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Research Article

RESEARCH ARTICLE

Microbiota is an essential element for mice to initiate a protective immunity against *Vaccinia virus*

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One sentence summary: The microbiota is essential for the effective immune response of mice against Vaccinia virus in intranasal inoculation and to control the virus at the primary site of infection.

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ABSTRACT

The gastrointestinal tract of vertebrates harbors one of the most complex ecosystems known in microbial ecology and this indigenous microbiota almost always has a profound influence on host–parasite relationships, which can enhance or reduce the pathology of the infection. In this context, the impact of the microbiota during the infection of several viral groups remains poorly studied, including the family *Poxviridae*. *Vaccinia virus* (VACV) is a member of this family and is the causative agent of bovine vaccinia, responsible for outbreaks that affect bovines and humans. To determine the influence of the microbiota in the development of the disease caused by VACV, a comparative study using a murine model was performed. Germ-free and conventional, 6- to 7-week-old Swiss NIH mice were infected by tail scarification and intranasally with VACV. Moreover, immunosuppression and microbiota reposition were performed, to establish the interactions among the host's immune system, microbiota and VACV. The data demonstrate that the microbiota is essential for the effective immune response of mice against VACV in intranasal inoculation and to control the virus at the primary site of infection. Furthermore, this study is the first to show that Swiss conventional mice are refractory to the intranasal infection of VACV.

Keywords: microbiota; *Vaccinia virus*; virus–microbiota interactions; germ-free mice

INTRODUCTION

The human gastrointestinal tract harbors one of the most complex ecosystems known in microbial ecology, with bacterial populations reaching 10^{10}–10^{12} viable cells per gram of contents in its lower portions. In healthy hosts, the presence of this microbiota has a very large impact on various aspects of function and metabolism, such as metabolic rate, gastrointestinal function, specific and quantitative aspects of immune function, and the many aspects of biochemical homeostasis. Only the predominant species have population levels that are sufficient to be considered responsible for the three main functions of the intestinal microbiota that are particularly important for host health: (i) colonization resistance, (ii) immunomodulation, and (iii) a nutritional contribution to the host (Sekirov *et al.* 2010; Conlon and Bird 2014; Marietta *et al.* 2015).

Currently available data also indicate that this indigenous microbiota almost always has a profound influence on host–parasite relationships during bacterial and protozoan infections (Stecher and Hardt 2008). As examples, it is well known that

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the presence of the indigenous microbiota is essential for the pathogenicity of some protozoa and helminthes (Phillips and Wolfe 1959; Wescott and Todd 1964; Wescott 1968; Przyjalkowski and Wescott 1969; Visco and Barnes 1972; Johnson and Reid 1973; Owen 1975; Rutter and Beer 1975; Gouet *et al.* 1984; Vieira *et al.* 1987; Torres *et al.* 2000). In contrast, the microbiota can reduce the pathological consequences of other infectious diseases, as described for experimental infections with protozoa, fungi and helminthes (Salkowski *et al.* 1987; Silva *et al.* 1987; Martins *et al.* 2000) and almost all enteropathogenic bacteria (Wilson 1995). There are very few cases where the indigenous microbiota has no influence on the course of an infectious disease (Reide and Botero 1967; Harleman and Meyer 1984).

