VENEZUELAN EQUINE ENCEPHALITIS VIRUS REPLICON PARTICLES:
MUCOSAL VACCINE VECTORS AND BIOLOGICAL ADJUVANTS

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ABSTRACT

JOSEPH MICHAEL THOMPSON: Venezuelan Equine Encephalitis Virus Particles:
Mucosal Vaccine Vectors and Biological Adjuvants
(Under the direction of Dr. Robert E. Johnston)

Vaccination is the most effective control measure in the fight against infectious
diseases, and represents an opportunity to intercede in the spread of dangerous organisms
through prophylactic intervention. Viral vectors, including alphavirus vectors, have proven
to be powerful vaccine delivery vehicles and a promising platform for vaccines against
multiple pathogens. Specifically, as demonstrated here, Venezuelan equine encephalitis
virus (VEE) replicon particles (VRP) induced strong humoral, cell-mediated, and mucosal
immune responses directed against heterologous antigens expressed from the viral genome,
as well as against antigens simply mixed with VRP. These observations established a dual
function of VRP as both vaccine expression vectors and vaccine adjuvants, demonstrating
that VRP possess intrinsic immunostimulatory properties. When utilized as adjuvants, VRP
systemic humoral adjuvant activity was as strong as the activity of CpG DNA. In addition,
the mucosal responses induced by VRP adjuvants were superior to those induced by CpG, an
effect that was dependent upon VRP RNA replication.   The induction of mucosal immune
responses is critical for vaccine-mediated protection following challenge with mucosal
pathogens. Delivery of antigens directly to mucosal lymphoid tissues, as occurs following
mucosal delivery, results in the strongest mucosal immune responses. While this has revealed

the components of the natural mucosal inductive pathway, little is known regarding the lymphoid structures responsible for mucosal immune induction following nonmucosal delivery. Here we demonstrate that following nonmucosal VRP vaccination, several markers of mucosal lymphoid tissues were present in the draining lymph node (DLN). This included the presence of antigen-specific polymeric IgA antibodies, upregulated expression of the $\alpha_4\beta_7$ integrin on DLN lymphocytes, expression of the mucosal addressin, MAdCAM-1, and the production of IL-6 and other mucosal cytokines. The presence of these markers is consistent with a model in which the DLN is converted by VRP infection into the functional equivalent of a mucosal inductive site. Furthermore, while type I interferon (IFN) signaling was not required for VRP adjuvant activity, it was critical for the induction of mucosal IgA responses induced by VRP expression vectors. Together, these findings may significantly improve both our knowledge of viral immunology and the efficacy of viral-based vaccines.

For Team Thompson,

My parents, Mark and Joanie, my twin brother, Brian, my sister-in-law, Jennifer, and my nephew, Gabriel

For your constant and unwavering belief in me...
your love and support have shaped me into who I am, and inspire me to be more.

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LIST OF ABBREVIATIONS

Ad	Adenovirus
APC	Antigen presenting cell
ASC	Antibody-secreting cell
AST	Average survival time
CCR	Chemokine (C-C motif) receptor
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DLN	Draining lymph node
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunospot assay
ER	Endoplasmic reticulum
FAE	Follicle associated epithelium
FDA	Food and Drug Administration
GFP	Green fluorescent protein
HA	Hemagglutinin
HEV	High endothelial venules
Hib	Haemophilus influenzae type b

HIV	Human immunodeficiency virus
hpi	Hours post infection
HRP	Horseradish peroxidase
HS	Heparin sulfate
I-Flu	Inactivated influenza virus
IEL	Intraepithelial lymphocytes
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
im	Intramuscular
IPV	Inactivated polio vaccine
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
IU	Infectious units
kb	Kilobase
kDA	Kilodaltons
KO	Knockout
LC	Langerhans cell
LOD	Limit of detection
LPL	Lamina propria lymphocyte
LRT	Lower respiratory tract
LT	Labile toxin

MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MALT	Mucosal-associated lymphoid tissue
MHC I	Major histocompatibility complex I
MLN	Mesenteric lymph node
MVA	Modified vaccinia Ankara
MyD88	Myeloid differentiation primary response gene 88
nsP	Nonstructural protein
nt	Nucleotide
OPV	Oral polio vaccine
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
pIgR	Poly Ig receptor
PIV	Parainfluenza virus
PNAd	Peripheral lymph node addressin
PP	Peyer's patches
PRR	Pattern recognition receptor
RKO	Receptor knockout
RNA	Ribonucleic acid
RRV	Ross River virus
RT	Room temperature
SC	Secretory component
SEM	Standard error of the mean

SFV	Semliki Forest virus
SHIV	Simian human immunodeficiency virus
SIgA	Secretory IgA
SIN	Sindbis virus
SIV	Simian immunodeficiency virus
TAP	Transporter associated with antigen processing
TB	Mycobacterium tuberculosis
TCI	Transcutaneous immunization
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TLR	Toll-like receptor
URT	Upper respiratory tract
UTR	Untranslated region
UV	Ultraviolet light
VAPP	Vaccine-associated paralytic poliomyelitis
VEE	Venezuelan equine encephalitis virus
VRP	VEE replicon particles
YFV	Yellow fever virus

CHAPTER ONE

INTRODUCTION

GENERAL PRINCIPLES OF VACCINATION

Historical Perspective

The earliest recorded accounts of variolation, or vaccination, date back as far as 1000 *A.D.* in China, where smallpox scabs or pustules from infected individuals were used to inoculate unexposed people and afforded a significant degree of protection (207, 294). It was stated that following this procedure, "not one in 10, not one in 100 does not recover." (98, 207). Reports of parenteral smallpox variolation surfaced in India, Asia, and Africa as early as the sixteenth century (207, 291). In eighteenth century Europe, smallpox was a devastating illness, responsible for 8-20% of all the deaths in both rural and urban populations (8). It was recognized by physicians and scholars of the time that milkmaids often acquired a pox-like infection from cows, and in turn, were spared from the most agonizing symptoms of smallpox (98, 207, 339). However, a direct relationship between the mild pox infection and protection from smallpox infection was not established until 1796, in the pioneering work performed by Edward Jenner.

On May 14, 1796, Jenner inoculated a 13-year-old boy, James Phipps, with the cowpox or vaccinia virus obtained from a woman named Sarah Nelmes, who was accidentally infected by a cow named Rosebud (8). James Phipps was deemed "secure" or immune to a subsequent smallpox challenge delivered "some months afterwards." (8, 207). Thus, the science of vaccine inoculation (159), or vaccination, as coined by Louis Pasteur many years later (207), was born. As a result of these discoveries, and as predicted by Jenner (8, 207), smallpox has been eradicated from its natural environment in the world by a vaccine developed approximately 200 years before (98).

The Jennerian approach to vaccination was first deliberately applied by Louis Pasteur, following his discovery of the principle of attenuation (291, 294). Pasteur returned to the laboratory following a vacation over the summer of 1881 to find a culture of a *Pasteurella* species, the causative agent of fatal chicken cholera, which had been left out on the bench over the summer. Pasteur discovered that the aged culture was avirulent in chickens, and in fact, protected chickens from lethal disease following challenge with fresh cultures (207, 280, 291). These results served as the groundwork for the hypothesis that organisms could be rendered attenuated by exposure to external insults. This hypothesis was confirmed by his work on anthrax and rabies over the next several years (207, 281, 291), a remarkable achievement given the fact that he was unable to cultivate the rabies virus *in vitro*, as cell culture for the purposes of virus growth was not adapted until the middle of the twentieth century (379). The concept of attenuation was applied to a number of organisms over the next century (291, 294), including *Mycobacterium bovis* by Calmette and Guerin (49) and yellow fever virus by Max Theiler (357). To date, live attenuated vaccines still serve as some of the most immunogenic vaccines ever produced, most likely due to the fact that they most closely mimic the events which occur following a natural infection (109, 179, 260, 372).

Immunological Correlates of Protection and Vaccines

The development of new vaccine technologies in the twentieth century has brought about vaccines for lethal diseases such as diphtheria, tetanus, paralytic poliomyelitis, pertussis, measles, mumps, rubella, *Haemophilus influenzae* type b (Hib), and Hepatitis A and B [reviewed in (8, 291, 294, 295)]. These vaccines are based on live attenuated

vaccines, subunit or whole-cell bacterial vaccines, and/or recombinant production of individual vaccine components [reviewed in (8, 291, 294, 295)]. These advances have significantly increased overall life expectancy over the last few decades (29, 290), with widespread vaccination efforts in infants estimated to save the lives of 3 million children a year (8). Systematic administration of existing vaccines would save millions of additional lives worldwide (7, 384).

The overall goal of vaccination is to generate memory B and T cell responses sufficient to protect the individual from a natural infection (196). While the concept of protective immunity is a relative quantity/quality of immune induction, much effort has been exerted to define protective factors (288). The nature and magnitude of the immune response required to provide protection clearly varies from pathogen to pathogen (100, 200, 203, 277). Knowledge of the correlates of protection for a specific organism is central to the design of an efficacious vaccine (288). In general terms, vaccines activate B cell responses (antibody secretion or other activities), $CD8^+$ T cell responses (usually as cytotoxic lymphocytes or CTLs), and/or $CD4^+$ T cell responses to provide cytokine help for either B cell responses or $CD8^+$ T cell responses (288, 315, 349).

A role for systemic antibody responses. Protection from challenge after immunization with most traditional vaccines is mediated by the reduction in systemic spread of the organism, as manifested by a decrease in serum viremia or bacteremia, in the case of viral and bacterial pathogens, respectively. This is normally facilitated by vaccine-induced specific serum IgG antibodies [reviewed by Stanly Plotkin (288)]. Here we provide support for the role of vaccine-induced systemic antibody responses in protection. For example,

serum IgG responses directed against hepatitis A virus protect individuals from the onset of hepatitis. In fact, gamma-globulin preparations from naturally-infected patients provides protection, allowing the establishment of a minimally protective dose (62, 338). Studies in primates suggested that serum antibodies also protect from the paralytic manifestations of polio infection, presumably by limiting viremic spread to the CNS, even though viral delivery and replication occur in the gut. (26). Systemic IgG antibodies also provide various levels of immunity to rabies virus infection (289), yellow fever virus infection (222), meningococcal polysaccharides (392), pneumococcal polysaccharides (180), and typhoid polysaccharides (292). While controversy exists regarding which component of the pertussis vaccine is the dominant protective antigen (54, 242, 293, 360), it is clear that serum IgG antibodies play an important role in protection from this important pathogen as well (288). Serum IgG responses directed against bacterial toxins have also been shown to provide protection; such as is the case for vaccines against both diphtheria and tetanus (227, 228).

A role for local mucosal antibody responses. The examples provided above clearly demonstrate the importance of systemic antibody responses in vaccine-induced protective immunity (288). However, what role do vaccine-induced systemic antibodies play in limiting infection at the local mucosal surface? For example, polio virus replicates in the gut epithelium, and delivery of the oral polio vaccine (OPV) induces local mucosal immunity to subsequent challenge (348). Interestingly, parenteral delivery of the inactivated polio vaccine (IPV) does not induce equivalent levels of local antibody production in the gut (94, 253). However, IPV does appear equally capable as OPV at reducing extraintestinal virus titers (219). The effectiveness of IPV appears to be affected by previous natural or OPV

exposure (270, 288). IPV vaccination only induced mucosal immune responses in previously infected individuals (141), suggesting that mucosal antigen delivery is required for the induction of mucosal immunity.

It is also important to consider the relative contributions of both systemic and mucosal immunity antibodies in protection from infectious agents that either do not have a systemic replication stage in their life cycle, or have a very limited systemic phase. Such is the case for both rotavirus infection as well as influenza virus infection. The definitive correlates of protection from rotavirus infection are not completely understood; however, resistance to natural infection appears to correlate with both systemic IgG and IgA antibody production (265). It is likely that systemic IgA merely serves as a surrogate for mucosal IgA, as local IgA antibody production likewise appears to predict outcome of infection (223). The inactivated parenteral influenza vaccine provides a strong degree of protection form mortality, although it does not block replication at the local mucosal surface. Serum anti-hemagglutinin titers correlate with protection (57, 71, 72, 144); however, it is thought that such antibodies are transudated onto the local lung mucosa to mediate protection, as influenza does not replicate to substantial levels outside the lung (90). In a later section of this review, the anatomical basis and molecular mechanisms responsible for the induction of mucosal immune responses are discussed in detail (see below).

A role for T cell responses. Many natural infections with organisms and/or vaccine strains that replicate systemically and at the local mucosal surface result in the activation of efficient $CD8^+$ T responses. Such is the case with both rotavirus infection (267-269) and influenza virus infection (25, 106, 226). As mentioned above, both antibody and T cell

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responses may influence the outcome of infection with these mucosal viruses; however, T cell immunity alone, in the absence of an antibody response, is necessary and sufficient for protection with other pathogens. The activation of CD4$^+$ T cells and CD8$^+$ T cells are critical for protection from *Mycobacterium tuberculosis* (TB) (69, 70, 274, 288), and the live attenuated vaccine (BCG) is known to induce protective T cell immunity in humans and animal models (99, 275).

T cell responses also play an important role with infections agents other than TB. CD8$^+$ T cells are capable of killing virus- and bacterial-infected cells and represent one of the most critical clearance mechanisms, especially with chronic infections (83, 93, 208).   Cell-mediated immune responses provide protective capacity either with primary infection or reactivation from latency with several herpesviruses including varicella zoster (13), herpes simplex virus type 1 (211), herpes simplex virus type 2 (243), Epstein-Barr virus (48), cytomegalovirus (168), as well as other chronic infections such as hepatitis C virus (305).

Herd Immunity.   This review has focused thus far on the effects of vaccination within a given vaccinated individual. However, when considered on a population level, vaccination provides significant benefits even to the unvaccinated individuals within the population. These benefits are manifested in at least 3 separate ways (288). First, within a highly vaccinated population, there are fewer infected individuals, decreasing the likelihood of an exposure event. Secondly, some vaccines, such as the Hib vaccine (245), reduce carriage of the organisms, further decreasing the chance of exposure. Lastly, live vaccine strains have the potential to spread to, and directly induce immunity in, unvaccinated individuals, as was observed with OPV (154). While controversy exists regarding the

nomenclature for herd immunity (164), the protective effect of vaccination in unvaccinated individuals is unquestioned, and should be considered in vaccine design.

   The status of human immunodeficiency virus vaccines. While this review is not intended to be comprehensive in terms of vaccine-induced immunity to all human pathogens, we would be remiss not to mention the current status of human immunodeficiency virus (HIV) vaccine development. There are approximately 5 million new HIV infections each year and the virus is spreading at an increased rate in numerous locales worldwide (203). The development of an efficacious vaccine would save the lives of millions of individuals. Unlike the scenario described above for many of the infectious agents in which the exact correlates of protective immunity are known, definitive correlates of protection from HIV infection have not been clearly established (203). To date, the most promising protective efficacy has been demonstrated with live attenuated vaccines in the simian immunodeficiency (SIV) and simian human immunodeficiency virus (SHIV) models (190). In fact, live attenuated SIV vaccines not only provide protection from systemic challenge, but also from challenge by the natural mucosal route (317). While this approach harbors the potential to define immunological correlates, delivery of live viruses is not feasible in humans because of the potential of generating a pathogenic revertant virus in vaccinees.

   Conflicting evidence exists regarding the ability of specific immune components to provide protection from SIV infections. For example, passive antibody transfer studies have implicated humoral immune responses in protection from SIV following both systemic (128, 220) and mucosal (221) challenge. However, B-cell-depleted monkeys displayed normal virus clearance at early times following SIV challenge, suggesting that humoral immunity

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may not play a dominant role (327). In contrast, SIV was not controlled in monkeys lacking CD8^{+} T cells, directly implicating a role for cell-mediated immunity in protection from SIV infection (326). Additionally, local mucosal immune responses are proposed to play an important role in protection as well (241). As definitive proof regarding precise correlates of protection is lacking, a successful HIV vaccine most likely will need to stimulate humoral, cell-mediated, and mucosal immunity.

New Vaccine Technologies.

Traditional vaccine approaches have relied upon parenteral delivery of either live attenuated organisms, subunits/proteins derived from infectious organisms, or inactivated particles. While these approaches have resulted in a plethora of efficacious vaccines against important human pathogens, it is estimated that infectious diseases still account for almost 25% of deaths worldwide, particularly in developing countries (192, 272). The failures associated with HIV vaccine development alone exemplify the need for new vaccine platforms. Here we outline several of the promising new techniques currently under development at various stages from early preclinical studies to efficacy-stage clinical trials, including viral/bacterial gene transfer systems, adjuvants, DNA vaccines, differential prime/boost systems, and new delivery methods. Again, this is not intended to serve as a comprehensive list, but instead provide a survey of the state of this new area of vaccinology.

DNA vaccines. The utilization of plasmid DNA as an antigen delivery system is a relatively recent development, first utilized in the 1980s [reviewed in (80, 365)]. Early studies with DNA vaccines demonstrated activation of both B cell and T cell responses in

small rodents and non-human primates (313, 363). In humans, DNA vaccines elicit potent T cell-mediated immune responses (373); however, they appear inefficient at stimulating an antibody response directed against the encoded antigen (66). DNA vaccines are traditionally delivered by the intramuscular (i.m.) route, which results in DNA uptake and antigen expression in myocytes. Myocytes present the expressed antigen in the context of major histocompatibility complex (MHC) I molecules on the cell surface; however, as myocytes do not upregulate costimulatory molecule expression, other cell types most likely function as antigen-presenting cells (APCs) for immune activation (146) [reviewed in (189)]. Both local and recruited APCs, especially dendritic cells (DCs), become transfected and activate costimulatory molecules for direct activation of CD8$^+$ T cells (389), or activation by cross priming (364). DNA vaccines activate components of the innate immune system through the presentation of unmethylated CpG motifs present in bacterial DNA (182). Bacterial DNA is recognized by toll-like receptor (TLR) 9 (see below), and stimulation of this pathway plays a pivotal role in the immunogenicity of DNA vaccines (14).

DNA vaccines may be advantageous under various experimental conditions, including as a tool in neonatal or early childhood vaccines, when maternal antibodies are present. Traditional vaccines which are reliant upon the authentic particles initiating infection or presenting antigen to the immune system are susceptible to interference by placentally-transferred antibodies (334). As DNA delivery does not deliver any antigens which maternal antibodies can recognize, DNA vaccines may circumvent maternal antibody interference (333). DNA vaccines have proven effective in numerous infectious disease and cancer models [reviewed in (80, 365)] either alone, or as components of an alternative prime

boost system; and therefore may enable the production of safe, efficacious vaccines in the future.

Adjuvants. Adjuvants were originally defined by Ramon as, "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone" (306) [reviewed in (264, 335)]. Adjuvants can function to improve vaccine-induced immunity in numerous different ways, including 1) increasing immunogenicity of weak antigens, 2) enhancing the speed and/or duration of the immune response, 3) modulating antibody avidity, specificity, isotype, or subclass distribution, 4) stimulating cytotoxic T lymphocytes (CTLs), 5) promoting mucosal immune induction, 6) increasing immunity in immunologically immature or senescent individuals, 7) decreasing the antigen dose in a vaccine required to induce a protective response, or 8) helping to overcome antigen competition in combination vaccines (264, 335). Here we briefly introduce several common and new adjuvant systems and what is known regarding their mechanism/s of action.

Alum. While the adjuvant activity of aluminum-based mineral salts, or alum, was discovered over 80 years ago (117), it is, to this day, the only adjuvant approved in licensed vaccines by the Food and Drug Administration (FDA) in the United States (174). It has been extremely difficult to precisely determine the mechanism/s of adjuvant activity associated with alum, as it has profound effects on numerous systems following *in vivo* delivery (42). The original hypothesis suggested that alum exerted its adjuvant activity by means of a "depot" effect, that is the deposition of antigen in the immune system for continued immune

stimulation (116); however, this notion has been challenged recently (124). The major effect of alum appears to be the regulation of the Th1/Th2 environment (247) by promoting the production of Th2 cytokines, particularly interleukin (IL)-4 (362) and antibodies of the Th2 IgG subtype, IgG1 (210). Interestingly, alum still exerted a significant adjuvant effect in IL-4 knockout (KO) mice (43), IL-13 KOs (43), IL-6 KOs (41), and tumor necrosis factor (TNF) receptor KO mice (41). The production of IL-4 following alum delivery was markedly reduced in IL-18 KO animals; however, the production of IgG1 antibodies was not affected (296). Alum has not proven an effective inducer of Th1 cytokines nor activation of delayed type hypersensitivity (DTH) reactions (127), consistent with a strong skewing towards Th2 responses. Together, these observations typify the complexity of the cytokine networks active following adjuvant delivery and underscore the difficulty in attributing adjuvant effects to a single immunological mechanism.

Freund's adjuvant/s. Similar to the alum story, incomplete Freund's adjuvant (IFA) and complete Freund's adjuvant (CFA) were discovered over fifty years ago, and the mechanism of adjuvant activity remains elusive to this day (101) [reviewed in (22)]. IFA is composed of a paraffin oil surfactant mixture that, when mixed with antigen, forms a viscous water-in-oil emulsion with antigen in the water phase (101). CFA also contains preparations of heat-killed Mycobacterium (M. *tuberculosis* and/or others) which play a significant role in immunogenicity (22)]. Like alum, CFA and IFA provide a potent adjuvant signal for the activation of antibody responses to co-delivered antigens (386). Interestingly, CFA, but not IFA, induces cell-mediated immune responses in addition. This observation suggests that the presence of Mycobacterium preparations drives T cell activation. This is the case, as

cytokine profiles are dramatically shifted towards a Th1 phenotype with CFA as compared to IFA (137, 390). Both CFA and IFA upregulate the phagocytic activity of APCs (261) and have dramatic effects on antigen localization (22). Interestingly, both CFA and IFA delivery appear to trap antigens, at least at early times post delivery, in the subcapsular sinuses of the draining lymph nodes, potentially providing an immunological locale for lymphocyte activation (177, 209). This suggests that, unlike the induction of cell-mediated immunity, the antigen entrapment function of Freund's adjuvant is attributable to the oil-in-water emulsion alone.

While IFA and CFA have provided valuable tools as a vaccine adjuvants, there are detrimental effects of their use as well. Both adjuvants are associated with moderate to extreme local inflammation in all species tested to date, including small rodents and humans (22, 340). Additionally, both IFA and CFA are associated with the formation of granulomas, or tubercles in treated animals, especially following repeated treatments (374). Granulaoma formation was dependent upon adjuvant-induced TNF-α (176), which may also play a role in the adjvanticity of Freund's (22). Together, these observations support the notion that Freund's adjuvants are potent immunomodulators and illustrate the balance which must be struck between immunogenicity and toxicity for the widespread use of vaccine technologies in humans.

TLR ligands. It has been suggested that the overall goal of adjuvants, in the context of vaccines for infectious diseases, is to ensure that the vaccine mimics natural infection accurately enough to promote induction of protective immunity (96, 157) [reviewed in (192, 279, 301, 335)]. Therefore, stimulation of the receptors which first "sense" natural infection

may serve as a potent adjuvant strategy. TLRs are among the pattern recognition receptors (PRRs), which recognize conserved pathogen-associated molecular patterns (PAMPs) (158). The Toll proteins were originally discovered in *Drosophila*, recognized for their role in the induction of anti-fungal responses (202). Eleven different TLRs have been identified to date (279), each receptor recognizing a distinct PAMP or set of PAMPS (382): TLR1 and TLR 2 recognize ligands from gram-positive bacteria (129, 330, 367); TLRs 4 and 5 recognize products from gram-negative bacteria (105, 136, 298); TLR 9 recognizes bacterial DNA (353); and TLRs 3, 7, and 8 recognize viral RNA (5, 138). TLR ligation triggers a complex intracellular signaling cascade (266) involving the adaptor molecule myeloid differentiation factor 88 (MyD88), ultimately culminating in the activation of the transcription factor NF-kB and the release of pro-inflammatory cytokines such as IL-6, IL-12, and TNF-α (3, 352). Signaling via TLR3 and TLR4 also utilize additional adaptor molecules to mediate cellular responses (387).

TLR ligands in general have pleiotropic effects following *in vivo* delivery, and while commonalities exist in terms of responses induced by all TLR agonists, stimulation with a given TLR agonist may promote a slightly different response than others (301). For this reason, here we consider the adjuvant activity of CpG DNA as a representative TLR agonist, mindful that different TLR agonists may regulate immunity to co-delivered antigen in a different manner. Delivery of CpG DNA has dramatic effects *in vivo*, including increased cellular migration towards the draining lymph node (368), increased costimulatory molecule expression on DCs (135), increased cytokine production (156), and increased immunity directed towards co-delivered antigen (73, 183). CpG DNA promotes the production of IgG2a antibodies (56), as well as an increase in cell-mediated immune responses (55)

directed towards a co-delivered antigen, consistent with a Th1 phenotype. CpG DNA provides a potent adjuvant signal either when used alone, or in combination with other traditional adjuvants (153).

The promising results obtained with CpG DNA as a vaccine adjuvant have raised the question of involvement of TLR triggering as a general mechanisms of action of adjuvant activity. Querec *et al*. recently proposed multiple TLR stimulation as a critical mechanism responsible for the efficacy of the yellow fever virus (YFV) 17D vaccine, one of the most successful and safe vaccines utilized in humans (304). Indeed TLR signaling plays a critical role in mediating the adjuvant effect associated with CFA, evidenced by the observation that T cell proliferation and IFN-γ production were abrogated in MyD88 knockout mice, which are defective in TLR-mediated signaling (328). However, a generalized role for TLR signaling with traditional adjuvants has been called into question recently, given the fact that some traditional adjuvants including CFA do retain activity in MyD88 knockout animals (104). It is clear TLR ligands in general, and CpG DNAs specifically, possess potent immunomodulatory effects which may be harnessed as tools in vaccinology; however, the details of how generalized a role TLR signaling plays with traditional adjuvants remains to be fully elucidated.

Authentic infections provide adjuvant effects. As mentioned above, adjuvants have a place is vaccinology as a means to more accurately mimic the events which occur following natural infection which lead to potent immune induction (96, 157) [reviewed in (192, 279, 301, 335)]. Delivery of TLR ligands is an example of a reductionist approach to defining the individual components amongst a complex infection environment which are responsible for

immune induction, and harnessing them separately for use as immunomodulatory modalities. While potentially less satisfying from a definitive mechanistic point of view, it is possible that the holistic approach of simply relying on natural infection to provide an adjuvant signal may in fact provide an even stronger adjuvant effect that any one constituent alone, as proposed for the YFV vaccine (304). While this principle has not been tested vigorously in animal models, it is clear that natural infection with several viruses and bacteria do provide an adjuvant signal. For example, bacterial DNA provides an adjuvant signal in the form of CpG DNA described above, as does infection with bacteria from which the DNA was isolated. This is demonstrated by the observed adjuvant activity of Mycobacterium *tuberculosis* (22), Corynebacterium *parvum* (375), and Bacillus *firmus* (244).

It is possible that the adjuvant effect observed following bacterial infection is due entirely to the presence of CpG DNA present in the bacterial genome; however, such an explanation does not account for the adjuvant effect observed following virus infection. Various forms of adjuvant effects have been observed with influenza virus (44), poxviruses (152), adenovirus (152), oka varicella zoster virus (287), lactic dehydrogenase virus (263), and alphaviruses (65, 151). Interestingly, adjuvant effects were observed with non-replicating virus particles derived from parvovirus (27) as well as the alphaviruses (142, 358), suggesting that early events in the virus replication cycle are capable of triggering an adjuvant signal. Consistent with this idea, adjuvant activity in the alphavirus system was dependent upon RNA replication (358) and type I interferon signaling (142). Together these examples are supportive of a role for natural viral and bacterial infections as adjuvant tools.

Viral expression vectors. As mentioned above, considerable effort has gone into understanding the specific mechanisms by which natural infections promote immune induction. While much is known regarding the general principles, there is paucity of knowledge regarding the specifics. Viruses have evolved highly efficient mechanisms for both infecting cells and generating new copies of the viral genome and structural components. While the strategies employed by different viruses vary widely, cell entry and genome replication are commonalities of all successful infections. This concept is exploited by live attenuated vaccines as described above. It is also exploited by viral-based expression vector systems. In this case, molecular genetic approaches are employed to generate recombinant viruses which express a heterologous gene as a vaccine antigen [reviewed in (40, 84, 198, 337)]. Vaccine expression systems have also been developed based upon a number of bacterial genomes [reviewed in (82, 139)]; however, here we focus on viral-based delivery systems. Here we describe the salient features of some of the most promising viral expression vector systems.

Poxviruses. Poxviruses are large, complex viruses which contain a double-stranded DNA genome ranging in size from 130-300 kilobases (kb) (165) and exhibit a number of advantageous characteristics as vaccine vectors. First, these vectors are very stable and retain effectiveness following lyophilization, a real advantage as cold chain problems represent a significant impediment to vaccine distribution, especially in the developing world (337). Second, poxvirus vectors are relatively easy to produce, cost effective, and versatile, as they are effective following delivery via multiple routes, including mucosal delivery (see below). Pre-existing immunity, which can be a problem with some vaccine vectors, can be

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overcome with mucosal delivery (337), as the systemic and mucosal immune systems are distinct (145). The most widely used vectors are based on attenuated strains such as modified vaccinia Ankara (MVA) (346), NYVAC (based on the Copenhagen strain of vaccinia) (354), as well as viruses which naturally infect alternative species such as canarypox (355) or fowlpox (356).

Poxvirus vectors have proven to be potent inducers of humoral and cellular immunity in numerous experimental systems, as well as inducers of mucosal immunity following mucosal delivery (see below) (107). Poxviruses induce measurable protection in several infectious disease models including SHIV (6), influenza (347), measles (341), and others. Another advantage of the poxvirus system is the ability to incorporate large amounts of foreign DNA into recombinant viruses. Up to 10 kb of heterologous DNA can be inserted; in fact, poxvectors induced protection/immunity to up to 3 foreign genes from 3 separate pathogens in the same recombinant vector (282). These observations support the pursuit of poxvirus vaccine vectors for improved human vaccines.

Adenovirus. The adenoviruses (Ad) are members of the Adenoviridae family, and have been used extensively as live vaccines in the US Army (337). Ad contains a double-stranded DNA genome of approximately 35 kb and display a number of important features which promote their use as vaccine vectors including stability, ease of manipulation, feasibility of high titer production, and simple purification (337). As with poxviruses, Ad is active following lyophilization and functions as a potent gene delivery vector following delivery by the intranasal, oral, intratracheal, intraperitoneal, intravenous, intramuscular, or subcutaneous routes, allowing tailored use based upon the specifics of the desired immune

response (337). Replication-incompetent Ad vectors have been engineered which either lack structural components required for virion assembly all together or harbor mutations in the structural components. These replication-incompetent constructs are likewise extremely immunogenic as vectors (191).

Potential concerns regarding the use of Ad-based vaccines that have arisen are the potential for inhibition by pre-existing immunity to the virus, the relatively small payload for heterologous sequence, especially with replication competent vectors, and the virus-induced inflammatory response (40, 337). Strategies to deal with these concerns have been developed including the use of alternative strains of Ad to circumvent pre-existing immunity and oral delivery to limit the systemic inflammatory reaction (40, 337). Recombinant Ad expression vectors have demonstrated protective efficacy as vaccines for important pathogens such as SHIV(332), Ebola (345), measles (323), and malaria (314). Ad provide a powerful tool in vaccinology and an opportunity to study virus-induced immunity.

Alphaviruses.   Alphaviruses are small, single-stranded RNA viruses of the Togaviridae family. Alphaviruses contain a message sense genome of approximately 12 kb and have proven to be effective vaccine vectors. Expression vectors based on Sindbis virus (SIN), Semliki forest virus (SFV), and Venezuelan equine encephalitis virus (VEE) have all shown promise in preclinical studies in both infectious disease and cancer models [reviewed in (77, 214, 215, 297, 307, 312, 325)]. Both replication-competent and replication-defective vector systems have been developed. Alphavirus vectors are active following many parenteral delivery routes as well as following mucosal delivery. Replication-defective

vectors, or replicon particles, have the structural gene cassette replaced with the heterologous gene and therefore infect cells, but fail to produce new progeny virions. (324, 342)

The alphavirus display numerous advantages as vaccines in that they are simple to work with, produce large amounts of heterologous transgene product, target DCs *in vivo* (103, 218) , and induce robust humoral, cellular, and mucosal immunity (77, 214, 215, 297, 307, 312, 325). Additionally, as the majority of the human and veterinary population has not been exposed to most of the alphaviruses, pre-existing immunity is not predicted to be prohibitive. Protective immune responses have been demonstrated with a number of important pathogens such as influenza virus (74, 303, 329), Ebola virus, (302), SIV (75, 248), as well as a number of bacterial toxins (197, 199). Alphavirus vectors have demonstrated promise as vaccine expression vectors and are under development for vaccines in numerous additional model systems. A more extensive review of alphavirus-induced immunity, especially in the context of mucosal immune induction is found in the VEE section of this review.

Novel Delivery Techniques

As mentioned above, the majority of vaccines are delivered by a parenteral route (288). The most common methods employ either intramuscular or subcutaneous delivery. Under these conditions, antigens and vaccines access the skin DC system and are reliant upon the function of skin-derived APCs and lymphatics to deliver antigens to the draining lymph node/s to initiate immune induction (351). While parenteral delivery results in efficient stimulation of systemic immunity, this technique typically fails to stimulate a response that is active at the local mucosal surface (291). Additionally, parenteral delivery

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relies on the use of sterile syringes and needles, which can serve as an impediment, especially is resource-poor countries. Still other complications with parenteral delivery exist, such as reactogenicity at the injection site, training of vaccinators, and contamination with blood after multiple users of the same syringe (108). Therefore, additional delivery methods are under development in an attempt to circumvent some of the pitfalls associated with traditional delivery approaches. For example, mucosal antigen delivery is an efficient method for stimulating systemic, but more importantly mucosal immunity, and various needle-free delivery techniques are being explored. Here we briefly summarize some of the promising non-parenteral delivery methods in use.

Mucosal immunization. The term mucosal immunization refers to antigen or vaccine delivery across a mucosal surface (252). The ideal mucosal delivery system should satisfy a set of important requirements. Effective systems should be protected from both physical and enzymatic barriers which exist at the mucosal surface, target the vaccine to the appropriate mucosal lymphoid environment for immune induction (see below), and orchestrate the induction of the appropriate innate signals to fully activate antigen-specific adaptive immunity at the mucosal surface (148). In theory, all mucosal surfaces are equally good candidates for mucosal vaccine delivery; however, some practical limitations impede antigen delivery across the vaginal and rectal surfaces as well as the conjuctiva (204). Thus, delivery of antigens by the oral and nasal routes represent two of the most promising mucosal delivery strategies (205). Here we discuss some of the successful mucosal vaccines as models of mucosal immunization.

Oral vaccines. Oral vaccine delivery has proven effective in several systems. The most widely used mucosal vaccine is OPV, developed some 45 years ago (348). Following oral delivery, OPV replicates in the gut and efficiently delivers viral antigens to the gut-associated lymphoid tissue. This local infection triggers the induction of a strong intestinal humoral immune response (81, 170). Immunogenicity of OPV is one of the factors contributing to the largely successful eradication of polio, as is the fact that OPV is simply delivered by a single-dose disposable pipette and requires only a single immunization. These characteristics have proven important in the eradication campaign (108). The success of OPV has prompted hope for the development of other oral vaccines (273). However, the incidence of vaccine-associated paralytic poliomyelitis (VAPP), which causes approximately 1 case per 2.9 million vaccinations, is cause for caution and signifies the potential dangers of live attenuated vaccines (4, 175).

Two additional live oral vaccines were developed based on the knowledge gained from OPV for vaccines against cholera and typhoid fever (108). The live attenuated strain *Salmonella typhi* Ty21a was developed in the 1970s and has proven to be well tolerated and safe (108). This vaccine induces protection in 60-80% of vaccinees for at least 5-7 years, demonstrating its efficacy as a human mucosal vaccine (81, 206). Likewise, a single oral inoculation of the cholera strain, CVD 103-HgR, induced 60-100% efficacy and few adverse effects (81, 359). An additional oral vaccine, a human-rhesus rotavirus reassortant was developed as a rotavirus vaccine; however, it was withdrawn from the market due to safety concerns with vaccine-induced intussusception (254, 255). While intussusception was clearly observed in vaccinees, the incidence was not significantly increased as compared to unvaccinated children of the same age group (284).

A number of new technologies have been developed in the arena of oral vaccines. As mentioned above, oral delivery of viral and bacterial expression vectors represents a promising area of oral vaccine development. Additionally, new successful oral vaccine technologies have been developed, such as strong mucosal adjuvants like the bacterial enterotoxins (149), the development of oral edible vaccine approaches (343, 344), and oral delivery of many of the established parenteral vaccines. The area of mucosal adjuvants will be reviewed in the next section in detail.

Nasal Vaccines. Nasal vaccine delivery is an active area of inquiry, and here we highlight one of the more recent success stories. The traditional influenza vaccine consists of a trivalent inactivated preparation which is delivered parenterally. This vaccine induces a strong serum IgG response which protects the lungs from complications associated with lower respiratory tract (LRT) infection; however, it does not inhibit virus replication in the upper respiratory tract (URT) (57, 71, 72, 144). Therefore, a cold-adapted influenza virus was selected for its ability to grow at the colder temperatures of the URT, but that would not replicate at the higher temperatures of the LRT (64). The cold-adapted vaccine appears to be safe and much more immunogenic, due to the fact that it more accurately mimics natural infection (1, 64). Other methods for increasing the immunogenicity of nasal vaccines include the use of mucosal adjuvants, as will be discussed below.

Transcutaneous immunization. Transcutaneous immunization (TCI) refers to a novel vaccine delivery technique in which vaccine antigens and adjuvants are delivered through the skin using a skin patch [reviewed in (111, 113, 114)]. Recently, the skin has

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been appreciated not only as a barrier to antigen entry, but also as one of the largest immunological organs of the body, and a promising target for vaccine delivery (112, 113). The epidermis of the skin contains a significant number of powerful APCs called Langerhans cells (LCs), which take up skin derived antigens and transport them to the draining lymph node/s for immune induction (155). The inclusion of strong adjuvants in skin patches dramatically increased LC migration and activation (63, 64). TCI has proven to be a potent inducer of humoral and cell-mediated immune responses in numerous experimental systems and in rodent and human models (112-115). Interestingly, TCI also results in potent mucosal antibody and T cell induction (17, 89, 118). The mechanism by which TCI is capable of stimulating mucosal immunity in the absence of mucosal delivery is not completely clear; however, it is proposed that the TCI-activated skin DCs are induced to migrate to mucosal inductive sites to present antigen (17, 89). TCI represents a promising new delivery technique which may enable the production of new efficacious vaccines against multiple pathogens, including mucosal pathogens.

Concluding Remarks

Here we have highlighted what is known regarding the components of the immune system that are activated by vaccines, the immune correlates of protection with a select group of important human pathogens, and the current tools under development in vaccinology. This is not intended to serve as a complete list in any one of the mentioned areas, but instead, to provide a context in which to place the development of new vaccines and vaccine technologies. We hope this affords the reader an appreciation of the simultaneous simplicity, and complexity of the interactions between vaccines and the immune system. The previous

studies described here provide a strong foundation for discussion of new vaccine technologies, such as those introduced in this dissertation. Hopefully, these new vaccine approaches will provide new tools in the fight against infectious diseases, and their study will lead to an improved basic understanding of pathogenesis and immunity associated with infectious organisms.

ORGANIZATION OF THE MUCOSAL IMMUNE SYSTEM

Overview

The mucosal surface is classically defined as the mucus-covered exterior which serves as the body's interface with the external world (231). The epithelial structures lining the respiratory, gastrointestinal, and urogenital tracts as well as the exposed cornea/conjuctiva provide simultaneous absorptive and barrier function for nutrient uptake, oxygen exchange, and a path for waste excretion (234). The mucosal immune system is charged with the daunting task of protecting the body's vast mucosal surfaces from invading organisms, while simultaneously facilitating the uptake of critical nutrients from food particles as well as air exchange (259). This objective is complicated by the fact that the mucosal immune system must differentiate harmful organisms from innocuous food and inhaled antigens (251, 257).

It is estimated that approximately 90% of all human infections are initiated at the mucosal surface (234), and the surface area of the mucosa is extensive, at approximately 400 m^2 in humans (67, 150). In turn, size and cellular makeup define the mucosal immune system as the largest immunological organ of the body (234). The gut alone contains approximately 10^{12} lymphoid cells per meter of small intestine, accounting for the greatest number of immunoglobulin (Ig)-secreting cells, more than in all other lymphoid organs (spleen, bone marrow, peripheral lymph nodes) combined (234, 236). IgA antibodies are by far the most prominent antibody isotype present in mucosal secretions (37, 231, 232, 237, 238). In fact, Conley and Delacroix estimated that adult humans produce between 2 and 4 grams of IgA per day (61).

The activation of adaptive mucosal immune responses, including IgA-producing B lymphocytes, occurs within the organized mucosal-associated lymphoid tissue (MALT) (259). Antigen transport into the mucosal lymphoid tissues initiates a complex cascade of events which ultimately culminate in the activation of lymphocytes with effector function at the mucosal surface (258, 259). Here we review the anatomical basis of mucosal immune induction, with emphasis on the gut immune system, the anatomy and function of mucosal antibody responses, the role of mucosal adjuvants in stimulating the natural pathway of mucosal immune induction, and the evidence supporting the existence of an alternative pathway for mucosal immune induction.

Anatomy of a Mucosal Immune Response

Organized mucosal lymphoid tissue. The mucosal immune system is organized into mucosal inductive and mucosal effector sites. Mucosal inductive sites, include the organized mucosal lymphoid tissues and are responsible for the activation of immune responses active at the diffuse mucosal surfaces, or the mucosal effector sites (181, 231). Organized mucosal lymphoid tissues are present at all of the mucosal surfaces including in the digestive (276), respiratory (16), and genital mucosa (34). While the specific organization of MALT differs between different mucosal surfaces, the aggregates of lymphoid cells at each surface perform the same function of directing luminal antigens into the correct anatomical context for the activation of antigen-specific immune responses (259). A commonality amongst the primary lymphoid structures at all of the mucosal surfaces is the presence of a specialized epithelial surface covering the lymphoid aggregates (181). In the Peyer's patches (PPs) of the gut, this specialized epithelium is termed the follicle-associated epithelium (FAE) and contains the

microfold cells, or M cells, specialized epithelial cells which function to transport luminal antigens into the underlying lymphoid compartment (169).

The lymphoid cell component underlying the FAE has been studied in a number of animal models. (24, 91, 92). The follicle contains B cells, T cells, DCs, and macrophages. Most B cells in the actual corona of the follicle are surface IgM positive, whereas the B cells present in the germinal centers within the follicle have switched to the IgA isotype (195, 233). Both $CD4^+$ and $CD8^+$ T cells are localized in the inductive sites; however, the $CD4^+$ T cells are predominantly located in the subepithelial dome, while the $CD8^+$ T cells are found in the parafollicular zones (24, 91). Both Th1 and Th2 $CD4^+$ T cells (as defined by IFN-γ and IL-5 secretion, respectively) reside within the inductive sites of the mouse intestine; however, a greater proportion of Th2 cells exist throughout the lamina propria (181, 350) (see below). DCs and macrophages, in addition to M cells, play a critical role in sampling luminal antigens; however, the relative contribution of the various APC subsets varies significantly based upon the structure of the epithelial cell anatomy at the specific mucosal surface (259, 322).

