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## Vaccinia virus infection induces dendritic cell maturation but inhibits antigen presentation by MHC class II

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### Abstract

Vaccinia virus (VV) infection is known to inhibit dendritic cells (DC) functions *in vitro*. Paradoxically, VV is also highly immunogenic and thus has been used as a vaccine. In the present study, we investigated the effects of an *in vivo* VV infection on DC function by focusing on early innate immunity. Our data indicated that DC are activated upon *in vivo* VV infection of mice. Splenic DC from VV-infected mice expressed elevated levels of MHC class I and co-stimulatory molecules on their cell surface and exhibited the enhanced potential to produce cytokines upon LPS stimulation. DC from VV-infected mice also expressed a high level of interferon- $\beta$ . However, a VV infection resulted in the down-regulation of MHC class II expression and the impairment of antigen presentation to CD4 T cells by DC. Thus, during the early stage of a VV infection, although DC are impaired in some of the critical antigen presentation functions, they can promote innate immune defenses against viral infection.

### Keywords

vaccinia virus; dendritic cell; MHC class II; gene regulation; cytokines; type I interferon; innate immunity

### INTRODUCTION

Vaccinia virus (VV) belongs to the poxviridae family of DNA virus and has been used as a vaccine against smallpox as well as a vector to deliver novel vaccines [1;2]. Unfortunately, however, immunization with the vaccinia has been associated with significant complications, particularly in immunocompromised individuals [3]. Thus, an increased understanding of the

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host's immune responses in the context of viral pathogenesis during a VV infection is of critical importance. Although VV is known to be equipped with multiple strategies that can impair host immune responses [4;5], the effect of a VV infection on antigen presentation is not well understood.

A typical immune response against a viral infection is mediated by CD8 cytotoxic T cells that require major histocompatibility complex (MHC) class I. Because of this, many viruses down-regulate the level of surface MHC class I antigens following infection [6;7;8]. Defects in MHC class I-mediated antigen presentation have been observed with recombinant VV that was engineered to express a heterologous antigen [9;10]. In this case, however, the virus appears to have altered antigen processing in an epitope specific manner. Less is known regarding MHC class II mediated antigen presentation during a VV infection, although CD4 T cell responses are also thought to be critical in promoting viral clearance and immunity [11]. Mice deficient in MHC class II protein expression displayed slightly decreased cytotoxic T lymphocyte (CTL) responses against VV [12]. In addition, we have recently shown that a VV infection *in vitro* disrupts antigen presentation by MHC class II [13]. Thus, it is clear that VV can modulate antigen presentation by both MHC class I and class II molecules.

Dendritic cells (DC) are specialized antigen-presenting cells (APC) derived from bone marrow (BM) precursors, and carry out important functions during both innate and adaptive immune responses [14;15]. Upon encountering infectious agents, immature DC resident in peripheral tissues capture antigens and migrate to secondary lymphoid organs while undergoing maturation [16]. Matured and thus activated DC secrete both inflammatory and anti-inflammatory cytokines that regulate the immune response. Mature DC also typically express high levels of MHC class I, class II, and co-stimulatory molecules. Therefore, mature DC are potent APC set to prime naive CD4 T cells and elicit CD8 T cell responses, bridging the innate and adaptive immune responses [17;18].

There are multiple DC subsets in both humans and mice, as characterized by surface marker expression and phenotypic functions [19]. In general, DC can be divided into two major populations: plasmacytoid DC (CD11c<sup>+</sup>B220<sup>+</sup>; pDC) and conventional DC (CD11c<sup>+</sup>B220<sup>-</sup>; cDC) that are preferentially associated with innate and adaptive immune responses, respectively [19;20]. cDC can be further divided into lymphoid DC (CD11c<sup>+</sup>CD8<sup>+</sup>) and myeloid DC (CD11c<sup>+</sup>CD11b<sup>+</sup>CD4<sup>+/-</sup>; mDC), within which CD8<sup>+</sup> DC are generally involved in priming CTL immunity to viruses [19;21]. On the other hand, pDC specialize in the secretion of type I ( $\alpha/\beta$ ) interferons (IFN), which play a pivotal role in viral clearance [22;23]. Therefore, it would not be surprising that one means by which viruses can evade the host's antiviral immune response is by altering the balance between these DC subsets. Indeed, a lymphocytic choriomeningitis virus infection has been shown to change the predominant DC subset from pDC to mDC to divert host immune response away from anti-viral immunity [24]. On the other hand, it was reported that pDC accumulate in the spleen after infection with murine cytomegalovirus [25], and that mDC present exogenous virus-like particles to CD8 T cells and subsequently become CD8<sup>+</sup> [26].

VV infection is also known to inhibit DC functions [4]. VV blocks cytokine signals by expressing decoy receptors for interleukin (IL)-1 $\beta$ , tissue necrosis factor (TNF)- $\alpha$ , and IFN [27;28]. The VV protein A52R disrupts the formation of Toll-like receptor (TLR) complexes, which are important in triggering some pathways of DC maturation [29]. A VV mutant lacking A52R was shown to be less virulent than the wild type (WT) VV *in vivo* in a murine intranasal infection model [29]. In addition, a VV infection induces apoptotic cell death and blocks maturation of human DC [30;31]. However, a majority of the studies demonstrating the impairment of DC function by a VV infection have been carried out *in vitro*. VV is known to be highly immunogenic, and thus has been successfully used as an attenuated vaccine to

eradicate human smallpox [2]. Nonetheless, it has remained unclear whether DC functions were compromised by a VV infection *in vivo*.

In the present study, we investigated the effects of a VV infection on DC function *in vivo* by focusing on aspects of innate immunity. Our data indicated that DC are activated upon an *in vivo* VV infection of mice. Splenic DC from VV-infected mice expressed elevated levels of MHC class I and co-stimulatory molecules on their cell surface and exhibited an enhanced potential to produce cytokines. However, a VV infection resulted in the down-regulation of MHC class II expression and an impairment of antigen presentation to CD4 T cells. Together, our results demonstrate that during early stages of a VV infection, the host's immune responses are geared toward anti-viral immunity but the host experiences a transient immunosuppression presumably due to deficits in MHC class II presentation by DC.

