was spun to pellet cells; cells were preserved in 10% DMSO in FBS and stored in liquid nitrogen while supernatant was aliquoted and stored separately at -80°C. In addition, cervicovaginal fluid (CVF) was collected from female animals at necropsy using Weck-Cel sponges and snap frozen at -80°C.

At the time of euthanasia, multiple tissues, including tonsil, lymph nodes (LN: axillary, mesenteric, retropharyngeal and submandibular), spleen, intestinal tissues (colon and ileum) and oral mucosal tissues (sublingual mucosa and gingiva) were collected. Tissues were divided into multiple aliquots and i) formalin-fixed/ paraffin-embedded, ii) preserved in RNAlater or iii) placed in complete media for fresh tissue analysis as previously described (208). Single cell suspensions for cellular immunogenicity assays were prepared using

gradient centrifugation (whole blood, spleen), gentle homogenization (lymph nodes, tonsil) or collagenase digestion and gradient centrifugation (mucosal tissues) as described previously (208).

SIV-specific Antibody Measurements.

ELISA was used as previously described (208) to measure anti-SIV IgG and IgA antibodies in plasma, saliva and fecal extracts, as well as BAL fluid and CVF secretions from the terminal timepoint. Briefly, plates were coated overnight with 100ng per well of SIV Env rgp120mac251 (Immune Technology, New York, NY) or 100µl per well of 1/400 SIVmac239 viral lysate (Advanced Biotechnologies, Columbia, MD) in PBS. Antibodies against the lysate are termed Gag, Pol-specific because the lysate lacked detectable Env protein at the dilution used. Total IgA or IgG in extracts was similarly measured by ELISA (24, 152) using goat anti-monkey IgA (AlphaDiagnostics, San Antonio, TX) or goat anti-monkey IgG (MP Biomedicals), respectively, to capture Ig. The following day, plates were washed, blocked and loaded with diluted samples and a monkey serum standard containing known amounts of Ig, SIV-specific IgA or IgG. After overnight reaction at 4˚C, the plates were developed with biotinylated goat anti-monkey IgA (AlphaDiagnostics) or anti-human IgG antibody (Southern Biotech, Birmingham, AL), avidin-labeled peroxidase (Sigma), TMB and sulfuric acid stop solution (Sigma). The SIV Env or Gag, Pol-specific IgA or IgG antibody concentration measured in each secretion was normalized in accordance with the total IgA or IgG concentration. The specific activity (ng IgA or IgG antibody per µg total IgA or IgG) was deemed significant if it was greater than the mean + 3SD of negative

controls. Anti-SIV Env IgA values for assayed mucosal secretions and plasma at select timepoints (Table 4.2).

Antigen-specific T Cell Responses.

1-2x10^6 mononuclear cells (MNCs) from blood or tissue single cell suspensions were added to round-bottom tubes and treated with 0.5 μg purified anti-CD28 and anti-CD49d (BD Biosciences, San Jose, CA) in 1ml supplemented RPMI plus 1) no stimulant, 2) PMA (5 ng/ml)/Ionomycin (500 ng/ml) (Sigma, St. Louis, MO) 3) 10 μg SIVmac239 p27 Gag peptide pool (peptides 5243-5299, AIDS Research and Reference Reagent Program #6204), 4) 10 μg SIVmac239 Env peptide pool (peptides 6708-6719, AIDS Research and Reference Reagent Program #6883), 5) 25 μg purified protein derivative (PPD) (Tuberculin PPD, Statens Serum Institute, Copenhagen, Denmark) or 6) 25 μg recombinant Antigen 85b protein reference standard (Ag85b) (NR-14870, NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH). Appropriate antigen concentrations were determined by prior titration experiments using known antigen-reactive donor cells. Due to small infant sample volumes and corresponding low cell counts, priority for SIV- and *M. tuberculosis*-specific T cell responses was given to SIV Gag and to PPD antigens, respectively.

Stimulated cells were incubated at 37°C and 5% CO_2 for one hour, then treated with 1x Brefeldin A (eBioscience, San Diego, CA) and incubated for an additional five hours. Cells were then washed and stained using a fixable live/dead discriminator (Invitrogen) and antibodies against rhesus macaque CD3, CD4, CD45RA, CCR7 IL-2, TNF-α, IFN-γ, granzyme B (all BD Biosciences, San Jose, CA) and IL-17A (eBioscience). Cells were fixed in 1% paraformaldehyde and stored in the dark at 4°C until flow cytometric analysis. When

cell counts permitted, unstimulated MNC suspensions were stained with antibodies against markers for T cell activation (CD69), T cell exhaustion (PD-1) and intestinal/mucosal homing (CD103). In a retrospective analysis, expression of activation markers was measured on cryopreserved PBMC and tissue cell suspensions from historical animals (151, 152). Cells were quickly thawed at 37°C, immediately washed 2x in 37°C AIM-V media supplemented only with antibiotics and allowed to rest overnight at 37°C/5% CO_2. Cells were rewashed the next day prior to staining for CCR5, CD69 and Ki-67 (all BD Biosciences). All staining panels were evaluated using fluorescence-minus-one (FMO) controls and assays were run in parallel with isotype-specific controls when cell counts permitted. Samples were acquired on a LSRII instrument and analyzed using FlowJo software with Boolean gating (TreeStar, Ashland, OR). Antigen-specific T cell responses are represented as the percentage of positive cells within the $CD4^+$ or $CD8^+$ T cell populations after correction for background responses in unstimulated controls.

Histopathology and Immunohistochemistry.

Formalin-fixed, paraffin-embedded tissues were cut into 5µm serial sections and H&E stained to evaluate SIV-induced pathology by a nonhuman primate pathologist blinded with respect to treatment groups. A tissue disease score scale was used to measure disease severity: 0- no indications of SIV-associated pathology, 1- mild disease, multifocal, 2- mild disease, diffuse, 3- moderate disease, multifocal, 4- moderate disease, diffuse, 5- severe disease with mild T cell depletion and 6- severe disease with massive T cell depletion. Similarly, tissues were evaluated in a blinded manner for TB-indicative pathologies.

In addition, colon sections were analyzed by immunohistochemistry to quantitate the

frequencies of CD3 (BD Biosciences) and IL-17 (eBioscience) single- and double-positive T cells. Sections were treated with Target Retrieval Solution pH 6.1 (Dako, Carpinteria, CA), blocked using Background Snipper (Biocare Medical, Concord, CA) and incubated with either rabbit anti-CD3 (1:100, Thermo Scientific, Rockford, IL) or mouse anti-IL-17 (1:50, eBioscience) antibodies at 4°C overnight. Sections were stained using secondary fluorescent antibodies (goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 594 at 1:200, Invitrogen, Grand Island, NY), counterstained with DAPI and coverslipped with Prolong Gold Antifade (Invitrogen) before imaging with a Leica DMI6000B inverted microscope equipped with rotating excitation filter wheel, photometrics HQ2 camera and high performance imaging software (Simple PCI, Hamamatsu Corporation, Sewickley, PA). Image processing and analysis was carried out using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) and Image J program (Wayne Rasband, NIH).

***M. tuberculosis* Isolation.**

Multiple tissues were snap frozen and stored at -80°C until shipment to the National Animal Disease Center (USDA-ARS, Ames, IA) to recover viable mycobacteria using three different culture methods: i) the Fast Indicator Tube test (MGIT), ii) Middlebrook 7H12 media (BacTec), and iii) Solid Culture media to determine colony forming units (CFU). Because vaccine strains are auxotrophic, pantothenate and leucine supplemented media was used to ensure maximal bacterial recovery. A tissue was considered positive if one of three culture methods yielded mycobacterial growth. *M. tuberculosis* positive control samples were run in parallel for quality assurance. The analysis was performed in a blinded manner.

MHC Class I Allelic Typing.

Snap frozen splenic cell pellets were typed for common MHC Class I variants *Mamu-A*01, A*02, A*08, A*11, B*01, B*03, B*04, B*08, B*17*, chosen for their possible roles in restricting SIV infection and/or control of viremia (Table 4.1). Genotyping was completed by the MHC Genotyping Service at the University of Miami Miller School of Medicine and the established methods have been previously reported(104, 156, 195, 390).

Statistical Analysis.

Data were analyzed using GraphPad Prism and InStat software (GraphPad Software, Inc., La Jolla, CA) with assistance provided by the UNC CFAR Biostatistics Core. The probabilities of infection per SIV exposure for mock or vaccinated animals were used to calculate the relative risk of infection per exposure and data were plotted using a Kaplan Meier plot and analyzed by logrank test. Nonparametric one-way ANOVA (Kruskal-Wallis test) was used to compare three or more groups and nonparametric, two-tailed Mann-Whitney tests using 95% confidence intervals were used to compare data between only two groups. Whisker plots always represent 10-90% confidence with the central line at the sample mean. Error bars indicate the standard error of the mean (SEM) except in the weight plot in which error bars indicate standard deviation (SD). Correlations between IgA in various compartments (plasma, saliva, intestine) were analyzed to determine the effect of antibody transudation versus mucosal antibody production. P values ≤ 0.05 were considered statistically significant.

RESULTS

Vaccine efficacy following repeated low-dose oral SIV challenge

Previous challenge studies have exposed animals to relatively high doses of SIV to ensure infection but such dosing may not recapitulate the lower viral loads found in breast milk. Although HIV viral loads in human breast milk are highly variable by individual and can fluctuate considerably throughout the breastfeeding period, the establishment of a low-dose challenge model was important to more closely mimic HIV transmission in human infants by breast milk (388). In the current study, $5x10^3$ $TCID_{50}$ $SIVmac_{251}$ was administered once weekly by the oral route to establish a repeated low-dose oral SIV infection model in infant rhesus macaques.

Among the mock-vaccinated animals (Group A), two infants became infected following a single exposure and one animal each became infected after two, five, and seven exposures (Table 4.1 and Figure 4.1A). The animal (#42409) that became persistently infected after 7 low-dose oral challenges experienced transient plasma viremia at two consecutive time points with plasma SIV RNA levels of 550 and 380 copies/ml, followed by undetectable viral RNA at the subsequent time point. To ensure the validity of this finding, a second plasma sample from the same time point was tested and confirmed to be SIV RNA negative by PCR. Peak viremia in mock-vaccinated animals ranged from $7.3x10^6$ - $3.4x10^8$ viral RNA copies/ml plasma and viral replication was maintained at high levels in all animals (Figure 4.1A). One mock-vaccinated animal (#42380) was SIV-negative by PCR after 10 low-dose challenges (Table 4.1). Beginning at week 11, subsequent challenge of this infant with a higher dose of $SIVmac_{251}$ ($2x10^4$ $TCID_{50}$) resulted in detectable plasma viremia (Figure 4.1A). Similarly, one animal each in Group B and Group C remained uninfected after

10 low-dose oral SIV exposures; these animals also became infected after the high dose challenge (Table 4.1; Figure 4.1B-C).

Although statistically not significant, vaccinated animals in Group B and Group C appeared to require fewer low-dose oral SIV exposures for persistent infection (Figure). In fact, four of the six Group B animals became infected after a single exposure and one additional animal after two exposures. Similarly, five and two of eight Group C animals required only one or two low-dose SIV challenges, respectively, to become systemically infected (Figure 4.1B-C). Only 50% of Group A animals, but 83% (Group B) and 88% (Group C) of vaccinated animals required ≤2 challenges for transmission. Using the number of challenges required for SIV transmission (Table 4.1), the probability of SIV infection per exposure was 0.23 for Group A animals compared to 0.45 in the combined vaccinated animals of Group B and Group C, resulting in a transmission risk per exposure of 1.96 in vaccinated compared to unvaccinated infant macaques. Thus, with the caveat of small group sizes, vaccination nearly doubled the risk of infection with each challenge dose compared to the unvaccinated animals (Figure 4.1D). No difference in SIV transmission efficiency was observed between Group B and C animals, suggesting that the vaccine prime, and not the vaccine boost, was primarily associated with the observed enhanced infectivity.

Once infected, the peak viremia in animals primed and homologously boosted with attenuated *M. tuberculosis*-SIV (Group B) was comparable to mock-vaccinated animals ($2.4x10^6$ - $1.4x10^8$) (Figure 4.1A-B). In Group C animals that were heterologously boosted with MVA-SIV, two distinct patterns of plasma viremia were observed (Group C) (Figure 4.1C). While five animals had peak viremia ($1.6x10^7$ – $4.3x10^7$) that were indistinguishable from Group A and Group B animals, three animals (#42924, #42944 and #42958) had lower

peak viremia ($6.5x10^5$ - $7.8x10^6$ copies/ml) and continued to show reduced or controlled levels of virus in plasma. A similar trend was observed in the one Group B animal (#42918, peak viremia $2.4x10^6$) that required a high dose oral SIV challenge to establish infection. The differences in virus levels at peak viremia and at viral set point (week 20) were significant between the 'controller' and 'non-controller' Group C animals with p= 0.0154 and p= 0.0357, respectively (Figure 4C).

Thus, overall, 16 of the 20 animals in Groups A, B and C maintained high plasma SIV levels throughout the study, as is typically observed for SIV-infected infant macaques. To determine whether partial control of SIV replication could be maintained in the four vaccinated animals with reduced peak viremia, we followed these animals for 34 weeks. Although viremia was consistently reduced until week eight post-infection, animal #42958 lost control of viral replication after eight weeks and plasma viremia began to increase (Figure 4.1C). Two animals, #42944 and #42918, maintained reduced viremia and plasma viremia steadily decreased in animal #42924 (Figure 4.1B-C). Therefore, the attenuated *M. tuberculosis*-SIV vaccination and boost regimen may have provided some benefit resulting in reduced SIV replication in a subset of animals.

Resistance to low-dose SIV challenge was not associated with protective MHC alleles (Table 4.1). Because animals were assigned to treatment groups within three days of birth, MHC allele genotypes could not be evaluated prior to group assignment. However, retrospective MHC genotyping revealed no bias towards protective MHC alleles in animals with partial control of SIV replication or in animals requiring high-dose challenges for infection. For example, animals #42376 and #42380 (Group A) both expressed the 'protective' variants A*01 and B*17, but animal #42376 became infected following a single

SIV dose while animal #42380 remained uninfected after 10 low-dose challenges and required a high dose to become infected. The Group B animal #42918 that required a high-dose SIV challenge expressed the protective A*01 allele but also the non-protective B*01 allele. Trim5α genotyping was not performed because a recent study demonstrated that mucosal SIVmac251 challenges are not influenced by the Trim5α alleles (86).

Clinical markers of disease progression and vaccine safety

The four vaccinated infant macaques that were able to partially control viremia (termed 'controllers'), animals #42918 (Group B), #42924, #42944 and #42958 (all Group C), showed normal weight gain and did not experience significant $CD4^+$ T cell loss in peripheral blood (Figure 4.2A). In contrast, animals unable to suppress viral replication plateaued or even lost weight with time (p=0.0017 by one-way ANOVA, Figure 4.2A) and showed $CD4^+$ T cell loss in peripheral blood and in multiple tissues, shown longitudinally in PBMC and for mesenteric LN and submandibular LN (Figure 4.2B-D). This difference in peripheral blood $CD4^+$ T cell frequencies was statistically significant at 7-8 weeks after SIV infection and also at necropsy (p= 0.0095 and 0.028, respectively, by t-test).

Consistent with this finding, immunohistochemical analysis of colon tissues from controller and non-controller vaccinated animals confirmed that animals with better control of virus replication had $CD3^+$ T cell numbers comparable to those of age-matched SIV-uninfected animals, whereas T cell frequencies were significantly lower in non-controller animals (Figure 4.3A). Furthermore, we observed greater numbers of IL-17^+ and $CD3^+$IL-17^+ dual positive cells in the colons of controller animals (quantified in Figure 4.3B). IL-17 positive $CD4^+$ T cells have been suggested to be primary target cells of HIV and SIV because

terminal differentiation via the T_h17 lineage may promote higher CCR5 and α4β7 integrin expression (11, 34, 43, 47, 51, 80, 213). In the current study, the Group C animals with controlled viremia had significantly higher frequencies of $CD3^+IL\text{-}17^+$ cells in colon tissues than animals with uncontrolled high viremia (p=0.0014 by one-way ANOVA; Figure 4.3B). The SIV-induced depletion of IL-17-producing T cells contributes to the loss of intestinal mucosal integrity, a phenotype commonly associated with HIV/SIV infection. IL-17A, a proinflammatory cytokine important for sustaining the integrity of the mucosal barrier, commonly declines after HIV/SIV infection (23, 24) but is maintained in long-term non-progressor patients (292).

Breakdown of mucosal surface immunity could lead to the establishment of intestinal opportunistic infections. Histopathological analyses of the ileum and colon tissues indicated that mock vaccinated and non-controller animals experienced more opportunistic infections, as was evident by cryptosporidiosis. While 17% of mock vaccinated, 60% of Group B non-controllers and 100% of Group C non-controllers were positive for cryptosporidiosis in the ileum, this infection was not detected in controller animals. The difference in the occurrence of opportunistic infections per group was statistically significant only for the ileum (one-way ANOVA p= 0.0107) (Figure 4.4A), but similar data were observed in the colon.

Overall SIV-induced pathology though was comparable across all groups. In lymphoid tissues, SIV-induced pathology ranged from mild hyperplasia to severe disease with massive T cell depletion (Figure 4.4B). The average tissue pathology disease score in both vaccinated groups was lower than the average score in mock vaccinated animals, but this difference did not reach statistical significance unless the Group B/C non-controller animals were combined prior to comparison to mock-vaccinated animals (p=0.02 by two-

tailed t-test), because two animals in Group B had low tissue disease scores despite high plasma viremia,. Therefore, we could not detect an apparent correlation between partial control of plasma virus replication and overall tissue pathology.

Consistent with our previous data in healthy and SIV-infected infant macaques vaccinated with $mc^2$6435, no pathology indicative of tuberculosis disease was detectable (152). Furthermore, viable mycobacteria could not be recovered from the axillary LN, mesenteric LN, spleen, lung and bronchial LN tissues tested (data not shown). Importantly, we have now demonstrated that attenuated *Mtb*-SIV vaccines can be safely administered to neonates prior to SIV infection, as shown in the current study, and after SIV infection (152) with no risk of adverse safety concerns.