Diffuse mucosal lymphoid tissue. Following antigen delivery to the inductive tissues and subsequent lymphocyte activation, lymphocytes migrate through the general circulation and home to the diffuse mucosal effector tissues of the corresponding mucosal surface (reviewed below). Once present at the mucosal surface, local signals drive further differentiation of activated cells where they mediate their effector functions at the local surface (91, 181). Two distinct subsets of lymphocytes exist at the mucosal surface according to their specific localization; the intraepithelial lymphocytes (IELs) and the lamina

propria lymphocytes (LPLs). The majority (approximately 80%) of the gut IELs in mice and humans are CD8$^+$ T lymphocytes (181). There are fewer IELs present at the nasal and respiratory mucosal surfaces, and a higher proportion of CD4$^+$ T cells (385). Within the IEL population, two distinct subsets of CD8$^+$ T lymphocytes exist based on expression of the CD8 molecule. Both CD8 α/β and CD8 γ/δ T cell populations are present (181). γ/δ T cells are proposed to play a critical role in mediating defense to common bacterial infections in the gut (28).

The second population of gut lymphocytes is the LPLs. LPLs consist of approximately 40% B cells and 25% T cells (mostly CD4$^+$). Approximately 90% of the LPL B cells produce IgA antibodies as well as secrete Th2 cytokines, along with the CD4$^+$ T cells, and epithelial cells, promoting IgA class switch (39, 331, 350). It is thought that Th1 cells in the LPL compartment produce IL-2, which activates proliferation of all T cells, including Th2 cells, which in turn secrete large amounts of IL-5, IL-6, transforming growth factor beta (TGF-β), and other cytokines to orchestrate the efficient IgA class switch signal (224, 336). Together, these lymphocyte populations provide the first line of adaptive defense at the local mucosal surface, and are critical for protection from mucosal pathogens (35).

The Natural Pathway for Mucosal Immune Induction

Mucosal immune responses are most efficiently induced following antigen/pathogen delivery at the local mucosal surface (231). In turn, the natural pathway for mucosal immune induction is defined as the cellular mechanism by which adaptive mucosal immune responses are activated following mucosal delivery. Much is known regarding the programming that is initiated in mucosal tissues and culminates in the localization of antigen-specific cells at the

mucosal surface. Here we outline those events and the characteristics of mucosal IgA antibodies at mucosal surfaces.

Mucosal lymphocyte homing. Following activation in the mucosal inductive tissues lining each mucosal surface, a complex cascade of events is initiated which directs those activated cells to migrate back to effector sites at that mucosal surface. This differs from the homing program initiated following antigen encounter in a peripheral lymph node. In general terms, lymphocytes activated in a mucosa-draining lymphoid tissue are programmed to migrate back to effector sites at that mucosal surface, whereas lymphocytes activated in a non-mucosa-draining lymphoid tissue (i.e. peripheral lymph node or spleen), as occurs following parenteral vaccine delivery, are directed elsewhere (46). In this regard, the regulation of homing to the mucosal surface represents a critical checkpoint in the natural pathway of mucosal immune induction (187).

Classical adoptive transfer studies demonstrated the propensity of cells isolated from mucosal lymphoid tissues to preferentially repopulate the mucosal compartment following adoptive transfer (229, 316). Since then, many of the mechanisms responsible for lymphocyte homing to the mucosal surface have been discovered and recently reviewed (46, 186, 187). Here we present an overview of the regulation of mucosal homing by endothelial cell-integrin interactions as well as specific chemokines and chemokine receptors (CCRs).

Regulation of mucosal homing by lymphocyte-endothelial cell recognition. Transendothelial lymphocyte migration can be broken down into four key steps: 1) initial tethering; 2) activation; 3) arrest; and 4) diapedesis (46, 47, 234). The initial tethering steps

are mediated through the interaction of lymphocytes with the high endothelial venules (HEVs). The HEVs of peripheral lymph nodes express peripheral lymph–node addressins (PNAd), whereas the HEV of mucosal lymphoid tissues express mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) (46, 47, 234). L-selection (CD62L) is responsible for binding to PNAd, while the $\alpha_4\beta_7$ integrin binds to MAdCAM-1. The tethering and rolling of lymphocytes on the endothelium mediated by these interactions serve to slow lymphocyte movement and allow the establishment of more permanent interactions (234). In the gut, rolling is mediated by both L-selectin and $\alpha_4\beta_7$ integrin binding to MAdCAM-1 (20, 130). The activation stage involves signaling via a number of chemokine-CCR interactions and promotes firm arrest on the endothelium (50). Following the establishment of firm contacts, lymphocytes next undergo transendothelial migration by diapedesis into the mucosal lymphoid area (46, 47). This step is also regulated by the activities of a number of factors including $\alpha_4\beta_7$ integrin-MAdCAM-1 interactions and numerous chemokines (95, 173, 311). Interactions between $\alpha_4\beta_7$ integrin and MAdCAM-1 regulate mucosal homing at numerous levels and therefore are proposed to be critical regulators of mucosal homing, especially in the gut (37, 38, 46, 47).

Chemokine regulation of mucosal homing. Recently, a number of chemokine-CCR interactions have been demonstrated to play a role in mucosal homing, especially in the recruitment of IgA-secreting cells to the gut mucosa. Both thymus-expressed chemokine (TECK/CCL25)-CCR9 interactions, as well as mucosae-associated epithelial chemokine (MEC/CCL28)-CCR10 interactions play a critical role in the recruitment of IgA-secreting cells to the gut mucosa (31, 143, 194). In fact, the $\alpha_4\beta_7$ integrin, TECK, and MEC, all play a

role in the local IgA response to an important gut pathogen, rotavirus (97, 185). Thus, CCR9 and CCR10 expression, along with $\alpha_4\beta_7$ integrin appear to regulate mucosal homing of IgA-secreting cells (187, 188).

Architecture of mucosal IgA antibodies. The respiratory and gastrointestinal surfaces of humans comprise approximately $100m^2$ and $300m^2$ respectively (161). These surfaces are vulnerable to attack from various mucosal pathogens and require active effort for protection. A number of innate protective mechanisms play a critical role in protecting the mucosal surfaces (300, 320); however, here we focus on the adaptive responses that are activated by vaccination and their roles in protection from mucosal challenge. Secretory IgA (SIgA) is the most prominent antibody isotype in mucosal secretions and mediates protection of the mucosal surface in a number of experimental systems. Here we review the structure of mucosal IgA, the mechanism by which IgA antibodies are transported into mucosal secretions, and the evidence for IgA-mediated protection at the mucosal surface.

Structure of mucosal IgA. As mentioned above, IgA antibodies are the most abundant antibodies present in mucosal secretions; although IgG and IgM antibodies are also present and do play a role in mucosal defense (34, 239). IgA antibody-secreting-cells (ASCs) present at mucosal sites are distinguishable from systemic IgA ASCs, as over 90% of mucosal IgA cells concomitantly express the joining chain, or J chain (20). J chain is a 15 kilodalton (kDa) protein incorporated into IgA molecules, most likely during trafficking through the endoplasmic reticulum, that facilitates the production of dimeric and/or polymeric forms of IgA (36, 37, 239). Intracellular J chain expression has been observed in

IgG-secreting-cells as well; however, the J chain is not incorporated into secreted IgG and the reason for J chain expression in these cells is unclear (23). IgA molecules form mostly dimers in the presence of J chain; however, trimeric and tetrameric polymers also have been observed (369).

In humans, serum contains between 80-100% monomeric IgA, while dimeric and polymeric forms of IgA dominate in mucosal secretions (238, 239). Increased levels of polymeric IgA has been noted in the serum of rodents, although the exact importance of this finding is unclear (318). The importance of J chain in polymeric IgA production is evidenced by the observation that J-chain knockout mice exhibited a significant increase in the ratio of monomeric-to-dimeric forms of IgA. J chain is also incorporated into pentameric IgM complexes (262). J-chain-containing IgA and IgM antibodies are preferentially transported into mucosal secretions by an active transport mechanism (see below). Thus, the physiological structure of mucosal IgA molecules facilitates their role in mucosal defense.

Transport of IgA molecules into mucosal secretions. The unique ability of IgA and IgM antibodies to polymerize into higher order structures is determined by the ability of the α and μ heavy chains to interact with J chain. Two cysteine residues present in the human J chain form a disulfide bridge with the penultimate cysteines in the α and μ chain to form polymeric IgA and pentameric IgM respectively (161, 240). Support for a role of J chain in the transport of IgA molecules into mucosal secretions comes from analysis of J chain knockout mice. Decreased levels of fecal IgA antibodies were observed in J chain-deficient animals, suggesting that polymeric forms of IgA were transported across mucosal surfaces (140).

The original description of dimeric IgA at the mucosal surface demonstrated not only the addition of the J chain to IgA, but also an 80 kDa protein, designated as the 'secretory piece' (361). We now know this component as the secretory component (SC), a proteolytic fragment of the poly Ig receptor (pIgR) which is retained in SIgA following transport through mucosal epithelial cells. Much is known regarding the mechanism of IgA transport via the pIgR (167, 250). The pIgR is a 100 kDa protein expressed on the basolateral surface of mucosal epithelial cells which binds to IgA in a J chain-dependent manner (162, 249). The polymeric IgA-pIgR complexes are endocytosed and transported to the apical surface of mucosal epithelial cells. During transport, disulphide bridges hold the complex together and proteolytic cleavage at the apical surface cleaves off the transmembrane domain of pIgR and releases SIgA into the mucosal lumen (10). Support for the importance of this pathway comes from the observation that animals lacking the pIgR have severe defects in the levels of IgA and IgM antibodies in mucosal secretions (163). Analysis of the structure and function of mucosal IgA transport suggests a clear relationship between the form and function of IgA molecules within the mucosal compartment.

Role of IgA antibodies in mucosal defense. As mentioned above, mucosal antigen delivery is the most efficient method of generating a mucosal IgA response. Evidence from a number of experimental systems has demonstrated a correlation between the appearance of IgA antibodies in mucosal secretions and protection from challenge with influenza virus (58), rotavirus (63), polio virus (270), and cholera (160) [reviewed in (234, 319)]. While this correlation is strong and appears to be broadly applicable, the correlation alone does not prove that IgA provides a critical, non-redundant role in protective mucosal immunity.

Two lines of evidence support the notion that IgA antibodies provide a critical protective effect at the local mucosal surface. First, systemic passive transfer of IgA antibodies is protective in several mucosal challenge models [reviewed in (319)]. Following systemic IgA transfer, IgA antibodies can be detected at the mucosal surface, suggesting they mediate protection at the local level; however, such antibodies are also present systemically, complicating that clear interpretation. Interestingly, nasal administration of IgA antibodies mediated protection from an intranasal influenza challenge in mice (225), and intranasal challenge with respiratory syncytial virus in both mice (380) and monkeys (381) provides clear evidence for a role for IgA at the local mucosal surface.

The second line of evidence comes from studies performed in IgA, and/or mucosal IgA deficient mouse models. A number of IgA deficiency models exist including IgA knockouts (12), J chain knockouts (217), and pIgR knockouts (388), all of which exhibit increased susceptibility to mucosal challenge. However, compensatory mechanisms exist in these models such as increased serum IgA (J chain and pIgR KOs), serum IgG/IgM, and increased mucosal IgG, clouding the interpretation of such studies (234). Further definitive evidence for the protective capacity of mucosal IgA comes from an additional model system. In an elegant study, Renegar and Small (310) depleted IgA, IgG, and IgM antibodies specifically in the URT of influenza virus-immune animals by local delivery of specific anti-immunoglobulin antibodies prior to/during a nasal influenza challenge. The local depletion of nasal IgA, and not IgG or IgM, abrogated the protection observed in immune animals, providing strong evidence that mucosal IgA antibodies directly at the mucosal surface are critical for mucosal defense. Together, these studies are consistent with a model in which

IgA antibodies are produced at the local mucosal surface following mucosal delivery, are transported into mucosal secretions, and provide a protective effect.

__Stimulation of the natural pathway by bacterial enterotoxins.__ In earlier sections of this review we have introduced the concepts of adjuvants as well as mucosal antigen delivery. As we have now reviewed the anatomical basis of mucosal immune induction, here we summarize what has been learned regarding the natural pathway for mucosal immune induction using mucosal delivery of the bacterial enterotoxins: cholera toxin (CT) and labile toxin (LT). The use of CT and LT as mucosal adjuvants has set the benchmark by which mucosal immune induction is measured, and serves as the "gold standard" for the evaluation of mucosal immunity in new experimental systems (33). CT is used as an example of the mechanisms regulating mucosal immune induction, as well as a further example of the paucity of knowledge regarding the critical mechanisms of adjuvant activity.

CT, from *Vibrio cholerae*, is comprised of an A subunit with adenosine-diphosphate (ADP)-ribosly-transferase activity, and a pentameric B subunit (CTB) which mediates receptor binding to the cellular receptor, GM1-ganglioside (87). The ADP ribosyl-transferase activity of the A subunit has dramatic effects on vesicular membrane trafficking and organelle integrity *in vivo* (193).   Adjuvant experiments performed with CT enzymes harboring mutations in either the A subunit active site, B subunit active site, or both, suggested that both subunits were required to generate a full adjuvant effect (88); however other studies have demonstrated adjuvant activity with the B subunit alone, albeit not as strong as with the complete enzyme (149).

As mucosal CT delivery is the most potent stimulator of the natural pathway, its adjuvant activity is typically studied under the assumption that its immunomodulatory effects are likewise the most efficient means of stimulating mucosal immune responses. Therefore, the characterization of CT-induced immunomodulation provides a target level for induction with new adjuvants, in lieu of a single mucosal inductive mechanism. CT, like most adjuvants, affects multiple aspects of the immune environment which alone, or in combination are responsible for immune induction. These effects likely occur by multiple, overlapping mechanisms, as outline for systemic adjuvants (see above). CT appears to induce a Th2-biased cytokine profile with strong production of IL-4, IL-5, IL-6, IL-10 and the production of IgA and IgG1 antibodies (147). Further analysis has led to the conclusion that the B subunit has a more profound effect on Th2 polarization than either the A subunit alone, or delivery of the holoenzyme (32). A role for Th2 cytokines in the adjuvant effect of CT is supported by the observation that CT failed to exert a mucosal adjuvant effect in IL-4 deficient animals (371); however, IL-6 appears not to be necessary, as normal adjuvant effects were observed in IL-6 deficient mice (45). As is true in IgA deficiency models, caution must be exercised in the interpretation of adjuvant activity in specific cytokine deficient models, as compensatory effects may occur in such animals.

In addition to cytokine production, CT induces recruitment and activation of DCs at the mucosal surface (9). These effects may partially explain the strong effect of CT by modulating the cellular antigen presentation pathway. In humans, CT is quite toxic and to date is inappropriate for inclusion in human vaccines. However, much work has gone into the development of genetically detoxified mutants which lack the toxic effects while retaining adjuvant activity (2, 216). The utilization of CT as a mucosal adjuvant has shed

new light on the mechanisms by which mucosal immune responses are activated, and if non-toxic mutants still retain adjuvant activity in humans, should enable the development of mucosal vaccines in humans.

An Alternative Pathway for Mucosal Immune Induction

There is no disputing that, to date, the strongest mucosal immune responses are observed following stimulation of the natural pathway for mucosal immune induction. Indeed, it has been suggested that localization of antigen to the MALT following mucosal antigen delivery is a prerequisite for mucosal immune induction (230-232). However, protection from mucosal challenge, and production of local mucosal immune responses following nonmucosal delivery has been demonstrated in a number of experimental systems, suggesting the presence of an alternative pathway(s) for mucosal immune induction [reviewed in (30, 366)]. Unlike the natural pathway, antigen delivery across a luminal barrier appears not to be required for immune induction via this alternative pathway. Here we briefly review the evidence supporting the existence of such a pathway and attempt to identify the commonalities amongst the individual examples as a means to determine the specific immunological mechanism(s) at work.

The examples presented here are extremely varied, and a common unifying mechanism responsible for mucosal immune induction under all circumstances is not readily evident upon initial review. Both virus infections as well as bacterial infections appear capable of promoting mucosal immunity through the stimulation of an alternative mucosal inductive pathway. Peripheral, or nonmucosal delivery of a diverse class of viruses or viral vaccine vectors including rotavirus (59, 60), canarypox vectors (256), Venezuelan equine

encephalitis virus (51) and replicon particles (131, 212, 358), and Rubella virus (271) results in the presence of antigen-specific antibodies in mucosal secretions and/or the activation of antigen-specific T cells located at the mucosal surface. Likewise, bacterial antigens/vectors derived from Listeria *monocytogenes* (285) and Haemophilus *influenzae* type b (171) stimulate immunity at the mucosal surface following nonmucosal delivery.

The examples cited above suggest that traditional nonmucosal delivery methods are capable of stimulating mucosal immunity. In addition, several specialized nonmucosal delivery approaches have been developed which likewise promote mucosal immune induction. One example is parenteral targeting of mucosal lymphoid tissues by the use of anti-MAdCAM-1 antibodies, which dramatically augment mucosal IgA production (235). As mentioned above, TCI in the presence of CT also induces potent mucosal immunity following delivery through the skin in both mice and humans (113, 115, 391). Interestingly, an approach termed targeted lymph node immunization, which relies on direct antigen inoculation into a peripheral lymph node, also stimulates mucosal immunity in non-human primates (172, 201).

Whether mucosal immune induction, following either traditional nonmucosal delivery or one of the specialized approaches outlined above, is in fact due to an intrinsic signal provided by a direct effect of the infectious agent/antigen itself or, instead, is due to an indirect effect, such as the induction of an immunoregulatory agent is unclear at this point. A number of candidate immunomodulatory factors have been identified including various forms of vitamin D3 (89) as well as a group of specific cytokines and chemokines (85) which potentially provide a mechanistic explanation for mucosal immune induction following nonmucosal antigen delivery.

The exact mechanisms underlying mucosal immune induction following nonmucosal antigen delivery have yet to be clearly elucidated. The identification of such inductive mechanisms is an active area of inquiry in our laboratory, as well as several others. In our estimation, the definition of the exact lymphoid tissues which serve as the critical components of the alternative pathway for mucosal immune induction is a crucial first step in defining the operative mechanisms. In this report (Chapter 3), we provide supportive evidence for the inclusion of the draining lymph node in the alternative pathway for mucosal immune induction following footpad delivery of VEE replicon particles. Whether the involvement of this lymphoid tissue in such an alternative pathway is common to the other examples remains to be determined. In the next section of this review, we describe our current understanding of a peripherally-induced mucosal inductive pathway, as revealed by nonmucosal delivery of alphaviruses and alphavirus-derived vectors (see below). The elucidation of the immunological mechanisms operative in an alternative mucosal inductive pathway may allow mucosal immune induction with parenterally-delivered vaccines, and therefore more efficacious vaccination protocols.

VENEZUELAN EQUINE ENCEPHALITIS VIRUS

Overview

VEE is a member of the *Togaviridae* family and the alphavirus genus. Further, VEE is a member of the New World alphaviruses, along with Eastern equine encephalitis virus and Western equine encephalitis virus. These viruses possess the capability to cause a febrile illness and encephalitis in equines and humans (123). The Old World alphaviruses, including SIN, SFV, Ross River virus (RRV), Chikungunya virus, and O'nyong-nyong virus, produce an arthritic and/or arthralgic disease characterized by fever and rash in humans (123). All of the alphaviruses are arthropod-borne infections and are transmitted in nature by the bite of an infected mosquito (376).

VEE is maintained in nature in an enzootic cycle between mostly Culex mosquitoes and small rodents in North and south America (377). Epizootics and epidemics are possible, especially with specific subtypes (I-AB and I-C) and can have a dramatic effect on equines, including as an amplification host for the generation of a high titer viremia (377). VEE was first identified as the etiologic agent of equine encephalitis in Venezuela in 1938 when it was isolated from the brains of fatal cases of encephalitis in horses (184). The first documented cases of naturally acquired human disease was identified in the 1950s in Colombia (321). Infection in equines results in a severe disease with mortality rates ranging between 20-80%; however; severe disease is much less common in humans with a neurological symptoms apparent in 5-15% of infections and mortality in less than 1% (377). The implementation of the TC-83 vaccine, a live attenuated I-AB strain passaged in guinea pig heart cells 83 times (18), has gone a long way to curb VEE outbreaks where vaccination of equines is extensively

utilized (377). The potential of epizootic outbreaks, as evidenced by the 1995 epidemic which affected up to 100,000 people in South America (378), along with the previous history of VEE as a potential agent of biological warfare (377), has stimulated great interest in understanding the biology of VEE infection.

Genome Organization and Replication

Much of the knowledge of VEE biology and replication has stemmed from comparative studies with SIN in cultured cells. VEE contains a message-sense, single stranded RNA genome of 11, 477 nucleotides which contains a 5' methylguanosine cap and a 3' polyadenlyated tail. The genome has considerable secondary structure and is divided into two regions: the viral non-structural proteins (nsPs 1-4), responsible for RNA replication, are encoded at the 5' two-thirds of the genome, while the structural components, the capsid, E3, E2, 6K, and E1 proteins, are encoded at the 3' one-third of the genome, expressed from a separate subgenomic 26S promoter (342). The genome is encapsidated into an icosohedral nucleocapsid containing 240 copies of the capsid protein with T=4 symmetry (50) following specific interactions with the capsid protein and the encapsidation signal found in the nsP1/2 gene region (102). The nucleocapsid is engulfed in a lipid bilayer which harbors the viral glycoprotein spikes, composed of trimers of E1/E2 heterdimers, also with T=4 symmetry (342).

The receptor for VEE is currently unknown; however, c-type lectins (178), the laminin receptor (213), and heparin sulfate (HS) proteoglycans (21) have all been proposed as attachment receptors for VEE. Binding to HS represents a tissue culture adaptation and most likely is not a natural receptor, as HS-binding viruses are all attenuated *in vivo* (21).

Following receptor-mediated endocytosis and fusion of viral and endosomal membranes triggered by low pH, or alternatively, the direct release of the genome through the plasma membrane after attachment (278), the viral RNA is directly translated by the host translation machinery into the precursor P123 or P1234, depending upon a low efficiency read through event at an opal termination codon just prior to nsP4 (342). The P123 precursor and free nsP4, the RNA-dependent RNA polymerase, mediate the production of minus sense RNA during the first 3-4 hours post infection (342). Following cleavage of the P123 complex into the individual nsP proteins, a shift occurs, driving the production of positive sense messages, including 5-10 fold molar excess of the subgenomic RNA over the genomic. This ensures adequate production of the structural components required for assembly of new virions (342). Budding of newly assembled virions occurs at the plasma membrane following encapsidaton of full length genomic RNA and transport of newly made E1 and E2 glycoproteins through the endoplasmic reticulum (342). Infection has dramatic effects on host cells including cessation of host RNA and protein synthesis and eventual apoptosis (119, 123).

Pathogenesis and Control

A range of symptoms are exhibited in humans following VEE infection from little or no disease, to a fatal encephalitis (377). A number of factors affect disease severity including age and genetic factors of the patient as well as the virus serotype (86). Infection of humans with VEE results in fever, malaise, vomiting, and acute retro-orbital pain, following a 2-5 day incubation period. In most cases, these symptoms subside within one week; however, in a minority of cases severe complications develop including convulsions, coma, and neurological sequelae. In the <1% of fatal human cases, disease is accompanied

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by diffuse congestion, edema with hemorrhage in the brain, lungs, and gastrointestinal tract (79, 86). Interestingly, VEE infection causes a severe lymphopenia in humans, equines, and rodents (86, 377), which may result from either lymphocyte apoptosis or lymphocyte retention in peripheral tissues. (79, 86, 377).

Several animal models of VEE pathogenesis have been developed over the years including macaques (246, 309), rabbits (68), and guinea pigs (68); however, the mouse model, in combination with the development of the infectious clone of the Trinidad donkey strain (78), has proven the best model to date. In the mouse model, much like the disease course in equines, VEE exhibits a bi-phasic disease, with an early lymphotropic phase involving replication in peripheral lymphoid tissues, followed by a neurotropic phase, which ultimately culminates in a lethal encephalitis by day 6-8 post-infection (110). The stepwise pathogenesis of virulent VEE, as well as molecularly cloned mutants, was recently demonstrated by Johnston and colleagues in the mouse model of VEE disease (11, 76, 121).

The initial targets of VEE infection following footpad inoculation were identified by MacDonald and Johnston as DCs; specifically, LCs (218). Infected DCs migrated to the draining lymph node (DLN) as early as 4 hours post infection (hpi) following footpad infection, where viral replication was detected at 6 hpi (121). Viremia peaked at 12 hpi, and by 18 hpi, virus replication was rampant in numerous peripheral organs including the spleen, kidney, lung, and heart; although viral RNA was restricted to a subset of these tissues, suggesting the VEE serum viremia is a major source of virus at early times (121). Peripheral titers peaked between 24-48 hpi; a time at which the virus begins to seed infection in the central nervous system (CNS) (76, 121). CNS invasion likely occurs through infection of the

olfactory neuroepithelium and the trigeminal nerve (53), resulting in fatal encephalitis by day 6-7 post infection (121).

Both innate and adaptive components of the immune system play a critical role in mediating protection from natural VEE infection. A role for the type I interferon system is demonstrated by the observation that mice genetically deficient in the type I interferon receptor succumb to lethal VEE disease between 24-48 hpi (122, 383). VEE-specific IgM and/or IgG antibodies appear to play an important role in clearance of VEE from the periphery (52) as well as the induction of protection following vaccination (123); however, antibody responses initiated during infection are not sufficient to prevent neuroinvasion and a lethal outcome following primary infection (123). Infection with VEE, SIN, and SFV results in immunopathology in the CNS; although VEE, unlike the other alphaviruses, induces a lethal spongiform encephalopathy in animals devoid of functional lymphocytes, demonstrating the ability of VEE to directly damage neurons in the CNS (52).

Protection from Mucosal Challenge and Mucosal Immune Induction

Mucosal immune induction and/or protection from mucosal challenge have been described in several VEE systems including attenuated virus mutants, as well as both replication-competent and replication-deficient viral vectors. Here we review the evidence of mucosal immune induction, and discuss the putative inductive mechanisms under all three conditions.

Venezuelan equine encephalitis virus vectors. In addition to attenuated VEE, two additional VEE vectors have been described. The first, termed double promoter vectors,

contains a second cloned 26S subgenomic promoter expressing a foreign antigen, downstream of the authentic 26S promoter expressing the viral structural proteins (74). These replication-competent vectors express the foreign antigen in virus-infected cells and induce strong humoral and cell-mediated immune responses (74). The other vaccine vectors are VRP, which are engineered such that the heterologous antigen is cloned in place of the viral structural genes. These vectors express high levels of the heterologous transgene in infected cells; however, they fail to spread beyond the first infected cell as no new progeny virions are produced due to the lack of structural components (303). VRP have proved to be effective inducers of protective immunity in several experimental models [reviewed in (77, 214, 215, 297, 307, 312, 325)]. Here we describe the ability of VEE, VEE-based double promoter vectors, and VRP to induce protection from mucosal challenge and to induce mucosal immune responses, as well as discuss the putative inductive mechanisms in each scenario

Attenuated VEE mutants. In nature, VEE is spread through mosquito bite, representing a subcutaneous challenge (377). However, aerosol exposure of laboratory workers as well as threats of VEE as a bioterrorism agent warrant, the development of vaccines which are capable of protecting from a mucosal VEE exposure as well. Interestingly, nonmucosal delivery of attenuated mutants of VEE have been shown to induce protection from lethal aerosol challenge with virulent VEE in mice (51, 132-134, 286, 299), hamsters (299), and monkeys (299, 308). While attractive to speculate that peripheral delivery of attenuated VEE induced protection from mucosal challenge through the induction of a local mucosal immune response, this is not necessarily demonstrated by this observation.

However, VEE-specific IgG and/or IgA antibodies were in fact present in mucosal secretions following subcutaneous delivery of VEE mutants in mice (51, 132-134, 286) and monkeys (299), providing strong support for the notion that VEE stimulates an alternative pathway for mucosal immune induction which does not require delivery across a mucosal surface. Hart *et al*. demonstrated that two attenuated mutants of VEE, the vaccine strain TC-83, as well as a virus harboring a mutation in the PE2 cleavage domain, V3526, both induced protection from aerosol challenge with virulent VEE, and likewise both induced VEE-specific IgA antibodies in bronchial lavage samples (133). Interestingly, V3526 induced a stronger mucosal IgA response than TC-83, which correlated with stronger protection from mucosal challenge, suggesting that virus-induced mucosal IgA antibodies may mediate protection from virulent mucosal VEE challenge (133).

VEE-based double promoter vectors. The examples cited above demonstrate that VEE induces local mucosal immune responses directed against itself following nonmucosal delivery, a response that likely mediates protection from a lethal mucosal challenge. Double promoter vectors derived from VEE likewise induce protection from mucosal challenge and IgA antibody production at mucosal surfaces following nonmucosal delivery. Davis *et al*. demonstrated that a VEE double promoter construct expressing the hemagglutinin (HA) gene from influenza virus not only protected mice from overt signs of clinical disease following a mucosal challenge with influenza virus, but also significantly reduced challenge virus replication in the URT, suggesting that local mucosal immune induction inhibited the earliest stages of challenge virus infection (74). Additionally, Caley *et al*. demonstrated induction of matrix capsid (MA/CA)-specific IgA antibodies present in vaginal lavage fluids following

subcutaneous delivery of a double promoter VEE vector expressing the MA/CA gene from HIV-1. Thus, VEE appears to be capable of inducing mucosal immune responses to the virus itself, as well as to antigens which are expressed from genetically modified virus vectors.

The mechanisms by which VEE and replicating VEE vectors induce mucosal immunity remains to be fully determined; however, Charles *et al*. (51) recently proposed a model to explain such an observation. The central thesis of the natural pathway for mucosal immune induction suggests mucosal antigen delivery, as occurs following nasal or oral inoculation, is required to target antigen to the MALT, and to induce mucosal immunity. However, VEE induces mucosal immunity without the necessity of mucosal delivery. Charles *et al*. suggested that VEE induces mucosal immunity by delivering viral antigens to the MALT not across the mucosal barrier, but instead from the anatomical interior of the animal (51). Consistent with this hypothesis, VEE efficiently replicates in the MALT tissues of the gut, providing a plausible mechanism by which viral antigens gain access to the mucosal immune system in the absence of mucosal delivery (51). In further support of this notion, attenuated VEE mutants efficiently replicate in the PPs and mesenteric lymph node following subcutaneous vaccination, and not only protect animals from nasal challenge with virulent VEE, but also reduce challenge virus replication in the mucosal epithelium to below background levels (Richmond E. M., and Johnston, R. E., unpublished). These observations are consistent with mucosal immune induction from the "inside out" as opposed to the natural "outside in" pathway. Further experimentation will be required to validate such an hypothesis.

VEE replicon particles. While studies with replicating VEE mutants and double promoter vectors have clearly demonstrated mucosal immune induction following nonmucosal delivery, the VRP system provides an opportunity to study mucosal immune induction with VEE under conditions in which the infection is limited to the first infected cells. Protection from mucosal challenge with VRP has been demonstrated with influenza virus in mice (303) and chickens (329), Simian immunodeficiency virus in non-human primates (166), and Equine arteritis virus in horses (15). Additionally, results from intranasal challenge of HA-VRP-immunized mice suggested that VRP reduced challenge virus replication in the URT to below the limits of detection (Richmond, E. M., Davis, N. L., Brown, K., West, A. C., and Johnston, R. E., unpublished). These observations are consistent with a model of local mucosal immune induction; however, they are not definitive. Recently, mucosal immune responses have in fact been observed in animals immunized parenterally with VRP. Antigen-specific IgG and IgA antibodies were present in gut mucosal secretions in two separate studies utilizing VRP expressing the major capsid protein from Norwalk virus (131, 212). Likewise, analysis of immune responses in animals immunized in the footpad with VRP expressing influenza virus HA revealed production of HA-specific IgG and IgA antibodies both in the URT (358) and in vaginal secretions (Thompson, J. M., Richmond, E. M., Davis, N. L., and Johnston, R. E., unpublished) as well as antigen-specific IFN-γ-secreting cells in the URT (Thompson, J. M., Whitmore, A. C., Heise, M. T., and Johnston, R. E., unpublished).

Replicon particles derived from SIN and SFV also have demonstrated induction of mucosal antibody and/or T cell responses, but only after a protocol involving mucosal delivery, not following nonmucosal delivery alone (19, 370). Comparative immunogenicity

studies between SIN and VEE replicon particles have been performed recently. Stronger antigen-specific $CD8^+$ T cell responses were present in animals immunized with VEE replicon particles as compared to animals immunized with SIN replicon particles expressing the same antigen (283). Interestingly, analysis of systemic immune induction with chimeric particles derived from VEE and SIN suggested that the increased immunogenicity phenotype was dependent upon the VEE RNA, regardless of whether the VEE RNA was enveloped in VEE capsid and glycoproteins or the SIN capsid and glycoproteins (283).

Nonmucosal delivery of chimeric particles in which the RNA was derived from VEE, and the structural components derived from SIN resulted in antigen-specific IFN-γ-secreting cells at the local vaginal mucosal surface following heterologous vaginal boost (125), as well as strong protection in a mouse HIV challenge model (125). Combined parenteral/mucosal delivery of VEE/SIN chimeras results in antigen-specific mucosal immunity in macaques as well (126). Interestingly, Greer *et al.* recently demonstrated that parenteral delivery of chimeric particles expressing parainfluenza virus (PIV) antigens not only protected animals from clinical signs associated with mucosal challenge with PIV, but also reduced early challenge virus replication in the URT and LRT to levels below the limits of detection in both mice and hamsters (120), consistent with immune induction at the local mucosal level. While these examples warrant the further investigation of VEE/SIN chimeras, local mucosal immune induction following nonmucosal delivery has as yet only been observed with alphavirus replicon particles derived from VEE.

Understanding the mechanism by which replication-defective VEE vectors promote mucosal immune induction represents an active area of inquiry. Replication-competent VEE appears to access the MALT following nonmucosal delivery; it is possible that VRP-infected

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DCs also target VEE antigens to the mucosal lymphoid tissues. VRP promote the migration of infected DCs to the DLN. It is possible that free VRP, or VRP-infected DCs, could migrate to the MALT and present antigen to the mucosal inductive sites in a manner similar to replication competent virus. However, to date, there is no evidence of VRP-infected cells within mucosal lymphoid tissues following footpad inoculation (Thompson, J. M., Richmond, E. M., and Johnston, R. E., unpublished).

It is plausible that instead of targeting MALT following peripheral delivery, VRP set in motion the events required to activat mucosal immune responses in a non-mucosa-draining lymph node. If this hypothesis were true, one would predict such a lymph node to exhibit markers of mucosal lymphoid tissues following VRP infection. In fact, recent work supports such a notion (Thompson *et al.*, in preparation, Chapter 3). The DLN of VRP-immunized animals appears to serve as the earliest site of antigen-specific IgA antibody production, including production of polymeric forms of IgA. Additionally, B cells present in the VRP DLN upregulate expression of the mucosal homing receptor, and MAdCAM-1 is expressed on the HEVs of the VRP-targeted DLN. Moreover, analysis of cytokines present in the VRP DLN suggests the activation of a massive inflammatory response, with expression of several cytokines with documented roles in mucosal immune induction, including IL-6 and TNF-α. While further experimentation is required to validate such an hypothesis, all of these observations are consistent with a model in which the VRP-infected draining lymph node is converted into the functional equivalent of a mucosal inductive site.

Adjuvant effects. Studies performed as early as the 1960s suggested that viral infection could dramatically affect the magnitude and duration of a concomitant immune

response to an unrelated antigen (263). At the same time, a similar effect was demonstrated with an attenuated VEE in both mice (151) and guinea pigs (65). This work demonstrated the adjuvant effect of replicating VEE on the generation of a humoral immune response to an antigen present in virus-infected animals. Interestingly, the strongest adjuvant effect was observed when attenuated VEE was delivered 24 hours prior to antigen delivery (65). In fact only a very modest increase in humoral immunity was observed when VEE was delivered 5 minutes prior to antigen inoculation. These observations suggest that VEE possess inherent immunostimulatory properties and strong adjuvant activity for the induction of humoral immunity.

Little, to no follow up work has been performed with replicating VEE adjuvants; however, we have recently demonstrated that the adjuvant effect of VEE is not dependent upon propagation and spread *in vivo*, as VRP likewise possess adjuvant activity (358). Further characterization of this effect demonstrated robust adjuvant activity for both systemic and mucosal antibody responses (358) as well as T cell responses in mice (Thompson *et al*, in preparation, Chapter 4). The adjuvant effect was sensitive to ultraviolet (UV) light, suggesting that elements of VRP RNA replication play a critical role in immune induction (358). Recently, replicon particles derived from SFV were also shown to possess adjuvant activity for systemic humoral immunity (142). While the mechanistic explanation for the adjuvant effect appears to differ between the two systems in terms of sensitivity to UV, and dependence upon type I interferon signaling (discussed in Chapter 5), these observations suggest that alphavirus replicon particles possess intrinsic immunostimulatory properties independent of antigen expression and provide the basis for a novel vaccine technology.

DISSERTATION OBJECTIVES

The purpose of the studies described here are the following:

1) Identify the critical immunological and virological components responsible for VRP systemic and mucosal adjuvant activity,

2) Define the mechanism(s) by which VRP promote mucosal immune induction following nonmucosal delivery, and

3) Evaluate the efficacy of VRP adjuvants in protective vaccines

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CHAPTER TWO

MUCOSAL AND SYSTEMIC ADJUVANT ACTIVITY OF ALPHAVIRUS REPLICON PARTICLES

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ABSTRACT

Vaccination represents the most effective control measure in the fight against infectious diseases. Local mucosal immune responses are critical for protection from, and resolution of, infection by numerous mucosal pathogens. Antigen processing across mucosal surfaces is the natural route by which mucosal immunity is generated, as peripheral antigen delivery typically fails to induce mucosal immune responses. However, we demonstrate in this article that mucosal immune responses are evident at multiple mucosal surfaces after parenteral delivery of Venezuelan equine encephalitis virus replicon particles (VRP). Moreover, coinoculation of null VRP (not expressing any transgene) with inactivated influenza virions, or ovalbumin, resulted in a significant increase in antigen-specific systemic IgG and fecal IgA antibodies, compared with antigen alone. Pretreatment of VRP with UV light largely abrogated this adjuvant effect. These results demonstrate that alphavirus replicon particles possess intrinsic systemic and mucosal adjuvant activity and suggest that VRP RNA replication is the trigger for this activity. We feel that these observations and the continued experimentation they stimulate will ultimately define the specific components of an alternative pathway for the induction of mucosal immunity, and if the activity is evident in humans, will enable new possibilities for safe and inexpensive subunit and inactivated vaccines.

INTRODUCTION

The control of a number of important infectious diseases by immunization is arguably one of the most significant accomplishments of the 20th century (2). However, other infectious diseases remain intractable, causing devastating morbidity and mortality in human

populations, especially in resource-poor countries. Control of these diseases will depend on an expanded array of affordable and effective vaccine technologies, such as propagative and nonpropagative expression vectors based on viral and bacterial genomes. One such technology uses replicon particles based on the alphavirus Venezuelan equine encephalitis virus (VEE). VEE replicon particles (VRP) are potent inducers of antigen-specific immune responses and/or protection after pathogen or toxin challenge in various animal species including mice (31, 45), rabbits (17), cats (9), chickens (47), horses (3), guinea pigs (44), and nonhuman primates (25). Currently, VRP expressing the *gag* gene from HIV clade C are in phase-I clinical trials in the United States and Africa.

VEE virions contain a positive sense RNA genome of ≈11.5 kb. The four viral nonstructural proteins, which constitute the enzymatic activity required for RNA replication, are encoded in the 5' two-thirds of the genome, whereas the viral structural proteins (capsid, E1, and E2) are expressed from a 26S subgenomic mRNA and encoded in the 3' one-third of the genome (27, 49). VRP are propagation-defective viral particles carrying a modified VEE genome. The VRP system takes advantage of the high-level expression of 26S mRNA by replacing the viral structural genes with a cloned antigen gene (45). Progeny virions are not produced in VRP-infected cells, as the viral structural genes are absent from the replicon RNA; however, the replicon RNA and the mRNA encoding the antigen are expressed at high levels after infection (20, 45). To facilitate assembly of VRP, the replicon RNA is coelectroporated into permissive cells with two defective helper RNAs that lack the viral packaging signal and provide the structural genes in trans (20, 45).

VRP display a number of attractive features as vaccine delivery vehicles, including high-level antigen expression in infected cells (45), efficient *in vivo* targeting of mouse (34),

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and primate (A. West and R.E.J., unpublished work) dendritic cells (DCs), efficient *ex vivo* infection of human DCs (40), and safety, as the vectors are incapable of synthesizing new virion particles in infected cells (20, 45). One of the most intriguing properties of VRP is their ability to induce significant protective immunity in mucosal challenge models, even when the immunization is at a nonmucosal site (3, 22, 25, 45, 47).

The natural pathway of mucosal immune induction involves the direct delivery of immunogen to a mucosal surface and local processing of antigen in specialized aggregates of lymphoid tissue, termed mucosal inductive sites (35, 55). Stimulated lymphocytes then migrate to the corresponding mucosal surface where antigen-specific IgA and IgG are locally produced, and specific T cells reside to protect that mucosal surface from pathogen attack (10, 30). We show in this article that, unlike many vaccine vector systems that rely on mucosal delivery to access the natural inductive pathway, VRP are capable of inducing mucosal immune responses after nonmucosal delivery. Moreover, we demonstrate that this property is experimentally separable from VRP-driven immunogen production, as soluble or particulate immunogens can be simply mixed with VRP expressing an irrelevant transgene, or no transgene at all, to induce a mucosal response. Therefore, VRP exploit an alternative pathway for mucosal immune induction that is distinct from the natural pathway and suggest important applications of VRP as mucosal and systemic adjuvants in protein subunit or whole inactivated prophylactic vaccines and in immunomodulatory therapies for chronic diseases.

MATERIALS AND METHODS

VEE Replicon Constructs. The construction and packaging of VRP have been described (15, 45). Briefly, monolayers of baby hamster kidney (BHK) 21 cells (American Type

Culture Collection, passages 55-65) were coelectroporated with three RNAs transcribed *in vitro*: one replicon RNA, and two defective helper RNAs that lack the viral-specific packaging signal and drive the expression of the viral structural genes. The replicon constructs used in this study were (*i*) replicons expressing GFP (GFP-VRP), (*ii*) replicons expressing the HA gene from mouse-adapted influenza virus A/PR/8/34 (HA-VRP), and (*iii*) replicons that lack a functional transgene downstream of the 26S promoter (null VRP). Null VRP contain the viral nonstructural genes, 14 nt of VEE sequence downstream of the 26 mRNA transcription start site, an inserted 43-nt-long multiple cloning site, and the 118-nt 3' UTR. All VRP were quantitated by infection of BHK cells followed by detection of infected cells by using either immunofluoresence (GFP-VRP), immunocytochemistry with antisera against HA for HA-VRP, or sera collected from animals immunized with null VRP for null VRP. Titers are expressed as IU. All replicon particles used in this study were packaged in the wild-type (V3000) envelope (45).