## MATERIALS AND METHODS

### Mice, viruses and infection

C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME), and maintained under specific pathogen-free conditions at the Indiana University School of Medicine. BM cells from mice deficient in type I IFN receptor (IFNAR<sup>-/-</sup>) were kindly provided by Dr. Mary O'Riordan (U. Michigan, Ann Arbor). All procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Stocks of WT VV (Western Reserve strain) were generated in the human osteosarcoma 143B cell line, followed by sucrose purification and titer determination as described [13]. The recombinant mKO mutant VV expressing orange fluorescent protein (OFP) has been previously described [32].

Female C57BL/6 mice were injected i.p. with  $1 \times 10^6$  pfu (plaque-forming unit) of VV in HBSS with 0.1% BSA (HBSS/BSA) and sacrificed up to two days after injection. Mice receiving HBSS/BSA alone were used as a mock-infected control.

To prepare splenic DC, CD11c positive cells were isolated from splenocytes using anti-CD11c magnetic beads (Miltenyi Biotec, Auburn, CA). In some experiments, the CD11c negative flow through was used to isolate B cells and macrophages with anti-B220 and anti-CD11b magnetic beads, respectively. To activate DC, CD11c<sup>+</sup> enriched DC were cultured for 24 hr at  $1 \times 10^6$  cells/ml in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) in the presence of 1  $\mu$ g/ml lipopolysaccharide (LPS, *E. coli* O55:B5 serotype, Sigma, St. Louis, MO). BMDC were generated as previously described [33].

### In vitro infection

Immature BMDC or total splenocytes were infected with VV at different multiplicities of infection (MOI,  $10^{-4}$  –  $10^{-1}$ ) for 90 min, followed by washing and culturing in fresh RPMI 1640 supplemented with 5% FBS in the presence or absence of 10 ng/ml murine recombinant IFN- $\beta$  (Endogen) for 24 or 48 hr. To activate BMDC, mock- or VV-infected BMDC were cultured in the presence of 1  $\mu$ g/ml LPS for 24 hr.

To investigate the bystander effect of VV infection, conditioned medium (CM) were collected from mock- or VV-infected BMDC 24 hr post-infection, either filtered or unfiltered with a 0.2  $\mu$ m syringe driven filter unit (Millipore, Bedford, MA), and added to uninfected BMDC followed by culturing them for 24 hr.

### Fluorescence-activated cell sorter (FACS) analysis

Cells were preincubated for 15 min with the anti-FcγR monoclonal antibody (Ab) 2.4G2 to block non-specific binding before staining with the following fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll a protein (PerCP)-, or allophycocyanin (APC)-conjugated Abs for 30 min at 4 °C: CD11c (clone HL3), CD11b (clone M1/70), CD49b (pan-NK, clone DX5), CD45R (B220, clone RA3-6B2), CD4 (L3T4, clone RM4-5), CD8α (Ly-2, clone 53-6.7), CD40 (clone 3/23), CD80 (B7-1, clone 16-10A1), CD86 (B7-2, clone GL-1), MHC class I (H2-K<sup>b</sup>, clone AF6-88.5), MHC class II (I-A<sup>b</sup>, clone AF6-120.1), and mouse IgG<sub>2a</sub>, κ (isotype control), all of which were obtained from BD Biosciences (San Diego, CA). Cells were then washed and fixed with 2% paraformaldehyde. To identify VV-infected cells, the cells were fixed, permeabilized with 0.2% saponin (Sigma), and stained with a biotinylated mouse IgG<sub>2a</sub>, κ isotype control (eBioscience, San Diego, CA) or a biotinylated TW2.3 monoclonal Ab specific for the E3L viral protein [34], followed by the addition of PerCP- or APC-conjugated streptavidin (BD Biosciences). Flow cytometric analyses were performed using a FACSCalibur and analyzed using CellQuest software (BD Biosciences).

### Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations in the culture supernatants were detected by ELISA as previously described [35]. Purified anti-mouse capture and biotinylated detection Abs, respectively, were: IL-6 (clone P5-20F3 and MP5-32C11), IL-10 (clone JES5-2A5 and SXC-1), IL-12p70 (clone 9A5 and C17.8), and TNF-α (clone G281-2626 and MP6-XT3). All Abs were purchased from BD Biosciences.

### RT-PCR and quantitative real-time PCR (qRT-PCR)

Total RNA preparation, cDNA synthesis, and PCR were conducted as described [36]. The primers used for E3L and hypoxanthine guanine phosphoribosyl transferase (HPRT) were described previously [13;37].

qRT-PCR was performed by the comparative threshold cycle ( $\Delta C_T$ ) method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used for IFN-β, IFN regulatory factor (IRF)-7, MHC class II (I-A<sup>b</sup>) and GAPDH were as described [37;38].

### Antigen presentation assays

CD11c-enriched splenic DC ( $1 \times 10^5$ ) from VV- or mock-infected mice were incubated with increasing concentrations of the HEL<sub>74-88</sub> peptide for 16 hr, followed by washing and fixation (0.5% paraformaldehyde, 10 min, 4°C). Peptide-loaded DC were cultured with the HEL-peptide-specific T cell hybridoma, B04 ( $5 \times 10^4$ ) for 24 h. IL-2 production was measured using HT-2, an IL-2-dependent T cell line, as previously described [13]. HT-2 cell proliferation was quantitated via [<sup>3</sup>H]thymidine incorporation into DNA and the data are expressed as the net cpm (counts per minute).

### Statistical analysis

Differences between VV- and mock-infected groups were examined by a two-way ANOVA followed by Bonferroni-Dunn post hoc test. Statistical significance was defined as a *p* value < 0.05.