Maintenance of vaccine-induced *Mtb*-specific T cell responses despite SIV infection

M. tuberculosis-specific immune responses were longitudinally measured by cytokine production following *in vitro* stimulation with *M. tuberculosis* purified protein derivative (PPD). Vaccination at birth with strains $mc^2$6208 and $mc^2$6435 induced *M. tuberculosis*-specific T cell responses in peripheral blood (Figure 4.5), which persisted despite SIV-induced immunosuppression and could be detected in multiple tissues at the time of necropsy (data not shown). The magnitudes of *M. tuberculosis*-specific T cell responses were consistent with previous studies (151). Interestingly, i.d. boosting with strains $mc^2$6208, $mc^2$6435 and $mc^2$6440 in Group B animals did not appear to increase *M. tuberculosis*-specific T cell responses compared to Group C animals that were not boosted for *M. tuberculosis* responses (Figure 4.5).

Role of SIV-specific antibodies in the control of SIV replication

Consistent with previous results, Groups B and C animals in the current study developed SIV Gag, Pol- plasma IgG and IgA antibodies in response to vaccination (151). In addition, Group C animals developed plasma anti-SIV Env IgG and IgA antibodies, and intestinal and salivary anti-SIV Env IgA in saliva. The induction of the SIV Env-specific antibody response appeared to be a result of the MVA-SIV boosts only, because Group B animals did not develop SIV Env-specific antibodies prior to SIV infection despite being primed and boosted with SIV Env expressing $mc^2$6208. Thus, although vaccine cultures were tested in mice, it will be necessary to optimize strain $mc^2$6208 for in vivo production and secretion of SIV Env in macaques.

Group C plasma SIV Env-specific IgG antibodies at the time of challenge (week 9) were associated with reduced viremia in the first few weeks post-SIV infection (Figure 4.6A). Thus, the controller animals in Group C had higher SIV Env-specific plasma IgG antibody levels than non-controller Group C animals and these inversely correlated with viremia at 2 and 4 weeks post-infection ($p= 0.0368$ and $p= 0.0154$, respectively). In addition, a trend of higher SIV Env-specific plasma IgG antibody avidities at the time of challenge inversely correlated with plasma viremia during the first few weeks after SIV infection (Figure 4.6B). Although greater concentrations and higher avidities of anti-Env plasma IgG were insufficient to prevent SIV infection, these antibodies might have helped to control early viral replication, as suggested by reduced peak viremia and viral set points (see Figure 4.1C). Antibody function was insufficient to prevent SIV acquisition likely because avidity indices were relatively low (Figure 4.6B) and we could not detect antibody-dependent cell-mediated virus inhibition (ADCVI) (data not shown).

Plasma IgA antibodies specific for SIV Env or SIV Gag, Pol were detectable only in Group C animals at the time of challenge. The plasma SIV-specific IgA antibody levels differed significantly between Group A, Group B, and Group C animals ($p< 0.0001$ for anti-SIV Env IgA and $p= 0.0018$ for anti-SIV Gag, Pol IgA; Figure 4.7AB), but there was no difference in IgA responses between controller and non-controller animals. Unlike the inverse correlations between plasma SIV-specific IgG antibodies and viremia, SIV Env-specific plasma IgA antibodies were not associated with reduced viremia (data not shown). In contrast to plasma IgA antibodies, the salivary and intestinal SIV Env-specific IgA levels at the time of challenge inversely correlated with plasma peak viremia in Group C animals ($p= 0.0154$ and $r^2= -0.8333$; Figure 4.7C; $p= 0.0028$ and $r^2= -0.9636$; Figure 4.7D). There was a strong positive correlation between SIV Env-specific salivary and intestinal IgA ($p= 0.0123$ and $r^2= 0.8895$), but not with plasma SIV Env IgA ($p= 0.911$) indicating that the SIV Env IgA antibodies in saliva and intestine were produced locally and were not due to transudation. Inverse correlations of peak viremia with i) plasma IgG and ii) mucosal IgA are indicative of a contribution from both systemic and mucosal antibodies for partial control of SIV replication in the controller animals.

To assess the capacity of infant macaques to secrete antibodies at mucosal surfaces distal to the site of vaccination, we measured SIV Env-specific IgA in CVF of female animals and in BAL of all vaccinated animals at the terminal timepoint only (Table 4.2). BAL samples could not be acquired at the time of challenge for risk of introducing microabrasions in the oral mucosa. The single measurement of SIV Env IgA at the terminal time point did not allow any conclusion regarding their induction by vaccination or in

response to SIV infection, and whether these antibodies played any role in controlling SIV replication.

Vaccine-induced T cell responses in blood and tissues

Consistent with our previous results (151), all vaccinated animals in Groups B and C had detectable SIV Gag-specific T cell responses in peripheral blood prior to SIV infection, but responses were not consistently detected at all time points and varied in magnitude (data not shown). Although there might have been an anamnestic SIV Gag-specific $CD8^+$ T cell response in Group B and Group C animals compared to mock-vaccinated infants at 2 weeks post-oral SIV challenge, there was no correlation between peak viremia and SIV-specific $CD4^+$ T cell (Spearman r= -0.0707; p= 0.7671) or $CD8^+$ T cell responses (Spearman r= 0.0425; p= 0.8587).

After SIV infection and at the time of necropsy, differentiation between vaccine-induced and SIV infection-induced SIV-specific T cell responses was not possible. In general, oral priming with $mc^2$6435 and $mc^2$6208 followed by systemic MVA-SIV boosts induced comparable SIV-specific T cell responses in tissues in SIV-infected and SIV-naïve infant macaques (151) (data not shown). There were no statistically significant differences in SIV-specific T cell responses in tissues between vaccine Groups B and C animals. Interestingly though, vaccinated animals showed greater frequencies of granzyme B-expressing peripheral blood $CD8^+$ T cells at the time of challenge compared to mock-vaccinated animals and this enhancement was maintained post-SIV infection ($p \leq 0.002$; Figure 4.8A). This finding is noteworthy because infants generally show reduced production of cytotoxic granules and immature effector cell degranulation compared to adults (22, 271).

The enhanced cytotoxic function was confirmed by a more pronounced granzyme B release following *in vitro* SIV Gag stimulation in the peripheral blood $CD8^+$ T cells of vaccinated animals compared to Group A animals (Figure 4.8B), but due to the large variations across animals, differences in SIV-specific CTL function were only statistically significant at week 13 ($p= 0.0094$; Figure 4.8B). In addition to improved granule production and degranulation by SIV-specific $CD8^+$ T cells, vaccinated animals had higher frequencies of T cells expressing the mucosal homing marker CD103 (Figure 4.9). Enhanced mucosal tissue trafficking by effector cell populations following vaccination could translate into improved effector cell function against pathogens encountered at mucosal sites. In fact, among the vaccinated animals, controller animals had significantly higher CD103-positive $CD4^+$ and $CD8^+$ T cells in intestinal tissues, and this trend, although not significant, was conserved in mesenteric LN, submandibular LN and tonsil T cell populations.

Cytotoxic function of $CD8^+$ T cells in vaccinated animals might have been negatively impacted by enhanced frequencies of PD-1-positive $CD4^+$ and $CD8^+$ T cells in vaccinated compared to mock animals at the time of challenge (Figure 4.10A). Interestingly, frequencies of PD-1-positive peripheral blood $CD4^+$ T cells of controller animals were similar to mock animals and less similar to vaccinated non-controller animals. Increased populations of exhausted PD-1-expressing T cells in the periphery of vaccinated animals prompted us to ask whether T cell exhaustion was associated with vaccine-induced immune activation. Indeed, more peripheral blood $CD4^+$ T cells from vaccinated compared to unvaccinated animals expressed the activation marker CD69 (Figure 4.10B). At the time of necropsy, T cells from the mock-vaccinated animals expressed greater frequencies of CD69

in all tissues to vaccinated animals (Figure 4.11B), but PD-1 positive T cells were still higher in vaccinated animals (Figure 4.11A).

We, therefore, further evaluated non-specific immune activation in peripheral blood and tissues at the time of necropsy, and attempted to determine whether specific immune activation profiles would stratify with better control of viremia. We already alluded to the fact that controller animals showed better preservation of IL-17 producing T cells in the colon (Figure 4.3). *Ex vivo* analysis of basal IL-17 production by T cells of blood and tissue showed that controller animals seemed to produce predominantly IL-17 whereas non-controller animals showed higher frequencies of TNF-α-positive T cells. In fact, the ratio of TNF-α:IL-17 producing T cells was low in controller animals, indicating conserved expression of IL-17 and low levels of pro-inflammatory TNF-α production (Figure 4.12). In contrast, vaccinated animals unable to control viral replication exhibited higher ratios of TNF-α:IL-17 producing T cells (Figure 4.12). In every tissue analyzed, controller animals had the lowest ratios, followed by mock-vaccinated Group A animals, then vaccinated non-controller animals exhibited the highest ratios of TNF-α:IL-17-producing T cells (Figure 4.12). Differences in the ratio of TNF-α:IL-17 producing T cells were statistically significant by one-way ANOVA, except for the submandibular $CD4^+$ T cells (p values ranged from 0.0063-0.3695 for $CD4^+$ and 0.0110-0.0373 for $CD8^+$ T cells).

In addition to the distinct TNF-α:IL-17 ratios, the expression of the single cytokines IL-2, IFN-γ, IL-17, and TNF-α in unstimulated, *ex vivo* T cells at the terminal time point was characteristic between the four controller animals and the other vaccinated animals (shown for PBMC and colon in Figures 4.13 and 4.14, respectively). To determine the vaccine effect on basal cytokine expression, historical samples from SIV-naïve, orally $mc^2$6435 primed /IM

MVA-SIV boosted infant macaques were evaluated for basal cytokine expression profiles at the terminal timepoint (151). The data show that controller animals exhibited cytokine expression profiles resembling those observed in vaccinated, SIV-naïve animals (Table 4.3, Group D). Again, most noteworthy was the robust colon IL-17 production by SIV-naïve and controller animals compared to Group B and C non-controller animals (Figure 4.14). Without additional studies, it is difficult to speculate if these expression patterns would change over time, but the 'snapshot' of total nonspecific cytokine production suggests that even partial control of viremia limited SIV-induced immune activation and prevented the loss of IL-17-expressing T cells in the colon of controller animals.

Potential immune correlates of vaccine-induced enhanced SIV acquisition

The observed high background cytokine responses in unstimulated cell suspensions at necropsy suggested unresolved immune activation, a hallmark of SIV infection. However, as we had observed enhanced infectivity in the vaccinated animals compared to unvaccinated controls, we retrospectively tested for immune activation at the time of challenge. Indeed, quantification of *basal* cytokine production in PBMC T cell populations at the time of challenge revealed that vaccinated animals had significantly greater numbers of both $CD4^+$ and $CD8^+$ T cells actively producing IL-2, TNF-α and IFN-γ (Figure 4.15). In contrast, mock-vaccinated animals showed very low amounts of background activation by comparison. Only IL-17-expressing T cell frequencies did not differ between vaccinated and unvaccinated animals (Figure 4.15). The non-specific production of IL-2, TNF-α and IFN-γ was indicative of an environment promoting $CD4^+$ T cell activation, which could potentially translate into enhanced HIV/SIV infectivity (56, 83, 341). This assumption was further strengthened by

the fact that T cells expressing the activation marker CD69 were also found at higher frequencies in vaccinated compared to unvaccinated animals at the time of challenge (Figure 4.16). Non-specific T cell activation was observed independent of the boost regimen in both Group B and Group C animals suggesting that it was predominantly the common $mc^2$6435 and $mc^2$6208 prime that was responsible for the immune activation phenotype. Likely as a result of persistent immune activation, PD-1-expressing T cell populations were more pronounced in vaccinated animals at the time of challenge (Figure 4.16).

To confirm vaccine-induced T cell activation in the absence of SIV-induced immune activation, we retrospectively measured markers of T cell activation in SIV-naïve, orally $mc^2$6435 primed /systemically MVA-SIV boosted infant macaques. These samples were derived from animals of our previous immunization studies (151, 152) and their immunization regimens are listed in Table 4.3. Group D most closely mirrored the immunization schedule used in the current SIV challenge study with oral $mc^2$6435 priming and MVA-SIV boosting. Group F animals were also orally primed with strain $mc^2$6435 but were boosted instead using adenovirus 5-SIV (Ad5-SIV). Finally, to test for route-specific effects of neonatal *Mtb*-SIV vaccination, Group G animals received intradermal priming with strain $mc^2$6435 and an Ad5-SIV boost.

Indeed, CD69- and Ki67-positive peripheral blood T cell frequencies were higher in all vaccinated compared to unvaccinated historical animals (Figure 4.17A). Importantly, increased activated $CD4^+$ T cell frequencies were also observed in retropharyngeal LN and colon of all SIV-naïve, $mc^2$6435-vaccinated animals compared to mock-vaccinated animals (Figure 4.17CD). Although only shown for $CD4^+$ T cells, CD69 and Ki-67 expression patterns were duplicated in $CD8^+$ T cell populations. To test whether this vaccine-induced

immune activation resulted in increased target cell frequencies for SIV, we additionally quantitated CCR5-positive $CD4^+$ T cell frequencies in SIV-naïve, orally $mc^2$6435-primed infant macaques. Indeed, significantly greater frequencies of $CD4^+$ T cells in the peripheral blood, retropharyngeal LN and colon expressed the HIV/SIV co-receptor and activation marker CCR5 (Figure 4.17). It should be reiterated that elevated CCR5-positive T cell populations were observed in all $mc^2$6435 primed infant macaques independent of the route of $mc^2$6435 priming and the type of boost. The samples used for this retrospective analysis match the prime in the current study, but are representative of a later time point post-prime as these animals were followed for 16-18 weeks. However, we rationalized that any immune activation induced by the prime should decrease over time. Therefore, the fact that we could detect immune activation at these later time points, similar to activation observed in the current study at 9 weeks post-prime, even further underlines the potential importance of our findings of increased target cell availability at the time of oral SIV challenge, and indicates the need for more thorough analysis of immune activation after vaccination.

DISCUSSION

Previous work characterized the development of a novel combination vaccine using attenuated *Mtb* engineered to co-express SIV antigens to elicit dual immunogenicity against both SIV and *Mtb*. We demonstrated that oral and intradermal vaccination with strain $mc^2$6435 at birth was safe in healthy and immunosuppressed, SIV-infected neonatal macaques (152), and that vaccination could elicit SIV and *Mtb* cellular and humoral immune responses (151). This current study aimed to evaluate the ability of the vaccination regimen to protect against repeated low-dose oral SIV challenge.

Two unexpected intriguing results were obtained. First, the majority of vaccinated animals required fewer low-dose oral SIV exposures to become infected than the mock-vaccinated animals, and this enhanced risk of oral SIV infection was associated with vaccine-induced immune activation. Secondly, although vaccination did not protect animals from SIV acquisition, a subset of vaccinated animals developed more robust SIV Env-specific IgA in mucosal secretions and higher avidity Env-specific plasma IgG antibodies that correlated with reduced viremia. In addition, we observed persistent immune activation in vaccinated animals, independent of the boost regimen, suggesting that vaccination may have enhanced infectivity.

Consistent with our previous studies, the oral $mc^2$6435 prime/IM MVA-SIV boost regimen induced both SIV and *Mtb*-specific immune responses (151). The addition of strain $mc^2$6208 to the vaccine prime to improve immune response breadth did not appear to affect either immunogenicity or efficacy in the current study. The Group B animals, despite priming *and* homologous boosting with *Mtb* strains individually expressing SIV Gag, Pol and Env, made little-to-no SIV Env- or Gag, Pol-specific plasma IgG. In fact, the lack of plasma SIV Env-specific antibody responses in Group B suggests that the SIV Env insert was unstable and not expressed at sufficient levels to induce immune responses in infant macaques despite *in vivo* expression in mice (Ranganathan et al., manuscript in preparation). Therefore, improved *Mtb*-SIV vaccine strains optimized for persistent *in vivo* SIV antigen expression need to be developed for forthcoming macaque studies as antibody responses are critical for the prevention of HIV acquisition in humans (82).

Our results showed that the avidity of SIV Env specific plasma IgG antibodies at the time of challenge inversely correlated with reduced peak viremia. Thus, animals with lower

peak viremia and reduced viremia throughout the course of infection had developed SIV Env specific IgG antibodies with higher avidity than vaccinated animals with persistently high viremia. Interestingly, HIV Env specific plasma IgG antibodies were an immune correlate identified in the RV144 HIV vaccine trial (110, 188, 398) and although could not prevent HIV acquisition through neutralization, instead likely prevented infection through a mechanism of infectious virion capture or ADCVI (193). The SIV Env IgG antibodies elicited in the current infant study did not mediate ADCVI (data not shown). Furthermore, the avidity indices of the plasma SIV Env-specific IgG antibodies were relatively low when compared to SIV challenge studies with partial protection in adult macaques in which avidity indices were 3 to 4-fold higher (272, 397). Thus, while our vaccine regimen was able to induce SIV Env-specific antibodies, their functional capacity was severely limited. Additionally, the current vaccine regimen was not designed to elicit neutralizing antibodies.

It remains unclear what vaccine characteristics are important for driving the development of antibodies that are broadly-neutralizing, have high binding avidities and are persistently secreted at mucosal surfaces. Antibody development early during acute HIV infection appears to be a dynamic process, one that we are just beginning to understand. For example, Yates *et al*. recently reported that early after HIV infection, gp41, and not gp120, anti-Env IgA is produced both systemically and mucosally, but that gp41 antibodies were rapidly depleted due to ablated production and a short half-life (391). The ontogeny of antigen-specific mucosal B cell responses, whether induced following vaccination or infection, remains uncharacterized, but its understanding is essential for prior to designing immunogens to induce the development of robust, broadly-neutralizing and/or high-avidity mucosal antibodies.

Interestingly, our study revealed that SIV Env specific mucosal IgA antibodies in saliva and intestinal secretions contributed to reduced viremia in some animals. The controller animals showed the highest mucosal SIV Env specific IgA activities at the time of challenge, and these higher IgA antibodies in mucosal secretions were correlated with reduced peak viremia and continued reduced virus replication compared to non-controller animals. In contrast, plasma IgA antibodies did not appear to influence virus acquisition or replication. This data supports the conclusion that mucosal IgA might be critical in controlling early virus replication at mucosal entry sites (20, 230). It should be pointed out that the specific SIV Env IgA activities induced by the current vaccination regimen have not been achieved by other pediatric SIV vaccination regimen (217, 360). In fact, with the caveat of different immunogens and study parameters, the specific activities of anti-Env mucosal IgA antibodies was comparable to measurements in SIV vaccine study adults (272, 397, (82, 110, 188, 193, 333, 398). This is a remarkable finding because infants produce very little IgA at birth and due to limited VDJ rearrangements, epitope recognition by IgA antibodies is highly restricted (121, 303, 327). Improved understanding of the vaccine components best able to enhance the development of antibody breadth, avidity and secretion at mucosal surfaces will dramatically improve the protective efficacy of vaccines against mucosally transmitted pathogens. It will be important to more thoroughly understand the implications of age-dependent antibody development for vaccine design. Our results here provide proof-of-concept that mucosal IgA can be robustly induced by infant vaccination.