The interrelationships between virus, indigenous microbiota and the host immune system show differences in relation to those observed with bacteria and protozoa (Wilks *et al.* 2013). Where viruses come into contact with the local microbiota at the beginning of their infectious processes on the host mucosal surfaces, these microbial communities can modulate the viral infection. However, information concerning the influence of the indigenous microbiota on viral infections is scarce. Viruses and commensal bacteria have co-evolved and some viruses are dependent on the presence of the microbiota or its products. For example, the host's microbiota facilitates viral replication and pathogenesis in poliovirus (*Enterovirus C*), *Murine leukemia virus* and *Mouse mammary tumor virus* infections. (Kouttab and Jutila 1972; Isaak, Bartizal and Caulfield 1988; Kane *et al.* 2011; Kuss *et al.* 2011). Conversely, as described above for pathogenic bacteria, fungi and protozoa, the microbiota can play a protective role against viral replication and virally induced disease, as observed for the influenza virus (Ichinohe *et al.* 2011; Abt *et al.* 2012). Additionally, *Murine norovirus*, a common murine enteric virus, can replace the maturating function of commensal bacteria when introduced into germ-free (GF) mice, reverting intestinal morphology and lymphocyte function to phenotypes similar to those observed in conventional (CV) animals (Kernbauer, Ding and Cadwell 2014). These data exemplify the different relationships between the host microbiota and diverse viral clades. The study of the interaction between viruses, microbiota and the host immunity system is an important field of research, which might lead to the discovery of improved treatments and the prevention of infectious diseases. Nevertheless, the impact of the microbiota on infection by several viral groups, such as the *Poxviridae* family, remains unknown.

Smallpox is a severe infectious disease caused by the *Variola virus* (VARV), a member of the *Orthopoxvirus* (OPV) genus (Fenner *et al.* 1988; Damon 2013). Considering only the 18th century, smallpox caused the death of more than 400 000 people annually in Europe. This highly lethal and contagious disease was declared to have been eradicated in 1980, after an intensive vaccination campaign promoted by the World Health Organization (WHO) (Fenner *et al.* 1988). *Vaccinia virus* (VACV), a virus closely related to VARV that also belongs to the OPV genus and can induce serological cross-reactivity against other OPV members, was used in the WHO campaign (Fenner *et al.* 1988; Damon 2013). Currently, species of the OPV genus have a great impact on human and veterinary health, due to emerging and re-emerging zoonotic agents around the world, such as *Cowpox virus* (Kalthoff *et al.* 2014) in Europe, *Monkeypox virus* primarily in Africa (Reynolds, Carroll and Karem 2012), and VACV in Asia and South America (Trindade *et al.* 2003; Singh *et al.* 2007; Franco-Luiz *et al.* 2014; Kroon *et al.* 2011).

Vaccinia virus is the causative agent of bovine vaccinia (BV), which is responsible for outbreaks that primarily affect bovines and humans and cause economic losses and public health problems. This exanthematous disease starts with erythema, which evolves to vesicles, papules and pustules, and finally forms scabs. In cattle, these lesions occur primarily on the teats of cows and on the nose and mouth of calves (Trindade *et al.* 2003; Kroon *et al.* 2011). In humans, VACV infection is characterized by the development of skin lesions, principally on the hands, and systemic signs include headache, myalgia, fever and lymphadenopathy (Trindade *et al.* 2003; Kroon *et al.* 2011; Damon 2013). Virulence studies in a murine model using Balb/c showed the existence of two distinct populations of Brazilian VACV; one group caused disease and death, but the other did not cause any clinical symptoms or death in infected mice (Ferreira *et* al. 2008).

Despite the eradication of smallpox, bioterrorism and the emergence of OPVs are concerns for the population worldwide. Given the social and economic impact of the diseases caused by OPV viruses and the unknown relationships between the host microbiota and these pathogens, we evaluated the importance of the host's microbiota in VACV infection.

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney BSC-40 (ATCC-CRL-2761) and Vero cells (ATCC-CCL-81) were maintained in a 5% CO_2 atmosphere at 37°C in Eagle's minimum essential medium (MEM) (Gibco BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Cultilab, Brazil), 25 μg/mL fungizone (Amphotericin B) (Cristália, São Paulo, Brazil), 500 U/mL penicillin (Cristália) and 50 μg/mL gentamicin (Schering-Plough, São Paulo, Brazil). Vero cells were used for viral replication. The BSC-40 cells were used for titration assays. The VACV Western Reserve (VACV-WR) virus was kindly provided by Dr C. Jungwirth (Universität Würzburg, Würzburg, Germany) and was used for mice assays. The virus was purified on a sucrose gradient as described elsewhere (Joklik 1962).

