

Research Article

Immunogenicity of a Recombinant Influenza Virus Bearing Both the CD4+ and CD8+ T Cell Epitopes of Ovalbumin

Bruno Garulli,^{1,2} Giuseppina Di Mario,¹ Ester Sciaraffia,¹ Yoshihiro Kawaoka,^{3,4,5} and Maria R. Castrucci¹

¹ Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, 00161 Rome, Italy

² Department of Biology and Biotechnology "Charles Darwin", University of Rome "La Sapienza", 00185 Rome, Italy

³ Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, WI 53706, USA

⁴ Division of Virology, Department of Microbiology and Immunology, and International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

⁵ ERATO Infection-Induced Host Responses Project, Japan Science and Technology Agency, Saitama 332-0012, Japan

Correspondence should be addressed to Maria R. Castrucci, mariarita.castrucci@iss.it

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Recombinant influenza viruses that bear the single immunodominant CD8+ T cell epitope OVA_{257–264} or the CD4+ T cell epitope OVA_{323–339} of the model antigen ovalbumin (OVA) have been useful tools in immunology. Here, we generated a recombinant influenza virus, WSN-OVA_{I/II}, that bears both OVA-specific CD8+ and CD4+ epitopes on its hemagglutinin molecule. Live and heat-inactivated WSN-OVA_{I/II} viruses were efficiently presented by dendritic cells *in vitro* to OT-I TCR transgenic CD8+ T cells and OT-II TCR transgenic CD4+ T cells. *In vivo*, WSN-OVA_{I/II} virus was attenuated in virulence, highly immunogenic, and protected mice from B16-OVA tumor challenge in a prophylactic model of vaccination. Thus, WSN-OVA_{I/II} virus represents an additional tool, along with OVA TCR transgenic mice, for further studies on T cell responses and may be of value in vaccine design.

1. Introduction

Recombinant influenza viruses represent a promising delivery system of foreign antigens that can be fused to viral proteins, such as hemagglutinin (HA), neuraminidase (NA), or nonstructural protein 1 (NS1), or can be expressed as an individual whole protein flanked with NA segment-specific packaging signals [1–5]. Influenza viruses bearing model antigens have been used as tools to address several questions and long-standing issues in immune biology. In particular, recombinant influenza viruses bearing the single immunodominant CD8+ T cell epitope OVA_{257–264} or the CD4+ T cell epitope OVA_{323–339} of chicken ovalbumin (OVA) have been used in several studies to characterize T cell responses [6–12].

HA engineered to carry small polypeptides fused to its N-terminus is efficiently expressed and stably incorporated into retroviral particles without affecting its fusion activity [13].

Recently, this strategy was adopted to generate recombinant influenza viruses bearing relatively large domains of the protective antigens of *Bacillus anthracis* toxin (domain 1', the region responsible for binding to the lethal factor and the edema factor (LEF) and domain 4, the receptor-binding domain (RBD)) fused to the N-terminus of the HA of A/Aichi/2/68 (H3N2) in the genetic background of A/WSN/33 virus (H1N1) [14, 15]. The chimeric fusion proteins, named HA/LEF and HA/RBD, were functionally expressed during virus infection in mice and elicited antibody responses specific to both the viral HA and the protein domains of the *Bacillus anthracis* toxin. Here, by using a similar approach, we generated a recombinant influenza virus, WSN-OVA_{I/II}, bearing both OVA-specific CD8+ and CD4+ epitopes in its HA, and characterized the OVA-specific T cell immune response *in vitro* and *in vivo* as well as its ability to protect mice from challenge with the OVA-expressing tumor cells B16-OVA in a prophylactic model of vaccination.

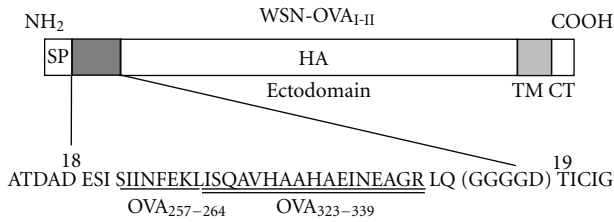


FIGURE 1: Construction of the mutant hemagglutinin (HA) segment. Schematic representation of the HA/OVA_{I/II} construct, containing the OVA epitopes inserted after the HA signal peptide (SP). CT, TM = cytoplasmic, transmembrane domains of HA, respectively.

