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Strategic Design of a Vaccine for Tumor-Associated Herpesviruses

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular and Medical Pharmacology

by

Gurpreet Brar

2018

ABSTRACT OF THE DISSERTATION

Strategic Design of Vaccine for Tumor-Associated Herpesviruses

by

Gurpreet Brar

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2018

Professor Ting-Ting Wu, Chair

Human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV) are associated with malignancies, which are directly linked to viral latency. Therefore, the goal of vaccination for gammaherpesviruses is to prevent the establishment of latent infection. There are urgent needs for effective and safe vaccines against gammaherpesviruses, particularly for high risk groups, such as naïve transplant patients and individuals at high risk for HIV-1 infection. Vaccines based on live attenuated viruses enable the presentation of a full repertoire of antigens and generation of long-lasting memory responses by mimicking an infection. A safe live gammaherpesvirus vaccine candidate should be highly attenuated and unable to persist in the host. We propose a rational design by eliminating viral antagonists of type I interferon (IFN-I) to achieve attenuation while maintaining immunogenicity. IFN-I not only provides immediate defense against infection but also regulates the development of memory immunity. In this proof of principle study, we used murine gammaherpesvirus-68 (MHV-68), closely related to EBV and KSHV, as a model system to test the vaccine candidate

and study the underlying protective immune mechanisms against the establishment of viral persistence. We engineered a recombinant MHV-68 by targeted mutations at immune evasion genes, including the viral antagonists of IFN-I, and eliminated the latency locus responsible for persistent infection. This design results in a highly attenuated virus that demonstrates no measurable replication capacity, latent infection or persistence in immunocompetent hosts, C57BL/6. This highly attenuated virus stimulates robust innate immunity, drives the differentiation of antiviral memory T cells and elicits neutralizing antibodies. Vaccination using this live attenuated virus induces durable protective immunity that can inhibit the establishment of latent infection from challenge infection six months post vaccination. In this study, we establish the first strategy to inactivate viral IFN-I antagonists as a rational design of a herpesvirus vaccine to achieve both safety and immunogenicity.

The dissertation of Gurpreet Brar is approved.

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2018

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Qi H, Chu V, Wu NC, Chen Z, Truong S, **Brar G** et al. Systemic Identification of anti-interferon function on hepatitis C virus genome reveals p7 as an immune evasion protein. *Proceedings of the National Academy of Sciences* **114**, 2018–2023 (2017).

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CHAPTER 1

INTRODUCTION

Herpesviruses

Herpesviruses are enveloped double stranded DNA viruses, which are capable of establishing lifelong persistent infections in humans. The herpesvirus life cycle consists of two phases: lytic replication and latency. Lytic replication is characterized by the initiation of a viral gene expression program, which ultimately generates infectious particles. During latency, minimal viral gene expression allows the virus to evade immune detection. Viral proteins expressed during latency drive the survival and proliferation of virally infected cells. These two distinct phases of the life cycle allow herpesviruses to maintain the presence of viral genomes in the host.

Herpesviruses are classified into three groups based on the host cell type that maintains the latent virus. Alpha herpesviruses establish latency in the sensory nerve ganglia. Alphaherpesviruses that are known to infect humans include Herpes simplex virus-1 (HSV-1/HHV-1), Herpes simplex virus-2 (HSV-2/HHV2) and Varicella zoster virus (VZV/HHV-3). Betaherpesviruses establish latency in monocytes, macrophages, lymphocytes and the salivary glands. Betaherpesviruses that infect humans include Cytomegalovirus (CMV/HHV-5). Roseolovirus (HHV-6) and HHV-7. Gammaherpesviruses establish latency in B lymphocytes, dendritic cells, macrophages and lung epithelial cells[13]. Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) and Epstein-Barr virus (EBV/HHV-4) are the two members of the gammaherpesviruses known to infect the human population.

Tumor-Associated Herpesviruses

KSHV and EBV present a severe global health burden. EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's and non- Hodgkin's lymphomas [1-3]. KSHV is associated with KS, as well as primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), three tumors that frequently develop in AIDS patients [4,5]. Although

there is a low incidence of disease in the majority of infected people, it is estimated more than 200,000 annual cancer cases are associated with EBV and KSHV [6-8]. Gammaherpesvirus associated cancers and malignancies manifest primarily in individuals with compromised immune systems[9]. A viral vaccine against EBV and KSHV is urgently needed for high risk groups, such as Southeast Asian populations at high risk for NPC, central African populations at high risk for Burkitt's lymphoma or KS, naïve transplant patients, and high-risk groups for HIV-1 infection.

Latency associated tumorigenesis

A hallmark of herpesviruses is the biphasic lifecycle, characterized by short lived lytic replication followed by latent infection. Lytic replication of alpha- and beta-herpesviruses results in disease pathologies, such as varicella and herpes zoster for VZV, cold sores, and genital lesions for HSV and congenital defects for CMV. In contrast, both phases of the viral cycle, latency and lytic replication, contribute to the pathogenesis for gammaherpesviruses. It is widely accepted that viral latency is directly linked to tumorigenesis, however lytic replication plays a key role in driving tumor development. Lytic replication can produce infectious virions that can infect naïve cells which can replenish the pool of latently infected cells which subsequently give rise to transformed cells. In addition, lytic replication may enhance latent infection in a tumor environment in a paracrine manner by expressing viral homologues of chemokines and cytokines[10,11].

Targeting lytic replication has clinical efficacy against the onset and development of gammaherpesvirus associated malignancies. Acyclic nucleoside analogs, acyclovir and ganciclovir, are antiviral drugs that are activating upon phosphorylation by viral thymidine kinase during lytic replication. Phosphorylated nucleoside analogs compete with GTP for the substrate binding region of the viral DNA polymerase and terminate DNA elongation. Nucleoside analog

efficacy is higher in malignancies with greater lytic KSHV, such as PEL and MCD, than in malignancies with low lytic KSHV such as KS[12,13]. However, in most virus-associated malignancies, EBV and KSHV are found in their latent state. Inducing latent cells to become lytic has been a rationale to improve the efficacy of nucleoside analogs. Radiation and chemotherapeutic agents such as a gemcitabine and arginine butyrate have been combined with nucleoside analogs to show efficacy in post-transplant lymphoproliferative disease[14].

Viral proteins expressed during latency are anti-apoptotic or growth promoting to ensure the survival and proliferation of latently infected cells. This strategy ensures the viral genome persists in the host but also predisposes latently infected cells to transformation[12-14]. Latency is maintained by EBNA1 (EBV), LANA (KSHV), and ORF73 (MHV-68) which ensure the viral episome is distributed evenly amongst dividing cells[15-17]. KSHV LANA also functions to promote survival of latent cells through binding to p53 and inhibiting p53-mediated apoptosis[18]. EBV LMP1 employs many strategies to promote survival of latent cells including upregulation of anti-apoptotic protein, BCL-2, ligand independent induction of NF-kB to promote cell proliferation and activation phosphatidylinositol 3-kinase (PI3K) to induce the activation of Akt to promote cell survival[19-21]. The integral role of LMP1 in transformation is observed in a meta-analysis that positively correlated the expression of LMP with NPC metastasis[22]. EBV LMP2 mimics the B cell receptor and is essential for the transformation of germinal center B cells[23]. KSHV ORF71 (vFLIP) maintains latency by inhibit lytic replication through the suppression of the AP-1 pathway, ORF72 (vcyclin) inducing cellular transformation of latent cells by constitutively activating cellular cyclin-dependent kinase 6 (CDK6), ORF74 (viral GPCR) and vbcl2 promotes transformation and angiogenesis in KSHV associated malignancies[24-26]. Thus, the vaccine strategy for tumor associated herpesviruses must focus on preventing the establishment of latent infection.

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Murine herpesvirus 68 as a laboratory model for gammaherpesviruses

A critical barrier in the gammaherpesvirus field is the lack of an *in vivo* immune system to study KSHV and EBV infection in the laboratory. To investigate how gammaherpesviruses interacts and modulates the immune response *in vivo*, a closely related murine gammaherpesvirus, MHV-68, is used as a laboratory model system. MHV-68 consists of 80 ORFs and is genetically more homologous to KSHV than EBV. 63 ORFs in MHV-68 are homologous to KSHV[27]. Upon entry of a host, gammaherpesviruses undergo transient lytic replication in epithelial cells or other cell types for amplification before establishing latency in B cells. Following intranasal inoculation, MHV-68 first replicates in the lung and produces infectious virions. Subsequently, the virus establishes latency in B cells, reaching peak latency at two weeks post inoculation accompanied by splenomegaly. The massive lymphoproliferation results in splenomegaly at the peak of viral latency which resembles several features of infectious mononucleosis that some humans experience with primary EBV and KSHV infection [28]. While lytic replication is resolved, the virus is able evade immune detection and persist in the host by establishing latency. Viral latency in B cells is the critical event associated with tumorgenesis[15].

Previous vaccine strategies

Due to the oncogenic potential of gammaherpesviruses, vaccine development has focused on subunit vaccines. Subunit vaccines against EBV are based on the major viral envelope protein, gp350. gp350 is the target of neutralizing antibodies that block EBV infection of B cells, the long-term latency reservoir[29,30]. The gp350-based vaccines, while protecting against symptomatic primary infection, infectious mononucleosis (IM), do not have an impact on the infection rate, and therefore, is unlikely to prevent EBV from establishing latency [31] [32]. This is also the case for subunit vaccines against HSV-2, which can reduce genital lesions but has limited efficacy in preventing infections [33]. All herpesviruses have co-evolved with their hosts for

millions of years and evolved various strategies to evade/modulate the host immunity for persistent infections. Thus, it is likely that vaccines eliciting a broad and durable immunity will be required to prevent infections of herpesviruses, such as EBV and KSHV.

Glycoproteins have garnered significant interest as targets for neutralizing antibodies. gp350, the most abundant glycoprotein on the surface of EBV, is the primary immunogen associated with neutralizing antibody titers[34,35]. Studies have shown that vaccination with purified gp350 with or without an adjuvant is sufficient to prevent EBV induced lymphomas in cotton top tamarins. In human trials, EBV vaccines using recombinant gp350 cannot reduce primary infection despite the induction of neutralizing antibodies [36,37]. A self-assembling nanoparticle vaccine displaying domains of gp350 can generate significantly higher neutralizing antibody titers compared to soluble gp350 [38] . However, it is important to note that EBV deficient in gp350 does not abolish B cell infectivity but simply lowers the efficacy of infection suggesting other viral proteins can mediate infection. An effective vaccine to prevent gammaherpesvirus infection might have to target several glycoproteins involved in cell entry such as gB, gH, gL, and gp70.

So far, the only vaccination strategy that can reduce the long-term latent load is based on live attenuated viruses designed to be deficient in either the establishment of latency or reactivation from latency[39-43].

A single subdermal injection of 10^7 PFU of heat inactivated MHV-68 is able to reduce the lytic viral titer in the lungs 6 days after $4x10^5$ PFU intranasal challenge of WT MHV-68. However, two injection of heat inactivated MHV-68 are required to reduce the number of latently infected cells in the spleen at 14 days post intranasal challenge. Two injections of heat inactivated MHV-68 fails to reduce the number of latently infected cell when the spleen was assayed at 28 days post challenge by infectious center assay. A heat inactivated MHV-68 strategy demonstrates an ability to limit acute infection, splenomegaly and peak latency titers

but is unable to reduce long term latent infection[44]. To determine if the IFN response could improve the vaccination efficacy of a heat inactivated virus, $2x10^5$ IU of IFN-α/β injections were administered in associated with 10^7 PFU of heat inactivated MHV-68. The viral lytic titer in the lungs was assessed 6 days post intranasal challenge of $4x10^5$ PFU WT. The addition of IFN-α/β did not enhance protection from viral challenge compared to heat inactivated MHV-68 alone[45].

Switching to live attenuated viruses, Arico et al. constructed a recombinant live attenuated MHV-68 expressing mIFNα1 and a Psorlaen-mediated UV crosslinking (PSUV)-inactivated MHV-68 expressing mIFNα1[46]. Intranasal inoculation using $1x10^5$ PFU of either vaccine virus is followed by a booster inoculation 2 weeks later using the same virus. Vaccinated mice were challenged intranasally two weeks post booster with $4x10^5$ PFU WT MHV-68. The viral titer in the lungs were significantly reduced by the PSUV-inactivated MHV-68 expressing mIFNα1 while mice vaccinated with live attenuated MHV-68 expressing mIFNα1 had no lytic titers in the lungs 4 days post challenge. Both vaccine strategies are able to limit the number of latently infected splenocytes at 14 days post challenge but unable to reduce the number of latently infected cells at 80 days post 10^5 PFU WT MHV68. Both strategies were able to protect against WT only when the challenge dose was lowered to 10^4 PFU WT MHV-68. A key finding in this study was establishing the ability of the live attenuated MHV-68 expressing mIFNα1 to establish long term latency. This feature of the vaccine virus is a significant safety drawback which propels the PSUV inactivated MHV-68 expressing mIFNα1 as the safer candidate for prophylactic vaccination.

In attempts to create live attenuated viruses, several labs have removed essential genes to create an effective prophylactic vaccine. The immediate early gene, ORF6, is a single stranded DNA binding protein. Deletion of ORF6 (γHV68.ΔssDNABP) results in a virus that is lytic replication defective but can still establish long term infection in the spleen and peritoneal cells at frequencies similar to WT MHV-68 infection *in vivo*. Mice were inoculated i.p. with 1000

TCID50 γHV68.ΔssDNABP and then intranasally challenged with 1000 PFU WT MHV-68. At 14 days post challenge, spleens from γHV68.ΔssDNABP inoculated mice show no lytic virus in the spleen[47]. Much like ORF6, a mutation in immediate early gene ORF50 (G50.stop), results in a replication defective virus that is still able to infect the B cells in the lungs long term upon intranasal inoculation[48]. Mice were inoculated intranasally with 940 PFU of G50.stop and challenged 100 PFU WT MHV-67 i.n. G50.stop is unable to limit the acute infection in the lungs at 7 days post challenge and was unable to prevent the establishment of latency at 16 days post challenge[49]. Another replication deficient virus was generated by inserted a stop codon into ORF31 (ORF31STOP). ORF31 is essential for replication and late lytic protein expression [50]. Mice were inoculated using 10^5 PFU of ORF31STOP i.p and challenged using 400 PFU WT MHV68 i.n. ORF31STOP inoculation was unable to reduce the number of latently infected cells 30 days post challenge. However, once again the vaccine virus is able to establish latent infection in splenocytes upon i.p. inoculation[51]. Tibbetts et a. interrogated whether it is possible to vaccinate against long term latency. γHV68.v-cyclin.LacZ is a live attenuated vaccine which establishes a normal acute and latent infection but is unable to reactivate from latency[39]. γHV68.v-cyclin.LacZ protects against a 100 PFU i.p. challenge when spleens were tested 42 days post challenge. Adoptive serum transfer from γHV68.v-cyclin.LacZ immunized mice to naïve mice was able to protect against splenic latency but had no effect on the latency in peritoneal cells. When B cell $^{/-}$ mice were immunized with γHV68.v-cyclin.LacZ , the antibody response was not required to protect against latency. This data suggests the T cells play a significant role in mediating protection. CD4+ T cells and CD8+ T cells were depleted from HELMET mice, which are unable to generate an antibody response. Upon immunization with γHV68.v-cyclin.LacZ, T cell depleted HELMET mice were no longer protected against latent infection[52]. CD4+ T cells, CD8 T cells, and antibodies alone are not essential for protection but either CD4 or CD8 T cells is required in conjunction with antibodies to provide protection.

γHV68.v-cyclin.LacZ informed that an attenuated virus can protect against long term latency but the oncogenic potential from the vaccine establishing latency is a safety concern.

In order to limit the ability of a vaccine virus to persist, several groups employed strategies to create latency deficient viruses. Loss of expression mutants, Δ73 and 73.STOP are defective for the establishment and maintenance of long-term latency when mice are inoculated intranasally[17,48]. Vaccination with 10^5 PFU i.p. Δ73 is able to protect a mouse 57 days post intranasal challenge using 10^4 PFU WT MHV-68[40]. Importantly, when LANA null was inoculated i.p. into C57BL/6 mice, the virus was able to persistently infect the mice for 6 months post inoculation but unable to efficiently reactivate from latency[53]. In contrast to eliminating ORF73, Rickabaugh et al. attempted to create a latency deficient virus by constitutively overexpressing RTA (C-RTA). An RTA expression cassette controlled by a CMV promoter was cloned into the left end of the MHV-68 genome. C-RTA demonstrated faster replication kinetics than WT and reduced ability to establish latency, though not latency was not completely abolished. 500 PFU of C-RTA is able to protect against the establishment of latency 20 days post intranasal challenge using 500 PFU WT MHV-68. However, *in vivo* the overexpression cassette was unstable in the viral genome and often deleted during the course of infection, resulting in a recombinant virus able to establish latency [41]. To overcome this problem, Jia et al inserted the RTA overexpression cassette into the major latency locus, replacing viral genes ORF72, M11, and ORF73, creating AC-RTA. AC-RTA is lytically attenuated and latency deficient *in vivo*. Intranasal inoculation of 500 PFU AC-RTA is followed by intranasal challenge using 500 PFU WT MHV-68. 14 days post challenge, spleens from AC-RTA inoculated mice show no establishment of latency [43]. Unfortunately AC-RTA is ruled out as a safe vaccine candidate but it acquired to infect the brain not only in antibody deficient mice (AID$^{-/-}$us$^{-/-}$) but also in immune competent C57BL/6 mice [54]. AC-RTA requires further attenuations to fulfill safety requirements for a prophylactic vaccine.

Immunogenicity is often compromised to ensure safety of vaccines. Subunit vaccines are non-infectious, non-persistent, and have no risk of recombining genetic material. However, subunit vaccines against a single epitope have been ineffective at preventing latent infection by tumor-associated herpesviruses. The advantage of an attenuated vaccine strategy is that antibodies can be generated against multiple epitopes. In order to create a safe live attenuated vaccine for tumor-associated herpesviruses, our strategy employs a virus that demonstrates no measurable replication capacity, no latent infection and no persistence in immunocompetent (C57BL/6) hosts. Other strategies have utilized a reactivation deficient vaccine virus. However, reactivation deficient viruses have the ability to persist in the host and can be detected months after vaccination [39].

Eliminating viral immune evasion genes to attenuate vaccines

This study presents a vaccine strategy that aims to address two fundamental design aspects, immunogenicity, and safety. A genome-wide screen identified several gammaherpesvirus genes that counteract IFN-I responses [55]. We hypothesize that a recombinant herpesvirus lacking multiple IFN-I evasion genes, while attenuated in replication, will trigger a robust innate response that can then efficiently prime the adaptive immune cells to generate immunological memory. For safety, this vaccine strategy also employs a design that would eliminate the possibility of persistence and being oncogenic. In this study, we evaluate the safety and efficacy of a recombinant gammaherpesvirus vaccine, which lacks immune evasion proteins and is unable to establish latency.

Construction of the DIP Vaccine

In a genome-wide screen of MHV-68 open reading frames (ORF), each ORF was co-transfected with a reporter construct containing firefly luciferase driven by an interferon

stimulated response element (ISRE). Transfected 293T cells were treated with IFN-a, and eight

genes were identified to suppress the ISRE reporter by 50% (Figure 3-1). These eight candidate

genes could be mutated to create a vaccine virus unable to evade the IFN response[55]. We

eliminated candidate genes M2 and M8 because they do not have homologs in EBV and KSHV.

We then considered the genes that are not essential for viral replication, eliminating the need of

creating complementary cell lines to amplify the recombinant viruses. This consideration

removed ORF50 and ORF64, as they are essential for replication. The remaining four genes are

non-essential for viral replication and conserved among MHV-68, KSHV, and EBV: ORF10,

ORF11, ORF36, and ORF54[29-32]. These 4 genes were selected to be inactivated in the vaccine

virus. ORF10 binds JAK1 and TYK2 to block IFN signal transduction [56]. ORF11 prevents IFN

production by binding IRF-7 and preventing its dimerization[57]. ORF36 limits the IFN response by

binding to IRF-3 and preventing the transcriptional activation of the IFNβ promoter. ORF54

suppressed the IFN pathway by causing the degradation of the type I interferon receptor

(IFNAR) [58] [55,59]. Furthermore, their removal could also allow IFN-I responses to efficiently mount,

facilitating the development of adaptive immunity. In addition to 4 IFN evasion genes, K3 was

also inactivated to increase antigen presentation from the vaccine virus. K3 is a viral gene

responsible for downregulating MHCI on infected cells [60]; without K3, viral latency is decreased

and more virus-specific CD8 T cells are generated[61]. While attenuated viral replication would

indicate reduced virus-specific immune responses, inactivation of these five immune evasion

genes was hypothesized to was hypothesized to attenuate the vaccine virus replication while

maintaining its immunogenicity.