The induction of mucosal immunity by our vaccine regimen was further support by the presence of increased frequencies of CD103 positive T cells in lymph nodes draining the oropharynx and in intestinal tissues. Although we could not detect any apparent correlations

between SIV-specific T cell responses and control of viremia, vaccinated animals did show improved cytotoxic T cell function. CTL responses have been identified as an important mechanism of HIV/SIV control (96, 117-119, 194, 247, 279, 305). In infants, CTLs/ are generally reduced compared to the older individuals (6, 311, 312), and thus it is essential that a pediatric HIV vaccine will induce cytotoxic effector function. Despite improved granzyme expression and release in vaccinated animals in the current study, the percentage of granzyme B expressing $CD8^+$ T cells in vaccinees never even reached levels typically observed in healthy unvaccinated adult macaques (Jensen, unpublished data). Together these data demonstrate that the oral *Mtb*-SIV prime/IM MVA-SIV boost regimen was able to effectively induce SIV-specific T and B cell responses at mucosal and systemic sites, but SIV immunity lacked sufficient functional competence to prevent oral SIV acquisition.

In fact, despite partial control of viremia in a subset of animals, the risk of oral SIV acquisition per low-dose oral exposure seemed to be enhanced in the vaccinated compared to the unvaccinated infant macaques. This finding was indicative of potential vaccine-induced immune activation that could have promoted SIV infection. Mycobacteria are known to have intrinsic adjuvant activity, best illustrated by their inclusion in Freund's complete adjuvant. A recent study reported that human infants vaccinated with BCG at birth showed peripheral immune activation that peaked about 10 weeks post-vaccination (320). This time point is similar to when repeated low-dose oral SIV exposures were started at 9 weeks after *Mtb*-SIV priming. However, in contrast to BCG, the double auxotroph *Mtb* strains used in this study are considered replication-incompetent within a mammalian system. In fact, we could never recover live mycobacteria from tissues in supplemented cultures. Still, we clearly

demonstrated that activated peripheral blood T cells occurred at greater frequencies in vaccinated than unvaccinated animals at the time of oral SIV challenge initiation.

Importantly, using historic samples from animals in our previous *Mtb*-SIV immunization studies, we could document that vaccination induced an increase in CCR5-positive $CD4^+$ T cells and $CD14^+$ monocytes in blood and tissues. Thus, vaccination with *Mtb*-SIV resulted in increased frequencies of potential SIV target cells at anatomic sites indicated as entry sites following oral SIV exposure (208, 360, 361, 389). If our findings can be repeated and confirmed, they would have major global health implications. It should be clearly stated that unvaccinated and vaccinated animals did not statistically differ in their relative risk of SIV infection per oral exposure, but we observed a trend towards enhanced risk of SIV infection in vaccinated infant macaques and could associate increased infection risk with vaccine-induced persistent immune activation and increased SIV target cell availability. South African researchers have previously voiced concern about early BCG vaccination and its potential for immune activation that could promote HIV acquisition (126, 127, 150). Conclusive studies, however, are missing.

The goal of a successful pediatric HIV vaccine to prevent breast milk transmission of HIV relies on striking the delicate balance between vaccine-induced immune activation to prompt the development of innate immune responses to effectively prime adaptive immune responses without inducing an inflammatory milieu that can persist in the infant host and accelerate HIV acquisition. This will be a difficult and challenging task, requiring a deep understanding of pediatric immune development, the kinetics of immune responses to mycobacterial vaccine strains, the optimization of relevant immunogens and the mechanisms of vaccine-induce immune activation. Further, improved quantitative read-outs to evaluate

the potential of T and B cell responses effective for preventing HIV/SIV acquisition or limit virus replication will positively impact immunogen optimization. Our data provide insight into the complex mechanisms underlying successful vaccine design. We do not consider the observed lack of vaccine efficacy a defeat, but instead as a stepping stone that will guide us in our future pediatric HIV vaccine design.

TABLES

Group	Animal	Sex	MHC Class I[a]	Immunization (Route)[b]			SIV Challenge (5x10[3] $TCID_{50}$, PO)[c]														Nx (wk)
				Week 0	3	6	9	10	11	12	13	14	15	16	17	18	19	20	21	No.[d]	
A	42376	F	*A*01, A*11, B*17, B*29*	PBS (PO)	PBS (ID + IM)	PBS (IM)	+													1	21
	42380	F	*A*01, A*11, B*17, B*29*												+		c	c		9[c]	27
	42386	F						+												2	22
	42388	F	*A*11, B*17, B*29*								+									5	25
	42409	M	*A*08*										+							7[e]	25
	42434	M					+													1	21
B	42903	F		mc²6435 + mc²6208 (PO)	mc²6435 + mc²6208 + mc²6440 (ID)		+													1	21
	42918	M	*A*01, B*01*														+ c	c	c	11[d]	34
	42925	F					+													1	21
	42943	M	*A*01*					+												2	21
	42947	F	*A*01*				+													1	20
	42950	M					+													1	20
C	42899	F	*A*01, B*01*	mc²6435 + mc²6208 (PO)	MVA-SIV (IM)	MVA-SIV (IM)	+													1	20
	42906	F	*B*01*				+													1	22
	42924	F	*A*01*					+												2	34
	42929	F	*A*02, B*01*					+												2	20
	42937	M	*A*11, B*01, B*08*				+													1	21
	42944	M						+												2	33
	42949	M	*A*11*				+													1	21
	42958	F										+					c	c		6[d]	33

Table 4.1. Study outline and challenge schedule. [a] Animals not expressing alleles in this list are intentionally blank. [b] Vaccine doses for strains mc²6435, mc²6208 and mc²6440 were 10^9 CFU and 10^8 IU for MVA-SIV boosts. p.o. = oral, i.d.= intradermal, i.m.= intramuscular. [c] Grey boxes indicate the duration of weekly challenges by animal. Positive viremia for three consecutive time points was required to confirm systemic infection. Increased SIV challenge doses ($2x10^4$ $TCID_{50}$) were administered weekly after 10 low-dose exposures failed to result in infection; high-dose challenges are indicated in dark grey boxes. The inherent lag between bleed date and viral PCR data resulted in additional challenges that were not required for infection, which is why some higher dose challenges occur after determined infection age. [d] Denotes the number of weekly oral challenges required for persistent SIV infection as determined by viral RNA in plasma. A (+) indicates timepoint one week prior to the first detection of virus and is the probable time of infection. [e] Transient viremia was observed in animal #42409 for two consecutive weeks (13 and 14, indicated by hashing), followed by undetectable viral RNA in plasma at week 15; at week 16, SIV RNA was observed in plasma.

			IgA (ng/ml)		Specific Activities (ng Env IgA/ug total IgA)				
			Plasma		Saliva		Intestine	CVF	BAL
Group	Animal	Sex	Week 6	Week 9	Week 9	Nx	Week 9	Nx	Nx
A	42376	F	ND	ND	ND	8.10	ND	1.00	0.57
	42380	F	ND	ND	ND	74.40	NT*	76.45	19.34
	42386	F	ND	ND	ND	281.80	ND	5.25	35.72
	42388	F	ND	ND	ND	29.10	ND	2.52	27.97
	42409	M	ND	ND	ND	6.30	ND	NT	20.48
	42434	M	ND	ND	ND	6.50	ND	NT	0.00
B	42903	F	ND	ND	ND	10.80	ND	0.84	0.00
	42918	M	ND	ND	ND	29.20	ND	NT	110.06
	42925	F	ND	ND	ND	92.60	ND	19.34	125.04
	42943	M	ND	ND	ND	10.10	ND	NT	34.93
	42947	F	ND	ND	ND	14.70	ND	4.82	13.00
	42949	M	ND	ND	ND	4.50	ND	NT	0.00
C	42899	F	31.7	381.0	35.07	24.10	NT*	83.20	46.30
	42906	F	76.7	471.6	39.25	7.80	ND	3.74	41.39
	42924	F	66.8	415.1	70.70	28.00	11.22	0.00	44.04
	42929	F	132.5	1335.8	29.69	29.70	ND	78.79	1226.33
	42937	M	99.1	224.1	12.79	23.20	ND	NT	78.42
	42944	M	18.8	278.6	34.69	7.00	7.45	NT	9.51
	42947	M	45.8	259.6	10.46	30.10	ND	NT	30.33
	42958	F	346.8	3626.3	97.80	41.80	8.26	13.25	34.09

Table 4.2 Anti-Env IgA in plasma and mucosal secretions. Plasma, saliva and stool samples were collected longitudinally from study animals when possible; cervicovaginal fluid (CVF, female animals only) and bronchoalveolar lavage (BAL) samples were collected only at terminal timepoints. Intestine IgA values are secretory antibody produced within the intestinal mucosa. Note that plasma samples are recorded as total anti-Env IgA (ng/ml) while mucosal antibodies are reported as specific activities (anti-Env IgA/total IgA; ng/µg). NT*= not tested, sample tube broke; NT (grey box)= not tested because animals were male; ND= not detected or sample was not significantly greater than assay background (concentrations ≥ background +3SD).

Group	Group Size	Vaccination								Necropsy Age (wks)
		Prime				Boost				
		Strain	Dose	Route	Age (wks)	Strain	Dose	Route	Age (wks)	
D	8	$mc^2$6435	10^9 CFU	PO	0	MVA-SIV	10^8 PFU	IM	3 + 6	18
E	3	mock		PO	0	mock		IM	3 + 6	16
F	6	$mc^2$6435	10^9 CFU	PO	0	Ad5-SIV	$9x10^9$ PFU	IM	3	16
G	6	$mc^2$6435	10^6 CFU	ID	0	Ad5-SIV	$9x10^9$ PFU	IM	3	16
H	4	mock		PO / ID	0	mock		IM	3	16

Table 4.3 Study outline for retrospective CCR5 quantification experiment in SIV-naïve, vaccinated animals. Vaccination schedule from historical animals orally primed with strain $mc^2$6435 at birth and heterologously boosted with either MVA-SIV (Group D) or Ad5-SIV (Groups F/G). Frozen PBMC, retropharyngeal lymph node and colon cell suspensions from animals in Groups D-H were assayed for the presence of activation markers CCR5, CD69 and Ki-67 on T cell and monocyte populations to evaluate possible vaccine-induced activation in the absence of SIV infection. Responses from mock animals (Groups E and H) are reported together as 'mock'.

FIGURES

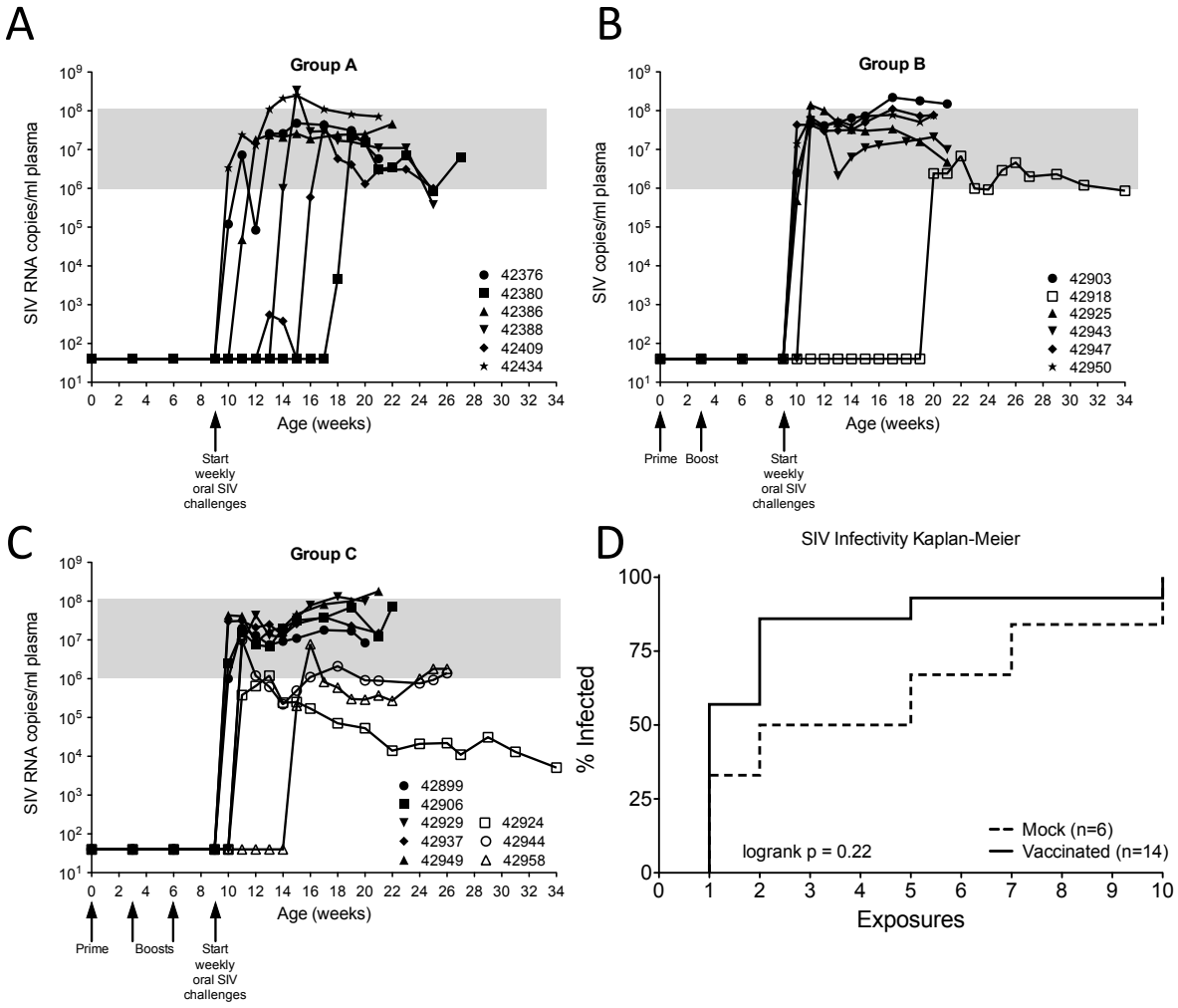


Figure 4.1. Viral Loads. SIV Gag RNA copies per ml plasma were quantified at weekly intervals beginning at week 10 of age, with the assay detection threshold reported to be around ≤30 copies/ml. A) Viral loads in mock-vaccinated Group A animals, B) viral loads in homologously-boosted Group B animals and C) viral loads in heterologously-boosted Group C animals. Arrows indicate when weekly low-dose challenges were initiated for all groups, and vaccination interventions for animals in Groups B and C. In Group B (n=1) and Group C (n=3), four animals exhibited reduced peak viremia and were able to maintain/reduce viral loads compared to other cohort animals. Due to their ability to reduce/control viremia, these four animals were followed longer and are represented in open symbols. The grey bars are arbitrary and are included only as points of reference. D) Kaplan-Meier plot comparing the number of SIV exposures required for animals in either the mock-vaccinated Group A (dashed line, n=6) or $mc^2$6435-vaccinated Groups B and C (solid line, n=14). While not statistically significant by log-rank (p=0.22), there was a trend towards enhanced infectivity in the vaccinated animals compared to mocks, with a relative infectivity rate of 1.96 following vaccination.

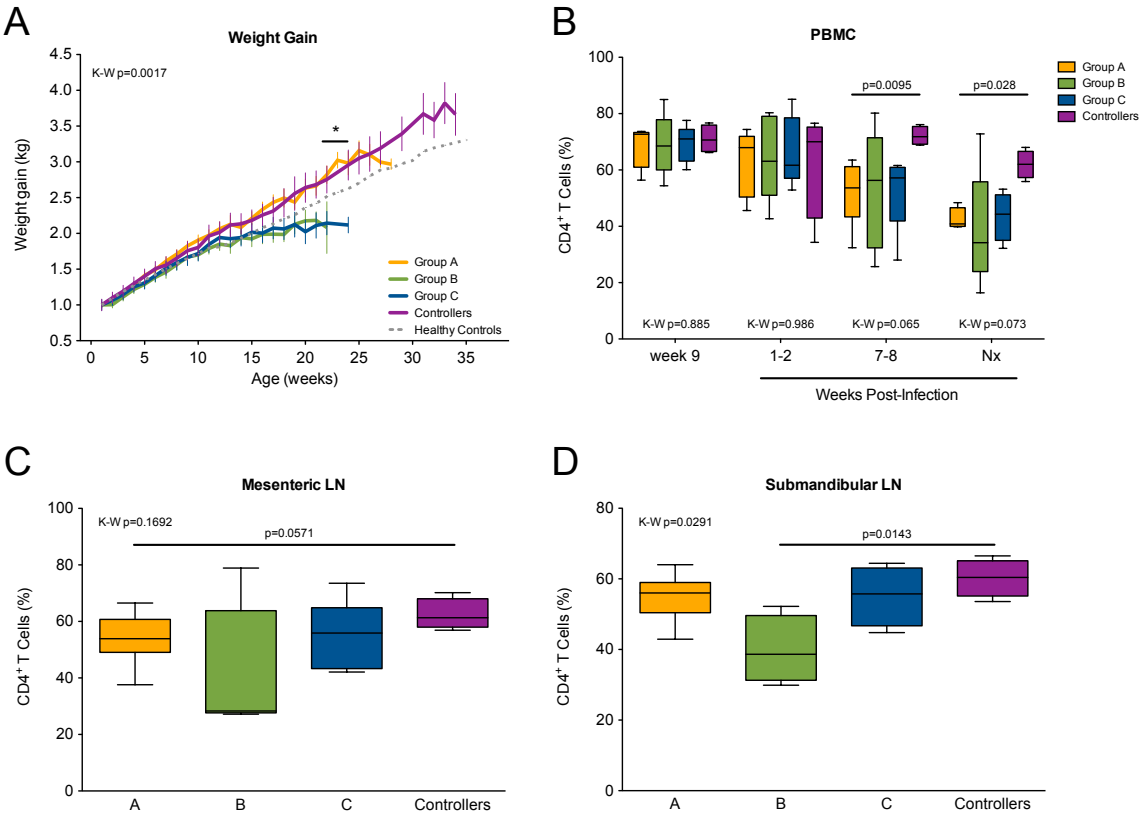


Figure 4.2. Clinical parameters of SIV infection. A) Failure to maintain and/or gain weight was observed in vaccinated animals that were unable to control SIV viremia (Groups B (green) and C (blue)). No weight gain in Groups B and C was observed in contrast to Group A animals (yellow), which continued to gain appropriately until the final two weeks. The four vaccinated Controller animals (#42918, #42924, #42944 and #42958, purple) showed excellent weight gain throughout. Weights at week 22 were statistically different between Groups B/C and Group A/Controller animals by one-way ANOVA (p=0.0017). Error bars indicate the standard deviation within each cohort and weight differences were normalized for each animal to birth weight (kg). The dashed grey line represents the mean of healthy male and female nursery-reared neonatal macaques (n=69-2083, depending on age). B) $CD4^+$ T cells as a percentage of total $CD3^+$ T cells in peripheral blood by age. Although there is no difference in cell frequencies at the time challenges were initiated (week 9), Controller animals exhibit less $CD4^+$ T cell loss with time compared to non-controller animals in peripheral blood (p=0.0095 and p=0.028 at weeks 7/8 post-infection and at necropsy, respectively). Controller animals also exhibited a trend of reduced $CD4^+$ T cell loss in specific tissues at terminal timepoints (illustrated for C) mesenteric LN and D) submandibular LN) compared to non-controller animals in Groups B and C.