## RESULTS

### Splenic DC are infected by VV at a low efficiency *in vivo*

To study the effects of a VV infection on DC function *in vivo*, we infected C57BL/6 mice with  $1 \times 10^6$  pfu VV i.p. Mice were then sacrificed one or two days post-infection, and splenic DC

(SPDC) were prepared as described in the Materials and Methods. To determine whether DC were infected with the virus *in vivo*, we examined total splenocytes from mock- and VV-infected mice using an Ab recognizing E3L, a VV-encoded early gene product. As expected, E3L-positive SPDC were not detectable from mock-infected mice (Figure 1A, top panel). However, SPDC from VV-infected mice had a very small population expressing E3L (Figure 1A, top panel). The low levels of E3L detected after an *in vivo* infection suggest either a low level of SPDC viral infection or reduced virus synthesis of this antigen in DC. To assess the infection efficiency, we performed *in vitro* VV infection using both BMDC and SPDC with a range of MOI. A distinct E3L positive BMDC population was present after an *in vitro* infection with an MOI as low as  $10^{-3}$  and E3L expression correlated with the dose of VV used for infection (Figure 1A, middle panel). SPDC were also infected by VV *in vitro*, although the efficiency was less than that of BMDC (Figure 1A, bottom panel). The E3L signal appears to be specific since a control Ab with the same IgG<sub>2a</sub>,  $\kappa$  isotype did not show a positive cell population from mock- or VV-infected BMDC (Figure 1B).

To further confirm that SPDC were infected by VV *in vivo*, we analyzed mRNA levels of E3L. SPDC from VV- but not mock-infected mice expressed E3L mRNA (Figure 1C). To test whether other APC are also infected, we prepared B cells and macrophages by enriching CD11c<sup>-</sup>B220<sup>+</sup> and CD11c<sup>-</sup>CD11b<sup>+</sup> cells, respectively, and compared E3L mRNA levels among all three APC types. As shown in Figure 1D, both B cells and macrophages expressed E3L mRNA, albeit at lower levels than DC, suggesting that splenic APC including DC are infected by VV *in vivo*. While E3L mRNA was detected in splenic B cells and macrophages, little, if any, E3L protein was observed in these APC using E3L staining and flow cytometry (data not shown).

### Splenic DC from VV-infected mice exhibit an activated phenotype yet display reduced MHC class II expression

It is well established that activated and thus matured DC are potent APC expressing elevated cell surface molecules, such as MHC class I and II and co-stimulatory molecules, as well as producing cytokines. Previously, it has been shown that DC infected with VV *in vitro* are compromised in their ability to undergo maturation in response to stimulation with TLR ligands [31;39]. Therefore, we tested whether SPDC were capable of becoming activated after an *in vivo* VV infection of mice and *in vitro* exposure to LPS. We assessed the amount of cytokines produced by SPDC that were prepared from mock- and VV-infected mice followed by stimulation with LPS overnight. Unexpectedly, SPDC from VV-infected mice produced a greater amount of IL-10, IL-12, IL-6 and TNF- $\alpha$  than those from mock-infected mice (Figure 2A). Cytokine production peaked on day 1 post-infection, which correlated with the virus load in the DC populations. Thus, SPDC appear to be activated by a VV infection *in vivo*.

The fact that SPDC from VV-infected mice produced more cytokines prompted us to examine the response of *in vitro*-infected DC. Since the infection efficiency *in vivo* appears to be equivalent to that of an *in vitro* infection at an MOI of  $10^{-3}$ – $10^{-4}$  (Figure 1A), we infected BMDC with VV at various MOI and stimulated them with LPS. We observed that an *in vitro* infection with a low dose of VV did not inhibit the BMDC's potential to produce IL-10 or IL-12 upon LPS stimulation (Figure 2B). However, unlike the SPDC infected *in vivo* that produced more cytokines in response to LPS, BMDC infected *in vitro* with a low dose of VV had a similar level of cytokine production as compared to that of mock-infected DC upon LPS exposure (Figure 2B).

Next, we examined the levels of MHC and co-stimulatory molecules. SPDC from VV-infected mice as early as one day following infection up-regulated MHC class I, MHC class II, CD40, and CD86 on their cell surface (Figure 2C, top panel). However, two days post-infection, the

amount of MHC class II on the cell surface was decreased although the levels of MHC class I and co-stimulatory molecules remained elevated (Figure 2C, bottom panel).

### A change in the splenic DC populations in VV-infected mice

Several DC subsets are present in the spleen and each subset is known to perform different functions [19]. Therefore, it is possible that the relative levels of the DC subpopulations could have been altered upon VV infection, which may have been responsible for their apparently activated phenotype. To assess the DC subset composition post-infection, total and CD11c<sup>+</sup> enriched splenocytes were prepared from mock- or VV-infected mice and analyzed for the presence of different cell types.

The total number of splenocytes in mice was comparable with or without VV infection (data not shown). However, the proportion of CD4<sup>+</sup> T cells was decreased gradually after VV infection, while CD8<sup>+</sup> T cell levels remained relatively unchanged at these early stages of infection (Figure 3A). In addition, there was a small but significant increase in the percentage of B cells (B220<sup>+</sup>CD11c<sup>-</sup>) and macrophages (CD11b<sup>+</sup>CD11c<sup>-</sup>) two days after a VV infection (Figure 3A). The DX5<sup>+</sup> natural killer (NK) cell and CD11c<sup>+</sup> SPDC population were not altered (Figure 3A).

We next analyzed CD11c<sup>+</sup> cells in detail. It is known that NK cells express CD11c upon virus infection or activation [40;41]. In agreement with these reports, CD11c<sup>+</sup> NK cells increased in VV infected mice although the CD11c level of NK cells was lower than that of DC (Figure 3B). A similar observation was made in total splenocytes (data not shown), suggesting that these NK cells might have been activated upon VV infection. We also found that DC subsets expressing CD8 or B220 that are known to increase upon virus infection [25;26] were indeed enhanced two days post-infection, while CD4<sup>+</sup> or CD11b<sup>+</sup> DC remained at a comparable level (Figure 3B).

### Impaired APC function of DC after a VV infection

Reduced MHC class II on SPDC from VV-infected mice led us to test their potential to present antigens to CD4 T cells. To do this, CD11c<sup>+</sup> SPDC were prepared from mock- or VV-infected mice, loaded with a different amount of HEL<sub>74-88</sub> peptide, fixed, and used to stimulate HEL peptide-specific CD4 T cells. As expected, SPDC from mock-infected mice stimulated T cells in the presence of the peptide. However, DC from VV-infected mice showed a substantially impaired ability to present antigens to CD4 T cells as early as day 1 post-infection (Figure 4).