Animals and Immunizations. Seven- to 8-week-old female BALB/c mice (Charles River Laboratories) were immunized with a 10-μl volume containing various VRP and/or soluble antigens either in the rear footpad by using a Hamilton syringe and a 30-gauge needle or intranasally by using a micropipette. Grade V chicken egg albumin (OVA) was purchased from Sigma, CT was purchased from List Biological Laboratories (Campbell, CA), and CpG DNA (ODN 1826) was purchased from Invivogen (Montreal). Formalin-I-Flu (Charles River Laboratories) was dialyzed against PBS in a Slidalyzer cassette (Pierce) according to the manufacturer's guidelines before immunization. Diluent consisted of endotoxin-free, filter-sterilized PBS, except for the experiment described in Fig. 1, in which 110 mM Ca^{2+}, 50 mM

Mg^{2+}, and 0.1% (vol/vol) donor calf serum were included. All animals were primed and then boosted 4 weeks later, either in the footpad or intranasally.

Inactivation of VRP by UV Treatment. Null VRP preparations were diluted to a concentration of 10^6 units/ml, and 0.2-ml aliquots were placed in individual wells in a 48-well tissue culture plate. The plates were exposed to a UV lamp (Sun-Kraft, Chicago) at a distance of 5 cm for 20 min. This procedure ablated infectivity of two related alphaviruses (R. Shabman and M. Heise, personal communication). The effect of UV treatment was assessed by immunocytochemistry after infection of BHK cells. No VRP-infected cells were detectable *in vitro* after infection of baby hamster kidney cells with undiluted UV-VRP (data not shown).

Sample Collection. Animals were bled either from the tail vein or after cardiac puncture, and sera were analyzed by ELISA (see below). Preparation of fecal extracts was modified from Bradney *et al.* (7). Briefly, ≈100-150 mg (5-8 pellets) of fecal material was freshly isolated from individual animals and placed into fecal extract buffer [PBS containing10% (vol/vol) normal goat serum and 0.1% (vol/vol) Kathon CG/ICP (Supeleco)] and vortexed for 10-20 min until pellets were completely disrupted. Samples were then centrifuged at $12,000 \times g$ for 20 min and supernatants were transferred to new tubes and stored at -20°C. Although sampling variability may have been introduced by not directly weighing each sample, this variability was small relative to differences in immune responses between experimental groups and is considered in the statistical analysis comparing inoculation groups. Vaginal lavage was performed by washing the exterior vaginal opening with 0.07 ml of PBS 8–10

times. Wash fluid was stored at -20°C and centrifuged at 12,000 x g for 10 min before analysis.

Lymphoid Organ Cultures. Lymphoid cultures, originally developed by Cebra and colleagues (33), were modified from Coffin *et al.* (12). Briefly, spleen and nasal tissue (tissue remaining after removal of nasal-associated-lymphoid tissue) were dissected from immunized animals and placed into Eppendorf tubes containing 1 ml of wash buffer (Hanks' balanced salt solution containing 100 units/ml penicillin, 100 μg/ml streptomycin, 110 mM Ca^{2+}, 50 mM Mg^{2+}, and 15 mM Hepes) and washed three times by aspiration and resuspension. Nasal tissue from each individual animal was placed in a well of a 48-well tissue culture plate containing 300 μl of media [RPMI medium 1640 (GIBCO) containing 15 mM Hepes, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 mM L-glutamine (GIBCO), and 0.25 μg/ml amphotericin B]. Plates were incubated at 37°C for 7 days to allow antibody secretion from B cells localized in the given tissue. After incubation, supernatants were collected, clarified, and analyzed for the presence of antigen-specific antibodies by ELISA (see below).

ELISA. ELISAs for influenza- and OVA-specific antibodies were performed according to standard ELISA methods (45). Briefly, antigen solutions (either 250 ng/ml of influenza virus in carbonate buffer, or 1 mg/ml of OVA in PBS) were used to coat 96-well plates (Costar) overnight at 4°C. Antigen was removed, and blocking solution [PBS containing 5% milk for flu, or 1× Sigmablock (Sigma) for OVA] was added for 2 h for flu or overnight for OVA at room temperature. Blocking solution was removed, and plates were incubated at room temperature for 2 h (flu) or overnight (OVA) with 2-fold serial dilutions of samples. Plates

were washed and incubated for 1 h with horseradish peroxidase-conjugated secondary goat anti-mouse γ or α chain-specific antibodies (Southern Biotechnology Associates or Sigma). Plates were washed, and *O*-phenylenediamine dihydrochloride substrate was added for 30 min and then stopped with the addition of 0.1 M NaF. Antibody endpoint titers are reported as the reciprocal of the highest dilution that resulted in an $OD_{450} \geq 0.2$. In lymphoid culture supernatants, endpoint titers for flu-specific IgA are reported as the reciprocal of the highest dilution that results in an OD_{450} reading at least 2 SDs greater than values obtained from mock-vaccinated animals. Data are presented as the geometric mean ± SEM.

ASC ELISPOT. To evaluate the presence of OVA-specific ASCs, single-cell suspensions were prepared from both spleen and the nasal epithelium. Whole spleens were disrupted between frosted glass slides, and red blood cells were lysed either under hypoosmotic conditions or after addition of ammonium chloride buffer. Cells were washed and placed on a Lympholyte-M density gradient (Accurate Scientific, Westbury, NY). Banded cells were harvested, washed, and counted. For preparation of nasal lymphocytes, nasal tissue from the tip of the nose to just anterior of the eye sockets was harvested from immunized animals, and the upper palate, including the nasal-associated lymphoid tissue, was carefully removed. Nasal tissue was physically disrupted and incubated at 37°C for 2 h in a 50-ml Erlenmeyer flask in complete R-10 media [RPMI medium 1640 containing 10% (vol/vol) FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 mM Hepes] containing 2.5 mg/ml Collagenase A (Roche Applied Science), 17 μg/ml DNase I (Roche Applied Science), and glass beads. After digestion, cells were filtered through a 40-μm cell strainer (BD Falcon), washed, resuspended in 44% Percoll (Amersham Pharmacia), and layered on Lympholyte-M as described for spleen cells above. Banded cells were

harvested, washed, and counted. Cells were pooled from two animals, and typical yields were $\approx 2.5 \times 10^5$ to 1×10^6 cells per animal. ASC ELISPOT analysis was modified from previous reports (14, 53). Briefly, 96-well nitrocellulose membrane plates (Millipore) were incubated with 1 mg/ml OVA in PBS overnight at 4°C. Plates were then washed and blocked for 2 h with complete R-10 (10% serum). Two-fold dilutions of single-cell suspensions were then added to plates in duplicate in R-10 and incubated overnight. Plates were washed, and bound spots were detected by the addition of horseradiush peroxidase-conjugated secondary goat anti-mouse γ or α chain-specific antibodies (Southern Biotechnology Associates), followed by addition of 3-amino-9-ethylcarazole (Sigma), and enumerated with a computerized ELISPOT plate reader (Immunospot). Data are presented as the number of antigen-specific ASCs per 10^6 cells plated.

Statistical Analysis. Antibody titers and ASC numbers were evaluated for statistically significant differences by either the ANOVA or Mann–Whitney tests (INSTAT; GraphPad, San Diego). $P \leq 0.05$ was considered significant.

RESULTS

VRP Induce Mucosal Immune Responses. Previous reports have documented the ability of peripherally inoculated VRP to induce significant protection from virulent mucosal challenge with influenza virus in mice and chickens (45, 47), simian immunodeficiency virus in macaques (25), and equine arteritis virus in horses (3). Also, results obtained with intranasal influenza virus challenge of hemagglutinin (HA)-VRP-immunized mice showed significantly decreased influenza virus replication in the nasal epithelium, as determined by influenza-specific plaque assay and *in situ* hybridization. (N.L.D., K. Brown, E.M.B.R., A. West, and

R.E.J., unpublished work). Although VRP induced protection of the mucosal tissue, it was not directly determined whether local mucosal immune responses contributed to the observed protection. Typically, mucosal immunity is induced only when antigens are processed and presented across mucosal surfaces (36); however, VRP induced protection in these mucosal challenge models after immunization by a nonmucosal route.

We wanted to determine whether nonmucosal VRP delivery resulted in the induction of locally produced, mucosal immunity. Groups of female BALB/c mice were immunized in the rear footpad at weeks 0 and 4 with diluent, 10^5 infectious units (IU) of HA-VRP or 10 µg of formalin-inactivated influenza virus (I-Flu), as a non-VRP-vectored influenza antigen. Another group of animals was immunized in the rear footpad with 10 µg of I-Flu mixed with 10^5 IU of GFP-VRP, as an irrelevant VRP control. At various times after the second inoculation (days 3, 7, 10, 14, 18, 21, and 28), groups of three animals were killed, and the nasal mucosa were harvested for analysis in a lymphoid culture assay originally developed by Cebra and colleagues (33). Detection of flu-specific antibody in supernatant fluids from *ex vivo* nasal epithelium organ cultures was used as a measure of mucosal immune induction. Significant antibody production was not observed in supernatants from nasal epithelium until day 7 postboost and was detectable from day 7 to day 28 postboost. In comparing nasal antibody production across the range of time points, we found that VRP-containing inocula induced a statistically significant increase in flu-specific IgA antibodies in organ cultures from the nasal epithelium, compared with cultures from animals inoculated with I-Flu alone (HA-VRP compared with I-Flu, $P < 0.001$; GFP-VRP + I-Flu compared with I-Flu, $P < 0.001$, data not shown). Shown in Fig. 1 is the day-21 time point. All three antigen delivery methods were capable of stimulating local flu-specific IgG antibody production in nasal

mucosa as detected in the *ex vivo* supernatants, although VRP-induced responses were significantly increased compared with responses induced by delivery of I-Flu alone (Fig. 1*A*). HA-VRP and the delivery of I-Flu mixed with GFP-VRP, but not delivery of I-Flu alone, induced flu-specific, mucosal IgA antibodies (Fig. 1*B*). Also, VRP induced a statistically significant increase in flu-specific IgG and IgA antibodies present in nasal washes of immunized animals compared with inoculation of I-Flu alone (data not shown). These results indicate that (*i*) VRP are capable of inducing local, antigen-specific antibody production in mucosal tissues after nonmucosal delivery, (*ii*) mucosal immune induction is a property of VRP, as antigen alone fails to induce significant mucosal IgA responses, and (*iii*) VRP are capable of inducing mucosal immunity either when the immunogen is expressed by the VRP, or when the immunogen is simply mixed with an irrelevant VRP that appears to serve as an adjuvant.

The mucosal response observed in the nasal epithelium did not result from an inordinately high systemic response in the VRP-containing groups. The experimental system was designed such that the systemic IgG response induced in I-Flu-immunized animals, as measured by flu-specific IgG antibodies in *ex vivo* spleen cultures, was statistically equivalent to the systemic responses induced by VRP-containing inocula (Fig. 1*C*). Therefore, any differences in the mucosal responses could not simply be attributed to higher immune responses in general. However, HA-VRP and I-Flu mixed with GFP-VRP induced significantly greater levels of flu-specific, systemic IgA antibodies than I-Flu alone, as measured in spleen culture supernatant fluids (Fig. 1*D*). Preliminary results with analogous vectors based on Girdwood virus and A.R.86 virus, alphaviruses in the Sindbis group, also

suggest induction of mucosal immune responses (J.M.T., A.C.W., and M. Heise, unpublished work).

VRP Possess Systemic and Mucosal Adjuvant Activity. The results reported in Fig. 1 strongly suggest that VRP themselves, independent of the expressed gene, are capable of serving as both a systemic and mucosal adjuvant after nonmucosal delivery. To confirm this hypothesis, groups of eight animals were immunized in the rear footpad with 10^6 IU of VRP not expressing any transgene (null VRP) mixed with either 0.1 or 1.0 µg of I-Flu at weeks 0 and 4. Although null VRP do not express an inserted gene behind the 26S promoter, a short 175-nt noncoding mRNA is predicted from the sequence. Animals were bled 2 weeks postboost, and flu-specific serum IgG antibodies were analyzed by ELISA. As shown in Fig. 2, the presence of null VRP in the inoculum increased the flu-specific systemic antibody response by up to 44-fold (1.0 µg dose of I-Flu). To assess mucosal antibody responses, fecal extracts were prepared and analyzed for the presence of flu-specific mucosal antibodies by ELISA (Fig. 2 *B* and *C*). Antibodies present in fecal extracts are almost exclusively locally produced, with minimal contribution from serum-derived antibodies (38). Flu-specific fecal IgA antibodies were barely detectable after immunization with I-Flu alone; however, the inclusion of null VRP as an adjuvant augmented those responses by ≈60 fold (1.0-µg dose of I-Flu, IgA). These data confirm that VRP possess systemic and mucosal adjuvant activity for a particulate antigen.

To further characterize the adjuvant properties of VRP, the following experiments used null VRP and a soluble test antigen, ovalbumin (OVA), rather than a particulate antigen (I-Flu). Groups of six female BALB/c mice were immunized at weeks 0 and 4 with 10 µg of OVA, either alone or coinoculated with 10^6 IU of null VRP, by both parenteral (footpad) and

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mucosal (intranasal) delivery. As shown in Supplemental Fig. 2-1, both footpad and nasal delivery of OVA alone resulted in detectable OVA-specific serum IgG titers 3 weeks postboost. The coinoculation of null VRP with OVA increased OVA-specific serum IgG responses by ≈60- and 1,400-fold after footpad and nasal delivery, respectively. To assess mucosal antibody responses, fecal extracts were prepared from vaccinated animals before the booster inoculation and at weeks 1, 2, and 3 postboost, and analyzed for the presence of OVA-specific mucosal IgG and IgA antibodies by ELISA. Delivery of OVA alone failed to consistently induce detectable levels of OVA-specific fecal antibodies over background after either footpad or nasal immunization 3 weeks postboost. However, the inclusion of null VRP in the inoculum resulted in an ≈20- to 60-fold increase in OVA-specific fecal IgG and IgA antibody titers (Supplemental Fig. 2-1 *B* and *C*), regardless of the route of immunization. Taken together, the observations using I-Flu and OVA confirm the systemic and mucosal adjuvant activity of VRP after either mucosal or nonmucosal delivery of soluble or particulate immunogens.

VRP RNA Replication Is a Trigger for Adjuvant Activity/Immune Induction. The critical VRP-specific parameters that mediate adjuvant activity are currently undefined. Numerous molecular sensors are capable of recognizing viral products in virus-infected cells (48), including members of the toll-like receptor family (1, 23), and a number of IFN-inducible proteins (46, 54). We hypothesize that one or more of these pathways might be involved in recognizing RNA products produced after VRP infection and might play a critical role in VRP adjuvant activity. To test the hypothesis that VRP RNA replication is necessary for adjuvant activity, we treated null VRP with UV light before inoculation. UV treatment causes the formation of uridine dimers in the replicon RNA, which blocks both RNA

replication and translation of the input RNA, and allows evaluation of replication-defective VRP as molecular adjuvants.

Groups of six BALB/c mice were inoculated in the rear footpad at weeks 0 and 4 with 10 µg of OVA alone or 10 µg of OVA mixed with (*i*) 1.0 µg of cholera toxin (CT), a known systemic and mucosal adjuvant used here as a positive control (52), (*ii*) 10^4 IU of null VRP, (*iii*) 10^4 IU of null VRP treated with UV light (UV-VRP), or (*iv*) 10^6 IU of null VRP. At 1 week postboost, serum was harvested from immunized animals and analyzed for the presence of OVA-specific IgG antibodies by ELISA. OVA-specific serum IgG titers were increased by ≈64- and 114-fold after the codelivery of OVA plus 10^4 or 10^6 IU of VRP, respectively (Supplemental Table 2-1). In contrast, codelivery of OVA and 10^4 IU of UV-VRP failed to induce a statistically significant increase in OVA-specific serum IgG antibodies ($P > 0.05$). These results suggest that viral RNA replication was required for the immune stimulation observed with null VRP. Importantly, the adjuvant effect of VRP was comparable to responses induced by 1.0 µg of the control adjuvant, CT, under these conditions.

To quantitate the number of OVA-specific IgG- and IgA-secreting cells in spleen and nasal epithelium of the same animals, single-cell suspensions were prepared and analyzed in an antibody-secreting cell (ASC) enzyme-linked immunospot assay (ELISPOT). Increased levels of IgG (Fig. 3*A*) and IgA (Fig. 3*B*) ASCs were present in spleen and nasal epithelium in the OVA-plus-VRP inoculated animals, compared with the OVA-alone group, again demonstrating a clear systemic and mucosal VRP adjuvant activity leading to the local production of antigen-specific antibodies in both systemic and mucosal tissues. UV treatment of VRP before inoculation largely abrogated this effect, indicating the importance of VRP RNA function and also suggesting that contaminants potentially present in the VRP

preparations (such as LPS) were not responsible for the observed adjuvant activity. Again, VRP adjuvant activity as measured by ASC ELISPOT was comparable with that of CT. These results demonstrate that null VRP can act as a true mucosal adjuvant, and VRP RNA replication is likely the molecular trigger for the adjuvant activity.

VRP Adjuvant Activity as Compared with Adjuvant Activity of CpG DNA. We sought to determine how the VRP adjuvant compared with another known adjuvant, CpG DNA. Unmethylated CpG motifs found in bacterial genomes are recognized by the innate immune system through interactions with TLR9 and increase immunity to coimmunized antigens in numerous experimental systems [reviewed in ref. (28)]. To further characterize the relative strength of VRP adjuvant activity, groups of eight BALB/c mice were inoculated in the rear footpad at weeks 0 and 4 with 10 µg of OVA alone, 10 µg of OVA mixed with 10^5 IU of null VRP, or 10 µg of OVA mixed with 1.0 µg of CpG DNA. Two weeks after the second inoculation, sera, fecal extracts, and vaginal lavage samples were prepared from individual animals and analyzed for the presence of OVA-specific antibodies by ELISA. Also at 2 weeks postboost, single-cell suspensions were prepared from spleen and nasal epithelium and analyzed for OVA-specific ASCs by ASC ELISPOT. As shown in Fig. 4, both VRP and CpG augmented OVA-specific spleen IgG ASCs compared with OVA alone ($P < 0.001$ and $P < 0.05$, respectively). Although VRP adjuvanted systemic OVA responses to a greater extent than CpG, as measured by spleen ASC, measurement of OVA-specific serum IgG titers suggested that the CpG and VRP systemic adjuvant effects were comparable (Supplemental Table 2-2). However, VRP induced a significant adjuvant effect on mucosal IgA responses in fecal extracts and vaginal washes and in IgA ASCs in the nasal epithelium (Fig. 4 and Supplemental Table 2-2). By each of these assays, VRP-adjuvanted OVA responses in

mucosal tissues were superior to OVA plus CpG. These data suggest that the systemic adjuvant activity of VRP is at least as strong as that of CpG and that VRP possess significantly stronger mucosal adjuvant activity.

DISCUSSION

Alphavirus replicon vectors expressing pathogen-derived immunogens have been used extensively as vaccine delivery vehicles and have proven effective at inducing significant protection from challenge with a number of important pathogens in experimental and natural hosts. However, the mechanisms that govern immune induction after vector delivery remain largely unexplored.

We demonstrate in this article that VRP possess inherent immunostimulatory properties that are independent of protein production. Either irrelevant or null VRP, simply codelivered with soluble OVA protein or inactivated influenza virions, dramatically augmented antigen-specific antibody production in both the systemic and mucosal compartments, compared with inoculation of antigen alone. In work not presented here, VRP systemic and mucosal adjuvant activity also has been demonstrated with Norwalk virus-like particles (A. LoBue, J.M.T., R. Baric, and R.E.J., unpublished work), cowpox B5R protein (N. Thornburg, J.M.T., and R.E.J., unpublished work), and simian immunodeficiency virus gp120 (A. West, J.M.T., and R.E.J., unpublished work), suggesting that the VRP adjuvant functions without respect to the antigen. In the present study we have measured only short-term immunity with VRP adjuvants. However, VRP used as expression vectors elicited responses that endured throughout the lifetime of the animal. If we assume that the immunological parameters that govern VRP as expression vectors are the same as those that

govern immune induction with VRP as adjuvants, then it is likely that adjuvant-induced immunity will be equally long-lived.

We demonstrate the adjuvant property of alphavirus replicon particles for both systemic and mucosal immunity, even when administered by a nonmucosal route. A number of recent reports have identified other viral (5, 8, 24, 43) and bacterial (39) particles that possess various types of adjuvant activity when codelivered with antigen. We speculate that such activity is also likely to play an important role in immune induction under conditions in which such particles (including VRP) are engineered as vectors to express a given immunogen. Although those other reports document the ability of microbial particles to serve as adjuvants, no other system has demonstrated mucosal immune induction after nonmucosal delivery, as is observed with VRP. It will be of interest to determine whether other viruses are capable of augmenting mucosal antibody responses after nonmucosal delivery, or if this property is unique to VEE.

The natural pathway of mucosal immune induction relies on antigen processing and presentation at mucosal surfaces and results in the local production of IgA antibodies at those surfaces (29, 36). VRP were capable of immune induction via the natural pathway, as nasal delivery resulted in the induction of mucosal immunity. However, VRP were also capable of exploiting an alternative pathway that resulted in mucosal immunity after nonmucosal inoculation. Although there have been a limited number of examples where induction of mucosal immunity occurred after inoculation at a parenteral site [reviewed in refs. (6) and (50)] there is little consistency among the several examples, and none of them is analogous to the null VRP adjuvant activity described here (11, 13, 16, 18, 19, 21, 26, 37, 41, 42). Likewise, induction of mucosal immunity has been demonstrated with alphavirus expression

vectors, but only after immunization (4, 51) or boost (22) at a mucosal surface, and in none of these instances was the potential for mucosal adjuvant activity examined.

The mechanism by which VRP trigger mucosal immunity after nonmucosal delivery is undefined at present. One potential explanation is that either free VRP, or cells infected by VRP in the skin (34) or lymph node migrate to a traditional mucosal inductive site, such as Peyer's patches or mesenteric lymph node, and induce local antibody production (6). However, experiments using GFP-VRP have failed to consistently demonstrate VRP-infected cells in such tissues (E.M.B.R., J.M.T., and R.E.J., unpublished work). We favor the hypothesis that the lymph node draining the site of VRP inoculation develops at least some functions characteristic of a mucosal inductive site. In support of this idea, preliminary experiments demonstrate the production of antigen-specific, multimeric IgA in the draining lymph node (DLN) in response to inoculation of VRP (J.M.T. and R.E.J., unpublished work). It needs to be determined whether additional characteristics of a true mucosal inductive site are present in the DLN of VRP-inoculated mice. We feel that detailed examination of this alternative pathway for the induction of mucosal immunity in the VRP experimental system will contribute to a greater understanding of alphavirus-induced immunity, in particular, and mucosal immunity in general.

The molecular basis for the adjuvant activity likely resides in the ability of the VRP genome to replicate, given the sensitivity of adjuvant activity to UV inactivation. We suggest that an element present during virus replication is recognized in infected host cells and that this recognition initiates a cascade of events that ultimately leads to the induction of immunity to codelivered antigens. The most prominent candidates include viral RNA and/or replicative intermediates and their interactions with components of the innate immune

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system. A variety of cellular sentinel molecules exist, such as TLR3 (1), TLR7 (23), RIG-I, MDA-5 (54), protein kinase R, and RNaseL (46), which are capable of recognizing viral replicative molecules. In fact, a recent report (32) implicates RNaseL in immune induction to a tolerant melanoma antigen in an alphavirus replicon system.

Both transgene-expressing particles and particles lacking a transgene possess adjuvant activity, suggesting that adjuvant activity neither depends on, nor is inhibited by, the presence of a particular transgene protein. The VRP constructs lacking a transgene are predicted to express a short, noncoding RNA. It is unlikely that this truncated subgenomic RNA, or the presence or activity of the 26S promoter itself, is responsible for the observed adjuvant activity. Another formal possibility is that translation of the replicase proteins is responsible for the activity.

One potential trivial explanation for the adjuvant effect is that it is mediated by a contaminant present in VRP preparations (such as LPS). However, two observations strongly suggest that a contaminant is not the predominant mechanism of immune activation: (*i*) no adjuvant activity was observed after codelivery of identically treated media from a mock VRP preparation (data not shown), and (*ii*) UV treatment of VRP ablated adjuvant activity.

We have compared VRP adjuvant activity to that of CT and CpG DNA. Results from such comparisons suggest that systemic responses induced by VRP are at least equivalent to that of both CT and CpG DNA. Moreover, after nonmucosal delivery VRP mucosal adjuvant activity appears to be comparable to that of CT and superior to CpG DNA. A number of important questions regarding VRP adjuvant activity remain to be answered, such as how VRP-induced systemic and mucosal immune responses compare with those of other peripherally delivered adjuvants, such as alum, and mucosally delivered CT and whether

VRP act as a systemic and mucosal T cell adjuvant. These additional comparisons will allow more accurate evaluations of the relative efficiency of VRP-induced immune stimulation.

In summary, we have demonstrated two activities of alphavirus-derived viral vectors: (*i*) induction of local mucosal immune responses after inoculation at a remote, nonmucosal site and (*ii*) systemic and mucosal adjuvant activity with codelivered soluble and particulate immunogens. We feel that these observations and the continued experimentation they stimulate will advance a search for adjuvant activity among other viruses and viral vectors, will ultimately define the specific components of an alternative pathway for the induction of mucosal immunity, and if the activity is evident in humans, will enable new possibilities for safe and inexpensive subunit and inactivated vaccines.

ACKNOWLEDGEMENTS

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Figure 2-1
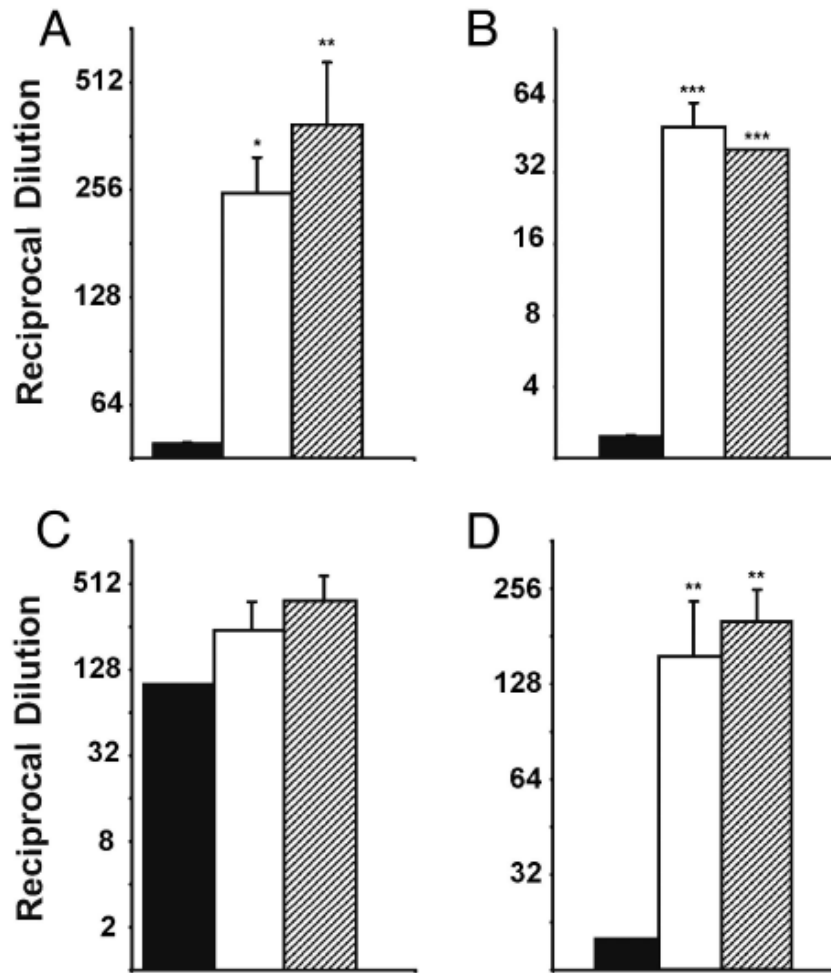
Fig. 2-1. VRP induce mucosal immune responses. Groups of animals were immunized in the rear footpad with diluent, 10 µg of I-Flu (solid bars), 10^5 IU of HA-VRP (open bars), or 10 µg of I-Flu plus 10^5 IU of GFP-VRP (hatched bars) at weeks 0 and 4. Three weeks after the second inoculation, lymphoid organ cultures were established from the nasal epithelium (*A* and *B*) and spleen (*C* and *D*). Culture supernatants were evaluated for flu-specific IgG (*A* and *C*) and IgA antibodies (*B* and *D*) by ELISA. Data are presented as the geometric mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with I-Flu alone, as determined by ANOVA.

Figure 2-2
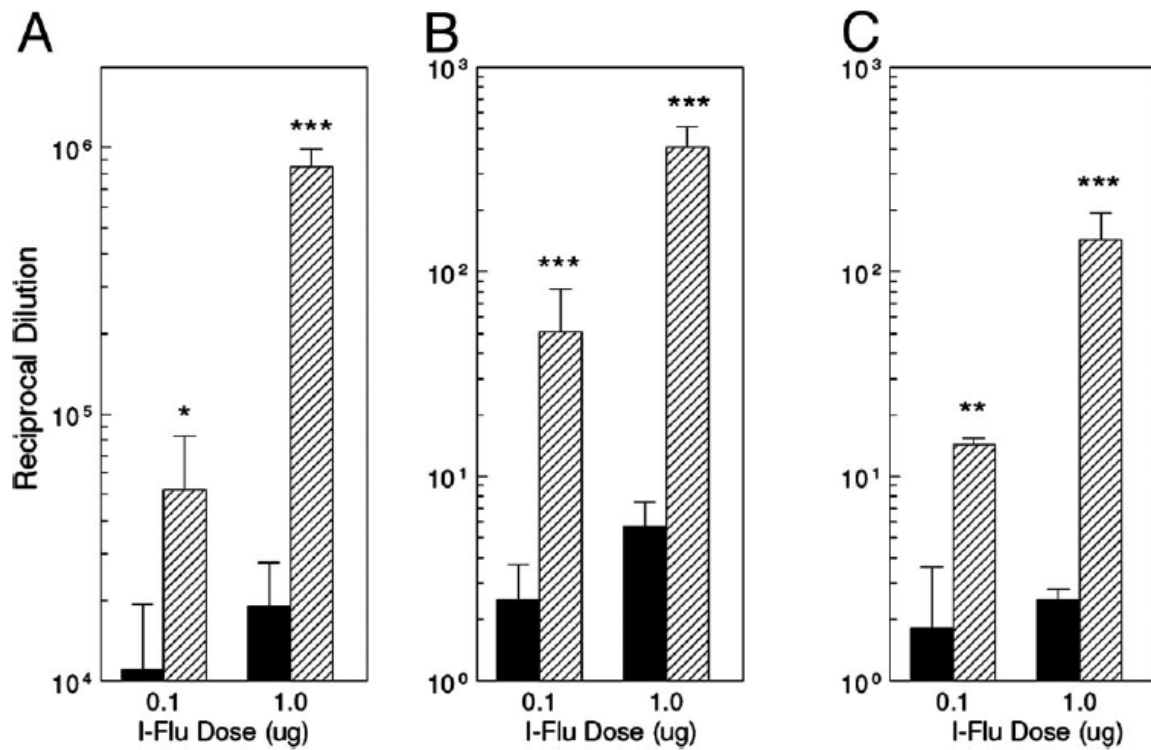
Fig. 2-2. VRP adjuvant activity for particulate antigens. Groups of eight animals were immunized in the rear footpad with 0.1 or 1.0 µg of I-Flu in the presence (hatched bars) or absence (solid bars) of 10^6 IU of null VRP at weeks 0 and 4. Two weeks after the second inoculation, flu-specific IgG antibodies were measured in sera (*A*) and fecal extracts (*B*), and flu-specific IgA antibodies were measured in fecal extracts (*C*) by ELISA. Data are presented as the geometric mean ± SEM. *, $P < 0.02$; **, $P < 0.005$; ***, $P < 0.0003$ compared with I-Flu alone, as determined by Mann–Whitney.

Figure 2-3
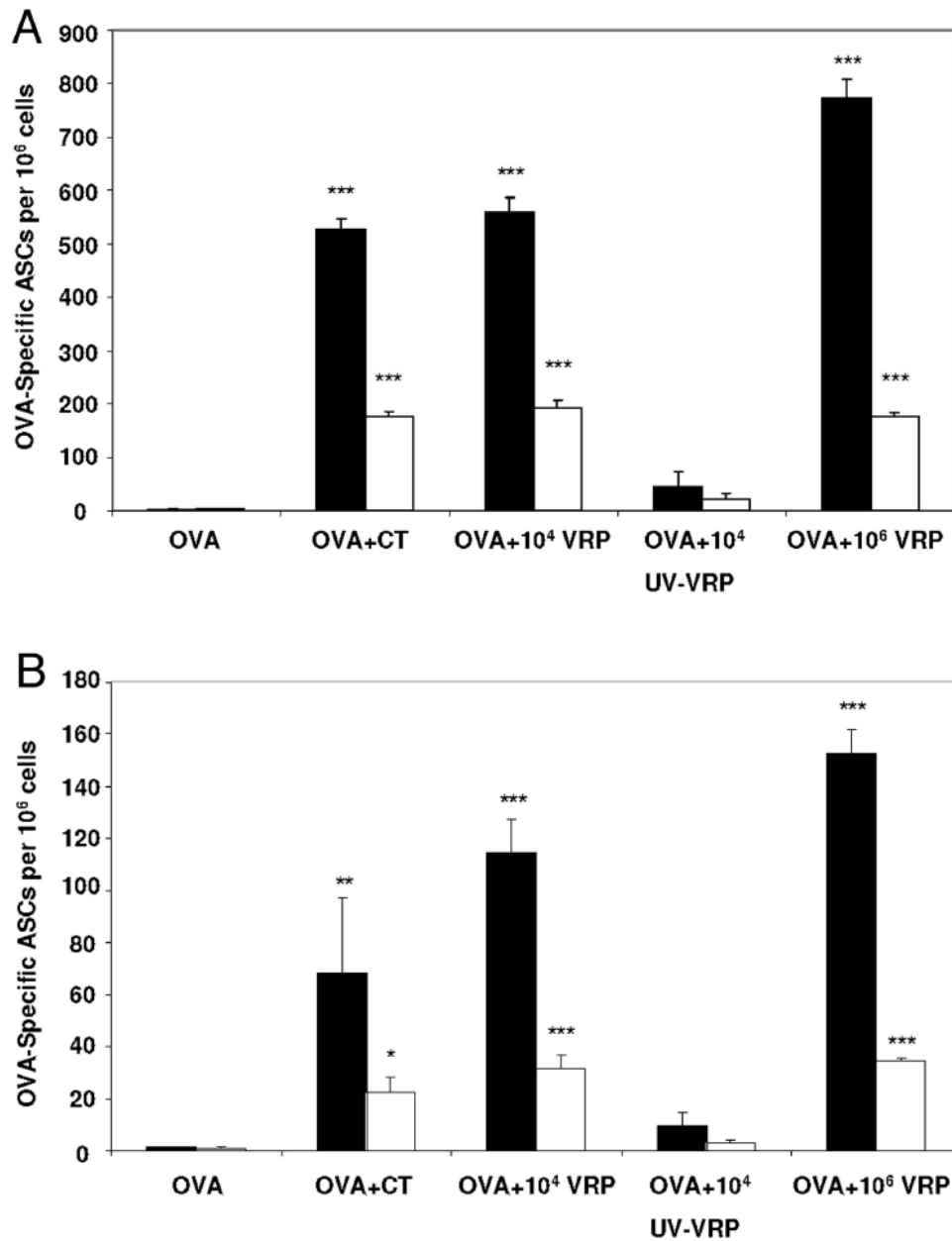
Fig. 2-3. **Systemic and mucosal adjuvant activity of UV-treated VRP.** Groups of six animals were immunized in the rear footpad with 10 µg of OVA alone or coinoculated with 1.0 µg of CT, 10^4 IU of null VRP, 10^4 IU of UV-VRP, or 10^6 IU of null VRP at weeks 0 and 4. One week after the second inoculation, splenocytes (open bars) and nasal lymphocytes (solid bars) were isolated from immunized animals and analyzed for the presence of OVA-specific IgG-secreting cells (*A*) and IgA-secreting cells (*B*) by ELISPOT. Data are presented as the geometric mean ± SEM. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ compared with OVA alone, as determined by ANOVA.

Figure 2-4

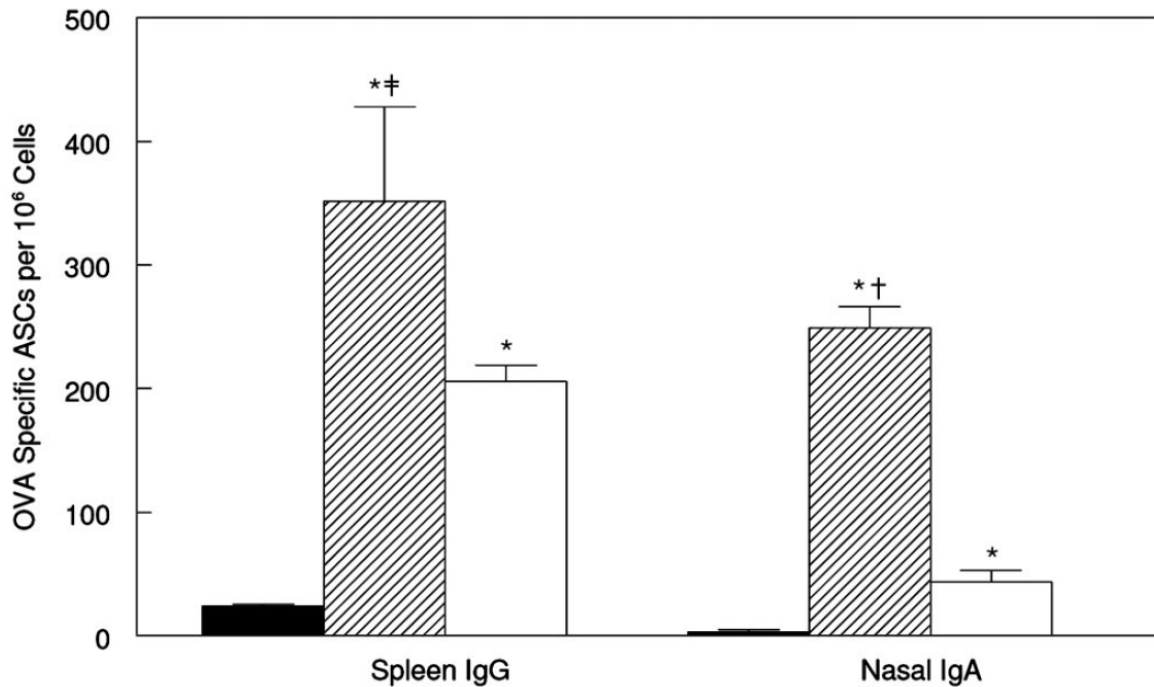**Fig. 2-4. Systemic and mucosal adjuvant activity of VRP compared with CpG DNA.** Groups of eight animals were immunized in the rear footpad with 10 µg of OVA alone (solid bars) or coinoculated with 10^5 IU of null VRP (hatched bars) or 1.0 µg of CpG DNA (open bars) at weeks 0 and 4. Two weeks after the second inoculation, splenocytes were isolated and analyzed for the presence of OVA-specific IgG ASCs, and nasal lymphocytes were isolated and analyzed for the presence of OVA-specific IgA ASCs by ELISPOT. Data are presented as the geometric mean ± SEM. ∗, $P < 0.001$ compared with OVA alone; †, $P < 0.01$ compared with CpG; ‡, $P < 0.05$ compared with CpG.

Supplemental Figure 2-1
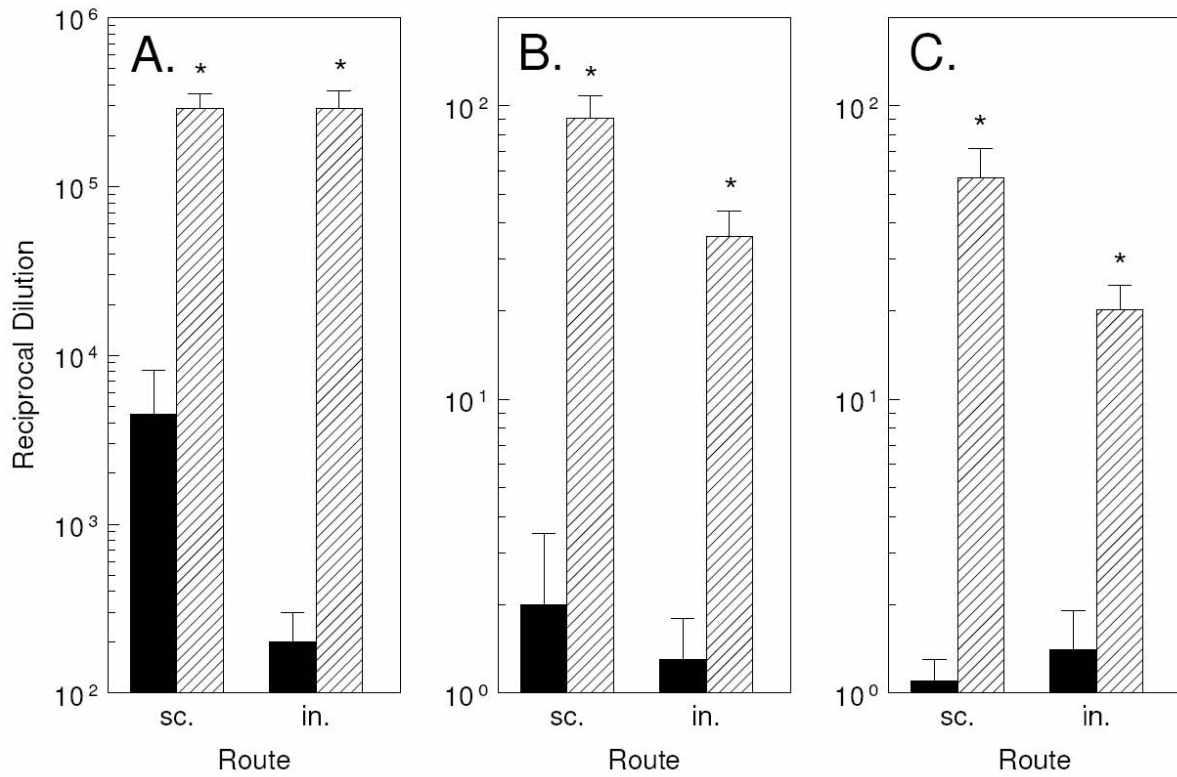
Supplemental Fig. 2-1. VRP adjuvant activity for soluble antigens. Groups of six animals were immunized in the rear footpad or intranasally with 10 μg of OVA in the presence (hatched bars) or absence (solid bars) of 10^6 IU of null VRP at weeks 0 and 4. Three weeks after the second inoculation, OVA-specific IgG antibodies were measured in sera (*A*) and fecal extracts (*B*), and OVA-specific IgA antibodies were measured in fecal extracts (*C*) by ELISA. Data are presented as the geometric mean ± SEM. *, *P* < 0.003 compared with OVA alone, as determined by Mann-Whitney.