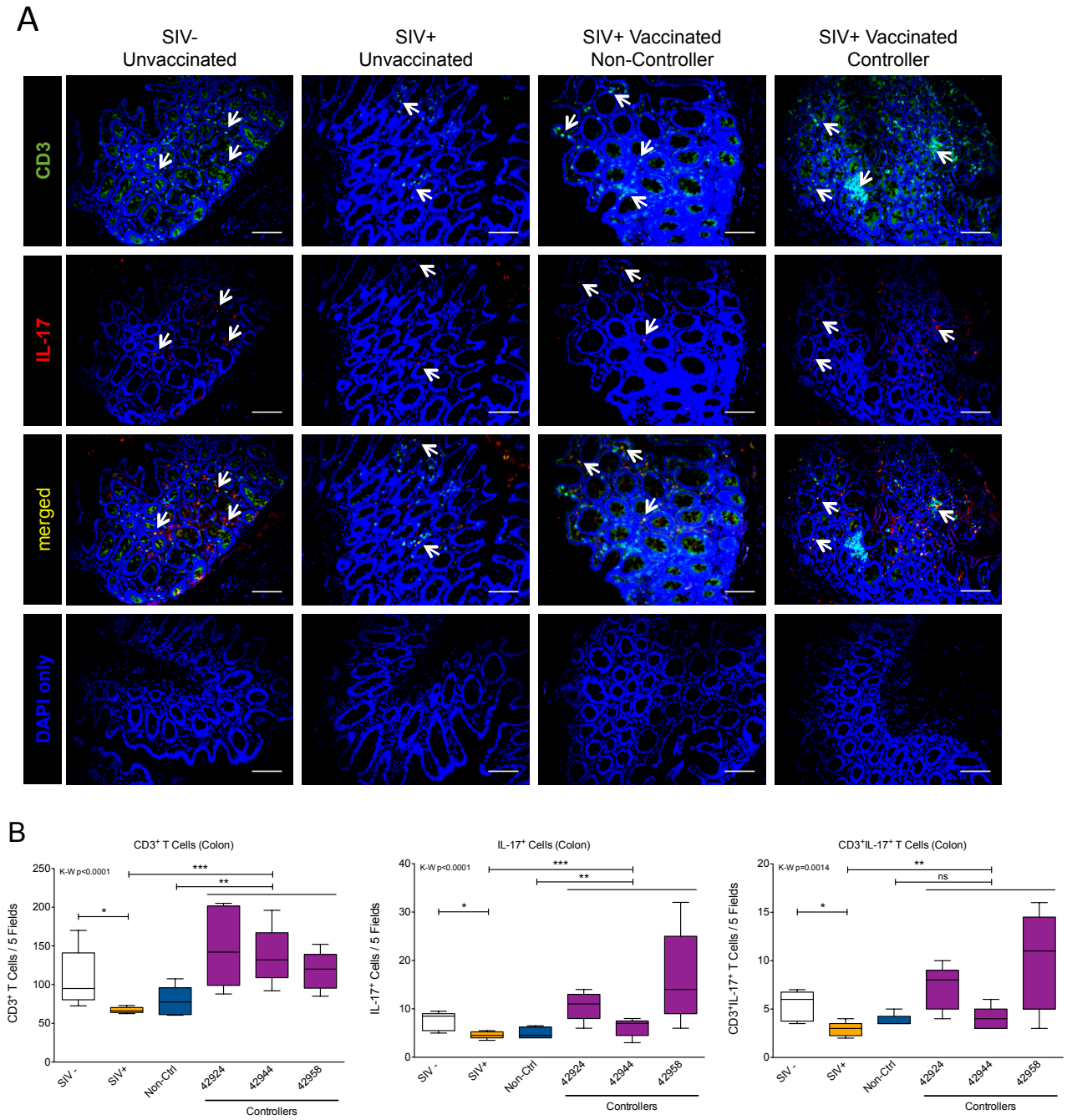


Figure 4.3. Immunohistochemical analysis of IL-17 expression and T cell maintenance in colon. A) IHC was used to evaluate the integrity of the intestinal mucosa (colon) following SIV infection. Controller animals in Group C (#42924, #42944 and #42958) exhibited improved CD3 T cell maintenance, IL-17 expression and $CD3^{+}IL\text{-}17^{+}$ co-expression in colon tissues at the time of necropsy. White arrows highlight $CD3^{+}$ (green), $IL\text{-}17^{+}$ (red) and $CD3^{+}IL\text{-}17^{+}$ dual-positive events (yellow). DAPI (blue); scale 100 µm. B) Expression patterns from five fields per tissue sample were quantified and plotted to enumerate T cell loss in colon tissue following infection with SIV. Total $CD3^{+}$ events (left plot), $IL\text{-}17^{+}$ cells (center plot) and dual positive cells (right plot) are shown for i) SIV-naïve unvaccinated infants (SIV-), ii) SIV-infected unvaccinated infants (SIV+), iii) SIV-infected,

vaccinated non-controller infants (Non-Ctrl) and individually for the 3 controller infants in Group C iv) #42924, v) #42944 and vi) #42958. Controller animals exhibited significantly more CD3 T cells and IL-17^+ cells compared to non-controllers and SIV+ unvaccinated animals, although not statistically more than healthy unvaccinated controls by one-way ANOVA ($p \leq 0.0001$). Two animals (#42924 and #42958) exhibited greater frequencies of $CD3^+IL\text{-}17^+$ dual cells compared to non-controllers but dual-positive cells in the colon of #42944 were similar to non-controllers, making this difference not statistically significant. The controller animals did, however, have greater frequencies of dual positive cells compared to SIV-infected unvaccinated animals. Box and whisker plots indicate median values and standard deviation. K-W= Kruskal-Wallis one-way ANOVA test, with p values reported by plot.

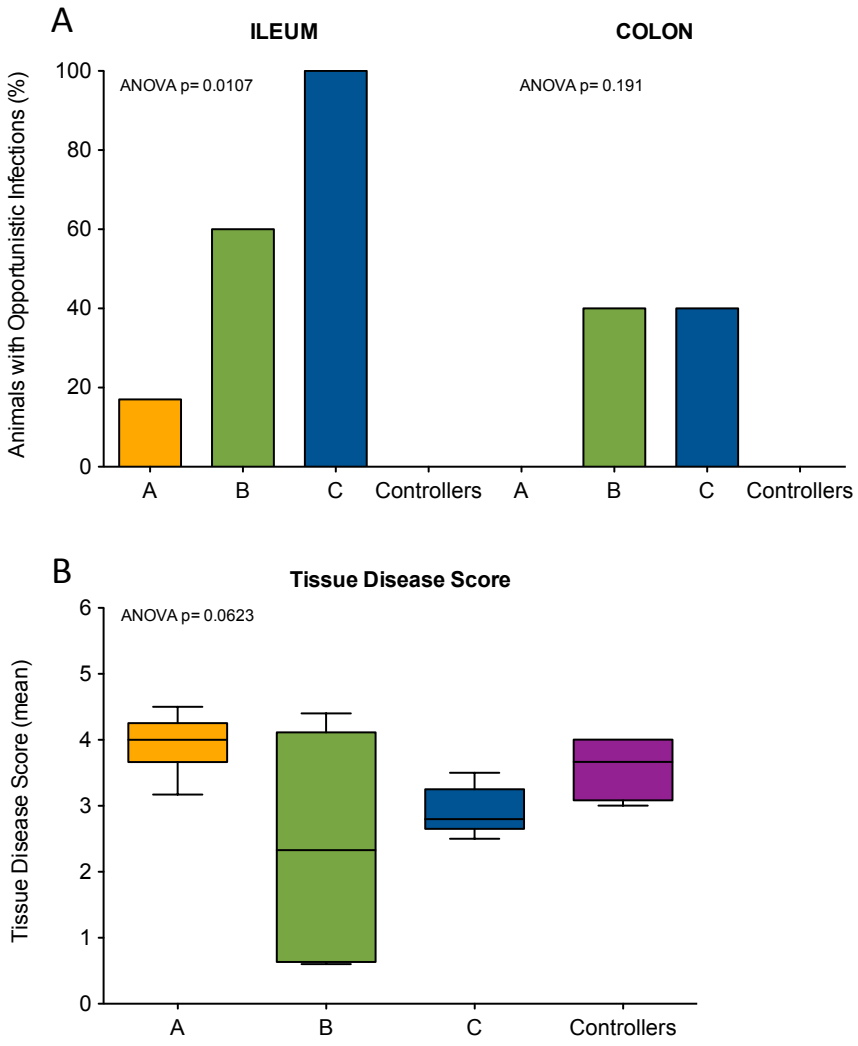


Figure 4.4. SIV-induced opportunistic infections and pathology. A) The presence of opportunistic infections (primarily cryptosporidium) in the ileum and colon tissues is enhanced in vaccinated animals unable to control viremia (Groups B an C) compared to mock-vaccinated (Group A) and Controller animals. There was no indication of opportunistic infections in either the ileum or colon of Controller animals. All Group C animals, 3 of 5 Group B animals and 1 of 6 Group A animals had indications of secondary infections in the ileum (one-way ANOVA p=0.0107) and 2 of 5 Group C animals, 2 of 5 Group B animals and no Group A animals had indications of secondary infections in the colon (not significant). B) Blinded pathology reports generated across multiple individually-scored tissues (axillary LN, bronchial LN, mesenteric LN, retropharyngeal LN, submandibular LN, ileum, colon, lung and spleen) were compiled for a total disease score (TDS) mean value to indicate the degree of SIV-induced disease at the time of necropsy. Scoring rubric: 0- no indications of SIV-associated pathology; 1- mild disease, multifocal; 2- mild disease, diffuse; 3- moderate disease, multifocal; 4- moderate disease, diffuse; 5- severe disease with mild T cell depletion; 6- severe disease with massive T cell depletion.

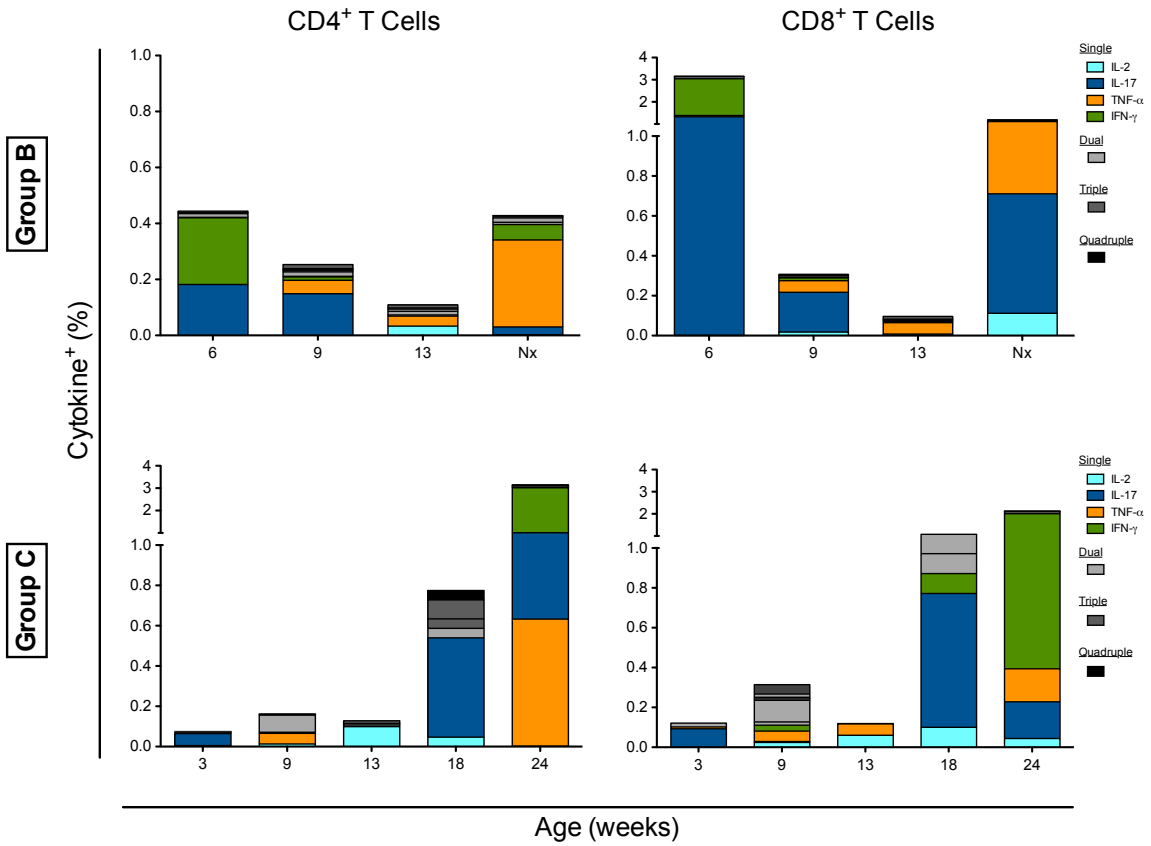


Figure 4.5. *Mtb*-specific T cell responses are maintained despite SIV infection. A) Longitudinal CD4 (left) and CD8 (right) peripheral T cell responses in direct response to stimulation with purified protein derivative (PPD) in a representative Group B animal ('non-controller'; top panels) and Group C animal ('controller'; bottom panels). Combinations of single and polyfuctional cytokine responses are represented by the legend at right. Responses have previously been corrected for background expression in unstimulated cultures.

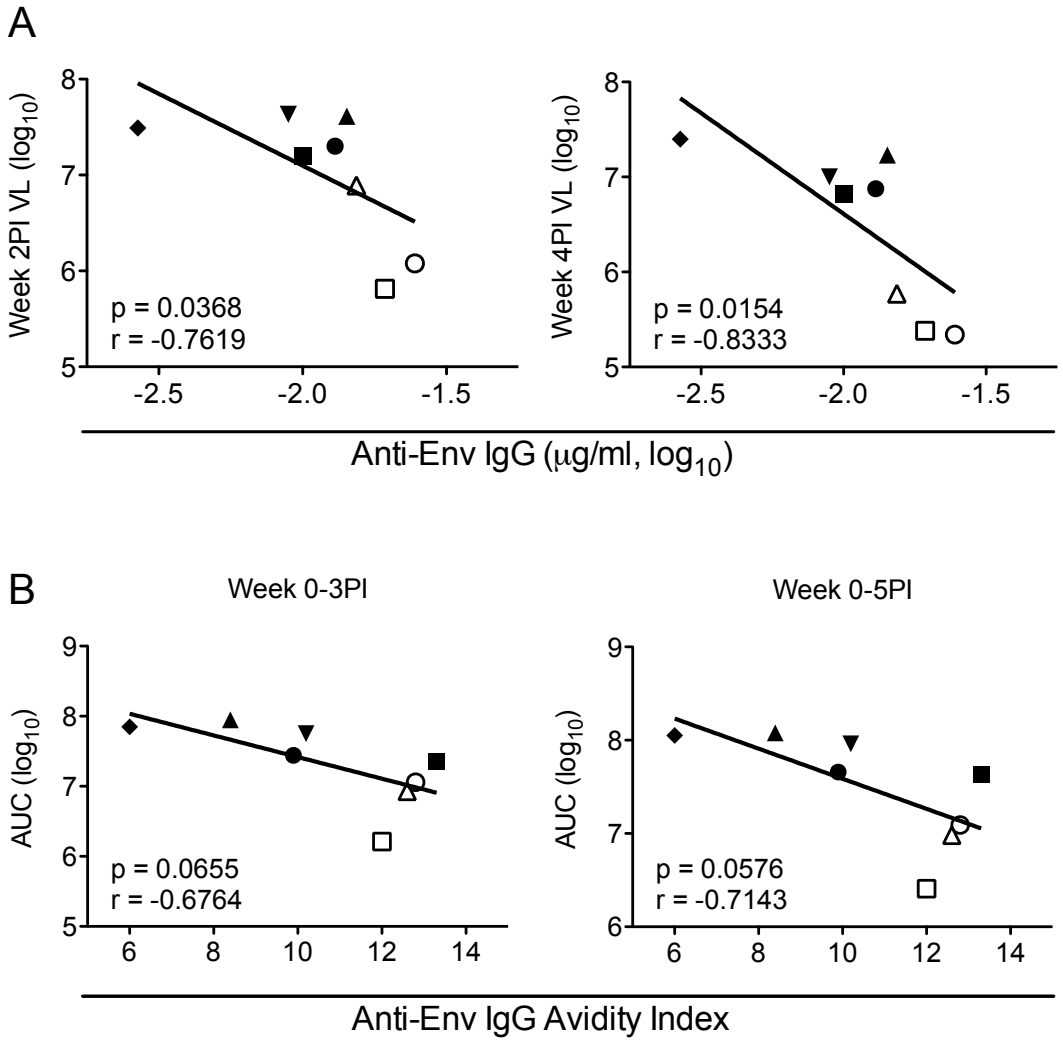


Figure 4.6. Env-specific IgG antibodies inversely correlate with peak viremia. A) The viral loads (VL) at 2 (left panel) and 4 (right panel) weeks post-infection (PI) for Group C animals inversely correlates with the plasma concentrations of anti-gp140 Env IgG binding antibody at week 9. Both viral load and IgG concentrations have been log-transformed. The correlation is statistically significant and strengthens between weeks 2 and 4 post-infection (p= 0.0368 and p= 0.0154, respectively). B) Area-under-the-curve (AUC) calculations represented for 0-3 weeks PI (left panel) and 0-5 weeks PI (right panel) against anti-gp140 Env IgG avidity index values measured in plasma at week 9. Although not significant, there is a trend towards inverse correlations between avidity and viremia; controller animals (open symbols) produce antibodies with the strongest avidity. Dark symbols are non-controller animals and open symbols are controllers. Symbols correspond to those used per animal in Figure 4.1C for viral load.