### Role of type I IFN in MHC class II expression and viral replication in DC

So far, we have demonstrated that a VV infection appears to down-regulate the amount of MHC class II on the surface of SPDC and disrupts antigen presentation. To understand the mechanism responsible for these effects, we assessed the level of MHC class II mRNA in DC from VV-infected animals. As shown in Figure 5A, SPDC from VV-infected mice expressed less MHC class II mRNA compared to mock-infected mice, implying that the observed decrease in surface MHC class II after two days of infection could have resulted from reduced mRNA. In fact, MHC class II gene expression was gradually declined as the infection progressed during VV infection (Figure 5B). However, the level of MHC class II on the cell surface was increased despite the reduction of mRNA after one day of infection (Figure 2C). This is most likely due to the transport of pre-assembled MHC class II molecules from the endosomal compartments to the cell surface in activated DC [42]. Previous studies have shown that type I IFN can inhibit MHC class II expression in multiple cell types including macrophages [43; 44; 45; 46]. Virus infections are known to induce type I IFN gene expression, raising the possibility that the decline in MHC class II mRNA was linked to the action of type I IFN. Notably, IFN- $\beta$  expression by SPDC was greatly elevated in SPDC from VV-infected

mice (Figure 5A). The induction of IFN- $\beta$  mRNA was accompanied by the expression of IRF-7, a critical transcription factor for type I IFN [47] (Figure 5A).

Having observed an inverse relationship between IFN- $\beta$  and MHC class II gene expression in SPDC after VV infection, we next investigated a possible role for type I IFN in VV-mediated MHC class II mRNA down-regulation. BMDC from wild type and IFNAR<sup>-/-</sup> mice were mock- or VV-infected, or treated with IFN- $\beta$ . MHC class II mRNA levels were then assessed. Consistent with the SPDC data from VV-infected mice, the level of MHC class II mRNA was reduced in BMDC by a VV infection *in vitro* (Figure 5C, left side). In addition, and consistent with a report showing that a viral infection switches non-pDC to IFN producers [48], VV-infected BMDC *in vitro* also expressed IFN- $\beta$ , albeit at lower levels compared to SPDC (data not shown). However, IFN- $\beta$  treatment alone enhanced MHC class II gene expression in BMDC (Figure 5C, left side). When BMDC were infected with VV and then cultured in the presence of IFN- $\beta$ , they were still able to maintain MHC class II expression. Thus, IFN- $\beta$  induces an increase in the MHC class II mRNA level in DC, and VV-mediated down-regulation of MHC class II expression is unlikely caused by IFN- $\beta$  production by infected DC. In fact, the MHC class II mRNA level in IFNAR<sup>-/-</sup> DC was greatly reduced after VV infection even after receiving IFN- $\beta$  (Figure 5C, right side).

It has been shown that IFNAR<sup>-/-</sup> mice are more susceptible to a VV infection than are wild type mice, although DC in these mice were not examined [32;49]. Therefore, we investigated whether IFNAR<sup>-/-</sup> DC can control VV infection. To do this, we utilized a recombinant VV strain (mKO) that expresses OFP under the p7.5 early/late promoter of VV [32]. Infection with the mKO VV is comparable to that of wild type VV [32]. BMDC from WT and IFNAR<sup>-/-</sup> mice were infected with mKO and then cultured with or without IFN- $\beta$ . As shown in Figure 5C, both WT and IFNAR<sup>-/-</sup> DC had an equivalent population expressing E3L regardless of the dose of VV or the duration of infection. However, compared to WT DC, IFNAR<sup>-/-</sup> DC displayed higher OFP positive cells, suggesting that type I IFN is necessary to limit viral replication in this cell population (Figure 5D). In fact, we observed that IFNAR<sup>-/-</sup> DC were more susceptible to cell death upon VV infection even at a very low dose of VV (data not shown). Although WT DC also underwent cell death after 2 days of infection *in vitro*, adding IFN- $\beta$  to the culture prevented cell death and completely abolished virus replication in WT DC (Figure 5D, bottom panel). This effect was not observed in IFNAR<sup>-/-</sup> DC (Figure 5D, bottom panel).

We next sought whether the down-regulation of MHC class II by VV is a result of VV replication or an indirect effect exerted by host cell factors secreted to culture media. To test this, CM was collected from DC that were mock- or VV-infected at MOI of 0.1 or 0.01 for 24 hrs, filtered to remove virus particles, and then added to uninfected DC. As expected, unfiltered CM from VV-infected DC caused a secondary infection at a dose-dependent manner (Figure 5E). However, filtered and thus virus-free CM was not able to infect DC. Moreover, virus-free CM did not inhibit MHC class II expression, suggesting that the down-regulation of MHC class II expression is likely, at least in *in vitro* infected BMDC, caused by a VV gene product (s) not secreted host factors (Figure 5F).

## DISCUSSION

In the present study, we have demonstrated that SPDC are activated following a VV infection *in vivo*, as evidenced by elevated levels of cell surface MHC class I and co-stimulatory molecules, and by the greater potential of these cells to produce cytokines. However, despite their activated phenotype, SPDC exposed to VV *in vivo* are compromised in MHC class II gene expression and antigen presentation by MHC class II molecules.

To our knowledge, this is the first study showing an effect of VV infection on MHC class II gene expression. We propose that there are at least two possible mechanisms responsible for impaired MHC class II APC function in DC following VV infection. First, VV may directly interfere with ligand binding to MHC class II molecules. As the experiments in the current study used antigenic peptides rather than native antigens, the disruption in MHC class II function is unlikely caused by a defect in antigen processing. This hypothesis would be consistent with our previous report that *in vitro* VV infection disrupts MHC class II-restricted Ag presentation [13]. Secondly, similar to other viruses, VV appears to down-regulate MHC class II gene expression by DC at a low MOI and 1–2 days exposure to virus [6;50]. Although MHC class II levels were maintained on the cell surface, gene expression is reduced in DC after one day of VV infection (Figure 2C and 5A). The sustained level of surface MHC class II is likely attributed to the longer half-life of proteins than mRNA of MHC class II. Indeed, reduced amounts of MHC class II on the cell surface of DC were only observed after two days of infection.

