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# Infected dendritic cells are sufficient to mediate the adjuvant activity generated by Venezuelan equine encephalitis virus replicon particles

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## Abstract

Replicon particles derived from Venezuelan equine encephalitis virus (VEE) are infectious non-propagating particles which act as a safe and potent systemic, mucosal, and cellular adjuvant when delivered with antigen. VEE and VEE replicon particles (VRP) can target multiple cell types including dendritic cells (DCs). The role of these cell types in VRP adjuvant activity has not been previously evaluated, and for these studies we focused on the contribution of DCs to the response to VRP. By analysis of VRP targeting in the draining lymph node, we found that VRP induced rapid recruitment of TNF-secreting monocyte-derived inflammatory dendritic cells. VRP preferentially infected these inflammatory DCs as well as classical DCs and macrophages, with less efficient infection of other cell types. DC depletion suggested that the interaction of VRP with classical DCs was required for recruitment of inflammatory DCs, induction of high levels of many cytokines, and for stable transport of VRP to the draining lymph node. Additionally, in vitro-infected DCs enhanced antigen-specific responses by CD4 and CD8 T cells. By transfer of VRP-infected DCs into mice we showed that these DCs generated an inflammatory state in the draining lymph node similar to that achieved by VRP injection. Most importantly, VRP-infected DCs were sufficient to establish robust adjuvant activity in mice comparable to that produced by VRP injection. These findings indicate that VRP infect, recruit and activate both classical and inflammatory DCs, and those DCs become mediators of the VRP adjuvant activity.

### Keywords

Adjuvant; dendritic cell; replicon; alphavirus

## INTRODUCTION

Vaccines have been tremendously successful at limiting and even eradicating many diseases, yet techniques that were successfully used to develop existing vaccines have often been

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inadequate to induce protective immunity with new vaccine candidates. For this reason, new tools and approaches are necessary for the next generation of vaccines. In the development of new vaccines, dendritic cell (DC) activation and antigen uptake are major goals, for these cells are central mediators of the link between innate and adaptive immunity and thus have the capacity to strongly amplify the magnitude of an immune response [1, 2]. A past example of a highly effective DC-targeting vaccine is the yellow fever vaccine, in which an attenuated yellow fever virus infects DCs in humans, inducing robust immunity [3]. To achieve activation of DCs that will potently enhance an immune response one of the most promising approaches is to utilize novel adjuvants. Adjuvants are compounds that strengthen and expand the scope of the immune response to a co-delivered antigen, often by activation of pattern recognition receptors in DCs and other APCs [4]. Adjuvant selection is critical, as many adjuvants generate a skewed immune response [5], in contrast to live-attenuated vaccines such as the smallpox and yellow-fever vaccines which produce a more balanced response [6]. A highly effective adjuvant may therefore be one that resembles a live virus and is able to target and activate dendritic cells. Alphaviruses show promise as the basis for such an adjuvant, for many alphaviruses have a tropism for DCs [7] and the alphavirus Venezuelan equine encephalitis virus (VEE) can enhance the immune response to antigen delivered after infection [8, 9]. A promising VEE-based adjuvant is VEE replicon particles (VRP) [10–12], which consist of the wildtype VEE capsid and envelope encapsulating a truncated VEE genome encoding only the non-structural protein sequence [13]. Because their genome lacks the structural protein sequences, VRP are able to infect and replicate within cells but cannot form new particles and propagate, rendering them safe for use in humans [14, 15]. VRP were originally conceived as antigen expression vectors in which a vaccine antigen was encoded in the VRP genome [13, 16–19]. While VRP expression vectors have been used successfully to induce immunity, it is possible that innate immune shutdown of VRP replication will limit production of antigenic mass, whereas use of VRP as an adjuvant allows antigen dose to be controlled independently of the VRP dose. VRP have the further advantage that they can be rapidly combined with any antigen.

When injected into mice with a soluble antigen, VRP increase the level of antigen-specific serum IgG and mucosal IgA, and also augment the CD8 T cell response to antigen [10–12]. VRP adjuvant activity has also been demonstrated in rats (unpublished data) and non-human primates [20]. When included in an immunization, VRP improve vaccine-mediated protection to challenge by several pathogens including norovirus and influenza [20, 21]. Blocking mucosal entry of pathogens is key to protection from many diseases [22, 23], so the ability of VRP to establish a mucosal immune response to antigen is an outstanding feature of this adjuvant. Furthermore, mucosal immunity is rarely generated in response to parenteral delivery of antigen and adjuvant, and this non-classical mucosal immune induction may be a valuable tool to circumvent some of the obstacles encountered in mucosal antigen delivery [22]. These various qualities of VRP make them a promising candidate for use as an adjuvant in human vaccines.

DCs have been hypothesized to play a role in VRP adjuvant activity based on several observations of DC-VRP interactions in vitro and in vivo. Immunofluorescent histology and density centrifugation showed that after footpad injection VRP move rapidly to the draining popliteal lymph node and infect cells with a DC-like morphology that are in the DC density fraction [24]. Subsequent in vitro studies of both murine and human DCs revealed that DCs were infectable by VRP, leading to upregulation of costimulatory molecules and secretion of Type-I IFN, IL-6 and TNF [25–27]. These cytokines, as well as many others, are observed in the serum and in the draining lymph node of mice within hours of VRP injection [12]. Additionally, VRP injection causes a rapid increase in the cellularity of the draining lymph node, with a disproportionate increase of DCs after both prime and boost [12, 28]. While these findings indicate that DCs are very responsive to VRP, their impact on the VRP

adjuvant effect has remained undefined. We undertook a series of studies to evaluate the role that DCs play in VRP-mediated inflammation and adjuvant activity.

The work described here is the first to demonstrate that DCs are sufficient to mediate the adjuvant activity of VRP. By flow cytometric analysis, we established a more detailed picture of which cell populations are targeted by VRP in the draining lymph node. We found that while many cell types are targeted – it is the monocyte-derived inflammatory DC population which is most dramatically recruited to the lymph node and also the population most frequently targeted by VRP. We further found that DCs infected with VRP are sufficient to enhance the in vitro T cell response. Most importantly, upon injection into mice VRP-infected DCs are sufficient to enhance the in vivo systemic, mucosal and cellular responses. These findings support the conclusion that VRP infection of DCs causes those DCs to become mediators of the VRP adjuvant effect.

## MATERIALS AND METHODS

### VEE Replicon Particles

Production and packaging of VRP have been previously described [13, 29]. Briefly, VRP are packaged into functional particles by electroporation of BHK-21 cells with the replicon genome along with two helper RNAs. The helper RNAs produce the structural proteins in trans but lack the cis-acting packaging sequence, so that only the replicon RNA is incorporated into the viral particles. All replicon particles used in this study were packaged in the wild-type (V3000) envelope [30]. Three VRP genomes were used: VRP-GFP and VRP-RFP encode the sequence for GFP and RFP under the control of the 26S promoter, and VRP(-5) (subsequently referred to as simply VRP) contains the viral nonstructural genes but lacks the sequence between the nsP4 stop codon (5 nts before the 26S mRNA transcription start site) and the beginning of the 118-nt 3' UTR [12]. All VRP genomes contain all of the known cis-acting signals for RNA replication. After purification, the absence of detectable replication-competent virus was confirmed by the lack of cytopathic effect after two passages on BHK-21 cells, and VRP were titered by infection of BHK-21 cells as measured by immunofluorescent staining of VEE non-structural proteins. VRP genome equivalents (GE) were determined by RNA extraction with an Ambion MagMAX Viral RNA Isolation Kit followed by real time PCR using nsP1-specific primers and probe as previously described [31]. The ratio of VRP GE to BHK infectious units (IU) was approximately 200.

### Bone marrow dendritic cells

BALB/c or C57BL/6 bone marrow dendritic cells (BMDCs) were derived by culturing bone marrow progenitors in RPMI-10% FBS with the addition of GM-CSF (20ng/ml). The media volume was doubled by addition of an equal volume of fresh media containing GM-CSF and IL-4 on days 3 and 5 (the final concentration of both cytokines was 10 ng/ml on day 3 and 5 ng/ml on day 5). After 7 days BMDCs were harvested and frozen [32]. BMDCs were thawed one day before use in media containing GM-CSF (5ng/ml) and IL-4 (5ng/ml). Of the frozen cells, 40–60% were recovered as live cells. BMDCs were resuspended in 0.2 ml warm RPMI-1% FBS and cultured with or without VRP (MOI=2) for 2 hours. For in vitro T cell activation, BMDCs ($10^6$) were pulsed with 100 μg OVA in 0.5 ml media for 2 hours before VRP infection. BMDCs were then washed and either cultured in vitro or injected into the footpads of mice.