Supplemental Table 2-1.

|		Serum
Antigen	Adjuvant	IgG ELISA (x 10^4)
OVA		1.3 (+/-0.2)
OVA	CT	72.4 (+/-26.2)*
OVA	VRP (106)	144.8 (+/-24.1)**
OVA	VRP (104)	81.3 (+/-10.8)*
OVA	UV-VRP (104)	4.0 (+/-3.9)

Systemic adjuvant activity of UV-treated VRP. Groups of six animals were immunized in the rear footpad with 10 µg of OVA alone or coinoculated with 1.0 µg of CT, 10^4 IU of null VRP, 10^4 IU of UV-VRP, or 10^6 IU of null VRP at weeks 0 and 4. One week after the second inoculation, sera were analyzed for OVA-specific IgG antibodies by ELISA. Data are presented as the geometric mean +/- SEM. *, $P < 0.05$; **, $P < 0.001$ compared with OVA alone, as determined by ANOVA.

Supplemental Table 2-2.

		Systemic IgG	Mucosal IgA	
		Serum	Fecal	Vaginal
Antigen	Adjuvant	IgG ELISA (x10^5)	IgA ELISA	IgA ELISA
OVA	–	1.4 (+/-0.3)	1.3 (+/-0.2)	5.2 (+/-7.6)
OVA	VRP	37.6 (+/-6.7)*	117.4 (+/-70.8)*[†]	332.0 (+/-134.5)*[‡]
OVA	CpG	41.0 (+/-0.0)*	10.4 (+/-7.0) *	90.5 (+/-42.3) *

Systemic and mucosal adjuvant activity of VRP compared with CpG DNA. Groups of eight animals were immunized in the rear footpad with 10 µg of OVA alone or coinoculated with 10^5 IU of null VRP or 1.0 µg of CpG DNA at weeks 0 and 4. Two weeks after the second inoculation, sera, fecal extracts, and vaginal wash samples were prepared and analyzed for OVA-specific antibodies by ELISA. Data are presented as the geometric mean +/- SEM.

CHAPTER THREE

INDUCTION OF A MUCOSAL INDUCTIVE ENVIRONMENT IN THE PERIPHERAL DRAINING LYMPH NODE FOLLOWING NONMUCOSAL VACCINATION

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ABSTRACT

The strongest mucosal immune responses are induced following mucosal antigen delivery and antigen processing in the lymphoid tissues of the mucosal immune system. Much is known regarding the immunological parameters which regulate immune induction via this pathway. Recently, a number of experimental systems have been identified in which mucosal immune responses are induced following nonmucosal antigen delivery. One such system, footpad delivery of Venezuelan equine encephalitis virus replicon particles (VRP) led to the production of IgA antibodies directed against both expressed and co-delivered antigens at multiple mucosal surfaces in mice. In contrast to the mucosal delivery pathway, little is known regarding the lymphoid structures and immunological components which are responsible for mucosal immune induction following nonmucosal delivery. Here we have utilized peripheral VRP delivery to probe the inner workings of this alternative pathway for mucosal immune induction. Following nonmucosal VRP delivery, IgA antibodies, and polymeric or mucosal forms of IgA antibodies, were first detected in the peripheral draining lymph node (DLN), prior to IgA detection at the mucosal surfaces. Further analysis of the DLN revealed upregulated expression of the $\alpha_4\beta_7$ integrin on DLN B cells, expression of MAdCAM-1, the ligand for the $\alpha_4\beta_7$ integrin, and production of IL-6 and CC chemokines, all characteristics of mucosal lymphoid tissues. Taken together, these results implicate the peripheral DLN as an integral component of an alternative pathway for mucosal immune induction. An understanding of the critical immunological and viral components of this pathway may significantly improve both our knowledge of viral-induced immunity and the efficacy of viral-based vaccines.

INTRODUCTION

The mammalian immune system has evolved two minimally overlapping arms: the systemic immune system which relies upon lymphoid cells and structures in the skin, spleen, bone marrow and peripheral lymph nodes for immune induction; and the mucosal immune system with its coupled mucosa-associated lymphoid tissue (MALT) responsible for immune activation at the mucosal level (8, 10, 23, 30). This distinctive segregation allows focused immune induction in the compartment in which the relevant antigen predominates. As the vast majority of harmful pathogens rely on penetration of a mucosal barrier as an integral step in the initiation of infection, innate and adaptive immune mechanisms which uphold the integrity of the mucosal surface are paramount for mediating protection from invading microbes (48). To date, vaccination has proven to be one of the most effective strategies of prophylactic immunomodulation against mucosal pathogens; however, mucosal infections with agents such as human immunodeficiency and highly virulent influenza viruses still pose a significant threat to human health. Therefore, the development of vaccine regimens capable of stimulating protective mucosal immunity would represent a powerful opportunity to intercede in the disease course of many infectious organisms (57).

It is well established in the literature that the strongest mucosal immune responses are induced following mucosal antigen delivery and antigen processing in the lymphoid tissues of the mucosal immune system (6, 48, 57). This natural pathway of mucosal immune induction following mucosal antigen delivery in the presence of immunomodulatory agents, such as the enterotoxins from various gram negative bacteria, has been studied extensively (1, 12, 48, 57, 65). Following nasal or oral delivery, antigens are captured by specialized epithelial cells termed M cells, or microfold cells of the follicle associated epithelium (FAE)

(25, 35). The FAE envelopes the lymphoid cell component of the mucosal inductive sites such as the Peyer's patches (PPs) in the gut. PPs contain dendritic cells (DCs), macrophages, B lymphocytes, T lymphocytes, and endothelial cells which all work in concert to chaperone luminal antigens from harmful organisms into an immunostimulatory environment, culminating in the activation of antigen-specific B cells and T cells which mediate protection of the mucosal surface (38, 39, 56).

A cascade of events is initiated following lymphocyte activation in mucosal inductive tissues in the presence of appropriate inflammatory signals (49). As a consequence of B cell activation in mucosa-draining-lymphoid tissues, activated B cells migrate from the PPs through the mesenteric lymph node and ultimately enter systemic circulation via the thoracic duct. These B cells differentiate along an activation pathway as a result of priming signals received in the mucosal lymphoid tissues, and upregulate a group of surface molecules which direct their migration back to the mucosal surface from which they were originally activated (7, 8). One of the most important mediators of mucosal homing is the $\alpha_4\beta_7$ integrin, also termed the mucosal homing receptor (9). This heterodimeric integrin pair is upregulated on mucosally-activated B and T cells and binds to its ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is expressed on the high endothelial venules (HEVs) of mucosal lymphoid tissues (2, 9). MAdCAM-1 expression is a discriminating characteristic of mucosal lymphoid tissues and is abundant on the HEVs of PPs and MLN. In contrast, very little MAdCAM-1 is detectable in systemic lymphoid structures such as the spleen and peripheral lymph nodes. Instead, the HEVs of systemic lymphoid tissue express the peripheral lymph node addressin (PNAd), which binds to CD62L and mediates migration of lymphocytes throughout the systemic lymphoid tissues (7). Additionally, chemokine

receptors CCR9 and CCR10 mediate lymphocyte migration into the mucosal compartment following migration towards mucosal chemokines TECK and MEC respectively (29, 40, 41).

Following integrin/chemokine-mediated migration back to the mucosa lamina propria, mucosally-activated B cells undergo further differentiation and activation in the mucosal environment directed by numerous cytokines and growth factors (31). The mucosal inflammatory milieu promotes the activation of antigen-specific B cells and orchestrates antibody isotype class switch towards the IgG and IgA isotypes (47). IgA is the most prominent antibody isotype present at mucosal surfaces (52). In fact, in humans more IgA is produced in the intestinal tract each day than all other isotypes in the body combined (15). Mucosal IgA antibodies exist as dimeric or polymeric forms by inclusion of the joining chain, or J chain protein during antibody secretion from mucosally-activated B cells (32). J chain-containing IgA molecules are transported onto mucosal surfaces following a specific interaction with the poly immunoglobulin receptor (pIgR) expressed on the basolateral surface of mucosal epithelial cells (32). Following transport through mucosal epithelial cells, dimeric/polymeric IgA is released into/onto the mucosal surface and retains a portion of the pIgR. This complex is subsequently termed secretory IgA (33).

As mentioned above, mucosal antigen delivery is the most efficient method of inducing local mucosal antibody responses; however, a growing body of evidence supports the existence of an additional pathway capable of stimulating mucosal antibody synthesis following nonmucosal antigen delivery [reviewed in (5, 71)]. The examples are varied and a common unifying mechanism responsible for mucosal immune induction under all circumstances is not readily evident upon initial review. Peripheral, or nonmucosal delivery of a diverse class of viruses or viral vaccine vectors including rotavirus (13, 14), canarypox

vectors (55), Venezuelan equine encephalitis virus (11) and replicon particles (28, 45, 70), and Rubella virus (58) potentate either mucosal antibody or T cell activation. Likewise, nonmucosal delivery of bacterial antigens/vectors derived from Listeria *monocytogenes* (59) and Haemophilus *influenzae* type b (36) promote mucosal immunity, suggesting that both viral and bacterial particles are capable of stimulating this pathway.

While traditional nonmucosal delivery methods are clearly capable of stimulating mucosal immunity, several specialized nonmucosal delivery approaches have been developed which likewise promote mucosal immune induction. One example is parenteral targeting of mucosal lymphoid tissues using anti-MAdCAM-1 antibodies, which dramatically augment mucosal antibody responses (50). In an alternative strategy, antigens are delivered through the skin in the presence of known mucosal adjuvants in a technique termed transcutaneous immunization (26). This technique results in significantly increased mucosal antibody responses both in mice and in humans (27, 72). Interestingly, an approach termed targeted lymph node immunization, which relies on direct antigen inoculation into a peripheral lymph node, also stimulates mucosal immunity in non-human primates (37, 42).

At this point it is unclear whether mucosal immune induction following either traditional nonmucosal delivery or following one of the specialized approaches outlined above of is in fact due to an intrinsic signal provided by the viral/bacterial particle itself or instead is due to the induction of a specific cellular factor or group of factors following. A number of candidate immunomodulatory factors have been identified including various forms of vitamin D3 (22) as well as a group of specific cytokines and chemokines (19) which potentially provide a mechanistic explanation for mucosal immune induction following nonmucosal antigen delivery.

The identification of the components of a peripheral mucosal immune induction pathway will provide valuable insights into the regulatory networks involved in mucosal immune induction, as well as the potential to significantly improve mucosal vaccines. We have recently demonstrated the ability of modified viruses derived from the alphavirus, Venezuelan equine encephalitis virus (VEE), to promote IgG and IgA antibody synthesis at multiple mucosal surfaces (28, 45, 70). The particles utilized in these studies, termed VEE replicon particles (VRP), express only the viral non-structural, or replicase components responsible for replication of the genomic RNA (60). VRP efficiently infect dendritic cells (DCs) following footpad inoculation in mice and replicate the viral genome to high levels (46). However, VRP fail to propagate beyond the first infected cell, as progeny virions are not produced following infection (24). In this study, we have utilized nonmucosal delivery of VRP as a model system to dissect the individual components of the peripheral mucosal immune induction pathway. Here we demonstrate several markers of mucosal lymphoid tissues including antigen-specific multimeric IgA antibodies, increased levels of mucosal cytokines such as IL-6 and TNF-α, a population of B cells with upregulated mucosal homing receptor expression, and expression of MAdCAM-1 on the HEVs of the DLN of VRP-inoculated animals. These observations are consistent with a model in which, following nonmucosal VRP delivery, the DLN is converted into the functional equivalent of a mucosal inductive site and serves as a component of an alternative pathway for mucosal immune induction. These studies provide a framework for the identification of the critical components of the alternative pathway for mucosal immune induction and the potential to improve mucosal vaccination strategies.

MATERIALS AND METHODS

VEE replicon constructs. The construction and packaging of VRP was performed as previously described (16, 60). Briefly, confluent monolayers of BHK-21 cells were co-electroporated with three separate *in*-vitro-transcribed RNAs, the replicon RNA, and two defective helper RNAs which drive the expression of the viral structural genes *in trans*. Only the replicon RNA is packaged into VRP as the helper RNAs lack the viral packaging signal. In this study, three different replicon constructs were utilized: 1) replicons expressing GFP (GFP-VRP); 2) replicons expressing the HA gene from influenza virus (HA-VRP); and 3) replicons which lack a functional transgene downstream of the 26S promoter (null VRP) (70). Null VRP contain the viral nonstructural genes, a 14 nt stretch of VEE sequence downstream of the 26S mRNA transcription start site, a heterologous 43-nt long cassette containing multiple restriction sites for cloning into the replicon backbone, and the 118-nt 3' UTR. HA-VRP and null VRP were quantitated by immunocytochemistry of infected BHK cells with anti-sera against HA (60) and null VRP (70), respectively. GFP-VRP were quantitated by immunofluoresence of infected BHK cells. All replicon particles utilized in this study were packaged in the wild-type (V3000) envelope.

Animals and immunizations. Seven-to-10-week-old female BALB/c mice were immunized in a 0.01 ml volume in the rear footpad/s as previously described (70). Briefly, animals were immunized at week 0 and week 4 with antigen alone or antigen co-inoculated with either VRP or CpG DNA as an adjuvant.   Chicken egg albumin (OVA) was purchased from Sigma; inactivated influenza virus (I-Flu) was purchased from Charles River Laboratories and was dialyzed against PBS in a Slidalyzer cassette (Pierce) according to manufacturer's guidelines prior to use. CpG DNA (ODN 1826) was purchased from Invivogen. Diluent

consisted of low endotoxin, filter-sterilized PBS, except for the lymphoid organ culture experiments (see below), in which 110 mM Ca^{2+}, 50 mM Mg^{2+}, and 0.1% (vol/vol) donor calf serum were included.

Lymphoid Organ Cultures. Lymphoid cultures were prepared as previously described (45, 70). Briefly, spleen, nasal tissue, and draining popliteal lymph nodes were harvested from immunized animals and placed in Eppendorf tubes containing 1 ml of wash buffer (Hanks' balanced salt solution containing 100 units/ml penicillin, 100 μg/ml streptomycin, 110 mM Ca^{2+}, 50 mM Mg^{2+}, and 15 mM Hepes) and washed three times by aspiration and resuspension. Spleen and nasal tissue were placed in individual wells of a 48-well tissue culture plate in 0.3 mls of media [RPMI medium 1640 (GIBCO) containing 15 mM Hepes, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 mM L-glutamine (GIBCO), and 0.25 μg/ml amphotericin B], and DLNs were placed in individual wells in a 96-well tissue culture plate in 0.1 mls of media. Plates were incubated at 37ºC for 7 days to allow antibody secretion from tissue-resident B cells into the supernatant. Following incubation, supernatants were collected, clarified by centrifugation at 4ºC, and analyzed for the presence of antigen-specific antibodies by ELISA and/or large molecular weight IgA antibodies by western blot. A limited set of tissue samples from an additional timepoint (day 21 post-boost) was previously published in Thompson et al (70) (figure 1) as evidence of VRP-induced mucosal immune induction.

Preparation of DLN extracts. Draining popliteal lymph nodes were dissected from immunized animals, and each lymph node was placed in a 1.5 ml tube (Kontes) with 0.1 mls of PBS containing protease inhibitors (PIs) [complete mini protease inhibitor cocktail tablet (Roche)]. DLNs were physically homogenized with a plastic pestle (Kontes) with the aid of

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a hand-held motor and were frozen at -20ºC. Following thaw, debris was pelleted by centrifugation at 4ºC, and supernatants were analyzed for IgA antibodies by ELISA, or for cytokine production by Beadlyte multiplex LUMINEX custom analysis performed by Millipore/Upstate (see below). Extracts were compared from individual lymph nodes across the various immunization groups. As a control, lymph node extracts were prepared from individual Peyer's patches (PPs) exactly as described for the popliteal lymph nodes (homogenized in 0.1 mls of PBS).

Sera, Fecal Extracts, and Vaginal Washes. All sample collection was performed as previously described (70). Blood was harvested from either the tail vein, following cardiac puncture, or from the submandimular plexus from individual animals, and sera were collected following centrifugation in microtainer serum separator tubes (Becton Dickinson). For fecal extracts, fresh fecal pellets (5-8, ≈ 100-150 mg) were isolated from individual animals and placed in a 1.5 ml Eppendorf tube containing 1 ml of fecal extract buffer [PBS containing10% (vol/vol) normal goat serum and 0.1% (vol/vol) Kathon CG/ICP (Supeleco)]. Samples were vortexed for at least 10 mins until all pellets were disrupted into a homogenous mixture. Samples clarified by centrifugation at 4ºC, and supernatants were transferred to fresh tubes and stored at -20 ºC prior to analysis by ELISA assay (see below). Vaginal washes were performed by lavage of the exterior vaginal opening with 0.07 mls of PBS 8-10 times. Lavage samples were stored at -20 ºC and clarified at 4ºC prior to ELISA analysis (see below).

Flow Cytometric Analysis. DLNs were harvested from immunized animals, and the overall mass of the lymph nodes was determined by weighing individual lymph nodes on an analytical balance (Mettler). Each lymph node was next disrupted with a razor blade and a

hemostat, and single cell suspensions were created by agitating each lymph node in complete RPMI media [RPMI medium 1640 containing 10% (vol/vol) FBS, 2 mM L-glutamine, 50 μg/ml gentamicin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 mM Hepes] containing 2.5 mg/ml Collagenase A (Roche Applied Science), 17 μg/ml DNase I (Roche Applied Science) for 30 minutes at 37ºC. Single cell suspensions were then stained with antibodies directed against CD3, CD19, CD45 (B220), CD11c, CD11b (all purchased from ebioscience) as well as $\alpha_4\beta_7$ integrin (LPAM-1, clone DATK32) purchased from BD Pharmingen and examined on a Becton Dickinson Facscaliber Flow cytometer. Stained cells were analyzed using Cellquest software.

Enzyme Linked Immunosorbant Assay (ELISA). ELISAs for influenza- and OVA-specific antibodies were performed on serum, fecal extracts, vaginal washes, and lymphoid culture supernatants as previously described (70).   Briefly, antigen solutions (either 250 ng/ml of influenza virus in carbonate buffer, or 1 mg/ml of OVA in PBS) were incubated in 96-well plates (NUNC Immulon 4) overnight at 4°C to allow antigens to bind to the plate. Excess antigen was removed, and blocking solution [PBS containing 5% milk for flu, or 1× Sigmablock (Sigma) for OVA] was added for 2 h for flu or overnight for OVA at room temperature. Following removal of blocking solution, plates were incubated at room temperature for 2 h (flu) or overnight (OVA) with serial dilutions of individual samples diluted in the appropriate blocking buffer. Plates were washed with a multi-channel plate washer (NUNC) and incubated for 1 h with horseradish peroxidase-conjugated secondary goat anti-mouse γ or α chain-specific antibodies (Southern Biotechnology Associates or Sigma). Finally, plates were again washed, *O*-phenylenediamine dihydrochloride substrate was added for 30 min, and the reaction was stopped with the addition of 0.1 M NaF.

Antibody endpoint titers are reported as the reciprocal of the highest dilution that resulted in an $OD_{450} \geq 0.2$. In lymphoid culture supernatants, endpoint titers for flu-specific IgA are reported as the reciprocal of the highest dilution that results in an OD_{450} reading at least 2 SDs greater than values obtained from mock-vaccinated animals (figure 1). Data are presented as the geometric mean ± standard error of the mean (SEM).

Analysis of Polymeric IgA. Lymph node (popliteal and PP) extracts were assayed for the presence of polymeric IgA antibodies by non-reducing western blot analysis. Proteins were separated in Laemmli buffer in the absence of reducing agent (no β-ME) by 6% SDS-PAGE and transferred to polyvinlyidene difluoride membrane (Bio-Rad) in transfer buffer (48mM Tris, 39mM Glycine, 10% Methanol) at 12V for 1 hour. Membranes were subsequently blocked in PBS with 5% dry milk, 0.1% Tween-20 (Sigma) at room temperature overnight. Blocked membranes were next washed in PBS with 1% dry milk, 0.1% Tween-20 and incubated with a goat anti-IgA antibody (Sigma and/or Southern Biotechnology Associates) at room temperature for 2 hours. Membranes were again washed and then incubated with a rabbit anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) at room temperature for 1 hour. Membranes were washed again, and HRP-conjugated antibodies were detected via chemiluminesence with ECL detection reagents (Amersham Pharmacia). For analysis of influenza-specific, polymeric IgA, lymphoid culture supernatants or lymph node extracts were mixed with either influenza virus or an irrelevant virus (Girdwood virus) for 2 hours at 4ºC, and virus-antibody complexes were centrifuged at 100,000 x g for 1 hour through a sucrose cushion. Pelleted virus-antibody complexes were resuspended in non-reducing sample buffer and analyzed by IgA western blotting as described above.

Statistical Analysis. Antibody titers and cytokine values were evaluated for statistically significant differences by either the ANOVA or Mann-Whitney test (GraphPad INSTAT). A *P* value of ≤0.05 was considered significant.

Immunofluoresent staining of DLNs. To examine the addressin profile present in the DLN of immunized animals, DLNs were harvested, snap frozen in liquid nitrogen and sectioned. Frozen sections were stained with antibodies against PNAd and MAdCAM-1 as previously described (67, 73) and analyzed by confocal microscopy (Zeiss LSM510; Oberkochen, Germany).

Cytokine/Chemokine analysis PBS homogenates of lymph nodes (see above) were analyzed for the presence of IL-1β, TNF-α, IL-5, IL-6, IFN-γ, RANTES, GM-CSF, MIP-1 β, TGF-β1, TGF-β2, and TGF-β3 on a Luminex machine. Samples were analyzed by the Upstate/Millipore Custom Multiplex cytokine analysis service. DLN samples were diluted 1:10 in PBS plus PIs (see above) and were analyzed by Upstate. At least 4 individual lymph nodes for each inoculaum and timepoint were analyzed. For statistical purposes, any analyte with a value below the assay limit of detection (LOD) was assigned a value of the LOD minus 1 pg/ml.

RESULTS

The DLN is an early site of IgA production following VRP infection. In this study we have utilized nonmucosal delivery of VRP to probe the cellular components and molecular basis of an alternative pathway for mucosal immune induction. IgA antibodies are the most prominent antibodies present at mucosal surfaces in both small rodents and humans, and

represent a critical component of the mucosal immune system (52). Therefore, the kinetics and anatomical localization of IgA production following VRP delivery were determined.

Groups of female BALB/c mice were immunized in a rear footpad at week 0 and week 4 with diluent, 1×10^5 infectious units (IU) of HA-VRP, or 10 μg of formalin-inactivated-influenza virus (I-Flu) which serves as a non-VRP-vectored antigen control. In order to evaluate the ability of VRP to induce mucosal immunity to a co-delivered antigen, an additional group of animals received 10 μg of I-Flu mixed with 1×10^5 IU of VRP expressing green fluorescent protein (GFP) as an adjuvant (GFP-VRP). Groups of 3 animals were sacrificed on days 3, 7, 14, and 28 post-boost, and lymphoid organ cultures (45, 70) were established from the spleen, as a characteristic systemic lymphoid tissue, the nasal epithelium, as a characteristic mucosal surface, as well as the draining popliteal lymph node, as a candidate component of an alternative mucosal immune induction pathway. Organ culture supernatants were evaluated for the presence of influenza (flu)-specific IgG and IgA antibodies by ELISA. In general, the appearance of flu-specific IgG and IgA antibodies in a given tissue were similar in terms of kinetics of induction (Fig 3-1). The dose of I-Flu utilized in this study (10 μg) was chosen so as to induce an antigen-specific systemic IgG response similar to that of HA-VRP, allowing assessment of the role of VRP in IgA production under conditions of equivalent overall immune stimulation. Following delivery of I-Flu alone, flu-specific IgG (Fig. 3-1A) and IgA (Fig 3-1B) antibodies were detectable in spleen organ culture supernatants, however, flu-specific IgA antibodies were significantly increased following either delivery of HA-VRP or when GFP-VRP were co-administered with I-Flu. Likewise, significantly increased flu-specific IgG (Fig. 3-1C) and IgA (Fig. 3-1D) antibodies also were detected in the nasal epithelium of animals immunized with VRP-

containing inocula. Flu-specific IgG (Fig. 3-1E) and IgA (Fig. 3-1F) antibodies also were detected in the supernatants from the DLN following delivery of antigen alone. These values were again significantly increased in animals immunized with VRP as expression vectors and as adjuvants. Taken together, these results further validate the use of VRP as both expression vectors and adjuvants for the induction of pathogen-specific immunity

Additionally, in this study we sought to determine the anatomical location in which IgA antibodies were first produced in VRP-immunized animals. Flu-specific IgA antibody responses peaked in the DLN at day 3 post-boost (Fig. 3-1F), a time at which such antibodies were essentially undetectable at the mucosal surface (Fig 3-1D). These results suggest that VRP-induced IgA production occurs in the DLN prior to production at mucosal surfaces and provides a foundation for the further study of VRP-stimulated IgA synthesis in the DLN in the context of mucosal immune induction.

The data presented in figure 3-1 suggested that IgA antibodies were produced in the DLN following VRP delivery, and that such production was dependent upon signals provided by the VRP and/or VRP infection, as equivalent levels of antigen-specific IgA antibodies were not produced in the DLN following delivery of antigen alone. However, the issue of whether DLN IgA antibodies are produced *in vivo* in the absence of *ex vivo* culture was not addressed in the previous study. Therefore, groups of female BALB/c mice were immunized in a rear footpad at week 0 and week 4 with 1 μg I-Flu alone, or 1 μg I-Flu alone co-delivered with 1×10^5 IU of null VRP. Null VRP genomes express the viral nonstructural genes which drive RNA replication. However, they do not encode a heterologous transgene downstream of the subgenomic promoter, but nevertheless provide a strong adjuvant signal (70). To evaluate the kinetics of IgA synthesis following VRP delivery in the absence of *ex*

vivo culture, DLNs were harvested at day 1, 3, 7, and 14 post-boost, and homogenized in 0.1 mls of PBS. Supernatants were evaluated for the presence of flu-specific IgA antibodies by ELISA. Fecal extracts also were prepared from immunized animals as a measure of mucosal immune induction (28, 70). As demonstrated in figure 3-2, antigen-specific IgA antibodies were in fact produced in the DLN *in vivo*; and were detectable in DLN homogenates (Fig 3-2A). IgA antibody levels peaked at day 3 post-boost, similar to the kinetics of IgA production in the DLN in the lymphoid culture system, and likewise decreased over the timecourse. Conversely, antigen-specific IgA antibodies peaked in fecal extracts at day 7 post-boost, a time after peak production in the DLN (Fig 3-2B).

The contribution of components of the conventional pathway for the mucosal immune induction also were evaluated in the VRP system. As a first step, we harvested a single PP from each of six individual animals and prepared PBS homogenates as described for DLNs. PP homogenates were evaluated for the presence of flu-specific antibodies by ELISA; however, the mean values produced in PP homogenates at all four timepoints tested were below the limits of detection of the ELISA assay, and were not statistically distinct from the assay background (Fig 3-2C). Taken together, these results suggest that antigen-specific IgA antibodies are produced *in vivo* in the DLN prior to production at mucosal surfaces following stimulation of the alternative pathway by VRP.

VRP stimulate high molecular weight IgA antibody production in the DLN.   IgA-producing B cells in the mucosal lamina propria secrete large amounts of dimeric or polymeric forms of IgA, a defining characteristic of IgA antibodies produced at mucosal surfaces (52). IgA dimer/polymer formation is mediated by the inclusion of the J chain

protein during antibody secretion. Thus, mucosal IgA antibodies are high molecular weight antibodies as a result of J chain incorporation (32).

While delivery of VRP-containing inocula clearly increased the levels of antigen-specific-IgA antibodies produced in the DLN, antigen-specific IgA antibodies also were produced at detectable levels following delivery of I-Flu alone. Based on the idea that mucosal IgA antibodies are polymeric, the molecular size of the IgA species produced in the DLN was determined following delivery of both VRP and non-VRP inocula. Monomeric IgA antibodies run at a molecular weight of approximately 150 kilodaltons (kDa) (2 heavy chains of 50 kDa, and 2 light chains of 25 kDa), while polymeric IgA molecules have a molecular weight of 300 kDa or higher (32). Supernatants from day 3 DLN cultures were analyzed by SDS-PAGE under non-reducing conditions for the presence of IgA antibodies. As shown in Figure 3-3A, the IgA antibodies in the DLN following delivery of I-Flu alone were predominantly monomeric, with a molecular weight of approximately 160 kDa. Interestingly, the delivery of both HA-VRP and I-Flu in the presence of a VRP adjuvant resulted in the production of monomeric forms of DLN IgA as well as large molecular weight (greater than 250 kDa) forms not present at substantial levels following delivery of antigen alone. IgA antibodies were not produced in the contralateral popliteal lymph node.

To determine whether VRP-induced high molecular weight IgA antibodies were specific for the influenza antigen, day 3 DLN supernatants were incubated with influenza virus, or an irrelevant virus (Girdwood virus) to allow the formation of virus-antibody complexes. The complexes were pelleted through a sucrose cushion by ultracentrifugation. This procedure utilized co-sedimentation of influenza virions as a means to affinity purify virus-specific antibodies from non-flu-specific antibodies prior to western blot analysis. As

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shown in figure 3-3B, the flu-specific IgA antibodies present in the DLN following delivery of I-Flu were predominantly monomeric. Immunization with either HA-VRP or I-Flu plus GFP-VRP resulted in the production of both a monomeric species of flu-specific IgA, as well as two high molecular weight forms not present at appreciable levels following delivery of I-flu alone. Taken together, these results suggest that VRP provide a signal which promotes the production of high molecular weight IgA molecules in the DLN.

While production of polymeric forms of IgA in the DLN was clearly dependent upon the presence of the VRP, the question of whether such antibodies are produced in the DLN *in vivo* was not evaluated in the preceding study. To evaluate the presence of polymeric IgA antibodies in the DLN *in vivo*, groups of female BALB/c mice were immunized at week 0 and week 4 with 10 μg OVA alone, or OVA mixed with either $1x10^5$ IU of null VRP or 1 μg of CpG DNA. At day 3 post-boost, DLNs were harvested, and homogenates in PBS were prepared. Homogenates were evaluated for the presence of IgA antibodies by non-reducing western blot analysis as described above. As shown if figure 3-3C, IgA antibodies were not detectable in the DLN following delivery of OVA alone or following co-delivery of OVA with CpG DNA. In contrast, both monomeric and dimeric forms of IgA were produced in the VRP DLN, suggesting that high molecular weight IgA antibodies are in fact produced in the DLN *in vivo*.

Antigen stimulation is required for DLN polymeric IgA production. To determine whether the production of polymeric IgA antibodies in the DLN was dependent upon exogenous antigen stimulation, or if antigens naturally present in the DLN have the capacity to promote IgA production during a concomitant VRP infection an additional experiment was performed. Groups of female BALB/c mice were immunized at week 0 and week 4 with 10

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μg OVA alone, 10 μg of OVA mixed $1x10^5$ IU of null VRP, PBS, or $1x10^5$ IU of null VRP alone and DLN homogenates in PBS were prepared at day 3 post-boost and analyzed for the presence of IgA by western blot under non-reducing conditions. As shown in figure 3-4, VRP induced the production of both monomeric and polymeric forms of IgA following co-delivery with antigen. In contrast, only a monomeric species was present following delivery of the same VRP in the absence of exogenous antigen, suggesting that antigenic stimulation, in addition to VRP infection is required to promote polymeric IgA synthesis in the DLN.

Characterization of DLN cells following VRP delivery. The fact that the DLN appeared to serve as the earliest site of IgA production, and high molecular weight antigen-specific IgA was synthesized there following VRP delivery, led us to further characterize the general characteristics of the DLN under these conditions. The overall mass of the DLN, as a general marker of inflammation as well as the global cellularity of the DLN were determined. Groups of animals were immunized with 1 μg of I-Flu alone, 1 μg of I-Flu mixed with $1x10^5$ IU null VRP, or $1x10^5$ IU null VRP alone, and DLNs were harvested at days 0, 1, and 3 post-boost. DLNs were carefully weighed on an analytical balance to determine overall mass, and single cell suspensions were created by collagenase digestion. Total cell counts were performed by trypan blue exclusion, and cells were analyzed by flow cytometry for the presence of B cells, T cells, and dendritic cells (DCs) following staining with antibodies directed against the appropriate cell surface markers (CD19, CD3, and CDllc respectively). As shown in figure 3-5A, VRP delivery in the presence or absence of co-delivered antigen resulted in a 2-4 fold increase in the overall mass of the DLN depending upon the timepoint examined. Moreover, the total cellularity of the VRP DLN was increased by 4-6 fold (Fig 3-5B). The increase in cellularity most likely represents a significant proportion of the

increased mass of the lymph node. This is of interest as Soderberg *et al.* recently demonstrated that viral signals increase arterial blood feed to inflamed lymph nodes, which also would result in a lymph node with increased mass (68). VRP induced a proportional 3-10 fold increase in B cells, T cells, and CD11c positive, CD11b negative DCs in the DLN at days 1 and 3 post-boost (Fig 3-5C-3-5E). Conversely, VRP infection led to an approximately 20-40 fold increase in the number of CD11c positive, CD11b positive DCs in the DLN at days 1 and 3 post-boost, both in the presence and absence of exogenous antigen (Fig 3-5F). These results suggest that VRP delivery results in a significant increase of numerous cell types to the inflamed DLN independent of exogenous antigen delivery, as well as a preferential recruitment of a CD11b positive DC subset to the DLN.

VRP induce the expression of the mucosal homing receptor on DLN B cells. Analysis of the kinetics of IgA synthesis following VRP delivery suggested that the DLN serves as one of the earliest sites of IgA production following VRP immunization, and that a significant portion of DLN IgA displayed characteristics typical of mucosal IgA antibodies. Additionally, VRP induced a significant increase in the number of B cells present within the DLN concomitant with IgA production. Here we sought to further characterize the phenotype of DLN B cells for markers characteristic of mucosal B cells. A number of investigators have demonstrated that the $\alpha_4\beta_7$ integrin, also known as the mucosal homing receptor, plays a significant role in licensing lymphocyte migration into the mucosal compartment, especially in the gut (9). This heterodimeric integrin binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on the high endothelial venules of mucosal lymphoid structures such as Peyer's patches and the mesenteric lymph node, and retains $\alpha_4\beta_7$ positive cells at the mucosal surface (4). Therefore, we wanted to determine

whether B cells present in the DLN following nonmucosal VRP delivery expressed the $\alpha_4\beta_7$ integrin.

Groups of female BALB/c mice were immunized in a rear footpad at week 0 and week 4 with 10 μg OVA alone or co-inoculated with 1×10^5 IU null VRP, and DLNs were harvested at day 1 and day 3 post-boost. Single cell suspensions were generated by collagenase digestion, and DLN B cells were analyzed for expression of CD19, B220, and the $\alpha_4\beta_7$ integrin by flow cytometry. Additionally, single cell suspensions prepared from the MLN were also analyzed as a representative mucosal lymphoid tissue. As shown in figure 3-6A, the $\alpha_4\beta_7$ integrin was in fact expressed to similar levels on B cells isolated from the mesenteric lymph node at both day 1 and day 3, regardless of the immunizing inoculum. Analysis of DLN B cells revealed two populations of B cells according to B220 expression levels; a B220 high, CD19 high population, and a B220 low, CD19 high population. Expression levels of the $\alpha_4\beta_7$ integrin were analyzed on both populations at day 1 and day 3 post-boost. As shown in figure 3-6B, a small B220 low population of B cells was present in the DLN of animals inoculated with OVA alone at day 1 post-boost. Increased $\alpha_4\beta_7$ integrin expression was observed in a subset of these B220 low B cells (see inset). Delivery of OVA in the presence of VRP resulted in a significantly increased population of B220 low B cells with upregulated $\alpha_4\beta_7$ integrin expression at day 1 post-boost. The $\alpha_4\beta_7$ integrin levels were comparable to those of MLN B cells. Interestingly, while a B220 high population was still present in the DLN following delivery of OVA in the presence and absence of VRP at day 3 post-boost, this population of cells no longer expressed increased levels of the $\alpha_4\beta_7$ integrin. Increased $\alpha_4\beta_7$ integrin expression was detected on a subset of B220 high B cells at day 3 following delivery of OVA alone.

To determine the proportion of B220 low DLN B cells with upregulated $\alpha_4\beta_7$ integrin expression, as well as to evaluate the role of exogenous antigen delivery in the activation of mucosal homing receptor, an additional experiment was performed. Groups of mice were immunized at week 0 and week 4 with 1 μg of I-Flu alone, 1 μg of I-Flu plus 1×10^5 IU null VRP, or 1×10^5 IU null VRP alon, and $\alpha_4\beta_7$ integrin expression was evaluated on DLN B cells at days 0 (prior to boost), 1, and 3 post-boost. As shown in figure 3-6C, $\alpha_4\beta_7$ integrin expression was induced only in the B220 low population at day 1 post-boost and was again undetectable at day 3. Moreover, VRP induced an upregulation of the $\alpha_4\beta_7$ integrin either when co-inoculated with a viral antigen or when delivered in the absence of any exogenous antigen, suggesting that the signals which drive increased mucosal homing receptor expression are provided exclusively by the VRP. Taken together, these results suggest that peripheral inoculation of VRP results in a mucosal homing profile of DLN B cells that is similar to the homing profile of B cells isolated from mucosal lymphoid tissues (MLN).

VRP induce MAdCAM-1 expression on the HEVs of the DLN. MAdCAM-1 expression is characteristic of mucosal lymphoid tissues, and this marker also was present in the DLN of mice immunized with VRP. Female BALB/c mice were immunized in both rear footpads with 1 μg of I-Flu alone, 1 μg of I-Flu plus 1×10^5 IU null VRP, or 1 μg of I-Flu plus 1 μg of CpG DNA at weeks 0 and 4. DLNs were harvested from immunized animals at day 1, day 2, and day 3 post-boost, snap frozen in liquid nitrogen, sectioned, and analyzed for expression of MAdCAM-1 and peripheral lymph node addressin (PNAd) by confocal microscopy (67, 73) PNAd is expressed on the HEV of systemic and mucosal lymphoid tissues and binds to CD62L, or L-selectin (2, 9). Sections derived from MLN served as a mucosal lymphoid tissue positive control. As expected, expression of both PNAd (green) and MAdCAM-1

(red) were readily detectable on the HEV of the MLN, including double positive cells (yellow, figure 3-7A). PNAd staining was abundant in the DLN following delivery of I-Flu alone at all timepoints examined, consistent with a systemic lymphoid tissue phenotype. Interestingly, MAdCAM-1 expression was detected in the DLN following delivery of I-Flu in the presence of VRP beginning at day 2 post-boost, with increased expression at day 3 post-boost. Analysis of MAdCAM-1 staining suggested that endothelial cells lining the HEV upregulated MAdCAM-1, similar to expression in mucosal lymphoid tissues, such as the MLN (figure 3-7C). Both "1st type" staining, in which a single cell inside the vessel is detected (these cells could be endothelial cells), as well as "2nd type," in which MAdCAM-1 is clearly expressed by the endothelial cells is appearant in the VRP DLN (figure 3-7C). Positive staining was also detected in the DLN following delivery of I-Flu plus CpG DNA at day 3, however to a lesser extent. These results suggest that stimulation of an alternative mucosal inductive pathway by VRP results in an HEV profile in the DLN which is distinct from traditional systemic lymphoid tissues, but instead more accurately resembles the profile of mucosal lymph nodes.

VRP drive mucosal cytokine/chemokine production in the DLN. Mucosal antigen delivery in the presence of a number of important cytokines and chemokines significantly augments mucosal antibody and T cell responses (reviewed in (51). As VRP induced a mucosal phenotype in the DLN in terms of IgA antibody production, homing receptor expression, and addressin profile, the cytokine/chemokine profile in the DLN following VRP infection was analyzed for mucosally-relevant lymphokines. Groups of female BALB/c mice were immunized in the rear footpads at week 0 and week 4 with 10 μg of OVA alone, 10 μg of OVA co-inoculated with 1×10^5 IU null VRP, or 10 μg of OVA plus 1 μg of CpG DNA

and DLNs were harvested at 6 hrs, 12 hrs, and 24 hrs post-boost. DLN homogenates in PBS were prepared as described above. Homogenates were analyzed for expression of IL-1β, IL-5, IL-6, TNF-α, IFN-γ, MIP-1β, RANTES, TBF-β1, 2, and 3 in a LUMINEX assay by UPSTATE/Millipore (figure 3-8). In general, VRP-induced cytokine/chemokine production peaked at 6 hrs post-boost and decreased over the timecourse. However, subtle differences existed between the kinetics of expression of the individual proteins. The inclusion of VRP in the inoculum significantly increased expression of IL-1β (11 fold), IL-5 (3.5 fold), IL-6 (16.5 fold), TNF-α (7 fold), IFN-γ (5 fold), MIP-1β (27 fold), and RANTES (6.5 fold) at the 6 hr and/or 12 hr timepoints compared to delivery of antigen alone. Although significant levels of all 3 subtypes of TGF-β were present in the DLN at 6 hrs, VRP infection did not induce a statistically significant increase in these proteins, and responses were quite variable (data not shown). This analysis suggests that VRP significantly augment the production of numerous mucosally-relevant cytokines and chemokines in the DLN compared with immunization with antigen alone.

DISCUSSION

Of the infectious agents causing morbidity and mortality in humans, the overwhelming majority, including HIV, initiate infection at a mucosal surface. While the correlates of protection differ with individual diseases, it is logical that induction of immune responses active at the site of virus challenge would represent a substantial obstacle to infection (44, 66), and that the induction of mucosal immune responses by vaccination has the potential to dramatically curtail the spread of mucosal pathogens. Therefore, an understanding of the basic parameters which regulate mucosal immune induction in the

numerous existing experimental systems represents a meaningful accomplishment, and may considerably promote the efficacy of mucosal vaccines.

An extensive body of work supports the notion that the strongest mucosal immune responses are present following mucosal antigen delivery and antigen processing in the specialized mucosal lymphoid tissues lining the various mucosal surfaces (57). The strongest support for this comes from studies of mucosal antibody and T cell activation which have revealed the components and the immune inductive requirements of the natural pathway of mucosal immune induction. It has been suggested that mucosal delivery of foreign antigens in the presence of bacterial enterotoxins such as cholera toxin and labile toxin (20, 61) is required for the stimulation of the natural pathway of mucosal immune induction. However, it has become increasingly clear that nonmucosal antigen delivery also can result in mucosal immune induction under specific conditions. The molecular mechanisms which regulate peripherally-induced mucosal immunity are poorly understood at present. The examples are varied and suggest either a consistent unifying mechanism is responsible for such induction under all circumstances, or conversely, that numerous diverse signals have the capacity to drive mucosal immunity following nonmucosal delivery.

It is possible that mucosal immune induction following nonmucosal delivery is merely a result of stimulating components of the natural pathway, such as has been proposed by McKenzie *et al.*, following peripheral delivery of anti-MAdCAM-1 antibodies (50). Conversely, mucosal immune induction following nonmucosal delivery may be a result of stimulation of an alternative pathway for mucosal immune induction; a pathway with components distinct from those of the natural pathway. A third possibility is that specific vaccine signals promote the generation of a "mucosal-like" site outside of the mucosal

compartment. We hypothesize that this is the mechanism by which VRP promote mucosal immune induction following nonmucosal delivery, as several characteristics of mucosal lymphoid tissues are observed in the DLN of VRP-immunized mice.