The biphasic lifecycle is regulated by the expression of two essential proteins, replication

and transcription activator (RTA) and latency associated nuclear antigen (LANA) [62]. LANA is

required for latency establishment and RTA induces the expression of lytic genes [63-65].

Abolishing LANA expression and constitutively expressing RTA results in a latency deficient

virus [43]. The major latency locus, composed of ORF72 (v-cyclin), ORF73 (LANA), ORF74 (viral

GPCR) and M11, is replaced with a phosphoglycerate kinase 1 (PGK) promoter driving the

constitutive expression of RTA. This modification of the latency locus is a two-tiered approach to

prevent persistent infection. Combining the alteration of the latency locus and constitutive

expression of RTA with the removal of immune evasion genes resulted in a live attenuated

gammaherpesvirus vaccine, termed DIP (deficient in immune evasion and persistence) (Figure

3-2). Generating a latency deficient virus is a strategy to remove the oncogenic potential of a

live attenuated vaccine for tumor-associated herpesviruses. Latent infection functions to

maintain the viral genome and evade detection by the host immune system.
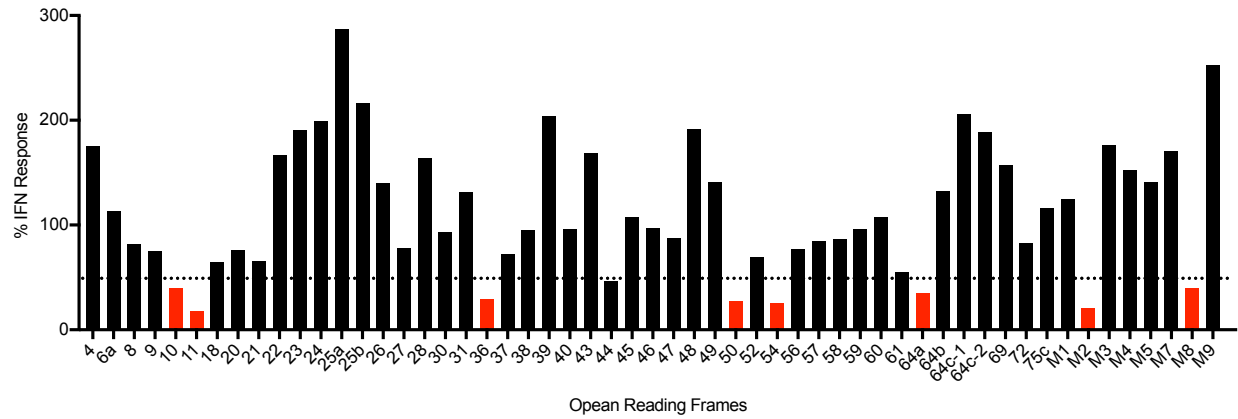
Figure 1-1. Genome wide screen to identify ORF that can inhibit IFN response

Each ORF was co-transfected with a reporter construct containing firefly luciferase driven by an interferon stimulated response element (ISRE). Transfected 293T cells were treated with 1000 units/mL IFN-α. Red bars indicate ORF able to inhibit the IFN response by 50% compared to transfection control.
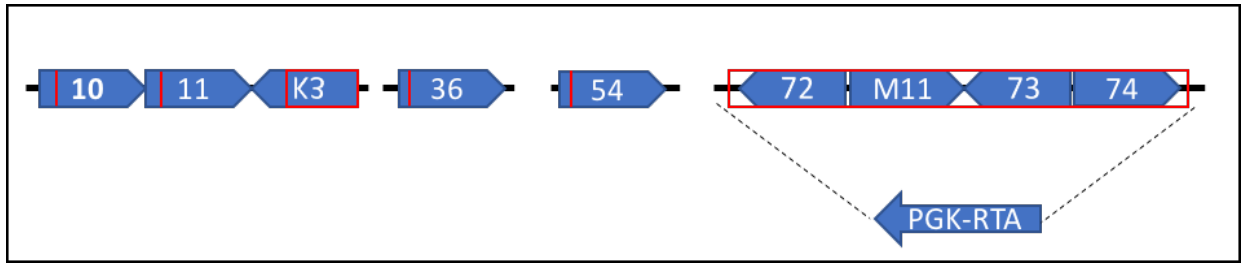
Figure 1-2. Schematic representation of DIP Vaccine

Schematic representation of mutations introduced in the MHV-68 genome to generate the DIP

vaccine. Red lines indicate the insertion of translation stop codons into ORF10, 11, 36 and 54.

Open red rectangles indicate deletion of coding sequences of K3 and the latency locus,

including ORF72, ORF73, ORF74 and M11. The latency locus is replaced by an RTA cassette

(indicated by the arrowhead) which is constitutively driven by the PGK promoter.

In order to create a safe live attenuated vaccine for tumor-associated herpesviruses, our strategy employs a latency deficient virus. Other strategies have utilized a reactivation deficient vaccine virus. However, reactivation deficient viruses have the ability to persist in the host and can be detected months after vaccination [39]. In the strategy, not only is the major latency locus deleted but a second measure to prevent long-term persistence is adopted by constitutively expressing RTA. We hypothesize the DIP vaccine will be latency deficient, therefore also reactivation deficient, and unable to persist in a host. This strategy aims to de-escalate the oncogenic potential of the vaccine. Inactivation of the five immune evasion genes was chosen to be the strategy to attenuate the viral replication because of the likelihood to maintain immunogenicity.

CHAPTER 2

MATERIALS AND METHODS

Viruses and cells

WT MHV-68 was originally obtained from American Type Culture Collection (ATCC; Vr1465). WT and DIP were propagated using 3T3 cells and vero cells and titers were determined by plaque assay. Viruses were concentrated using high speed centrifuge and resuspended in serum free DMEM. Vero cells are cultured in Dulbecco modified eagle medium (DMEM) containing 10% fetal bovine serum and supplemented with penicillin and streptomycin. 3T3 cells were cultured in DMEM containing 10% BCS and 1% Penicillin and Streptomycin.

Plaque assay

For plaque assays, each sample was serially diluted by 10-fold and incubated on Vero cells in duplicates on 12-well plates. The inoculum was removed after 1 hour, and cells were overlaid with 1% methylcellulose DMEM containing 10% FBS. Six days post infection, cells were fixed with 2% crystal violet in 20% ethanol. Virus titers were determined by counting plaque numbers. To determine the viral titers in the mouse tissues, the homogenates in 1mL obtained by dounce homogenizers were used for plaque assay. The number of plaques formed were counted and the viral titer in each tissue was expressed as PFU/mL.

In Vitro **Growth Curve**

3T3 cells were plated in media +/- 100 units/mL IFNβ for 24 hours. Cells were infected at MOI of 0.01 with the WT or DIP virus for 1 hour at 37°C. The inoculum was removed and cells were washed with media twice before adding fresh media +/- 100 units/mL IFNβ. Both cells and supernatant were harvested 24, 48 and 72 hours post infection for plaque assay.

Construction of DIP vaccine

The recA+ Escherichia coli strain GS500 harboring a BAC containing the WT MHV-68 genome was used to construct recombinant MHV-68 by allelic exchange with the conjugation competent E. coli strain GS111 containing a suicide shuttle plasmid pGS284, as previously described[66-68]. For each recombinant MHV-68, overlap extension PCR was used to construct a unique shuttle plasmid pGS284 harboring the desired mutation and an approximately 500 bp flanking region. The sequences upstream of the desired mutation (A fragments) were amplified by primers of AF and AR while the downstream sequences (B fragments) were by primers of BF and BR using wild-type MHV-68 virion DNA as the template. The A and B fragments were designed to have an >20 bp overlapping sequence. In a subsequent PCR reaction, the A and B fragments were used as templates and amplified by primers of AF and BR. The final PCR products were digested with appropriate enzymes and cloned into pGS284. To screen for the correct mutation, restriction enzyme digest was performed on PCR products produced with the primers of AF and BR on the BAC MHV-68 clones. We carried out sequential allelic exchanges to obtain the final recombinant clone containing all the mutations we designed (Fig. 1). Following selection of the desired recombinant clone, the MHV-68 BAC was purified and transiently transfected with Lipofectamine 2000 into 293T cells with equal amount of a plasmid expressing Cre recombinase to remove the BAC sequence. Three days post transfection, a single viral clone was isolated by limiting dilution and propagated for future studies. Produced viruses were quantified by plaque assay and limiting dilution.

Primers used for the construction of shuttle plasmids were described previously: ORF11, ORF36, and ORF54[55,59,66]. The primers used for other shuttle plasmids are listed in supplementary Table 1. Primers 1-8 were used to construct shuttle plasmids for the stop codon mutation. To construct the shuttle plasmid for replacing the latency locus with RTA expression driven by PGK promoter, we amplified four different fragments using primers 9-16, A (ORF72),

B (RTA coding sequence and poly A tail), C (PGK promoter), and D (ORF74), and then generate the ABCD fused fragment for cloning into pGS284.

Mice

Animal studies are approved by the Animal Research Committee at the University of California, Los Angeles. Female C57BL/6J, SCID, and B6.SJL-$Ptprc^a$ $Pepc^b$/BoyJ mice were obtained from Jackson Laboratory. IFNAR$^{-/-}$ mice were a gift from Genhong Cheng at UCLA. Mice 6-8 weeks of age, were infected intraperitoneally with 10^5 PFU of virus in a total volume of 200μL. Intranasal vaccinations and challenges were performed by anesthetizing mice using isoflurane and performing drop-wise administration of virus in a total volume of 20μL. At the end point, mice were euthanized and various tissues were collected in 1ml DMEM and homogenized subsequently using mesh filters and dounce homogenizers. The tissue lysates were clarified by centrifugation and subsequently used for DNA extraction with the DNeasy Blood and Tissue kit (Qiagen, Cat. 69504) and plaque assays. For infectious center assays and flow cytometry studies, single cell suspension were obtained from the spleens.

Phenotyping viral specific T cells

Prior to staining, splenocytes were incubated with FC block (BD, 553142). Tetramers were obtained from the NIH Tetramer Facility. Allophycocyanin-conjugated MHCI tetramers specific for MHV68 epitopes D^b/ORF6$_{487-495}$ (AGPHNDMEI), K^b/ ORF61$_{524-531}$ (TSINFVKI), K^b/ORF75c$_{940-947}$ (KSLTYYKL), and K^b/ORF8$_{604-612}$ (KNYIFEEKL) were incubated with splenocytes for 1 hour at room temperature (RT). Surface staining using the following antibodies were performed by incubation at 4°C for 30 minutes: anti-KLRG1 (eBioscience, 46-5893), anti-CD127 (eBioscience, 17-1273), anti-CD8 (eBioscience, 48-0081), anti-CD4 (eBioscience, 11-0042), anti-CD3 (eBioscience, 25-0031), anti-CD44 (eBioscience, 11-0441), anti-CD62L

(eBioscience, 83-062), anti-CCR7 (eBioscience, 47-1971), anti-CD45.1 (eBioscience, 47-0453), and anti-CD45.2 (eBioscience, 12-0454). For intracellular staining, BD Cytofix and Cytoperm (Cat. 554714) was used prior to incubating splenocytes with anti-IFNγ (eBioscience, 17-7311), anti-TNFα (eBioscience, 46-7321), and anti-IL-2 (eBioscience, 25-7021) antibodies at RT for 30 minutes. All samples were fixed using 1% PFA. All experiments were analyzed on SORP BD LSRII Analytic Flow Cytometer. Data analysis was performed using FlowJo.

Ex vivo T cell peptide stimulation

Splenocytes were ACK lysed and B cells were depleted by incubating in flasks coated with AffiniPure goat anti-mouse IgG(H+L) for 1 hour at 37°C. Splenocytes from vaccinated mice (CD45.2+) were incubated 1:1 with naïve splenocytes (CD45.1+) in CTM media containing 10units/mL IL-12, 10μg/mL BFA and 1 μg/mL peptide for 5 hours at 37°C. Splenocytes were than stained and processed for flow cytometry with indicated tetramers and surface marker antibodies.

Infectious Center Assay

Serial diluted splenocytes were plated on a monolayer of Vero cells and incubated for overnight at 37°C. Splenocytes were aspirated and then washed off with gentle agitation. Vero cells were overlaid with 1% methylcellulose DMEM containing 10% FBS for 6 days before fixing with 2% crystal violet in 20% ethanol. Infectious centers indicated by plaques were counted.

Quantitative PCR

All qPCR was performed on MJ Opticon 2 using PerfeCTA Fastmix (Quantabio). For viral genome copy number analysis, 150ng of extracted DNA and the primers annealed to the

upstream of ORF6 coding sequence (ORF6: 5'-TGCAGACTCTGAAGTGCTGACT-3' and 5'-ACGCGACTAGCATGAGGAGAAT-3') were used.

For RNA expression analysis, cells were harvested in Trizol (company name?) for RNA extraction according to the recommended protocol. Total RNA was treated with DNAse and used for reverse transcription with qScript cDNA synthesis Kit (Quantabio) to generate cDNA followed by qPCR.

Gene expression analysis by qPCR

Cell lysate is stored in Trizol at -80°C. Chloroform RNA extraction is followed DNAse treatment. qScript cDNA synthesis Kit (Quantabio) was used to generate cDNA, followed by gene expression analysis using PerfeCTa Fastmix. Primers designs for IL-1β, TNFα, IL-6, IL-12 and actin are listed in Supplemental Table 2.

BMDM Infections

Cells were harvested from bone marrows and differentiated into macrophages (BMDM) by incubating for 7 days in DMEM containing 20% FBS, 5% M-CSF, 1% Penicillin and Streptomycin, 1% glutamine and 0.5% sodium pyruvate. BMDM were infected with at MOI of 1. 24 hours post infection RNA was extracted using Trizol.

ELISA

Supernatants were collected 24 hours post infection of BMDM using MOI 1 WT or DIP. Protein abundance was measured using eBioscience mouse IL-12/IL-23 (Total p40) Uncoated ELISA Kit (Catalog 88-7120-22).

Western Blot

Virion proteins were prepared in 5% Triton solution in PBS. 180 μg of virion proteins were resuspended in Laemmli buffer and incubated at 95 ℃. 36 μg of protein was used per lane and resolved by 10% SDS-PAGE gel electrophoresis. Then it was transferred onto nitrocellulose membrane using Bio-Rad Turbo Blot System for 30 minutes at 25V. The membrane was blocked with 5% milk solution in PBS-T for 1 hour. 1:4000 serum dilution in PBS-T (2% milk) was applied overnight at 4°C. 3 10-minute washes with PBS-T were performed and 1:4000 mouse IgG solution in PBS-T (2% milk) was applied (Southern Biotech 4050-05) for 1 hour. A 30-minute wash with PBS-T was performed. Horseradish peroxidase-conjugated secondary antibodies was used and detection was performed with SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher). All incubations were performed at RT unless specified.

Immunofluorescence Assay

Heat inactivated sera obtained from mice 2 months post inoculation with 10^5 PFU WT, DIP or mock. MEF cells were infected with WT MOI1 for 24 hours. MEFs incubated in blocking buffer for 1 hr at room temperateure. Sera diluted 1:100 in IFA blocking buffer in the absence of Triton-100x. MEFs were incucbated with diluted sera for 2 hours at RT. Cells were washed with PBS, incubated for 1 hr at room temperature with conjugated secondary antibody and imaged.

Neutralizing Activity

Serially diluted serum was incubated with 100 PFU WT for 1 hour at 37°C. This mixture was plated on a monolayer of Vero cells for 1 hour at 37°C. The plate was overlaid with 1% methylcellulose DMEM containing 10% FBS for six days before fixing with 2% crystal violet in 20% ethanol. The neutralizing activity was determined by the most diluted serum that was still

able to reduce the number of plaques by 50% compared to the virus mixture containing four-fold diluted mock serum.

Viral specific IgG ELISA

5 μg/mL WT virion antigen solution was used to coat a 96-well plate and incubated overnight at 4°C. The plate was blocked overnight using a PBS solution containing 1% BSA and 0.5% Tween-20. The plate was washed twice using PBS-T. Mouse sera were diluted in PBS buffer containing 0.1% BSA and 0.025% Tween-20 and incubated on the plate for 1 hour at RT. The plate was washed three times with PBS-T and a final wash if preformed with PBS. Substrate solution consisting of 1 tablet of OPD and 1 tablet of urea hydrogen peroxide (Sigma P9187) in 10mL of ddH$_2$O, was added to the plate. The plate was incubated for 30 minutes in the dark at 4°C. The reaction is stopped by the addition of 4N H$_2$SO$_4$ and the plate is read at dual wavelength 490nm and 620nm.

Serum Transfer

Sera were obtained from mice 2 months post infection. 200μL of heat inactivated pool serum was i.p. injected into a naïve mouse. 24 hours post serum transfer, the naïve mice were challenged i.n. with 5000 PFU of WT MHV-68. A second dose of 200μL of heat inactivated pooled serum was i.p. injected 7 days post WT challenge. Splenocytes were harvested 14 days post challenge for infectious center assay.

Adoptive T Cell Transfer

Splenocytes were isolated from C57BL/6J mice 2 months post infection and pooled from multiple mice for T cell transfer. Splenocytes were negatively selected for CD4, CD8 or total T cells using Stemcell technologies EasySep isolation kits (Catalog # 19765, 19853 and 19851).

Negative selection was confirmed by flow cytometry analysis to >90% purity. Three million cells in 100μL were injected into the tail vein of B6.SJL-*Ptprc^a Pepc^b*/BoyJ mice. 24 hours post T cell transfer, the recipient mice were i.n. challenged with 5000 PFU of WT MHV-68. The spleens were harvested 14 days post challenge for infectious center assay and flow cytometry to confirm donor T cells present in the recipient mice using anti-CD45.1 and anti-CD45.2.

T Cell Depletion

2 months post i.p. inoculation with 10^5 PFU DIP, mice were i.p. injected with 250μg of anti-Thy1.2 (BioX Cell, Clone 20H12, Catalog BE0066) or isotype control (BioX Cell, Clone LTF-2, Catalog BE0090) every 48 hours. 24 hours after the second injection, mice were bled by tail vein to confirm depletion of T cells by flow cytometry. After confirming depletion, mice were challenged with 5000 PFU WT i.n. Mice continued to receive 250μg anti-Thy1.2 or isotype control antibody i.p. every 48 hours until sacrifice, 14 days post challenge. Splenocytes were assessed by flow cytometry to confirm T cell depletion at the endpoint.

Statistical Analysis

All statistical analyses were performed using the two tailed unpaired student's t-test, unless otherwise stated. $P<0.05$ = *, $P<0.01$ = **, $P<0.001$ = *** and $P<0.0001$ = ****.

TABLE 2-1. Primer sequences for the construction of DIP vaccine

1	ORF10 and deletion mutation in K3: ORF10SAF	ACGCGTCGACcctcagattcatgacagaatacc
2	ORF10SAR	CAGCTGTCAATTAATTAACCGGggtgtatccattccttctgtttag
3	ORF10BF	CCGGTTAATTAATTGACAGCTGactctggacctaccactgac
4	ORF10BR	TTAAGCATGCagagactgattggcacaggcc
5	K3delAF	GCCGAGATCTtcaggatctctgcaaccatg
6	K3delAR	tactacactaccGCGGCCGCaagactgaccttggattagc
7	K3delBF	aaggtcagtcttGCGGCCGCggtagtgtagtagctcctgg
8	K3delBR	GCTTgcatgcggagaacatataagcctgac
9	Del72-74+PGKRTA-AF	TCTCGTCGACctggtctaaccgcaaaccga
10	Del72-74+PGKRTA-AR	CTGCTCCCTTCCCTGTCCTTcctttcctcgttgccatag
11	Del72-74+PGKRTA-BF	ctatggcaacgaggaaaggAAGGACAGGGAAGGGAGCAG
12	Del72-74+PGKRTA-BR	TCATCTCCGGGCCTTTCGAAGAGCTCGTTTAGTGAACC
13	Del72-74+PGKRTA-CF	GGTTCACTAAACGAGCTCTTCGAAAGGCCCGGAGATGA
14	Del72-74+PGKRTA-CR	ttgttgggctaatctcacacACCGGGTAGGGGAGGCGCTTTC
15	Del72-74+PGKRTA-DF	GAAAAGCGCCTCCCCTACCCGGTgtgtgagattagcccaacaa
16	Del72-74+PGKRTA-DR	TCTCGCTAGCggaagggctactgctatgct

Table 2-2. Primer sequences for gene expression analysis by RT-qPCR

	qPCR Primers
Actin	5'-GTATCCTGACCCTGAAGTACC-3' 5'-TGAAGGTCTCAAACATGATCT-3'
IL-6	5'-CTGCAAGAGACTTCCATCCAG-3' 5'-AGTGGTATAGACAGGTCTGTT-3'
IFNβ	5'-CAGCTCCAAGAAAGGACGAAC-3' 5'-GGGAGTGTAACTCTTCTGCAT-3'
IL-1β	5'-TGTTCCTGAACTCAACTGTG-3' 5'-GGTCAAAGGTTTGGAAGCAG-3'
IL-12	5'-TGGTTTGCCATCGTTTTGCTG-3' 5'-ACAGGTGAGGTTCACTGTTCC-3'
TNFα	5'-AAATTCGAGTGACAAGCCTG-3' 5'-ACCACTAGTTGGTTGTCTTTGAG-3'

CHAPTER 3

DIP IS REPLICATION DEFICIENT AND LATENCY DEFICIENT *IN VIVO*

ABSTRACT

The replication capacity and persistence *in vivo* will be important measures to evaluate the safety of live viruses as vaccines against tumor-associated herpesviruses. DIP is unable to establish latent infection and does not persist in the host. Furthermore, upon i.n. and i.p. inoculation, DIP demonstrates no productive infection in immunocompetent hosts, C57BL/6 mice. DIP replication is also undetectable in immunosuppressed hosts, SCID mice. DIP replication is rescued in interferon-α/β receptor-deficient (IFNARα/β$^{-/-}$) mice upon i.p. inoculation. DIP attenuation is mediated by the IFN response *in vivo*. The inability to establish productive infection, latent infection, and persistence are significant features which suggest DIP is a safe vaccine candidate, even in an immunocompromised setting.