### Mice and Immunizations

Six to eight week old female BALB/c or C57BL/6 mice were purchased from Charles River, and OTI and OTII TCR-transgenic mice were purchased from Jackson Labs. CD11c-diphtheria toxin receptor (DTR) mice were provided by Michael Dee Gunn (Duke

University). Mice were housed either at the University of North Carolina Division of Laboratory Animal Medicine animal facility or the Global Vaccines Inc animal facility according to protocols approved by both Institutional Animal Care and Use Committees. The model antigen tested was endotoxin-free chicken egg albumin (OVA) (Hyglos). Injections were performed in the footpad in a 10 μl volume, using either 10 μg OVA alone or OVA mixed with the stated infectious units (IU) of VRP, as described in the text. In some cases mock or VRP-infected DCs ($5\times10^5$) were injected into the footpad alone or with OVA in a 20 μl volume. Mice were boosted the same way 4 weeks later. For some experiments, VRP-GFP or VRP-RFP was injected into footpads with 2 μg OVA that was conjugated to Alexa Fluor 647 (AF647) using the Alexa Fluor 647 Protein Labeling kit (Invitrogen). In CD11c-DTR mice, DCs were depleted by i.p. injection of 8 ng diphtheria toxin (DT) (List Biological Labs) per gram body weight, in combination with rear footpad injection of 10 ng of DT each, all 12 hours before VRP injection.

### Analysis of OVA-specific IgG and IgA in serum and fecal extracts

Serum was collected from mice 3 weeks after boost. Fecal pellets were collected 10 days after boost and vortexed at 4°C at 0.2 g/ml in PBS containing protease inhibitors (Roche) and 10% goat serum until pellets were disrupted. Samples were centrifuged and fecal extract supernatants were passed through 0.22 μm filters. OVA-specific IgG and IgA antibodies were detected by ELISA on 96-well high binding plates (Thermo Scientific) coated with OVA in PBS. Sera and fecal extracts were added to plates in serial dilutions. OVA-specific antibodies were detected with horseradish peroxidase conjugated antibodies specific for mouse IgG (Sigma) IgA, or IgG2a (Southern Biotech) followed by addition of either *o*-phenylenediamine dihydrochloride substrate (Sigma) or tetramethylbenzidine (TMB) (KPL) for 30 minutes. The TMB reaction was stopped with 1M HCl. Endpoint titers were determined as the last sample dilution that generated an $OD_{450}$ reading of greater than 0.2. For determination of total IgA levels in fecal extracts, 96 well plates were coated with 0.4 μg/ml goat anti-mouse IgA (Invitrogen), ELISAs performed as above, and a standard curve generated from dilutions of purified murine IgA (Sigma). This standard curve was used to determine the concentration of both OVA-specific IgA and total IgA in fecal extracts.

### Surface and intracellular staining of cells from spleen and lymph node

Spleens or draining popliteal lymph nodes were harvested at the time points indicated. Spleens and lymph nodes were homogenized through 40 μm cell strainers. For VRP-GFP analysis, lymph nodes were first diced with scalpels and incubated at 37° for 30 minutes in RPMI-10% FBS containing 1 mg/ml collagenase D (Roche) before homogenization. Live cells were counted by hemacytometer. For intracellular cytokine staining, cells were cultured in RPMI-10 10 containing brefeldin A (GolgiPlug, BD Biosciences). For evaluation of CD8 T cell response to OVA, cells were cultured alone or with OVA peptide (SIINFEKL) (2 μg/ml) for 5 h at 37°. Cells were washed and stained at 4°C for desired surface receptors with a selection of the following fluorochrome-conjugated antibodies specific for CD3, CD8, CD11b, CD11c, CD69 (eBioscience), B220 (Invitrogen), Ly-6g and Ly-6c (BD Bioscience) in 1% BSA/PBS. After staining, cells were washed and then fixed in 2% paraformaldehyde for 15 min at room temperature. For intracellular staining, fixed cells were washed and permeabilized in 0.5% saponin and stained with cytokines specific for IFN-γ or TNF (eBioscience) at 4°C, and then washed in saponin buffer. Cells were analyzed on a C6 (Accuri) or Cyan flow cytometer (Dako).

### Analysis of secreted cytokines after VRP injection

Twelve hours after VRP injection of DTR mice, one draining popliteal lymph node per mouse was harvested and placed in 100 μl of PBS containing 1X protease inhibitors (Roche). Lymph nodes were mechanically homogenized with a pestle, followed by

centrifugation at 4°C. Supernatant was transferred to another tube and frozen. Levels of 32 cytokines were determined by a Luminex-based assay using the Milliplex MAP Mouse Cytokine/Chemokine Premixed 32 Plex (Millipore). Samples were analyzed by the Immunotechnologies Core of the Center for Gastrointestinal Biology and Disease at the University of North Carolina Chapel Hill. For analysis of Type-I IFN, serum samples were tested by bioassay of protection of L929 cells from killing by EMCV as previously described [33].

### In vitro T cell activation by VRP-infected DCs

Splenic CD4 T cells from OT-II mice and CD8 T cells from OT-I mice were isolated by negative selection with CD4 and CD8 isolation kits (Miltenyi). T cells were cultured with OVA-pulsed and/or VRP-infected BMDCs at a 5:1 ratio in the presence of 100 U/ml recombinant human IL-2 (Peprotech). T cells were fed with fresh media and IL-2 (20 U/ml) on days 3 and 5. After 1 week, T cells were harvested. In some cases, T cells were labeled with 5 μM CFSE. T cells were restimulated with OVA-pulsed BMDCs at a 5:1 ratio. Supernatants from OTII T cells were harvested after 2 days for ELISA analysis of IFN-γ in the supernatant (eBioscience). CFSE dilution was measured on day 3 by flow cytometric analysis. For analysis of CD8 degranulation, C57BL/6 spleen cells were cultured +/− SIINFEKL peptide (1 μg/ml) for 2 hours. These spleen cells were cultured with the primed CFSE-labeled OTI T cells at a 1:1 ratio in the presence of anti-LAMP-1-AF647 antibody (eBioscience). After 2 hours of stimulation, cells were harvested and degranulated LAMP-1-positive OTI cells identified by flow cytometry.

### Statistics

Statistical significance was calculated using a two-tailed Student's t test, (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$)

## RESULTS

### VRP infect multiple cell types in the draining lymph node and preferentially recruit and target inflammatory DCs

After footpad injection, VRP cause an increase in lymph node cellularity with a preferential recruitment of dendritic cells after both prime and boost [12, 28]. In another study VRP were also found to move rapidly to the popliteal lymph node after footpad injection and infect a cell type that was phenotypically consistent with DCs [24], but limited staining for CD11c and other cell-specific markers left some ambiguity to the identity of the infected cells. We used flow cytometric analysis to better identify the cell types infected and recruited by VRP to the lymph node. Previous studies demonstrate that both footpad and intramuscular VRP injection result in comparable adjuvant activity [12], and we utilized footpad injection of VRP and DCs, because antigen delivered to the footpad drains to a single popliteal lymph node, simplifying analysis of lymph node activity. BALB/c mice were injected in the footpad with VRP-GFP at a low and high dose ($10^3$ or $10^5$ IU), both of which induce systemic and mucosal adjuvant activity [12]. Fluorescently labeled OVA antigen was injected alone or co-injected with VRP-GFP to assess how VRP affects the amount and location of antigen in the lymph node. Peak VRP-GFP expression in the draining lymph node was between 6 and 12 hours post injection (unpublished observations). In the results shown here, popliteal lymph nodes were harvested 12 hours post injection, stained for various surface receptors, and analyzed by flow cytometry. Lymph node cellularity was increased 2–3 fold by VRP-GFP injection (Fig 1A). The cells in the lymph nodes were divided into the following non-overlapping subsets (gating is shown in Fig S1 A and B in the supplemental material): monocyte-derived inflammatory DCs (CD11c+ CD11b+ Ly-6c-hi), CD11b- DCs (CD11c+ CD11b−), CD11b+ DCs (CD11c+ CD11b+), macrophages/

neutrophils (CD11c− CD11b+), B cells (CD11c−, CD3−, B220+), T cells (CD3+). Cells negative for all these markers were considered “undefined”. The inflammatory DCs are different from classical DC subsets in that they are not present in the steady state, but rapidly mature from monocytes upon migration to an inflammatory site [34–36]. It should be noted that both inflammatory macrophages and inflammatory DCs are both derived from Ly-6c-hi inflammatory monocytes and have similar phenotypes and overlapping definitions [37, 38]. In other models inflammatory macrophages are reported to reside in the inflammatory tissue, whereas the monocytes and/or macrophages develop a DC-like phenotype upon migration to the lymph node [39]. In the lymph nodes of VRP-injected mice the Ly-6chi cells are >95% CD11c+ and we have defined these Ly-6c-hi CD11c+ CD11b+ cells as inflammatory DCs (Fig S1 A, D and E). The few Ly-6c-hi CD11c- that we observed can be tentatively defined as inflammatory macrophages, although we have observed that CD11c expression increases uniformly on the Ly-6c-hi cells at later timepoints (data not shown), suggesting that the inflammatory monocytes recruited to the lymph node by VRP are all in the process of upregulating CD11c to become inflammatory DCs.