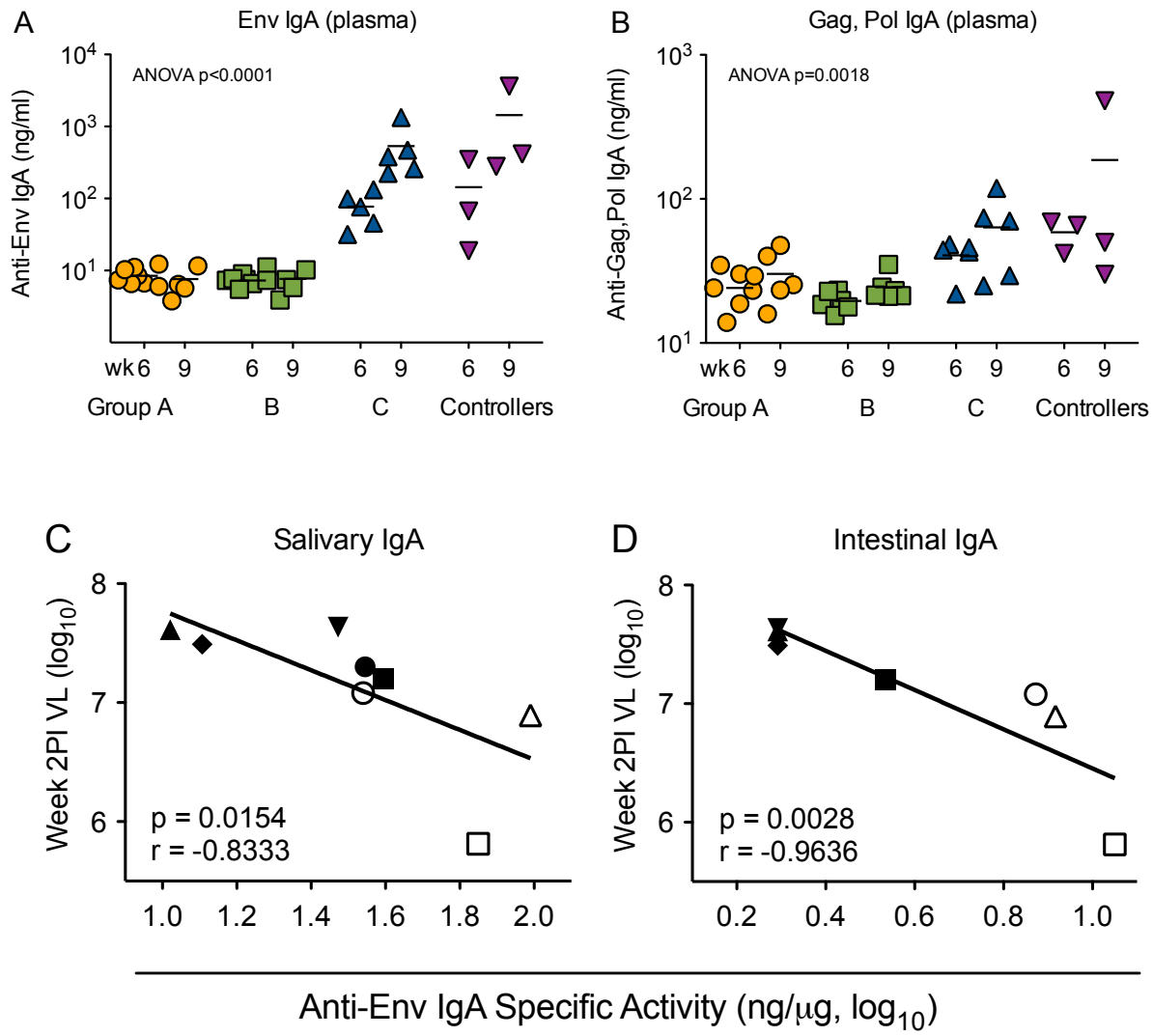


Figure 4.7 Plasma and mucosal SIV-specific IgA. A) Plasma anti-Env IgA and B) anti-Gag, Pol IgA (ng/ml) antibodies are improved in Group C animals following MVA-SIV boosting at week 6. Despite the ability of vaccine strains mc^{2}6435, mc^{2}6208 and mc^{2}6440 to express SIV Gag, Env and Pol respectively, Group B animals produce little to no SIV-specific plasma IgA. Group C animals all make anti-SIV IgA antibodies in plasma and concentrations are enhanced by week 9 following MVA-SIV boosting. There is not a statistically significant difference in the plasma IgA levels for Group C animals depending on ability to control viremia. C) Salivary and D) intestinally-secreted Env-specific IgA specific activities plotted against peak viremia (2 weeks PI). There was a significant, inverse correlation between peak viremia and salivary and intestinally secreted anti-Env mucosal IgA. Dark symbols are non-controller animals and open symbols are controllers. Symbols correspond to those used per animal in Figure 4.1C for viral load.

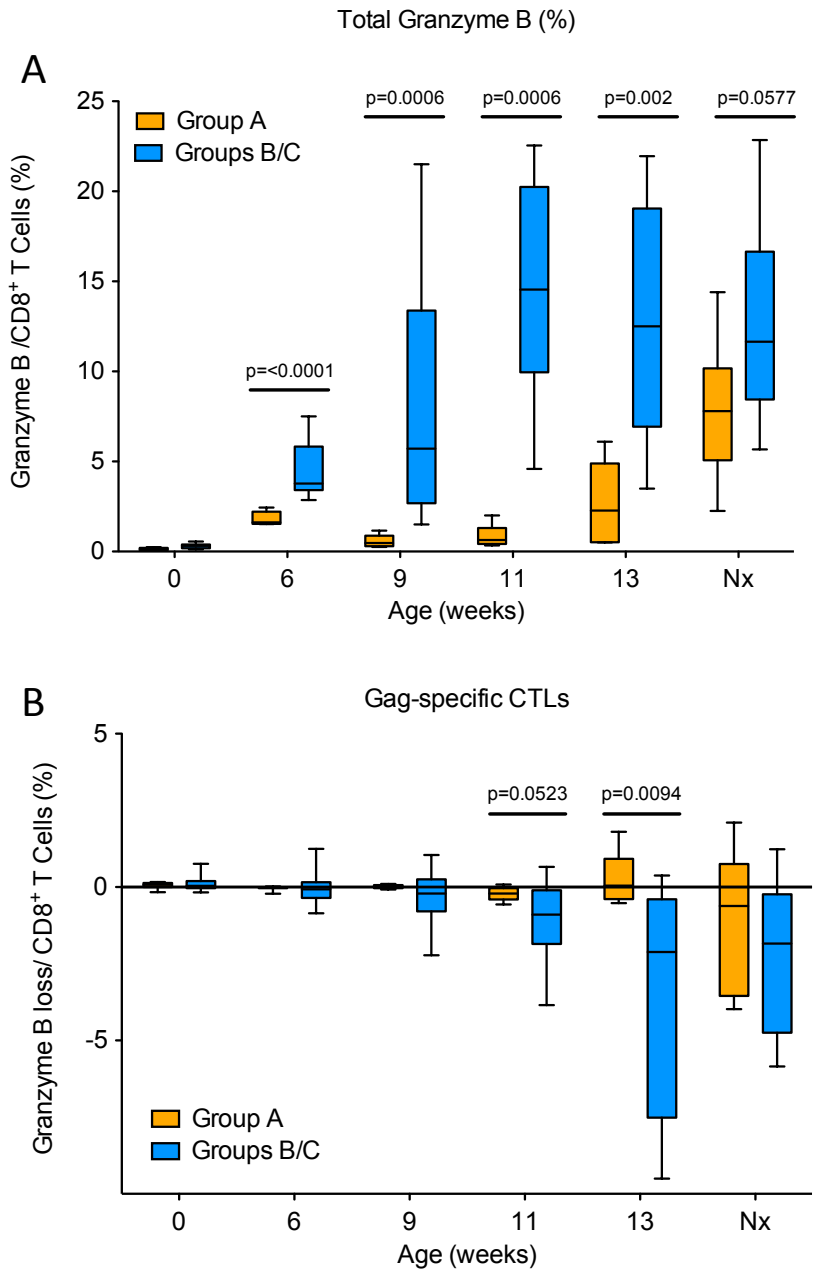


Figure 4.8. Granzyme B expression longitudinally in CD8$^+$ T cells. A) The percentage of unstimulated peripheral CD8$^+$ T cells expressing granzyme B over time developed more quickly in vaccinated animals (Groups B/C) than in mock-vaccinated animals (Group A), beginning by week 6. There is no statistical difference observed between Groups B, C or Controller animals in the ability of their CD8$^+$ T cells to produce granzyme B. The improved production of granzyme B following vaccination seemed unaffected by which boost was administered. B) The percentage of granzyme B loss in CD8$^+$ T cells following *in vitro* SIV Gag stimulation of PBMC. There is a trend of improved antigen-specific CTL degranulation in vaccinated animals (Groups B/C) compared to mock-vaccinated animals (Group A), although this difference only reached statistical significance at weeks 11 and 13.

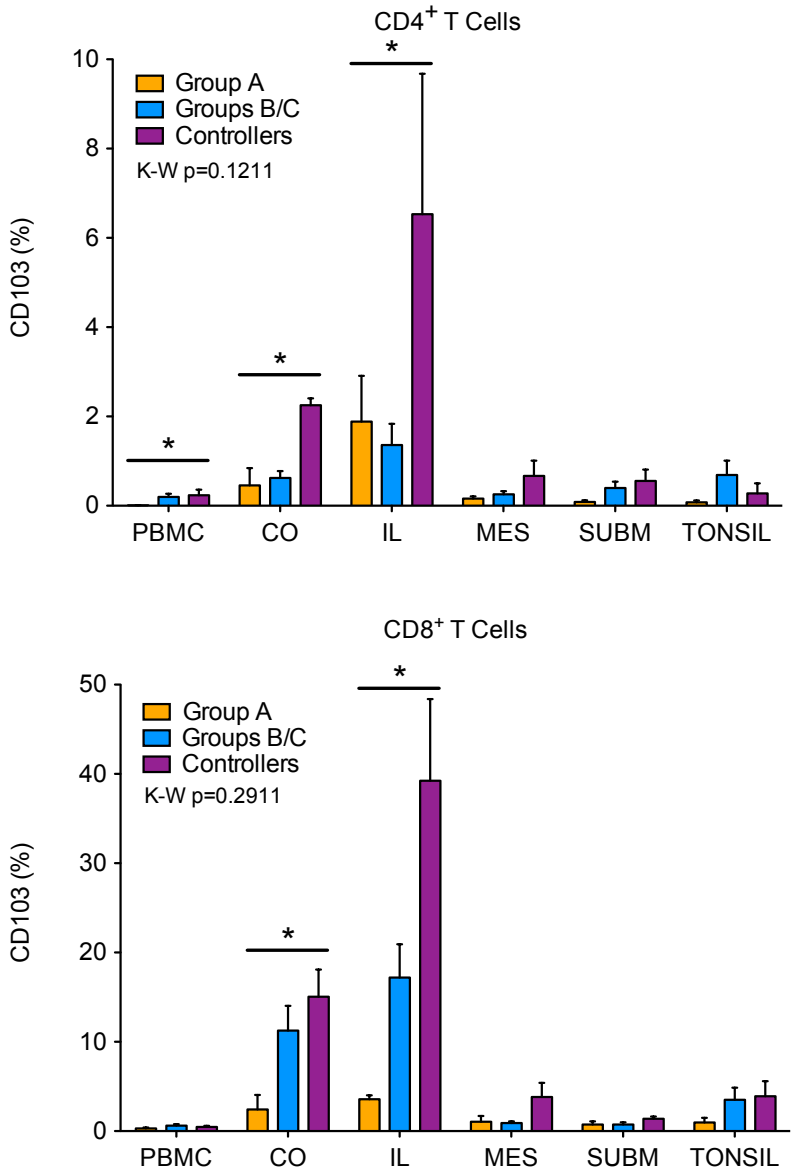


Figure 4.9. Expression of mucosal homing marker CD103 improved with vaccination. Vaccination improved $CD4^+$ (top panel) and $CD8^+$ (bottom panel) T cell expression of mucosal homing marker CD103. Improved expression of CD103 did not appear to correlate with the vaccination boost regimen (not shown) but expression was accentuated in Controller animals (purple) compared to non-controller Group B/C (blue) and mock vaccinated (Group A) animals. Group-specific expression differences were significant in the PBMC, colon and ileum ($CD4^+$ T cells) and in the colon and ileum ($CD8^+$ T cells), although several tissues exhibit a trend of vaccination-induced improved CD103 expression. PBMC= peripheral blood mononuclear cells; CO= colon; IL= ileum; MES= mesenteric LN; SUBM= submandibular LN.

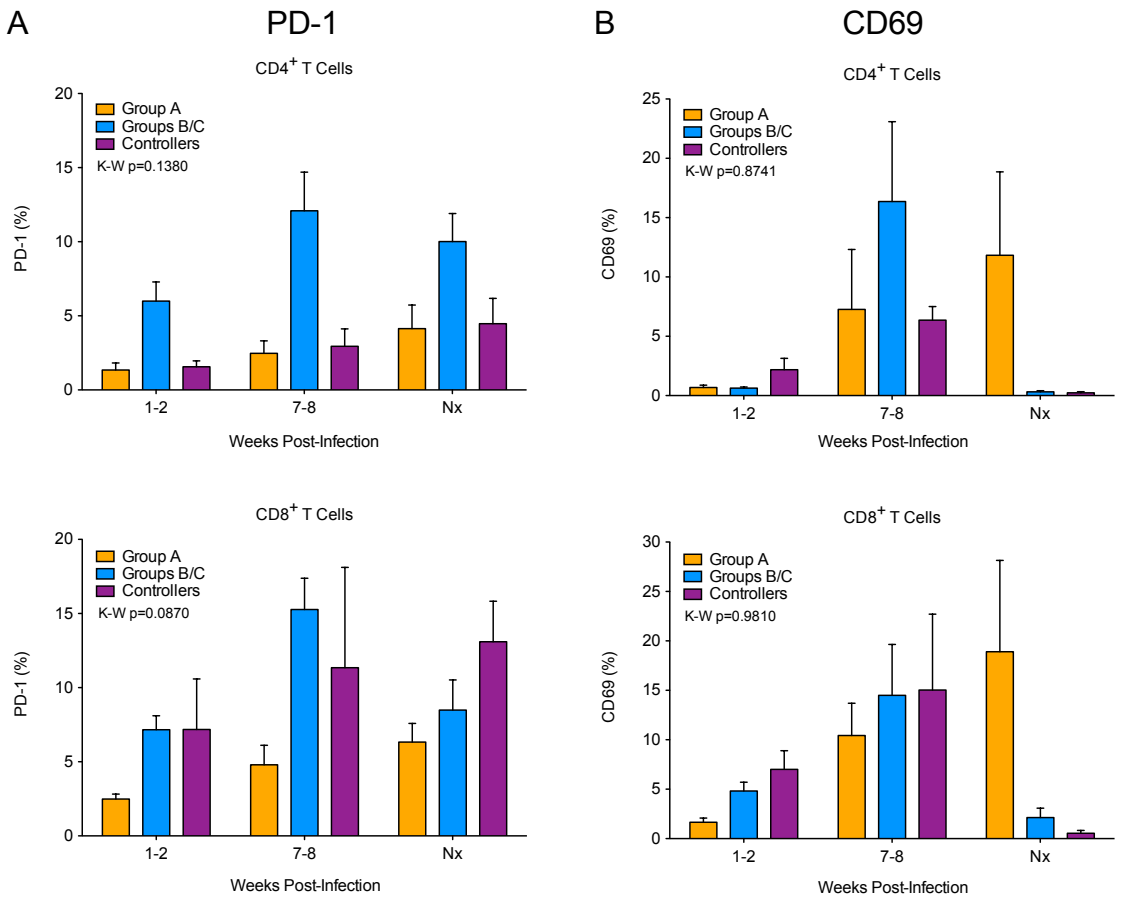


Figure 4.10. Expression of T cell activation and exhaustion markers in PBMC. A) PD-1 expression by PBMC $CD4^+$ (top left panel) and $CD8^+$ (bottom left panel) T cells. B) CD69 expression by PBMC $CD4^+$ (top right panel) and $CD8^+$ (bottom right panel) T cells was also enhanced in vaccinated. One-way ANOVA Kruskal-Walis p values are indicated per plot.