We feel that the combined examples of mucosal immune induction following nonmucosal delivery in the literature support the notion that other immunizations, other than VRP also stimulate an alternative pathway (5, 11, 14, 17, 19, 22, 27, 28, 36, 37, 42, 45, 55, 58, 59, 71). In order to test the "conversion" hypothesis in the VRP system, we first sought to determine the anatomical location in which IgA antibodies are produced following VRP delivery. Such analysis revealed that antigen-specific IgA antibodies were first detected in the popliteal lymph node draining the site of VRP infection, prior to antibody production at mucosal surfaces. Moreover, further characterization of DLN IgA antibodies revealed the presence of polymeric forms present in the DLN. Such antibodies are typically absent from systemic lymphoid tissues, and instead found at mucosal lymphoid sites. These results are supportive of a model in which the DLN serves as the primary inductive tissue for the activation of mucosal immune responses following footpad VRP delivery.

The peripheral draining lymph node has been implicated in three other models of mucosal immune activation following nonmucosal delivery. Enioutina *et al.* demonstrated the presence of IgA antibodies at multiple mucosal surfaces following co-delivery of antigen in the presence o the active form of vitamin D3 (22). Interestingly, IgA-producing cells were also present in the DLN following vitamin D delivery; however, the presence of polymeric IgA was not evaluated in that study. Following intramuscular rotavirus delivery, cells isolated from the draining lymph node induced gut IgA and IgG responses following adoptive transfer into naïve recipients (13). An additional approach, further implicates a

peripheral lymph node as a component of the alternative pathway. In targeted lymph node immunizations, antigens are inoculated directly into the lymph node itself, promoting antigen acquisition and processing by lymph node resident cells. This inoculation regimen induces IgA antibody synthesis in the local inoculated lymph node as well as in mucosal secretions in the absence of antigen processing in other peripheral lymphoid structures (42). The precise mechanism underlying mucosal immune induction in this system remains elusive. However, Lehner *et al.* demonstrated antigen-specific IgA antibody production in the targeted lymph node and proposed that the affected lymph node in fact serves as an inductive site for the generation of cells with protective properties at the local mucosal surface (43). These examples, together with the data presented here in the VRP system, are consistent with a model in which the DLN serves as an integral component of an alternative pathway for mucosal immune induction.

The role of the DLN in mucosal immune induction in the VRP system may derive from the cell populations induced in or recruited to the DLN. The DLN contains numerous cell types and a significant increase in B cells, T cells, and DCs occurs in the VRP-infected DLN. It was of interest that VRP infection resulted in a significant increase in the number of B cells present in the DLN, as IgG and IgA antibodies were also present in the DLN. Further characterization revealed a population of B cells which express the $\alpha_4\beta_7$ integrin in the DLN. This observation is significant as $\alpha_4\beta_7$ integrin expression is thought to be restricted to cells activated at a mucosal surface, and represents a characteristic marker of mucosal lymphocytes (9). These results potentially implicate the mucosal homing receptor in the alternative pathway for mucosal immune induction and suggest that components of the alternative pathway, $\alpha_4\beta_7$-MAdCAM-1 interactions, overlap with the natural pathway. It is

important to consider the commonalities between the natural and alternative pathways within the VRP experimental system, as well as the commonalities in the alternative pathway amongst the various examples of peripherally-induced mucosal immunity.

It is attractive to speculate that VRP infection provides a specific signal leading to increased mucosal homing receptor expression on DLN B cells, and as a result, such cells eventually migrate to the mucosal surface where they continue to secrete antigen-specific IgG and IgA antibodies. It will be important to determine definitively the relationship between the $\alpha_4\beta_7$ positive DLN B cells and the B cells secreting antigen-specific antibodies at the mucosal surface some days later. The exact viral signal responsible for $\alpha_4\beta_7$ integrin upregulation remains undefined to date; however, data presented here suggest that the signal is antigen-independent, as increased $\alpha_4\beta_7$ integrin expression was observed following delivery of both antigen plus null VRP and null VRP alone.

The observation that DLN B cells harbor a mucosal homing phenotype provides a potential mechanistic explanation for the inclusion of the DLN in the alternative pathway for mucosal immune induction. However, whether $\alpha_4\beta_7$ integrin positive B cells are generated in the DLN or are recruited to the DLN from another site remains to be determined. The ligand for the $\alpha_4\beta_7$ integrin, MAdCAM-1, also is expressed in the DLN at day 3 post-boost, consistent with a model in which the DLN serves as the functional equivalent of a mucosal inductive site, and a potential mechanism by which $\alpha_4\beta_7$ integrin positive cells are recruited to the VRP DLN. However, $\alpha_4\beta_7$ integrin expression was detected at day 1 post-boost, a time which MAdCAM-1 expression was not detected. Instead, MAdCAM-1 expression kinetics correlated with the kinetics of IgA antibody production in the DLN, suggesting that the

involvement of MAdCAM-1 may not be in the recruitment of IgA-producing cells to the DLN but rather in the retention of such cells in the DLN

An additional marker of mucosal lymphoid tissues, polymeric IgA antibodies, was also detected in the DLN of VRP-immunized animals. The production of polymeric IgA in the DLN again suggests that VRP provide a qualitatively distinct signal driving the production of large molecular weight forms of IgA than that provided by antigen alone. Whether the viral signal driving polymeric IgA production is the same as the signal for $\alpha_4\beta_7$ integrin upregulation remains to be fully determined; however, we speculate that such signals are distinct, as $\alpha_4\beta_7$ integrin upregulation occurred following VRP delivery in the absence of antigen but high molecular weight IgA antibodies were not detected following delivery of VRP alone. Polymeric IgA was produced following delivery of HA-VRP, suggesting that specific B cell activation or antigenic stimulation in some fashion is a prerequisite. It will be interesting to determine whether delivery of BCR cross-linking agents in the presence of null VRP rescue high molecular weight IgA production.

The $\alpha_4\beta_7$ integrin and high molecular weight IgA phenotype in the DLN potentially implicate DLN B cells as components of the alternative pathway in the VRP system. However, whether DLN B cells are programmed by soluble mediators present in the DLN alone or require additional signals provided by other DLN-resident cells is unclear. As presented here, VRP infection resulted in a significant increase in DLN B cells, T cells, and DCs. All cell populations increased in proportion to the total cell increase in the DLN with the exception of CDllb$^+$ DCs, which were increased by up to 40 fold.

A large body of evidence supports the notion that mucosal DCs are qualitatively distinct from systemic DCs in terms of their ability to prime mucosally-relevant B cell and T

cell responses (18, 34, 54, 69), reviewed in (63). For example, von Andrian and colleagues recently demonstrated that mucosal DCs co-cultured *in vitro* with CD8 T cells significantly increased $\alpha_4\beta_7$ integrin expression on antigen-specific T cells compared to systemic DCs, promoting homing to the mucosal surface (53). Likewise, Sato et al demonstrated the ability of mucosal DCs to promote increased IgA synthesis *in vitro* compared to systemic DCs (62). In fact, this ability was mapped to mucosal DC production of IL-6, as mucosal DCs cultured in the presence of an anti-IL-6 antibody failed to stimulate IgA production. VRP infection resulted in the production of strong IL-6 responses in the DLN; however, whether DCs, and in particular CDllb[+] DCs were fully responsible for DLN IL-6 production has not been determined.

Interestingly, the mucosal DCs responsible for driving IgA production in the experimental system described by Sato *et al.* were CDllb[+], the same population of DCs that were dramatically increased in the DLN of VRP-immunized mice. Indeed, preliminary experiments suggest that the CDllb[+] DC population represents the major target of VRP infection (data not shown); however, whether these cells represent a migratory population originating in the skin or elsewhere, or represent a lymph node-resident population remains to be determined. It is attractive to speculate that VRP not only rely upon the DLN as a component of the alternative mucosal immune induction pathway, but that VRP infection promotes a "mucosal DC-like" phenotype in DLN CD11b positive DCs, potentially through IL-6 production. It will be interesting to determine if VRP-infected DCs possess the capacity to drive both increased $\alpha_4\beta_7$ integrin expression as well as IgA production following *in vitro* lymphocyte co-culture.

VRP delivery significantly increased the levels of numerous cytokines and chemokines in the DLN, in addition to IL-6, which potentially play a role in the alternative mucosal immune induction pathway. It will be interesting to determine the exact role of such cytokine production in directly modulating the DLN mucosal environment. For example, Sikorski *et al.* demonstrated the ability of both Il-1 and TNF-α to upregulate MAdCAM-1 expression (64). Additionally, expression of RANTES and MIP-1β, both of which were highly upregulated in the DLN by VRP, correlated with lymph node IgA production in the DLN in the targeted lymph node immunization model (43). The question of which cell type is responsible for the DLN proinflammatory response has yet to be determined as many of the observed cytokines and chemokines examined in the present study can be secreted by numerous cell types which are present in the VRP DLN including endothelial cells, DCs, and infiltrating T lymphocytes. Irrespective of the cell type(s) responsible for the lymph node inflammatory environment, we propose that the VRP-induced DLN cytokine milieu is critical role for both the mucosal phenotype of the DLN, as well as the VRP-induced mucosal immune response.

The data presented here suggest that the DLN of VRP-inoculated animals develops numerous characteristics of a mucosal lymphoid tissue including antigen-specific polymeric IgA production, mucosal homing receptor expression, and MAdCAM-1 expression. However, it is possible that components of the natural pathway of mucosal immune induction also play a role following nonmucosal VRP delivery. VRP specifically target DCs following footpad delivery (46) and it is possible that VRP-infected DCs migrate beyond the DLN to PPs and/or MLN following footpad delivery. These VRP-infected DCs may initiate mucosal immune induction following migration to known mucosal inductive tissues as has been

proposed following both transcutaneous immunization (3) and antigen delivery in the presence of vitamin D3 (21). However, a series of experiments have failed to experimentally support such an hypothesis. Analysis of PP, MLN, and nasal-associated lymphoid tissue following peripheral delivery of GFP-VRP has failed to reproducibly detect VRP-infected cells in these tissues (Thompson, Richmond, and Johnston, unpublished). Additionally, antigen-specific antibodies were not detected in PP homogenates from VRP-immunized animals under conditions where antigen-specific polymeric IgA was readily detectable in the DLN. Thus, we feel it is unlikely that infection of mucosal lymphoid tissues represents the predominant mechanism of immune induction in the VRP system

In summary, nonmucosal delivery of alphavirus replicon particles has been employed as a model system to dissect the organization of an alternative pathway for mucosal immune induction. The draining peripheral lymph node appears to be the central component of this pathway, as the VRP-infected DLN produced polymeric forms of IgA, increased mucosal cytokines, a population of B cells harboring a mucosal homing phenotype, and 'mucosal-like' HEVs. Identification of the essential viral and immunological factors which regulate mucosal immune induction following nonmucosal VRP delivery may shed new light on an alternative pathway for mucosal immune induction. Moreover, such insights may allow for robust stimulation of this alternative pathway, resulting in vaccines with protective efficacy against mucosal pathogens and consequent reduced morbidity/mortality associated with such infections.

ACKNOWLEDGEMENTS

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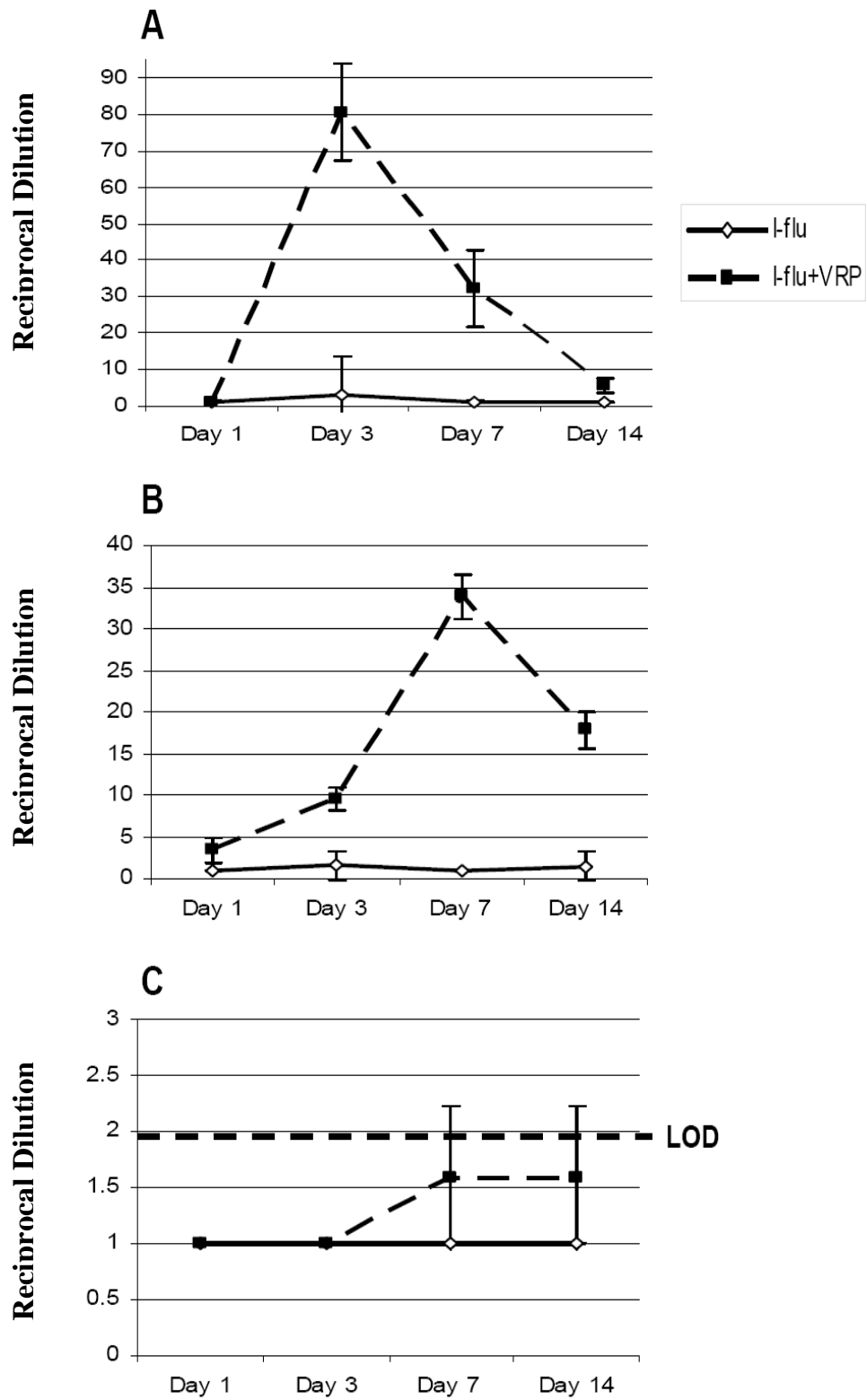
Figure 3-1
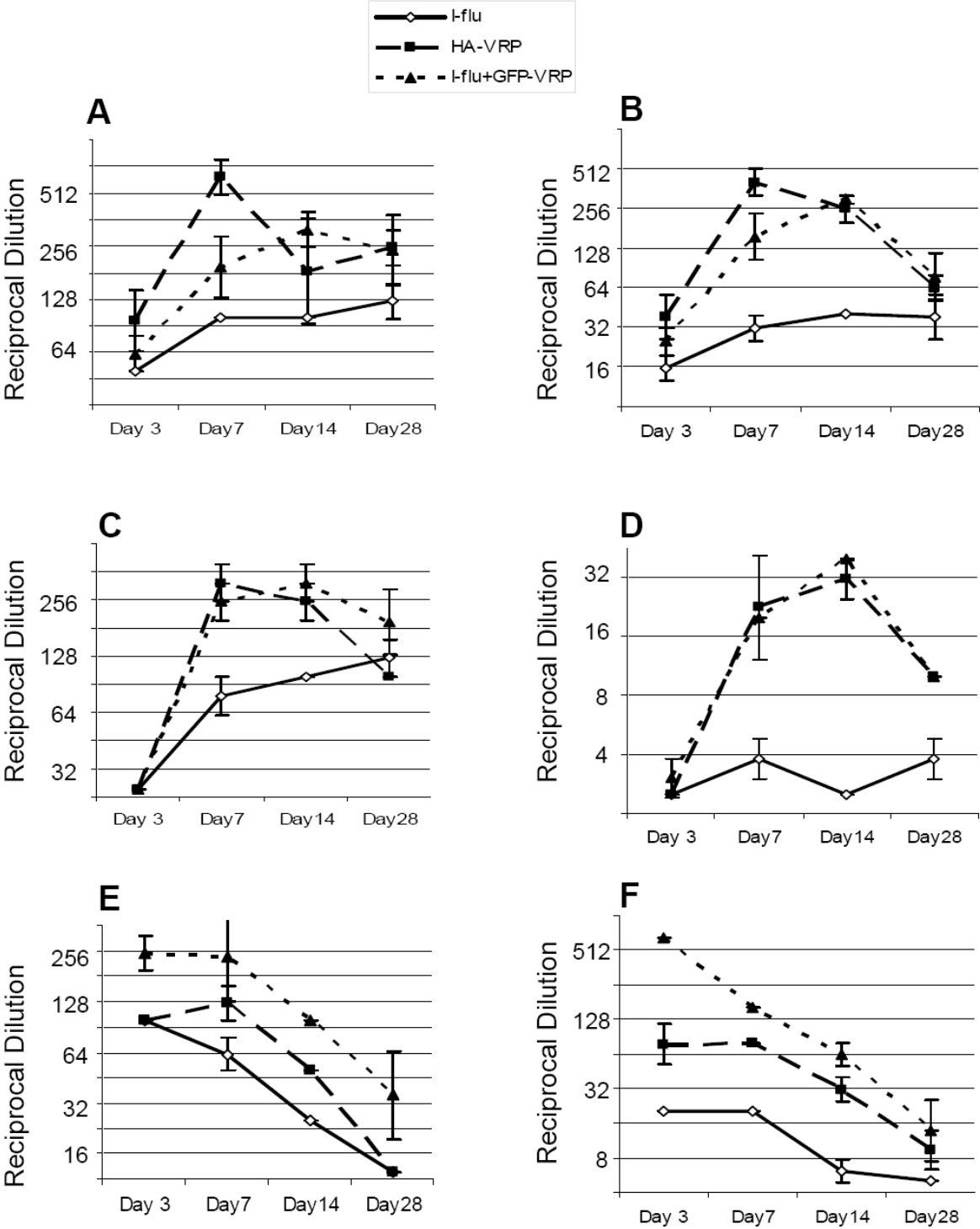
Figure 3-1. The DLN is an early site of IgA production following VRP infection.
Groups of BALB/c mice were immunized in the rear footpad with 1×10^5 IU of HA-VRP, 10 μg of I-Flu alone, or 10 μg of I-Flu plus 1×10^5 GFP-VRP at weeks 0 and 4. At the indicated timepoints, lymphoid organ cultures were established from the spleen (A and B), nasal epithelium (C and D), and DLN (E and F) and evaluated for the presence of flu specific IgG (A, C, E) and IgA antibodies (B, D, F) by ELISA. Values represent Mean+/- SEM.
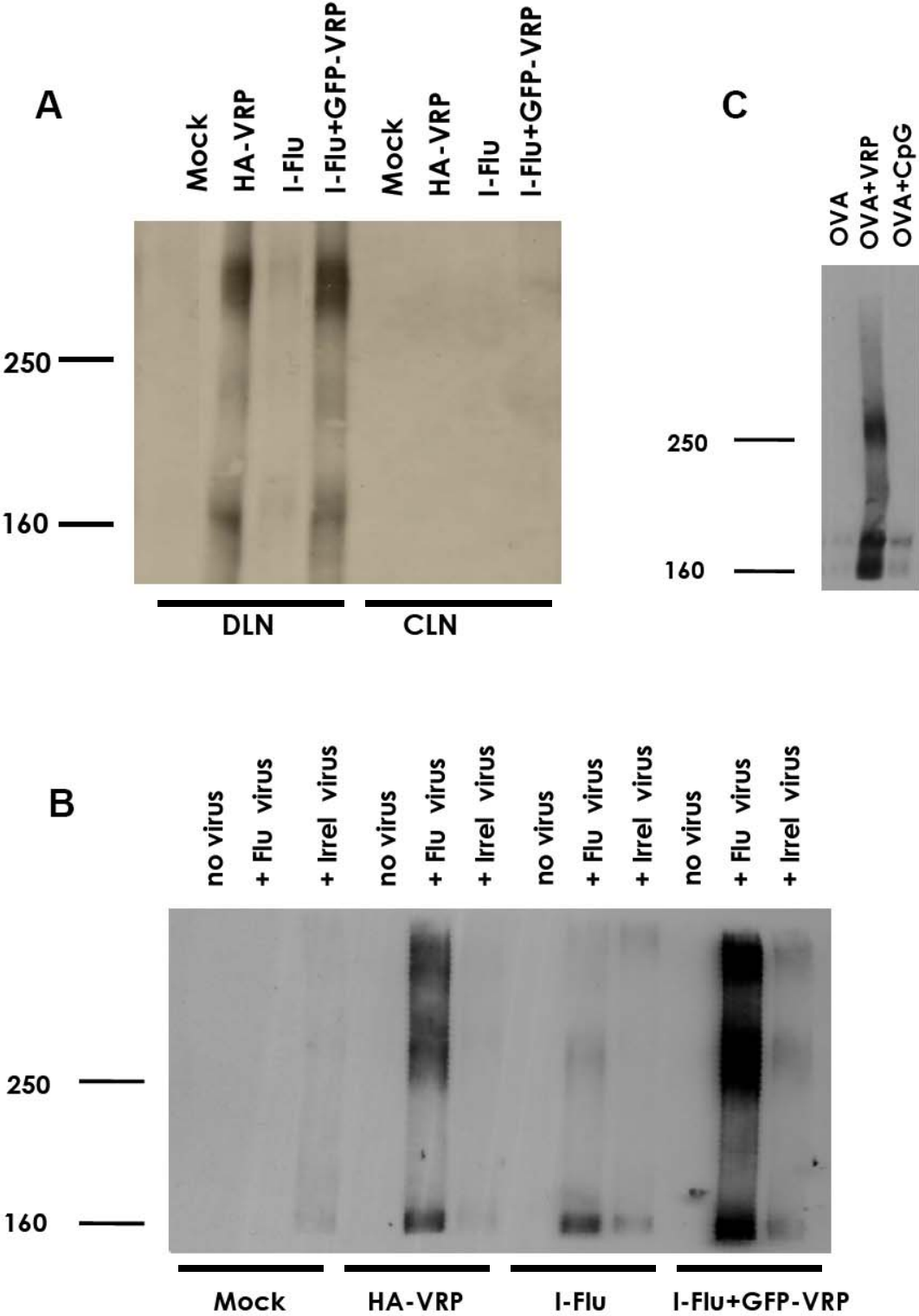
Figure 3-2

**Figure 3-2. VRP induce IgA antibody production in the DLN *in vivo.***
Groups of BALB/c mice were immunized in the rear footpad with 1 μg of I-Flu alone, or I-Flu plus of I-Flu plus $1x10^5$ null VRP at weeks 0 and 4. At the indicated timepoints, flu-specific IgA antibodies were evaluated in DLN homogenates (A), fecal extracts (B), and PP homogenates (C) by ELISA. Values represent Mean+/- SEM.
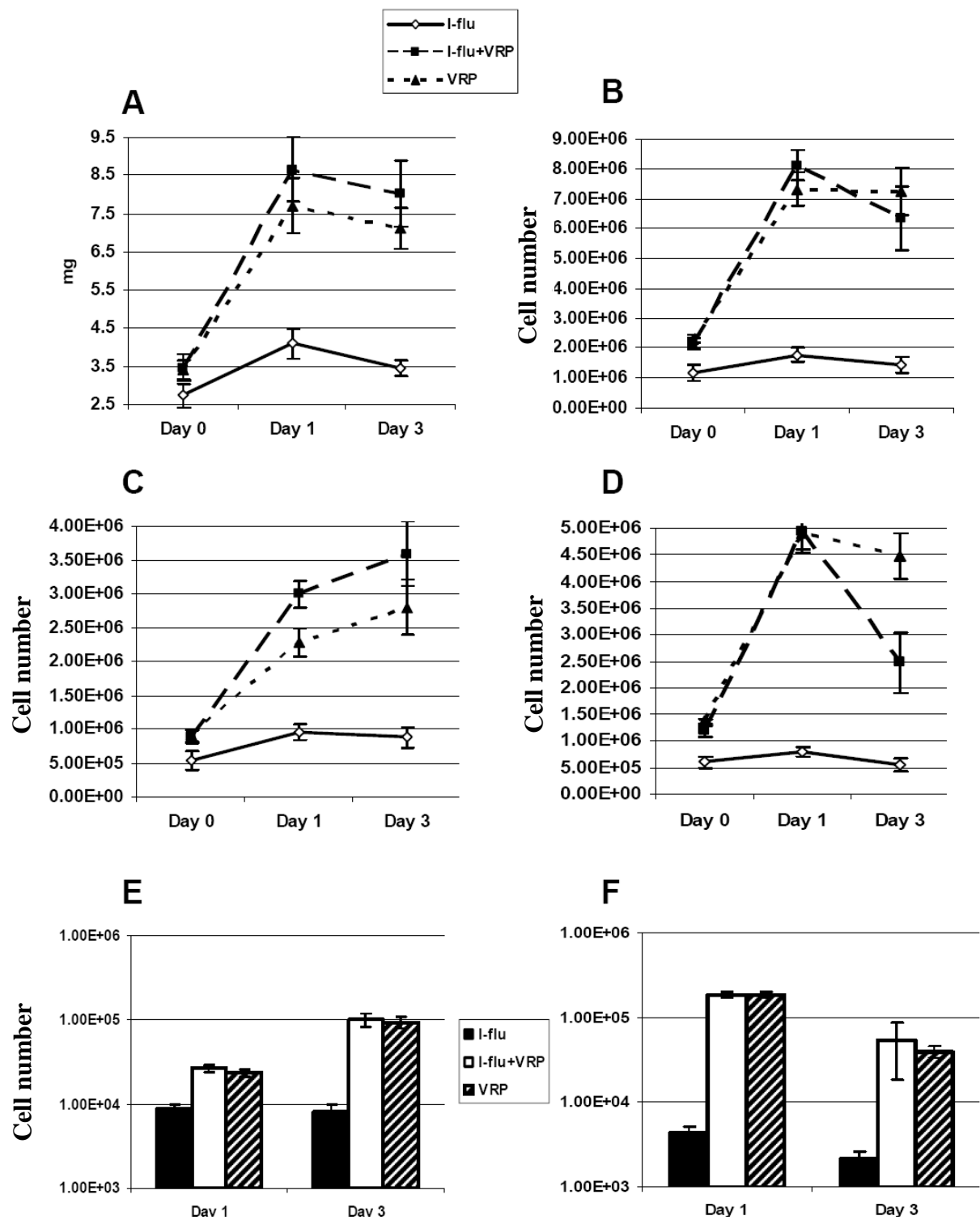
Figure 3-3

Figure 3-3. VRP induce the production of large molecular weight IgA antibodies in the DLN. Groups of BALB/c mice were immunized in the rear footpad with 1×10^5 IU of HA-VRP, 10 μg of I-Flu alone, or 10 μg of I-Flu plus 1×10^5 GFP-VRP at weeks 0 and 4. At day 3 post-boost DLN and contralateral lymph node (CLN) culture supernatants were evaluated for IgA antibodies by non-reducing western blot analysis (A). D3 DLN supernatants were then mixed with influenza virus to form virus-antibody complexes and complexes were purified via ultracentrifugation prior to non-reducing western blot analysis for IgA (B). Groups of BALB/c mice were immunized in the rear footpads with 10 μg of OVA alone, or co-inoculated with 1×10^5 null VRP, or 1 μg of CpG DNA at weeks 0 and 4 and DLNs were harvested at day 3 and DLN PBS homogenates were evaluated for IgA antibodies by non-reducing western blot analysis as in A (C).

Figure 3-4
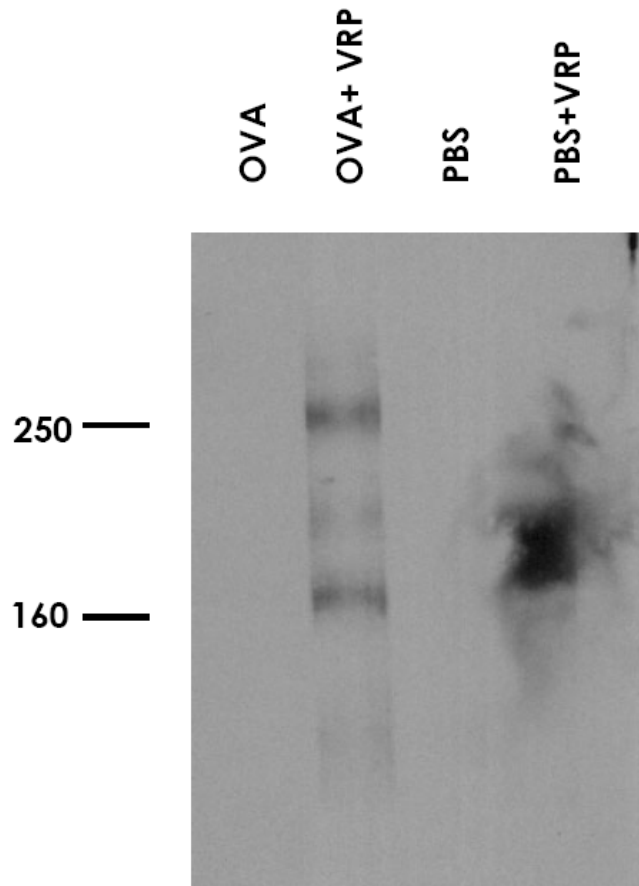
Figure 3-4. **Antigen stimulation is required for DLN polymeric IgA production.** Groups of BALB/c mice were immunized in the rear footpads with at weeks 0 and 4 with10 μg of OVA, 10 μg of OVA co-inoculated with $1x10^5$ IU of null VRP, PBS, or $1x10^5$ IU of null VRP alone. At day 3 post-boost, DLNs were harvested and PBS extracts were prepared and analyzed for the presence of IgA antibodies western blot under non-reducing conditions.

Figure 3-5

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Figure 3-5. **Characterization of VRP DLN cells.** Groups of BALB/c mice were immunized in the rear footpads with at weeks 0 and 4 with1 µg of I-Flu alone, 1 µg of I-Flu co-inoculated with $1x10^5$ IU of null VRP, or $1x10^5$ IU of null VRP alone. At day 0, 1, and 3 post-boost, DLNs were harvested and weighed on an analytical balance and single cell suspensions were prepared by collagenase digestion. The mass of each lymph node was determined (A), and total number of cells was determined by trypan blue exclusion (B). The number of B cells (C), T cells (D), CD11b- DCs (E), and CD11b+ DCs (F) was evaluated by flow cytometry. Values are presented as geometric mean +/- SEM.
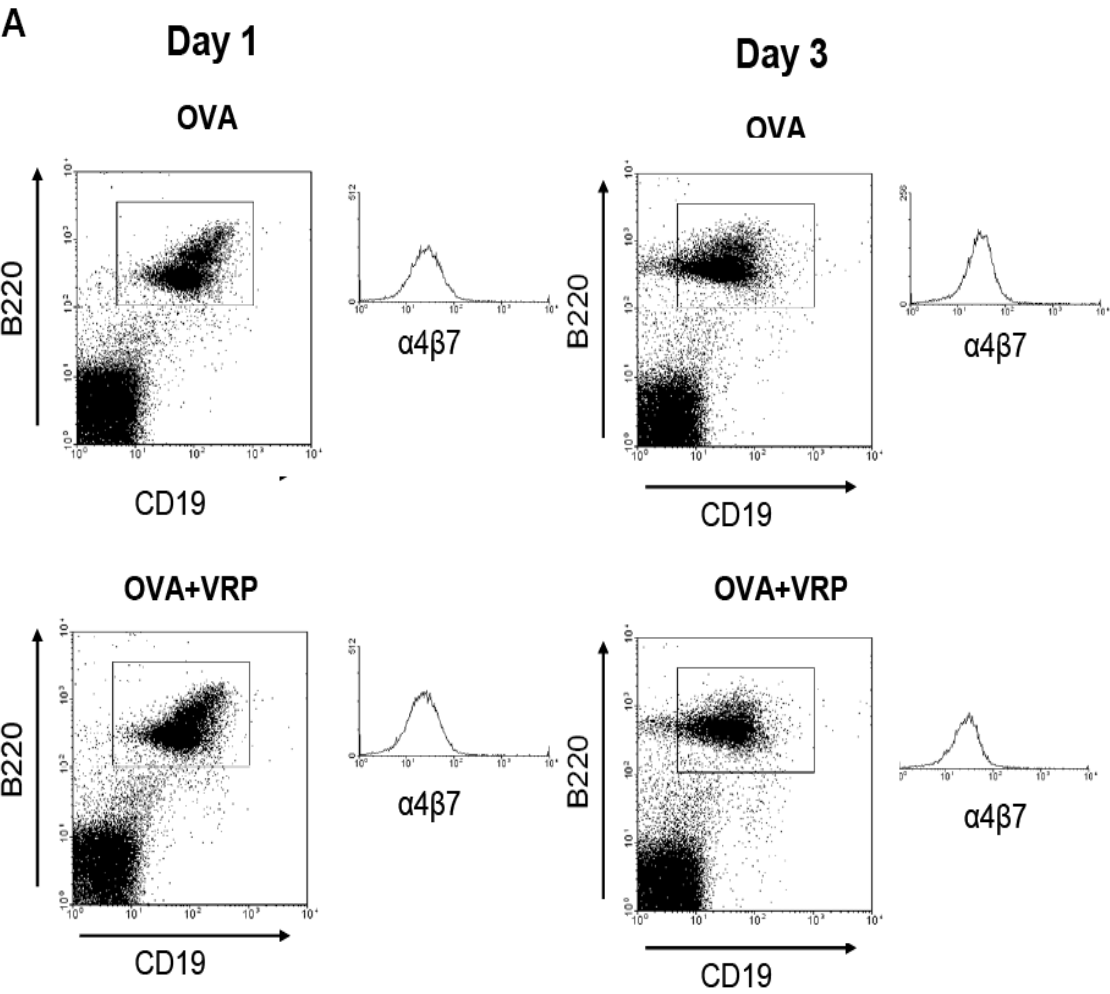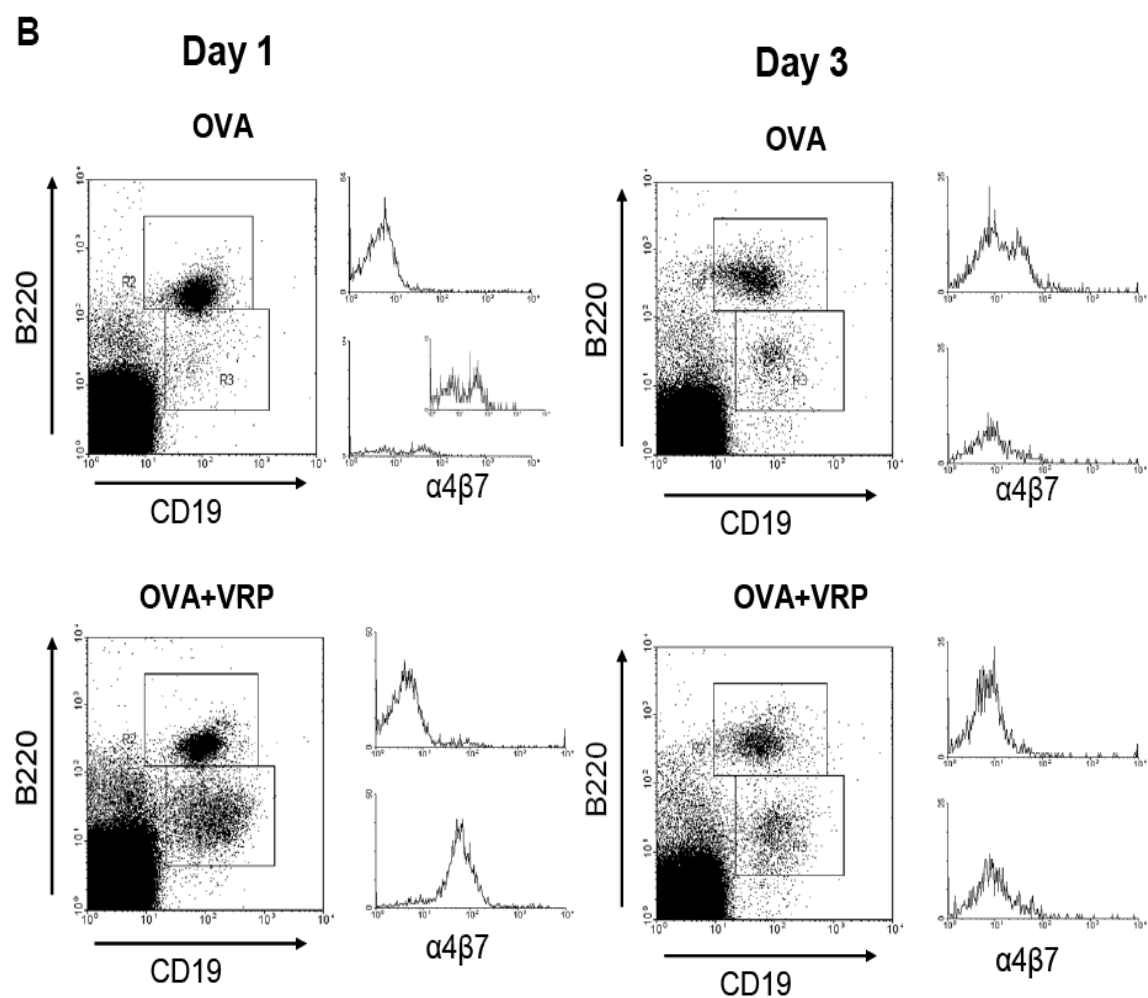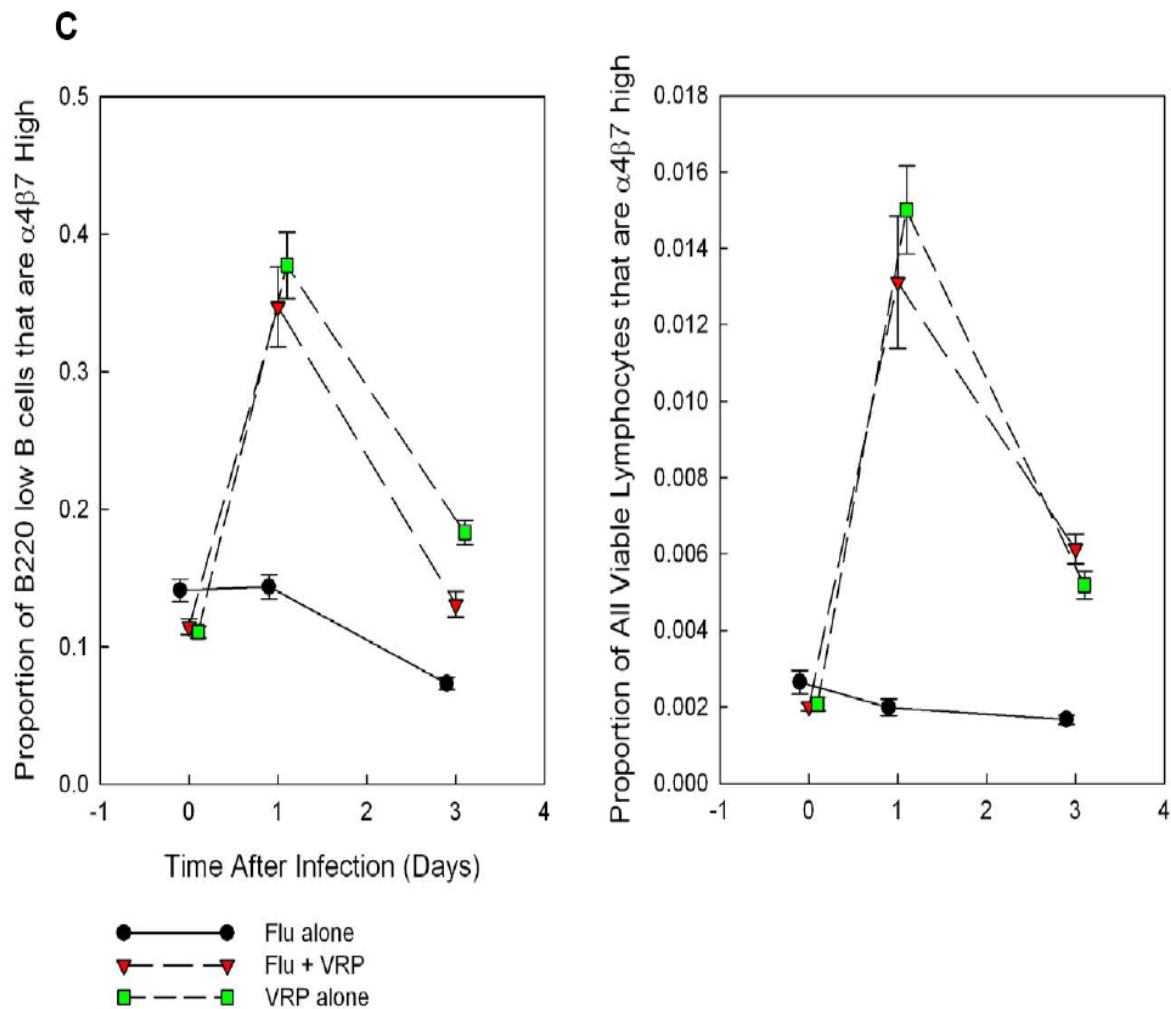
Figure 3-6

Figure 3-6

Figure 3-6

Figure 3-6. **VRP induce the expression of the mucosal homing receptor on DLN B cells.**
Groups of animals were immunized in the rear footpad at weeks 0 and 4 with 10 μg of OVA
alone of co-inoculated with $1x10^5$ IU null VRP. At days 1 and 3 post-boost, DLNs were
harvested and stained with antibodies directed against CD19, B220, and the $\alpha_4\beta_7$ integrin and
analyzed by flow cytometry. As a positive control, $\alpha_4\beta_7$ integrin expression was examined on
mesenteric lymph node (MLN) cells (A). $\alpha_4\beta_7$ integrin expression in both the B220 high and
B220 low populations in the DLNs is shown in (B). To confirm the upregulation of $\alpha_4\beta_7$
integrin expression specifically in the B220 low population following delivery of a viral
antigen, $\alpha_4\beta_7$ integrin expression was analyzed on DLN cells at day 0, 1, and 3 post-boost
following delivery of 1 μg of I-flu alone, 1 μg of I-flu plus $1x10^5$ IU null VRP, or $1x10^5$ IU
null VRP alone (C). Values are presented as geometric mean +/- SEM.