2. Materials and Methods

2.1. Mice. Female C57BL/6J mice and the TCR-transgenic mouse line OT-II, expressing a TCR that recognize the OVA₃₂₃₋₃₃₉ epitope, were obtained from Charles River, Calco, Italy. The TCR-transgenic mouse line OT-I, expressing a TCR that recognize an H-2^b-restricted OVA₂₅₇₋₂₆₄ epitope, SIINFEKL, was kindly supplied by Dr. M. Bellone (San Raffaele Scientific Institute, Milan, Italy). For all experiments, mice, aged 6–12 weeks, were maintained at the Istituto Superiore di Sanità by following institutional guidelines and approved protocols.

2.2. Generation of Recombinant WSN-OVA_{I/II} Virus. The coding sequences for the OVA₂₅₇₋₂₆₄ (SIINFEKL) and OVA₃₂₃₋₃₃₉ (ISQAVHAAHAEINEAGR) T cell epitopes were inserted into the HA gene of A/WSN/33 virus, between the coding regions that correspond to the C-terminus of the HA signal peptide (SP) and the N-terminus of the protein following signal peptidase cleavage. Specifically, a pair of complementary oligonucleotides encoding the OVA epitopes, designated OVA_{I/II}, with flanking Clal/PstI sites (encoding ESI and LQ amino acids, resp.) was inserted into the cloning cassette generated at the 3' end of the signal peptide coding sequence of the pPolI-WSN HA plasmid [16], and containing the GGGGD peptide arm, as described by Li et al. [14] (Figure 1). The recombinant influenza WSN-OVA_{I/II} virus bearing both of the OVA-specific CD8⁺ and CD4⁺ epitopes was generated by use of plasmid-driven reverse genetics, as described by Neumann et al. [16]. The presence of the inserted sequence was confirmed by sequencing.

2.3. Antigen Presentation In Vitro. For the antigen presentation assays *in vitro*, CD8⁺ and CD4⁺ T cells were isolated from spleens and lymph nodes of OT-I and OT-II OVA TCR transgenic mice, respectively, by use of magnetic sorting (Miltenyi Biotec), according to the manufacturer's protocol. CD11c-positive dendritic cells (DCs) were isolated from the spleens of C57BL/6 mice by using MACS beads (Miltenyi Biotec) and the purity of the enriched CD11c-positive cell preparation ranged from 90% to 95%. DCs were resuspended to 10⁷ cells/mL in serum-free RPMI 1640, and incubated with either live or heat-inactivated (HI) WSN-OVA_{I/II} virus (1000 HAU/mL) for 1 h at 37°C, 5% CO₂. Heat inactivation was performed by incubating WSN-OVA_{I/II} virus at 56°C for 30 min in a water bath, and

virus fusion activity and inactivation were determined as previously described [17]. After incubation, antigen-loaded DCs were washed and cultured with 10⁵ enriched OT-I or OT-II cells for 48 h, after which 0.5 μCi/well [³H]thymidine (Amersham Biosciences, UK) was added for 16 h before harvesting. For the carboxyfluorescein succinimidyl ester- (CFSE-) based lymphoproliferation assay, live virus-pulsed DCs were cocultured with 2 × 10⁶ CFSE-labelled OT-I or OT-II cells; 72 h later, the cells were stained with anti-CD8-phycoerythrin and anti-CD4-phycoerythrin (PE), respectively, for flow cytometry.

2.4. Experimental Infection. Five-week-old female C57BL/6J mice, anesthetized with Avertin given intraperitoneally, were inoculated intranasally (i.n.) with 50 μL of WSN-OVA_{I/II} virus at different dilutions (five mice per dilution) and observed for 21 days to determine the minimal dose lethal to 50% of mice (MLD₅₀). For virological analysis, mice were inoculated with 3 × 10⁵ PFU of WSN-OVA_{I/II} virus or 5 × 10² PFU of WSN virus, and virus titres in the lungs and nasal turbinates were determined 3 days and 6 days after infection by use of MDCK cells, as described previously [18].