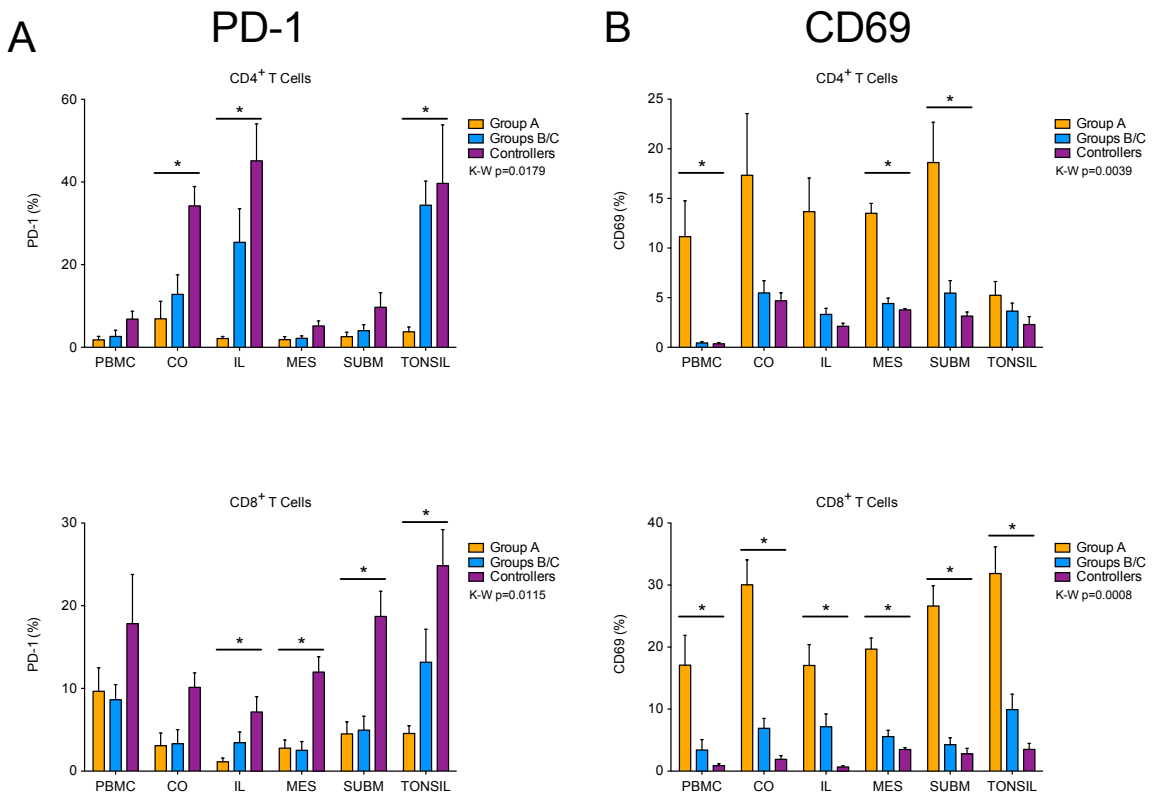


Figure 4.11. Expression of T cell activation and exhaustion markers in tissues. A) Tissue expression of PD-1 is distinctly inversed compared to CD69 with the highest levels of PD-1 expression in the tissues of Controller animals. Levels of PD-1 expression were statistically significant between groups in $CD4^+$ (upper left panel) T cells in the colon, ileum and tonsil and in $CD8^+$ (lower left panel) T cells in the ileum, mesenteric LN, submandibular LN and tonsil. In all tissues evaluated, the Controller animals had more expression of PD-1 in both T cell populations. Non-controller animals (Groups B/C) expressed similar levels of PD-1 compared to mock-vaccinated (Group A) animals, except in the ileum and tonsil, where non-controllers tended to express more PD-1. B) In contrast to the PBMC at week 9, CD69 expression at the terminal timepoint was enhanced in the tissues of mock-vaccinated (Group A, yellow) animals in both the $CD4^+$ (top right panel) and the $CD8^+$ (lower right panel) T cell populations. Statistical significance was observed in the PBMC, mesenteric LN and submandibular LN in the $CD4^+$ T cells and in all tissues in the $CD8^+$ T cells. No significant difference was observed between Groups B/C (blue) and Controller (purple) animals, although in every tissue evaluated, the Controller animals have equivalent or less CD69 expression compared to non-controller animals. All nonparametric one-way ANOVA tests were significant and p values are indicated below the legend. PBMC= peripheral blood mononuclear cells, CO= colon; IL= ileum; MES= mesenteric LN; SUBM= submandibular LN. Error bars indicate the SEM per group and $p<0.05$.

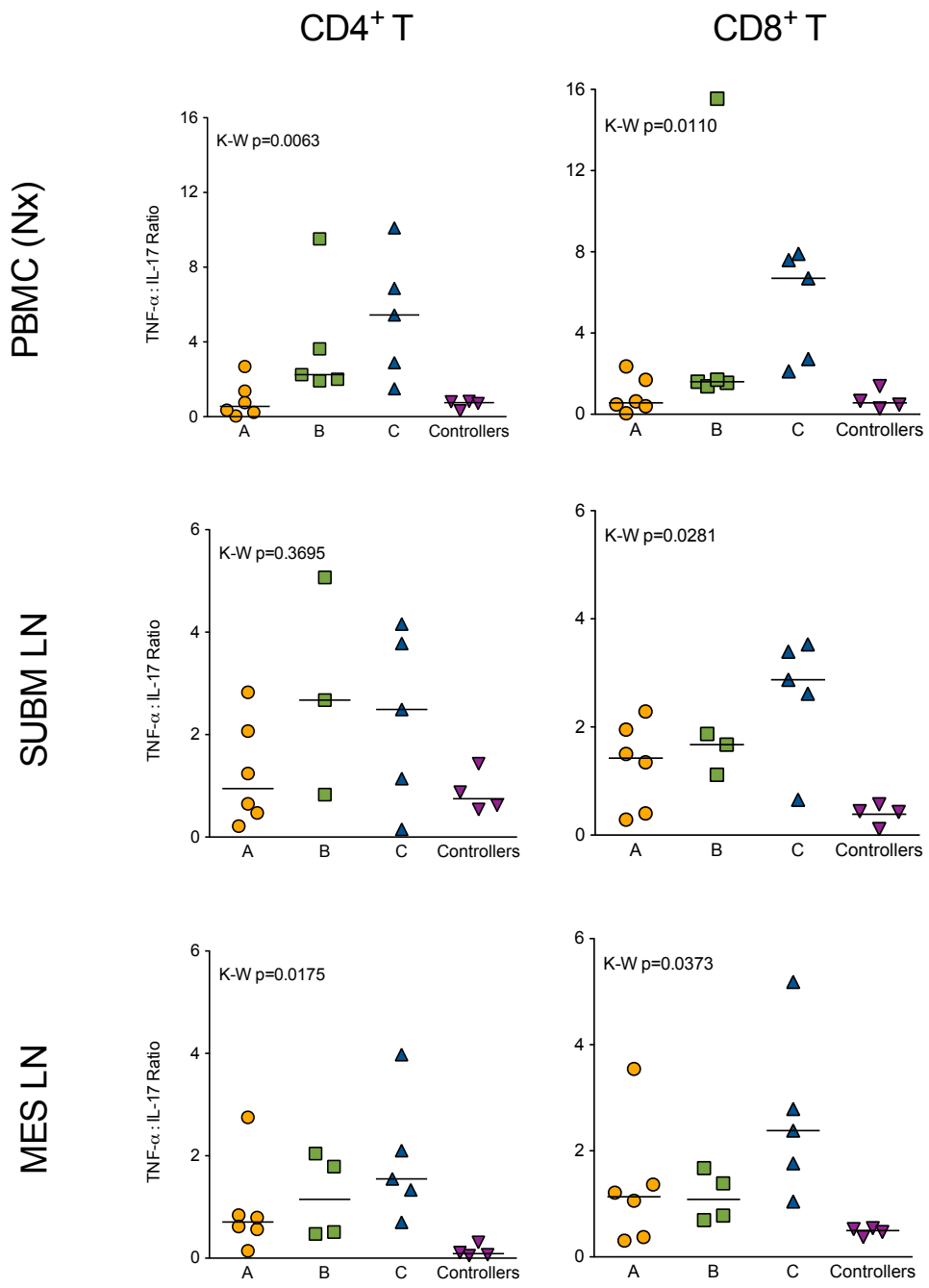
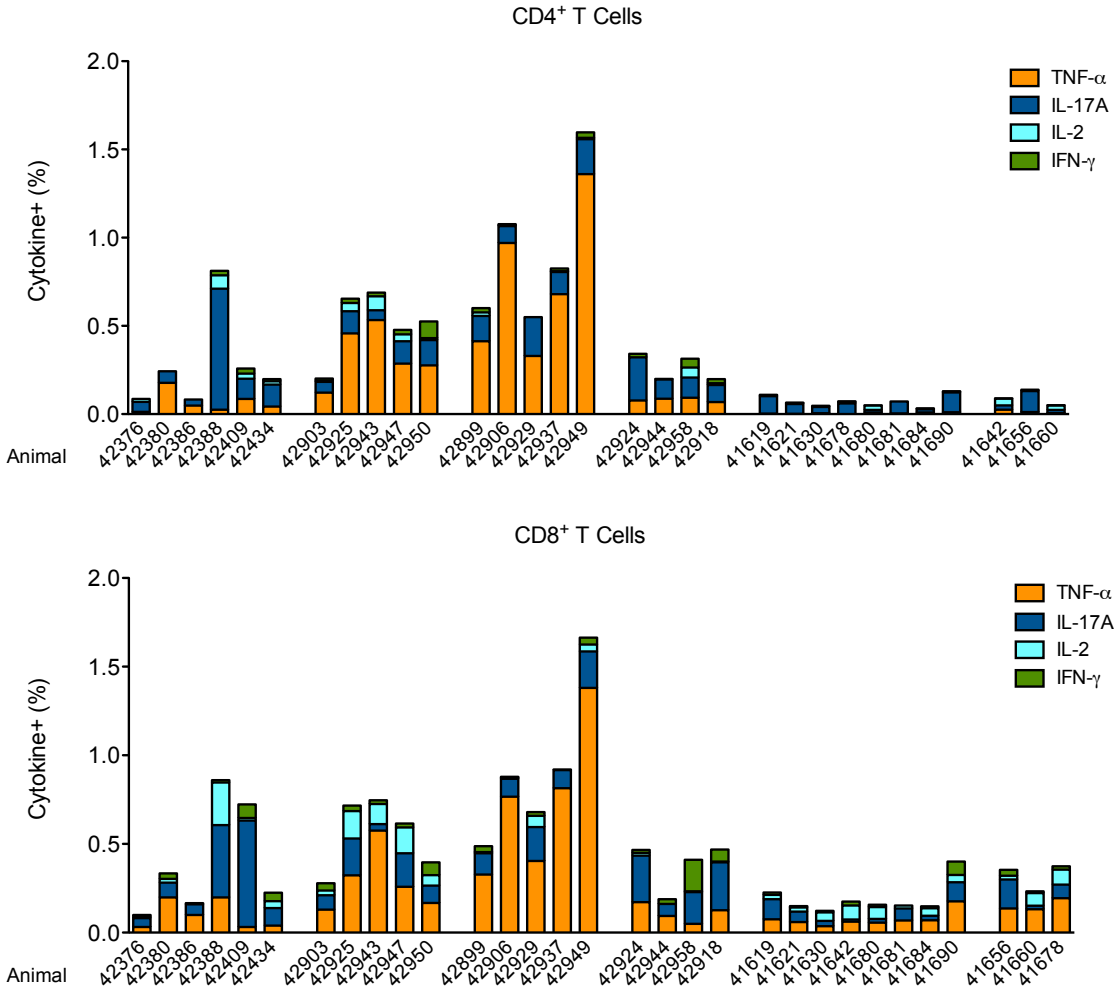
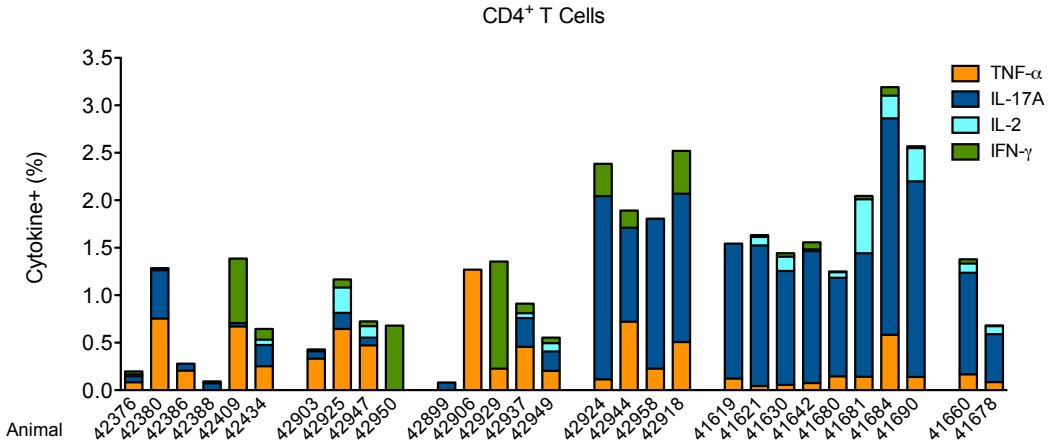


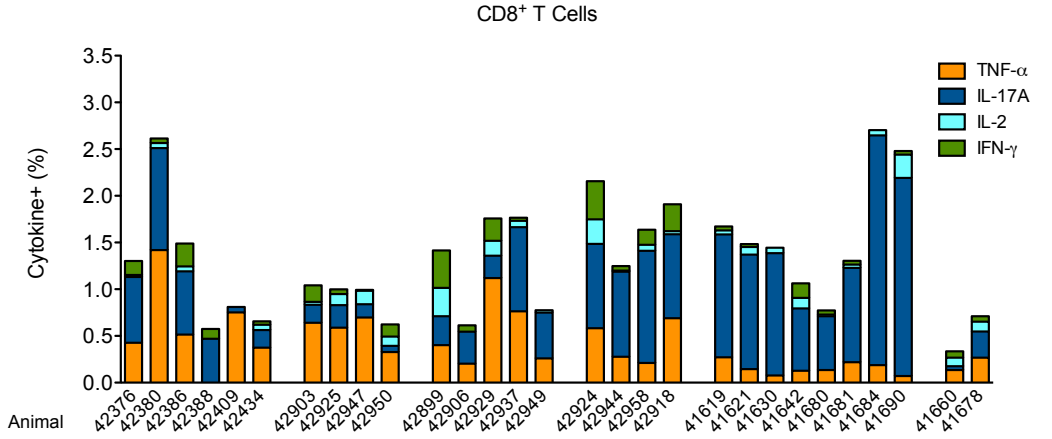
Figure 4.12. Ratio of TNF-α:IL-17 in unstimulated peripheral blood and tissue T cell populations. The ratio of TNF-α:IL-17 expressed basally in unstimulated $CD4^+$ (left) and $CD8^+$ (right) T cell populations in A) PBMC, B) submandibular LN (SUBM LN) and C) mesenteric LN (MES LN) cell suspensions from the terminal timepoint separated by mock-vaccinated (Group A, yellow), homologously-boosted vaccinated (Group B, green), MVA-SIV-boosted vaccinated (Group C, blue) and Controller (Group B/C, purple) animals. One-way ANOVA test significances are indicated within each plot. Only three tissues are represented but the trend of higher ratios was conserved in both T cell populations in all tissues assayed.



Group	A	B	C; non-controllers	B/C; controllers	D	E
Prime	mock	mc^{2}6435 mc^{2}6208	mc^{2}6435 mc^{2}6208	mc^{2}6435 mc^{2}6208	mc^{2}6435	mock
Boost(s)	mock	mc^{2}6435 mc^{2}6208 mc^{2}6440	MVA-SIV	varies[a]	MVA-SIV	mock
SIV	+	+	+	+	-	-

Figure 4.13. Basal cytokine production by PBMC T cells at the terminal timepoint. PBMC CD4$^+$ (upper) and CD8$^+$ (lower) T cell cytokine production in the absence of stimulation. The inherent cytokine production profiles sort according to vaccination group with the profile of the controller animals matching more similarly to SIV-naïve samples (Group D). Note that only single cytokine-positive events are represented for clarity.





Group	A	B	C; non-controllers	B/C; controllers	D	E
Prime	mock	$mc^2$6435 $mc^2$6208	$mc^2$6435 $mc^2$6208	$mc^2$6435 $mc^2$6208	$mc^2$6435	mock
Boost(s)	mock	$mc^2$6435 $mc^2$6208 $mc^2$6440	MVA-SIV	varies[a]	MVA-SIV	mock
SIV	+	+	+	+	-	-

Figure 4.14. Basal cytokine production by colon T cells at the terminal timepoint. Colon $CD4^+$ (upper) and $CD8^+$ (lower) T cell cytokine production in the absence of stimulation. The inherent cytokine production profiles sort according to vaccination group with the profile of the controller animals matching more similarly to SIV-naïve samples (Group D). Note that only single cytokine-positive events are represented for clarity.

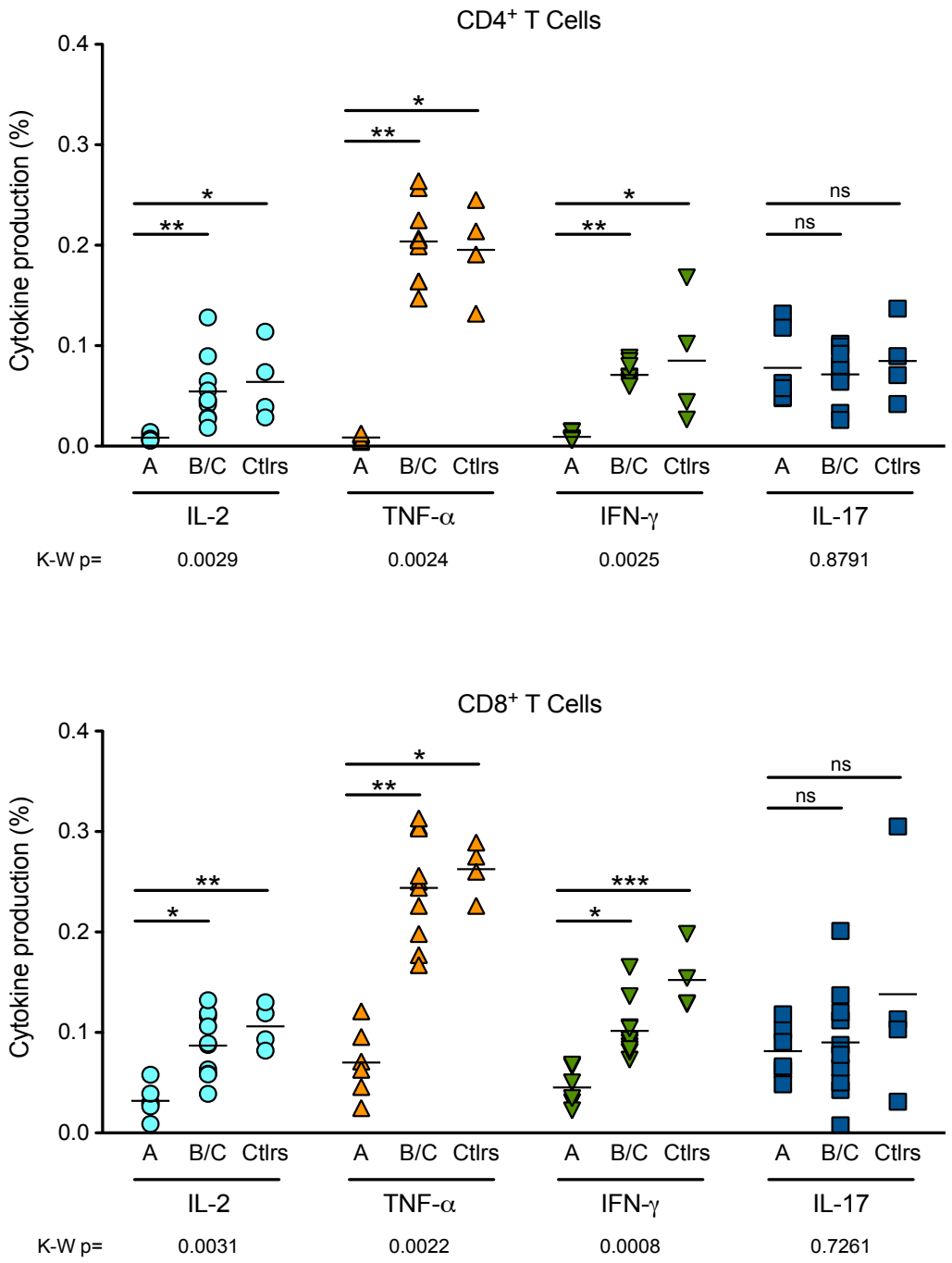


Figure 4.15. Basal cytokine production by T cells at the time of challenge. Increased immune activation following vaccination resulted in significantly greater levels of IL-2, TNF-α and IFN-γ in both $CD4^+$ (left) and $CD8^+$ (right) unstimulated T cells from blood at the time challenges were started (week 9). No differences were observed between groups in basal production of IL-17 in either T cell population. Mock vaccinated (Group A), homologously-boosted (Group B) and MVA-SIV-boosted (Group C) animals are indicated by A, B and C, respectively. Kruskal-Wallis nonparametric one-way ANOVA significance values are reported at the base of the figure for each cytokine.

