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THE CONTRIBUTION OF TYPE I INTERFERON SIGNALING TO IMMUNITY INDUCED BY ALPHAVIRUS REPLICON VACCINES

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Abstract

The type I interferon (IFN) system is critical for protecting the mammalian host from numerous virus infections and plays a key role in shaping the 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 (wt) and IFN α/β receptor knockout (RKO) mice. HA-VRP vaccination induced equivalent levels of flu-specific systemic IgG, mucosal IgG, and systemic IgA antibodies in both wt 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. In the VRP adjuvant system, co-delivery of null VRP with ovalbumin (OVA) protein significantly increased the levels of OVA-specific serum IgG, fecal IgG, and fecal IgA antibodies in both wt and RKO mice, suggesting that type I IFN signaling plays a less significant role in the VRP adjuvant effect. Taken together, these results suggest that, 1) at least in regard to IFN signaling, the mechanisms which regulate VRP-induced immunity differ when VRP are utilized as expression vectors as opposed to adjuvants, 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.

Keywords

viral immunity; Type I IFN; mucosal IgA; adjuvant activity

1. INTRODUCTION

The type I interferons (IFNs) are a family of pleiotropic cytokines which were originally identified for their ability to interfere with virus replication [1], and are now known to provide the first line of defense against numerous viral pathogens [2]. Type I IFNs, which include IFNα and IFNβ, signal through a common receptor, the type I IFN receptor, which is expressed on almost all cell types [3]. The importance of IFN signaling in antiviral defense is evidenced by the observation that animals with an engineered genetic deficiency

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in the IFNα/β receptor (IFNα/β receptor knockouts, or RKOs) are acutely susceptible to numerous viral infections [4–7].

In addition to its role in innate immunity, it has recently been appreciated that type I IFN signaling also plays an important role in the activation of adaptive immune responses [8–12]. Type I IFN provides a powerful activation signal to differentiated dendritic cells (DCs), promoting co-stimulatory molecule expression and their antigen-presenting-cell (APC) function [12]. Moreover, interferon-treated DCs, upon interacting with B cells, activate immunoglobulin (Ig) class switch recombination and Ig secretion [13] to multiple isotypes, including IgA [14]. In addition to effects on DCs, IFN signaling is directly required for complete activation of B cells [15], $CD4^+$ T cells [16], $CD8^+$ T cells [17], and natural killer cells [18].

Vaccine vectors based on the alphavirus, Venezuelan equine encephalitis virus (VEE), contain a message-sense, single-stranded RNA genome [19] and have proven to be efficacious inducers of antigen-specific immunity in several pre-clinical vaccination models. VEE replicon particles (VRP) function as antigen expression vectors, encoding a modified genome in which the structural genes are replaced with a heterologous antigen [20]. Following VRP infection, the replicon RNA encoding the transgene is expressed at very high levels in the first infected cells; however, progeny virions are not produced. VRP stimulate potent systemic and mucosal antibody responses directed against both the antigen carried in the viral genome and soluble antigens simultaneously delivered during a concomitant VRP infection ([21–23]; Thompson *et al.*, submitted).

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 [24]. Leitner *et al.* demonstrated a role for type I IFN signaling with an alphavirus 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 [25]. 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 interferon stimulated gene, RNaseL [26]. Moreover, Hidmark *et al.* recently demonstrated that the systemic IgG adjuvant effect of SFV replicon particles is dependent upon type I IFN signaling, as SFV replicons failed to augment serum IgG responses directed against co-delivered antigen 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 andc 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 vaccinated 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 offers a new perspective on the precise role of the IFN pathway in alphavirus-induced immunity.

2. MATERIALS AND METHODS

2.1. VEE replicon constructs

The construction and packaging of VRP was performed as previously described [20, 27]. Briefly, confluent monolayers of BHK-21 cells were co-electroporated with the *in*-vitro-transcribed replicon RNA and two defective helper RNAs which express the viral structural genes *in trans*. In this study, two different replicon constructs were utilized: 1) VRP expressing the HA gene from the A/PR/8/34 strain of influenza virus (HA-VRP); and 2) VRP which lack a functional transgene downstream of the 26S promoter (null VRP) [23]. HA-VRP and null VRP were quantitated by immunocytochemistry of infected BHK cells with anti-sera against HA [20] and null VRP [23], respectively. All replicon particles utilized in this study were packaged in the wild-type (V3000) envelope.