Animal assays

Ethical aspects

The present study was approved by the Animal Ethics Committee (Comitê de Ética no Uso Animal; CEUA), of the Universidade Federal de Minas Gerais (UFMG), Brazil, protocol number 207/2011.

Germ-free and conventional mice

Six-week-old germ-free (GF) NIH Swiss mice were obtained from Taconic Farms (Germantown, NY, USA) and were maintained in flexible plastic isolators (Standard Safety Equipment, McHenry, MD, USA) using classical gnotobiology techniques (Pleasants 1974). Conventional (CV) NIH Swiss mice are derived from GF matrices and are considered to be CV only two generations after conventionalization. Water and a commercial autoclavable diet (Nuvilab, Nuvital, Curitiba, PR, Brazil) were sterilized by steam and administered *ad libitum*. For experiments, animals were maintained in micro-isolators (UNO Roestvaststaal, The Netherlands) located in a ventilated animal caging system (Alesco Ltd, Campinas, SP, Brazil) with controlled lighting (12 h light–12 h dark), humidity (60–80%) and temperature (22 ± 1°C).

Immunosuppression

For immunosuppression experiments, dexamethasone (Decadron, ACHÉ Laboratorios LTDA, Guarulhos, SP, Brazil)

was administered intraperitoneally (i.p.) at a dose of 10 mg/kg/day, for 15 days in CV mice.

Conventionalization

Conventionalization was performed both by intragastric inoculation of diluted feces from CV donors (Day 0) and co-housing with CV mice for 21 days, as previously described (Pleasants 1974). Conventionalized (CVZ) animals were maintained in the same conditions as described above. After 21 days, CVZ animals were submitted to intranasal VACV infection or phosphate-buffered saline (PBS) inoculation, as described below. To assess whether microbiota colonization had occurred, a thioglycollate test was performed on feces from CVZ mice.

VACV infection by intranasal inoculation

The mice of all groups were anesthetized by i.p. injection of ketamine and xylazine (3.2 mg and 0.16 mg per mouse, respectively, in 0.9% PBS) before the procedure (dose: 80–120 mg/kg). The mice were inoculated intranasally with 10 μL of viral suspension containing 10^6 plaque-forming units (pfu) of VACV and the negative control group was inoculated with 10 μL of PBS as previously described (Ferreira *et al.* 2008). Mice were weighed daily, and other clinical signs were recorded for 10 days post-infection (p.i.). Mice were sacrificed on Day 10 p.i.

VACV infection by tail scarification

Mice were anesthetized as described above, and 10 scarifications were made with a 26 G syringe, 1 cm from the base of the tail, avoiding bleeding, as previously described (Melamed *et al.* 2007). Then, 10 μL of PBS containing 10^6 pfu VACV was spotted onto the area; PBS was inoculated in the same way for the control group after scarification.

Bronchoalveolar lavage

For bronchoalveolar lavage (BAL), mice were sacrificed with an overdose of ketamine–xylazine solution. Subsequently, a 1.7 mm catheter was inserted into the trachea and 1 mL PBS was inserted into the lungs and then re-aspired three times.

Tissue collection and processing, and viral titration

On Day 10 after VACV infection, three mice per group (GF, CV, CVZ) were sacrificed, and the spleen, lungs, ileum and blood were collected in pre-weighed 2.0-mL tubes (Axygen, USA). The tubes were weighed again to determine tissue weight and were then frozen at −80°C. On the day of processing, the tissues were thawed and macerated in MEM (Gibco, USA) and centrifuged at 20 000 *g* for 3 min, at 4°C. Supernatant fluids from macerated organs were collected and the virus titer (pfu/g) was determined by a plaque-forming assay in BSC-40 (Campos and Kroon 1993).