INTRODUCTION

Attenuated live viruses have the advantage of presenting a repertoire of viral antigens and generating long-lasting memory responses by mimicking an infection. A conventional strategy of attenuation is to remove one or more viral genes that are essential for replication in cell culture, which suffers from the risk of recombination to re-gain the removed essential genes. Moreover, sufficient attenuation for safety often compromises immunogenicity. Therefore, selective inactivation of viral immune evasion genes presents a compelling strategy of attenuation while maintaining immunogenicity. One important class of immune evasion genes to consider is viral antagonists of type I interferon (IFN-I) response, which is the first line of host defense against viruses. Subverting the IFN-I response is a critical step for viruses to establish infection in humans. The IFN-I response initiates a signaling cascade that results in a sophisticated transcriptional program of over ~300 genes which function to counteract a viral infection [69-72]. In addition to restraining an infection, the IFN-I responses promote the development of adaptive immune response. Approximately 25% of the genes encoded by herpesviruses have been identified to function in immune modulation, including counteracting the IFN response [73,74]. By selectively inactivating of viral immune evasion genes, we aim to create a vaccine that is deficient replication in immunocompetent hosts while potentially stimulating robust anti-viral immunity.

In a genome-wide screen of MHV-68 open reading frames (ORF), each ORF was co-transfected with a reporter construct containing firefly luciferase driven by an interferon stimulated response element (ISRE). Transfected 293T cells were treated with IFN-a, and eight genes were identified to suppress the ISRE reporter by 50% (Figure 1-1). These eight candidate genes could be mutated to create a vaccine virus unable to evade the IFN response [55]. We eliminated candidate genes M2 and M8 because they do not have homologs in EBV and KSHV. We then considered the genes that are not essential for viral replication, eliminating the need of

creating complementary cell lines to amplify the recombinant viruses. This consideration

removed ORF50 and ORF64, as they are essential for replication. The rest four are non-

essential for viral replication and conserved among MHV-68, KSHV, and EBV: ORF10, ORF11,

ORF36, and ORF54[29-32]. These 4 genes were selected to be inactivated in the vaccine virus.

ORF10 binds JAK1 and TYK2 to block IFN signal transduction [56]. ORF11 prevents IFN

production by binding IRF-7 and preventing its dimerization[57]. ORF36 limits the IFN response by

binding to IRF-3 and preventing the transcriptional activation of the IFNβ promoter. ORF54

suppressed the IFN pathway by causing the degradation of the IFNAR [58] [55,59]. We created a

recombinant virus that eliminated the expression of these four genes, and referred it as DI

(Deficient in IFN-I evasion). Removal of these four genes reduced the peak viral titer in the lung

>100 fold (Fig.), indicating a valid approach to attenuate the replication of a vaccine virus in

vivo. Furthermore, their removal could also allow IFN-I responses to efficiently mount, facilitating

the development of adaptive immunity. In addition to 4 IFN evasion genes, K3 was also

inactivated to increase antigen presentation from the vaccine virus. K3 is a viral gene

responsible for downregulating MHCI on infected cells [60]; without K3, viral latency is decreased

and more virus-specific CD8 T cells are generated{Stevenson:2002cw}. While attenuated viral

replication would indicate reduced virus-specific immune responses, inactivation of these five

immune evasion genes was hypothesized to attenuate the vaccine virus replication while

maintaining its immunogenicity.

Generating a latency deficient virus is a strategy to remove the oncogenic potential of a

live attenuated vaccine for tumor-associated herpesviruses. Latent infection functions to

maintain the viral genome and evade detection by the host immune system. The biphasic

lifecycle is regulated by the expression of two essential proteins, replication and transcription

activator (RTA) and latency associated nuclear antigen (LANA) [62]. LANA is required for latency

establishment and RTA induces the expression of lytic genes [63-65]. Abolishing LANA expression

and constitutively expressing RTA results in a latency deficient virus [43]. The major latency locus, composed of ORF72, ORF73 (LANA), ORF74 and M11, is replaced with a phosphoglycerate kinase 1 (PGK) promoter driving the constitutive expression of RTA. This modification of the latency locus is a two-tiered approach to prevent persistent infection. Combining the alteration of the latency locus and constitutive expression of RTA with the removal of immune evasion genes resulted in a live attenuated gammaherpesvirus vaccine, termed DIP (deficient in immune evasion and persistence) (Figure 3-2). Furthermore, removal of viral genes (ORF72, ORF74, M11) that are potentially oncogenic, increases the safety of the vaccine virus. Here we examined the *in vitro* and *in vivo* replication properties of DIP.

RESULTS

Attenuated DIP replication *in vitro*

We compared the *in vitro* growth kinetics of DIP in NIH3T3 fibroblasts to WT. DIP replication is significantly attenuated compared to WT. After infection at a MOI of 0.01, DIP yields 300-fold and 40-fold less viral production than WT at 48 and 72 hrs post-infection respectively (Figure 3-1A). Upon IFNβ pre-treatment of NIH3T3, the replication of WT virus is inhibited 10-fold while the DIP replication is inhibited 100-fold (Figure 3-1B). Vero cells also show a 40-fold reduction in DIP viral production at 72 hrs post-infection in Vero cells (Figure 3-1C). Vero cells are unable to produce IFNβ or IFNα. No further attenuation is observed by pre-treating Vero cells with IFNβ (Figure 3-1C). The infectious virion production by DIP suggests a higher susceptibility to the IFN-I response in the absence of viral IFN evasion genes.

Despite attenuated infectious virion production, cell viability decreases 40% upon DIP infection at 72 hours post infection compared to 1% by WT infection (Figure 3-2A). In Vero cells, cell viability decreases 67% upon DIP infection at 72 hours post infection compared to 37% by WT virus (Figure 3-2B). LANA has been shown to antagonize p53 mediated cell death

upon infection. The deletion of LANA from DIP could render the infected cells more susceptible
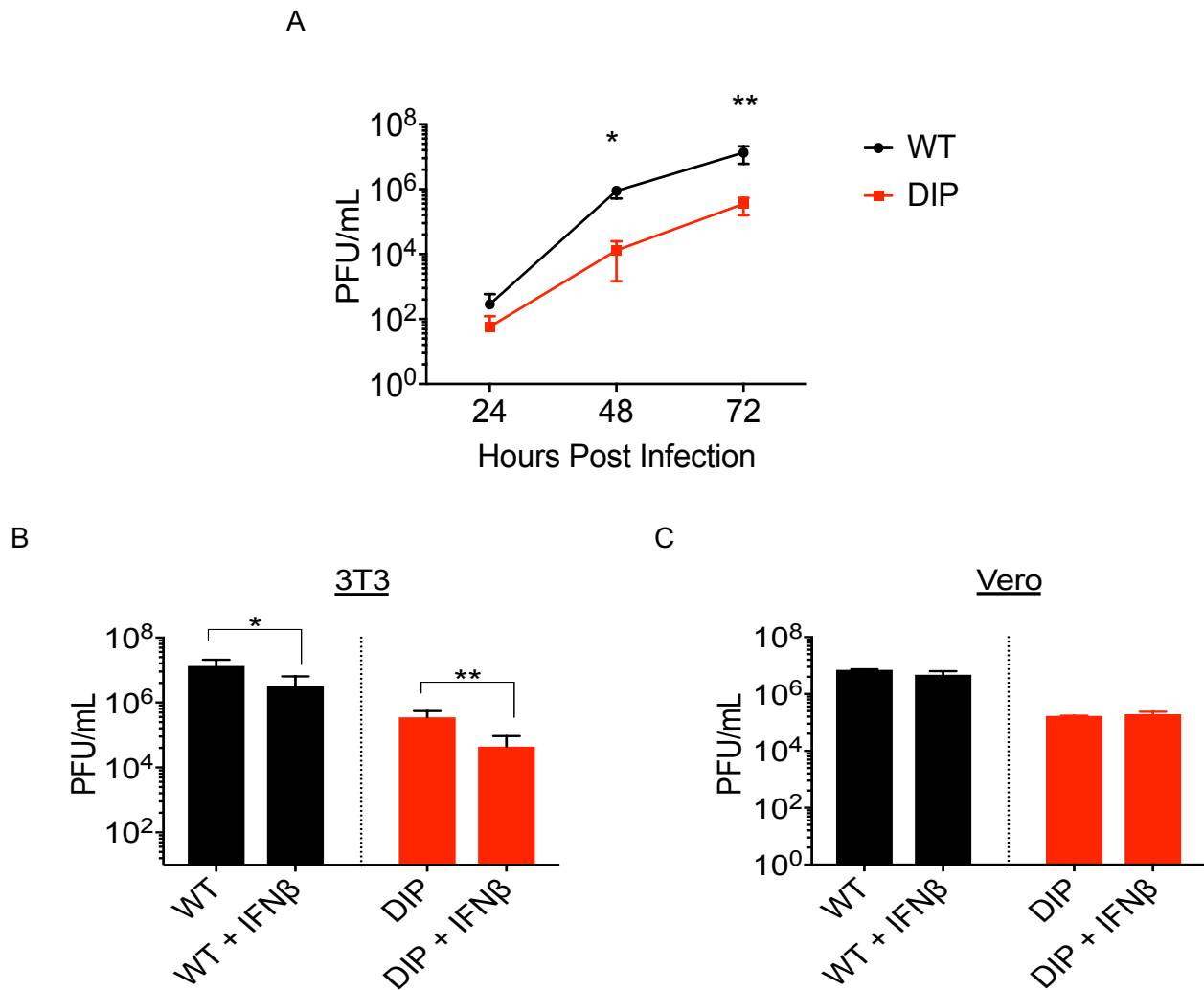
to cell death.

Figure 3-1. DIP replication is attenuated *in vitro*

(A) Growth curve of WT and DIP virus in NIH 3T3 cells using MOI 0.01, measured by plaque assays to quantify infectious virus production. (**B**) 3T3 cells and (**C**) Vero cells are either mock treated or treated with 100 units/mL IFNβ for 24 hours and then infected with either MOI 0.01 of WT or DIP virus for 72 hours. Infectious virus was quantified using plaque assay. All experiments were performed in triplicates and statistical significance was analyzed by two-tailed student t-test. The averages were graphed with standard deviations.
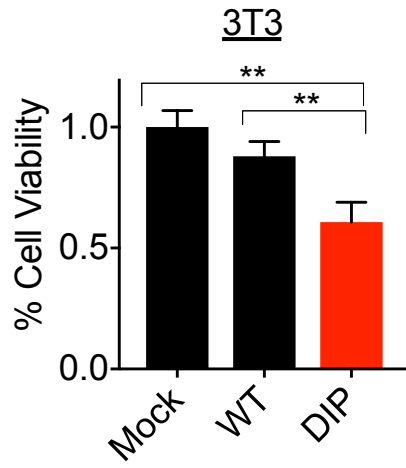
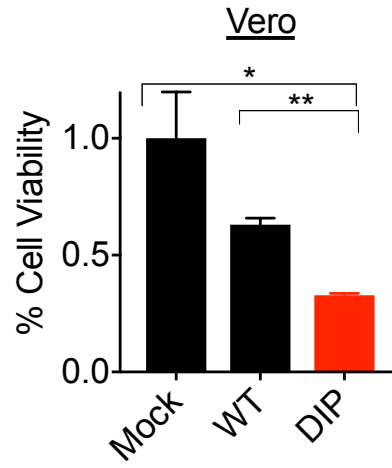**Figure 3-2. Cell Viability Post Viral Infection *in vitro***

(**A**) NIH3T3 cells and (**B**) Vero cells infected using MOI 0.01 of WT or DIP virus. Trypan blue

exclusion assay used to measure the cell count at 72 hours post infection.

DIP produces no infectious virions *in vivo*

We hypothesize removal of the viral IFN-I evasion genes will generate a highly attenuated vaccine virus *in vivo*. C57BL/6 mice were inoculated with 10^5 PFU of DIP via an intraperitoneal (i.p.) route, and viral replication was assayed by infectious virion production and viral DNA copy numbers three days post inoculation (Figure 3-3, A and B). WT virus produced 88 PFU/spleen while no infectious virus was detected in the spleens of DIP inoculated mice (Figure 3-3B). Furthermore, there is no presence of latent virus or reactivation of latently infected cells 14 days post DIP inoculation (Figure 3-3, C and D).

Additionally, no increase in spleen weight associated with splenomegaly is observed upon DIP inoculation (Figure 3-3E). Intranasal inoculation also confirmed no detectable infectious virion production or latency establishment in the spleen (Figure 3-4, A-D).

To determine whether the type I interferon response is responsible for the attenuation of the DIP virus, we inoculated IFNARα/β$^{-/-}$ (IFNAR$^{-/-}$) mice i.p. with 10^5 PFU DIP. DIP replication is rescued, and 4×10^5 infectious virions are detected in the spleen of IFNAR$^{-/-}$ mice (Figure 3-5). Infectious virions were also detected in the liver and lungs of IFNAR$^{-/-}$ mice. The brain was the only organ tested that did not have detectable virus. DIP could not be rescued in the IFNAR$^{-/-}$ mice when inoculated intranasally (Figure 3-6). In contrast, no DIP infectious virus is detected in the organs three days post infection in severe combined immune deficiency (SCID) mice, demonstrating that DIP replication is attenuated in the absence of a function T and B cell responses (Figure 3-5). When SCID mice were infected long-term with either WT or DIP, both viruses resulted in lethal infection. WT infection killed SCID mice by 3 weeks post infection and DIP infection killed SCID mice by 8 weeks post infection (Figure 3-7). Comprehensive analysis of spleen, liver, brain, lungs, and peritoneal showed no evidence of infectious virions in either C57BL/6 suggesting the DIP virus is a very safe vaccine candidate in healthy immune competent hosts.

DISCUSSION

In vivo data suggests the DIP vaccine is unable to replicate upon i.p. and i.n. inoculation in C57BL/6 mice. No productive lytic infection is measured and the viral genome is undetectable 48 hours post inoculation. The fast clearance of the viral genome is important to reducing the risk of genomic recombination. The possibility of the DIP vaccine replicating in tissues other than the lungs and spleen was investigated but no evidence of DIP replication is measured in the liver or brain. A previous vaccine design constitutively expressing RTA using a CMV promoter resulted in lethal lytic replication in the brains of mice deficient in antibody responses and immune-competent mice, C57BL/6 [54]. To attenuate the lytic replication of the DIP vaccine virus, a weaker PGK promoter was used to drive RTA expression and immune evasion genes were removed. DIP infection shows no lytic replication in the brain of inoculated mice at 3 days post infection. The attenuated replicated is rescued in the IFNAR$^{-/-}$ mice suggesting the IFN response is limiting lytic replication upon i.p. inoculation. Interestingly, the DIP vaccine is not rescued in the IFNAR$^{-/-}$ upon i.n. inoculation suggesting the attenuation is mediated differently depending on the route of inoculation. Identifying which cytokines are upregulated and which cell types are recruited upon i.n. inoculation may elucidate the mechanism of attenuation upon i.n. inoculation. While the IFN response plays a critical role in limiting DIP replication during early time points, adaptive immunity is still required to fully control DIP replication to avoid lethal consequences. The possibility of using the DIP vaccine virus as a therapeutic vaccine for HIV patients would require the patients to have a healthy CD4+ T cell count. The absence of viral genome in the spleen, no reactivating virus and the lack of splenomegaly in C57BL/6 mice all support the conclusion that the DIP vaccine is unable to establish latent infection and persist *in vivo*.
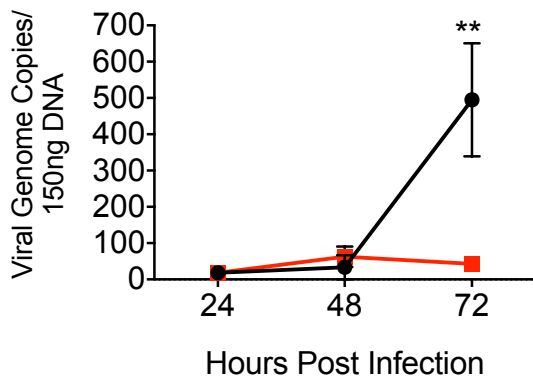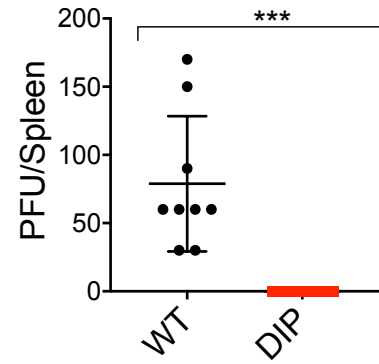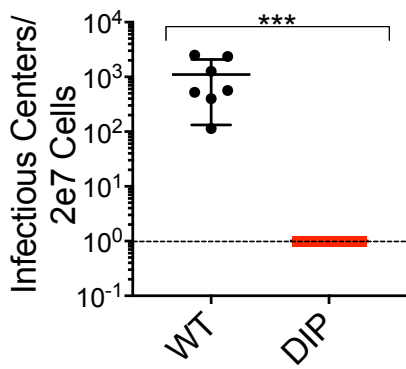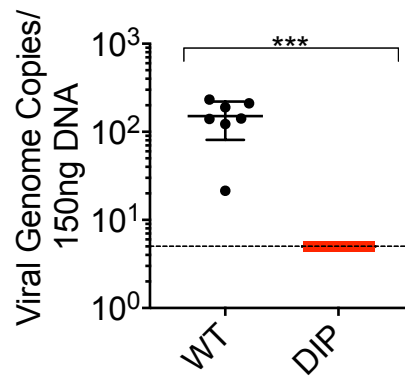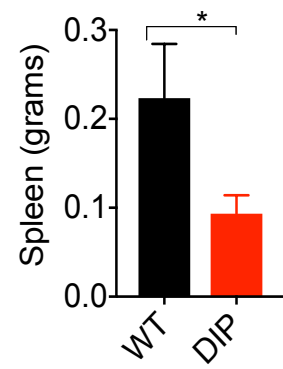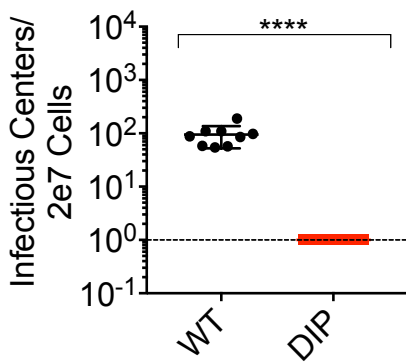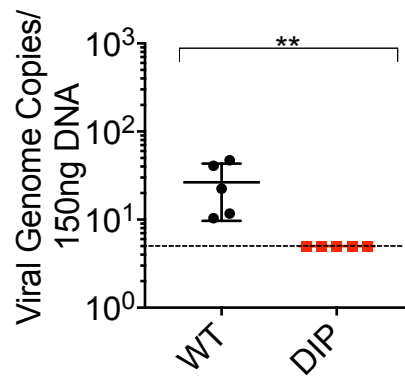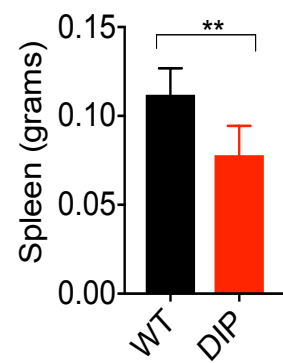
Figure 3-3. DIP produces no infectious virions and is latency deficient *in vivo*