2.5. Immunization and Tumor Challenge. Female C57BL/6J mice were i.n. immunized with 3 × 10⁵ PFU of WSN-OVA_{I/II} virus or 5 × 10² PFU of WSN virus. Nine days later, five mice from each group were sacrificed to obtain spleens and mediastinal lymph nodes (MLN), and epitope-specific T cell responses were determined by using a mouse IFN-γ enzyme-linked immunospot assay (ELISPOT) kit (Pharmingen, BD). Specifically, a single-cell suspension from lymphoid tissues was cultured with either OVA₂₅₇₋₂₆₄ or the viral nucleoprotein (NP₃₆₆₋₃₇₄) peptide, whereas enriched CD4⁺ T cells from the MLN were cultured for 48 h with the OVA₃₂₃₋₃₃₉ peptide and irradiated (2.500 rads) naïve splenocytes. Tumor challenge experiments were carried out by injecting intravenously (i.v.) 3 × 10⁵ B16-OVA cells into groups of mice that had been immunized three weeks earlier with WSN or WSN-OVA_{I/II} virus. Twelve days after tumor cell injection, eight mice from each group were sacrificed, and lung metastases were enumerated under a dissection microscope. Nonimmunized mice served as naïve controls. In addition, the frequency of SIINFEKL-specific IFN-γ-producing T cells was measured by using the ELISPOT assay on spleen-derived lymphocytes.

3. Results

The recombinant influenza WSN-OVA_{I/II} virus bearing both the OVA-specific CD8⁺ and CD4⁺ epitopes was generated by introducing the coding sequences into the HA gene of A/WSN/33 virus, after the coding region of the HA signal peptide (SP), and the GGGGD peptide arm, as described by Li et al. [14] (Figure 1). The rescued virus replicated in tissue culture with a titer ranging from 5 × 10⁶ to 10⁷ plaque-forming units (PFU)/mL.

We first determined whether the OVA-specific T cell epitopes of WSN-OVA_{I/II} virus were efficiently presented to antigen-specific T cells. To this end, the capacity of

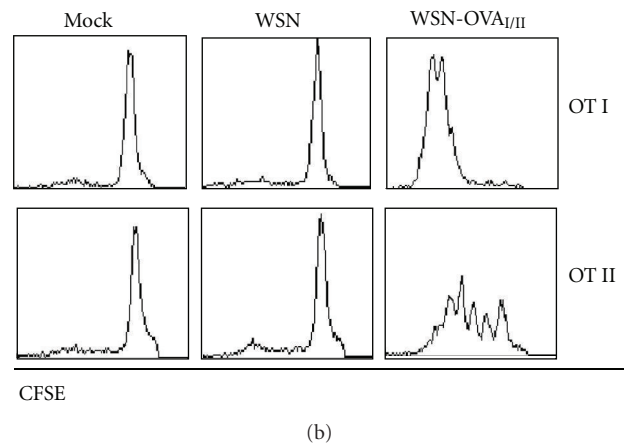
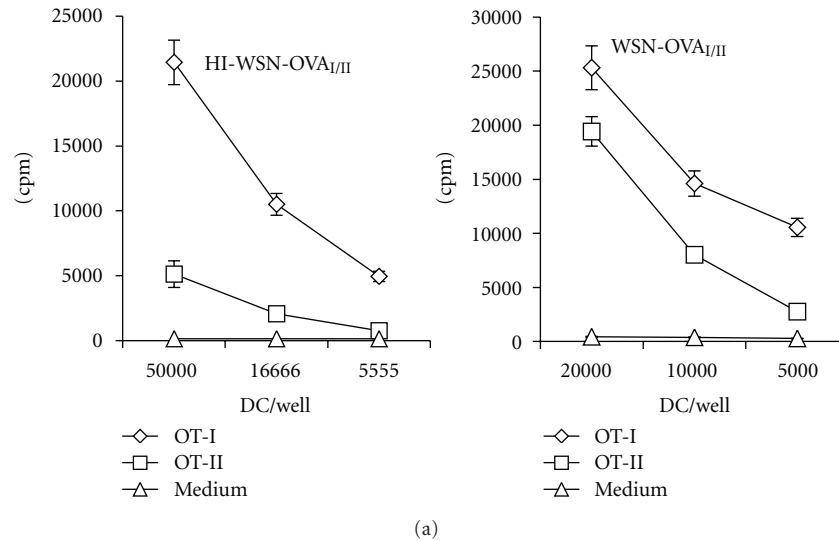