Figure 3-7

A
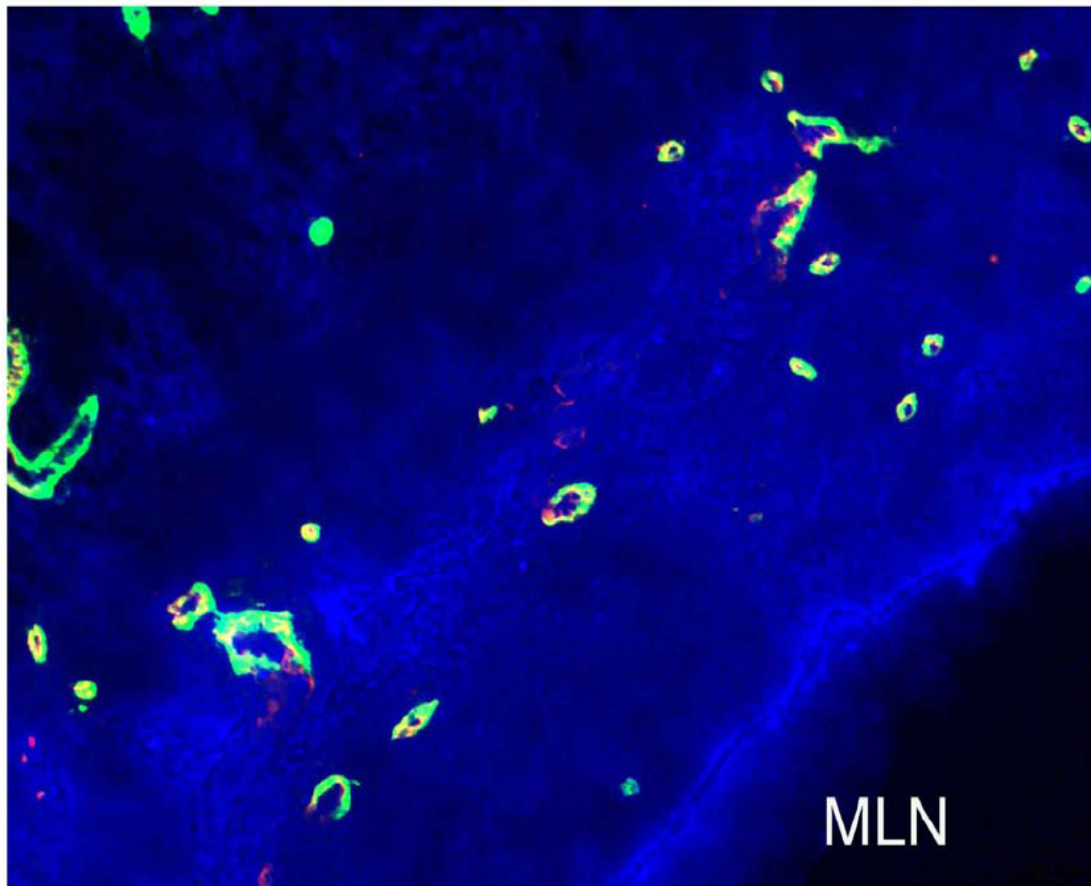
Figure 3-7

B
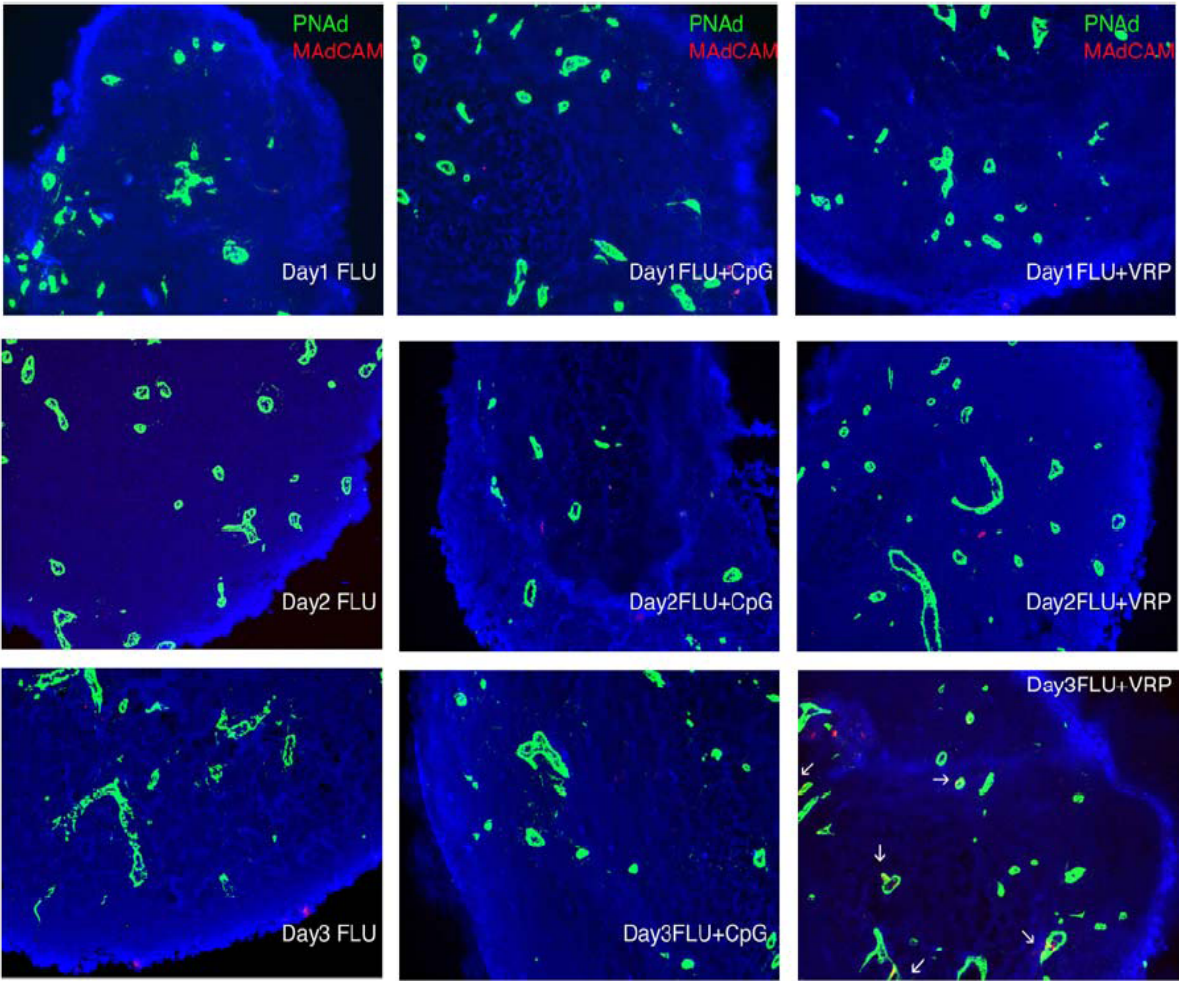
Figure 3-7
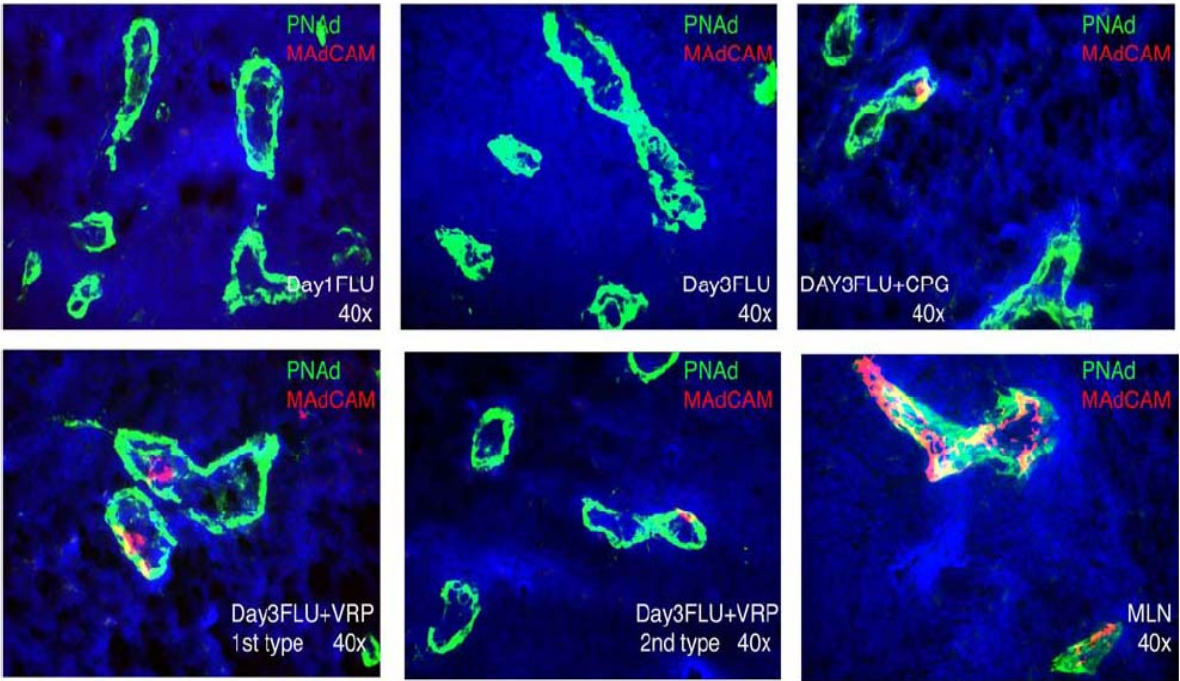
Figure 3-7. **VRP upregulate MAdCAM-1 in the DLN.**
Groups of female BALB/c mice were immunized with at weeks 0 and 4 with 1 μg of I-Flu alone, 1 μg of I-Flu co-inoculated with $1x10^5$ IU of null VRP, or 1 μg of I-Flu co-inoculated with 1 μg of CpG DNA and DLNs were harvested and snap frozen in OCT in liquid nitrogen at day 1, 2, and 3 post-boost. MLN (A) and DLN sections were stained with antibodies directed against MAdCAM-1 (red) and PNAd (green) analyzed by confocal microscopy and visualized at 10 x (B) and 40x (C).

Figure 3-8
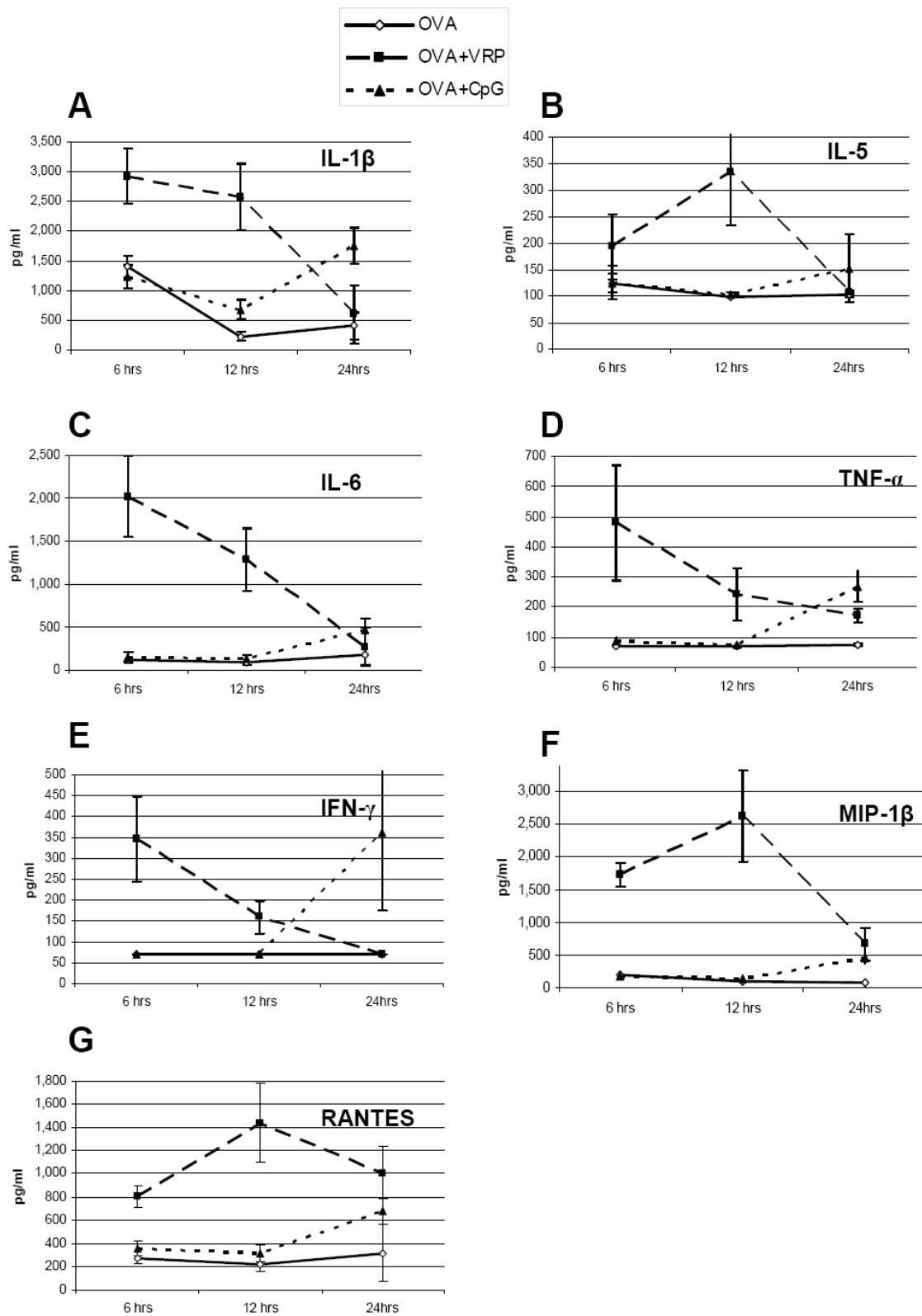
Figure 3-8. **VRP upregulate mucosal cytokine/chemokine production in the DLN.**
Groups of female BALB/c mice were immunized with at weeks 0 and 4 with 10 μg of OVA
alone, OVA co-inoculated with $1x10^5$ IU of null VRP, or 1 μg of CpG DNA and PBS
homogenates were created from the DLN at 6 hrs, 12 hrs, and 24 hrs post-boost. DLN
homogenates were evaluated for the presence of IL-1β (A), IL-5 (B), IL-6 (C), TNF-α (D),
IFN-γ (E), MIP-1 α (F), and RANTES (G) via LUMINEX. Values are presented as
geometric mean +/- SEM.

CHAPTER FOUR

ALPHAVIRUS REPLICON PARTICLES ACTING AS ADJUVANTS PROMOTE CD8^{+} T CELL RESPONSES TO CO-DELIVERED ANTIGEN

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ABSTRACT

Alphavirus replicon particles induce potent antibody and CD8^{+} T cell responses to expressed antigens in numerous experimental systems. We have recently demonstrated that Venezuelan equine encephalitis virus replicon particles (VRP) possess adjuvant activity for the activation of systemic and mucosal antibody responses. In this report, we demonstrate that VRP induced a balanced serum IgG subtype response, with simultaneous induction of antigen-specific IgG1 and IgG2a antibodies and increased both systemic and mucosal CD8^{+} T cell responses directed towards a co-delivered antigen. These responses significantly delayed the onset of tumor formation following challenge in a B16 melanoma model. Additionally, VRP further increased antigen-specific T cell immunity in an additive fashion following co-delivery with the TLR ligand, CpG DNA. Moreover, VRP-induced immune activation appeared to function downstream of antigen acquisition/processing, as increased immunity also was observed following delivery of VRP with a processed peptide as antigen. VRP infection led to recruitment of CD8^{+} T cells into the mucosal compartment, potentially utilizing the mucosal homing receptor, as this integrin was upregulated on CD8^{+} T cells in the draining lymph node of VRP-infected animals. This represents a novel activity of VRP, an adjuvant-mediated increased T cell response towards co-delivered antigen, and provides the potential to both define the molecular basis of alphavirus-induced immunity, as well as to improve alphavirus-based vaccines.

INTRODUCTION

Vaccination is the most effective strategy for protection against morbidity and mortality associated with numerous infectious agents (3). The exact immunological mechanisms which serve as the critical protective factor/s vary widely depending upon the specific pathogen (38). Likewise, the nature and makeup of the particular vaccine shapes the qualitative and quantitative aspects of the host immune response. In general terms, protection is traditionally associated with either the induction of a neutralizing antibody response, the induction of a cell mediated immune response, or both (38). There are a number of examples in which the immune correlates of protection has been identified [reviewed in (38)]; however, correlates have not been defined for important pathogens, such as human immunodeficiency virus (HIV) (23, 31). Therefore, vaccination regimens capable of stimulating both broadly active antibody and cell mediated immunity represent a potential opportunity to intercede in the spread of such diseases.

Immunogen delivery systems based upon the alphaviruses have proven to be potent inducers of both neutralizing antibody responses as well as cell mediated immune responses to multiple antigens, including HIV antigens, expressed from the viral genome [reviewed in (14, 33, 34, 39, 41, 42, 46)]. The most promising results have come from replicon systems based upon Sindbis virus, Semliki forest virus (SFV), and Venezuelan equine encephalitis virus (VEE). All three of these systems are actively under investigation as candidate HIV vaccine vectors in several laboratories. Replicon particles harbor a modified genome; the viral non-structural genes which encode the proteins required to replicate the RNA genome are expressed from the 5' two thirds of the genome, while the viral 26S subgenomic promoter catalyzes the transcription of the 3' one third of the genome into a subgenomic mRNA (20).

The genome of replication-competent virus contains the viral structural genes, the capsid and E1 and E2 glycoprotein genes, expressed from the 26S promoter. This structural gene cassette has been replaced with a cloned antigen of interest in the vaccine replicon constructs (48). In order to package replicon genomes into virus-like particles, the replicon RNA is co-electroporated into permissive cells with two defective helper RNAs which together drive the expression of the structural components *in trans*. However only the replicon RNA is incorporated into replicon particles, as the viral-specific packaging signal is deleted from the helper constructs (40). VEE replicon particles (VRP) are currently under development as an HIV vaccine platform in a animal model systems (8, 13, 15, 16, 27).

We have recently identified a novel activity of VRP; they act as adjuvants for both systemic and mucosal antibody responses to antigens that were simply mixed with VRP that encode either an irrelevant or not transgene (49). VRP significantly increased the systemic and mucosal IgG and IgA antibody responses to co-delivered antigen, however; the potential to increase T cell activation was not evaluated in that study. Here we sought to determine whether VRP were capable of a T cell adjuvant effect, augmenting T cell mediated immune responses to co-delivered antigens. As mentioned above, VRP induce potent T cell activation directed towards antigens expressed from the VRP genome. When VRP are utilized as expression vectors, essentially all antigen positive cells are also VRP positive, and vice versa. Following delivery of antigen mixed with VRP adjuvants, both VRP and antigen are likely to be sequestered to the same draining lymph node. However, antigen may or may not be taken up by the same cells as those that are targets of VRP infection. VRP target dendritic cells (DCs) following footpad delivery in mice (35); however, the precise subset of DCs that are initially infected has not yet been clearly determined. Theoretically, it is quite

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possible that co-delivered antigen could be processed in a very different manner compared to VRP-expressed antigen in the context of CD8^{+} T cell activation.

CD8^{+} T cells are activated following presentation of peptide fragments in the context of major histocompatibility complex I (MHC I) and co-stimulatory molecule expression on antigen presenting cells (APCs) (7). Understanding the factors which regulate antigen processing and presentation is an active area of investigation. In general terms, endogenous cytosolic proteins are degraded by the 26S proteosome and ubiquitin pathways and gain access to the endoplasmic reticulum (ER) following transport via the transporter associated with antigen processing (TAP) (21). Once in the ER these peptides are further processed by a number of ER-resident proteases, and are loaded into the peptide binding groove in MHC I molecules, displacing the peptide binding complex which protects "empty" class I molecules (28, 52). Loaded peptide-MHC (pMHC) complexes are then transported to the cell surface for presentation to cognate CD8^{+} T cells (52). An additional pathway of exogenous antigen loading, termed cross-presentation, also has been identified and involves the presentation of exogenous antigens into the class I pathway as a means to induce CD8^{+} T cell responses against pathogens which do not directly infect APCs (11, 44).

Toll-like receptors (TLRs) are pattern recognition receptors which recognize conserved motifs, or pathogen associated molecular patterns (PAMPs) (1). Recent evidence suggests that TLRs play a critical role in shaping both innate and adaptive immune responses (18, 26). Delivery of numerous TLR ligands promotes pro-inflammatory cytokine secretion and cross-priming (17). Interestingly, simultaneous delivery of multiple TLR ligands synergistically activates both cytokine production and/or cross presentation (4, 37, 45, 51) at least in part through the type I interferon (IFN) pathway (17). In this report we have assessed

the ability of VRP, either alone or in combination with a representative TLR ligand (CpG DNA), to activate cell-mediated immunity to a co-delivered antigen. VRP infection produced an adjuvant effect, promoting increased $CD8^+$ T cell immunity to co-delivered protein antigen, and an additive increase to both protein and peptide antigen in the presence of CpG DNA, as measured by IFN-γ secretion and antigen-specific pentamer analysis. Moreover, VRP-induced $CD8^+$ T cell activation significantly delayed tumor onset in a B16-OVA melanoma model. Interestingly, nonmucosal VRP delivery resulted in a recruitment of $CD8^+$ T cells into the mucosal compartment, as well as an increase in expression of the mucosal homing receptor on $CD8^+$ T cells in the peripheral draining lymph node (DLN). Taken together these results suggest that VRP promote not only increased antibody responses (49), but also T cell responses to co-delivered antigens in both the systemic and mucosal compartments.

MATERIALS AND METHODS

VEE replicon constructs. The VRP constructs utilized in this study were prepared and packaged as previously described (12, 40). Briefly, *in vitro*-transcribed replicon RNA, along with two defective helper RNAs, which drive the expression of the viral structural genes *in trans,* were co-electroporated into BHK-21 cells. The viral-specific packaging signal is absent from the helper RNAs, therefore, only the replicon RNA is packaged into particles. In this study, we have utilized a replicon which lacks a functional transgene downstream of the 26S promoter (null VRP) (49). Null VRP contain the viral nonstructural genes and the 26S promoter, a 14 nt stretch of VEE sequence downstream of the 26S mRNA transcription start site, a heterologous 43-nt long multiple cloning site, and the 118-nt 3' UTR (49). Null VRP

were quantitated by immunocytochemistry of infected BHK cells with sera from mice inoculated with null VRP (49). All replicon particles utilized in this study were packaged in the wild-type VEE (V3000) envelope.

Animals and immunizations. Seven-to-10-week-old female BALB/c or C57BL/6 mice were immunized in a 0.01 ml volume in the rear footpad(s) as previously described (49). Briefly, animals were immunized at week 0 and week 4 with antigen alone or antigen co-inoculated with either VRP or CpG DNA as an adjuvant. Chicken egg albumin (OVA) was purchased from Sigma and CpG DNA (ODN 1826) was purchased from Invivogen. Diluent consisted of low endotoxin, filter-sterilized PBS. For the peptide immunization experiments (presented in Figure 4-4), animals were immunized in both rear footpads in a 0.02 ml volume with the class I-restricted OVA peptide (SIINFEKL, New England Peptide) at weeks 0, 4, and 8. Single cell suspensions were prepared from immunized animals 2 weeks following the last immunization and were analyzed for the presence of OVA-specific CD8^{+} T cells by IFN-γ ELISPOT and pentamer staining (see below).

Serum collection. All sample collection was prepared as previously described (49). Blood was harvested from either the tail vein, following cardiac puncture, or from the submandibular plexus from individual animals and sera collected following centrifugation in microtainer serum separator tubes (Becton Dickinson).

Flow Cytometric Analysis. DLNs were harvested from immunized animals and the overall mass of the lymph nodes was determined by weighing individual lymph nodes on an analytical balance (Mettler). Each lymph node was next disrupted with a razor blade and a hemostat, and single cell suspensions were created by agitating each lymph node in complete RPMI media [RPMI medium 1640 containing 10% (vol/vol) FBS, 2 mM L-glutamine, 50

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μg/ml gentamicin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 mM Hepes] containing 2.5 mg/ml Collagenase A (Roche Applied Science), 17 μg/ml DNase I (Roche Applied Science) for 30 minutes at 37ºC. Single cell suspensions were then stained with antibodies directed against CD3, CD19, CD45 (B220), CD11c, CD11b (all purchased from ebioscience) as well as $\alpha_4\beta_7$ integrin (LPAM-1, clone DATK32) purchased from BD Pharmingen and examined on a Becton Dickinson Facscaliber Flow cytometer and analyzed using Cellquest software.

Enzyme Linked Immunosorbant Assay (ELISA). ELISAs for OVA-specific antibodies were performed on serum as previously described (49). Briefly, a 1 mg/ml OVA solution (in PBS) was incubated in 96-well plates (Costar) overnight at 4°C to allow antigen to bind to the plate. Excess antigen was removed, and blocking solution [PBS 1x Sigmablock (Sigma)] was added overnight at room temperature. Following removal of blocking solution, plates were incubated at room temperature (RT) overnight with serial dilutions of individual samples diluted in the appropriate buffer. Plates were washed with a multi-channel plate washer (NUNC) and incubated for 1 hr with horseradish peroxidase-conjugated secondary goat anti-mouse IgG1 or IgG2a chain-specific antibodies (Southern Biotechnology Associates or Sigma). Finally, plates were again washed, and *O*-phenylenediamine dihydrochloride substrate was added for 30 mins, and the reaction was stopped with the addition of 0.1 M NaF. Antibody endpoint titers are reported as the reciprocal of the highest dilution that resulted in an $OD_{450} \geq 0.2$. Data are presented as the geometric mean ± standard error of the mean (SEM).

IFN-γ enzyme-linked immunospot assay (ELISPOT). To evaluate the presence of OVA-specific IFN-γ-secreting cells, single cell suspensions were prepared from both spleen and

the nasal epithelium. Whole spleens were disrupted between frosted glass slides, and red blood cells were lysed either under hypo-osmotic conditions, or following addition of ammonium chloride buffer. Cells were washed and banded on a Lympholyte-M density gradient (Accurate). Banded cells were harvested, washed and counted.

Nasal lymphocytes were prepared as previously described (49). Briefly, nasal tissue from the tip of the nose to just anterior of the eye sockets was harvested from immunized animals, and the upper palate, including the NALT, was carefully removed prior to further processing. Nasal tissue was physically and enzymatically disrupted by incubation at 37ºC for 2 hrs in a 50 ml Erlenmeyer flask in complete R-10 media [RPMI-1640 containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 15mM HEPES] containing 2.5mg/ml Collagenase A (Roche), 17 µg/ml DNase I (Roche) and glass beads. Following digestion, cells were filtered through a 40 µm cell strainer (BD Falcon), washed, resuspended in 44% Percoll (Amersham) and layered on Lympholyte-M as described for spleen cells above. Banded cells were harvested, washed, and counted. Cells were pooled from two animals and typical yields were approximately $2.5x10^5$ to $1x10^6$ cells per animal. Nitrocellulose membrane plates (96 well; Millipore) were incubated with 5 µg/ml of an anti-IFN-γ antibody (AN18, Mabtech) sodium bicarbonate buffer (pH 9.6) overnight at 4˚C. Plates were then washed and blocked for 2 hrs with complete R-10 (10% serum) at RT. Single cell suspensions from either spleen or nasal epithelium were then added to plates in duplicate in R-10 and incubated in the presence and absence of the class I-restricted OVA peptide (SIINFEKL, New England Peptide) for 24 hrs. Cells were removed from plates, plates were washed, a biotinylated anti-IFN-γ antibody (R4-6A2, 1 µg/ml, Mabtech) was added to the plates, and the plates were incubated for 18 hours

at 4˚C. Membranes were again washed, incubated with a streptavidin-alkaline phosphotase conjugate for 2 hrs at RT. Plates were washed, and spots were developed following addition of BCIP/NBT substrate. Spots were enumerated with a computerized ELISPOT plate reader (Immunospot). Assay background values were obtained following incubation with either no peptide or an irrelevant peptide and were subtracted from specific peptide stimulated values. Data are presented as the number of antigen-specific IFN-γ-secreting-cells per 10^6 cells plated.

Intracellular cytokine staining. Single cell suspensions were prepared from spleen and nasal epithelium as described. 2×10^5 to 2×10^7 cells were incubated for 6-8 hours in 24 well or 48 well tissue culture plates with either media, 2 μg/ml of the class I-restricted OVA peptide (SIINFEKL), 2 μg/ml of an irrelevant peptide, or 5 μg/ml conA in the presence of 10 μg/ml of Brefeldin A. Cells were then washed and stained with antibodies against extracellular markers (CD8, CD4, CD3, etc) for 30 mins. Cells were washed, fixed and permeabilized (Bectin Dickinson cytofix cytoperm), and stained with an antibody against IFN-γ (Mabtech). Cells were washed and stored at 4˚C prior to analysis by flow cytometry.

Pentamer staining. Single cell suspensions prepared from animals immunized with the class I OVA peptide (SIINFEKL) were analyzed for the presence of OVA-specific cells via cell surface staining with antibodies against CD3, CD8, and the K^b-restricted SIINFEKL pentamer (Proimmune) according to manufacturer guidelines. Stained cells were examined on a Becton Dickinson Facscaliber Flow cytometer and analyzed using Cellquest software. Data are reported as the percentage of $CD3^+$, $CD8^+$ cells which also stain positive with the OVA pentamer. Values are presented as geometric mean +/- SEM.

Tumor challenge. Protective OVA-specific CD8$^+$ T cell responses were analyzed in the B16-OVA melanoma system. Briefly, animals were immunized with OVA and VRP constructs as described above, and subsequently challenged subcutaneously with 5 x 10^4 B16-OVA tumor cells in the flank. Animals were examined and scored for the presence of tumors.

Statistical Analysis. Antibody titers and cytokine values were evaluated for statistically significant differences by either the ANOVA or Mann-Whitney test (GraphPad INSTAT). Results are reported without adjustment for multiple comparisons. A *P* value of ≤0.05 was considered significant.

RESULTS

VRP promote a balanced Th1/Th2 antibody profile. We have previously demonstrated the ability of VRP to increase the systemic and mucosal antibody response to co-delivered antigens (Thompson *et al.*, in preparation; (49). Additionally, both Th1 and Th2 cytokines were induced in the VRP-draining lymph node at early times following VRP delivery (Thompson *et al.*, in preparation). Here we have further characterized the antigen-specific serum IgG antibody profile for the presence of IgG1 and IgG2a antibodies as an indirect measure of the Th1/Th2 cytokine profile following VRP immunization. Groups of female Balb/c mice were immunized in the rear footpad at week 0 and week 4 with 10 μg OVA alone, or 10 μg of OVA mixed with either 1x10^5 IU of null VRP or 1 μg of CpG DNA. Two weeks following the second inoculation, sera were collected and analyzed for the presence of OVA-specific IgG antibodies by ELISA. These results were previously published online as supporting information in (49) in a different format, and are reprinted here for clarity. As

shown in figure 4-1A, the inclusion of null VRP in the inoculum increased the OVA-specific IgG antibody response by approximately 40 fold. Delivery of OVA plus CpG DNA as an adjuvant likewise induced an approximately 40 fold increase in the systemic IgG antibody response. We next analyzed the same sera for the presence of OVA-specific IgG1 and IgG2a antibodies by ELISA. Delivery of OVA alone resulted in a strong OVA-specific IgG1 response relative to the IgG2a response, with an IgG1: IgG2a ratio of >20 (Figure 4-1B). In contrast, inclusion of VRP as an adjuvant significantly increased the OVA-specific IgG2a response, while still inducing an OVA-specific IgG1 antibody titer similar to that of antigen delivery alone. This resulted in a balanced IgG1: IgG2a ratio of 0.8 (Figure 4-1B). A similar IgG1: IgG2a ratio was observed following delivery of OVA and CpG DNA (0.5), indicating a balanced Th1/Th2 response induced by both adjuvants. These results suggest that VRP delivery significantly alters the systemic IgG subtype profile compared to delivery of antigen alone, and results in a balanced antibody response with the production of both Th1 and Th2 IgG profiles (6, 43).

VRP adjuvant activity for CD8$^+$ T cell responses to co-delivered protein antigen. As mentioned above, robust CD8$^+$ T cell-mediated immune responses are evident following delivery of VRP as expression vectors, where the antigen of interest is encoded within the viral genome and therefore, expressed in all infected cells. Here we sought to determine if VRP possess the ability to stimulate CD8$^+$ T cell responses to co-delivered antigens. Groups of female C57BL/6 mice were immunized in the rear footpad at week 0 and week 4 with 100 μg OVA alone, 100 μg of OVA mixed with 1×10^5 IU of null VRP, 1 μg of CpG DNA, or both 1×10^5 IU of null VRP and 1 μg of CpG DNA simultaneously. Two weeks following the second immunization, animals were sacrificed and single cell suspensions were prepared

from the spleen, as a characteristic systemic lymphoid organ, and from the nasal epithelium as a characteristic mucosal tissue. Splenocytes and nasal lymphocytes were evaluated for the presence of IFN-γ-secreting cells following stimulation with the class I-restricted OVA peptide (SIINFEKL) in an IFN-γ ELISPOT assay. As shown in figure 4-2, delivery of OVA alone resulted in low, but detectable levels of OVA-specific CD8$^+$ T cell responses in both the spleen (figure 4-2A) and the nasal epithelium (figure 4-2B). This response was increased approximately 12-fold in the spleen, and 7-fold in the nasal epithelium when VRP were included as an adjuvant. Coimmunization with VRP induced a significant increase in antigen-stimulated IFN-γ producing cells compared to immunization with antigen alone in both the spleen and the nose (Mann-Whitney, p=0.0286). CpG DNA also demonstrated an adjuvant effect in the systemic and mucosal compartments (Mann-Whitney, p=0.0286). When both VRP and CpG were combined as adjuvants, the OVA ELISPOT response was increased further, compared to either VRP or CpG DNA alone (Mann-Whitney, p=0.0286). Taken together, these results suggest that VRP as an adjuvant increases the CD8$^+$ T cell response to soluble protein antigen and that combining VRP and CpG DNA provides and additive adjuvant effect.

Functional activity of VRP-induced CD8$^+$ T cells. The data presented above, implicate VRP in the activation of CD 8 T cells specific for co-delivered antigens. To determine if the T cell response to VRP-adjuvanted antigens was functional in *vivo*, we employed a B16-OVA melanoma challenge model. Groups of female C57BL/6 mice were immunized with 100 μg of OVA protein alone, either 10 μg of OVA or 100 μg of OVA mixed with 1x10^5 IU of null VRP, or 1x10^5 IU of null VRP alone at week 0 and week 4. Two weeks following the second inoculation, animals were challenged subcutaneously in the flank with 5 x 10^4 B16-

OVA tumor cells and monitored for tumor formation. As shown in figure 4-3, 100% of the animals immunized with either OVA alone, or VRP alone formed palpable tumors by day 25 post challenge. However, co-delivery of either the low or high dose of OVA in the presence of VRP induced a significant delay in tumor formation, with either 25% of animals (low OVA dose) or 50% of animals (high OVA dose) remaining tumor free. These results suggest that VRP in fact induced a CD8$^+$ T cell adjuvant effect *in vivo* that was functional in tumor suppression.

VRP adjuvant activity for CD8$^+$ T cell responses to co-delivered peptide antigen. Potential mechanism(s) by which VRP augmented CD8 T cell immunity include modulating antigen acquisition by antigen-presenting cells (APCs), promoting expression of MHC class I molecules on APCs, processing of whole protein into the immunogenic peptide fragments, enhancing expression of costimulatory molecules on APCs required for robust T cell activation, increasing the precursor frequency/activation status of antigen-specific CD8$^+$ cells, as well as increasing the precursor frequency/activation status of Th1 CD4$^+$ cells. As a first step characterizing the T cell activation pathway in which VRP and CpG were active, the ability of VRP to augment CD8$^+$ T cell responses was examined under experimental conditions which do not require antigen processing to individual peptides. Groups of female C57BL/6 mice were immunized in the rear footpad at week 0 and week 4 with 20 μg of OVA peptide (SIINFEKL) alonefollowed by a third inoculation of 10 μg of peptide in hopes of boosting a CD8$^+$ T cell response with peptide antigen alone. In addition, mice were immunized with OVA peptide in the same amounts, and on the same dosing schedule mixed with either 1x10^5 IU of null VRP, 1 μg of CpG DNA, or both 1x10^5 IU of null VRP and 1 μg of CpG DNA. Two weeks following the third immunization, animals were sacrificed and

single cell suspensions were prepared from the spleen and nasal epithelium. Splenocytes were first examined for IFN-γ secretion by ELISPOT. As shown in figure 4-4A, delivery of OVA peptide alone failed to induce a significant IFN-γ response the spleen, as measured by IFN- γ ELISPOT. However, when VRP were co-inoculated with OVA peptide, IFN-γ-secreting cells were detectable in the spleen at approximately 10-fold higher levels then present following delivery of peptide alone (Mann-Whitney, p=0.0286). A similar increase was also observed following use of CpG as an adjuvant. Moreover, delivery of OVA peptide in the presence of both VRP and CpG DNA resulted in a significantly stronger response than delivery of either adjuvant alone (Mann-Whitney, p=0.0286). To further characterize the OVA-specific T cell response following peptide delivery, the OVA specific CD8^{+} T cells were evaluated by SIINFEKL/MHC I pentamer analysis. Delivery of OVA peptide in the presence of both VRP and CpG resulted in an increased proportion of OVA-specific CD8^{+} T cells in both the spleen (figure 4-4B) and nasal epithelium (figure 4-4C); however, neither adjuvant alone induced a significant increase in antigen-specific cells as compared to OVA peptide alone. Taken together, these results suggest that VRP, either alone or in combination with CpG DNA, increase the antigen-specific CD8^{+} T cell response to a peptide antigen.

Nonmucosal VRP delivery results in increased numbers of mucosal CD8^{+} T cells. Our initial experiments aimed at evaluating the role of VRP as T cell adjuvants entailed delivery of 10 μg of OVA in the presence and/or absence of 1×10^5 IU null VRP, and subsequent evaluation of spenocytes and nasal lymphocytes for the production of IFN-γ by intracellular cytokine staining following OVA peptide stimulation. IFN-γ positive CD8^{+} T cells were detected in the nasal epithelium following low dose OVA administration in the presence of

VRP; however, the proportion of IFN-γ positive CD8$^+$ T in the nasal epithelium was not significantly increased as compared to responses induced following delivery of OVA alone (0.010% +/- 0.004 vs. 0.016% +/- 0.002).

Although the proportion of OVA-specific IFN-γ$^+$ cells was not increased in the presence of VRP, the absolute number of such cells was increased. Groups of female C57BL/6 mice were immunized with 10 μg of OVA in the presence and/or absence of 1x10^5 IU null VRP at week 0 and week 4 and lymphocytes were prepared from the upper respiratory tract (URT) at two weeks post boost. Interestingly, the inclusion of VRP in the inoculum resulted in a an approximately 5-fold increase in the proportion of viable nasal lymphocytes which were CD8$^+$ (figure 4-5) (Mann-Whitney, p=0.0286). This would result in an increase in the absolute number of OVA-specific CD8$^+$ T cells in the nasal mucosa, even under conditions where the proportion of OVA-specific IFN-γ-secreting cells was not increased. The proportion of viable spleen cells that were CD8$^+$ was unaffected by VRP delivery (data not shown). These results suggest that CD8$^+$ T cells are recruited into the mucosal compartment when VRP are utilized as adjuvants.

Nonmucosal VRP delivery upregulates the mucosal homing receptor on CD8$^+$ T cells in the draining lymph node. Local production of mucosal IgG and IgA antibodies has been demonstrated at multiple mucosal surfaces, including the nasal epithelium, following nonmucosal delivery of VRP and antigen (49). Here we likewise demonstrate the stimulation of mucosal CD8$^+$ T cell responses directed towards co-delivered antigen following nonmucosal VRP delivery. VRP-induced IgA antibodies are first produced in the peripheral draining lymph node (DLN) following footpad inoculation (Thompson *et al.*, in preparation, Chapter 3). Furthermore, upregulated expression of the α$_4$β$_7$ integrin, or mucosal homing

receptor, also was demonstrated on DLN B cells, suggesting a potential mechanism by which VRP-activated cells gain access to the mucosal compartment. In this study, levels of the $\alpha_4\beta_7$ integrin expressed on T cells present in the DLN were evaluated. Groups of female Balb/c mice were immunized in the rear footpads with 10 μg of OVA in the presence and/or absence of 1×10^5 IU null VRP at week 0 and week 4. DLNs were harvested at day 1 and day 3 following the second inoculation ($\alpha_4\beta_7$ integrin expression was upregulated on DLN B cells at day 1), and single cell suspensions were created by collagenase digestion. Single cell suspensions were stained with antibodies against CD3, CD4, CD8, and the $\alpha_4\beta_7$ integrin (LPAM-1). As shown in figure 4-6, $\alpha_4\beta_7$ integrin expression was increased on DLN CD8$^+$ T cells at day 3 post boost following OVA plus VRP delivery, compared to delivery of OVA alone. In contrast, expression levels of the $\alpha_4\beta_7$ integrin were unchanged on DLN CD4 positive T cells. These results suggest that peripheral VRP immunization results in upregulation of proteins involved in licensing lymphocyte migration into the mucosal compartment.

DISCUSSION

Cell-mediated immune responses play a crucial role in protecting the host from invading pathogens. Thus, the development of vaccination strategies which are capable of activating CD8$^+$ T cells possess the potential to significantly influence the outcome of infection with harmful pathogens. Viruses, and viral vectors induce potent CD8$^+$ T cell-mediated immunity in a number of experimental systems. More importantly, gaining a mechanistic understanding of both the immunological and virological basis of T cell

activation may in the long run allow for the optimization of viral vectors as vaccine delivery tools.

Alphavirus expression vectors based on Sindbis virus, Semliki forest virus, and Venezuelan equine encephalitis virus induce strong CD8^{+} T cell responses against antigens which are expressed from the viral genome [reviewed in (14, 33, 34, 39, 41, 42, 46)]. Under these conditions, all productively infected cells would also be antigen positive, providing a means for virally-expressed proteins/peptide fragments to gain access to the MHC class I pathway in the same cells that receive viral activation signals. Viral signals in general, and alphavirus signals specifically (10, 47), appear to stimulate cross priming, or loading of exogenous antigens into the class I pathway in DCs. The activation of cross priming by SFV is dependent upon the TLR adaptor molecule MyD88, suggesting that TLR signaling is critical for licensing exogenous antigen delivery into the class I pathway. Production of type I IFN, which occurs following TLR ligation, appears to be at least partially responsible for viral-induced cross priming (17, 29, 30).

Here we demonstrate the ability of VRP, as an adjuvant, to induce activation of CD8^{+} T cell responses to a co-delivered antigen. It is possible that this T cell activation results from the interaction of CD8^{+} T cells with APCs that were both infected by VRP, and had taken up exogenous antigen. VRP are known to upregulate both co-stimulatory molecule expression and pro-inflammatory cytokine production in infected human DC cultures (36) and promote CD8^{+} T cell responses to antigens which are encoded in the VRP genome. Conversely, as VRP and OVA may target distinct APC subsets, VRP may provide an adjuvant signal for CD8^{+} T cells through the secretion of soluble mediators in the DLN. Consistent with this idea, VRP induce the production several inflammatory mediators in the

DLN including IFN-β, TNF-α, IL-6, RANTES, and MIP1-β (Thompson *et al*., in preparation, Konopka *et al*., in preparation). Virus-induced type I interferon is known to mediate cross-priming in DCs (30), suggesting a potential mechanism by which VRP may increase CD8^{+} T cell responses to co-delivered antigens.