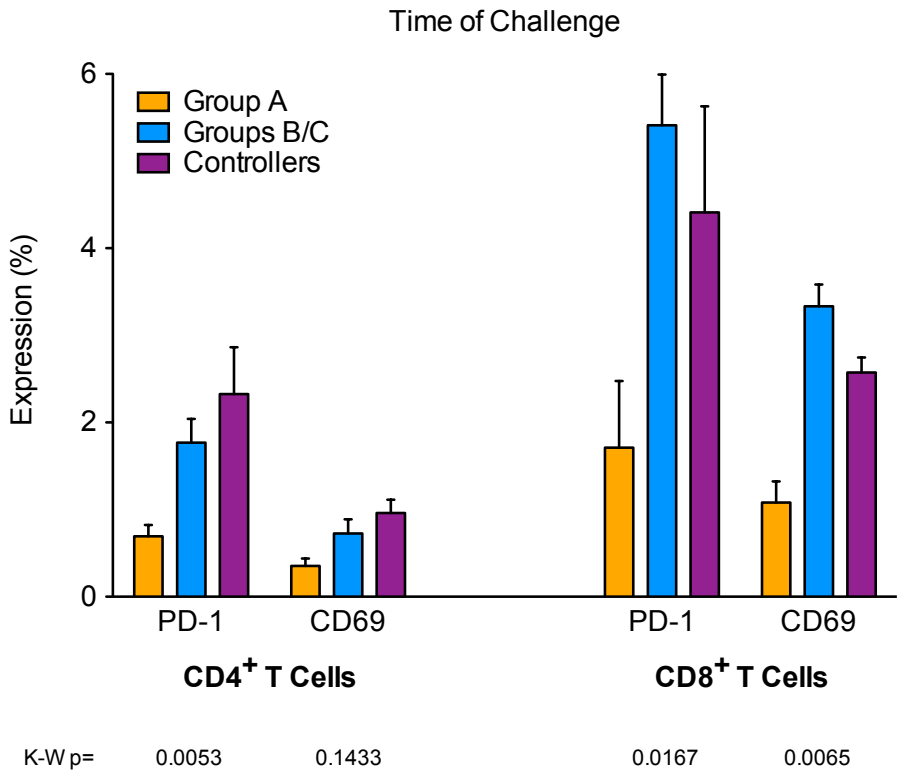


Figure 4.16. Vaccine-induced T cell activation and exhaustion at the time of challenge. Expression analysis of PD-1 and CD69 by PBMC T cells at week 9, the time of challenge. Greater frequencies of T cells from vaccinated animals, independent of whether they would later control viremia, expressed markers of activation and exhaustion compared to unvaccinated animals. One-way ANOVA indicated statistical significance for all comparisons except CD69 expression in $CD4^+$ T cells; p values are indicated above.

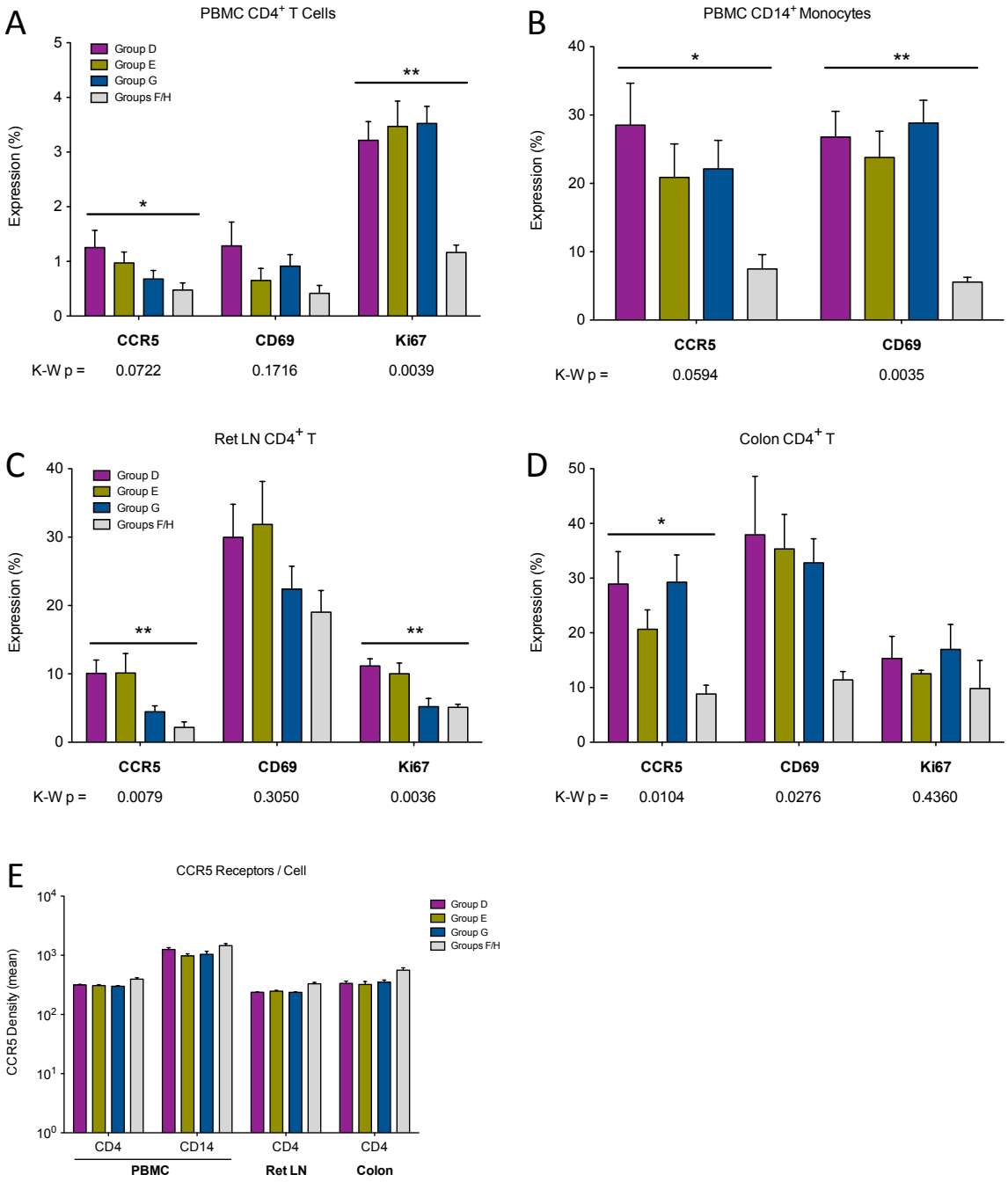


Figure 4.17. Vaccine-induced upregulation of CCR5 on T cells and monocytes. CCR5, CD69 and Ki-67 expression was assayed on cryopreserved cell suspensions of PBMC, retropharyngeal LN and colon from mc^{2}6435-vaccinated, SIV-naïve infant macaques (See Table 4.3 for Group assignments). Expression of CCR5 and T cell activation markers was enhanced in vaccinated animals in A) PBMC CD4$^+$ T cells (terminal timepoint), B) PBMC CD14$^+$ monocytes (terminal timepoint), C) Retropharyngeal LN CD4$^+$ T cells and D) Colon CD4$^+$ T cells. In every tissue, CCR5 expression was significantly enhanced after vaccination. Activation does not appear to be dependent on vaccine boost or route. Despite mc^{2}6435 vaccination at birth, activation does not resolve prior to necropsy at age 16-18 weeks. Nonparametric one-way ANOVA Kruskal-Wallis p values are reported under each figure;

two-tailed t-tests between treatment groups that were statistically significant are reported as asterisks (* $p < 0.05$, ** $p < 0.01$). Even in comparisons without statistical significance, trends of enhanced CCR5 and T cell activation marker expression are consistent with significant findings. E) Vaccination significantly expands the frequency of CCR5+ cells; the observed enhancement of CCR5 cannot be attributed to greater CCR5 expression on cells already expressing the receptor as calculated by quantifying the mean receptor expression per cell. There is no difference of CCR5 receptor density means across T cell and monocyte populations in different tissues. Although not statistically significant, there is a slight trend towards more CCR5 receptor density per cell in the mock animals (Groups F/H), not the vaccinated animals. Ki-67 was not evaluated in monocytes. Expression levels from Groups F and H mock-vaccinated animals were pooled for this analysis; there was no difference between Group F and H expression levels. Group D= $mc^2$6435 (i.d.) prime + Ad5-SIV boost; Group E= $mc^2$6435 (p.o.) prime + Ad5-SIV boost; Group G= $mc^2$6435 (p.o.) prime + MVA-SIV boost. i.d.= intradermal; p.o.= oral.

CHAPTER 5: DISCUSSION

INTRODUCTION

Infants represent an immunologically vulnerable population with an immature ability to recognize and respond to antigenic stimulation. A complete understanding of the mechanisms of immune ontogeny from birth through childhood remain uncharacterized, although studies in human cord blood, cross-sectional analyses in human infants and translational experiments using nonhuman primates at multiple ages have helped elucidate age-dependent changes in immune modulation. A comprehensive evaluation of the maturation of immune function with age will improve the quality of protective interventions targeted for young individuals.

The immaturity of the infant immune system also leads to increased disease susceptibility and morbidity following infections. Compared to an adult able to quickly and easily resolve an infection, infants progress more rapidly to severe symptoms and may not be able to clear an infection without medical intervention. For example, neonatal *Mycobacterium tuberculosis* infections can manifest with different pathologies than the same infection in adult. Infant *Mtb* infections can rapidly progress to miliary or meningeal TB, leading to complications in cognitive development, organ failure and death.

The administration of the BCG vaccine at birth offers protection for infants against more extensive TB disease. BCG, used ubiquitously around the globe to vaccinate >90% of newborns, can induce persistent cellular immunity via the T_h1 lineage even in the immature

immune environment of the infant. BCG-derived protection, however, wanes dramatically, offering little protection against adult pulmonary TB infections. Further, the live attenuated nature of the vaccine is not safe for use in immunosuppressed individuals, such as those infected with HIV and the WHO advises against BCG vaccination for infants infected with HIV or at high risk for infection. HIV infection in the infant, like *Mtb*, rapidly progresses to immunosuppression and commonly co-infections with *Mtb*. A safe and effective dual vaccine is urgently needed.

Borrowing effective components from BCG but improving elements of safety and immune persistence, a highly attenuated human *M. tuberculosis* strain may have improved immunogenicity in infants. Serial passaging of *M. bovis* attenuated the bacteria for pathogenicity and virulence, but also resulted in loss of immunodominant epitopes important for persistent immunity. Intentional deletions of genes important for replication, immune evasion and virulence in *Mtb*, with the insertion of a recombinant plasmid expressing conserved HIV genes could produce a dually immunogenic, safe infant vaccine to protect against both *Mtb* and HIV infections.

FINDINGS AND IMPLICATIONS

My central dissertation objective was to test the safety, immunogenicity and protective capacity of novel attenuated *Mycobacterium tuberculosis* strains that expressed SIV genes in SIV-naïve and SIV-infected, immunosuppressed rhesus macaques. Three *Mtb* strains with variable levels of attenuation were compared for safety and immunogenicity; two strains were further manipulated to co-express SIV genes in a first attempt to induce the development of dually immunogenic infant vaccine.

Because our vaccine strains share similarities with BCG, it was necessary to first

confirm complete safety in immunocompromised infants. First, we rigorously evaluated the safety of the live attenuated *Mtb* immunogens by vaccinating healthy and SIV-infected, immunosuppressed neonates. Animals were followed for up to six months and safety was evaluated using outgrowth cultures supplemented for auxotroph growth, acid-fast staining, gross pathology and clinical parameters. Safety varied according to the severity of the bacterial attenuations, with the least attenuated strain ($mc^2$5157) causing bacterial dissemination by six weeks of age. Strain $mc^2$6020 was moderately attenuated for bacterial replication and was mostly safe with a couple of bacilli detectable in only the draining LN of one SIV+ animal. The strain most attenuated for both bacterial replication, immune evasion and virulence ($mc^2$6435) was next tested and found to be completely safe, even in immunocompromised, SIV-infected neonatal macaques. Strain $mc^2$6435 has been evaluated most thoroughly of the three strains, tested for safety in healthy (n=20) and SIV-infected (n=17) infant macaques with no bacterial dissemination by outgrowth assays, acid-fast bacilli in tissues, pathological indications of *Mtb* infection or clinical symptoms. Further, vaccine safety was independent of whether vaccination occurred before SIV infection.

To optimize vaccine immunogenicity, we compared i) route of inoculation, ii) boost regimens and iii) prime-boost intervals. Although now infants are intradermally immunized, BCG was initially administered orally. Because we aimed to develop a combination vaccine to protect infants against vertical transmission of HIV in breast milk, we hypothesized that an oral vaccine would confer the most robust immune response at the site of vaccination and sought to compare intradermal and oral vaccination routes for immunogenicity. We observed that animals orally and intradermally vaccinated with strain $mc^2$6435 were capable of inducing SIV- and *Mtb*-specific cellular and humoral immune responses. Interestingly, we

did observe some small differences in antibody development depending on the route of vaccination. Intradermally vaccinated animals induced a more robust anti-PSTS1 *Mtb* plasma IgG antibody response compared to orally vaccinated animals (Figure 5.1); anti-PSTS1 responses also inversely correlated with bacterial attenuation in that the least attenuated strains induced the highest magnitude of antibodies.

While these differences appear trivial in that we observed vaccination route-specific differences for one anti-*Mtb* antibody, the more imperative point is that differences in vaccine regimens, such as route of administration, can have important implications in the quality and magnitude of immunity elicited in response to vaccination. It is essential to consider both how the vaccinated individual responds to the immunogen (e.g. age-associated immune immaturity or immunosuppression) *and* the type of immune response best able to induce sterilizing protection. For example, although there is still much unknown about immunomodulation during *Mtb* infection, TB is primarily mediated by cellular immunity and not by antibodies. An *Mtb* vaccine candidate, even if capable of inducing stellar humoral responses, likely will offer little protection.

Next, we evaluated the effect of a two or three-week prime-boost interval on the development of dual immunogenicity. While an effective infant vaccine must work quickly to protect against exposure of HIV in breast milk, boosting the immune response too soon after priming can result in activation induced cell death (AICD). AICD, Fas-FasL mediated programmed cellular death, occurs in instances of repeated exposure at specific T/B cell receptors as a mechanism of eliminating autoreactive T/B cells. However, AICD can also occur following secondary exposure to an immunogen for which the primary immune response has not yet completed clonal deletion. In the presence of expanded antigen-specific

lymphocytes, a vaccine boost can cause accelerated loss of the specific cells and essentially negate the effect of vaccination. Because we used live attenuated vaccine strains, we hypothesized that boosting three weeks post-prime might serve to avoid AICD. Although we did not directly test for AICD, we did not observe any differences in immune development in animals boosted at two weeks compared to three weeks, however, animals were only followed for a short time and differences may not have been apparent at that early timepoint.

After the route of vaccination and the prime-boost interval comparisons offered no distinct differences, we next aimed to elucidate the effect of different boost immunogens on the quality of the immune response. Because we primed animals with an attenuated bacterium expressing exogenous viral proteins, we hypothesized that the SIV-specific responses would likely require a systemic boost. Two replication-attenuated recombinant viral vectors expressing SIV *Gag, Pol* and *Env* were evaluated. Because of its excellent ability to prompt the development of the cellular immune response, we tested adenovirus 5-SIV (Ad5-SIV) following either oral or intradermal priming with $mc^2$6435. Secondly, animals orally primed with $mc^2$6435 were boosted with modified vaccinia Ankara-SIV (MVA-SIV). In both cohorts, we observe the development and persistence of SIV-specific cellular and humoral immune responses; subsequent studies used the MVA-SIV due to the challenges associated with Ad5 seroprevalence within the human population. Following MVA-SIV boosting, although there is not typically a distinct boost effect in the SIV-specific T cell response, we do observe improvements to the antibody responses.

After confirming safety and working to optimize vaccination regimens, we opted to evaluate the ability of oral $mc^2$6435 vaccination at birth plus MVA-SIV boosting at weeks 3 and 6 to protect infant macaques against low-dose oral SIV challenges, mimicking human

HIV exposure in breast milk. In addition, a group of animals was also homologously boosted with A*Mtb* strains for comparison.

The study responses were surprising and early viral load data suggested even possible vaccination-induced hyperinfectivity because the vaccinated animals were becoming infected more quickly than unvaccinated animals. Indeed, following statistical analysis, we observed a 1.96 relative infectivity rate, indicating that vaccination resulted in a nearly 2x enhancement in infection. Despite animals developing and maintaining cellular and antibody responses as expected according to previous immunogenicity optimization studies, vaccination was accelerating infection.