All infections were performed i.p. with 10^5 PFU of WT or DIP. (**A**) Spleens were harvested at 24, 48 and 72 hours, post-infection for qPCR analysis of viral DNA copy numbers. The averages of three mice were graphed with standard deviations. (**B**) Productive infection in the spleens 72 hours post-infection assessed by plaque assay. (**C**) Latent infection in the spleens at 14 days post-infection of 10^5 PFU assessed by infectious center assay and (**D**) qPCR analysis of viral DNA copy numbers. (**E**) Spleen weight is measured in grams at 14 days post infection. (**F**) Latent infection in the spleens at 2 months post-infection of 10^5 PFU assessed by infectious center assay and (**G**) qPCR analysis of viral DNA copy numbers. (**H**) Spleen weight is measured in grams at 2 months post infection. All the experiments, except (A), were repeated twice with different numbers of mice in each repeat. The data from individual mice, the averages, and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.
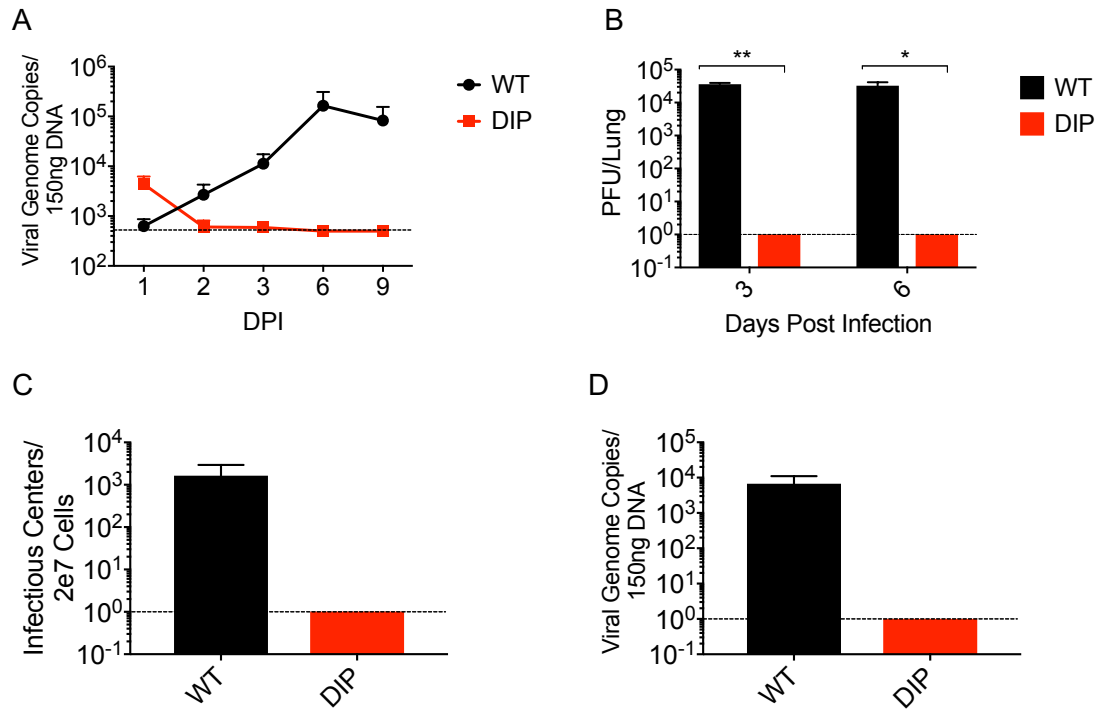
Figure 3-4. DIP is replication and latency deficient *in vivo* upon intranasal inoculation

Mice were i.n. inoculated with 10^5 PFU of DIP or WT. (**A**) Lungs (N=4) were harvested 1, 2, 3, 6, and 9 days post infection for copy number analysis by qPCR. (**B**) Lungs (N=3) were harvested 3- and 6-days post infection for plaque assays. Spleens (N=3) were harvested 14 days post infection for infectious center assay (**C**) and copy number analysis by qPCR (**D**). The averages and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.
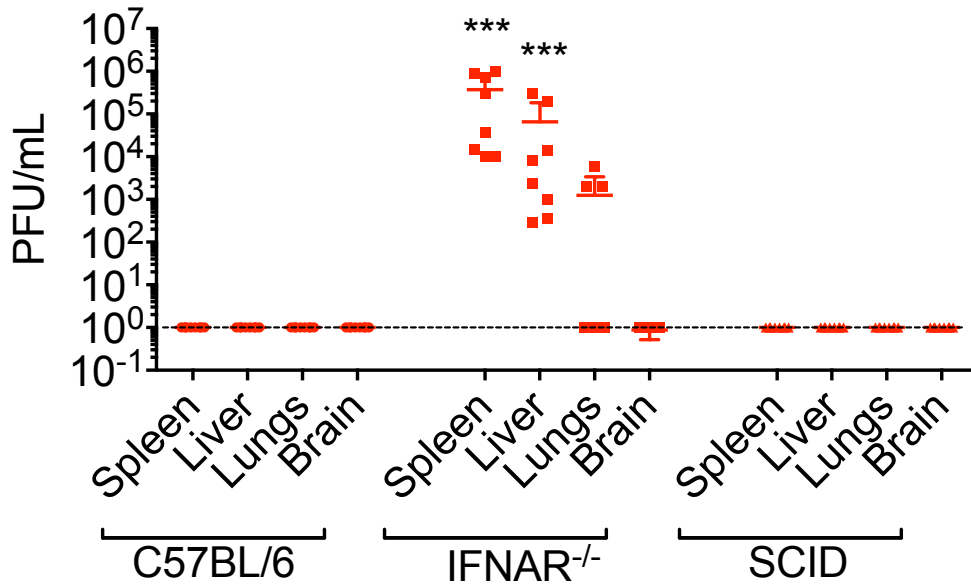
Figure 3-5. DIP attenuation is mediated by the type I IFN response.

Productive infection in the spleens, liver, lungs, and brains of DIP infected C57BL/6, IFNAR[-/-,] and SCID mice were harvested 3 days post-infection for plaque assay. All the experiments, except (A), were repeated twice with different numbers of mice in each repeat. The data from individual mice, the averages, and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.
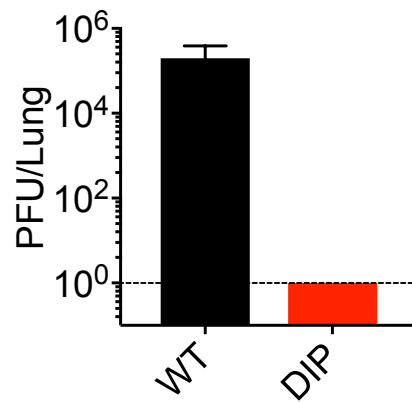
Figure 3-6. DIP cannot be rescued in IFNAR$^{-/-}$ mouse model upon intranasal inoculation

IFNAR$^{-/-}$ mice were intranasally inoculated with 10^5 PFU of WT or DIP virus. 6 days post inoculation, lungs were harvested for plaque assay (N=3 per group). The limit of detection, dotted line, is 1 PFU/lung.
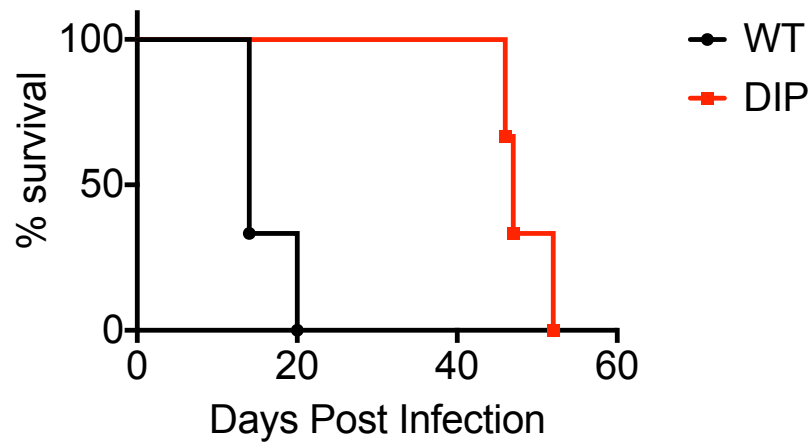
Figure 3-7. DIP causes delayed lethal infection in SCID mice.

SCID mice were inoculated i.p using 10^5 PFU WT or DIP virus. Mice were euthanized upon showing severe signs of weight loss, hunched back and/or paralysis according to Division of Laboratory Animal Medicine (DLAM) protocol.

CHAPTER 4

CHARACTERIZATION OF IMMUNE RESPONSES ELICITED BY DIP

ABSTRACT

Priming a durable memory response is critical for vaccination efficacy. Despite limited replication and consequentially limited antigen production, DIP is able to prime a cellular and humoral response. DIP generates functional viral specific T cells that display a higher frequency of MPEC viral specific population than WT generated T cells. DIP generates more viral specific IgG but lower neutralizing activity than WT generated sera. DIP antibodies are targeted to a limited repertoire of viral epitopes, none of which are unique to DIP vaccination compared to WT generated antibodies. DIP is highly immunogenic and induces several immunomodulatory and inflammatory cytokines which bridge the gap between innate immunity and adaptive immunity. The inoculation of DIP leads to a significant increase in leukocytes in the peritoneal, particularly neutrophil and plasmacytoid subsets.

INTRODUCTION

The cellular response plays a pivot role in controlling and clearing the acute infection in the lungs. Depletion studies outline the differential roles of CD8+ T cells and CD4+ T cells to control lytic infection in the lungs and latent infection of the lungs. CD8+ T cells are required to clear primary infection while depletion of CD4+ T cells results in extended period of primary infection that is eventually cleared[75]. In the spleen, CD8+ T cells reduce the number of latently infected cells while CD4+ T cells are unable to control latent infection. The function of CD4+ T cell is associated with controlling the lymphoproliferation and splenomegaly associated with latent infection. Mice depleted for both CD4+ and CD8+ T cells had significantly higher titers of virus in the lung and increased number of latently infected spleens in the spleen[75]. For mice that have undergone thymectomy, depletion of both subsets of T cells results in a fatal infection upon to i.n. WT MHV-68 inoculation[76]. CD4+ T cells can perform helper functions to drive the activation of CD8 T cells and B cells and also have direct cytolytic effects by producing IFNγ[77-79]. In the MHCII$^{-/-}$ model, the absence of functional CD4 T cells causes a loss of functional CD8 T cells over time and the mice succumb to viral infection[80].

To determine if the highly attenuated DIP vaccine can elicit a viral specific cellular and humoral immune response, the memory responses were characterized at 2 months post vaccination. WT MHV-68 elicits a T cell response with a broad range of epitopes from immediate early (IE), early (E) and late (L) genes[81,82]. Despite the broad repertoire, T cells against MHV-68 make up a small population of total T cells in the splenocyte. MHV-68 specific CD8+ T cell responses are identified using tetramers against two immunodominant epitopes derived from ORF6 and ORF61. ORF6, a single stranded DNA binding protein, and ORF61, a large ribonucleotide reductase subunit, are both early lytic genes and yet T cell responses to these two genes have different kinetic patterns during infection[83,84]. ORF6-specific T cell responses are predominant during early infection and decline rapidly afterwards. On the other hand, ORF61-specific T cell

responses are sustained over time, most likely due to restimulation during persistent infection[82].

We hypothesize that DIP will elicit less ORF61 specific T cells than WT because DIP is latency

deficient. In order to protect against WT challenge, DIP will need to generate T cells against a

broad repertoire epitope. Vaccinations against a single epitope, such as latent gene M2 by gene

gun vaccination or by vaccinia virus expressing ORF6, have been shown to be ineffective at

protecting against long term latent infection[85,86]. Similarly, generating T cells against a single

epitope by introducing dendritic cells pulsed with viral epitopes, including gp150, gB, ORF61

and ORF6, are also unable to prevent the establishment of latency[87]. To test whether T cells

against both early and late genes are being generated, we utilized peptides against ORF6 (E),

ORF61 (E), and ORF8 (L), in ex-vivo T cell stimulation experiments[88].

WT MHV-68 induces high titers of viral specific antibodies targeting several envelope

proteins, tegument proteins, and capsid proteins[89]. Viral specific antibodies limit MHV-68

reactivation in the spleen. T cell depletion in $CD28^{-/-}$ mice results in increased titers of lytic virus

in the spleen compared to T cell depletion in C57BL/6 mice. Serum from WT immunized mice

can be passively transferred to a T cell depletion $CD28^{-/-}$ to recover the ability to limit lytic

replication in the spleen[90]. EBV subunit vaccines focus on generating neutralizing antibody

titers against gp350. DIP sera will be tested for the presence of neutralizing activity and

antibody production against gp150, the MHV-68 homolog of EBV gp350.

RESULTS

DIP primes comparable virus-specific T cells responses to WT

Despite limited antigen expression due to its highly attenuated replication, DIP

immunization affords protection against WT. We hypothesize a robust and functional T cell

response is elicited by DIP immunization. To assess the ability of DIP to prime T cells, we

quantified the population of virus-specific $CD8^+$ T-cells using tetramers for the dominant

epitopes of MHV-68 derived from ORF6 and ORF61. At one-month post inoculation, similar frequencies of viral-specific T cells are induced by DIP and WT (Figure 4-1, A and B). However, the total number of the cells in the spleen are higher in WT infected mice compared to DIP infected mice (Figure 4-1D). We hypothesize that the smaller spleen is because DIP is unable to stimulate lymphoproliferation of the Vβ4+ T cells. At two months post inoculation, DIP generates 1.2% ORF6 specific T-cells compared to 0.6% generated by WT and similar frequencies of ORF61 specific T-cells were observed between DIP and WT (Fig. 4-2, A and B). We used the expression of CD127 (IL-7 receptor α-chain) and killer cell lectin-like receptor (KLRG1) to evaluate the phenotype of these T cells (Figure 4-1C) and (Figure 4-2C). CD127^{high}KLRG1^{low} CD8^{+} T cells, defined as memory precursor effector cells (MPECs), are capable of developing into long-lived memory cells, while CD127^{low}KLRG1^{high} cells, defined as short-lived effector cells (SLECs), are terminally differentiated[91] . Similar phenotypes were seen on ORF61-specific T cells between WT and DIP, 54% of ORF6 specific T cells primed by DIP are CD127^{high}KLRG1^{low}, compared to only 33% primed by WT (Figure 4-2C). At 2 months post inoculation, WT infected mice continue to maintain a larger spleen than mock and DIP infection (Figure 2-1D). This data suggests DIP is more effective than WT at driving differentiation of ORF6 specific T-cell towards MPECs. Virus-specific T-cells were also characterized by CD62L and CCR7 expression, both of which control homing to secondary lymphoid organs. DIP and WT immunization show no difference in the expression of these homing markers on viral-specific T cells (Figure 4-3). CD44, an activation marker also expressed to similar levels on T-cells elicited by WT and DIP (Figure 4-3).

To examine if there is a difference in effector function between T cells primed by WT and DIP, we carried out intracellular cytokine staining following *ex vivo* stimulation with ORF6, ORF61, and ORF8 peptides. Upon stimulation with ORF6 peptide, DIP primed T-cells producing IFNγ, TNFα, or IL-2 were two-fold more abundant than WT primed T-cells (Figure 4-4). Upon

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ORF61 peptide stimulation, similar frequencies of DIP primed T-cells produced IFNγ as WT

primed T-cells (Figure 4-5). A smaller proportion of DIP primed T cells produced TNFα after

ORF61 peptide stimulation, when compared to WT. Neither ORF61 nor ORF8 peptides

stimulated IL-2 production in DIP or WT primed T cells (Figure 4-5).
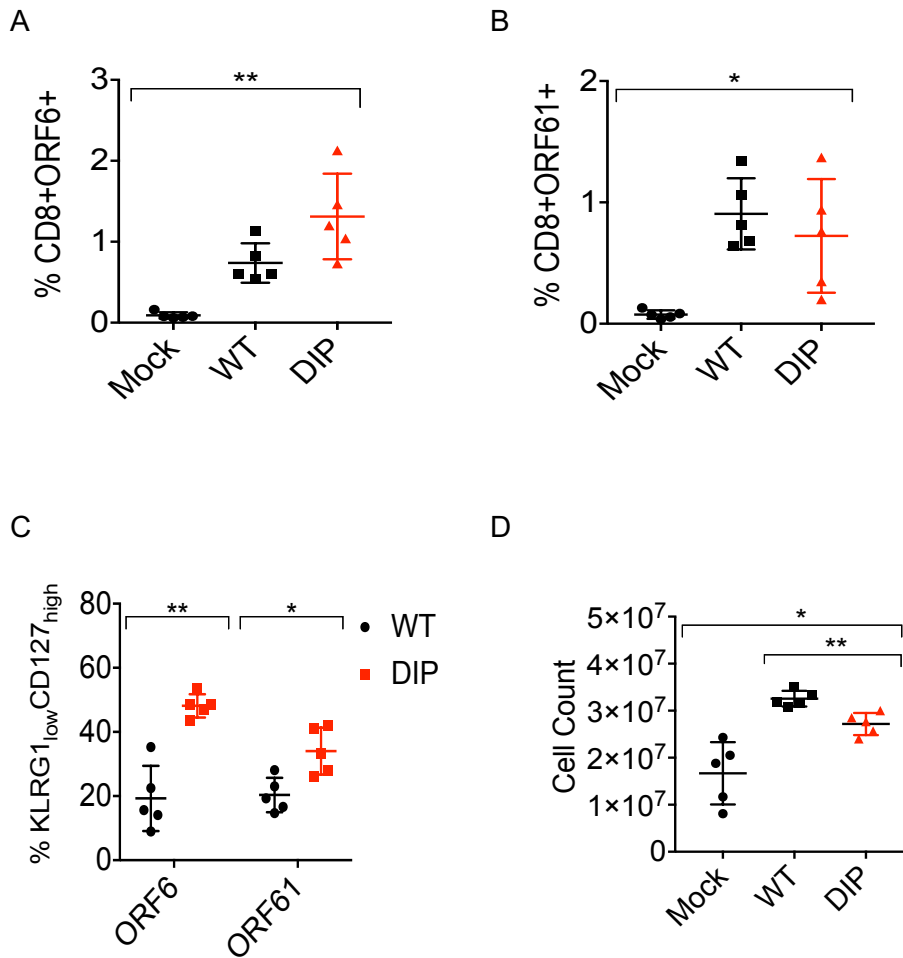
Figure 4-1. DIP generates higher frequency of viral specific MPEC compared to WT

One-month post vaccination, splenocytes were harvested and examined for virus-specific CD8+

T cells using tetramers $ORF6_{487-495}$/Db (**A**) and $ORF61_{524-531}$/Kb (**B**). Tetramer positive CD8^{+} T

cells were examined for KLRG1 and CD127 expression (**C**). The number of cells in spleen was

quantified using trypan blue (**D**). The averages and standard deviations were graphed (N=5).
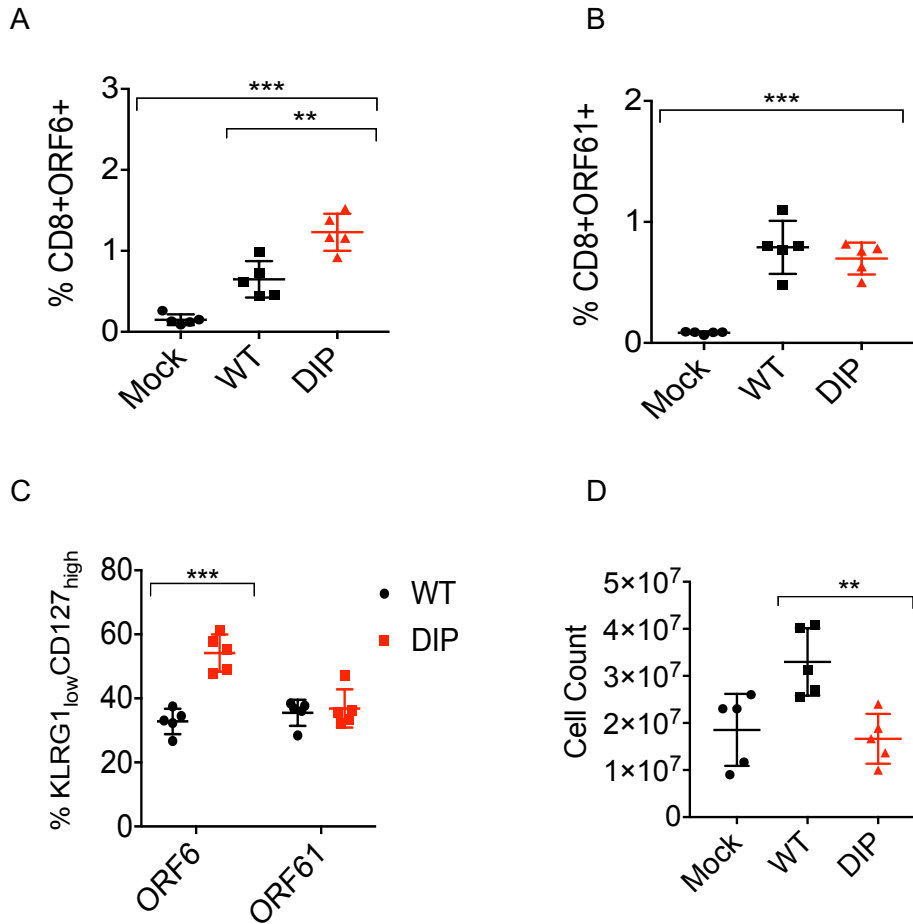
Figure 4-2. DIP generates higher frequency of ORF6 specific MPEC

At two months post i.p. infection of 10^5 PFU WT or DIP, splenocytes were examined for virus-specific CD8$^+$ T cells using tetramers ORF6$_{487-495}$/Db (**A**) and ORF61$_{524-531}$/Kb (**B**). Tetramer positive CD8$^+$ T cells were examined for KLRG1 and CD127 expression (**C**) The number of cells in spleen was quantified using trypan blue (**D**). The averages and standard deviations were graphed (N=5).