We have demonstrated that VRP specifically infect DCs in the lymph node draining the infection site (35), and preliminary experiments suggest that CD11b^{+} DCs represent the major target of VEE infection (West, A., Whitmore, A., and Johnston, R., unpublished). Interestingly, CD11b^{+} DCs have been implicated in the induction of Th1 CD4^{+} T cell responses in a number of model systems (19, 25, 53), [reviewed in (24, 26)], raising the possibility that VRP-infected CD11b^{+} DCs promote the activation of CD8^{+} T cell-mediated immunity by driving helper CD4^{+} T cells towards a Th1 phenotype *in vivo*. We have recently demonstrated that incubation of peripheral blood mononuclear cells with VRP-infected DCs *in vitro* represents a potent method for expanding antigen-specific CD8^{+} T cell responses (36). We are currently evaluating the capacity of various subsets of DCs isolated from the DLN of VRP-infected animals to stimulate co-cultured, antigen-specific CD8^{+} T cells, in an attempt to define the exact DC subsets involved in immune induction in the VRP system (2, 5).

The increased stimulation of CD8^{+} T cell responses in the VRP adjuvant system could occur at various steps in the activation pathway including enhanced antigen acquisition by APCs, increased expression of MHC class I molecules on APCs, enhanced antigen processing, increased expression of costimulatory molecules on APCs, increased precursor frequency and/or activation status of antigen-specific CD8^{+} T cells. While the precise explanation has yet to be elucidated, we suggest that VRP potentially act downstream of

antigen acquisition and antigen processing, as increased IFN-γ secretion by CD8^{+} T cells was observed following delivery of OVA peptide in the presence of VRP. This delivery circumvents both antigen uptake and processing to the immunogenic peptide, narrowing our focus on the events occurring subsequent to peptide loading into MHC molecules following delivery of VRP adjuvants.

Our studies clearly demonstrate that VRP possess intrinsic T cell adjuvant activity; however, the question of whether our experimental system is optimized for T cell activation has not been fully addressed. The inoculation regimen utilized in these studies was based upon our experience with using VRP to induce serum IgG responses directed against expressed antigens. We have not yet explored the possibility that the optimal dosing schedule for the induction of T cell adjuvant activity may be different than that for serum antibody responses; such experimentation may allow for the optimization of VRP T cell adjuvant activity. We are actively pursuing methods to augment the VRP T cell adjuvant effect.

One such method has been the utilization of VRP as adjuvants in combination with other known T cell adjuvants. Our studies demonstrated stronger T cell adjuvant effects following co-delivery of both VRP and CpG together, as compared to delivery of either adjuvant alone. Synergy has been documented following delivery of multiple TLR agonists in terms of either proinflammatory cytokine production or T cell activation (4, 37, 45, 51). However, increased CD8^{+} T cell responses appeared to be additive as opposed to synergistic following co-delivery of VRP and CpG DNA. One possible explanation of this result that VRP induced a more robust stimulation of the signaling pathways which lead to T cell activation in the presence of CpG DNA, suggesting that the signaling pathways induced by

both stimuli may be partially, or completely overlapping. Conversely, it is possible that the additive effect of VRP and CpG results from stimulation of intracellular signaling pathways by VRP which are distinct from those activated by CpG DNA. It will be interesting to determine whether VRP activate intracellular signaling pathways which are similar to those induced by CpG DNA. Another possibility is that VRP- and CpG-induced signaling occurs in distinct temporal waves, and delivery of VRP and CpG in a different temporal fashion may provide a synergistic effect. In support of this idea, the kinetics of proinflammatory cytokine production in the DLN appeared to differ between VRP and CpG DNA, with VRP-induced responses peaking approximately 18 hours prior to the peak induced by CpG DNA (Thompson *et al*., in preparation, Chapter 3) We are currently evaluating the stimulatory effect of antigen delivery in the presence of VRP and CpG when delivered simultaneously, as compared to staggering the timing of delivery of the two adjuvants. Additionally, it will be interesting to determine whether synergistic T cell activation occurs following delivery of VRP in the presence of other TLR ligands.

Here we demonstrate that nonmucosal VRP delivery resulted in a significant increase in the proportion of viable cells in the nasal mucosa that were CD8$^+$, suggesting that VRP mobilize CD8$^+$ T cell migration into the mucosal compartment. The mechanism underlying this phenomenon is unclear at present. One explanation is that VRP delivery induces the antigen-independent migration of CD8$^+$ T cells into the mucosal compartment. An alternative explanation is that the increase in mucosal CD8$^+$ T cells in the mucosal compartment is in fact due to the influx of antigen-specific cells, specific for antigens other than OVA. While VRP preparations are purified over a sucrose gradient, "contaminating" proteins may in fact be present in VRP preps at levels sufficient for such proteins to serve as an antigen. VRP

may in turn adjuvant the antigen-specific CD8^{+} T cell response to "contaminating" antigens, accounting for CD8^{+} cell influx into the mucosal compartment. Additional experiments will be required to distinguish between these possibilities. It will be interesting to determine the ratio of mucosal CD8^{+} T cells in the nasal epithelium following delivery of ultra purified VRP. Additionally, antigen-independent migration may be evaluated by determining the ability VRP to induce mucosal migration of adoptively transferred CD8^{+} T cells with a specificity distinct from that of the immunizing antigen. Regardless of mechanism, it will be important to determine whether this property is unique to the nasal mucosa, or if other mucosal surfaces likewise harbor increased numbers of CD8^{+} T cells.

Activation of mucosal IgA responses following nonmucosal VRP delivery has also been observed (22, 32, 49). Interestingly, IgA antibodies were first produced in the peripheral draining lymph node, consistent with a model in which tissue plays a role in the inductive process following nonmucosal VRP delivery (Thompson *et al*., in preparation, Chapter 3). Upon further characterization of the VRP DLN, a population of B cells with increased expression of the $\alpha_4\beta_7$ integrin was discovered, suggesting a role for the mucosal homing receptor in VRP-induced mucosal antibody activation (Thompson *et al*., in preparation). This integrin promotes lymphocyte migration into the mucosal compartment, especially in the gut (9).

Here we demonstrate that CD8^{+} T cells present in the VRP DLN also upregulated the $\alpha_4\beta_7$ integrin, here by approximately 3-fold. Furthermore, Gupta *et al*. demonstrated $\alpha_4\beta_7$ integrin expression on antigen-specific, IFN-γ-secreting cells both in the DLN and at the vaginal mucosal surface following nonmucosal prime and mucosal boost of an alphavirus replicon chimera encoding the VEE RNA, providing additional support for this pathway in

alphavirus replicon mucosal immune induction. Further experimentation, such as VRP adjuvant experiments in β_7 integrin knock out mice (50), will be required to definitively implicate this pathway in VRP-induce mucosal immunity; however, we speculate that the $\alpha_4\beta_7$ integrin pathway plays a significant role in VRP-induced mucosal T cell immunity.

In summary, we describe a novel activity of VRP to augment CD8^{+} T cell responses to co-delivered antigen alone and in concert with a TLR agonist. To our knowledge, this is the first demonstration of T cell adjuvant activity with alphavirus vectors. VRP-induced activation in the presence of CpG DNA appeared to occur downstream of antigen processing, as increased immunity was observed following delivery of peptide antigens. Additionally, VRP delivery resulted in increased homing of CD8^{+} T cells into the mucosal compartment, potentially via the mucosal homing receptor. These studies provide a framework which should allow for the identification of the critical viral factors and signaling pathways which are responsible for the activation of T cell responses to co-delivered antigens. In turn, such knowledge could lead to more efficacious vaccines based on viral vectors.

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Figure 4-1
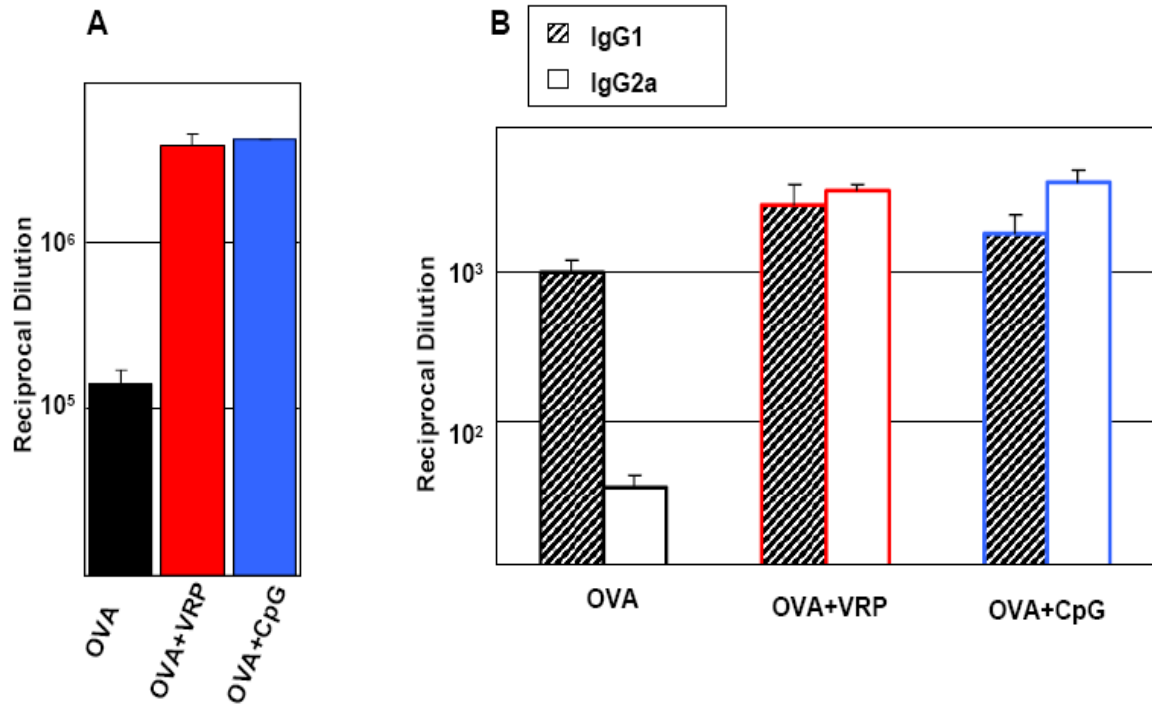
Figure 4-1. VRP promote a balanced Th1/Th2 antibody profile. Groups of female Balb/c mice were immunized in the rear footpad at week 0 and week 4 with 10 μg OVA alone, or 10 μg of OVA mixed with either 1×10^5 IU of null VRP or 1 μg of CpG DNA. Two weeks following the second inoculation, sera were collected and analyzed for the presence of OVA-specific total IgG antibodies (A) or OVA-specific IgG1 and IgG2a antibodies (B) by ELISA. Values represent the geometric mean +/- SEM.

Figure 4-2
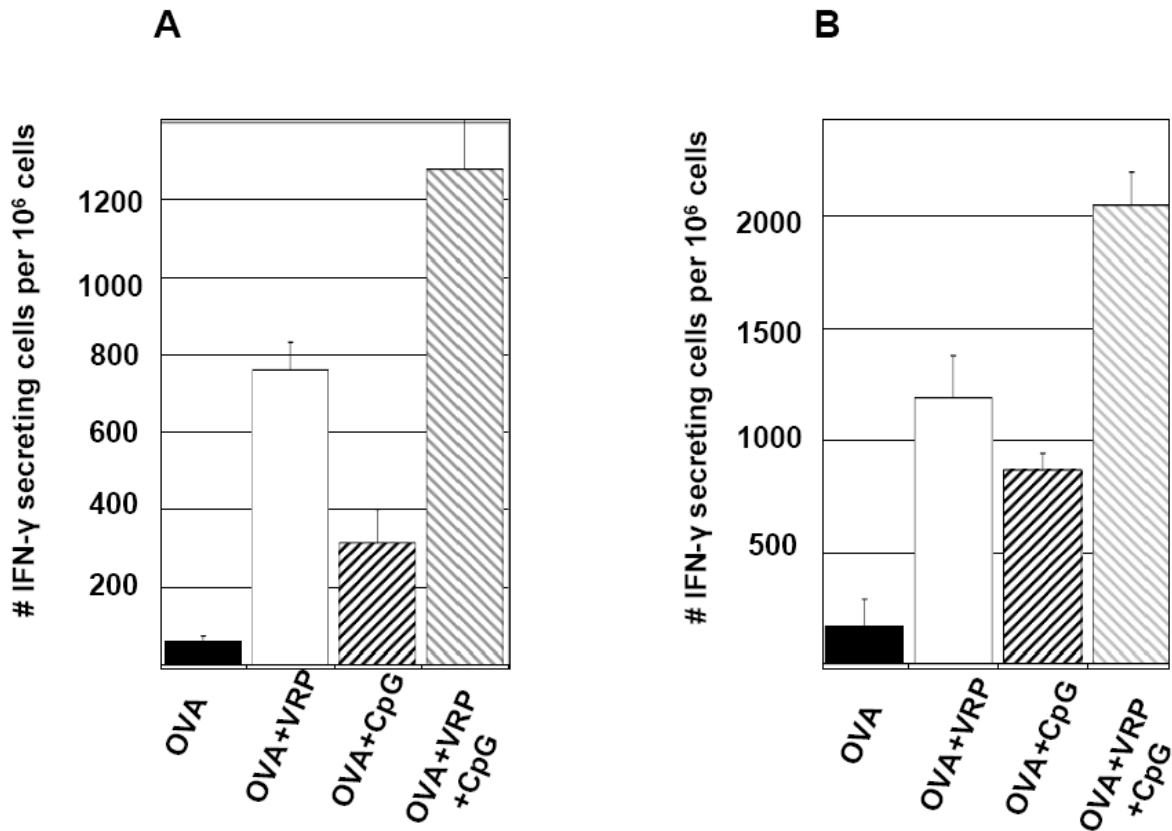
Figure 4-2. VRP promote CD8^{+} T cell immunity to co-delivered soluble antigen. Groups of female C57BL/6 mice were immunized in the rear footpad at week 0 and week 4 with 100 μg OVA alone, or 100 μg of OVA mixed with either 1×10^5 IU of null VRP, 1 μg of CpG DNA, both 1×10^5 IU of null VRP and 1 μg of CpG DNA . Two weeks following the second inoculation, splenocytes (A) and nasal lymphocytes (B) were analyzed for the presence of IFN-γ-secreting cells following stimulation with the OVA class I-restricted peptide in an IFN-γ ELISPOT assay. Values represent the geometric mean +/- SEM.

Figure 4-3

Figure 4-3. VRP-induced CD8^{+} T cells delay tumor onset. Groups of female C57BL/6 mice were immunized with 100 μg of OVA protein alone, both 10 μg and 100 μg of OVA mixed with 1×10^5 IU of null VRP, or 1×10^5 IU of null VRP alone at week 0 and week 4. Two weeks following the second inoculation, animals were challenged subcutaneously in the flank with TM 1/5 x 10^4 B16-OVA tumor cells and monitored for tumor formation.
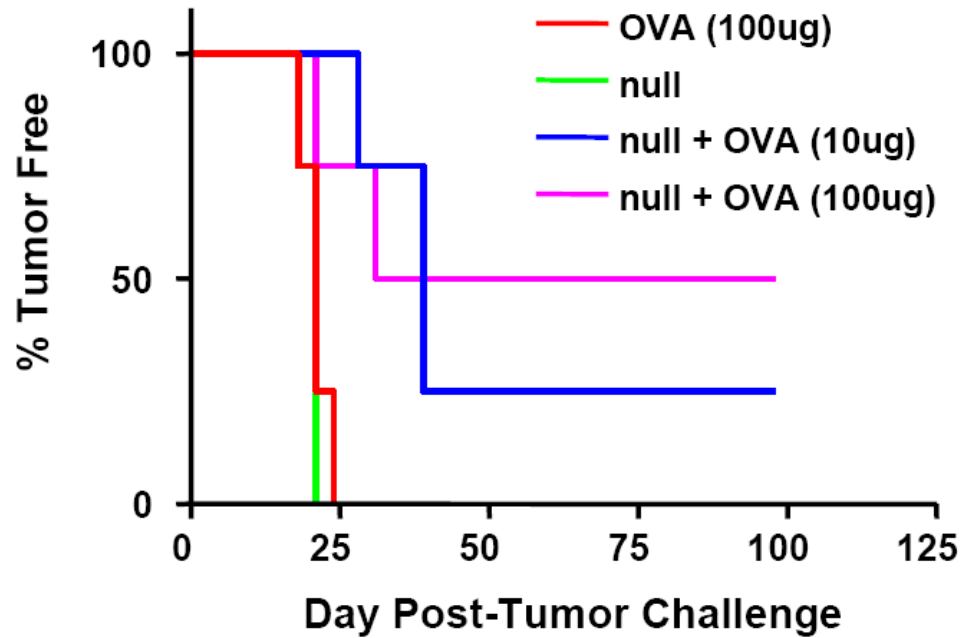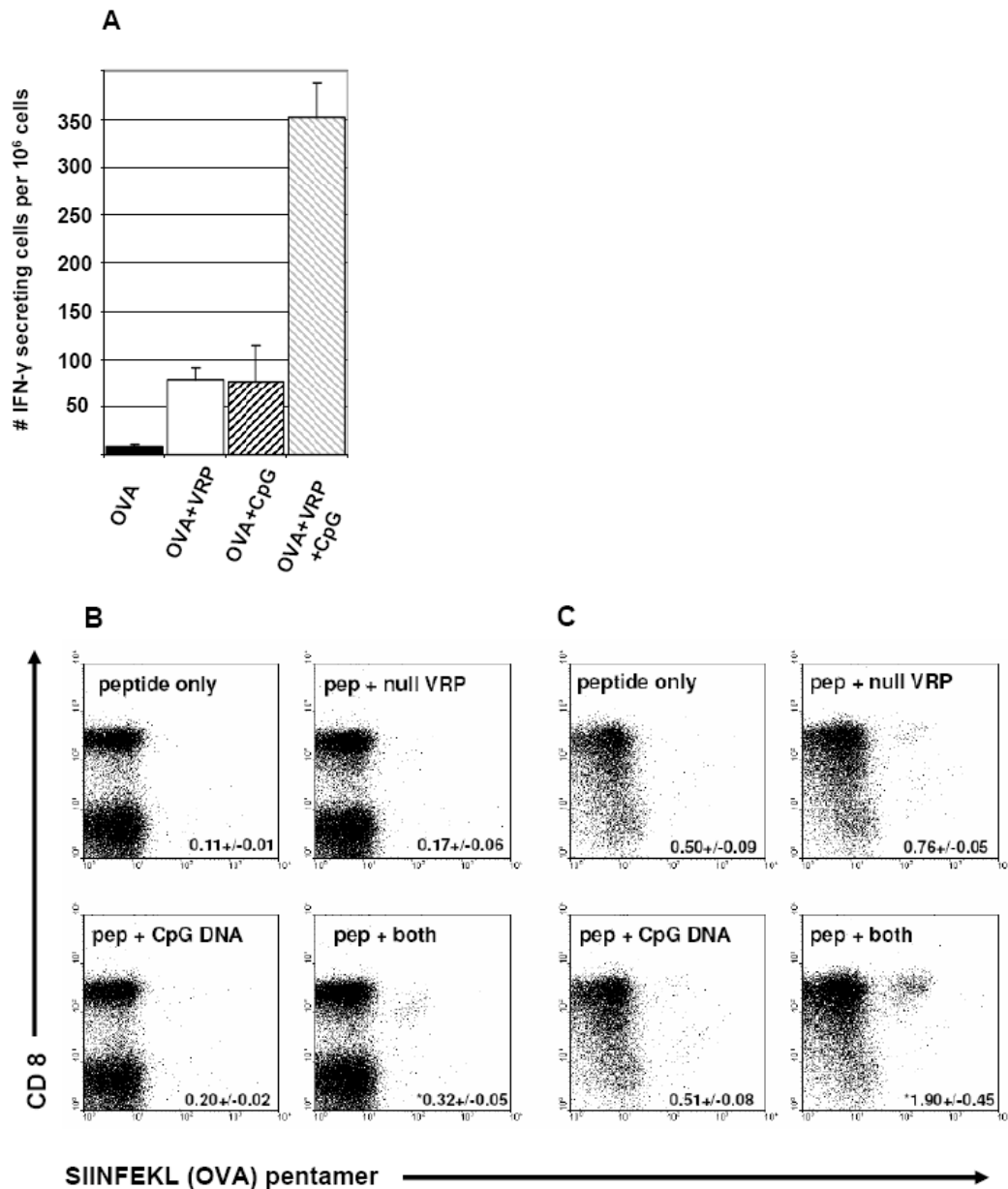
Figure 4-4

Figure 4-4. VRP promote CD8^{+} T cell immunity to co-delivered peptide antigen.
Groups of female C57BL/6 mice were immunized in the rear footpad at weeks 0, 4, and 8
with OVA peptide (SIINFEKL, 20 μg /20 μg/10 μg respectively) alone, or mixed with either
$1x10^5$ IU of null VRP, 1 μg of CpG DNA, both $1x10^5$ IU of null VRP and 1 μg of CpG
DNA. Two weeks following the last inoculation, splenocytes (A) were analyzed for the
presence of IFN-γ-secreting cells following stimulation with the OVA class I-restricted
peptide in an IFN-γ ELISPOT assay. Additionally, splenocytes (B) and nasal lymphocytes
(C) were analyzed for the presence pMHC-specific CD8^{+} T cells by pentamer staining.
Values represent the geometric mean +/- SEM.

Figure 4-5
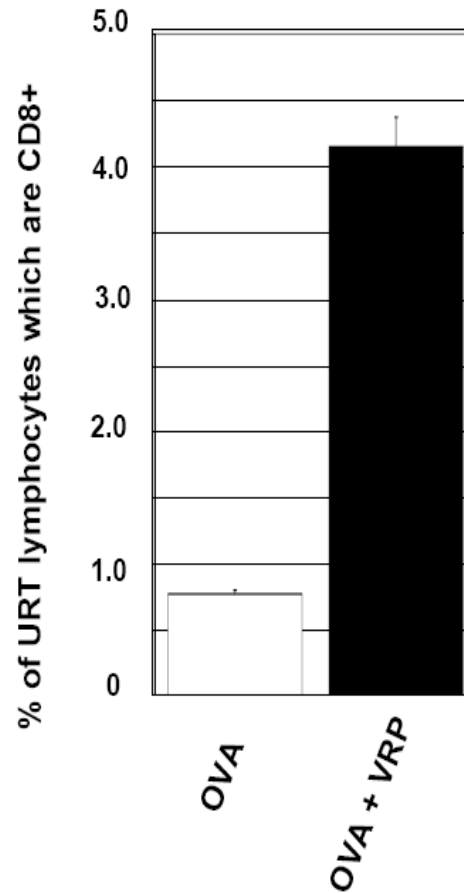
Figure 4-5. VRP recruit CD8$^+$ T cells to the upper respiratory tract (URT). Groups of Female C57BL/6 mice were immunized in the rear footpad at weeks 0 and 4 with 10 µg of OVA alone, or mixed with either 1x10^5 IU of null VRP, 1 µg of CpG DNA, both 1x10^5 IU of null VRP and 1 µg of CpG DNA . Two weeks following the last inoculation, splenocytes (A) and nasal lymphocytes (B) were analyzed for the presence pMHC-specific CD8$^+$ T cells by pentamer staining. Values represent the geometric mean +/- SEM.

Figure 4-6
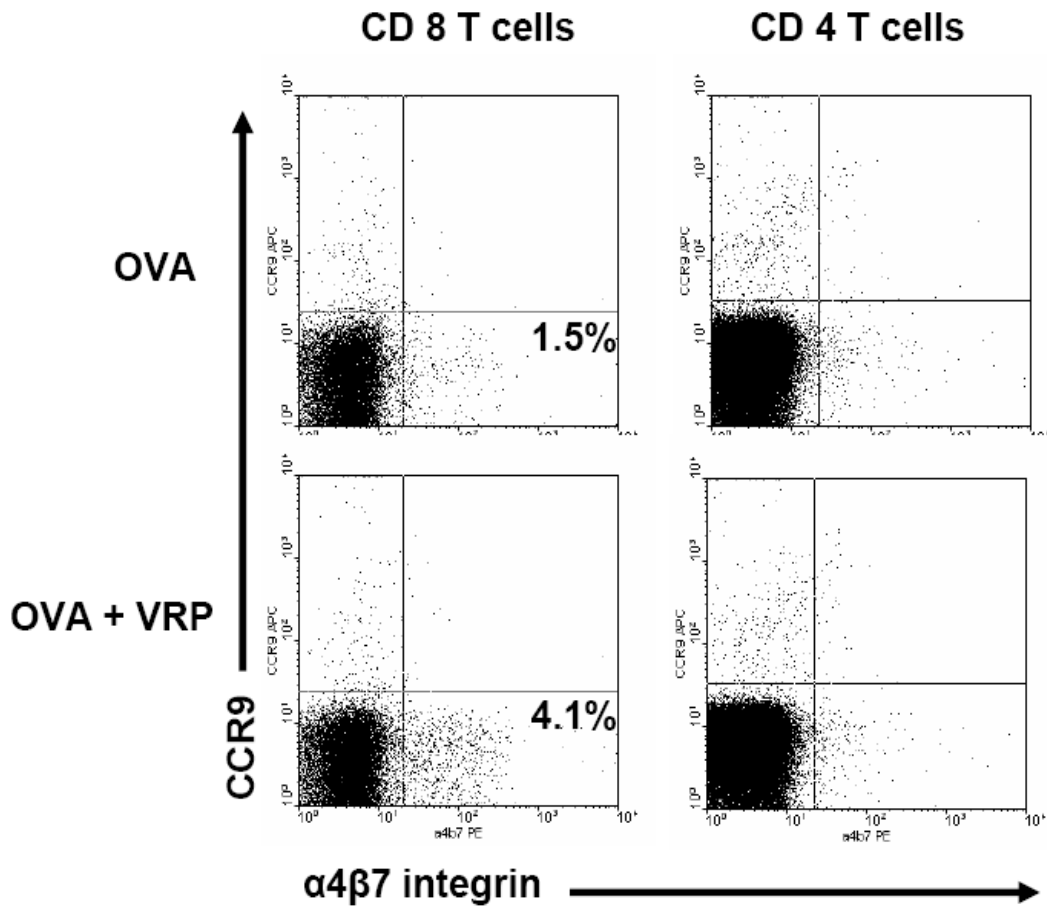
Figure 4-6. VRP upregulate the mucosal homing receptor on DLN CD8^{+} T cells.
Groups of female Balb/c mice were immunized in the rear footpads at weeks 0 and 4 with 10 μg of OVA alone, or mixed with either 1×10^5 IU of null VRP. Day 3 following the last inoculation, single cell suspensions were prepared from the DLN and CD4 and CD8^{+} T cells were analyzed for the presence of the $\alpha_4\beta_7$ integrin and CCR9 by flow cytometry. Shown are the proportion of $\alpha_4\beta_7$ integrin positive cells.

CHAPTER FIVE

THE CONTRIBUTION OF TYPE I INTERFERON SIGNALING TO MUCOSAL IgA RESPONSES INDUCED BY ALPHAVIRUS REPLICON VACCINES AND ADJUVANT PARTICLES

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ABSTRACT

The type I interferon (IFN) system is critical for protecting the mammalian host from numerous virus infections. Much is known regarding the signal transduction pathways and antiviral effector genes which are activated by IFN, and a growing body of evidence suggests that IFN also plays a key role in shaping anti-viral adaptive immune response. In this report, the importance of type I IFN signaling was assessed in a mouse model of alphavirus-induced humoral immune induction. Venezuelan equine encephalitis virus replicon particles (VRP) expressing the hemagglutinin (HA) gene from influenza virus (HA-VRP) were used to vaccinate both wildtype and IFN α/β receptor knockout (RKO) mice. HA-VRP vaccination induced equivalent levels of flu-specific systemic IgG antibodies, mucosal IgG antibodies, and systemic IgA antibodies in both wildtype and IFN RKO mice. In contrast, HA-VRP vaccination of IFN RKO mice failed to induce significant levels of flu-specific mucosal IgA antibodies at multiple mucosal surfaces, including the upper respiratory tract, the gastrointestinal tract, and the urogenital tract. We have recently demonstrated that VRP which do not encode a foreign antigen in the replicon genome (null VRP) activate systemic and mucosal immune responses to co-delivered antigens. The role of IFN signaling was also analyzed with respect to the VRP adjuvant effect. Delivery of ovalbumin (OVA) protein in the presence of null VRP increased levels of both OVA-specific systemic IgG antibodies and mucosal IgA antibodies in both wildtype and RKO mice, suggesting that type I IFN signaling is not required for the VRP adjuvant effect. Taken together, these results suggest that, 1) at least in regard to IFN signaling, the mechanisms which regulate VRP-induced responses in the expression vector system differ from those that regulate responses in the presence of VRP used as an adjuvant, and 2) type I IFN signaling is required for the induction of mucosal IgA

antibodies directed against VRP-expressed antigen. These results potentially shed new light on the regulatory networks which promote immune induction, and specifically mucosal immune induction, with alphavirus vaccine vectors.

INTRODUCTION

The type I interferons (IFNs) are a family of pleiotropic cytokines which were originally classified as proteins that interfere with virus replication (14), and are now known to provide the first line of defense against numerous viral pathogens (43).   Type I IFNs, which include IFNα and IFNβ, signal through a common receptor, the type I IFN receptor, expressed on almost all cell types (44). Signaling through the IFN receptor involves a complex cascade of events which translates extracellular signals into an increased antiviral state by upregulating the expression of interferon-stimulated genes, or ISGs (11). The importance of IFN signaling in antiviral defense is evidenced by the fact that animals with an engineered genetic deficiency in the IFNα/β receptor (IFNα/β receptor knockouts, or RKOs) are acutely susceptible to numerous viral infections (7, 13, 34, 49).

Recent evidence suggests that in addition to affecting innate immunity, type I IFN signaling also plays an important role in the activation of adaptive immune responses (2, 15, 22, 45, 47). Type I IFN promotes the differentiation of human monocytes into dendcritic cells (DCs) (40) and provides a powerful activation signal to differentiated DCs, promoting co-stimulatory molecule expression and their antigen-presenting-cell (APC) function (22). Interferon-treated DCs upon interacting with B cells, activate immunoglobulin (Ig) class switch recombination and Ig secretion (20) to multiple isotypes, including IgA. This effect is due, at least in part, to the release of TNF family ligands from IFN-activated DCs (25).   IFN

also promotes cross-priming, the activation of $CD8^+$ T cell responses directed against exogenous antigens, in DCs (19). In addition to effects on DCs, IFN signaling is directly required for complete activation of B cells (21), $CD4^+$ T cells (9), $CD8^+$ T cells (17), and natural killer cell activity (3).

The goal of vaccination is to stimulate an immune response within an individual which will protect that individual from morbidity and mortality associated with a natural pathogen challenge. Vaccine vectors based on the alphavirus, Venezuelan equine encephalitis virus (VEE), have proven to be potent inducers of antigen-specific immunity in several pre-clinical vaccination models (6, 27, 28, 35, 38, 39, 42), and are currently under evaluation in human clinical trials. The alphaviruses contain a message-sense, single-stranded RNA genome of approximately 12 kb (46). The viral nonstructural genes, which encode the enzymatic activity required for RNA replication are encoded at the 5' end of the genome, while the viral structural genes are expressed from a subgenomic 26S promoter at the 3' one third of the genome. The most well characterized members of this family are Sindbis virus (SIN), Semliki forest virus (SFV), and VEE. Several types of VEE-based vaccine technologies have been developed. One such technology, termed VEE replicon particles (VRP), functions as an expression vector, encoding a modified genome in which the structural genes are replaced with a heterologous antigen (37). Following VRP infection, the replicon RNA encoding the transgene is expressed at very high levels; however, progeny virions are not produced as the structural components are absent from the genome. Thus, VRP are single-cycle vectors capable of only one round of replication. VRP are potent inducers of both systemic and mucosal antibody responses directed against the antigen which is carried in the viral genome (8, 26, 48).

We have recently identified a novel activity of VRP; to serve as adjuvants for the activation of humoral immune and cell-mediated immune responses directed against a co-delivered antigen (48) (Thompson *et al*., in preparation, Chapter 4). Null VRP, which lack a heterologous transgene downstream of the 26S promoter, acting as adjuvants, were capable of stimulating systemic and mucosal immune responses following nonmucosal delivery, similar to our observations with VRP expression vectors (8, 26, 48). The finding that VRP, acting as both expression vectors and as adjuvants, stimulate local antibody synthesis following nonmucosal vaccination is significant, as nonmucosal vaccine delivery methods typically fail to induce authentic mucosal immunity (30-32). We have recently demonstrated the presence of several characteristics of mucosal lymphoid tissues, including antigen-specific polymeric IgA antibodies, in the draining lymph node (DLN) of VRP-vaccinated mice, suggesting that this lymphoid tissue may serve as a component of the mucosal inductive pathway stimulated by nonmucosal VRP delivery (Thompson *et al*., in preparation, Chapter 3). These results suggest that VRP are potent stimulators of the adaptive immune system.

A specific role for type I IFN signaling in alphavirus-induced adaptive immunity has previously been established. The activation of B and T lympocytes (as measured by upregulated CD69 expression) was significantly impaired in IFNα/β RKO mice, suggesting that alphavirus-induced lymphocyte activation is incomplete in the absence of IFN signaling (1). An additional alphavirus vaccine technology has been developed in which the replicon RNA is delivered, not from a virus particle, but instead encoded on a bacterial plasmid as a DNA replicase-based vaccine [reviewed in (41)]. These alphavirus vaccines are highly immunogenic in several mouse models, including murine models of tolerance. Leitner *et al*.

demonstrated a role for type I IFN signaling in a replicase-based vaccine, as this vaccine induced immunity to a "self" tolerant antigen in wildtype animals; however, failed to induce immunity to the same tolerant antigen in IFNα/β RKO mice (23). An additional study performed by Restifo and colleagues suggested that the ability of replicase-based vaccines to break immunological tolerance was dependent upon a single ISG, RNaseL (24). Moreover, Hidmark *et al.* recently demonstrated that the systemic IgG adjuvant effect of SFV replicon particles was dependent upon type I IFN signaling, as SFV replicons failed to augment serum IgG responses in IFNα/β RKO mice.

In this report we have evaluated the role of type I IFN signaling in the stimulation of systemic and mucosal antibody responses by VRP as expression vectors, expressing the hemagglutinin (HA) gene from influenza (flu) virus (HA-VRP), and as adjuvants, following co-delivery of null VRP with soluble ovalbumin (OVA). HA-VRP induced equivalent flu-specific systemic IgG and systemic IgA antibody responses in both wildtype (wt) and IFNα/β RKO mice. In contrast, while HA-VRP vaccinated, wt mice produced strong flu-specific IgA responses at several mucosal surfaces, mucosal IgA responses were barely detectable in IFNα/β RKO mice. Interestingly, null VRP significantly augmented OVA-specific serum IgG and fecal IgA antibodies in both wt and IFNα/β RKO mice. These results suggest that type I IFN signaling plays an important role in VRP expression vector-induced mucosal IgA responses; however, only a minimal role in the VRP adjuvant effect. This analysis should allow for a more basic understanding of the precise role of IFN in alphavirus-induced immunity.

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MATERIALS AND METHODS

VEE replicon constructs. The construction and packaging of VRP was performed as previously described (5, 37). Briefly, confluent monolayers of BHK-21 cells were co-electroporated with three separate *in*-vitro-transcribed RNAs; namely the replicon genome RNA, and two defective helper RNAs which drive the expression of the viral structural genes *in trans*. Only the replicon genome RNA is packaged into VRP, as the helper RNAs lack the viral packaging signal. In this study, two different replicon constructs were utilized: 1) replicons expressing the HA gene from the A/PR/8/34 strain of influenza virus (HA-VRP); and 2) replicons which lack a functional transgene downstream of the 26S promoter (null VRP) (48). Null VRP contain the viral nonstructural genes, a 14 nt stretch of VEE sequence downstream of the 26S mRNA transcription start site, a heterologous 43-nt long cassette containing multiple restriction sites for cloning into the replicon backbone, and the 118-nt 3' UTR. HA-VRP and null VRP were quantitated by immunocytochemistry of infected BHK cells with anti-sera against HA (37) and null VRP (48), respectively. All replicon particles utilized in this study were packaged in the wild-type (V3000) envelope.

Animals and immunizations. Eight-to-16-week-old 129 Sv/Ev and 129 Sv/Ev IFN α/β receptor knockout (RKO) mice were immunized in a 0.01 ml volume in the rear footpad as previously described (48). 129 Sv/Ev animals were bred under specific pathogen free conditions. Breeder pairs were obtained from Dr. Barbara Sherry, North Carolina State University, or were purchased from Taconic Laboratories. Breeder pairs of the RKO animals were obtained from Dr. Herbert Virgin, Washington University, and bred under specific pathogen free conditions.   Animals were immunized at week 0 and week 4 with either HA-VRP or antigen (chicken egg albumin, ovalbumin) in the presence or absence of null VRP as

an adjuvant. Ovalbumin (OVA) was purchased from Sigma. Diluent consisted of low endotoxin, filter-sterilized PBS.

Antibody-Secreting-Cell enzyme-linked immunospot assay (ASC ELISPOT). To evaluate the presence of OVA-specific ASCs, single cell suspensions were prepared from both spleen and the nasal epithelium. Whole spleens were disrupted between frosted glass slides, and red blood cells were lysed either under hypo-osmotic conditions, or following addition of ammonium chloride buffer. Cells were washed and placed on a Lympholyte-M density gradient (Accurate). Banded cells were harvested, washed and counted.

For preparation of nasal lymphocytes, nasal tissue from the tip of the nose to just anterior of the eye sockets was harvested from immunized animals, and the upper palate, including the NALT, was carefully removed prior to further processing. Nasal tissue was physically disrupted and incubated at 37°C for 2 hrs in a 50 ml Erlenmeyer flask in complete R-10 media [RPMI-1640 containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 15mM HEPES] containing 2.5mg/ml Collagenase A (Roche), 17 μg/ml DNase I (Roche) and glass beads. Following digestion, cells were filtered through a 40 μm cell strainer (BD Falcon), washed, resuspended in 44% Percoll (Amersham) and layered on Lympholyte-M as described for spleen cells above. Banded cells were harvested, washed, and counted. Cells were pooled from two animals, and typical yields were approximately 2.5×10^5 to 1×10^6 cells per animal. ASC ELISPOT analysis was modified from previous reports (24),(25). Briefly, 96-well nitrocellulose membrane plates (Millipore) were incubated with 1 mg/ml OVA in PBS overnight at 4°C. Plates were then washed and blocked for 2 hrs with complete R-10 (10% serum). Two fold dilutions of single cell suspensions were then added to plates in duplicate

in R-10 and incubated overnight. Plates were washed, and bound spots were detected by the addition of HRP-conjugated secondary goat anti-mouse γ or α chain-specific antibodies (Southern Biotechnology Associates), followed by addition of 3-amino-9-ethylcarazole (AEC, Sigma), and enumerated with a computerized ELISPOT plate reader (Immunospot). Data are presented as the number of antigen-specific ASCs per 10^6 cells plated.

Lymphoid Organ Cultures. Lymphoid cultures were prepared as previously described (26, 48). Briefly, draining popliteal lymph nodes were harvested from immunized animals and placed in Eppendorf tubes containing 1 ml of wash buffer (Hanks' balanced salt solution containing 100 units/ml penicillin, 100 μg/ml streptomycin, 110 mM Ca^{2+}, 50 mM Mg^{2+}, and 15 mM Hepes) and washed three times by aspiration and resuspension. Draining lymph nodes (DLNs) were placed in individual wells in a 96-well tissue culture plate in 0.1 mls of media [RPMI medium 1640 (GIBCO) containing 15 mM Hepes, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 mM L-glutamine (GIBCO), and 0.25 μg/ml amphotericin B] Plates were incubated at 37ºC for 7 days to allow antibody secretion from tissue-resident B cells into the supernatant. Following incubation, supernatants were collected, clarified by centrifugation at 4ºC, and analyzed for the presence of antigen-specific antibodies by ELISA (see below).

Sera, Fecal Extracts, and Vaginal Washes. All sample collection was prepared as previously described (48). Blood was harvested from individual animals either from the tail vein, following cardiac puncture, or from the submandibular plexus, and sera collected following centrifugation in microtainer serum separator tubes (Becton Dickinson). For fecal extracts, fresh fecal pellets (5-8, ≈ 100-150 mg) were isolated from individual animals and placed in a 1.5 ml Eppendorf tube containing 1 ml of fecal extract buffer buffer [PBS

containing10% (vol/vol) normal goat serum and 0.1% (vol/vol) Kathon CG/ICP (Supeleco)]. Samples were vortexed for at least 10 mins until all pellets were disrupted into a homogenous mixture. Samples were vortexed and clarified by centrifugation at 4ºC, and supernatants were transferred to fresh tubes and stored at -20 ºC prior to analysis in ELISA assay (see below). Vaginal washes were performed by lavaging the exterior vaginal opening with 0.07 mls of PBS 8-10 times. Lavage samples were stored at -20 ºC and clarified at 4ºC prior to ELISA analysis (see below).

Enzyme Linked Immunosorbant Assay (ELISA). ELISAs for influenza- and OVA-specific antibodies were performed on serum, fecal extracts, vaginal washes, and lymphoid culture supernatants as previously described (48). Briefly, antigen solutions (either 250 ng/ml of influenza virus in carbonate buffer, or 1 mg/ml of OVA in PBS) were incubated in 96-well plates (Costar) overnight at 4°C to allow antigens to bind to the plate. Excess antigen was removed, and blocking solution [PBS containing 5% milk for flu, or 1× Sigmablock (Sigma) for OVA] was added for 2 h for flu or overnight for OVA, at room temperature. Following removal of blocking solution, plates were incubated at room temperature for 2 h (flu) or overnight (OVA) with serial dilutions of individual samples diluted in the appropriate blocking buffer. Plates were washed with a multi-channel plate washer (NUNC) and incubated for 1 h with horseradish peroxidase-conjugated secondary goat anti-mouse γ or α chain-specific antibodies (Southern Biotechnology Associates or Sigma). Finally, plates were again washed, and *O*-phenylenediamine dihydrochloride substrate was added for 30 min. The reaction was stopped with the addition of 0.1 M NaF. Antibody endpoint titers are reported as the reciprocal of the highest dilution that resulted in an $OD_{450} \geq 0.2$. Data are presented as the geometric mean ± standard error of the mean (SEM).

Statistical Analysis. Antibody titers and cytokine values were evaluated for statistically significant differences by either the ANOVA or Mann-Whitney test (GraphPad INSTAT). A P value of ≤0.05 was considered significant.

RESULTS

Type I IFN signaling is not required for expression-vector-induced systemic immunity. Systemic and mucosal immunity can be induced by VRP used in two different modalities. In the first instance, VRP express an antigen encoded in the VRP genome (expression vectors). In the second, the antigen is supplied separately with the VRP contributing a mucosal and systemic adjuvant effect. We have examined the requirement for a functional type I IFN system for systemic and mucosal immunity induced by both VRP modalities. Groups of wildtype 129 Sv/Ev and IFN receptor knockout (RKO) mice were immunized in the rear footpad at weeks 0 and 4 with $1x10^5$ infectious units (IU) of VRP expressing the HA gene from influenza virus (HA-VRP). Two weeks following the second immunization, animals were sacrificed, and flu-specific systemic immune responses were measured by serum IgG ELISA and by IgG and IgA spleen ASC ELISPOT assay (figure 5-1). As shown in figure 5-1A, HA-VRP induced equivalent levels of flu-specific IgG antibodies in the serum of both wildtype and RKO mice. Consistent with this finding, similar numbers of flu-specific IgG- and IgA-secreting cells were evident in the spleens of both wildtype and RKO mice (figure 5-1B and 5-1C). Together, these results suggest that type I IFN signaling is not required for the induction of systemic immunity, both IgG and IgA, directed against VRP-expressed antigens.