Plaque reduction neutralization test

For the plaque reduction neutralization test (PRNT), serum samples were heat-inactivated at 56°C for 30 min, serially diluted in a 1:20 ratio in MEM and incubated at 37°C for 16 h with the same volume of MEM containing 150 pfu of VACV-WR. At the same time, the viral suspension was also incubated with a 1:20 dilution of MEM to serve as a virus control. Following this, 400 μL of this mixture was added to BSC-40 cells seeded in six-well plates, which were incubated for 1 h at 37°C and 5% CO_2 atmosphere. Then, 2 mL of the medium was added to each well and it was further incubated in the same conditions for 48 h. The cells were then stained with a solution of crystal violet for 20 min and the viral plaques were counted. The results are expressed as the highest serum dilution that was able to neutralize at least 50% of viral plaques ($PRNT_{50}$).

Molecular assays

The BAL was tested using an OPV-specific PCR reaction that amplified the C11R gene, which encodes the viral growth factor (*vgf*). The PCR conditions were 94°C for 10 min, 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, followed by 72°C for 10 min.

Statistical analysis

All results were plotted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and compared using Student's two-tailed *t*-test, one-way ANOVA, two-way ANOVA using the Bonferroni method and the Logrank test. In all tests, *P* values <0.05 were considered statistically significant.

RESULTS

Effect of microbiota determined by comparing CV and GF mice infected with VACV by intranasal inoculation

To determine the effect of the indigenous microbiota on VACV infection, CV mice and GF mice were infected with 10^6 pfu of VACV via an intranasal route. These animals were monitored for 10 days p.i. to evaluate clinical symptoms and survival rates (Fig. 1). All GF animals presented clinical symptoms and 60% of the animals died on the eighth day p.i. (Fig. 1A). Initial clinical symptoms, such as periocular alopecia and closed inflamed eyes, appeared in all animals on Day 5 p.i. (Fig. 1E), followed by the ruffling of fur, an arched back and labored breathing on Day 7 p.i. The infection in these animals was associated with severe weight loss starting on the fifth day p.i. (Fig. 1B). No clinical symptoms or death was observed in CV or control mice. All animals were sacrificed on the 10th day p.i. and their organs were collected, to investigate viral tropisms. Viral infectious particles were detected only in the lungs and spleen from GF mice. In these organs, viral titers were approximately 10^4 pfu/g (Fig. 1C and D). No viral infectious particles were detected in organs collected from CV and control mice (Fig. 1C and D). Blood was collected on the 10th day p.i. and sera were used to perform PRNT. Serum neutralizing activity was only detected in sera collected from the GF group (titers >1/40). Viral inoculation in CV animals was confirmed after viral DNA detection by qPCR performed in BAL collected from these animals (data not shown).

Effect of microbiota by comparing CV and GF mice infected with VACV by tail scarification

To investigate whether CV mice remained refractory by another route of infection, CV and GF mice were infected with 10^6 pfu VACV by tail scarification. These animals were monitored 10 days p.i. to evaluate clinical symptoms, survival rates and lesions. All animals presented lesions at the inoculation site, but no systemic clinical symptoms such as fur ruffling, an arched back or periocular alopecia were observed (Fig. 2A and B). All mice survived to infection, and no significant difference in weight was observed between the analyzed groups (Fig. 2C). However, the lesions at the inoculation site showed differences in appearance and were more inflamed in CV than in GF mice on Day 10 p.i. No clinical signals or weight loss was observed in CV