There is also a small population of Ly-6c-intermediate CD11c− CD11b+ cells that may represent inflammatory macrophages with downregulated Ly-6c expression, which has been observed in other models [40], but this small population contains very few VRP-GFP+ cells (Fig S1C). Except for the undefined cells, the number of cells in all subsets was increased by injection of either VRP-GFP dose, with the largest fold increase occurring in the inflammatory DCs which increased 6-fold over mice injected with OVA alone (Fig 1B). As expected, more VRP-GFP-positive cells were observed at the $10^5$ dose than at the $10^3$ dose, although the 100-fold increase in VRP-GFP dose only yielded a 4-fold increase in the number of VRP-GFP-positive lymph node cells (Fig 1C). VRP-infected cells detected in the footpad or in other lymphoid organs were not sufficient to account for this difference. Additionally, less than 5% of the positive cells contained both OVA and VRP-GFP (Fig 1C, Fig S1F in the supplemental material). Although all cell subsets contained some VRP-positive cells, calculation of the percentage of cells infected in a given subset demonstrated that inflammatory DCs were most readily infected, followed by CD11b+ DCs and the macrophage/neutrophil population (Fig 1D). Additional staining revealed that in the macrophage/neutrophil population, approximately 80% of the infected cells were macrophages. In the CD11b− DC population, 35% of the infected cells were plasmacytoid DCs, based on B220 expression (unpublished observations).

Finally, the total number of OVA and VRP positive cells in each injection condition was calculated (Fig 1 E,F). In all injection conditions approximately 75% of the OVA in the lymph node was contained within dendritic cells, the largest proportion of which was in inflammatory DCs. Surprisingly, up to 15% of the OVA-positive cells were CD3+ T cells. VRP targeted a wide range of cell types, but inflammatory DCs constituted the largest number of the VRP-GFP-positive cells, and lower levels of classical DCs were infected as well. After inflammatory DCs, T and B lymphocytes were the cell types most frequently infected at the higher VRP dose, despite their low rate of infection (Fig 1D). Lymphocyte infection by VRP has not been previously described, but comparison to lymph nodes of mice injected with null-VRP clearly demonstrated that GFP was induced in T and B cells after VRP-GFP injection (Fig S1 G & H in the supplemental material). Interestingly, at the lower VRP-GFP dose, T and B cells constitute a combined 15% of the VRP-GFP-positive cells, but at the higher $10^5$ IU dose the T and B cells constituted about 40% of the VRP-GFP-positive cells. This outcome indicates a preferential infection of DCs and macrophages by VRP, particularly at the lower dose. Even at the higher VRP dose the rate of infection of T and B lymphocytes by VRP-GFP was very low (Fig 1D), but due to the large size of these subsets the total number of cells infected is still substantial.

For some experiments it was useful to analyze VRP activity in C57BL/6 mice or transgenic mice bred on a C57BL/6 background. Cellular and systemic VRP adjuvant activity is intact in both of these strains (data not shown), so we compared draining lymph node activity after VRP-GFP injection in BALB/c vs C57BL/6 mice. Although popliteal lymph nodes are somewhat smaller in C57BL/6 mice, cellularity is significantly increased in all subsets, with a disproportionate increase in inflammatory DCs (see Fig. S2 in the supplemental material), similar to what is observed in BALB/c mice. A similar percentage of cells are infected with VRP-GFP, and the early activation marker CD69 is comparably upregulated. These findings led us to conclude that VRP induce very similar events in the lymph nodes of both BALB/c and C57BL/6 mice.

## Inflammatory DCs in the draining lymph node produce TNF after VRP injection

Robust TNF secretion is a hallmark of inflammatory DCs [41], and TNF is one of many cytokines which is produced in the draining lymph node in response to VRP [12]. To determine if inflammatory DCs were the source of this TNF, we harvested lymph nodes 12 hours after VRP injection and detected cells producing TNF by intracellular staining after ex vivo culture in the presence of brefeldin A. The majority of the specific TNF staining was found on Ly-6C-hi inflammatory DCs (Fig 2), demonstrating that this population is actively secreting cytokines after VRP injection and further indicating that this population plays a major role in the inflammatory response to VRP. These inflammatory DCs resemble monocyte-derived TNF/iNOS-producing (Tip) DCs [42, 43]. However, no iNOS producing cells were detected in the draining lymph node after VRP injection (unpublished observations). These are the first data to directly demonstrate cytokine production by lymph node DCs triggered by VRP injection.

## Effect of DC depletion on VRP activity in the draining lymph node

To assess the role of DCs in VRP-mediated inflammation in the lymph node, we depleted DCs before VRP injection by utilizing the CD11c-DTR mouse, in which GFP and the diphtheria toxin receptor are expressed as transgenes under the control of the CD11c-promoter. Treatment of these mice with diphtheria toxin (DT) results in transient depletion of the CD11c-hi dendritic cells [44], which was confirmed by analysis of spleen cells after 24 hours (Fig 3A). Interestingly, while classical DCs were depleted, there was a smaller population of Ly-6c-high inflammatory DCs present in the spleen that were unaffected by VRP or DT injection, likely due to the lower levels of CD11c on this population. Twelve hours after DC depletion with DT or mock depletion with PBS, mice were injected with OVA either alone or combined with VRP-RFP ($10^5$ IU). (VRP-RFP was used for identification of infected cells instead of VRP-GFP due to the transgenic expression of GFP in these mice; we have observed no differences in the inflammatory response to the two constructs). After another 12 hours lymph nodes were harvested, stained and analyzed by flow cytometry. In non-depleted mice, RFP+ cells were detected in the lymph node, while DC-depleted mice contained no detectable RFP+ cells above the level of background fluoresence (Fig 3B). Because many VRP-infected cell types other than DCs are normally found in the lymph node (Fig 1F) one interpretation of this outcome is that DCs are necessary for transport of VRP or retention of VRP in the lymph node. Transport of antigen to the lymph node was unaffected (see Fig S3 in the supplemental material).

Inflammatory DCs were recruited to the lymph node by VRP-RFP in CD11c-DTR mice, but were not recruited in DC-depleted mice (Fig 3C). While it is possible that inflammatory DCs were absent due to DT-mediated depletion, their persistence in the spleen indicates that this population was not significantly affected by DT treatment. VRP-RFP in DC-depleted mice still caused strong CD69 upregulation on all cell types in the draining lymph node, which was only slightly diminished from CD69 levels in DC-competent mice, whereas in the

spleen CD69 upregulation on leukocytes was dramatically reduced (Fig 3D), suggesting that the VRP retained some ability to activate a local innate immune response in these mice, whereas systemic leukocyte activation was abrogated by DT treatment.

Finally, we measured Type-I IFN in the serum and various cytokines in the lymph nodes of DC-depleted mice injected with VRP-GFP. Type-I IFN was robustly induced in response to VRP-GFP, but was undetectable in DC-depleted mice (Fig 3E). This reduction of serum IFN is most likely responsible for the weak CD69 induction in the spleens of these mice [45–47]. Although IFN is not required for VRP humoral adjuvant activity [48] it is a key indicator of an innate immune response to virus, so this finding demonstrates that DT treatment caused a loss of innate immune activation by VRP. We also found that DC depletion reduced the level of many but not all VRP-GFP-induced cytokines (Fig 3E and Table S1 in the supplemental material). Because DT treatment does not eliminate 100% of DCs, it is difficult to determine whether the remaining cytokine and CD69 upregulation is wholly in response to VRP activation of other cell types, or is driven by non-depleted DCs. The greatest fold reduction was for IL-5 and IL-6, both of which augment antibody secretion, particularly IgA antibody [49, 50]. We did observe induction of some cytokines such as IFN-γ in response to DT treatment, but less than was induced by VRP. This finding that DT induced an inflammatory response introduces the possibility that the effects we see in this model are due not to DC depletion but to the antiviral activity of factors such as IFN-γ induced by the DT. Such a possibility was not anticipated, because in several other studies of viral infection in CD11c-DTR mice, DT treatment did not generate an obvious antiviral response, for viral activity increased rather than decreased after treatment [51–55]. In contrast, a study of mouse mammary tumor virus did show decreased infection after DT treatment, although this was thought to be due to the preferential infectivity of DCs by the virus [56]. We are not aware of any previous use of alphaviruses in the CD11c-DTR model, which would be more relevant. VEE does have the ability to limit the activity of Type I and Type II interferons by suppression of STAT-1 signaling [57], but it has also been shown that IFN-γ has the ability to suppress alphavirus propagation [58]. To evaluate the effect of IFN-γ on VRP activity we treated C57Bl/6 bone marrow-derived DCs (BMDCs) with 0.2 or 1 ng/ml of IFN-γ (both doses exceed the 0.15 ng/ml detected in lymph node homogenates). These DCs were infected with VRP-GFP 12 hours later, and we found that IFN-γ caused a 20–30% reduction in the number of GFP+ cells (Fig S4A in the supplemental material). This finding indicates that IFN-γ might be inhibiting VRP activity in these mice, and while this effect is relatively weak at this dose range, it is difficult to predict how the in vitro activity will correlate to in vivo activity. We do not see Type I IFN induction after DT injection, but there is always a possibility that other antiviral factors induced by DT may be contribute to the absence of VRP in the draining lymph node. To address the potential antiviral effect of other factors induced by DT, we evaluated the effect of lymph node homogenates on VRP-GFP infection in BMDCs. While lymph node homogenates from PBS-injected mice had some suppressive effect on VRP infection, homogenates from DT-treated mice had no additional suppressive effect (Fig S4B in the supplemental material). Together, these in vitro data suggest that the cytokines induced by DT would be insufficient to fully suppress VRP activity, supporting the conclusion that the depletion of DCs resulted in poor VRP transport to the lymph node, reduced cytokine secretion, and poor recruitment of inflammatory DCs. Even so, such in vitro assays cannot disprove the possibility that the antiviral activity of DT-induced cytokines disrupted VRP activity in vivo. For subsequent experiments we evaluate the interaction of VRP and DCs in wildtype mice, avoiding any similar uncertainty.