Currently, it is not clear how MHC class II gene expression is down-regulated by VV. Studies here suggest a possibility that the down-regulation is not mediated by type I IFN, although it has been shown to inhibit IFN- $\gamma$ -inducible MHC class II gene expression in macrophages, fibrosarcoma, and astrocytoma cell lines [43;44;45;46]. Instead, MHC class II mRNA levels were induced by IFN- $\beta$  *in vitro* (Figure 5B). This difference in IFN- $\beta$  sensitivity could result from the different modes of MHC class II gene expression in DC as compared to these other cell types. DC express MHC class II constitutively, whereas macrophages, fibrosarcoma, and astrocytoma cell lines require an external stimulus, such as IFN- $\gamma$ , to activate the MHC class II promoter. Regardless, our data using IFNAR<sup>-/-</sup> DC support the idea that VV-induced type I IFN production is not responsible for the MHC class II down-regulation.

On the other hand, the addition of exogenous IFN- $\beta$  completely abolished virus replication and restored MHC class II gene expression. Moreover, unfiltered but not filtered CM from VV-infected BMDC caused secondary infection and MHC class II down-regulation. These data suggest that VV-encoded proteins either directly or indirectly, inhibit MHC class II gene expression in BMDC infected *in vitro*. In SPDC from *in vivo* infected mice, the down-regulation of MHC class II mRNA appears to be much greater than expected when compared with the relative level of virus detected in cells (Figure 1 and 5). Yet, viral infection in this case, was monitored by expression of the viral early gene product E3L, whose synthesis drops during the progression of infection. Thus, E3L protein levels may offer an underestimate of the amount of virus actually in cells. Some cells might have been infected with very low levels of VV, sufficient to decrease MHC class II gene expression but not detectable by E3L staining. Or, cells were infected but E3L expression was down-regulated during the infection. Lastly, during an *in vivo* infection, it is very likely that environmental host factors augment or promote down-regulation of MHC class II expression. Our *in vitro* infection data suggest that the DC-virus interactions lead to inhibition of MHC class II expression. Yet, we cannot rule out any bystander effect from secreted host cell factors in response to infection or stress, as factors driving the down-modulation of class II expression in *in vivo* infected DC. Future studies are warranted to dissect the specific mechanisms by which VV disrupts MHC class II gene expression. What critically important is our observation that while the virus can promote innate pathways for DC maturation, the loss of class II antigen presentation by these cells compromises or weakens, host protective immunity against VV. We also suspect that the enhanced susceptibility of IFNAR<sup>-/-</sup> mice to a VV infection is at least partly due to the limited capability of their DC to control virus replication. As the virus infection progresses *in vivo*, the viral load would increase likely causing more DC death. Consequently, the number of DC declines and T cell priming could thus be compromised.



Our data demonstrated that DC are activated during an *in vivo* infection, differing from studies suggesting *in vitro* the virus does not activate DC [30;31;39]. In concert with those reports, it has been reported that DC infected with a recombinant modified vaccinia Ankara (MVA) retain their immunogenicity *in vivo* despite *in vitro* dysfunction [51]. This is in contrast to another report suggesting that MVA can at least moderately activate human DC [52]. This inconsistency between *in vitro* and *in vivo* infection studies may be attributed to two factors: virus load and the DC subset infected. It is likely that low doses of VV activate DC, whereas a high virus load is detrimental. With i.p. delivery of virus, the amount of VV entering to the spleen directly, or indirectly carried by infected cells, appears to be low based upon the detection of virus in splenocytes. Delivery of  $10^6$  pfu via the i.p. route was equivalent to infecting cells with an MOI of  $10^{-3}$ – $10^{-4}$  *in vitro* (Figure 1A). At this low dose of VV, DC functions were not compromised and activation was observed (Figure 2B). However, an infection with an MOI greater than  $10^{-3}$  *in vitro* inhibited DC maturation (Figure 2B), consistent with published studies [13;31]. Therefore, it seems that DC with the activated phenotype observed from *in vivo* infected VV mice are likely bystanders that were not directly infected by VV.

The other factor possibly influencing maturation is the subtype of the infected DC. DC resident in the spleen are comprised of at least two subsets: cDC and pDC. This is in contrast to DC prepared from BM or blood that are primarily mDC (19). pDC are known to produce type I IFN that can control subsequent virus replication and induce DC maturation [53;54]. As we have shown (Figure 1A), SPDC were more resistant to VV infection *in vitro* than BMDC, likely due to the ability of DC to produce type I IFN. This idea is further supported by our data demonstrating that adding IFN- $\beta$  to VV-infected BMDC *in vitro* completely abolished virus replication (Figure 5C). However, other factor(s), in addition to type I IFN, seem to be required for proper DC activation because IFN- $\beta$  had no effect on the cytokine production potential of BMDC (data not shown). Moreover, virus-free CM from VV-infected BMDC did not alter the cytokine production by BMDC (data not shown). Together, the response of DC differs depending on the dose of virus, the subset of DC used, and other environmental factors. In this regard, it should be noted that lung DC from mice infected with VV two days previously by the intratracheal route also showed decreased antigen presentation potential (unpublished data). Although the type of DC in the lung and the levels of MHC class II mRNA still need to be analyzed in that model, it is clear that an *in vivo* VV infection appears to modulate antigen presentation function of DC regardless of the route of infection.

What would be the immunological significance of down-regulating antigen presentation by MHC class II during VV infection? Clinically, a transient decrease in human T cell proliferative responses has also been reported following immunization with the vaccinia vaccine [55]. Such suppressed immune responses could result in part from the decrease in DC's potential to present antigens by MHC class II. With this, it would be important to determine when DC re-express MHC class II genes and recover antigen presentation function to initiate CD4 T cell activation.

In sum, we showed that select DC functions can be activated by VV infection yet antigen presentation by MHC class II is compromised by this virus. Further investigations are needed to provide insights into the molecular mechanisms of host pathogenesis by VV, and improved vaccine design for poxviruses.