2.2. Animals and immunizations

Groups of 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 [23]. Breeder pairs of 129 Sv/Ev animals were obtained from Dr. Barbara Sherry, North Carolina State University, or were purchased from Taconic Laboratories and breeder pairs of the RKO animals were obtained from Dr. Herbert Virgin, Washington University. Animals were immunized at week 0 and week 4 with either HA-VRP or ovalbumin (OVA, Sigma) in the presence or absence of null VRP as an adjuvant in low endotoxin, filter-sterilized PBS.

2.3. Antibody-Secreting-Cell (ASC) enzyme-linked immunospot (ELISPOT)

Splenocytes and nasal lymphocytes were prepared from immunized animals as previously described [23] and evaluated in by ASC ELISPOT assay, modified from Thompson *et al* [23]. Briefly, purified influenza virus antigen (500ng/well, Charles River Spafas) was used to coat 96-well nitrocellulose membrane plates (Millipore) overnight at 4°C. Plates were blocked for 2 hrs with complete media (10% serum) and two fold dilutions of single cell suspensions were then added to plates in duplicate and incubated overnight. Plates were washed, and bound spots were detected by the addition of HRP-conjugated goat anti-mouse γ or α chain-specific antibodies (Southern Biotechnology Associates), followed by addition of 3-amino-9-ethylcarazole (AEC, Sigma). ASCs were enumerated with a computerized ELISPOT plate reader (Immunospot) and data are presented as the number of antigen-specific ASCs per 10^6 cells plated.

2.4. Sera, Fecal Extracts, and Vaginal Washes

All sample collection was prepared as previously described [23]. 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). Fecal extracts and vaginal lavage fluids were prepared from individual animals as previously described [23]. Samples were analyzed for the presence of antigen-specific IgG and IgA antibodies via ELISA (see below).

2.5. Enzyme Linked Immunosorbant Assay (ELISA)

ELISAs for influenza- and OVA-specific antibodies were performed on serum, fecal extracts, and vaginal washes as previously described [23]. Briefly, antigen solutions were incubated in 96-well plates (Costar) overnight at 4°C to allow antigens to bind to the plate and plates were blocked for 2 h for flu or overnight for OVA, at RT. 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 and incubated for 1 h with HRP-conjugated goat anti-mouse γ or α chain-specific antibodies (Southern Biotechnology Associates or Sigma). Finally, plates were washed, and developed with *O*-phenylenediamine dihydrochloride substrate for 30 min. 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).

2.6. Statistical Analysis

Antibody titers and ASC values were evaluated for statistically significant differences by the Mann-Whitney non-parametric test (GraphPad INSTAT). A p value of ≤0.05 was considered significant. The Bonferroni correction for multiple comparisons was applied to the data presented in Fig. 3, as appropriate.

3. RESULTS

3.1. 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 (wt) 129 Sv/Ev and IFN receptor knockout (RKO) mice were immunized in the rear footpad at weeks 0 and 4 with 1×10^5 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 (Fig. 1). As shown in Fig. 1A, HA-VRP induced equivalent levels of flu-specific IgG antibodies in the serum of both wt and RKO mice. Consistent with this finding, similar numbers of flu-specific IgG- and IgA-secreting cells were evident in the spleens of both wt and RKO mice (Fig. 1B, Fig. 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.

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

VRP expression vectors induce local mucosal IgA responses at multiple mucosal surfaces in mice including the upper respiratory tract (URT) [23], the gastrointestinal tract [21–23], and the urogenital tract [23], even when inoculated at a nonmucosal site. Therefore, mucosal antibody responses also were measured in wt and IFN RKO animals following HA-VRP delivery (Fig. 2). As observed with systemic IgG, the mucosal IgG response 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 (Fig. 2A). In contrast, HA-VRP failed to induce significant reduced levels of IgA-secreting cells in the upper respiratory tract of RKO mice as compared to wt mice (Fig. 2B). To determine whether 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 in mucosal samples derived from the gastrointestinal and urogenital tracts. HA-VRP-inoculated RKO mice also failed to generate significant IgA antibody responses in fecal extracts (Fig. 2C) and vaginal wash fluids (Fig. 2D). 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.