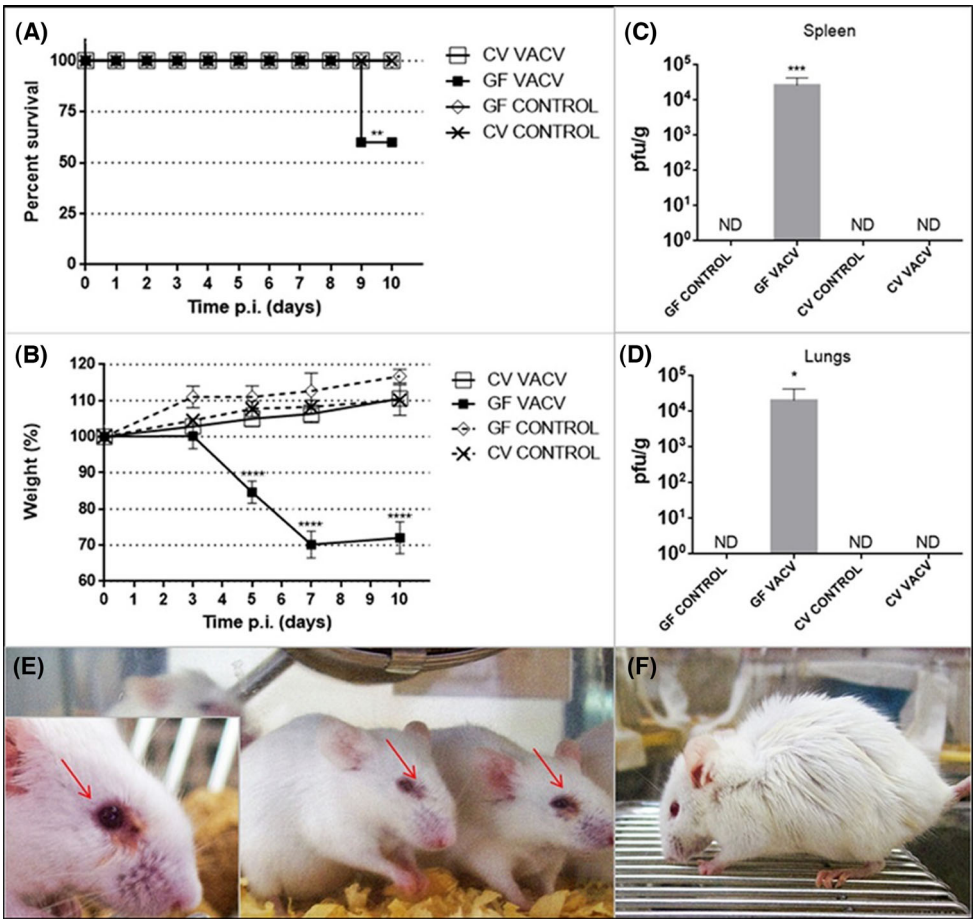

Figure 1. Intranasal infection of germ-free and conventional mice with *Vaccinia virus*. (**A–D**) Survival (A), relative mean weight (B) and viral titers in the spleen (C) and lungs (D) of the experimental and control groups ($n = 10$) of CV and GF mice inoculated with 10^6 pfu of VACV or PBS, respectively, via an intranasal route. The error bars indicate the standard deviations. For viral titers in lungs and spleens, organs ($n = 3$) were collected on Day 10 p.i. and were titrated. (**E**) Clinical aspects of mice on Day 5 p.i. (**F**) Facial edema and periocular alopecia (red arrows) were observed on Day 10 p.i., as well as fur ruffling and arching of the back. CV: conventional; GF: germ-free; ND: not detected; PBS: phosphate-buffered saline; VACV: *Vaccinia virus*. Asterisks indicate a statistically significant difference: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

or GF mice inoculated with PBS. The viral titers in lesions were similar for CV and GF groups 10 days p.i. ($\sim 10^6$ pfu/g) (Fig. 2D). Sera collected by Day 10 p.i. from CV and GF mice infected with VACV and submitted to PRNT were able to neutralize at least 50% of viral plaques (titers >1/40).