## VRP-infected DCs enhance the in vitro T cell response to antigen

Having demonstrated that VRP target and activate DCs in the draining lymph node, we devised an in vitro assay to test whether VRP-infected DCs directly improve T cell response

to antigen, or if interaction with other cell types is necessary. For this assay, we generated BMDCs in vitro which are CD11c+, CD11b+, and Ly-6c- (data not shown). BMDCs from C57Bl/6 mice were pulsed with OVA, followed by mock or VRP infection (MOI=2), and are referred to as DC-OVA and DC-OVA-VRP, respectively. At this MOI, between 3 and 10% of the BMDCs were infected by VRP-GFP (data not shown), depending on the BMDC lot. OVA-specific CD4 and CD8 T cells were isolated from spleens of TCR-Tg OTII and OTI mice, respectively. These T cells were then cultured with the BMDCs in the presence of IL-2. To test whether the effects of VRP-infected DCs can be conferred in trans, T cells cultured with DC-OVA were combined with either VRP-infected DCs (DC-VRP) or with filtered supernatant harvested from DCs 24 hours after VRP infection. In this primary stimulation we observed little effect of VRP on T cell activation (unpublished observations), so we examined the T cell recall response after one week by restimulation with fresh OVA-pulsed BMDCs. Analysis of supernatants by ELISA 48 hours after restimulation revealed that CD4 T cells produced more IFN-γ if they had been primed by DC-OVA-VRP (Fig 4A), whereas there was minimal effect on CD8 IFN-γ production (unpublished observations). IFN-γ production was also enhanced for CD4 T cells cultured with a mixture of DC-OVA and DC-VRP, and to a lesser degree when DC-VRP supernatant was added to the initial stimulation. This outcome indicates that the VRP need not be contained within the antigen presenting DCs in order to augment the T cell response.

For analysis of T cell proliferation, T cells were CFSE-labeled before restimulation with OVA. Three days after restimulation, cells were analyzed by flow cytometry, and CFSE dilution demonstrated that CD8 T cells had improved proliferation in response to antigen if they had been primed by DC-OVA-VRP (Fig 4B), whereas CD4 T cells did not (unpublished observations). We also measured CD8 degranulation by surface staining for LAMP-1 on OTI T cells restimulated with OVA-peptide. LAMP-1 is expressed on the cell surface after degranulation, and correlates with cytotoxic activity [59]. We found that CD8 T cells primed by DC-OVA-VRP had greater LAMP-1 expression than those primed by DC-OVA (Fig 4C). LAMP-1 expression was also increased for T cells primed by DC-OVA mixed with either DC-VRP or DC-VRP supernatant, indicating that VRP-infected DC-secreted factors enhanced CD8 cytotoxicity. These in vitro findings show that VRP-infected DCs have different effects on CD4 and CD8 T cells, but improve the responsiveness of both populations upon restimulation with antigen.

## Injection of VRP-infected DCs causes an inflammatory response in the draining lymph node

VRP injection induces rapid inflammation of the draining lymph node, as identified by increased cellularity, recruitment of dendritic cells, and dramatic upregulation of the early activation marker CD69 [12]. To evaluate whether VRP-infected DCs (DC-VRP) are sufficient to induce a comparable effect, we infected BALB/c BMDCs with VRP in vitro, and then injected those DCs ($5\times10^5$) into the footpads of BALB/c mice. As controls, some mice were injected with PBS, VRP ($10^5$ IU) or uninfected DCs. In some cases we labeled DCs with CFSE before injection and could detect transferred DCs in the draining lymph node after 18 hours (unpublished data). After 18 hours mice were sacrificed and draining popliteal lymph nodes harvested and stained for surface markers. Lymph nodes from VRP-injected and DC-VRP-injected mice had increased cellularity (unpublished observations). Injection of both VRP and DC-VRP, but not untreated DCs, caused a robust increase in the number of inflammatory DCs recruited to the lymph node (Fig 5). Additionally, VRP and DC-VRP induced significant upregulation of CD69 on the entire leukocyte population, although DC-VRP induced a lesser increase.

### VRP-infected DCs enhance the cellular response to antigen

To assess whether DCs are sufficient to augment the CD8 T cell response to antigen in vivo, we measured T cell responses after injection of OVA with VRP-infected BMDCs. It was previously shown with VRP expressing the tumor antigen neu that VRP-neu-infected DCs induced potent neu-specific immunity upon injection into mice [26], but this experiment only evaluated immunity to VRP-expressed antigen and did not address whether VRP-activated DCs enhanced the response to soluble antigen. We injected C57BL/6 mice in the footpad with OVA antigen alone or in the presence of VRP, DCs, or VRP-infected DCs ($5\times10^5$). One week after injection, mice were sacrificed and spleen cells stimulated with OVA peptide (SIINFEKL) in the presence of brefeldin A, and IFN-γ-producing cells identified by intracellular staining. Injection of both VRP and DC-VRP resulted in the generation of a comparable OVA-responsive IFN-γ-producing CD8 population, whereas untreated DCs had no effect (Fig 6), demonstrating that VRP-infected DCs are sufficient to generate a cellular adjuvant effect. This in vivo enhancement of T cell responses confirms the relevance of our observed in vitro effects of VRP-infected DCs on T cell activity (Fig 4).

### VRP-infected DCs enhance the systemic and mucosal antibody response to antigen

To further test whether DC-VRP can confer the same adjuvant activity as VRP in mice, we evaluated how DC-VRP affect the production of antigen-specific antibodies. VRP-infected or untreated BALB/c BMDCs were injected with OVA antigen into the footpads of BALB/c mice. Control mice were injected with OVA alone, or OVA and VRP ($10^5$ IU). After 4 weeks mice were boosted in the same way. After the boost we measured anti-OVA IgG in the serum and mucosal anti-OVA IgA in fecal extracts. Both VRP and DC-VRP caused a significant increase in the level of OVA-specific IgG (Fig 7A). Transfer of untreated DCs with antigen did increase the average OVA-IgG levels, but this increase was erratic and not statistically significant. Previous analyses have shown that VRP greatly increase the production of antigen-specific IgG2a [11]. In this experiment it is clear that VRP and DC-VRP, strongly increased the levels of OVA-specific IgG2a, whereas untreated DCs did not (Fig 7B). The increased amount of specific IgG2a antibody is consistent with an increased Th1 response. Most interesting was the ability of DC-VRP but not untreated DCs to augment the mucosal OVA-specific IgA response, comparable to the effect achieved by direct injection of VRP with antigen (Fig 7A). We also tested whether supernatant harvested from in vitro infected BMDCs could serve as an adjuvant by footpad injection with antigen, but observed no enhancement of IgG or IgA responses (unpublished data). While this may suggest that DC-secreted factors are insufficient to mediate the VRP adjuvant effect, it is also quite possible that the injected cytokines did not reach sufficient levels in the draining lymph node to enhance the immune response. Overall, our data demonstrate that VRP-infected DCs delivered to a non-mucosal site are sufficient to mediate the non-classical induction of mucosal immunity.

## DISCUSSION

These studies generated the first data to demonstrate that targeting of DCs by VRP is sufficient to drive their adjuvant activity and strongly contributes to the inflammatory response to VRP. We have clearly identified the cell types infected by VRP targeting in the draining lymph node, establishing that DCs are the predominant cell type targeted by VRP in vivo. We also made the novel finding that many other in vivo cell populations are infected by VRP to a lesser degree. Interestingly, at a lower VRP dose ($10^3$ vs $10^5$ IU), a smaller proportion of the VRP-positive cells are T and B lymphocytes, suggesting that the efficient infection of DCs and macrophages outcompetes the inefficient infection of lymphocytes until VRP-GFP are present in higher quantities. At higher doses VRP may saturate infection of DCs and macrophages, leaving more excess VRP free to infect other cell types. Because

adjuvant activity is intact at the lower VRP dose in which a smaller proportion of lymphocytes are infected, this suggests that these lymphocytes play a limited role in the adjuvant effect. The contribution of non-DCs to the VRP adjuvant effect remains undefined, but by focusing on DCs in this report we have demonstrated that VRP infection gives DCs the capacity to amplify the immune response to antigen.