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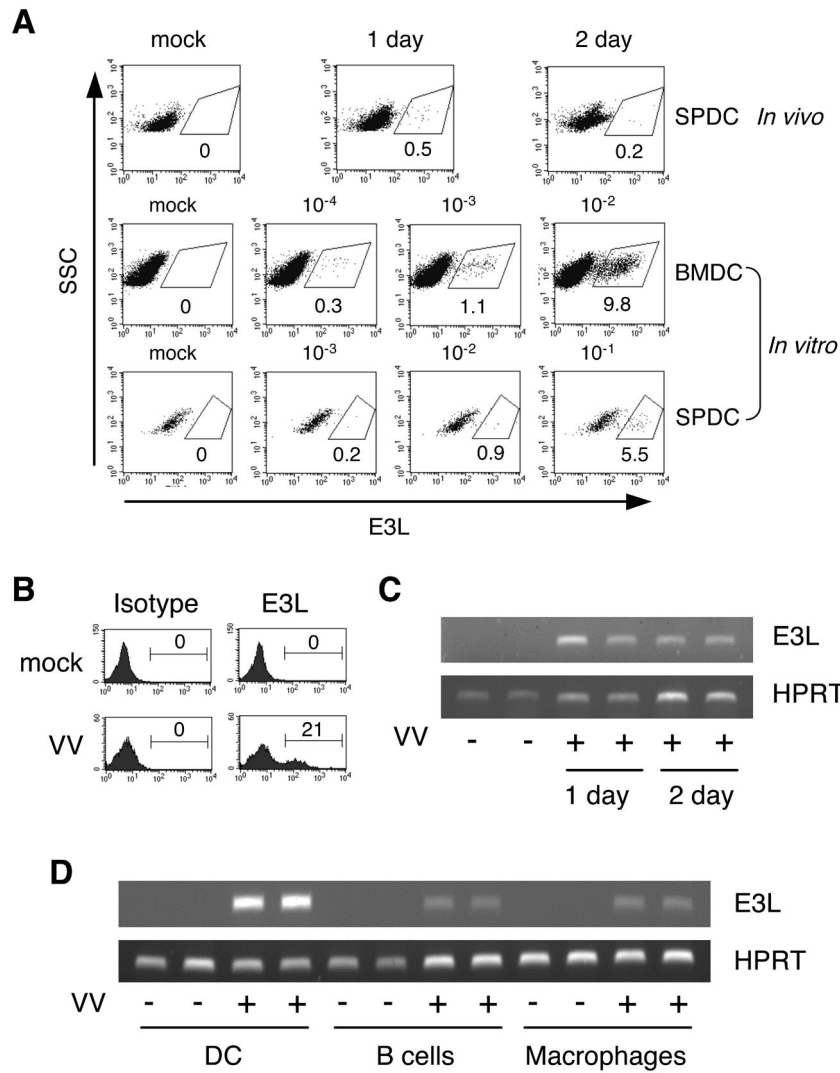
This work was supported in part by National Institutes of Health grants AI056097 (to C-H.C, J.S.B, S-C.H, and R.R.B), and an American Heart Association postdoctoral fellowship award 0520111z (to Y.Y.).

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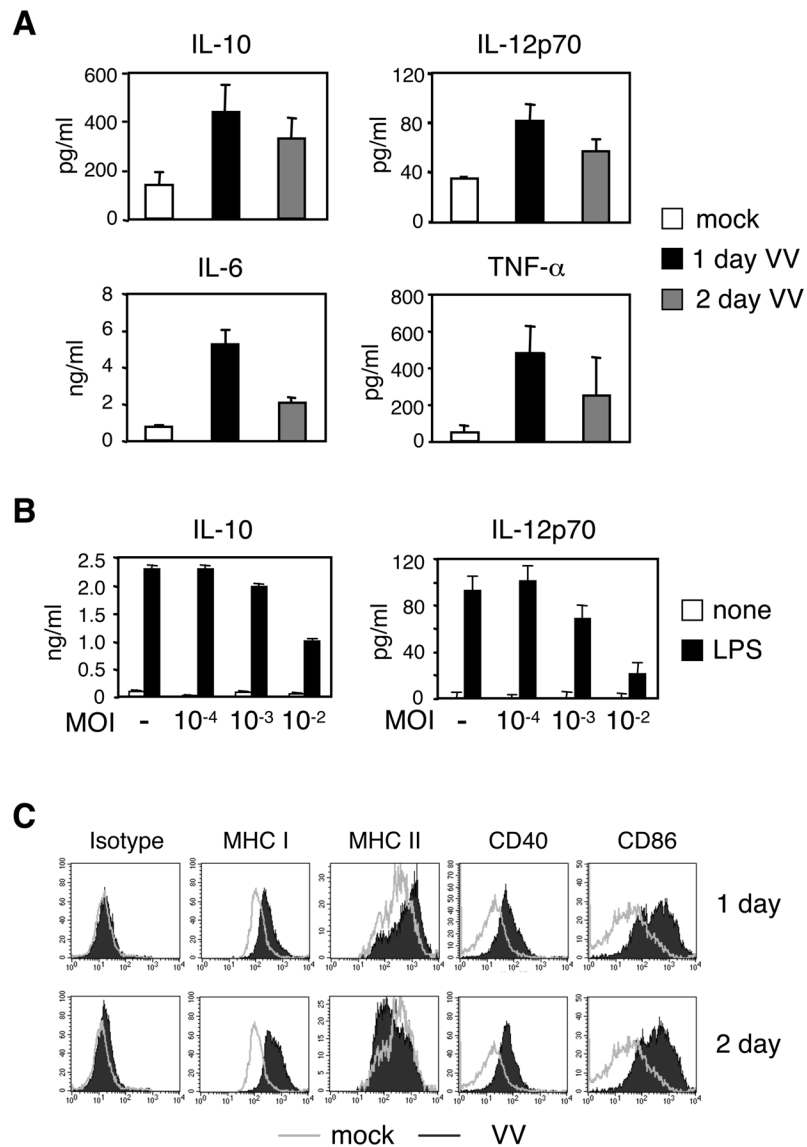
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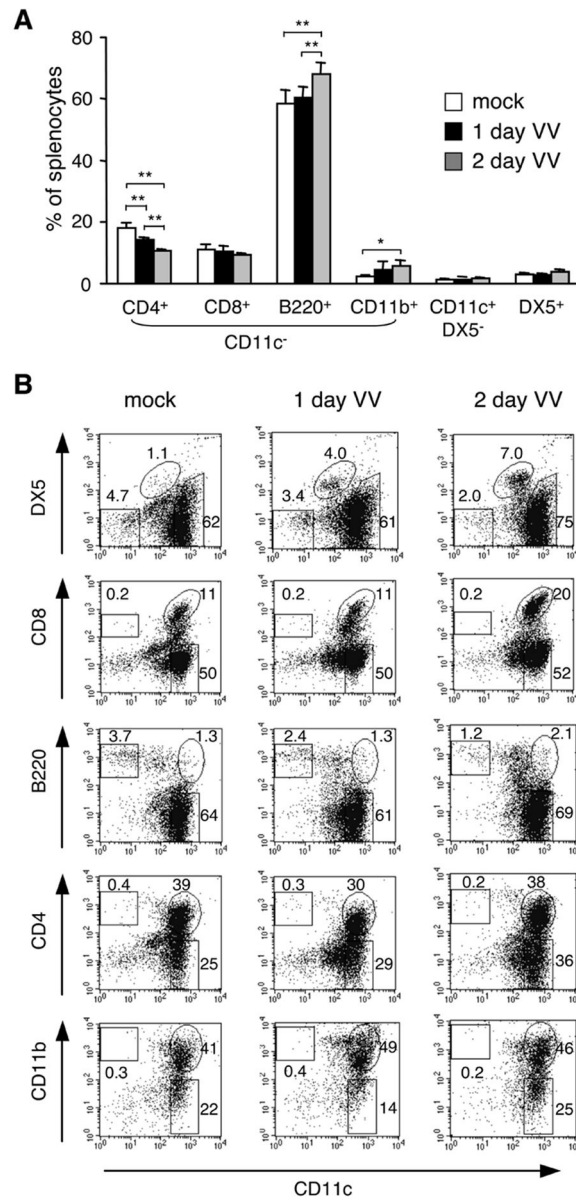
**Figure 1. Detection of VV infection**