Effect of immunosuppression on the resistance of CV mice to VACV infection

To evaluate the role of the immune system in the resistance of CV mice to VACV infection, 10 animals were submitted to immunosuppression by using dexamethasone (DEX) for 5 days at a dose of 10 mg/kg/d. An equal number of mice received PBS in the same period as a control group. After this treatment, animals were infected with 10^6 pfu of VACV and continued to receive the respective treatment with DEX and PBS until the end of the experiment. These mice were monitored for 10 days p.i. to evaluate clinical symptoms and survival rates (Fig. 3). Clinical symptoms such as fur ruffling, arching of the back and labored breathing were observed in all DEX-treated animals inoculated with VACV at 5 days p.i. (Fig. 3D). Two mice died on Day 8 p.i. and a severe weight loss was observed on Days 5 and 6 p.i. (Fig. 3A and C). In contrast, no clinical symptoms were observed in the control groups. All animals were sacrificed on Day 10 p.i. and their organs were collected for viral tropism analysis. Viral infectious particles were detected in the lungs of all animals and in the spleen of two mice from the immunosuppressed group inoculated with VACV. In these lungs, viral titers were $\sim 10^4$ pfu/g (Fig. 3B), whereas in spleens, the titers ranged from 10^2 to 10^3 pfu/g. No viral infectious particles were detected in any of the organs from mice of the control groups.

Effect of conventionalization of GF mice on the susceptibility to VACV infection

To confirm the influence of the indigenous microbiota on the susceptibility to VACV infection, GF mice were conventionalized both by intragastric inoculation of diluted feces of CV donors (Day 0) and by co-housing with CV mice for 21 days, with the aim of acquiring the skin and mucosal microbiota. The CVZ mice were then infected with 10^6 pfu VACV via an intranasal route .These mice were monitored for an additional 10 days p.i. to evaluate clinical signs and survival rates (Fig. 4). All animals inoculated with VACV presented clinical symptoms, but as

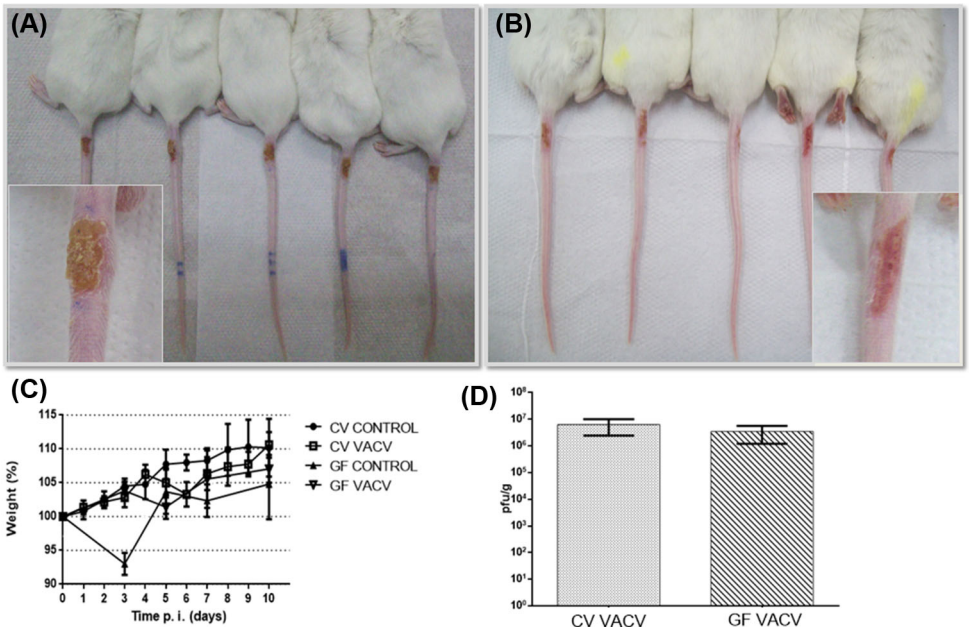

Figure 2. Tail scarification infection of germ-free and conventional mice with *Vaccinia virus*. Clinical aspects of CV (**A**) and GF (**B**) mice, relative mean weights (**C**) and viral titers in lesions (**D**) on Day 10 p.i. of CV and GF mouse groups ($n = 5$) inoculated with 10^6 pfu of VACV or PBS, respectively, by tail scarification. For viral titers in lesions, samples ($n = 5$) were collected on Day 10 p.i. and were titrated. CV: conventional; GF: germ-free; PBS: phosphate-buffered saline; VACV: *Vaccinia virus*. There were no significant differences in (C) and (D).