3.3. Role of type I IFN signaling on the VRP adjuvant effect

While IFN signaling has a clear effect on expression-vector-induced mucosal igA responses, we next sought to evaluate VRP adjuvant activity in RKO mice. Therefore, groups of wt 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 IgG and IgA antibodies in fecal extracts as a measure of systemic and mucosal immunity, respectively (Fig. 3). In the systemic compartment, the inclusion of VRP in the inoculum significantly adjuvanted the OVA-specific systemic IgG response in both wt (~100-fold, p=0.0044) and RKO (~30-fold, p=0.0044) mice, suggesting that IFN signaling does not play a critical role in systemic immune induction with VRP adjuvants (Fig. 3A). However, VRP adjuvanted responses were approximately 3-fold higher in wt mice as compared to RKO animals (p=0.025). In the mucosal compartment, VRP significantly increased fecal IgG (Fig. 3B) and fecal IgA (Fig. 3C) responses to the same extent in both wt and RKO animals, suggesting that, while IFN is critical for mucosal IgA induction when antigen is supplied from the VRP genome, IFN signaling plays only a minimal role in the VRP adjuvant effect.

4. DISCUSSION

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. 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, these authors also report a differential effect of the RKO defect on SFV-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 [28]. 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 versus adjuvants.

While work with replicon particles derived from VEE and SFV has unveiled their dual utility as both expression vectors and adjuvants, important differences exist between results obtained with the two viral systems. The SFV serum IgG adjuvant effect was completely abrogated in RKO mice [28]. This was not the case in the VEE system, as serum adjuvant activity was observed in RKO mice, albeit decreased approximately 3-fold compared to wildtype 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 dependence on IFN signaling for immune induction. VRP efficiently infect DCs both *in vitro* [29] and *in vivo* [30], (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 [31], and *in vivo* infection of DCs by SFV has not been 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 of IFN induced by both viruses has not been performed. Additionally, mammals encode multiple IFN α genes [8] 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 a distinction exists in the overall levels of IFN and/or the IFN α gene repertoire induced by VEE and SFV, and that this feature may account for the observed differential immunogenicity between the two systems. Future experimentation in both systems will be required to fully elucidate the role of IFN in alphavirus-induced immunity.

The observation that IFN directly serves as a an adjuvant for the induction of antibody responses, and IgA responses, to co-immunized antigen is consistent with a role for IFN in alphavirus-induced immunity [13, 32–34]. However, the mechanism by with IFN specifically promotes mucosal IgA responses following delivery of VRP expression vectors is unclear. Here we propose that IFN functions downstream of IgA class switch, as normal IgA responses were observed in the systemic compartment (spleen) and in the draining lymph node (data not shown) of HA-VRP-immunized RKO mice. These observations are consistent with a model in which IFN signaling promotes VRP-induced mucosal migration of IgA-secreting B cells under conditions in which the antigen expressed in VRP-infected cells. Further studies will be required to address the validity of this hypothesis.