FIGURE 2: Presentation of WSN-OVA_{I/II} virus by dendritic cells (DCs) *in vitro* to OVA-specific CD8⁺ and CD4⁺ T cells. (a) Splenic DCs were incubated with either live or heat-inactivated (HI) WSN-OVA_{I/II} virus and then washed and titrated in microtiter plates. OT-I cells and OT-II cells were added to the cultures (10^5 /well), and T cell proliferation was estimated by use of [³H]thymidine incorporation two days later. The data shown are the means (\pm SEM) from triplicate cultures. (b) CFSE-labelled naïve OT-I and OT-II cells were coincubated with live virus-pulsed or control DCs. Three days later, proliferation of CD8⁺ and CD4⁺ T cells was determined by using flow cytometry. The results shown are representative of three experiments.

splenic DCs to process and present both live and HI WSN-OVA_{I/II} viruses was assessed by using an *in vitro* proliferation assay of naïve OVA-specific CD8⁺ T and CD4⁺ T cells derived from OT-I and OT-II TCR-transgenic mice, respectively. Live virus-loaded DCs efficiently processed and presented both OVA-specific epitopes on MHC class I and II molecules, whereas there was no response with uninfected DCs (Figure 2(a)). CFSE-labelled transgenic T cells were also used to monitor antigen-specific proliferation due to the progressive halving of the dye fluorescence following cell division. WSN-OVA_{I/II} virus-loaded DCs were responsible for vigorous proliferation of OT-I and OT-II cells within the CD8⁺ and CD4⁺ gated populations, respectively, compared to unloaded- or WSN virus-loaded DCs (Figure 2(b)). Furthermore, our data indicate that the OVA-specific MHC class I-restricted epitope located on the HA of whole HI

WSN-OVA_{I/II} virus was efficiently cross-presented by DCs to CD8⁺ T cells (Figure 2(a)). The binding and fusogenic functions of HI WSN-OVA_{I/II} virus were intact as assessed by standard hemagglutinating and hemolytic assays, respectively, thus confirming the importance of a functional HA to access the cytoplasm of DCs [17]. The higher T cell proliferation of OT-I compared to OT-II cells induced by DCs loaded with HI WSN-OVA_{I/II} virus, bearing equimolar amounts of both epitopes, correlates with previous data showing that the cell-associated antigen is more efficiently presented to CD8⁺ than to CD4⁺ T cells [19].

To determine the immunogenicity of the WSN-OVA_{I/II} virus in C57BL/6J mice, we first determined its pathogenicity relative to the wild-type WSN virus. None of the inoculated mice died, even those inoculated with doses of WSN-OVA_{I/II} virus as high as 10^6 PFU, whereas the wild-type

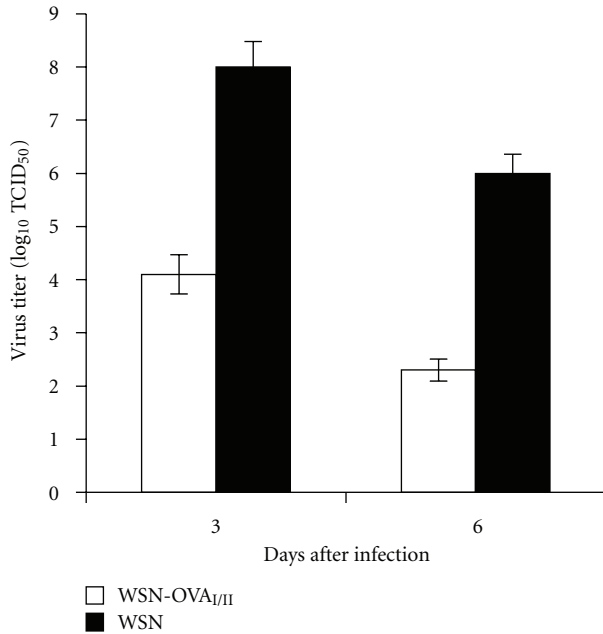


FIGURE 3: Replication of WSN-OVA_{I/II} virus in mice. C57BL/6J mice were inoculated i.n. under anaesthesia with 5×10^2 PFU of WSN virus or 3×10^5 PFU of WSN-OVA_{I/II} virus, and sacrificed on days 3 and 6 after infection for virus titration in lungs on MDCK cells. The values presented are averages of triplicate wells; the error bars indicate the standard deviation.