*A*, *In vivo* infection was carried out using C57BL/6 mice (four mice/group) that were injected with HBSS/BSA (mock) or  $1 \times 10^6$  pfu VV via i.p as described in the Materials and Methods. Mice were sacrificed at the indicated time. Total splenocytes were stained with TW2.3 Ab recognizing E3L. For *in vitro* infection, BMDC and total splenocytes were infected and cultured for 24 hrs with MOI as indicated. CD11c<sup>+</sup> cells were gated and shown. The percentage indicates % positive cells among CD11c<sup>+</sup> cells. *B*, Specificity of TW2.3 staining. BMDC were infected 24 hrs with VV at a MOI of 0.1 and stained with TW2.3 or its isotype-matched control Ab. The numbers indicate % positive cells. *C*, RNA was prepared from CD11c<sup>+</sup> enriched splenic DC from mock- or VV-infected mice as in (A) and analyzed for E3L expression. *D*, CD11c<sup>+</sup> DC cells were prepared as in (C), and B220<sup>+</sup> B cells and CD11b<sup>+</sup> macrophages were enriched from CD11c<sup>-</sup> splenocytes of one day infected mice. RT-PCR was performed to detect E3L gene expression. Data are representative of at least 2 independent experiments.

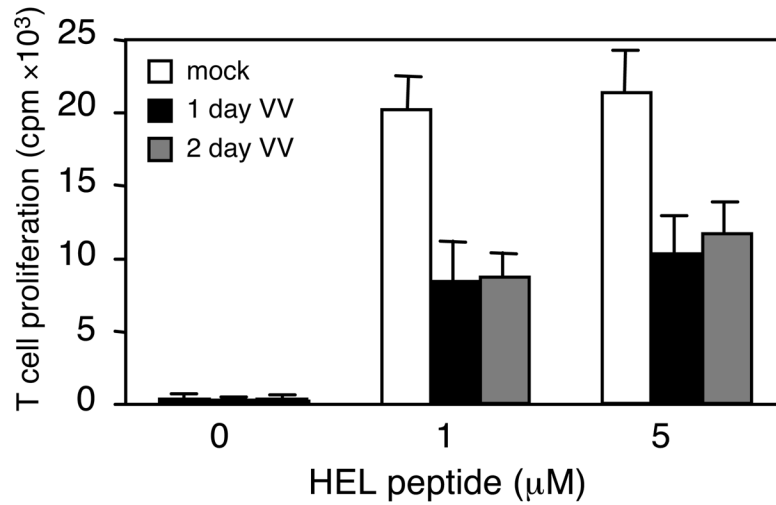


### Figure 2. Activated phenotype of SPDC except MHC class II expression

A, SPDC were prepared as in Figure 1 and stimulated with LPS (1  $\mu$ g/ml) overnight. ELISA was performed to measure the levels of IL-10, IL-12p70, IL-6, and TNF- $\alpha$ . B, BMDC were infected *in vitro* with VV at the indicated MOI for 24 h followed by LPS stimulation overnight. IL-10 and IL-12p70 production was measured by ELISA. C, SPDC from mock- or VV-infected mice were used to determine cell surface expression levels of MHC and co-stimulatory molecules. DX5<sup>-</sup>CD11c<sup>+</sup> or CD11c<sup>high</sup> cells were gated as in Figure 3B and shown. Open and filled histograms are DC from mock- and VV-infected mice, respectively. Data are the mean  $\pm$  SD (A and B) or representative (C) of at least 2 independent experiments.