Previous data indicate that VRP cause a disproportionate recruitment of CD11b+ DCs to the draining lymph node after both prime and boost [12, 28], and we have shown here that a majority of these cells are Ly-6c-hi monocyte-derived inflammatory-DCs. While these inflammatory DCs are rare in the steady-state, they are recruited in response to VRP and are also preferentially infected by VRP. It is somewhat of a paradox that VRP target a subset which is not present in the steady state. A straightforward explanation for this outcome is that VRP may infect monocytes in the inoculation site which then migrate to the lymph node and mature into inflammatory DCs. However, there are some limitations to this explanation which suggest that an alternative mechanism may be at play. In particular, monocytes are found in the blood and not in lymphoid or peripheral tissues in the steady state [36], and additionally, monocytes have been shown to be resistant to VRP infection [25]. We therefore favor an alternative explanation that VRP-mediated activation of other cell types (likely DCs) induces recruitment of inflammatory monocytes which mature into inflammatory DCs and become infected by VRP. The kinetics of such an explanation requires that some fraction of the VRP remains free to infect these inflammatory DCs as they are recruited. It is not yet certain whether the infection of the inflammatory DCs, as well as other cell types, occurs predominantly in the injection site or in the lymph node itself. One study of VRP indicated that essentially all VRP and/or VRP-infected cells move from the footpad to the lymph node within 30 minutes, whereas another study demonstrated by in vivo imaging the presence of VRP-infected cells in the footpad 8 hours after injection [24, 60]. While, these two findings have yet to be reconciled, the latter finding supports the possibility that VRP infect inflammatory DCs recruited to the injection site, which then migrate to the lymph node. The alternative is that VRP are transported to the lymph node by passive lymphatic drainage or by cell-mediated transport, allowing the VRP to infect inflammatory DCs as they arrive in the lymph node. We have observed some free VRP in the lymph node one hour after footpad injection, supporting this possibility (data not shown). As a whole, our data indicate that a first wave of VRP infection causes recruitment of inflammatory DCs to the draining lymph node, similar to what is observed following other viral infections [61, 62]. These inflammatory DCs are also infected by VRP, either in the injection site or in the lymph node itself. Further studies will be needed to work out the details of this process.

We have shown here that in VRP-injected mice, OVA is found in inflammatory DCs more than in any other cell subset, similar to what is observed after influenza infection [61], further suggesting a key role for this cell type in the enhancement of the immune response. Interestingly, despite the preferential targeting of both OVA and VRP to inflammatory DCs, OVA and VRP are rarely contained in the same cells. While it is possible that the few cells that contain both OVA and VRP are the key cells involved in the generation of an adjuvant effect, the rarity of this event suggests that the VRP-infected cells are able to augment the immune response in uninfected cells, likely by secretion of pro-inflammatory cytokines. This hypothesis is supported by the in vitro finding that VRP-infected DCs induce maturation in neighboring uninfected DCs [27]. Furthermore, T cell responses are enhanced when OVA-pulsed DCs are combined with VRP-infected DCs or supernatant from VRP-DCs, demonstrating that soluble factors secreted by those DCs play a role in amplifying at least some aspects of the T cell response. We have also observed adjuvant activity when VRP is injected 24 hours after antigen [12] further reducing the probability of in vivo targeting of VRP and antigen to the same cells, yet still allowing local cytokine signaling.

Our in vivo data also indicate that cytokine secretion is induced in non-infected cells, since nearly all of the recruited inflammatory DCs secrete TNF, while only around 10% are infected. This finding is consistent with previous demonstration of cytokine induction by VRP in neighboring cells [63].

Our in vitro analysis of VRP activity demonstrates that VRP-infected DCs are sufficient to enhance T cell responses to antigen without the involvement of additional in vivo interactions. While we cannot directly equate in vitro-generated BMDCs with DCs found in vivo, our findings demonstrate that the adjuvant and inflammatory effects generated in mice by injection of VRP can be mediated by VRP-infected BMDCs. It is a possibility that some VRP attach to BMDCs without infecting them, and these VRP can be shed after injection and infect and activate host cells. We have detected some VRP release into the supernatant by BMDCs after in vitro infection with VRP (unpublished observations), but the amount of shed VRP that was detected after washing is insufficient to generate the adjuvant activity observed upon DC-VRP injection, indicating that the impact of VRP release should be minor. It is more likely that the adjuvant activity is caused by the inflammatory milieu generated by the injected DCs. It is possible that injected DC-VRP take up and present antigen, but in vitro studies showed that VRP infection of DCs before pulse with antigen results in decreased T cell activation (unpublished data), presumably as a result of DC maturation which can reduce antigen uptake [64]. For this reason, we suspect that the injected DC-VRP enhance immunity by their action on uninfected host and donor DCs that take up and present antigen.

In summary, our findings demonstrate that after injection, VRP infect and activate DCs, causing a wave of cytokine secretion. These cytokines can enhance the T cell response and likely drive the recruitment of inflammatory DCs. The robust recruitment and preferential targeting of antigen and VRP to the inflammatory DCs further supports the conclusion that this DC subset in particular plays an important role in the VRP adjuvant effect. Inflammatory DCs are strong inducers of Th1 immunity [61], so their recruitment after VRP injection is likely responsible for the improved Th1 responses observed in these mice. Most significantly, these studies demonstrate that the ability of VRP to target and activate DCs enables those DCs to generate a robust adjuvant effect, strongly supporting the conclusion that infection of DCs is a key component of the adjuvant activity generated upon VRP injection. Notably, this indicates that targeting of non-mucosal DCs is sufficient to elicit non-classical mucosal immune induction under the appropriate activation conditions. We anticipate that the potent DC-mediated enhancement of the immune response will make VRP a valuable component of robust new human vaccines. In traditional vaccines, in vivo targeting and activation of DCs is a valuable quality that can enhance the immune response [65], and in DC-mediated therapy, which is under development, VRP could be used to activate DCs in vitro before those DCs are injected back into a patient [66].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- VEE replicon particles (VRP) recruit inflammatory DCs to the lymph node
- VRP preferentially infect and activate inflammatory DCs
- VRP-infected DCs enhance in vitro T cell responses to antigen
- VRP-infected DCs are sufficient to mediate VRP adjuvant activity