WSN virus killed all of the mice at this dose (data not shown). Pulmonary viral loads measured 3 and 6 days after infection in mice inoculated with 3×10^5 PFU of WSN-OVA_{I/II} virus (average titres of 4.1 and 2.3 log₁₀ TCID₅₀/lung, resp.) were about four logs lower than those observed for mice inoculated with 5×10^2 PFU of the wild-type WSN virus (Figure 3). Moreover, we could not detect virus in the nasal turbinates of the WSN-OVA_{I/II}-infected mice, whereas detectable viral titres were measured in the nasal turbinates of the WSN-infected animals on day 3 and 6 after infection (data not shown). Although the recombinant virus did not replicate as efficiently as the WSN virus, we used the above viral doses to immunize mice and measured comparably high levels of NP-specific CD8⁺ T cells for both viruses in the MLN and in the spleen at day 9 after infection (Figure 4(a)). The OVA-specific CD8⁺ T cell response was also vigorous, as determined by *ex vivo* IFN- γ release by lymphoid cells after the addition of specific peptides (Figure 4(a)) indicating that the attenuated recombinant virus was efficiently immunogenic for CD8⁺ T cells. To assess whether the engineered OVA-specific CD4⁺ T cell epitope was also presented in the context of infection, we measured the OVA_{323–339}-specific CD4⁺ T cells secreting IFN- γ in the enriched CD4⁺ T cell population from the MLN of these mice. WSN-OVA_{I/II}-infected mice produced an average of 120 spot-forming cells (SFC)/million cells, whereas WSN-infected mice gave no OVA-specific response (Figure 4(b)). Although the insertion of foreign epitopes into the HA of WSN-OVA_{I/II} virus was responsible for the attenuated

phenotype, the double OVA recombinant virus was still replicating and inducing a remarkable OVA-specific T cell response in mice.

Recombinant viral vectors expressing the immunodominant OVA-specific CD8⁺ T cell epitope induce protective immunity against challenge with lethal doses of malignant melanoma cells expressing OVA [20, 21]. Moreover, recombinant influenza viruses expressing a tumor-associated antigen have been previously shown to elicit efficient antitumor responses and to induce tumor inhibition in experimental murine cancer models [22, 23]. We, therefore, examined the ability of WSN-OVA_{I/II} virus to protect mice against tumors by using the tumor cell line B16-OVA (OVA-transfected B16 cells), which cause pulmonary metastases when given intravenously. Immunization with WSN-OVA_{I/II} virus protected mice from lung metastases, as measured 12 days after tumor challenge, compared to nonimmunized mice (Figure 5(a)). The antitumor immunity was OVA-specific, as all of the mice immunized with the parental WSN virus developed tumors. We also found that WSN-OVA_{I/II} virus-immunized mice produced a stronger OVA_{257–264} (SIINFEKL)-specific CD8⁺ T cell response than did either the nonimmunized mice or the wild-type WSN virus-immunized mice, as measured by *ex vivo* ELISPOT with pooled spleens on day 12 posttumor challenge (Figure 5(b)). The higher OVA_{257–264}-specific CD8⁺ T cell response detected in WSN-OVA_{I/II} virus-immunized mice that were subsequently challenged with B16-OVA cells correlated with the presence of OVA-specific memory T cells in these mice before tumor challenge.

4. Discussion

Here, we engineered a recombinant influenza WSN-OVA_{I/II} virus to bear the OVA-specific CD4⁺ and CD8⁺ T cell epitopes on its viral HA. This virus showed an attenuated phenotype and was highly immunogenic in mice. The effective proliferation of OVA-specific CD8⁺ T cells *in vitro* with HI WSN-OVA_{I/II} virus-loaded DCs is in agreement with previous data showing that inactivated influenza viruses that maintain their fusogenic properties are efficiently cross-presented by DCs [17]. The presence of both OVA T cell epitopes on the same viral HA protein provides an additional tool, along with OVA-specific TCR transgenic mice, to investigate antigen processing and presentation by nonreplicating influenza virus-loaded DCs, in the context of MHC class I and class II. The double OVA recombinant virus thus represents an attractive delivery system for use in studies that explore new vaccination strategies for inducing virus-specific memory CD8⁺ T cells. Moreover, the finding that foreign epitopes located on the HA of inactivated recombinant influenza virions are efficiently cross-presented to specific CD8⁺ T cells offers interesting opportunities to further explore the use of recombinant influenza virus-based nonreplicating immunogens. In particular, this feature could be used to develop virosomes, as antigen delivery systems, since antigen encapsulation inside the virosomal particles is usually required for cytosolic delivery upon membrane fusion and for proteasome processing to induce CTL responses [24].