To attempt to explain the mechanism by which this was occurring, we performed several evaluations. First, I observed that animals had high cytokine expression background in unstimulated samples. Next, the high background stratified into either TNF-α-producing or IL-17-producing. After comparing the ratios of TNF-α:IL-17, it became apparent that vaccinated animals were more likely to have TNF-α expression while the unvaccinated animals predominantly produced IL-17. Interestingly, the trend of increased cytokine production was apparent also at the time the SIV challenges were initiated, and not just in the tissues at the terminal timepoint. Although only blood samples were available at week 9 (start of challenges), TNF-α, IL-2 and IFN- γ were all significantly upregulated in vaccinated animals compared to mock-vaccinated controls. The cytokine production was not specific to the vaccine regimen, suggesting that the initial A*Mtb* prime at birth induced similar levels of activation in both vaccine groups. Further, despite subsequent SIV infection, increased cytokine production was maintained until the terminal timepoints, with vaccinated animals expressing greater levels of cytokines in unstimulated tissue cell suspensions as well.

To ensure that the observed non-specific immune activation was indeed an artifact of vaccination, I evaluated the levels of activation in cryopreserved cell suspensions from SIV-naïve animals in previous studies with *Mtb* strains $mc^2$6020, $mc^2$5157 and $mc^2$6435. In vaccinated animals, independent of the vaccine strain, T cells and monocytes were significantly more activated compared to mock vaccinated samples from the same study cohorts. Importantly, the $CD4^+$ T cells in retropharyngeal lymph node and colon, and $CD4^+$ and $CD14^+$ monocytes in PBMC cell suspensions expressed significantly more CCR5 compared to unvaccinated animals. The increase in CCR5 was actually an increase in the number of cells expressing the receptor and not just more CCR5 expression on cells already the receptor. Because HIV preferentially infects activated $CD4^+$ cells, most commonly using CCR5 as its co-receptor, the implications of increased CCR5 expression in an already activated cytokine milieu are incredibly problematic for infants with daily exposures to HIV.

Although vaccination conferred no protection against the acquisition of SIV, we did observe a strong, inverse correlation with the production of anti-Env mucosal IgA from both saliva and the intestine with peak viremia. Three of eight animals in Group C experienced reduced peak viremia, reduced viral set points and were able to control/reduce viremia for several months after infection. While other Group C animals experienced enhanced SIV-induced pathologies and opportunistic infections, the three 'Controller' animals had none, despite having been SIV-infected for about 10 weeks longer. Further, the Controller animals maintained T cell populations and IL-17 production at the intestinal mucosa better than non-controllers. While sterilizing protection is the gold standard of vaccine development, an intervention that could delay disease progression, maintain the integrity of the mucosal surface and limit immune cell depletion could prove beneficial, particularly in resource-

limited regions with limited access to ART.

REMAIMING QUESTIONS AND FUTURE DIRECTIONS

The experiments outlined as part of this dissertation have contributed to our understanding of infant immune development, antigen-specific responses to vaccination and the inability of attenuated *Mtb* vaccine strain $mc^2$6435 to protect against oral SIV challenge using our vaccination regimen in Chapter 4. The study questions below aim to specifically address the known limitations of completed experiments and help guide the further refinement of attenuated *Mtb* vaccine candidates as dual immunogens.

VACCINE SAFETY

How long-lived is the vaccine-induced immune activation? Is long-lived activation the mechanism of enhanced SIV infection? Would immune activation be resolved more quickly in an older individual? Does BCG induce long-lived activation as well?

We learned in Chapter 4 that vaccination with $mc^2$6435 induced persistent immune activation in both peripheral blood and tissues. Despite waiting to initiate SIV challenges until week 9, we hypothesize that the immune activation, including upregulation of CCR5 expressing-cells, led to enhanced infectivity in vaccinated infants, although future studies will be required to confirm if vaccination-induced immune activation is actually the mechanism by which we observed increased infectivity. If we could understand how long the non-specific immune activation persists and if there was an age-associated effect in that infants were less able to resolve immune activation compared to older individuals, it might help elucidate the length of time that infectivity could be enhanced in vaccinated infants. Of particular concern is that the A*Mtb* vaccine strains share significant homology to the live

attenuated BCG strain that is used to vaccinate >90% infants worldwide. If BCG shares the ability of A*Mtb* to induce persistent systemic immune activation and upregulation of CCR5 expression, BCG vaccination may also serve to enhance HIV transmission in infants exposed to the virus. While this hypothesis requires much additional study prior to validation, the potential implications of attenuated mycobacteria vaccine-enhanced HIV infection has important public health implications, justifying a thorough investigation in the near future.

VACCINE EFFICACY

Does vaccination with attenuated Mtb *vaccine strains induce immune responses comparable to BCG vaccination? How long are specific immune responses maintained?*

Despite the importance of a direct comparison of A*Mtb* vaccination to BCG, study group sizes were financially restricted from including a BCG group. We hypothesized that using a human-adapted *Mtb* vaccine strain could confer immune responses with improved persistence, particularly for older individuals who are at risk for pulmonary TB infections and for whom BCG-induced immunity has waned.

*Does vaccination with A*Mtb *strains protect against* Mtb *challenge? Is protection comparable to BCG?*

An *Mtb* challenge study to evaluate *Mtb*-specific protection will serve to test the dual immunogenicity of the recombinant vaccine approach. Importantly, this study should also aim to compare the protective capacities of A*Mtb* and BCG. Because the live attenuated vaccine prime is replication-limited mycobacteria and particularly because *Mtb* does not preferentially infect activated immune cells, we hypothesize that vaccination to protect

against *Mtb* infections could be more successful compared to SIV challenges. In addition, vaccinated animals induced persistent *Mtb*-specific cellular immune responses, even in the absence of boosting *Mtb*-specific immunity.

Does improved recombinant protein expression and/or secretion improve vaccine effectiveness?

In Chapter 4, we showed that Group B animals that received the homologous A*Mtb* strain boost developed no anti-Env or anti-Gag, Pol IgA antibodies prior to SIV infection but Group C animals boosted with MVA-SIV induced significant levels of both SIV-specific IgA antibodies. A similar trend was observed for SIV-specific IgG antibodies. We hypothesize that the failure of Group B animals to produce SIV-specific antibodies suggests that $mc^2$6435-SIV was not successfully producing and/or secreting SIV proteins. One way to potentially improve the antibody responses that we observed to inversely correlate with peak viremia would be to enhance the ability of the bacteria to express and secrete recombinant SIV proteins.

Although we observed no vaccine-induced protection, a caveat of the interpretation of these data is that the recombinant vaccine strains may not have been optimized to perform as expected. We can illustrate this with the SIV Env-specific antibody responses at weeks 6 and 9 in Groups B and C in the challenge study (Chapter 4). Group B animals, primed and homologously-boosted with recombinant *Mtb*-SIV strains were unable to produce any SIV-specific antibodies prior to SIV infection. Conversely, Group C animals, boosted instead with MVA-SIV vaccinations, all produced SIV-specific antibodies at weeks 6 and 9, prior to SIV challenging. Therefore, if the *Mtb*-SIV strains were producing SIV antigens as

efficiently as the MVA-SIV, we may have observed improved antibody levels in Group B animals. *Mtb*-SIV vaccination can induce the development of anti-SIV Gag, Pol antibodies, so we can pinpoint the failure of the *Mtb*-SIV Env strain, $mc^2$6208. Therefore, while we are unconvinced that homologous boosting with recombinant *Mtb* stains would be effective against SIV challenges, we can not confirm this hypothesis without further strain optimization and testing to improve Env expression and secretion.

As a further point, the efficacy of neutralizing antibodies for limiting HIV acquisition has been addressed multiple times in this thesis but not explicitly the concept of *Mtb*-SIV vaccine-induced neutralizing antibodies. We acknowledge that *Mtb*-SIV strains are not designed to elicit the development of Env-specific neutralizing antibodies. While the mechanisms of how broadly neutralizing antibodies are induced through vaccination are not fully elucidated, our vaccine regimen could be tailored to drive favorable antibody responses by including booster immunogens known to prompt the development of neutralizing antibodies, particularly at those mucosal surfaces exposed to virus. For example, the complexities of the HIV envelope protein, such as high rates of mutation, exposed epitopes with variable confirmations and concealed conserved sequences, demand intelligent immune design. As more broadly neutralizing antibodies are isolated in the clinic and their functional characteristics are deciphered, vaccine boosts can be designed to recapitulate their neutralizing function (48, 125, 211, 223, 301). While much attention has highlighted the importance of broadly neutralizing antibodies (bNAbs), the titers of neutralizing antibodies in HIV-infected individuals are typically quite low compared to non-neutralizing antibodies (273, 366). Perhaps the difficulties the immune response encounters inducing potent bNAbs

in natural infections is indicative of vaccine-induced bNAbs as well. Humoral mechanisms in addition to neutralization, such as ADCVI, provide protective benefits as well and should be included during vaccine design discussions (93). Antibodies recognizing linear epitopes also proved beneficial as the primary correlate of protection in the recent RV144 trial (110), highlighting an important role for abundant binding antibodies with high avidities for reducing HIV transmission. I hypothesize, however, that, especially in the unique immune system of the infant, an effective protective vaccine candidate will require a robust and activating prime, such as *Mtb*-SIV strains, followed by protein, subunit or recombinant viral boosting to drive the induction of humoral immunity. As we gain further understanding regarding the induction of neutralizing antibodies, vaccine regimens will only get more potent and effective.

MECHANISMS OF VACCINE ACTIVATION

*What mechanism(s) of A*Mtb *vaccination improve mDC function and cause immune activation?*

Vaccinated animals exhibit improved myeloid dendritic cell (mDC) function following treatment with TLR agonists compared to unvaccinated animals (Figure 5.2A). The trend of enhanced mDC function is observed longitudinally in peripheral blood and in tissues many weeks after vaccination but it is unclear by what mechanism mDC function is improved, considering that $mc^2$6435 is a double auxotroph strain and replication-incompetent *in vivo*. Interestingly, while we observed enhanced mDC cytokine production following TLR stimulation in vaccinated animals, mDCs do not exhibit non-specific cytokine production that occurs in the T cell populations in the same animals (Figure 5.2B).

If the bacteria strains are severely attenuated for replication and unable to propagate *in vivo*, the bacilli should be cleared, allowing the infant to dampen its immune activation and return to a state of equilibrium. Therefore, it is unclear why such robust immune activation persists for up to 34 weeks post-$mc^2$6435 vaccination. Elucidating the mechanisms of immune activation will be an important component to identify whether or not attenuated mycobacteria should be used as infant vaccines.

CONCLUDING REMARKS

Vaccinology is a tricky subject these days. Diseases like smallpox, measles and polio are so removed from our memories and our developed world that it hardly seems necessary to vaccinate; after all- out of sight, out of mind. Unfortunately, vaccines have been negatively discussed as the causative agents for a number of otherwise unexplained health complications, most notably for cognitive impairments including the autism spectrum disorders, attention deficit disorder (ADD) and attention deficit hyperactivity disorder (ADHD). While these allegations have been formally and scientifically discredited (60, 72), national media attention and celebrity endorsements have villainized vaccinations, particularly those administered during childhood. Despite public retractions, the popularity of vaccinations has not fully rebounded with more parents opting to delay or altogether forego advised vaccination regimens. At an individual level, this decision is seemingly negligible but, at a population level, declining vaccine-conferred immunity has dramatic implications for public health.

In recent years, we have observed this phenomenon in the U.S. with the reemergence

of pediatric pertussis and measles infections following declining levels of vaccine coverage. Pertussis and measles are two vaccine-preventable infections but parents choosing not vaccinate themselves and/or their children have resulted in epidemic levels of infections. For example, in August 2013, the anti-vaccination Eagle Mountain International Church in Newark, TX reported over 20 cases of measles within their congregation (313). The virus, brought to the megachurch by a visitor infected while traveling in southeast Asia, readily infected many congregants, most of whom were not vaccinated. Another example is the severe respiratory infection pertussis, or whooping cough, which is also vaccine-preventable and almost exclusively infects infants and young children. In 2012 the U.S. reported the highest number of cases of pertussis since 1955, with increases in 49 states due to failure to vaccinate (12, 42). It seems difficult to justify pediatric morbidity and mortality from vaccine-preventable infections.

The inherent difficulties of designing safe, effective and long-lived HIV vaccine candidates has been demonstrated by the lack of success by even the brightest and most innovative researchers across the globe. While we have learned an impressive amount since HIV was first identified- it's mechanisms of transmission, entry, replication and integration, the necessary immune responses required to combat infection and multiple designer therapeutic interventions that can essentially restore patient quality of life - it is apparent that HIV is a virus worthy of our utmost respect as virologists.

In 1984, following a conversation with the co-founder of HIV Robert Gallo, US Health and Human Services Secretary Margaret Heckler famously declared that a vaccine could be ready for testing in about two years. While this prediction was perhaps too optimistic, the unique challenges surrounding HIV vaccine development continue to elude

researchers three decades later. The best we can offer is our dedicated combinatorial approach towards preventing new infections, prolific testing and education, improved access to and distribution of HAART therapies alongside continued vaccine development efforts to someday live in a world no longer afflicted with HIV.

FIGURES

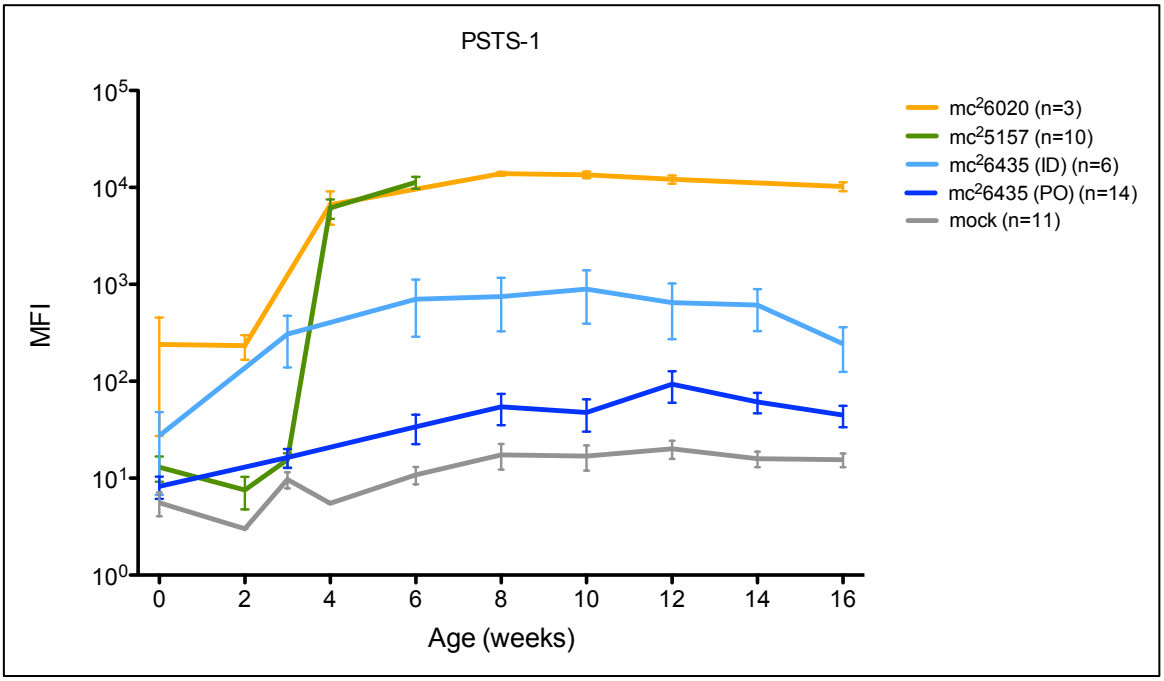


Figure 5.1. The development of anti-PSTS1 plasma IgG following vaccination. Vaccination with one of three distinct bacterial strains by both p.o./i.d. (mc^{2}6020 and mc^{2}5157) or by p.o. only (dark blue) or i.d. only (light blue) induced plasma PSTS1-specific IgG. Error bars show standard errors of the mean within a group. The grey line is age-matched, mock-vaccinated control animals. p.o.= oral; i.d.= intradermal. MFI is an arbitrary unit normalized within the Luminex assay.

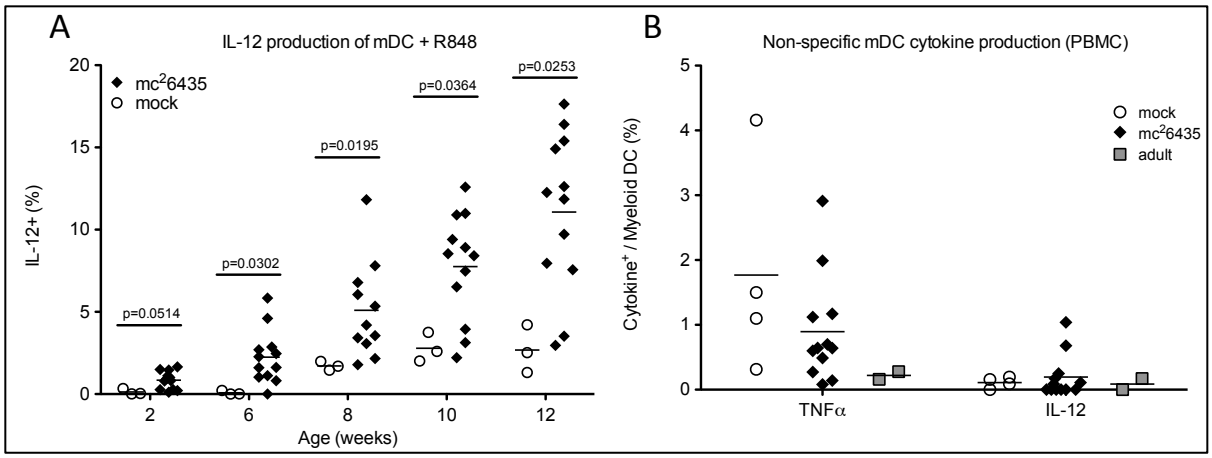


Figure 5.2. Effects of mc^{2}6435 vaccination on myeloid dendritic cells. A) Vaccination with mc^{2}6435 enhances the ability of mDCs to produce both IL-12 and TNF-α following stimulation with TLR agonists (IL-12 production shown longitudinally in PBMC mDC after stimulation with TLR7/8 agonist R848). p values calculated using Mann Whitney two-tailed t-test. B) Basal cytokine production in mDC at week 5; values were not statistically different between groups.

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