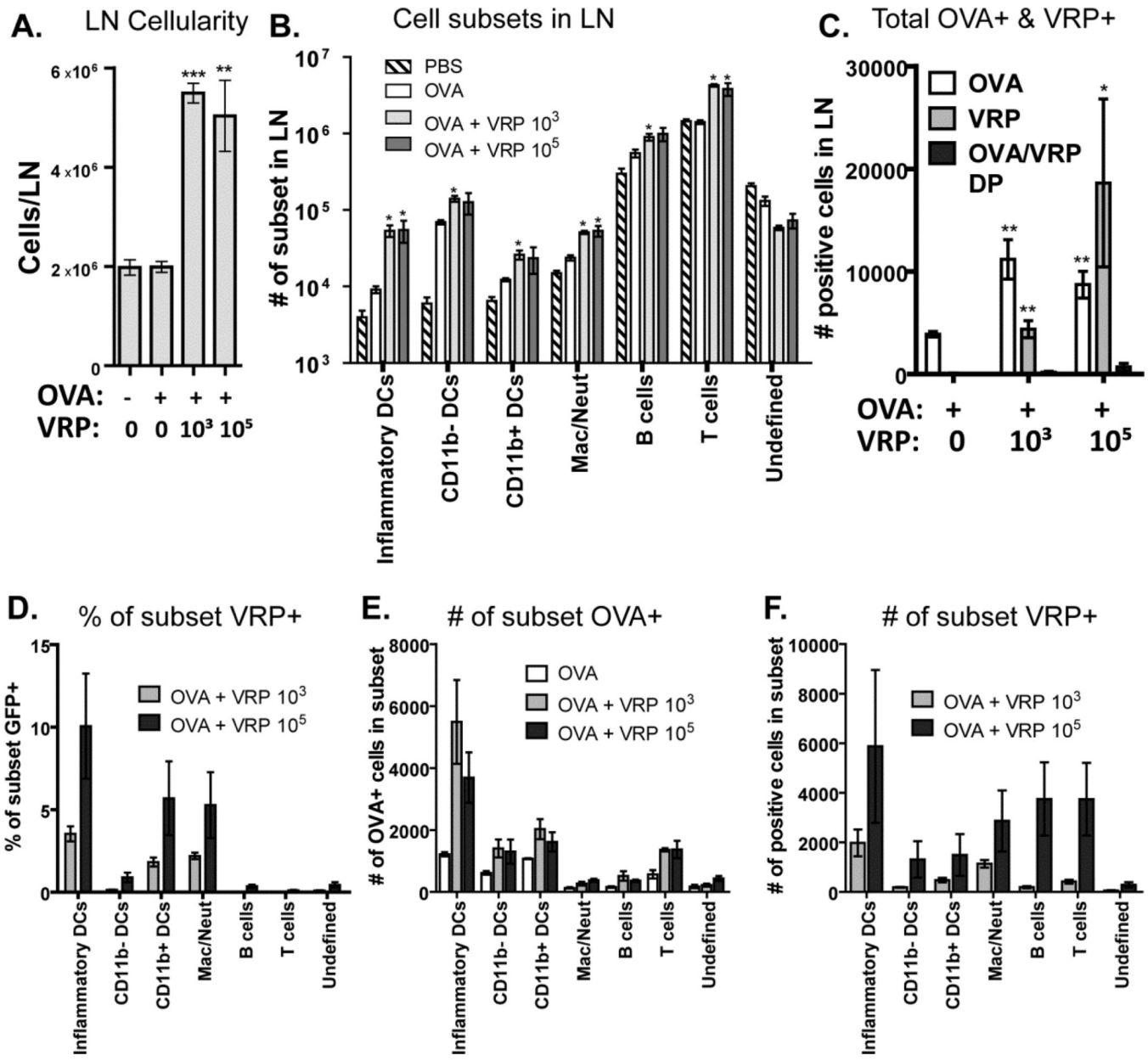


**Fig 1.**

VRP and OVA distribution in the draining lymph node. We injected BALB/c mice in the footpad with diluent or 2 μg OVA-AF647 alone or in the presence of VRP-GFP ($10^3$ or $10^5$ IU). (VRP-GFP is referred to as VRP in this figure). Lymph nodes were taken after 12 hours, stained, and then analyzed by flow cytometry. VRP-GFP-infected cells could be identified by the presence of GFP in those cells. (**A)** Lymph node cellularity (determined by hemacytometer) was increased in VRP-GFP-injected mice. **(B)** We divided all cells into the following cell subsets: inflammatory DCs (CD11c+ CD11b+ Ly6c-hi), CD11b− DCs (CD11c+ CD11b−), CD11b+ DCs (CD11c+ CD11b+), macrophages/neutrophils (CD11c− CD11b+), B cells (CD11c−, CD3−, B220+), T cells (CD3+). Cells negative for all these markers were considered “undefined”. **(C)** The total number of OVA+, VRP-GFP+, or double-positive (OVA/VRP-GFP DP) cells per lymph node are shown. **(D)** For each cell subset we determined the percent of that subset that was VRP-GFP-positive. **(E)** The total

number of OVA+ cells in each injection condition are shown. **(F)** The total number of VRP-GFP+ cells in each injection condition are shown. Significance shown is relative to levels in OVA-injected mice. Results shown are the average of 4 mice per group, and are consistent with 3 separate experiments.

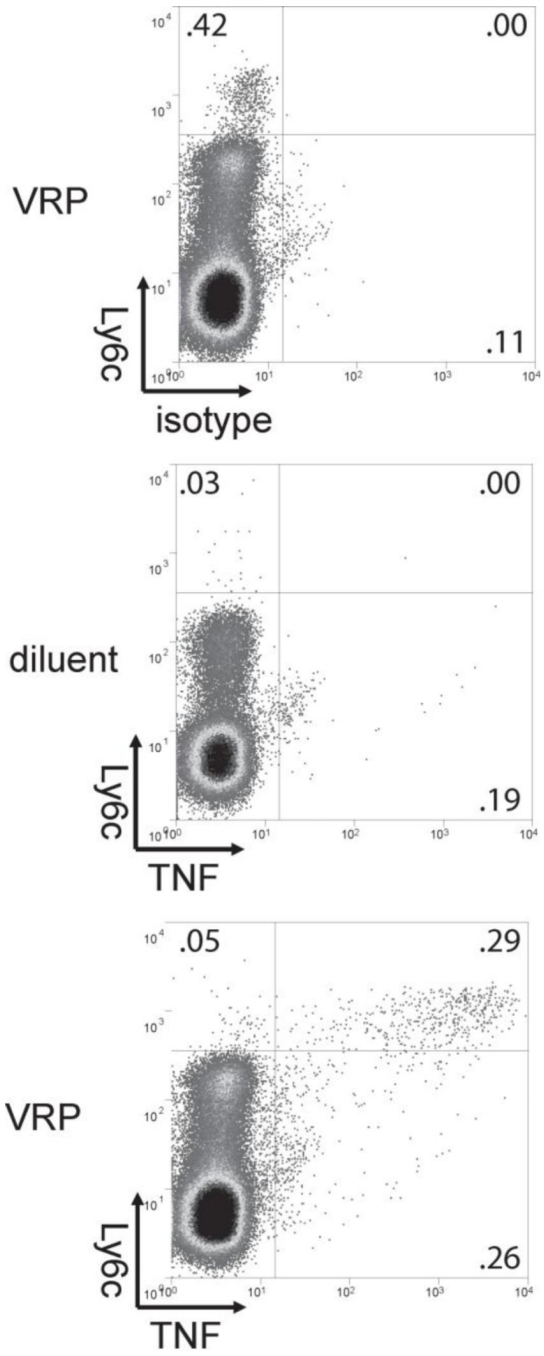


**Fig 2.**

VRP-recruited inflammatory DCs in the draining lymph node secrete TNF. Mice were immunized in the footpad with diluent or VRP ($10^5$ IU). After 12 hours draining popliteal lymph nodes were harvested and cultured in media containing brefeldin A for 5 hours. Cells were then stained for surface markers and intracellular TNF and analyzed by flow cytometry. Non-specific TNF staining was assessed with an isotype control antibody. In VRP-infected mice, the majority of the Ly6C-hi/CD11c+ inflammatory DCs were producing TNF. Results shown are representative of three separate experiments with 4 mice per group.

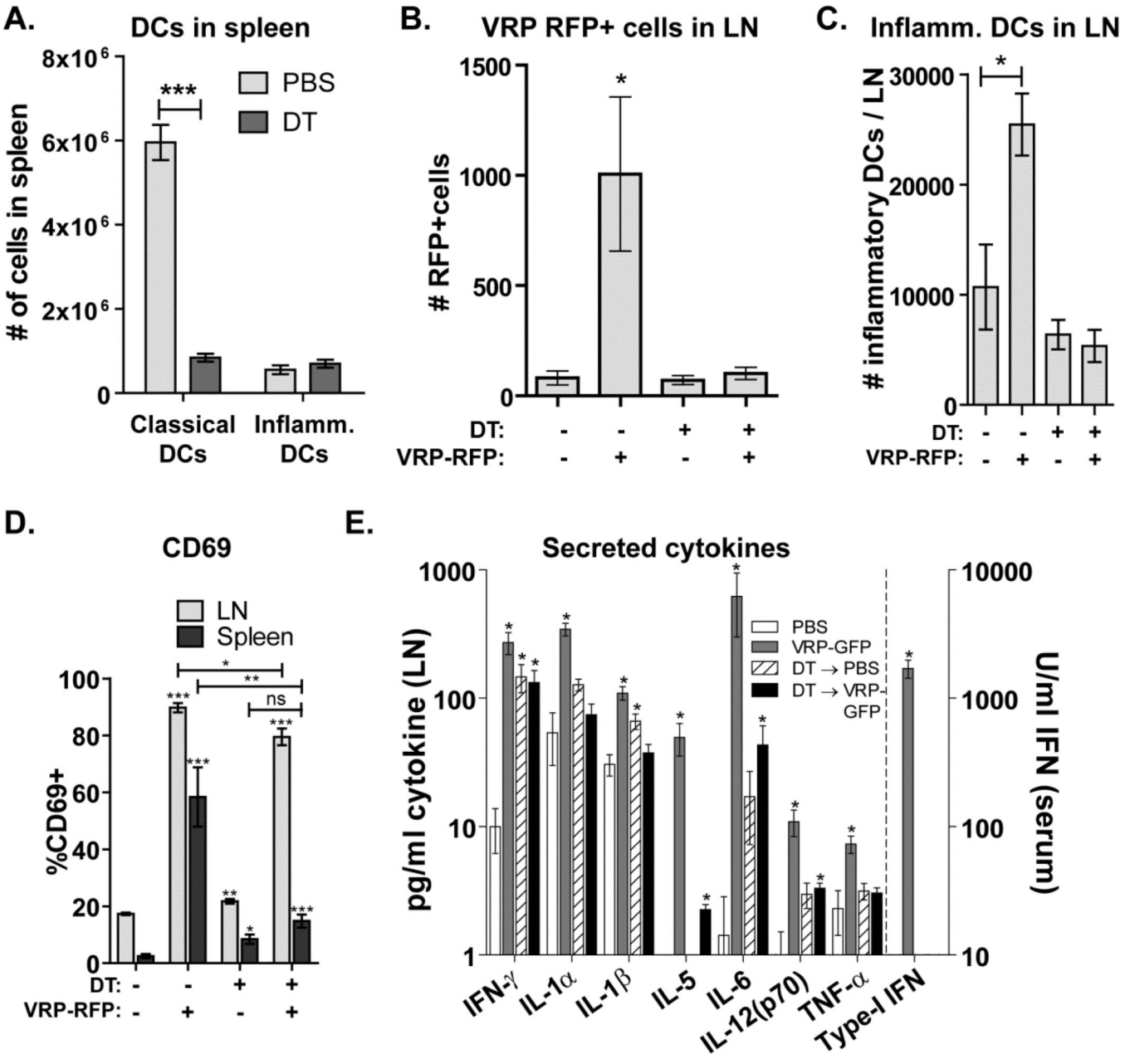


**Fig 3.**

Classical DCs mediate VRP transport and inflammatory DC recruitment to the lymph node. CD11c-DTR mice were injected with either PBS as a control or DT to deplete DCs. After 12 hours the same mice were injected with OVA alone or combined with VRP-RFP **(B–D)** or VRP-GFP **(E)** ($10^5$ IU). 12 hours after VRP injection, spleens and lymph nodes were harvested, stained and analyzed. **(A)** DT caused depletion of classical DCs (CD11c+ Ly-6c-lo/negative) but not inflammatory DCs (CD11c+ Ly-6c-hi). VRP injection did not affect DC numbers in the spleen, so data from PBS and VRP-injected mice are combined here. **(B)** VRP-RFP-infected cells were identified by RFP expression. **(C)** Total numbers of inflammatory DCs were identified by high expression of Ly-6c. **(D)** Activated leukocytes in the lymph node and spleen were identified by staining for CD69. **(E)** Cytokine levels in lymph node homogenates were measured by multiplex analysis. Type-I IFN in the serum

was measured by a bioassay. Significance shown is relative to PBS-injected mice without DT, or as indicated. Significance for changes in cytokine levels was based on log-transformed values. Results are shown from 1 of 3 repeats with 4 lymph nodes per group, with the exception of the cytokine multiplex, which was a single experiment.