An important point to consider in the interpretation of immune induction experiments in RKO mice is the amount of VRP-driven antigen expressed in both wildtype and RKO mice. It is known that alphavirus vector-expressed antigen is markedly increased in animals lacking the type I receptor ([25]; White *et al.*, in preparation), suggesting that, in wt mice, autocrine and paracrine IFN signaling limit alphavirus vector antigen expression [25]. 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 greatly increased in RKO mice. 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 a role of the type I IFN system in alphavirus-induced adaptive immunity. Type I IFN played a critical role in the activation of mucosal IgA responses following delivery of VRP expression vectors, possibly via regulating mucosal migtration. 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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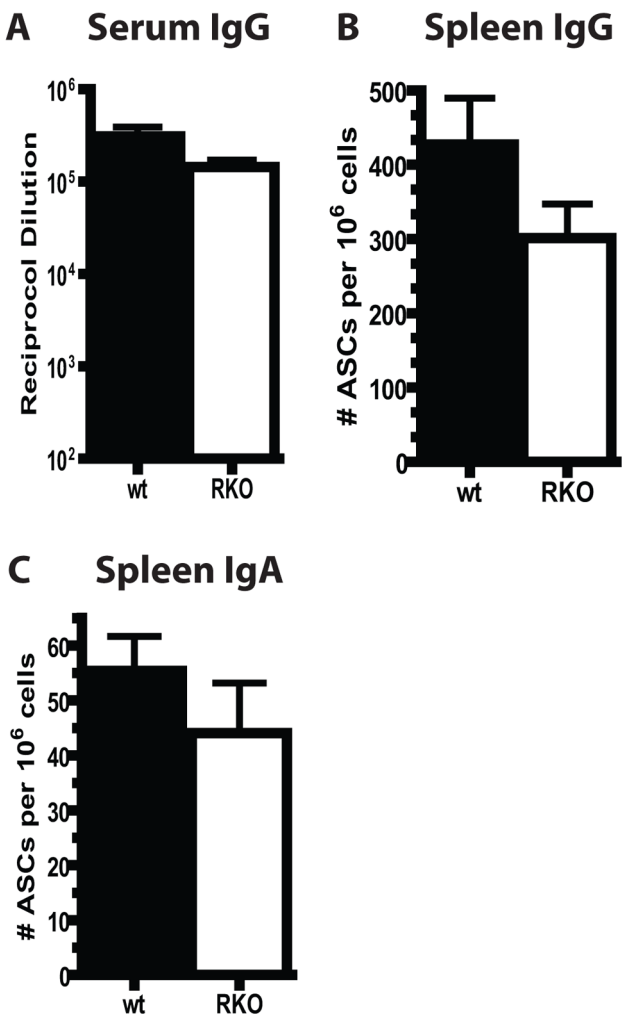

Figure 1. Type I IFN signaling is dispensable for expression-vector-induced systemic immunity

Groups of wt and RKO mice were immunized in the rear footpad at weeks 0 and 4 with 1×10^5 IU of HA-VRP. Two weeks following the boost, flu-specific serum IgG antibodies were evaluated by ELISA (A). Additionally, splenocytes were evaluated for the presence of flu-specific IgG- (B) and IgA- (C) secreting cells by ASC ELISPOT. Values represent the geometric mean ± SEM. No statistically-significant differences exist between responses induced in wt and RKO animals.