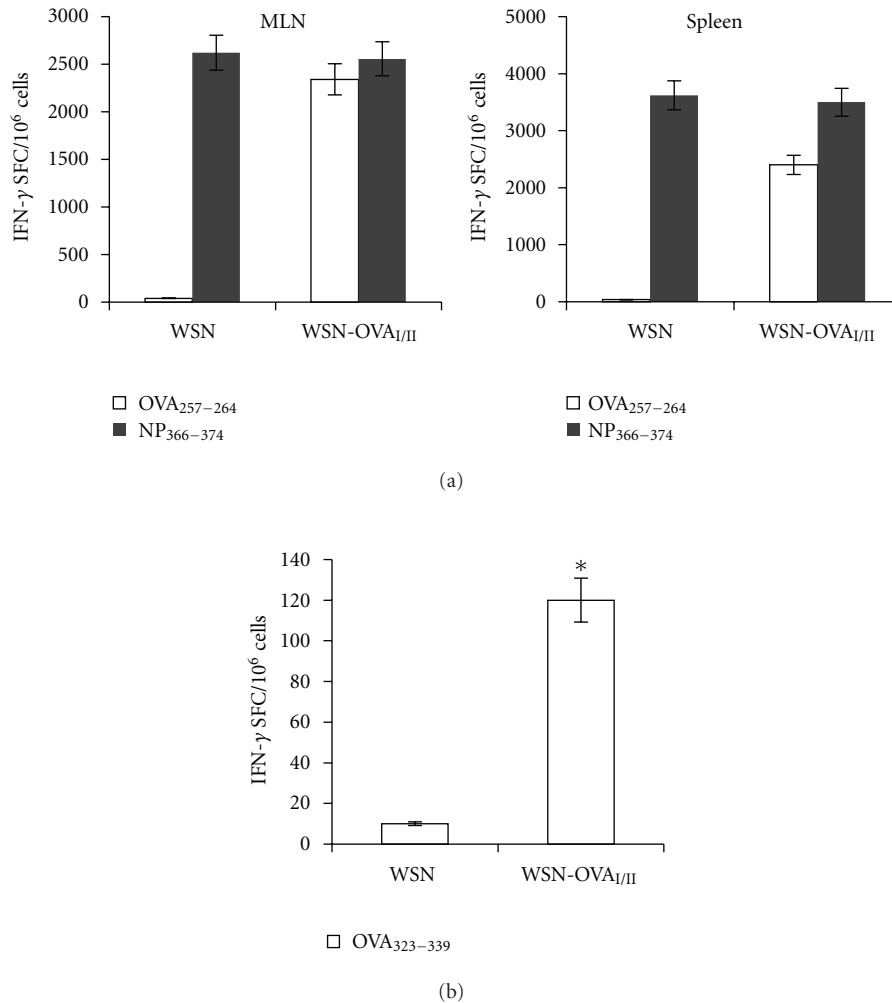


FIGURE 4: T cell responses in WSN-OVA_{I/II} virus-infected mice. C57BL/6J mice were immunized i.n. with 5×10^2 PFU of WSN virus or 3×10^5 PFU of WSN-OVA_{I/II} virus, and sacrificed on day 9 for measuring epitope-specific CD8⁺ T cell responses in mediastinal lymph nodes (MLN) and spleens (a) and CD4⁺ T cell responses in MLN (b), respectively, by use of an IFN- γ ELISPOT assay with the indicated peptides. The values presented are averages of triplicate wells; the error bars indicate the standard deviation. The data are representative of three experiments. * $P < 0.05$ comparing WSN- versus WSN-OVA_{I/II}-infected mice.