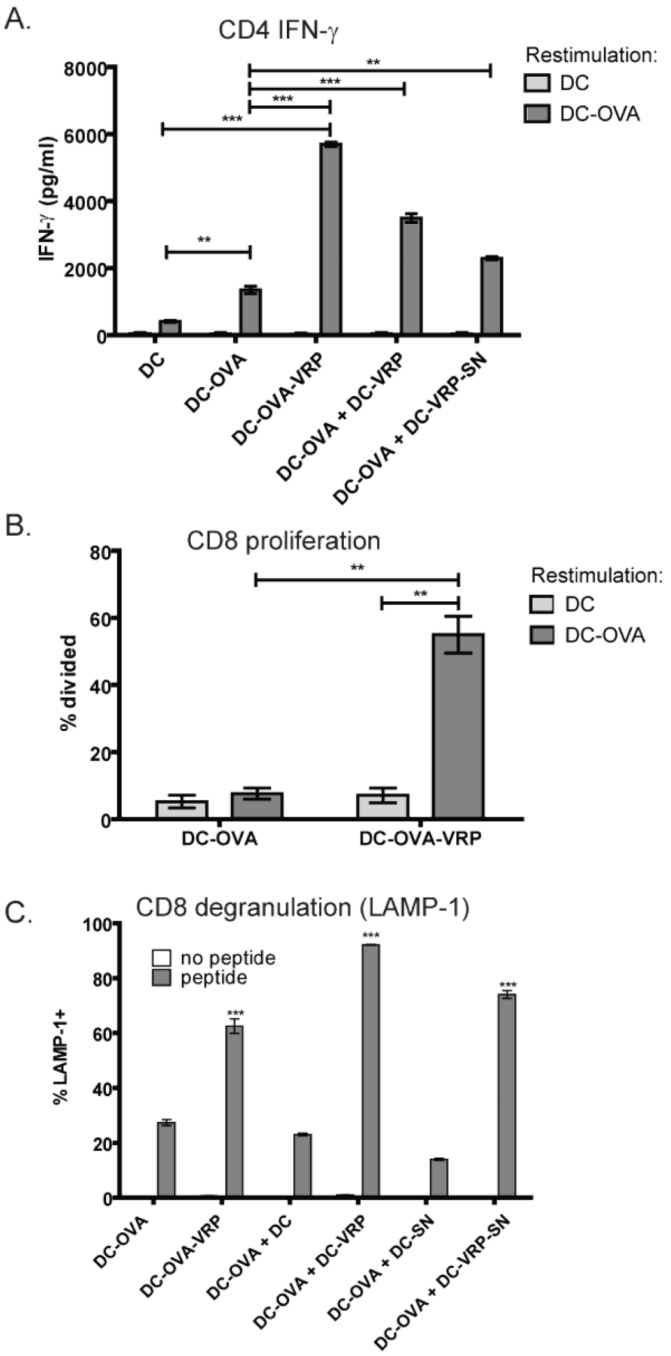


**Fig 4.**

VRP-infected DCs enhance in vitro T cell activity. C57BL/6 BMDCs were pulsed with or without OVA for 2 hours, and then infected with or without VRP for another 2 hours (MOI=2). These DCs were cultured with sorted OVA-specific splenic CD4 T cells (OTII) and CD8 T cells (OTI). In some cases the T cells were cultured with OVA-pulsed DCs (DC-OVA) mixed with mock (DC) or VRP-infected DCs (DC-VRP) or with filtered supernatant harvested from DCs 24 hours after mock (DC-SN) or VRP-infection (DC-VRP-SN). After 7 days the T cells were restimulated with fresh OVA-pulsed DCs. **(A)** IFN-γ secretion by OTII CD4 cells was measured 48 hours after restimulation with OVA-pulsed D48 hours.

Data are representative of 3 independent experiments. **(B)** The OTI T cells were labeled with CFSE and proliferation measured by CFSE dilution after 3 days. Data are combined from 3 separate experiments. **(C)** CTL degranulation was evaluated by measuring LAMP-1 upregulation on the surface of OTI CD8 T cells 2 hours after restimulation with spleen cells pulsed with OVA-peptide (SIINFEKL). Data are representative of three independent experiments. Significance shown is relative to cells primed with DC-OVA.

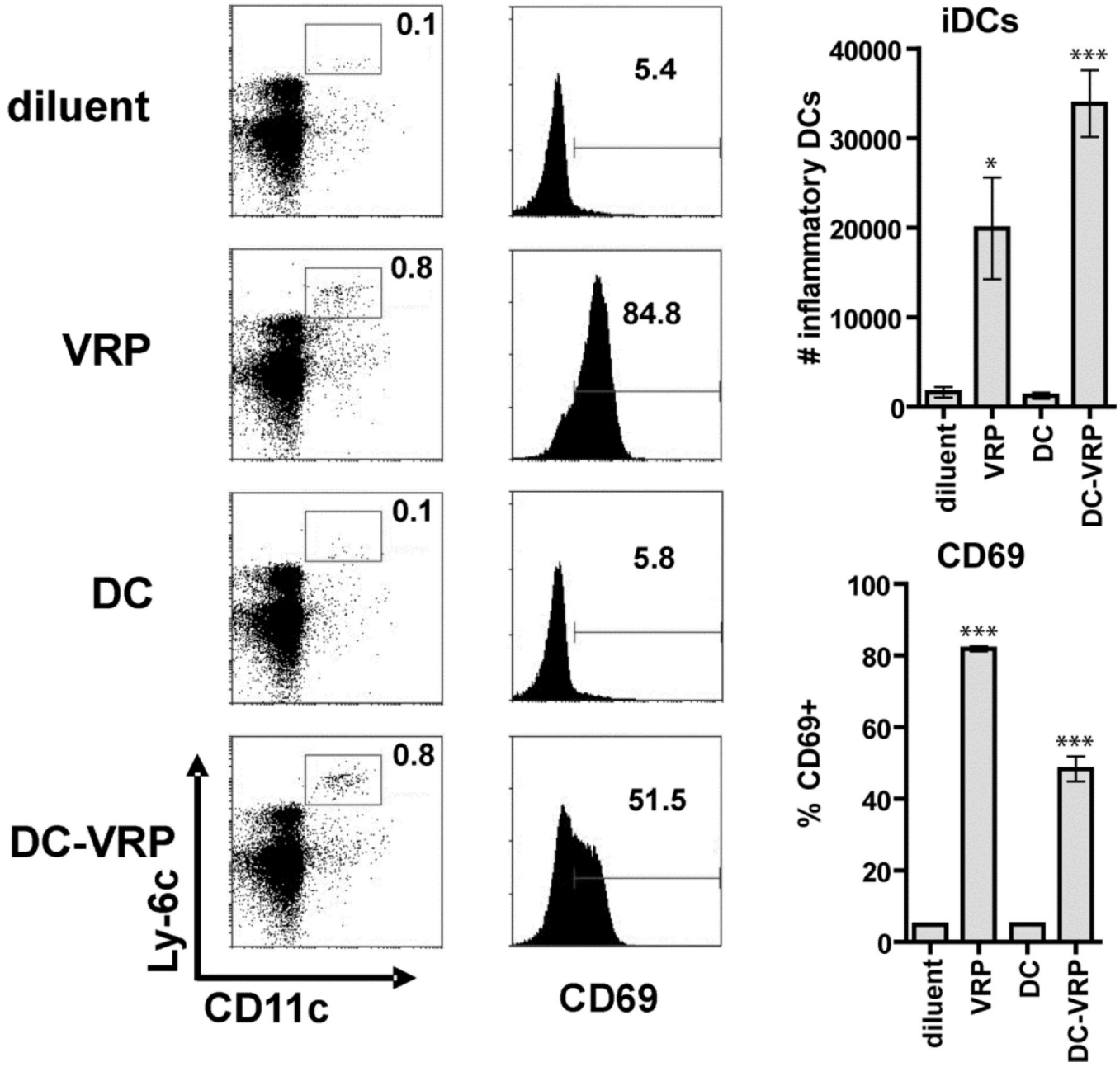


**Fig 5.**

VRP-infected DCs induce inflammation in the draining lymph node. BALB/c BMDCs were cultured +/− VRP (MOI=2) and then injected ($5\times10^5$) into the footpads of BALB/c mice. As controls, we injected mice with diluent or VRP ($10^5$ IU). After 18 hours we harvested draining popliteal lymph nodes and stained for surface markers. Representative dotplots show CD11c+ Ly-6c-hi inflammatory DCs (iDCs) and CD69+ cells as a percent of the total live leukocyte population. Average responses are shown as a graph. Significance is shown relative to diluent-injected mice. Data are representative of 4 independent experiments containing 4 mice per group.