Type I IFN signaling is required for expression-vector-induced mucosal IgA responses.

We have recently demonstrated that VRP expression vectors induce local mucosal IgA responses at multiple mucosal surfaces in mice including the upper respiratory tract (URT) (48), the gastrointestinal tract (8, 26, 48)**,** and the urogenital tract (48) even when inoculated at a nonmucosal site. Mucosal antibody responses were measured in wildtype and IFN RKO animals in the URT (ASC ELISPOT), gastrointestinal tract (ELISA on fecal extracts), and urogenital tract (ELISA on vaginal washes) (figure 5-2). As with systemic IgG, mucosal IgG appeared to be unaffected by the absence of type I IFN signaling with VRP expression vectors, evidenced by the observation that HA-VRP induced equivalent levels of flu-specific IgG-secreting cells in the URT in both wildtype and RKO mice. In RKO mice however, HA-VRP induced significantly reduced levels of IgA-secreting cells in the upper respiratory tract of RKO mice (figure 5-2B). To determine if the mucosal IgA defect in the URT of RKO mice was limited to this single mucosal surface or was also true of other mucosal surfaces, flu-specific mucosal IgA responses were analyzed at two additional mucosal surfaces. HA-VRP-inoculated RKO mice also generated reduced IgA antibody responses in fecal extracts (figure 5-2C) and vaginal wash fluids (figure 5-2D), representative of the gastrointestinal and urogenital tracts, respectively. These results suggest that type I IFN signaling plays a critical role in VRP expression-vector-induced mucosal IgA immunity, while playing only a minimal role, if any, in systemic immunity under the same experimental conditions.

VRP expression-vector-induced IgA production in the draining lymph node.   The immunological mechanism(s) underlying the induction of mucosal IgA after nonmucosal inoculation of VRP expressing an antigen, remain unclear. One hypothesis suggests that cytokines induced by the replication of VRP RNA convert the DLN into the functional

equivalent of a mucosal inductive site (Thompson *et al.*, in preparation, Chapter 3). A cardinal feature of such sites is the production of antigen-specific IgA antibodies, and this characteristic is observed in the DLN following inoculation of VRP expressing an antigen.

Here we sought to determine if type I IFN signaling affects the production of DLN IgA antibodies. Groups of wildtype 129 Sv/Ev and RKO mice were immunized in the rear footpad at weeks 0 and 4 with 1×10^5 IU of HA-VRP. At day 3 post boost (the timepoint at which VRP-induced IgA antibodies peaked in the DLN of BALB/c mice; Thompson *et al.*, in preparation), DLNs were harvested and incubated in media in a lymphoid organ culture assay as described (48). DLN culture supernatants were evaluated for the presence of flu-specific IgA antibodies by ELISA (figure 5-3). As shown in figure 5-3A, equivalent levels of flu-specific IgA antibodies were produced in wildtype and RKO mice. In the same experiment, HA-VRP also induced similar levels of flu-specific IgG antibodies in the DLNs of both wildtype and RKO mice (data not shown). These results suggest that the defect in mucosal IgA production at the mucosal surface following VRP expression vector delivery is not due to a lack of antigen-specific IgA antibodies in the DLN.

Type I IFN signaling is not required for VRP adjuvant-induced immunity. VRP, when used as an adjuvant, enhance systemic and mucosal antibody responses to co-delivered antigen (48). The systemic adjuvant effect of replicon particles derived from a related alphavirus, Semliki forest virus, was dependent upon type I IFN signaling (10). Therefore, the role of type I IFN signaling on VRP adjuvant activity was also evaluated. Groups of wildtype and RKO animals were immunized at week 0 and week 4 with 10 μg of OVA alone, or with 10 μg of OVA co-immunized with 1×10^5 IU null VRP (no transgene) as an adjuvant. Two weeks following the boost, animals were evaluated for the presence of OVA-specific

IgG antibodies in the serum and OVA-specific IgA antibodies in fecal extracts (Table 5-1). The inclusion of VRP in the inoculum as an adjuvant significantly increased the OVA-specific systemic IgG response in both wildtype and RKO mice, suggesting that IFN signaling does not play a critical role in systemic immune induction with VRP adjuvants. Likewise, VRP significantly increased fecal IgA responses to the same extent in both wildtype and RKO animals, suggesting that while IFN clearly plays a critical role in mucosal IgA induction when the antigen is expressed from the VRP genome, IFN signaling is not required for the induction of either mucosal IgA or systemic IgG when VRP are utilized as an adjuvant.

DISCUSSION

Alphavirus replicon particles are potent stimulators of adaptive immune responses, and provide a powerful model system for the study of host factors involved in the regulation of viral immunity. While the utility of VRP as a component of successful vaccines has clearly been established, little is known regarding the critical immunological factors which regulate VRP-induced immune induction. The type I IFN system has been implicated in several models of alphavirus-induced immunity. Here we present evidence that type I IFN signaling plays an important role in VRP-induced immune stimulation; specifically in the induction of mucosal IgA responses directed towards VRP-expressed antigen.

Our studies suggest that, at least in regard to the IFN system, the mechanisms which regulate immune induction to antigens expressed from alphavirus replicon particles are distinct from the immunoregulatory mechanisms operative when replicon particles are utilized as adjuvants. This notion is supported by the results presented here that mucosal IgA

responses induced by VRP expression vectors are significantly impaired in RKO mice; however, mucosal IgA responses directed against VRP adjuvanted antigen in RKO were equivalent to wildtype mice. Although Hidmark *et al*. did not measure mucosal IgA responses, they also report a different effect of the RKO defect on replicon expressed compared to replicon adjuvanted vaccines. The authors demonstrated that serum IgG responses directed against expressed antigen were equivalent between wildtype and RKO animals; however, the serum IgG adjuvant effect with SFV replicon particle was abrogated in RKO mice (10). Together, these results are consistent with a model in which alphavirus replicon particles rely on distinct mechanisms for immune induction when utilized as expression vectors as compared to adjuvants.

While work with both the VEE replicon particles and SFV replicon particles reveals a distinction between their use as expression vectors and adjuvants, there are important differences between results obtained with the two systems as well. The SFV replicon adjuvant effect in serum IgG was abrogated in RKO mice (10). This was not the case in the VEE system, as serum adjuvant activity was observed in RKO mice. To date, the mechanistic explanation regarding the differences between VEE and SFV have yet to be determined; however, several possibilities exist. One plausible explanation is that the initial targets of infection shape the IFN dependence for immune induction. VRP efficiently infect DCs both *in vitro* (33) and *in vivo* (29), (West, A., Whitmore, A., Moran, T., and Johnston, R., unpublished). In contrast, SFV does not appear to efficiently infect DCs *in vitro*, even at an MOI as high as 1000 (12). Additionally, while SFV infection has been shown to induce DC migration *in vivo* (16), infection of DCs by SFV has yet to be reported.

Other more subtle effects could also account for the differences observed between VEE and SFV. For example, both viruses induce type I IFN, however a careful comparison of the absolute levels induced by both viruses has not been carried out. Additionally, mammals encode multiple IFN α genes (47) and it is unclear exactly what role each individual α gene plays both in antiviral defense and activation of adaptive immunity. It is possible that both the absolute amount of IFN induced by VEE and SFV, as well as the distribution of the different α genes is different between the two viruses. Future experimentation will be required to fully elucidate the role of IFN in vaccine-induced immunity with VEE and SFV replicon particles.

Further study is required to discover the mechanism by which IFN specifically promotes mucosal IgA responses following delivery of VRP expression vectors. We have recently demonstrated that nonmucosal VRP delivery induces numerous characteristics of mucosal lymphoid tissues in the peripheral draining lymph, node including antigen-specific polymeric IgA antibodies, a population of B cells which express upregulated levels of the mucosal homing receptor, increased mucosal cytokine/chemokine expression, and expression of the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Thompson *et al.*, in preparation, Chapter 3). These results are supportive of a model in which the draining lymph node is converted into the functional equivalent of a mucosal inductive site.

The question of whether IFN exerts its effect at the level of the draining lymph node is still unclear; however, results presented here suggest that IFN is operative downstream of IgA class switch, as levels of both antigen-specific systemic IgA and DLN IgA were unaffected in RKO mice following VRP delivery. Instead, IFN may play a role in regulating VRP-induced cytokine responses in general, upregulation of the $\alpha_4\beta_7$ integrin, MAdCAM-1,

or a yet unidentified critical component. These results are consistent with a model in which IFN regulates the migration of VRP-activated IgA-secreting cells, promoting migration of IgA-secreting cells into the mucosal compartment. It has been reported that IgA-secreting cell migration occurs via the selective upregulation of specific integrins and chemokine receptors which may be affected by the production of type I IFN (18). It will be important to examine the VRP DLN in RKO mice for additional characteristics of mucosal lymphoid tissue, such as expression of MAdCAM-1 on the high endothelial venules and the $\alpha_4\beta_7$ integrin on DLN B cells as a means to identify the precise mechanism by which IFN promotes VRP expression vector-induced mucosal IgA responses.

The observation that IFN serves as a potent adjuvant for the induction of antibody responses to co-immunized antigen is consistent with a role for IFN in alphavirus-induced immunity (4, 20, 36); however, whether IFN plays a direct or indirect role in the VRP system remains to be determined. The direct role of IFN has been examined in studies using recombinant IFN as an adjuvant. In one report, increased IgA responses were not observed following IFN adjuvant treatment (20). It is possible that a specific subset of IFN α genes directly promote mucosal IgA responses in the VRP system, and this subset was not present in the recombinant preparations studied as an adjuvant in Le Bon *et al*. (20). In another study, recombinant IFN did promote mucosal IgA responses following nasal delivery in an influenza vaccine model (4, 36). It will be interestesting to examine the role of individual IFN α genes in the activation of mucosal IgA responses in general, and specifically in the VRP expression vector system.

An important point to consider in the interpretation of immune induction experiments in RKO mice is the differences in the level of antigen expression between wildtype and RKO

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mice. It is known that alphavirus vector-expressed antigen is markedly increased in animals lacking the type I receptor [(23) and White *et al*., in preparation], suggesting that, in wt mice, autocrine and paracrine IFN signaling limit alphavirus vector antigen expression (23). In experiments presented here, we concluded that IFN was not required for expression-vector-induced systemic immunity; however, this interpretation includes the caveat that equivalent systemic immune responses against the encoded antigen were induced under conditions in which antigen expression levels were markedly different. However, in the case of mucosal IgA responses induced in RKO mice with VRP expression vectors, a significant defect was observed in RKO mice even though much more antigen was present, demonstrating that increased antigen levels cannot replace the function of IFN signaling in this system.

In this report we provide further evidence for the role of the type I IFN system in regulating virus-induced adaptive immunity. Type I IFN played a critical role in the activation of mucosal IgA responses following delivery of VRP expression vectors. These results suggest a specific role for type I IFN in VRP-induced immunity, potentially the regulation of migration of IgA-secreting-cells into the mucosal compartment. Identification of the precise mechanism by which IFN promotes VRP-induced mucosal IgA should lead to both a basic understanding of the factors involved in virus-induced immunity as well as new strategies to increase the efficacy of VRP as mucosal vaccine vectors.

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Figure 5-1
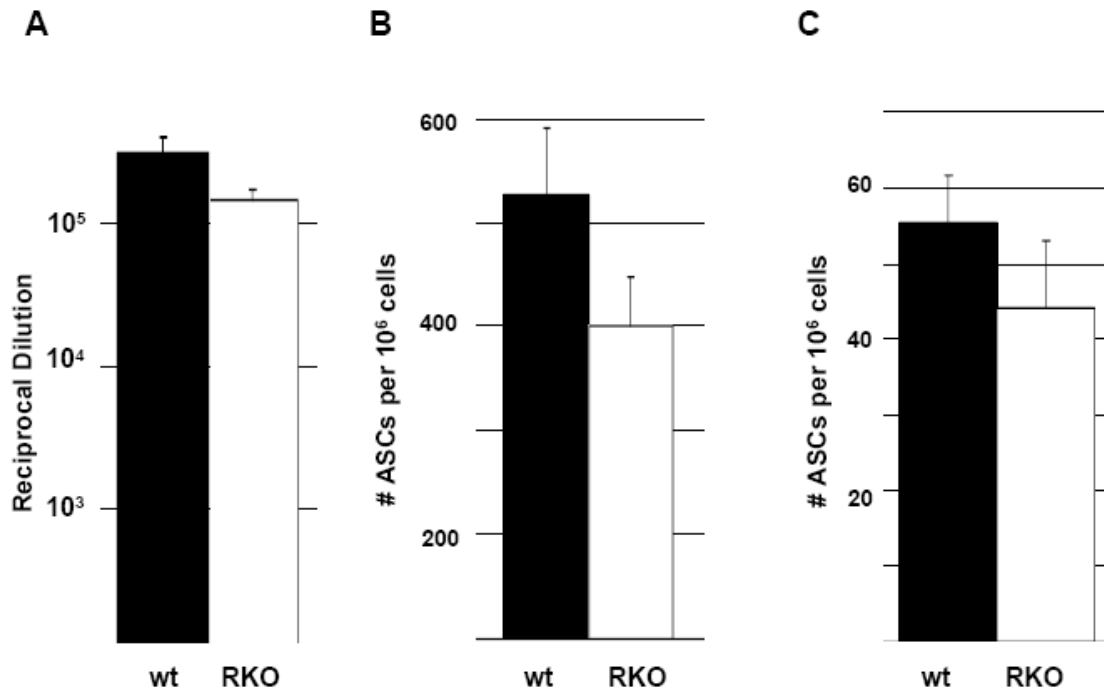
Figure 5-1. Type I IFN signaling is dispensable for expression-vector-induced systemic immunity.   Groups of wildtype (wt) and IFN α/β receptor knockout (RKO) mice were immunized in the rear footpad at weeks 0 and 4 with $1x10^5$ IU of HA-VRP. Two weeks following the boost, flu-specific serum IgG (A) antibodies were evaluated by ELISA. Additionally, flu-specific IgG- (B) and IgA (C) -secreting cells were evaluated by ASC ELISPOT. Values represent the geometric mean +/- SEM. No statistically-significant differences exist between responses induced in wt and RKO animals.

Figure 5-2
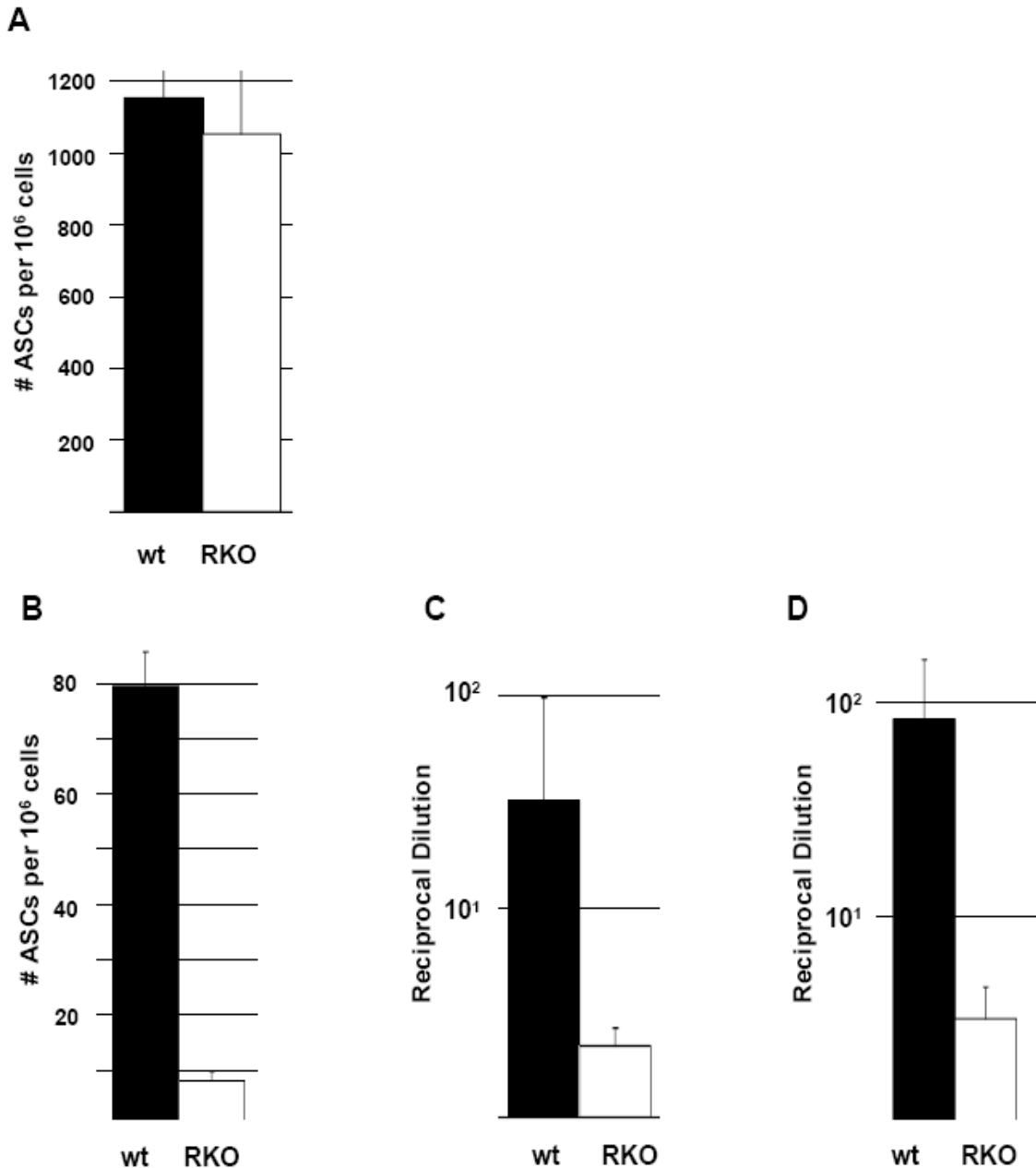
Figure 5-2. Type I IFN signaling is required for expression-vector-induced mucosal IgA responses. Groups of wildtype (wt) and IFN α/β receptor knockout (RKO) mice were immunized in the rear footpad at weeks 0 and 4 with 1×10^5IU of HA-VRP. Two weeks following the boost, flu-specific IgG- (A) and IgA- (B) were analyzed by ASC ELISPOT of URT cells. Also, flu-specific IgA antibodies were evaluated in fecal extracts (C) and vaginal washes (D) by ELISA. Values represent the geometric mean +/- SEM. IgA responses in wt mice are all statistically greater than IgA responses in RKO mice (Mann-Whitney).

Figure 5-3
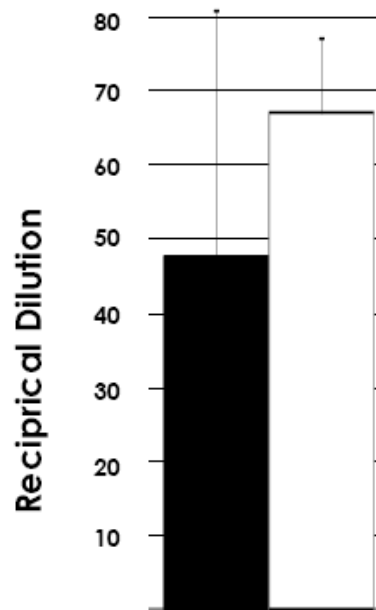
Figure 5-3. Type I IFN signaling is not necessary for expression-vector-induced DLN IgA. Groups of wildtype (wt) and IFN α/β receptor knockout (RKO) mice were immunized in the rear footpad at weeks 0 and 4 with $1x10^5$ IU of HA-VRP. Three days following the boost lymphoid cultures where established from the DLNs and supernatants were assayed for the presence of flu-specific IgA antibodies by ELISA. Values represent the mean +/- SEM.

Table 5-1

	OVA-Specific Serum IgG (x 10^4)		OVA-Specific Fecal IgA	
	129	RKO	129	RKO
OVA	4.5+/-2.1	5.0+/-1.6	3+/-1	4+/-2
OVA+VRP	>100	>100	64+/-18	72+/-46

Table 5-1. Type I IFN signaling is not required for VRP adjuvant-induced immunity. Groups of wildtype (wt) and IFN α/β receptor knockout (RKO) mice were immunized in the rear footpad at weeks 0 and 4 with OVA alone, or OVA plus 1x10^5 IU of nullVRP. Two weeks following the boost, OVA-specific serum IgG and fecal IgA- antibody responses were evaluated by ELISA. Values represent the geometric mean +/- SEM.

CHAPTER SIX

DISCUSSION AND FUTURE DIRECTIONS

The development of successful vaccines against human pathogens, such as smallpox and polio, is one of the most significant accomplishments in the field of medical intervention in the last 200 years. While traditional vaccination methods have proven effective in numerous cases, new vaccine technologies will be required to combat the continued threats posed by infectious diseases. Here we describe one such technology, alphavirus replicon particles, and present the viral and cellular components involved in alphavirus-induced immunity. A paucity of information exists regarding the definitive mechanisms by which vaccines induce protective immunity. Thus, we have attempted to delineate the viral trigger(s) of immune induction as well as the immunological pathways which lead to protection/immunity following vaccination.

Molecular Mechanisms of Alphavirus-induced Immunity

Role of DC targeting in VRP-induced immunity. Infection with alphaviruses, as is the case with most infectious agents, initiates a multi-pronged, overlapping cascade of events in infected cells, and in the animal or vaccinee. Because of this, defining a causative relationship between a single cellular pathway and the induction of an immunological response has proved elusive. Venezuelan equine encephalitis virus replicon particles display several features which potentially contribute to their potent immunogenicity. Targeting experiments in the mouse model suggest that VRP preferentially infect DCs following parenteral inoculation (26). Indeed, MacDonald and Johnston proposed that skin-derived Langerhans cells represent the early targets of VEE infection. An understanding of the specific cellular targets of VEE infection may provide valuable information regarding the critical immune inductive mechanisms operating in the context of VRP vaccines. The studies

presented here suggest that VRP infection promotes a massive influx of DCs into the VRP-infected draining lymph node. VRP infection promoted a preferential recruitment of CD11b$^+$ DCs to the DLN, although CD11b$^-$ DCs were also significantly increased. Interestingly, this increase was manifested by a significant increase in both the total cellularity of the DLN (8-fold), as well as a 10-fold increase in the proportion of CD11b$^+$ DCs in the DLN. Combined, these effects result in a dramatic increase in the total number of CD11b$^+$ DCs in the VRP-infected DLN.

While VRP infection dramatically affects the APC makeup in the DLN, it will be important to determine which cells are targeted by VEE. Our initial studies suggest that CD11b$^+$ DCs represent the major early target of VRP infection. Next, it will be important to determine the relationship between the VRP-infected DCs in the DLN and the DCs which present viral antigen and activate viral immunity. Interestingly, following skin infection of mice with HSV, influenza virus, and vaccinia virus, Langerhans cells, while critical for transferring viral antigens to the DLN, were incapable of stimulating antigen-specific CD8$^+$ T cells *in vitro*. Instead, the CD8$^+$ dermal DCs were found to be the only DC subset in the lymph node capable of stimulating antigen-specific CD8$^+$ T cells *in vitro* (2, 3). Belz *et al*. suggested a model in which Langerhans cells take up and transport viral antigens to the DLN following cutaneous viral infection and transfer these antigens to the dermal DCs where they initiate adaptive immunity (5). We are currently testing this model in the VRP system by evaluating the subset of DCs which are targeted by VRP and comparing this to the DC subset(s) capable of stimulating *in vitro* CD8$^+$ T cell responses specific for both encoded and co-delivered antigens. It is possible that the DC subsets involved in the activation of CD8$^+$ T cell responses under conditions in which antigens are encoded in the viral genome are

different than when VRP are used as an adjuvant, and antigen is not necessarily present in every VRP-infected cell. Such analysis should shed new light on the role of DC targeting and DC APC function in alphavirus-induced immunity.

Immunostimulatory properties of VRP. In addition to targeting vaccine antigens to DCs *in vivo*, VRP possess several other properties which may also play a role in their potent immunogenicity, including high level antigen expression in infected cells. Alphavirus replicons express heterologous antigens under the control of the 26S subgenomic promoter, which is significantly stronger than the commonly utilized cytomegalovirus (CMV) promoter (13). It is possible that high level transgene expression is the dominant mechanism of replicon-induced immune induction. However, here we demonstrate that VRP possess intrinsic immunostimulatory properties which are independent of antigen expression, as VRP promote potent immune induction to non-VRP-expressed antigens. These observations suggest that while high level antigen expression most likely does play a role in alphavirus expression-vector-induced immunity, the host innate immune response to the infection is critical for immune induction to both expressed and co-delivered antigens.

The induction of strong innate immune responses as a means to stimulate adaptive immunity is a developing theme in several experimental systems (15, 20, 23, 33). Here we have begun to characterize the early inflammatory pathways which are activated by alphavirus infection. VRP infection resulted in a significant increase in several proinflammatory and immunoregulatory factors in the DLN such as IL-1β, IL-6, TNF-α, IFN-γ, MIP-1β, and RANTES, consistent with the idea that VRP activate a strong innate immune response. Whether immune induction with VRP is dependent upon any or all of

these cytokines and/or chemokines remains to be determined. It is possible that VRP-induced immunity is dependent upon the induction of a single cytokine or chemokine, or conversely, it is more likely that immune induction is a cumulative property of a suite of inflammatory responses. It is also important to consider in this context that an individual cytokine or group of cytokines may play a more dominant role in the activation of one arm of the adaptive response versus another. For example, VRP-induced IFN-γ, while critical for the activation of T cell responses to either VRP-expressed or co-delivered antigens, may or may not be required for activation of humoral immunity. An additional consideration in this regard is the idea that a single cytokine or group of cytokines may have differential requirements for immune induction when using VRP as expression vectors as compared to VRP adjuvants. Consistent with this idea, type I interferon signaling was required for the induction of mucosal IgA responses with VRP expression vectors, but not with VRP adjuvants. The next step in the process should involve further immune induction experiments with both alphavirus expression vectors and adjuvants in animals either genetically devoid, or antibody depleted, of candidate cytokines involved in immune induction. These studies should allow the identification of critical host responses involved in alphavirus-induced immune induction. Any host immunological factors implicated in alphavirus-induced immunity in these studies would also serve as prime targets for cellular components which contribute to alphavirus pathogenesis (see below).

Alphavirus PAMP recognition. While we are beginning to understand the inflammatory mediators that are induced by alphavirus infection, the upstream recognition and signaling pathways which initiate these responses have yet to be determined. We have

speculated that an alphavirus-specific pathogen-associated molecular pattern (PAMP) is contained within replicon particles, or produced within replicon-infected cells (39). We first sought to address this question by evaluating the role of VRP RNA replication in the adjuvant effect. It is not feasible to evaluate RNA replication as a component of immune induction in the expression vector system, as mutations and/or treatments which would affect RNA replication, would also impact antigen expression, clouding the interpretation of such studies. We found that UV-treated VRP failed to induce an adjuvant effect in both the systemic and mucosal compartments. These results are consistent with a model in which VRP RNA replication is recognized by a cellular sentinel molecule which, once activated, initiates an immunological cascade which ultimately culminates in immune induction to co-delivered antigen. We are currently evaluating VRP-induced immunity, both in the adjuvant and expression vector systems, in animals genetically deficient for several candidate sentinel molecules such as TLR3 (1), MyD88 (7, 17), MDA5 (43), and MAVS (37). Such analysis should allow us identify an alphavirus PAMP(s) and to assess the role of both the major intracellular viral RNA recognition pathways in alphavirus-induced immunity, as well as in alphavirus pathogenesis.

Our studies with UV-treated VEE replicon particles suggest that the alphavirus adjuvant effect is UV sensitive, and therefore most likely dependent upon VRP RNA replication as a trigger for immune induction. However, Hidmark *et al*. recently demonstrated a serum IgG adjuvant effect with UV-treated replicons derived from the related alphavirus, Semliki forest virus (18). The mechanistic explanation for this discrepancy in the VEE and SFV systems is unclear at this point; however, several explanations are plausible. A significant complication in the interpretation is the fact that it is impossible to determine

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the exact amounts of UV applied to both replicon particles, as they were treated under different experimental conditions. One potential explanation is that the UV treatment in the VEE system was sufficiently strong as to affect not only the structure of the nucleic acid, but also the conformation of the glycoproteins on the particle surface, functionally inhibiting binding, entry, and/or uncoating. Conversely, it is possible that the UV treatment in the SFV system, while sufficient to inhibit the viral protein expression *in vitro*, did not fully abrogate RNA replication *in vivo*. In support of this idea, we have demonstrated that the mouse is at least 10-fold more sensitive to VRP than cell lines *in vitro*. This conclusion stems from the observation that, following subcutaneous delivery of 0.1 BHK cell IUs of HA-expressing VRP, seroconversion occurred in 100% of the immunized animals. This observation is consistent with a model in which animals did not seroconvert to less then 1 IU of VRP, but instead, at least 10 times more mouse infectious units are present in VRP preparations than BHK cell infectious units. Therefore, it will be important to assess the effects of a UV dose titration on VEE and SFV replicon RNA replication *in vivo* to clarify this issue. A third explanation is that New World and Old World alphaviruses rely on distinct immunological pathways for immune induction. Support for this idea stems from the VEE and SFV studies in type I interferon receptor knockout mice. In the SFV system, type I interferon signaling was critical for the serum IgG adjuvant effect (18); however, VEE replicons exerted an adjuvant effect in type I interferon receptor knockout mice (Thompson *et al*., in preparation, Chapter 5). It is possible that VEE induces additional cytokines which substitute for type I interferon whereas SFV does not. Further experimentation will be necessary to clarify these issues and should provide important insights into alphavirus-induced immune induction in general, and the potential to optimize alphavirus-vectored vaccines.

The VRP Alternative Pathway for Mucosal Immune Induction

While we have performed the initial characterization of both the early targets of VRP infection, as well as the phenotype of the VRP-induced mucosal immune response, the molecular details of the intervening events have yet to be fully elucidated. We are currently attempting to define the individual steps which occur following infection of DCs and prior to the presence of antibodies in mucosal secretions. Although mucosal antigen delivery appears to be the most efficient method for generating a mucosal immune response (28, 29), a growing body of evidence supports the existence of a pathway capable of inducing mucosal immune responses following nonmucosal delivery [reviewed in (8, 40), (Thompson *et al*., in preparation, Chapter 3)]. Our studies suggest that the VRP DLN is a critical component of the alternative mucosal immune induction pathway (Thompson *et al*., in preparation, Chapter 3). Several markers of mucosal lymphoid tissues were present in the VRP-infected DLN including antigen-specific polymeric IgA antibodies, B and T lymphocytes bearing the mucosal homing receptor, expression of MAdCAM-1 on the high endothelial venules, and increased mucosal cytokine secretion. However, the precise mechanism by which the VRP DLN activates mucosal immune responses has not been fully elucidated.

VRP-induced mucosal homing. The migration of lymphocytes into the mucosal compartment represents a critical regulatory step in the mucosal inductive pathway (10). Much is known regarding the individual components which promote mucosal homing (10). As mentioned previously, the $\alpha_4\beta_7$ integrin, termed the mucosal homing receptor plays a dominant role in homing to the gut mucosa. Our studies demonstrate that VRP induce a significant increase in $\alpha_4\beta_7$ integrin expression on B cells and $CD8^+$ T cells present in the

DLN. This suggests that the $\alpha_4\beta_7$ integrin-MAdCAM-1 pathway plays a role in mediating mucosal migration in the VRP system; however, this has not been proven. It will be interesting to determine whether VRP promote mucosal immune induction in β_7 integrin-deficient animals (41) or if this pathway is not functionally required, despite the fact that it appears to be activated in the DLN. If β_7 integrin-deficient animals do in fact have defects in VRP-induced mucosal immunity, it would suggest that at least some components of the peripheral VRP pathway and the natural pathway are overlapping. Other mediators of mucosal migration, especially in the gut, include CCR9-TECK and CCR10-MEC interactions. Evaluation of VRP mucosal immune induction in both CCR9 and CCR10 knockouts would likewise provide important insights into the VRP alternative pathway. Again, the finding of a defect in mucosal immune induction with VRP in one or more of these mouse models would warrant the further evaluation of such proteins in VEE pathogenesis.

Classical mucosal homing experiments involved harvesting lymphocytes from mucosal inductive tissues, such as the PPs and/or MLN, and monitoring homing to the mucosal compartment following adoptive transfer. In fact, lympocytes of mucosal origin do repopulate the mucosal compartment following adoptive transfer (27, 35). If the VRP DLN is truly converted into the functional equivalent of a mucosal inductive site, then one might predict that cells isolated from the DLN of VRP-immunized mice may have the capacity to home to mucosal tissues following adoptive transfer. These experiments are underway and, if positive, will provide a strong functional significance for the role of the VRP-infected DLN in mucosal immune induction.

VRP-induced DLN IgA. Our studies suggest that the DLN is a very early source of VRP-induced IgA antibodies, as antigen-specific IgA is detected in the DLN prior to detection in mucosal secretions. A significant proportion of the DLN IgA antibodies were found to be dimeric or polymeric in nature. These are characteristics of IgA antibodies produced at mucosal sites (6, 12, 31) and further support the notion of the DLN as part of a mucosal inductive pathway. It will be interesting to determine if the VRP-induced DLN polymeric IgA antibodies are functionally transported into mucosal secretions. We are currently evaluating the ability of DLN IgA to be recognized and transported through polarized epithelial cells expressing the pIgR (19) as a model system for *in vivo* mucosal transport. Additionally, it would be interesting to measure the ability of VRP to induce IgA antibodies in both the DLN as well as at the local mucosal surface in animals which have been genetically engineered to produce an increased monomeric to polymeric IgA ratio, such as J chain knockout mice or pIgR knockout mice. The results of such studies should further characterize the mucosal characteristics of DLN IgA and provide an additional test to the VRP alternative pathway model (21).

While the presence of IgA in the DLN of VRP-vaccinated mice is not disputed, the anatomical location in which IgA class switch occurs has yet to be determined. It is theoretically possible that the cells producing IgA antibodies in the DLN at day 3 post boost received the IgA class switch signals somewhere other then the DLN (such as the PP) and then migrated back to the inflamed DLN to initiate IgA secretion. Or conversely, that IgA class switch occurred in the DLN just prior to IgA secretion. As a first step in addressing whether IgA class switch occurs in the VRP-infected DLN, we are evaluating whether the cytokines present in the DLN of VRP-immunized mice possess the capacity to promote IgA

class switch *in vitro*. Several cytokines which we have shown to be present in the DLN, such as IL-5 (22), IL-6 (22), and TNF-α, are known to promote either IgA class switch or secretion from IgA-switched cells. We are currently utilizing DLN PBS extracts as a source of cytokines driving IgA class switch of LPS-treated B cells in culture (4). If VRP DLN extracts in fact promote IgA class switch, the stimulating cytokine(s) will be identified in a series of blocking studies with monoclonal antibodies directed against specific cytokines (32).

VRP-induced mucosal IgG/IgA ratio. A large body of evidence supports the notion that antibodies of the IgA isotype are the most abundant antibodies present in mucosal secretions, although IgG and IgM antibodies are also found in mucosal secretions at much reduced levels compared to IgA (11, 28-30). Our studies clearly demonstrate that VRP stimulate the production of antigen-specific IgA antibodies at the mucosal surface. However, an interesting finding in our analysis of VRP-induced mucosal immune induction is that VRP consistently stimulate the production of greater than 10-fold more antigen-specific IgG antibodies than IgA antibodies in mucosal secretions. This observation holds true in the upper respiratory tract (nasal washes, nasal lymphocyte ASC ELISPOT, nasal epithelium lymphoid cultures), the gastrointestinal tract (fecal extracts, LPL ASC ELISPOT, gut lymphoid cultures), the urogenital tract (vaginal lavage fluids) (16, 24, 39), (Thompson J. M., Richmond, E. M., and Johnston, R. E., unpublished) as well as in the VRP DLN (Thompson *et al.*, in preparation, Chapter 3). It has been proposed that IgG antibodies in mucosal secretions may play an important role in mucosal defense (9, 36). In fact, significant levels of IgG antibodies are found in secretions from the female reproductive tract (9, 36). The

exact signals which promote an increased ratio of mucosal IgG to IgA in the VRP system as compared to the IgG to IgA ratio following, for example, nasal antigen delivery in the presence of CT remain to be determined.

Irrespective of the inductive mechanism(s) responsible for mucosal IgG production, we speculate that VRP-induced mucosal IgG antibodies may play a significant role in mediating protection from mucosal challenge in the alphavirus system. We have demonstrated recently that HA-expressing VRP not only protect animals from the overt signs of disease following intranasal challenge with influenza virus, but also limit challenge virus replication to levels below the limits of detection by plaque assay as well as HA-specific *in situ* hybridization (Richmond E. M., Thompson, J. M., Davis, N. L., Brown, K., West, A. C., and Johnston, R. E., unpublished). It will be interesting to determine what respective roles nasal IgG and IgA antibodies play in mediating this protective effect. Several models of mucosal IgA deficiency exist including J chain knockouts (25) and pIgR knockouts (21); however, we propose to utilize local depletion of either nasal IgG and/or IgA by intranasal installation of anti-mouse IgG and/or IgA antibodies as developed by Renegar and Small (34), in combination with our nasal influenza challenge model, to identify the protective isotype(s). The results of these studies should significantly improve our knowledge of VRP-induced protective immunity.

Implications for VEE Pathogenesis

Role of DC targeting in VEE pathogenesis. The studies described here clearly demonstrate the utility of VRP as components of successful vaccines and provide a powerful model system for the study of viral immunology. Our findings may also have implications

for the pathogenesis of VEE as well. While a thorough examination of all the individual APC subsets present in the lymph node following VRP delivery has yet to be performed, our studies clearly demonstrate that VRP infection leads to a significant recruitment of DCs to the DLN. One possibility is that recruitment is a generalized host response to an inflammatory environment in the DLN. Consistent with this idea, VEE promotes the strong induction of several inflammatory mediators in the DLN such as IL-1β, TNF-α, and IL-6. A second possibility is that VEE specifically recruits its own cellular targets of infection to the DLN as a means to promote spread in the animal. However, it is not immediately apparent exactly why this would be advantageous to the virus, as virus replication occurs in nearly every organ in the animal at later times post infection, suggesting the cellular targets of infection are not limiting. Why the first round of infection, as modeled by VRP delivery, demonstrates preferential infection of DCs and not infection of the downstream visceral organs is unclear at this point. It is possible that DC-derived viruses are somehow better suited for either infection of, or replication within, downstream tissues *in vivo*. Glycosylation patterns in DCs could potentially give rise to viruses with an altered infectivity profile for downstream tissues, an enhanced ability to cross the blood-brain barrier, or the capability to evade the next wave of host innate immune responses. Shabman *et al.* recently demonstrated that the cell type in which alphaviruses are grown dramatically affects both the infectivity of progeny virions for DCs as well as their ability to interfere with the type I interferon response (38). This hypothesis presents a testable model. It would be interesting to evaluate the replication kinetics and *in vivo* pathogenesis of DC-grown virus as compared to fibroblast-grown virus following intraveneous inoculation to bypass the DC targeting steps.

It is also possible that VEE recruits DCs to the lymph node as a means to cripple their APC function. VEE causes significant apoptosis in DC cultures (Moran *et al.*, submitted), and recruitment of DCs to the pro-inflammatory environment of the DLN could hinder APC function directly through the activation cell death pathways in infected cells, or indirectly through cytokine and/or chemokine signals to neighboring, uninfected cells. Alternatively, virus-induced recruitment may not affect the viability or functionality of DCs, but instead provide a mechanism to sequester DCs in the DLN, blocking their function in anti-viral defense by limiting access to viral antigen and cells of the adaptive immune system in downstream tissues. Consistent with this idea, recruitment of DCs to the DLN following VRP delivery continued through day 3 post infection; a timepoint in which downstream tissues are already infected following delivery of replicating virus. A more careful examination of the kinetics of DC recruitment to the DLN following VRP delivery may help to determine what role, if any, viral recruitment of DCs to the DLN plays in VEE pathogenesis.

Role of lymphocyte migration in VEE pathogenesis. One of the most common clinical manifestations of VEE disease in humans, horses, and mice is an acute lymphopenia (42). The exact mechanisms responsible for this clinical outcome have not been clearly elucidated (14). One potential explanation is that lymphocytes undergo programmed cell death as a result of direct or indirect signals provided by virus infection. Alternatively, clinical presentation of lymphopenia could also occur following virus-induced lymphocyte migration to sites which would preclude their detection systemically. Our studies suggest the VRP infection dramatically affects cellular migration and trafficking patterns of lymphocytes

in *vivo*. VRP induced a significant influx of lymphocytes into the draining lymph node at early times post infection (Chapter 3) and migration of lymphocytes into the mucosal compartment at later times post infection (Chapter 4). These results are consistent with the idea that VEE infection dramatically affects the migratory properties of lymphocytes and begs the question of whether VRP promote the mobilization of lymphocyte migration into the mucosal compartment in a non-specific manner. It remains to be determined if this is really the case. It will be interesting to determine if VRP infection promotes mucosal migration of lymphocytes with a defined, unrelated specificity, to the VRP-associated antigen or if the observed migration is activation of cells specific for "contaminating" antigens in VRP preparations. While the quantitative analysis of cells present in the URT following VRP delivery does suggest that the URT is the single reservoir of systemic lymphocytes, it is possible that mucosal lymphocyte migration contributes to the lymphopenia observed following natural VEE infection. We are currently evaluating whether VRP infection promotes lymphocyte migration to other mucosal surfaces in addition to the URT. Further experimentation will be required to evaluate the role of mucosal lymphocyte homing in VEE pathogenesis; however, these studies typify the interplay between pathogenesis and vaccine development in the alphavirus system.

Optimization of VRP Vaccines

The primary focus of the studies presented here has been the identification of the viral and immunological factors which regulate alphavirus-induced immunity. The discovery of the alphavirus adjuvant effect has provided a powerful experimental system which has turned out to be extremely useful in this regard. We are also pursuing VRP as vaccines in a number

of systems. In fact, a phase I human trial has recently been completed utilizing VRP expressing HIV antigens. Therefore, regardless of the immune inductive pathways stimulated by VRP, the ability of VRP to promote protective immunity may significantly improve human vaccines. Thus, the optimization of VRP-induced immunity represents an important area of development.

The dosing schedule utilized in these studies was based upon our experience optimizing serum IgG responses induced by VRP expression vectors. Additional schedules including multiple dosing as well as combining several routes should be explored as a means to improve the utility of VRP vaccines, with special emphasis on induction of mucosal immunity. Additionally, here we have focused on the characterization of VRP adjuvant activity. Future work should combine the various alphavirus vaccine platforms (expression vectors, adjuvants, DNA-launched expression vectors) into a single immunization protocol. Furthermore, in order to identify immunological mechanisms involved with alphavirus-mediated immune induction we have focused on single modality studies; however, VRP may prove to perform better as components of successful combination vaccines. In support of this idea, VRP and CpG worked together in an additive manner to increase immunity to co-delivered antigen. Future studies should expand to test other modalities in combination with VRP in search of synergistic effects. This search should include other TLR agonists, both old and new vaccine technologies, as well as potentially delivering VRP in the skin through transcutaneous immunization. We believe that further dosing and combinational optimization has the potential to significantly improve the utility of VRP-based vaccines and adjuvants, and may enable successful vaccination protocols for the production of anti-pathogen immunity in humans.

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