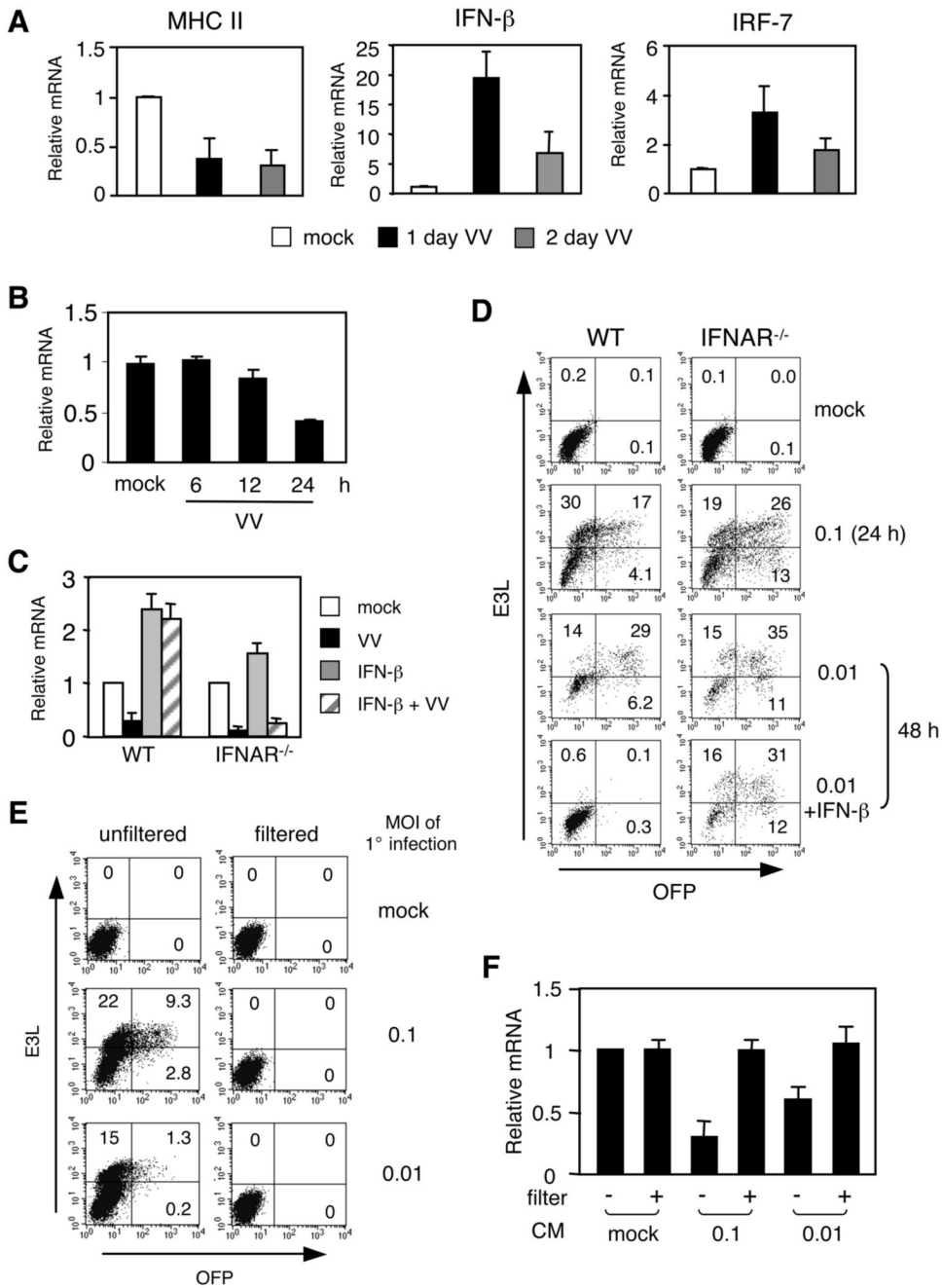
**Figure 3. Changes in cell composition and DC subset in the spleen from VV-infected mice**  
**A**, Total splenocytes from mock- or VV-infected mice were stained for cell surface markers and analyzed for different cell types. \* $p < 0.01$ , \*\* $p < 0.001$ . **B**, CD11c<sup>+</sup> enriched splenocytes were analyzed for purity and DC subpopulations. Numbers indicate each subpopulation as a percentage of live cells. Note that DX5<sup>+</sup> cells are CD11c<sup>low</sup>. Data are the mean  $\pm$  SD (**A**) or representative (**B**) of at least 2 independent experiments.



**Figure 4. Impaired APC function of SPDC from VV-infected mice**

CD11c<sup>+</sup> enriched splenic DC from mock- or VV-infected mice were incubated with increasing amounts of the HEL<sub>74-88</sub> peptide for 16 h, followed by washing, fixation and cultivation with Ag-specific T cells for 24 h. T cell activation was assessed by measuring IL-2 production using HT-2 cell proliferation. Data are expressed as cpm and are representative of 3 independent experiments with the SD for triplicated samples indicated.





**Figure 5. Role of type I IFN in MHC class II expression and viral replication in DC**  
 A, SPDC were enriched from mock- or VV-infected mice to prepare RNA. qRT-PCR was performed to assess MHC class II, IFN-β, and IRF-7 mRNA. The relative mRNA levels were normalized to GAPDH gene expression. B, MHC class II mRNA levels in SPDC enriched from mock- or VV-infected mice at indicated time points were measured by qRT-PCR as in (A). C, BMDC from WT or IFNAR<sup>-/-</sup> mice were infected with the mKO mutant VV (MOI of 0.01) for 90 min and cultured with or without IFN-β for 48 h. MHC class II mRNA levels were measured by qRT-PCR. D, BMDC were prepared, infected (MOI of 0.1 for 24 hrs and 0.01 for 48 hrs), and cultured as in (C) for 24 or 48 hrs. E3L and OFP expression were analyzed by flow cytometry. Numbers indicate each subpopulation as a percentage of live cells. E and F,

BMDC from WT mice were infected with the mKO mutant VV (MOI of 0.1 and 0.01) for 24 hrs (indicated as 1° infection). Conditioned medium (CM) from infected BMDC were collected, filtered or unfiltered, and then added to uninfected BMDC. Cells were analyzed an additional 24 h post-infection. Efficiency of virus depletion was monitored by flow cytometry (E). MHC class II mRNA levels were measured by qRT-PCR (F). Data are the mean  $\pm$  SD (A, B, C and F) or representative (D and E) of at least 2 independent experiments.