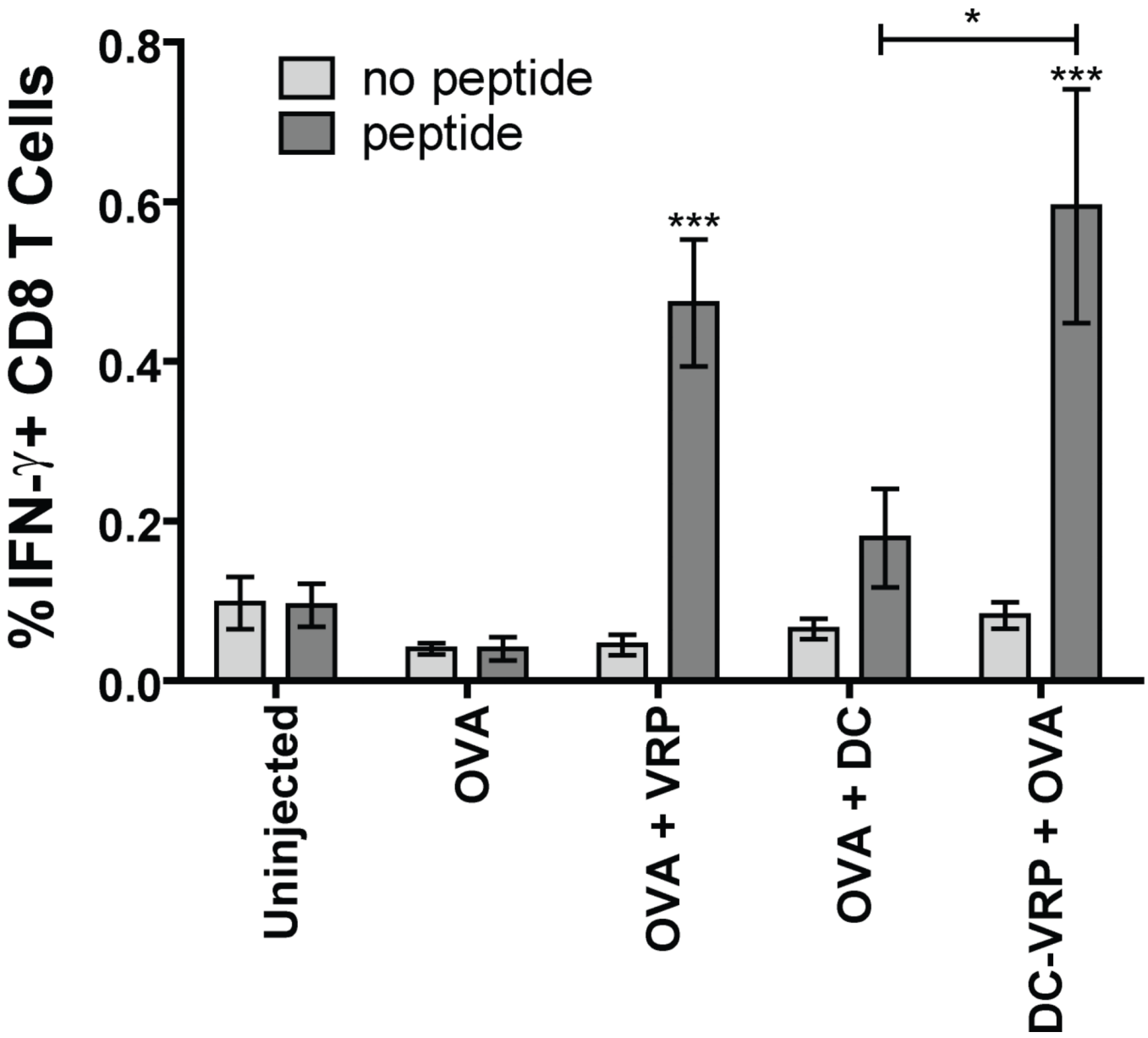


**Fig 6.**

VRP-infected DCs are sufficient to potentiate cellular adjuvant activity. BMDCs from C57BL/6 mice were cultured +/− VRP (MOI=2) for 2 hours and $5\times10^5$ DCs coinjected with OVA (10 μg) into the footpads of C57Bl/6 mice. As controls, mice were injected with OVA or OVA + VRP ($10^5$ IU). After 7 days we harvested spleens and stimulated with OVA peptide (SIINFEKL) for 5 hours in the presence of brefeldin A and then stained for surface markers and intracellular IFN-γ. Significance shown is relative to uninjected mice, except where indicated. Data are compiled from 5 independent experiments with at least 4 mice per group.

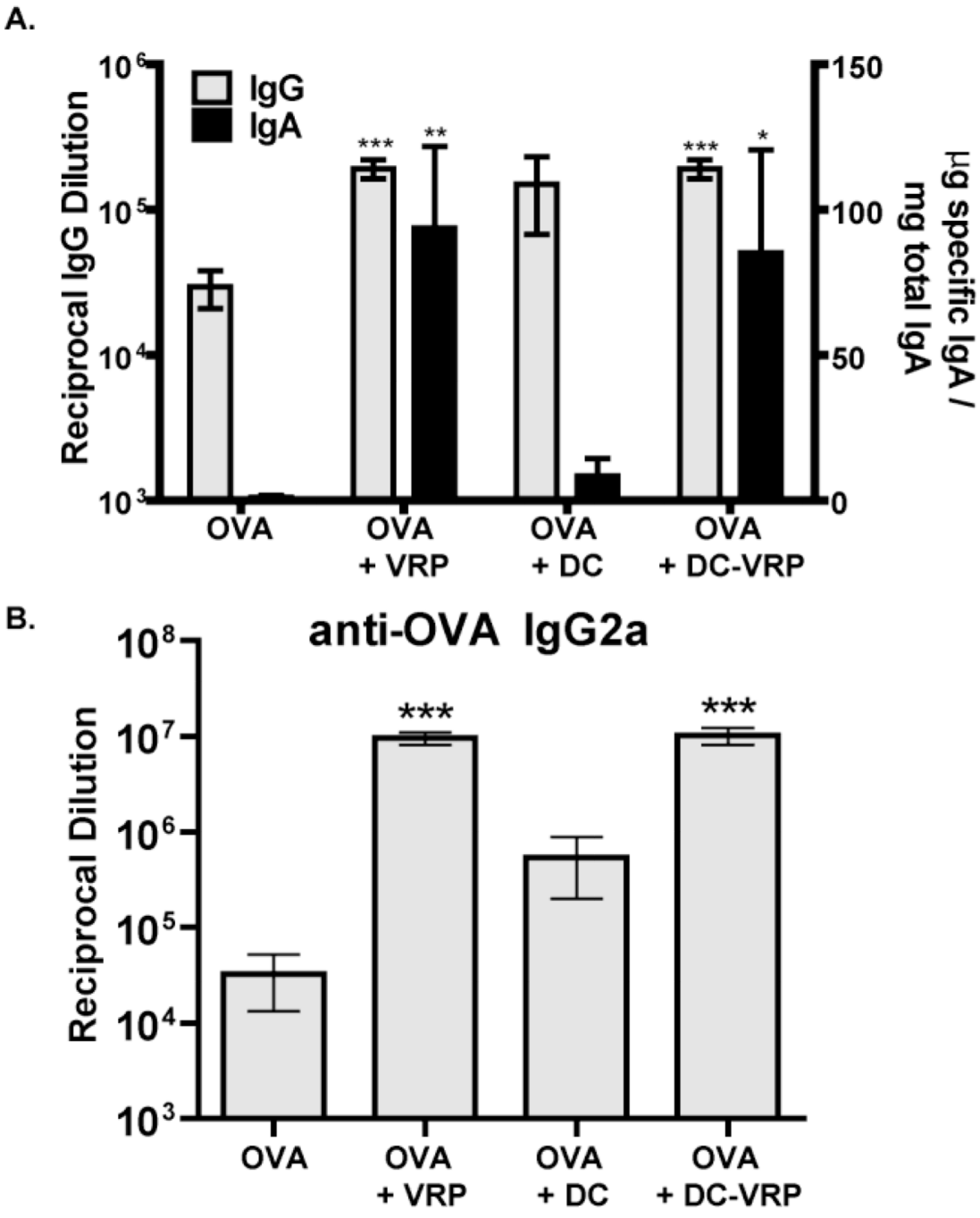


**Fig 7.**

VRP-infected DCs are sufficient to induce humoral adjuvant activity. BMDCs from BALB/c mice were cultured +/− VRP (MOI=2) for 2 hours and then washed and co-injected ($5\times10^5$) with OVA (10 μg) into the footpads of BALB/c mice. As controls, we injected mice with OVA or OVA + VRP ($10^5$ IU). Six mice per group were injected. Mice were boosted the same way 4 weeks later. (A) Mucosal OVA-specific IgA was measured in fecal samples 10 days after boost and systemic OVA-specific IgG was measured in serum 3 weeks after boost. (B) Levels of OVA-specific IgG2a in the serum were measured after boost. Significance shown is relative to OVA-injected mice.