ORF6$_{487}$

ORF61$_{524}$

KLRG1

CD127
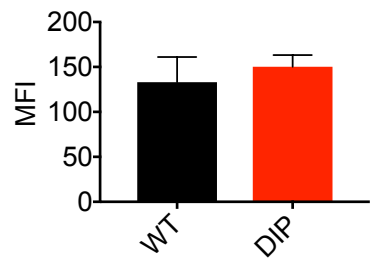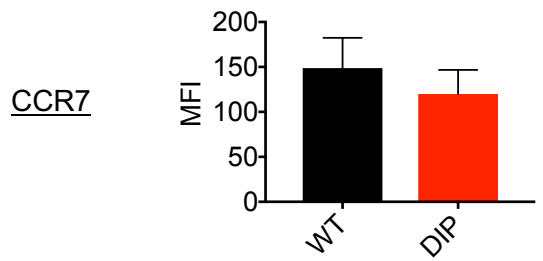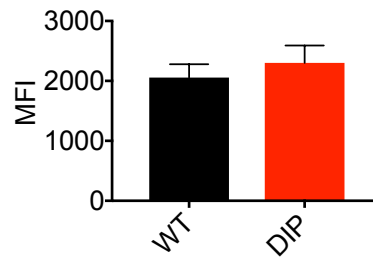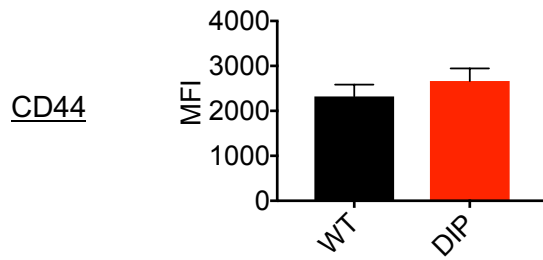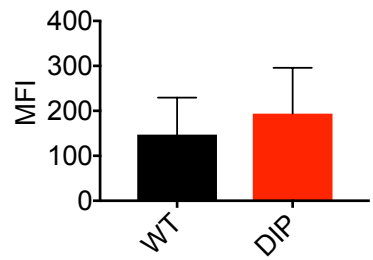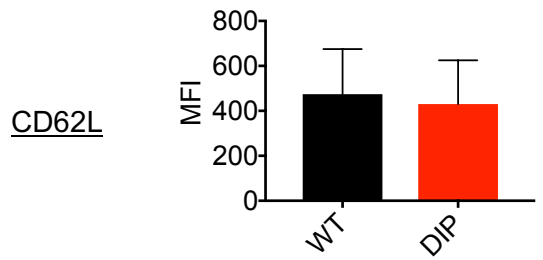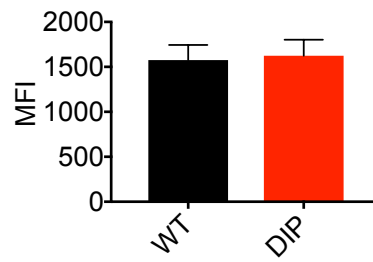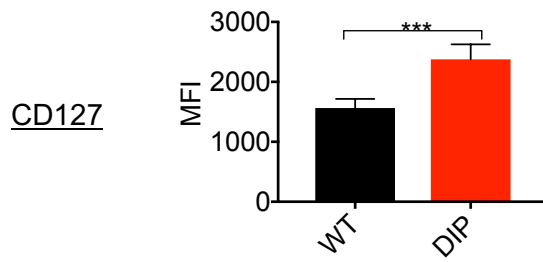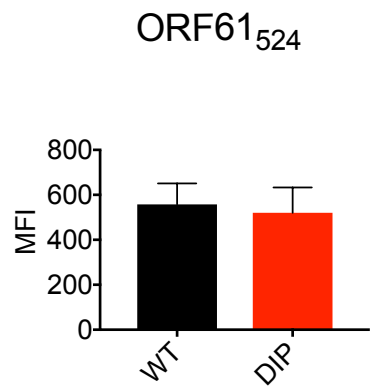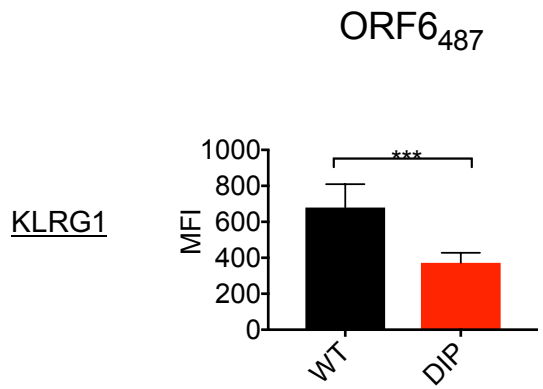
CD62L

CD44

CCR7

Figure 4-3. Phenotyping viral specific T cells using cell surface markers

Two months post vaccination, viral specific CD8$^+$ T cells were phenotypically characterized by the expression of KLRG1, CD62L, CD127, CD44, and CCR7. The averages and standard deviations were graphed (N=5). The averages and standard deviations were graphed (N=5).
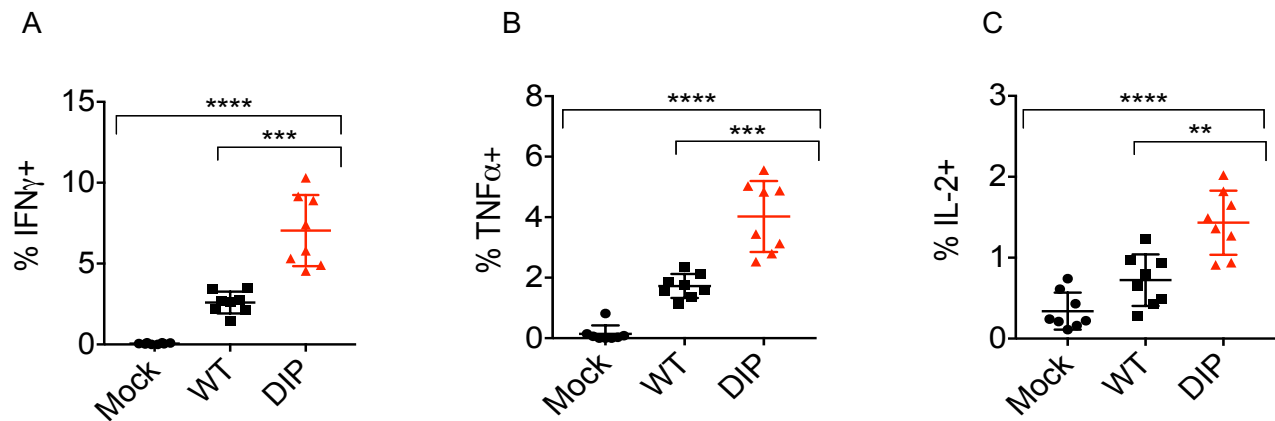
Figure 4-4. DIP vaccination elicits functional T cell immunity

Splenocytes harvested two months post i.p. infection were stimulated with the ORF6$_{487-495}$ peptide and stained for IFNγ (**A**), TNFα (**B**), and IL-2 (**C**). All the experiments were repeated twice with different numbers of mice in each repeat. The data from individual mice, the averages, and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.

Figure 4-5. DIP generates functional viral specific T cells

Two months post vaccination, splenocytes were stimulated with peptides: ORF61$_{524}$, ORF8$_{604}$ and negative control, Sendai$_{324}$. 16 hours post stimulation, splenocytes were intracellularly stained with IFNγ, TNFα and IL-2. The individual mice, averages and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.

DIP immunization generates humoral responses against multiple herpesvirus proteins

Although levels of virus-specific IgG were significantly higher in DIP primed mice than WT infected mice, DIP induced sera contained lower virus-neutralizing activity when compared to WT elicited serum (Figure 4-6, A and B). We further examined antibody binding to viral proteins using Western blot analysis, which revealed that antibodies generated by DIP and WT immunization recognize different viral proteins (Figure 4-7). We investigated whether antibodies generated by DIP vaccination would bind to epitopes found on the surface of infected cells using immunofluorescence assay (Figure 4-8). While WT sera binds predominately to the cell surface, DIP primed antibodies predominantly bind to intracellular epitopes. This data suggests that DIP antibodies may be targeting different epitopes and functioning in a mechanism distinct from WT generated antibodies. Mass spectrometry was used to identify the targets of DIP elicited antibodies (Figure 4-9 & 4-10). The results did not identify unique viral proteins were targeted by DIP antibodies. Both WT and DIP antibodies bound specifically to M9 (Small Capsid Protein), BALF4 (Transmembrane protein), ORF64 (Large tegument protein) and BALF3 (DNA replication protein). Unlike DIP antibodies, WT antibodies were also able to 8 other viral proteins including the following glycoproteins H, glycoprotein L, ORF28, and glycoprotein150.

Figure 4-6. DIP vaccination elicits protective antibodies

(**A**) Viral specific IgG titers in the sera collected from mice 2 months post-infection with 10^5 PFU WT or DIP were measured using ELISA. (**B**) Neutralizing activities were assessed by incubating 2-fold serially diluted serum with 100 PFU WT prior to plaque assay. All the experiments were repeated twice with different numbers of mice in each repeat. The averages and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.

Figure 4-7. DIP elicits antibodies targeting different epitopes compared to antibodies elicited by WT virus.

Western blots analysis of de-glycosylated WT virion preparations was performed using sera collected from infected mice as the primary antibodies. All the experiments were repeated twice with different numbers of mice in each repeat. The averages and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.
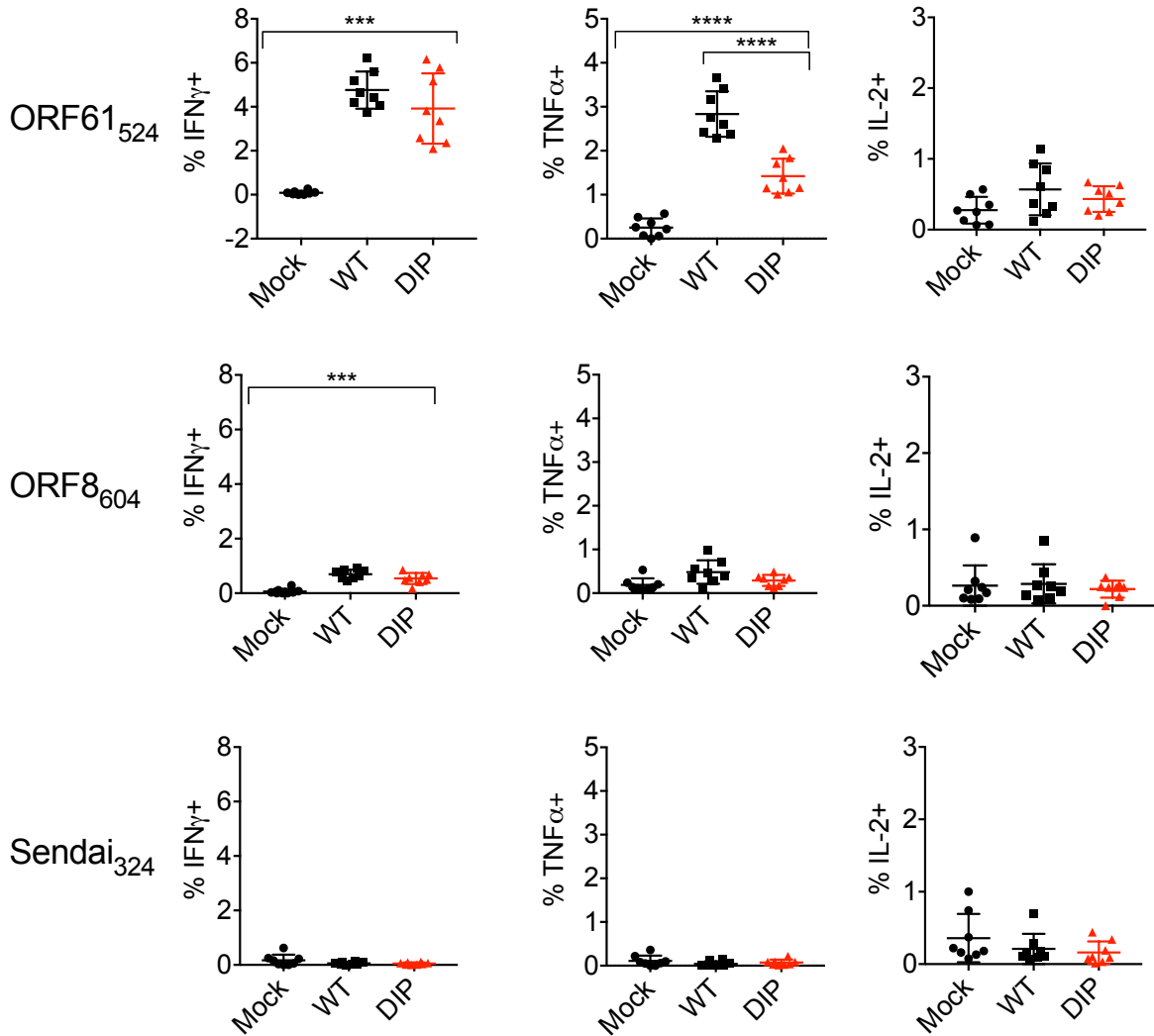
Control
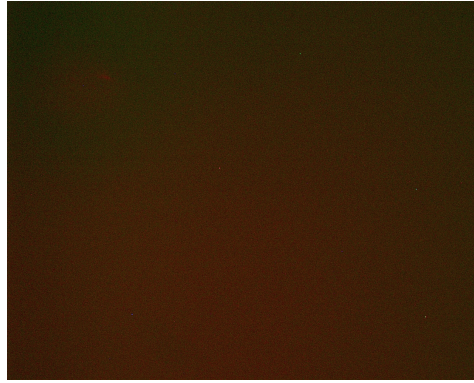
Mock Serum
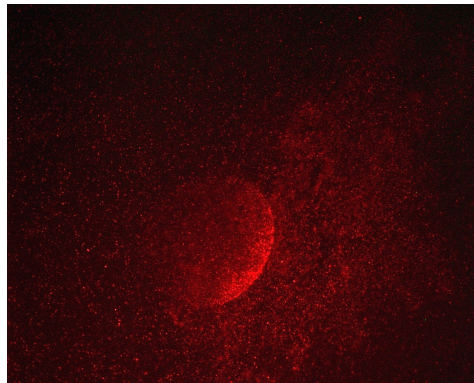
WT Serum
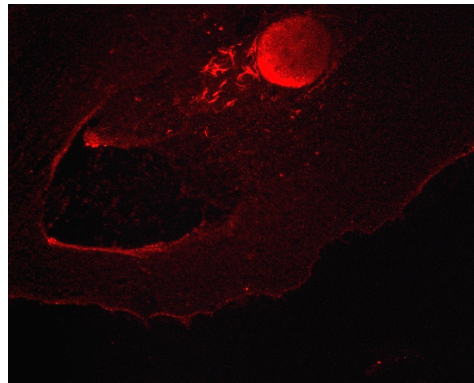
DIP Serum
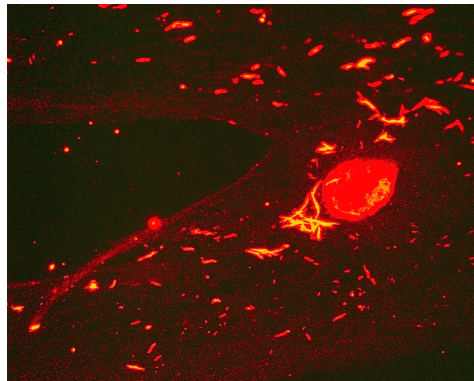
Figure 4-8. DIP antibodies bind cell surface and intracellular targets

Sera was collected from mice 1-month post inoculation with 10^5 PFU WT, DIP or mock. MEF

cells were infected with a MOI 1 of WT virus. Sera was diluted 1:100 in IFA blocking buffer in

the absence of Triton-100x.

IP using DIP serum	IP using WT serum	Protein Type
M9	M9	Small Capsid Protein
	ORF27	Transmembrane glycoprotein
ORF11	ORF11	Virion Protein
BALF4	BALF4	Transmembrane
ORF75c	ORF75c	Tegument Protein
ORF21	ORF21	Thymidine kinase
ORF75b	ORF75b	Tegument Protein
	ORF28	Glycoprotein
	ORF22	Glycoprotein H-like protein
ORF4	ORF4	Complement regulatory protein
ORF8	ORF8	Envelope glycoprotein B
	ORF47	Glycoprotein L
	ORF63	Tegument Protein
	M7	Glycoprotein 150
	ORF6	Major DNA-binding protein
	M3	
ORF64	ORF64	Large tegeument protein deneddylase
BALF3	BALF3	DNA replication protein
BQLF2	BQLF2	Tegument Protein

Figure 4-9. Proteins identified as significant targets of antibodies using IP-mass spectrometry

Sera collected from mice 2 months post-infection with 10^5 PFU WT or DIP was pooled from multiple mice.
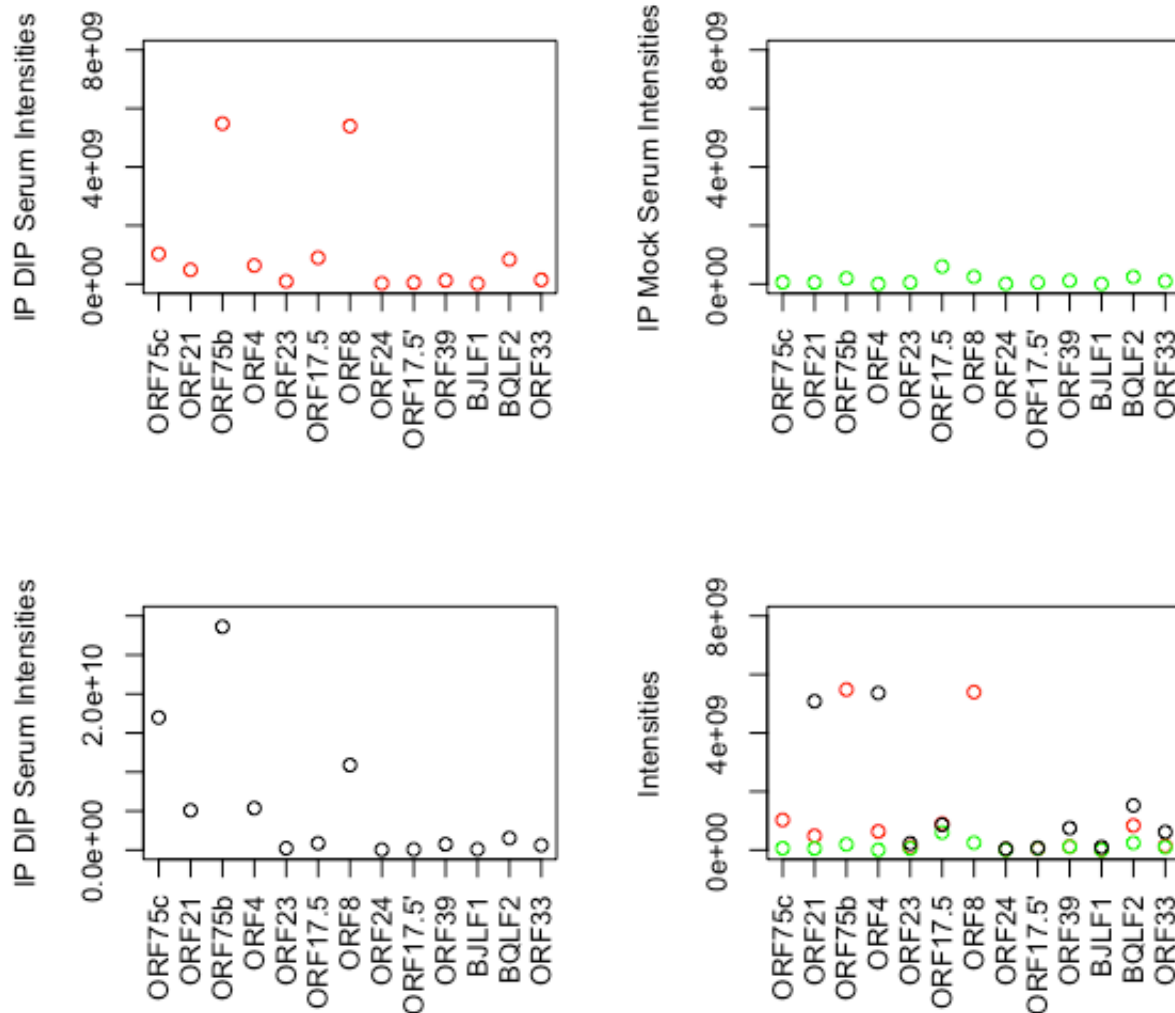
Figure 4-10. Signal intensities for identified protein targets of DIP antibodies using IP-mass spectrometry

Sera collected from mice 2 months post-infection with 10^5 PFU WT or DIP was pooled from multiple mice.