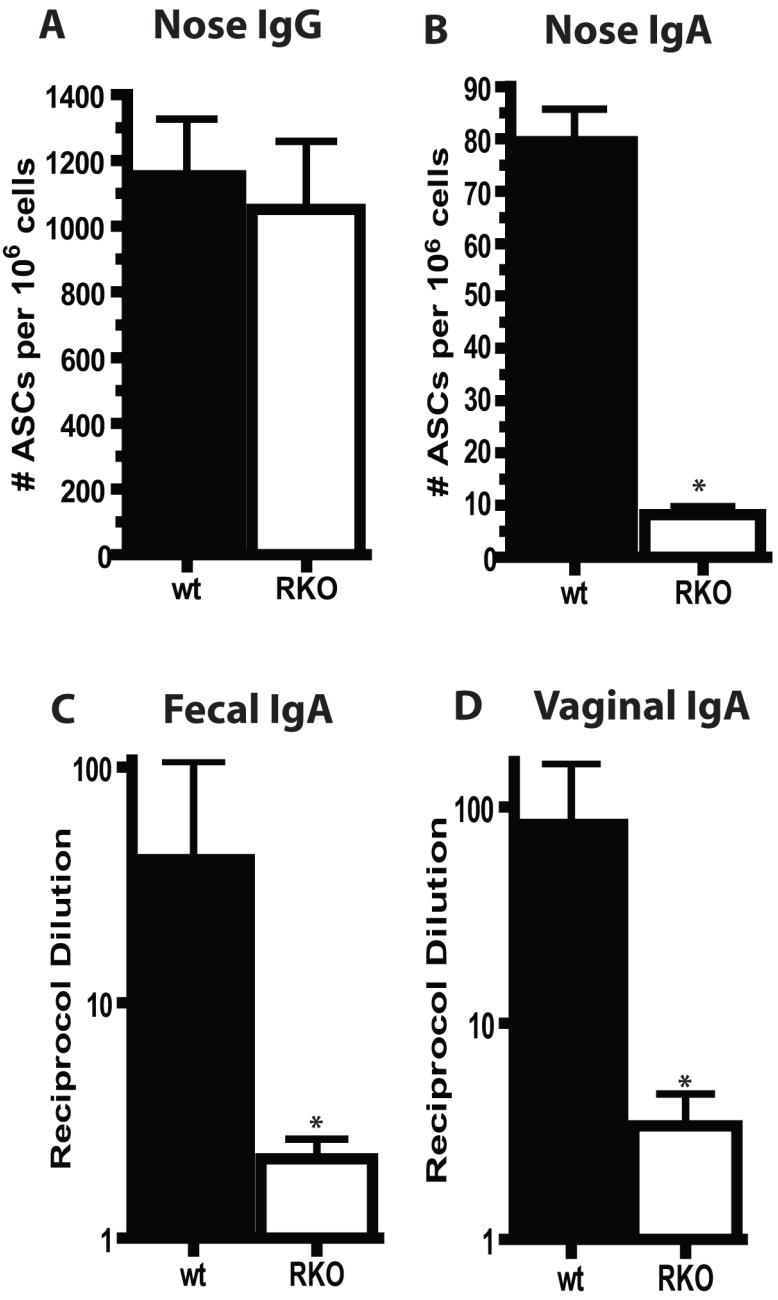

Figure 2. Type I IFN signaling is required for expression-vector-induced mucosal IgA responses

Groups of wt and RKO mice were immunized in the rear footpad at weeks 0 and 4 with 1×10^5 IU of HA-VRP. Two weeks following the boost, nasal lymphocytes were evaluated for the presence of flu-specific IgG- (A) and IgA- (B) secreting cells by ASC ELISPOT. Additionally, levels of flu-specific IgA antibodies were evaluated in fecal extracts (C) and vaginal lavage fluids (D) by ELISA. Values represent the geometric mean ± SEM. *, p=0.0286 compared to wt mice, as determined by Mann-Whitney.

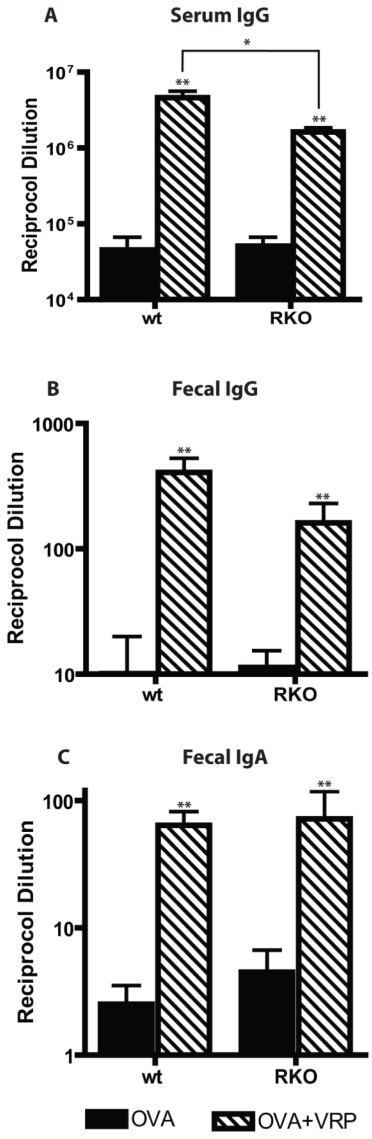

Figure 3. Role of type I IFN signaling on the VRP adjuvant effect

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 1×10^5 IU of null VRP. Two weeks following the boost, OVA-specific serum IgG (A), fecal IgG (B), and fecal IgA (C) antibody responses were evaluated by ELISA. Values represent the geometric mean ± SEM. *, p=0.025 compared to OVA plus VRP in wt mice; **, p=0.0044 compared to OVA alone, as determined by Mann-Whitney. The Bonferroni correction for multiple comparisons was applied to the reported p values.