Our results also show that immunization with WSN-OVA_{I/II} virus protects mice from B16-OVA tumor challenge. Here, we did not directly address the question of whether the double OVA recombinant virus has advantages over the existing recombinant viruses that bear the single OVA epitopes. Although the induction of OVA₂₅₇₋₂₆₄ (SIINFEKL)-specific CD8⁺ T cells in the context of viral infection may be sufficient to provide protective immunity, it is plausible that the simultaneous induction of tumor-specific CD4⁺ T cells may participate in interactions that shape the protective response against tumors [21]. This is particularly true if tumor-specific immune responses are induced with nonreplicating immunogens and thus in the presence of a poor inflammatory response. Recent studies suggest that abnormalities of the immune response in tumor-bearing hosts correlate with the induction of immune tolerance toward tumor-associated antigens and with the

functional suppression of antitumor T cell effectors due to myeloid-derived suppressor cells [25, 26]. Thus, combining WSN-OVA_{I/II} virus infection and adoptive transfer of OVA-specific TCR transgenic T cells in mice bearing OVA-expressing tumors may help dissect the immunoregulatory mechanisms responsible for tumor-induced tolerance and immune suppression and may provide useful information for rational design of cancer immunotherapy.

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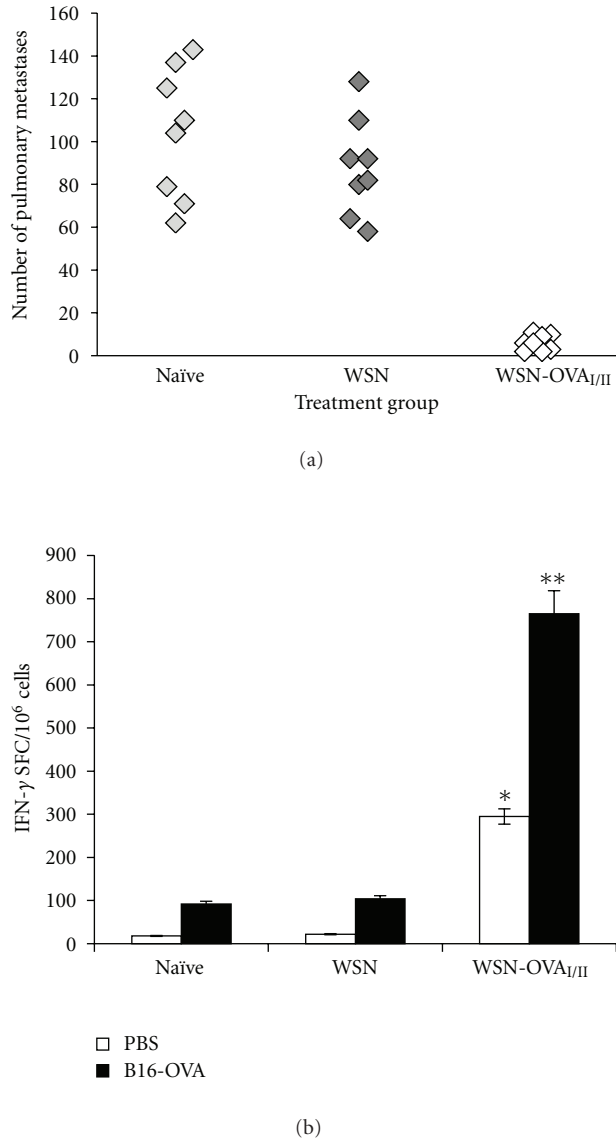


FIGURE 5: Protective and antigen-specific immunity to melanoma B16 cells expressing OVA. C57BL/6J mice were immunized once with either the parental WSN virus or the recombinant WSN-OVA_{I/II} virus. Three weeks after immunization, mice were challenged i.v. with 3×10^5 B16-OVA cells or with PBS. The number of pulmonary metastases (a) and the frequency of SIINFEKL-specific IFN- γ -producing T cells, as measured by using the ELISPOT assay on spleen-derived lymphocytes (b) was determined 12 days after the tumor cell inoculation. All of the experiments included 8 mice per group and were repeated three times with similar results. * $P < 0.001$ and ** $P < 0.0001$, comparing WSN-OVA_{I/II}-versus WSN-infected and naïve mice.

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