DIP vaccine elicits production of immunomodulatory cytokines

Despite highly attenuated replication, DIP is able to prime a broad and durable memory response. Activation of innate immune responses is critical to prime long-term memory responses [92-94]. WT MHV68 efficiently avoids the induction of inflammatory cytokines as part of its strategy to evade the immune system. *In vitro*, a high MOI (100 PFU) is required to elicit a measurable cytokine response in bone marrow derived macrophages (BMDM) and dendritic cells[95]. Here, we ask if the DIP virus becomes an inducer of cytokine production in BMDMs. We infected BMDMs with WT and DIP at a MOI of 1 and examined cytokine RNA expression at 24 hours post-infection. Several cytokines, including IFNβ, IL-1β, TNFα, IL-6, and IL-12p40, were significantly up-regulated by DIP infection compared to WT (Figure 4-11A). We confirmed this result by performing an ELISA for IL-12 p40 production. DIP induced 30-fold more IL-12 protein than WT infection (Figure 4-11B). The DIP virus prep is known to have five-fold more non-infectious particles than WT virus by copy number analysis. The induction of cytokines may be due to more debris and noninfectious virion proteins in the DIP preparation. To test this hypothesis, BMDM were infected with higher MOIs of WT virus. Higher MOIs of WT virus is able to induce similar induction of IFNβ. However, five-fold and 10-fold more WT virus infecting BMDM is unable to induce similar upregulation of IL-1β, TNFα, and IL-12 (Figure 4-12). The robust induction of IL-1β is confirmed to be induced by DIP *in vivo* (Figure 4-13). Spleen tissue was assessed by IL-1β ELISA 48 hours post inoculation with mock, WT or DIP.

We hypothesize that the removal of immune evasion genes results in a highly immunogenic virus that recruits innate immune cells and elicits immunomodulatory and inflammatory cytokines to effectively prime the adaptive immune response. Inflammatory responses in the peritoneal cavity (PEC) were examined between WT and DIP infected mice. Significantly more cells were detected in the PEC of DIP infected mice at day 2 post-infection

compared to the PEC of WT infected cells. DIP inoculated mice had 4.8×10^6 cells in the PEC while WT inoculated mice had 2.8×10^6 cells in the PEC (Figure 4-14).

The cells recruited in the PEC were characterized into leukocyte populations by flow cytometry (Figure 4-15). There was no significant increase in natural killer (NK) cells. DIP inoculation resulted in a 1.3% population of neutrophil identified in the PEC, while WT PEC and mock inoculated PEC consisted of 0.5% and 0.7% neutrophil populations, respectively. As dendritic cells (DCs) are the major innate immune cell type that prime the adaptive immunity, we examined the recruitment of DCs in the PEC. While no significant difference in the numbers of conventional DCs (cDCs), DIP inoculation resulted in a 31% population of plasmacytoid DC (pDC) expressing PDCA-1 compared to 4-5% population induced by WT and mock inoculation. pDCs can be identified by the expression of PDCA-1 and also the downregulation of MHCII (Figure 4-16). Unlike cDCs, which are professional antigen presenting cells (APCs), pDCs produce large amount of IFN-I to control viral replication as well as inflammatory cytokines, such as IL-6, TNF-α, and IL-12 to regulate the development of adaptive immunity[96]. pDCs make up 5×10^5 cells in the PEC upon DIP infection while only 1.7×10^5 cells in the PEC are pDC upon WT infection (Figure 4-17A). To determine if there is a correlated increase in the IFN response in the PEC, we measured the ISG, IFIT1. DIP inoculation led to a 3-fold increase in ISG production relative to mock and WT inoculation (Figure 4-17B). Taken together, these results suggest DIP superior ability to induce inflammatory responses.
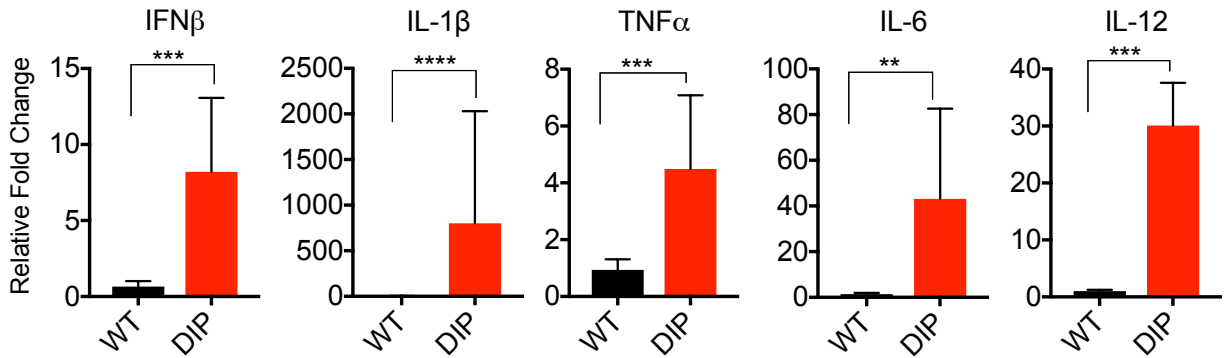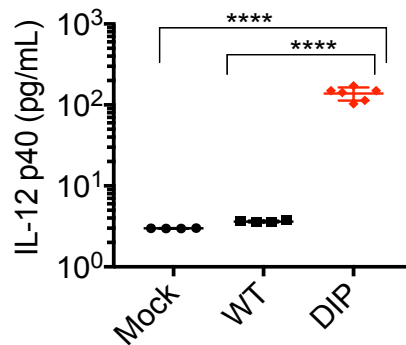
Figure 4-11. DIP elicits inflammatory and immunomodulatory cytokines

(**A**) Murine BMDM are infected with MOI 1 of WT or DIP in triplicates. Total RNAs were

extracted 24 hours post infection for reverse transcription and qPCR for IFNγ, IL-1β, TNFα,IL-6,

IL-12, and actin. The cytokine RNA expression was normalized against actin and fold changes

calculated by comparing to mock infection of BMDM were graphed. (**B**) The supernatants were

collected 24 hours post viral infection of BMDM using MOI 1 of WT or DIP. IL-12p40 ELISA kit

used to quantify secreted cytokine in the supernatant.
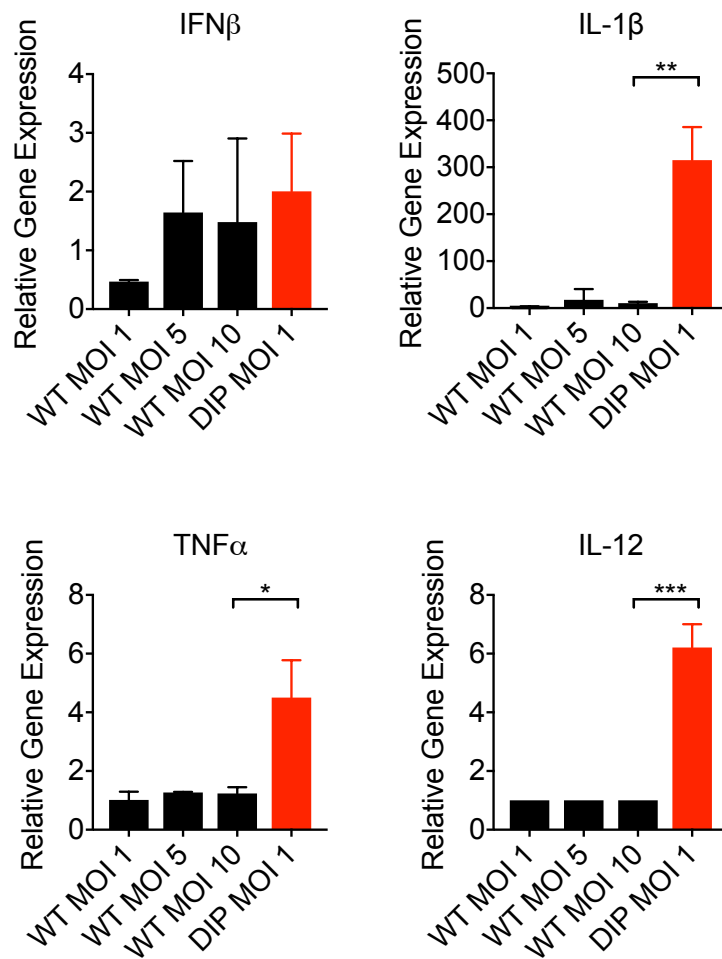
Figure 4-12. DIP mediated induction of cytokine is significantly more robust than WT induction

Murine BMDM are infected with MOI 1, 5, or 10 of WT or MOI 1 of DIP in triplicates. Total RNAs were extracted 24 hours post infection for reverse transcription and qPCR for IFNγ, IL-1β, TNFα,IL-6, IL-12, and actin. The cytokine RNA expression was normalized against actin and fold changes calculated by comparing to mock infection of BMDM were graphed.
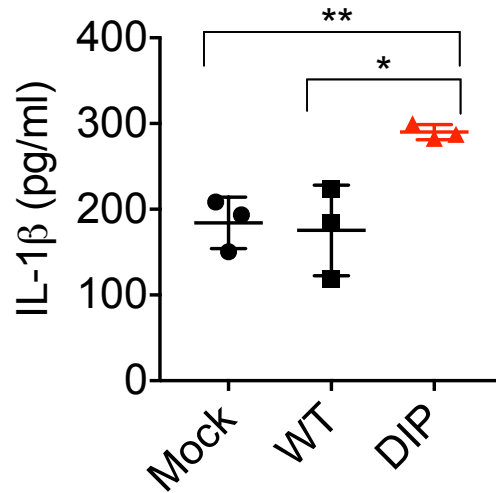
Figure 4-13. DIP robustly induces inflammatory cytokine, IL-1β, *in vivo*

48 hours post inoculation with either 10^5 PFU WT, DIP or mock, spleens were harvested.

Spleen tissue was processed for IL-1β ELISA. The data from individual mice, the averages, and

standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-

test.
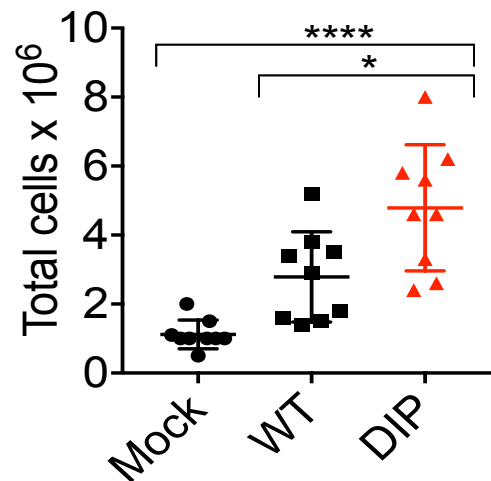
Figure 4-14. DIP recruits more cells to the peritoneal upon inoculation than WT MHV-68

PECs were collected and counted 48 hours post inoculation i.p. with either 10^5 PFU of WT, DIP
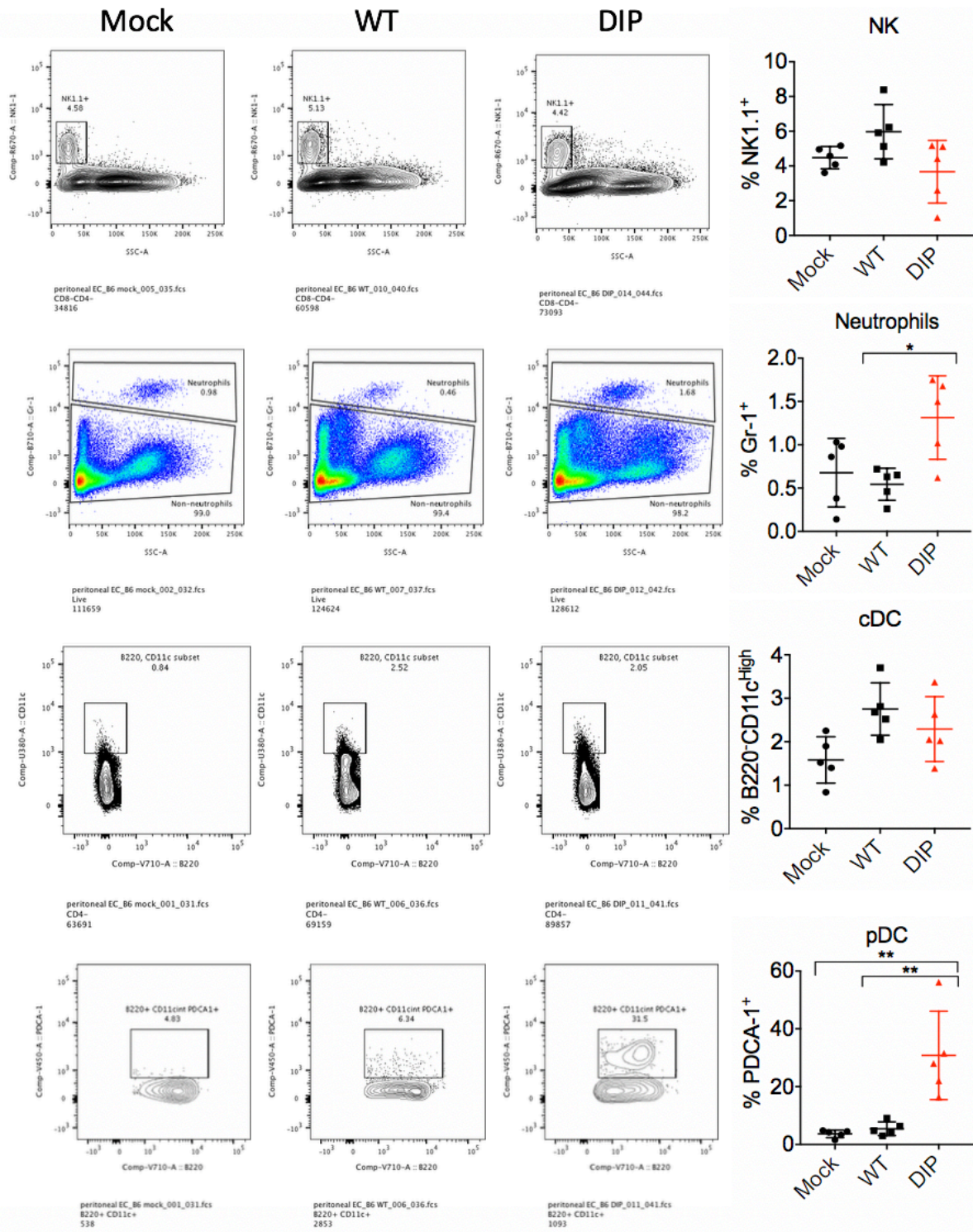
or DMEM.

Figure 4-15. Phenotyping cell types in the PEC upon DIP inoculation

PECs were collected and stained for flow cytometry 48 hours post inoculation i.p. with either 10^5 PFU of WT, DIP or DMEM (mock). NK cells are $B220^-CD4^-CD8^-NK1.1^+$ cells, neutrophils are $CD45^+Gr-1^+$ cells, cDCs are $B220^-CD11c^{high}$, and pDCs are Lin^- ($CD3^-CD19^-NK1.1^{-)}$ $B220^+CD11c^{Int}PDCA-1^{+.}$
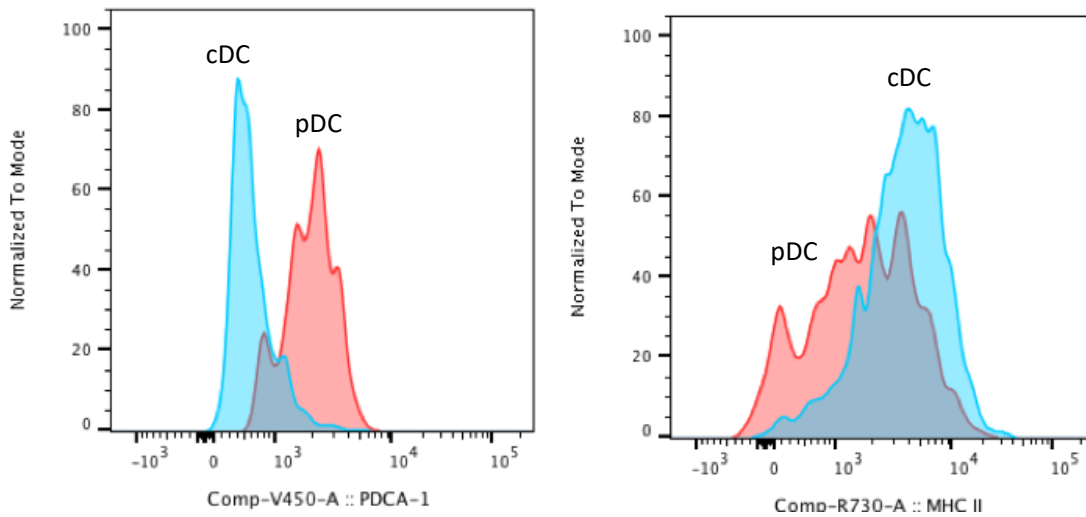
Figure 4-16. Characterizing conventional and plasmacytoid dendritic cells in the PEC

PECs were collected and stained for flow cytometry 48 hours post inoculation i.p. with either 10^5 PFU of WT, DIP or DMEM (mock). cDCs are $B220^-CD11c^{High}$, and pDCs are Lin^- ($CD3^-CD19^-NK1.1^{-)}$ $B220^+CD11c^{Int}PDCA-1^+$. cDCs and pDCs populations are subsequently gated for MHCII expression.
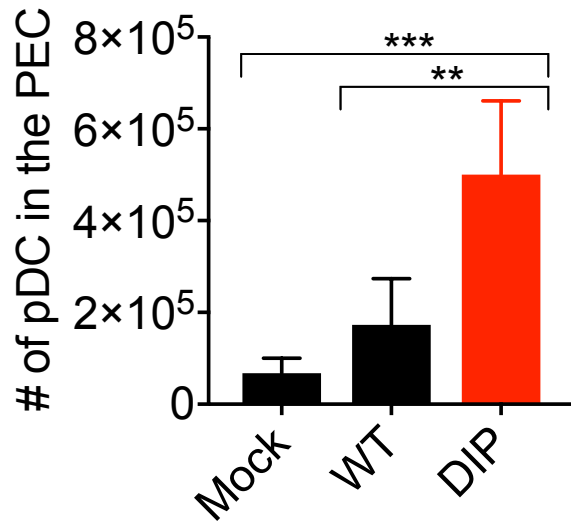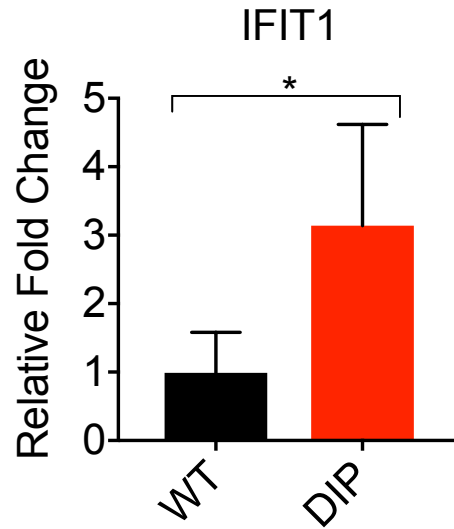
Figure 4-17. IFN producing dendritic cells elicited upon i.p. DIP inoculation

PECs were collected 48 hours post inoculation i.p. with either 10^5 PFU of WT, DIP or DMEM (mock). (**A**) # of pDC in the PEC is calculated using flow cytometry and trypan blue total cell count. Total RNAs were extracted for reverse transcription and qPCR for IFIT1 and actin. The cytokine RNA expression was normalized against actin and fold changes calculated by comparing to mock infection were graphed (**B**). Statistical significance was analyzed by two-tailed student t-test.

DISCUSSION

There is a significant divergence between ORF6 specific T cells and ORF61 specific T cells generated by DIP. Both genes are immunodominant during WT infection but have distinct kinetics over the course of infection. ORF6 specific T cells increase during lytic infection and decrease in frequency during latent infection. In contrast, ORF61 specific T cells increase in frequency during lytic replication and maintain that population size during long term latency. The kinetic pattern associated with ORF61 is believed to be caused by the continued antigen expression during persistent infection which re-stimulates the T cell response. Because DIP does not establish latency, the reduced number of ORF61 specific T cells compared to WT is predicted. An interesting phenomenon is why DIP generates a higher frequency of ORF6 specific T cells at early time points but equivalent frequency of ORF61 specific T cells compared to WT inoculation. Both ORF6 and ORF61 are early genes that should be overexpressed by the constitutively active RTA in the DIP vaccine, yet only ORF6 specific T cell frequency is higher than those induced by WT. The MPEC phenotype induced by the DIP vaccine virus may allow for the maintenance of viral specific T cells instead of decline over time, resulting in a higher frequency of ORF6 specific T cells.

Sera analysis shows high levels of viral specific IgG but reduced neutralizing activity compared to WT. Passive serum transfer from DIP immunized mice to naïve mice failed to protect against WT latent infection. A higher volume of DIP primed sera may be required to make up for the reducing neutralizing activity. The immunoblots and IFA suggest traditional surface proteins such as glycoproteins are not the predominant epitopes of DIP generated antibodies. Importantly, DIP generates very little antibodies against gp150, the homolog of EBV gp350. Neutralizing activity is the only functional antibody assay performed but the role of antibody dependent cell mediated cytotoxicity (ADCC), opsonization, complement and intracellular antibody neutralization via TRIM2 will be interrogated in the future1[97].

DIP is able to prime a broad and durable memory response despite limited replication. We hypothesize that the removal of immune evasion genes results in a highly immunogenic virus that recruits innate immune cells and elicits immunomodulatory and inflammatory cytokines to effectively prime the adaptive immune response. Several cytokines are upregulated in BMDM and require further investigation to elucidate impact on vaccine protection. The increase in the IFN producing pDC population may play a role in directly limiting the DIP replication upon inoculation. pDCs can prime cellular immune response by supporting the cytotoxic functions of CD8$^+$ T cells and polarizing CD4$^+$ T cells to become T helper 1 (TH1) cells[98,99]. pDCs also support the humoral response by activating B cells, promoting plasma cell differentiation and antibody production[100]. pDC depletion prior to i.p. WT MHV68 infection resulted in no significant increase in latently infected cells 5 days post infection. WT MHV-68 has several IFN evasion genes that could be limit the effect of pDCs[101]. Depletion of pDCs prior to DIP vaccination may result in lower T cell priming and antibody production as the innate immunity is unable to activate the adaptive immune response as efficiently. KSHV and EBV have been shown to infect pDC despite the upregulated anti-viral states maintained in pDC cells by constitutive IRF7 expression[102-104]. pDC depletion may be also be compensated by other innate immune cells as pDC depletion does not result in a systemic decrease in IFN production[105,106]. The role of pDC in DIP immunogenicity will need to be further investigated by depletion studies *in vivo*.

CHAPTER 5

DIP VACCINE EFFICACY AND MECHANISM OF PROTECTION

ABSTRACT

The vaccination goal for tumor-associated herpesvirus is to prevent the establishment of latency. The DIP vaccine is able to protect against lytic replication in the lungs 7 days post challenge. DIP immunized mice show protection against WT latent infection when spleens were analyzed 14- and 28-days post challenge. Robust and durable protection is sustained 6 months post vaccination. Spleens showed no presence of latent infection or viral genome in DIP vaccinated mice. Adoptive transfer of individual immune subsets was unable to confer protection. Total T cells were able to reduce the number of latently infected cells but were not able to prevent the establishment of latency. Passive transfer of serum to a naïve host was unable to limit the number of latently infected cells upon WT challenge. Adoptive studies suggest CD8$^+$ T cells, CD4$^+$ T cells and serum are required to confer full protection against latent infection.

INTRODUCTION

There is currently no approved vaccine to prevent infection by tumor-associated herpesviruses. An important advantage of using a live attenuated vaccine is that the full repertoire of viral epitopes are being expressed. Replication deficient viruses present a limited number of epitopes to the immune system. Heat inactivated virus is unable to transcribe and present on MHCI to CD8 T cells. Vaccines focused on a single target such as subunit vaccines do not take into consideration that herpesviruses have evolved several redundant mechanisms to evade immune detection and clearance. While some strategies are able to limit the lytic infection and peak latency, long term latent infection is not impacted. In this chapter, we assess the ability of DIP of provide protection against WT MHV-68 challenge.

DIP is a live attenuated virus that demonstrates no measurable replication capacity *in vivo*. The DIP vaccine is also latency deficient, reactivation deficient, and unable to persist in immune competent host. The efficacy of this highly attenuated vaccine candidate will be tested at several time points. The ability to inhibit lytic infection (7 days post challenge), peak latent infection (14 days post challenge) and long-term latency (28 days post challenge) are essential for a successful vaccine candidate. In additional to the efficacy of protection, the durability of the protection will also be assessed. This information is critical to inform whether a prime-boost strategy will be required to maintain long term protection.

Most licensed live attenuated vaccines provide protection by eliciting antibody responses[107,108,109]. Measles, mumps, rubella and varicella all have live attenuated vaccines. The measles vaccine induces neutralizing antibodies and T cell responses but the antibodies are able to control infection in the absence of a T cell response[110]. The mumps vaccines are also able to induce antibodies and T cell response but the prevalence of multiple strains of mumps vaccines makes it difficult to pinpoint the immune correlate of protection[111,112]. The rubella vaccine elicits high levels of neutralizing antibodies and generates antibodies in the

nasopharynx to prevent viral infection at the mucosa[113]. The only human herpesvirus vaccine is against VZV. The VZV vaccine induces antibodies to prevent varicella but the T cell response is believed to be correlated to protection against herpes zoster[114,115]. The correlate of protection for live attenuated influenza vaccines is strongly associated with the hemagglutinin (HA) neutralizing titers[116]. To determine the mechanism of protection of the attenuated DIP vaccine virus, serum and T cells from DIP vaccinated mice will be transferred to naïve mice prior to WT MHV-68 challenge. In conjunction, T cell depletion studies will be used to assess the ability of serum to prevent the establishment of latency upon WT MHV-68 infection.

RESULTS

DIP immunization protects against latent infection

We assessed the protective efficacy of the DIP vaccine by measuring the lytic replication in the lungs of vaccinated mice 7 days post challenge with WT virus. Lungs were assessed by plaque assay (Figure 5-1A) and qPCR for viral genome copy number (Figure 5-1B). Mock vaccinated mice produce 10^3 PFU/lung while DIP vaccinated mice have no detectable viral replication in the lungs.

Gammaherpesvirus associated malignancies are linked to latency; therefore, the ultimate goal of vaccination against gammaherpesviruses is to prevent the establishment of latency. We assessed the protective capability of DIP immunization against latent infection from WT challenge. Spleens were harvested from vaccinated mice 14 days post-challenge for an infectious center assay that measures reactivating virus and for quantification of viral genome copy numbers by RT-qPCR. Mock immunized mice exhibit an average of 6×10^2 infectious centers/2e7 cells, while no viral reactivation is detected 6/7 DIP-immunized mice (Figure 5-2A). Further confirmation by viral copy number analysis also indicates protection against latent infection from DIP immunization (Figure 5-2D).

While several vaccine strategies can lower the latent viral load at peak latency measured at d14, they fail to modify latent infection when assessed at later time points [51,85,87,117,118]. So far, the only vaccination strategy that can reduce the long-term latent load is based on live attenuated viruses designed to be deficient in either the establishment of latency or reactivation from latency[39-43]. To determine whether DIP can reduce latency at later times, spleens from WT-challenged mice were harvested at 28 days post challenge for analysis. Five out of six (83.3%) DIP vaccinated mice were completely protected from latent infection (Figure 5-2B) with no detectable reactivating virus or viral DNA (Figure 5-2E). The sole DIP vaccinated mouse that was not fully protected had a 100-fold reduction in reactivating latently infected cells compared to mock immunized mice. To determine whether protection is long term we challenged the mice six months post vaccination and assay viral latency at 28 days post challenge. All six DIP-immunized mice were protected against latent infection from WT virus challenge (Figure 5-2, C and F).

T cells are not able to confer protection in the absence of viral specific antibodies

To determine whether DIP primed T-cells can confer protection, we adoptively transferred 3 million cells of either CD4+, CD8+ or total T-cells from mice that were previously infected with WT or DIP two months earlier to naïve mice and challenged the recipient mice with WT one day after transfer. The percentage of donor CD4+ T cells at day 15 post transfer is 0.4% for both WT and DIP primed T cells (Figure 5-3). However, transfer of CD4+ T-cells had little impact on the number of latently infected cells despite evidence showing CD4+ T cells have cytotoxic capability against herpesviruses (Figure 5-4A) [79] [78,119] . The percentage of donor CD8 T cells is 2% for both WT and DIP-primed cells (Figure 5-3). Transfer of WT-primed CD8+ T-cells led to a 5-fold reduction in reactivating latently infected cells while CD8+ T-cells primed by DIP did not affect the pool of latently infected cells (Figure 5-4B). When total T-cells were

transferred, the donor cell population, CD45.2+, in the spleen is 2% with CD8 and CD4 T-cells composing 78% and 12%, respectively (Figure 5-3). WT-primed T cells were able to reduce the number of reactivating latently infected cells by 20-fold, and a 30-fold reduction was obtained from the transfer of DIP-primed T cells (Figure 5-4C). To further examine the role of T cell immunity in DIP-mediated protection, we carried out T-cell depletion *in vivo*. The depletion was confirmed in the periphery of DIP-vaccinated mice prior to challenge. T cell depletion did not negatively impact on DIP induced protection. Only one of eight mice showed very low-level detectable virus after T cell depletion (Figure 5-5). Together these results indicate that despite severely attenuated DIP vaccination elicits robust cellular immunity that protects against challenge infection but other adaptive immune components can also provide protection when T cells are absent.

Antibodies are not able to confer protection in the absence of viral specific T cells.

Protection in the absence of T-cells suggests DIP generated antibodies contribute to protection (Figure 5-5). We investigated whether antibodies are sufficient to confer protection against WT challenge by passively transferring DIP primed serum to naïve mice. Unexpectedly, only 1 of 6 DIP serum recipients was fully protected from WT challenge (Figure 5-6). To determine whether full protection can be conferred by the presence of both antibodies and T cells, we performed a transfer of DIP primed serum and total T cells to naïve mice prior to challenge. This transfer combination can fully protect 5/6 (83%) mice from WT challenge (Figure 5-7). The only unprotected mouse had a significantly reduced number of reactivating latently infected cells compared to the control mice. The transfer of fractionated T cells simultaneously with serum also failed to protect the recipient mice from WT latent infection (Figure 5-8, A-C).
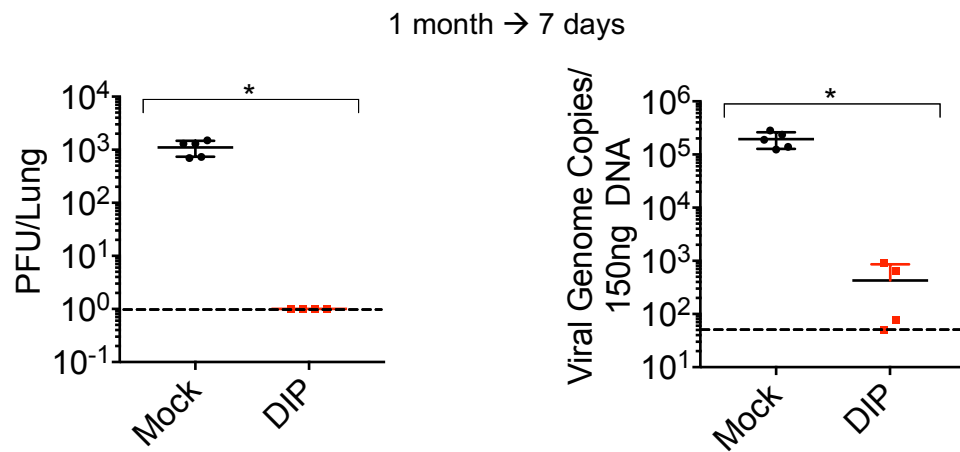
Figure 5-1. DIP vaccination confers protection against lytic infection

Vaccinations were performed i.p. with 10^5 PFU of DIP or 200μL of DMEM for mock. 1-month post vaccination, mice were challenged i.n. with 5000 PFU WT. Lytic replication in the lungs were assessed at 7 days post challenge by (**A**) plaque assay and (**B**) qPCR for viral DNA copy number.
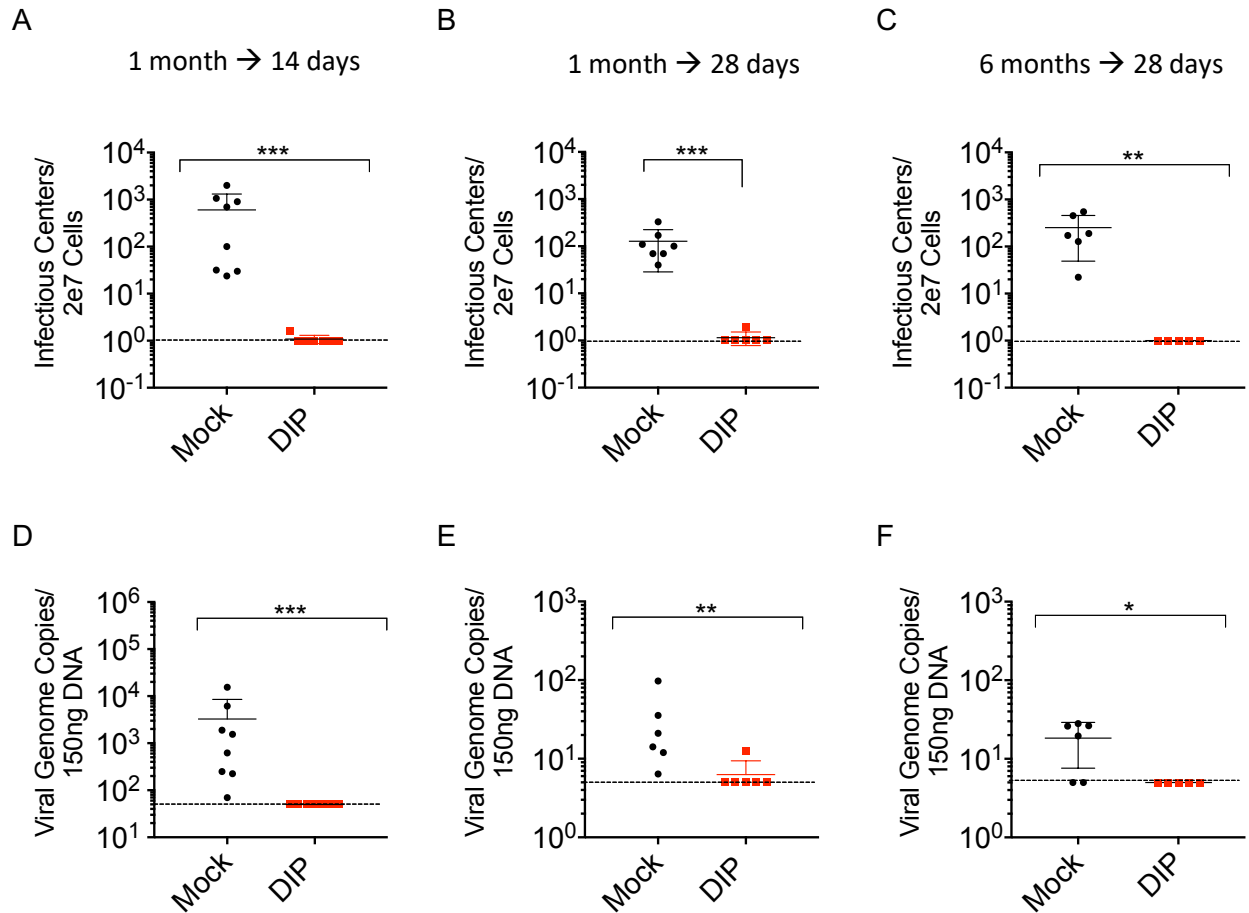
Figure 5-2. DIP vaccination confers durable protection

Vaccinations were performed i.p. with 10^5 PFU of DIP or 200μL of DMEM for mock. 1-month post vaccination, mice were challenged i.n. with 5000 PFU WT. Latent infection in the spleens were assessed at 14 days post challenge by (**A**) infectious center assay and (**D**) qPCR of viral DNA copy number, and at 28 days post challenge by (**B**) infectious center assay and (**E**) qPCR of viral DNA copy numbers. Six months post vaccination, mice were challenged i.n. with 5000 PFU of WT. Latent infection in the spleens 28 days post challenge were assessed at (**C**) infectious center assay and (**F**) qPCR of viral copy number analysis. All the experiments were repeated twice with different numbers of mice in each repeat. The data from individual mice, the

averages, and standard deviations were graphed. Statistical significance was analyzed by

Mann-Whitney t-tests.
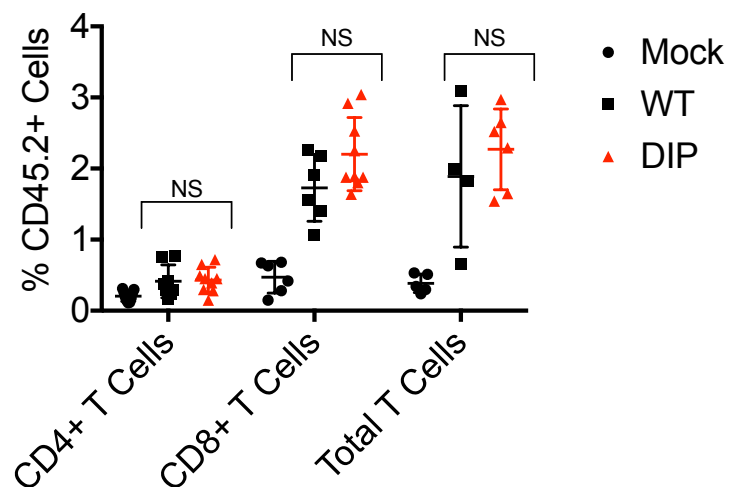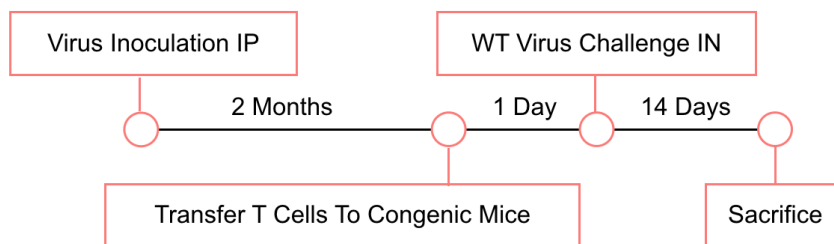
Figure 5-3. Schematic representation of adoptive transfer experiments

Schematic representation of adoptive transfer procedure and validating comparable transfer of donor cells into recipient mice by flow cytometry. The frequency of donor cells (CD45.2$^+$) in congenic mice (CD45.1$^+$) is quantified 15 days post transfer. The data of individual mice, the averages, and the standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.
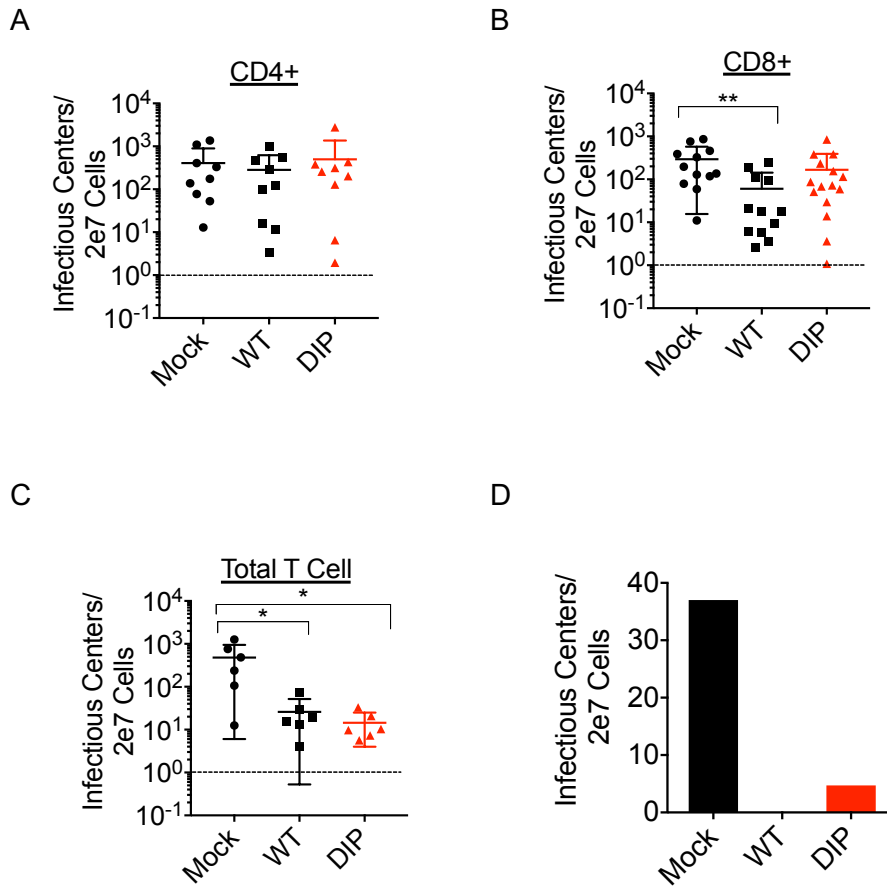
Figure 5-4. DIP primed T cells are not sufficient to confer protection against the establishment of latency

For adoptive transfers, CD4$^+$, CD8$^+$, or total T cells were purified via negative selection from the spleen of mice 2 months post-infection.   Three million of CD4+ (**A**), CD8+ (**B**), or total T (**C**) cells were transferred to a congenic mouse by tail vein injection. (**D**) 30 million total T cells were transferred to congenic mice by tail vein injection. The recipient mice were i.n. challenged with 5000 PFU WT 24 hours post transfer and the spleens were harvested 14 days post challenge for infectious center assays. All the experiments were repeated twice with different numbers of mice in each repeat (except D). The data from individual mice, the averages, and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.
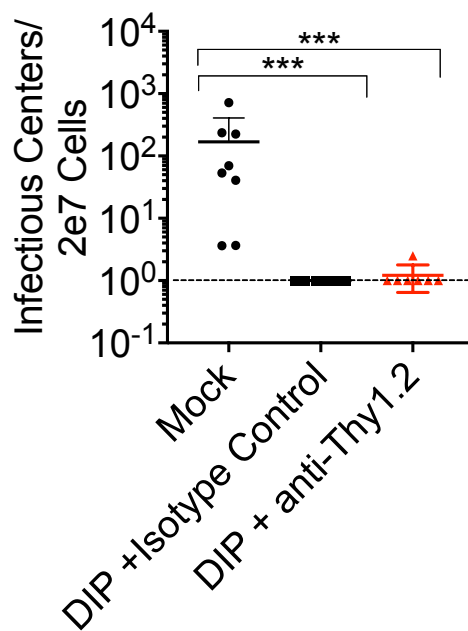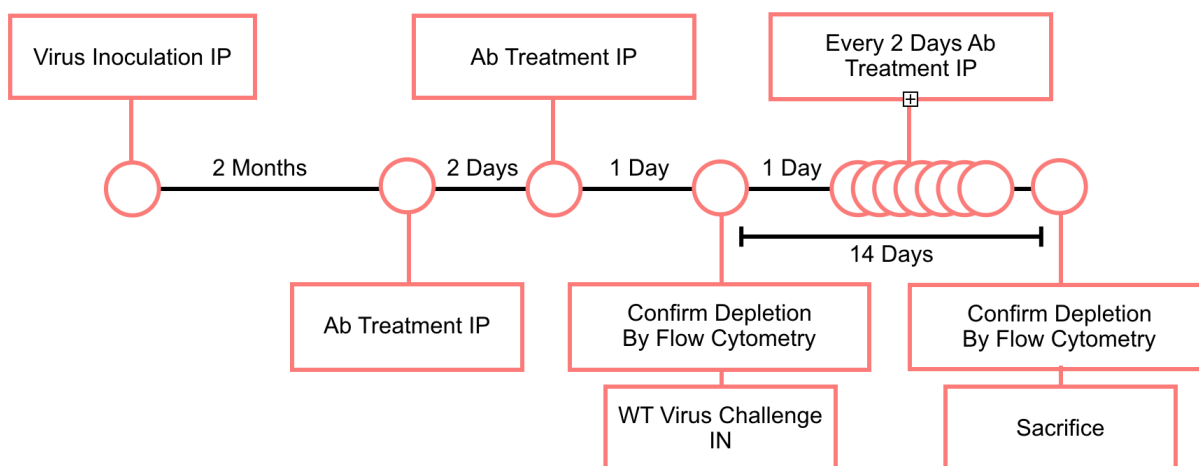
Figure 5-5. T cells are not essential for DIP mediated protection

Schematic representation of the depletion study and latent infection in the spleens, 14 days post

challenge, examined by infectious center assay. All the experiments were repeated twice with

different numbers of mice in each repeat. The data from individual mice, the averages, and

standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-

test.
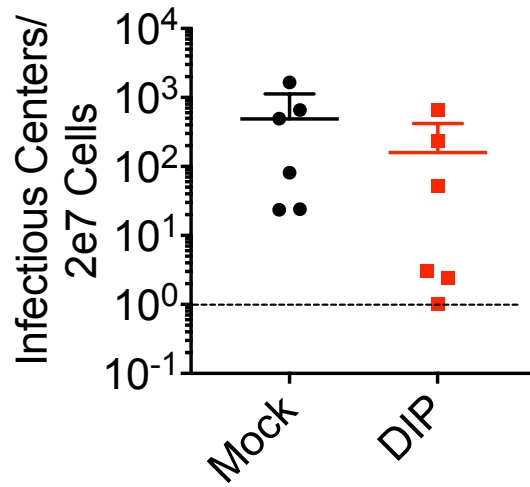
Figure 5-6. Passive transfer of DIP sera is unable to protect against latency

Sera collected from mice 2 months post-infection were transferred to naïve mice and i.n. challenged 24 hours later with 5000 PFU of WT. 14 days post challenge, the spleens were harvested for infectious center assay.
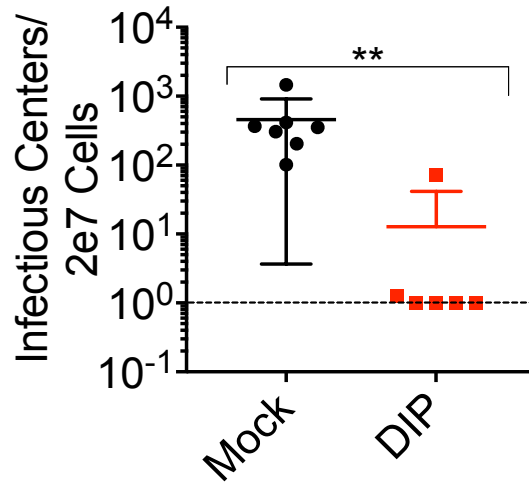
Figure 5-7. Adoptive Transfer of DIP primed serum and T cells can confer protection against latency

Total T cells and sera isolated from mice 2 months post infection with 10^5 PFU WT or DIP were transferred to congenic naïve mice by tail vein and i.p. injections, respectively. The recipient mice were i.n. challenged 24 hours later with 5000 PFU of WT and the spleens were harvested 14 days post challenge for infectious center assays. All the experiments were repeated twice with different numbers of mice in each repeat. The data from individual mice, the averages, and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.

A

CD8 T Cells + Serum

B

CD4 T Cells + Serum

C
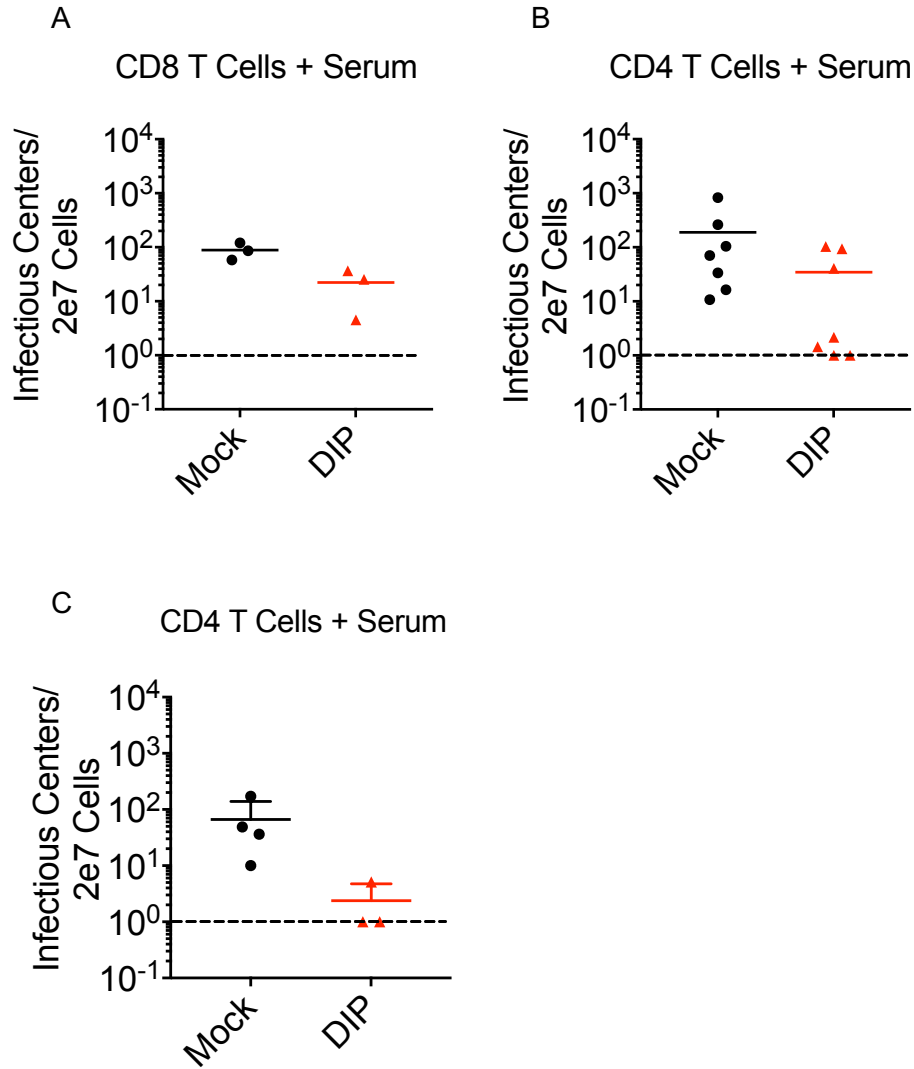
CD4 T Cells + Serum

Figure 5-8. Protection cannot be adoptively transferred using fractionated T cells and serum.

Three million of CD8+ (**A**) or CD4+ (**B**) T cells were transferred to a congenic mouse by tail vein injection. (**C**) Six million CD4+ T cells were transferred to a congenic mouse by tail vein injection. Heat inactivated serum was injected i.p. The recipient mice were i.n. challenged with 5000 PFU WT 24 hours post transfer of T cells and serum. 7 days post challenge, mice were given another dose of serum i.p. The spleens were harvested 14 days post challenge for infectious center assays. The data from individual mice, the averages, and standard deviations were graphed. Statistical significance was analyzed by two-tailed student t-test.

DISCUSSION

Despite significant attenuation in lytic and latent replication, the DIP vaccine is able to provide protection against WT latent infection. DIP prevents lytic replication at 7 days post challenge and prevents latent infection at 14- and 28-days post challenge. At 7 days post challenge (Figure 5-1), the mice had no lytic virus in the lungs as measured by plaque assay. However, 3/4 mice had detectable viral genomes in the lung by qPCR. This data suggests that the protection may not be sterilizing protection. Extending the vaccination period from one month to two months prior to challenge would allow antibodies to continue maturating and increase in titer. By extending the vaccination period, the immune system may be able to clear the viral genome at a faster rate. Alternatively, a prime-boost strategy should be considered to improve the memory response to eliminate WT challenge entirely. The 14-day protection study (Figure 5-2A), includes 1/7 DIP vaccinated mice that has reactivating virus in the infectious center assay while 0/7 DIP vaccinated mice have viral DNA present in the spleen. This discrepancy is due to the limit of the detection of the assays. The infectious center assays utilize 2×10^7 splenocytes while the qPCR utilizes 150ng DNA which is approximately 2.3×10^4 splenocytes establishing the infectious center assay as the more sensitive assay for viral detection during latent infection.

Durable protection is observed up to 6 months post vaccination, yielding complete protection against WT challenge. Time points extending beyond 28 days post challenge are required to address the vaccine's efficacy to inhibit long term latency. WT latency can be pushed below the limit of detection and recrudesce at later time points when the memory immune responses is declining. All DIP vaccinated mice show complete protection from latent infection by infectious center assays and by qPCR for viral genome copies in the spleen.

The adoptive transfer of fractionated CD4$^+$ and CD8$^+$ suggests that neither cell type is able to confer protection independently. The reduction in latently infected cells upon

simultaneously transfer of CD4$^+$ and CD8$^+$ suggests helper T cell function may be required by cytotoxic T cells. Depletion of T cells from DIP immunized mice suggest that while T cells have anti-viral activity, they are not essential for protection against latent infection. Full protection can be conferred to a naïve mouse if total CD4$^+$ T cells, CD8$^+$ T cells, and serum are simultaneously transferred supporting a complementary function for every immune component in preventing the establishment of latency.

CHAPTER 6

SUMMARY AND PERSPECTIVE

SUMMARY

Herpesviruses are enveloped double stranded DNA viruses, which are capable of establishing lifelong persistent infections in humans. The herpesvirus life cycle consists of two phases: lytic replication and latency. These two distinct phases of the life cycle allow herpesviruses to maintain the presence of viral genomes in the host. Gammaherpesviruses establish latency in B lymphocytes, dendritic cells, macrophages and lung epithelial cells[13]. KSHV and EBV are the two members of the gammaherpesviruses which present a severe global health burden. EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's and non- Hodgkin's lymphomas [1-3]. KSHV is associated with KS, as well as primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), three tumors that frequently develop in AIDS patients [4,5]. A viral vaccine against EBV and KSHV is urgently needed for high risk groups, such as Southeast Asian populations at high risk for NPC, central African populations at high risk for Burkitt's lymphoma or KS, naïve transplant patients, and high-risk groups for HIV-1 infection.

This study presents a vaccine strategy that aims to address two fundamental design aspects, immunogenicity, and safety. A genome-wide screen identified several gammaherpesvirus genes that counteract IFN-I responses [55]. We hypothesize that a recombinant herpesvirus lacking multiple IFN-I evasion genes, while attenuated in replication, will trigger a robust innate response that can then efficiently prime the adaptive immune cells to generate immunological memory. For safety, this vaccine strategy removes the major latency locus and constitutively expresses RTA in order to eliminate the possibility of persistence and being oncogenic. In this study, we evaluate the safety and efficacy of a recombinant gammaherpesvirus vaccine, which lacks immune evasion proteins and is unable to establish latency.

Chapter 1 describes the genome wide screen of MHV-68 ORFs to identify IFN evasion genes. Each ORF was co-transfected with a reporter construct containing firefly luciferase driven by an interferon stimulated response element (ISRE). Transfected 293T cells were treated with IFN-a, and eight genes were identified to suppress the ISRE reporter by 50% (Figure 1-1). The candidate genes needed to inhibit the IFN response by 50%, non-essential for replication and conserved amongst EBV, KSHV, and MHV-68. Using these criteria, we chose to inactivate ORF10, 11, 36, and 54 to create a vaccine candidate that is unable to evade the IFN response. Additionally, K3 was inactivated to increase the presentation of viral epitopes on MHC I. To create a safe vaccine, the latency locus consisting of ORF72, ORF73, M11, and ORF74 is replaced by a cassette that drives the constitutive expression of RTA by a PGK promoter. The vaccine candidate is design to be deficient in immune evasion and persistence and is termed DIP.

Chapter 3 elucidates the replication capacity of the DIP vaccine *in vitro* and *in vivo*. *In vitro*, the DIP vaccine is replication competent and attenuated compared to WT. DIP can be grown in Vero and NIH3T3 cells to high titers. No productive infection is measured in the spleen, liver, lungs and brain. The DIP viral genome is undetectable after 48 hours post inoculation. No latently reactivated cells were detected in the spleen by infectious center assay and qPCR for viral genome copy number. The vaccine also shows no splenomegaly further supporting DIP is unable to establish latency. DIP is attenuated in immunosuppressed SCID mice compared to WT MHV-68. DIP replication is rescued in the spleen and liver of IFNAR$^{-/-}$ mice. Unlike previous strategy AC-RTA, DIP is unable to establish a productive infection in the brain of C57BL/6 mice. *In vivo*, DIP is replication deficient with no measurable productive infection, no latent infection and no persistence in C57BL/6 mice.

Chapter 4 characterizes the vaccine elicited cellular and humoral response. The T cell response is characterized by an increased frequency of ORF6 specific T cells with a memory

precursor effector phenotype of KLRG1^{low}CD127^{high} compared to WT primed T cells. DIP primed T cells are able to limit latent infection when adoptively transferred to a naïve mouse prior to challenge but are unable to prevent the establishment of latency. DIP primed sera passively transferred a naïve mouse prior to challenge is also unable to prevent latent infection. DIP primed sera have higher viral specific IgG titers but lower neutralizing activity compared to WT primed sera. DIP sera target distinct viral epitopes compared to WT primed sera. In addition, DIP induces several immunomodulatory and inflammatory cytokines which play a role in priming adaptive immune responses.

Chapter 5 assess the efficacy of the DIP vaccine to protect against latent infection and elucidates the mechanism of protection. DIP provides protection against lytic infection in the lungs 7 days post challenge. DIP provides protection against latent infection in the spleen 14- and 28-days post challenge. This durable protection is able to completely prevent latent infection 6 months post vaccination. Total T cells and sera must simultaneously be transferred to a naïve mouse prior to challenge to protect against latent infection.

PERSPECTIVE

IFN-I is the first line of the host's defenses against viruses and is critical for the development of anti-viral immune responses. IFNs bridge the gap between innate and adaptive immunity by several mechanisms that include activating dendritic cells, inducing a Th1 response, eliciting potent antibody titers and promoting IgA production for mucosal immunity[120-122]. IFN-I as an adjuvant has been tested against several viral pathogens including influenza, HIV, Ebola, CMV, and gammaherpesviruses [123 45,124-126]. Using MHV-68, Aricò et al. demonstrated an increase in viral-specific antibody titers when heat-inactivated virus is co-administered with IFNα/β[45]. In another study, the IFNα gene is inserted into the MHV-68 genome to create a live attenuated vaccine strategy [46]. The IFNα-expressing virus still replicates and establishes long-term latency similar to WT. Our strategy does not aim to induce excess IFN-I; instead, we focus our efforts on disarming the viral IFN-I evasion genes thus allowing the IFN-I response to be mounted.

In this study, we demonstrate that removal of viral IFN evasion genes is an effective approach to attenuate a vaccine virus while maintaining immunogenicity. The DIP vaccine is unable to replicate or persist *in vivo*. Despite the attenuated replication, the vaccine elicits a robust innate immune response, memory T cells and virus-specific antibodies with neutralizing antibody titers. The durable memory response fully protects all immunized mice 6 months post vaccination.

The advantage of an attenuated vaccine strategy is that a broad immune response can be generated against multiple viral targets. Although antibodies play a role in preventing gammaherpesvirus infection, it remains unclear what type of antibody responses are required. Viral envelope proteins that are the major targets of neutralizing antibodies have garnered significant interest in vaccine development. gp350, the most abundant glycoprotein on the surface of EBV, is the primary target of neutralizing antibodies that blocks infection of B-cells [127].

In human trials, EBV vaccines using recombinant gp350 cannot reduce infection despite the induction of neutralizing antibodies [36,37]. A self-assembling nanoparticle vaccine that displays domains of gp350 can generate significantly higher neutralizing antibody titers compared to purified gp350 inoculation [38]. However, EBV deficient in gp350 does not abolish B cell infectivity but simply lowers the efficacy of infection suggesting other viral envelope proteins can mediate infection[128]. Furthermore, antibodies against gp350 can enhance viral entry of epithelial cells[129]. An effective vaccine to prevent gammaherpesvirus infection may need to generate broad spectrum of antibodies against an array of viral proteins rather a single one[130,131]. Depletion studies suggest T cells are not necessary for DIP mediated protection but may work together with antibodies to provide optimal protection.

Effective vaccination requires induction of inflammatory responses that activate antigen-presenting cells for T cell priming. Thus, one major advantage of using a live attenuated virus as a vaccine is its ability to stimulate multiple arms of innate immune responses culminating in a robust induction of inflammatory and immunomodulatory cytokines. Nevertheless, herpesviruses have acquired many strategies to evade the host immune surveillance, effectively establishing lifelong persistent infections. Our study demonstrates that rational design to remove viral immune evasion genes from a herpesvirus can generate an immunogenic vaccine that is capable of activating the innate immune system and eliciting inflammatory cytokines, providing adjuvanticity needed in a vaccine. Furthermore, such a rational design simultaneously compromises the ability of a vaccine virus to replicate in a host, even in an immune-deficient setting, providing an effective approach for constructing a safe and yet immunogenic live vaccines.

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