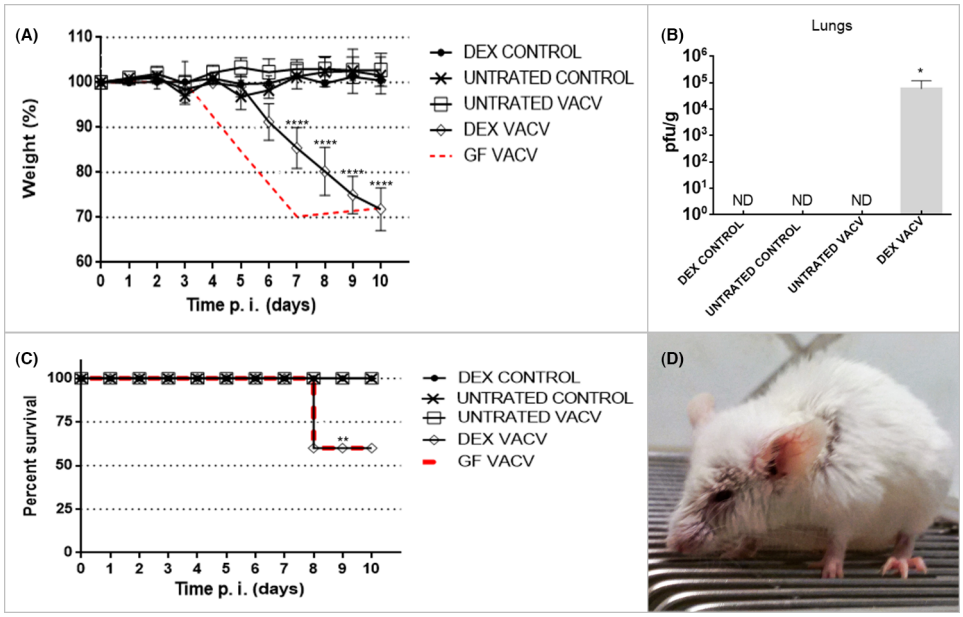

Figure 3. Intranasal infection of conventional immunosuppressed mice with *Vaccinia virus*. Relative mean weights (**A**), survival (**B**), viral titers in lungs (**C**) and clinical aspects (**D**) of experimental and control groups ($n = 5$–10) of CV immunosuppressed mice inoculated with 10^6 pfu of VACV or PBS, respectively, via an intranasal route. Mice were immunosuppressed by daily dexamethasone administration for 15 days. The control group was treated with PBS. For viral titers in lungs, organs ($n = 5$) were collected on Day 10 p.i. and were titrated. In red, data from Fig. 1 for GF mice intranasally infected with VACV are shown Asterisks indicate a statistically significant difference: $^*P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$. CV: conventional; DEX: dexamethasone; GF: germ-free; PBS: phosphate-buffered saline; VACV: *Vaccinia virus*.

expected, the lethality rate was lower in CVZ (12.5%) than GF mice (40%). All animals were sacrificed on Day 10 p.i. and their organs were collected for viral tropism analysis. Viral particles were detected in lungs from only two CVZ mice (ranging from 10^2 to 10^3 pfu/g), whereas all GF animals contained ~10^4 pfu/g VACV in their lungs. No viral infectious particles were detected in the organs of animals from the control group.

DISCUSSION

Previous studies have established varied interactions between the host's immune system, microbiota and viruses (Wilks *et al.* 2013). Similar to pathogenic bacteria and protozoa, the microbiota can potentially facilitate or hinder viral infections (Wilks *et al.* 2013), and each different virus studied to date shows diverse interactions with the host microbiota (Kouttab and Jutila

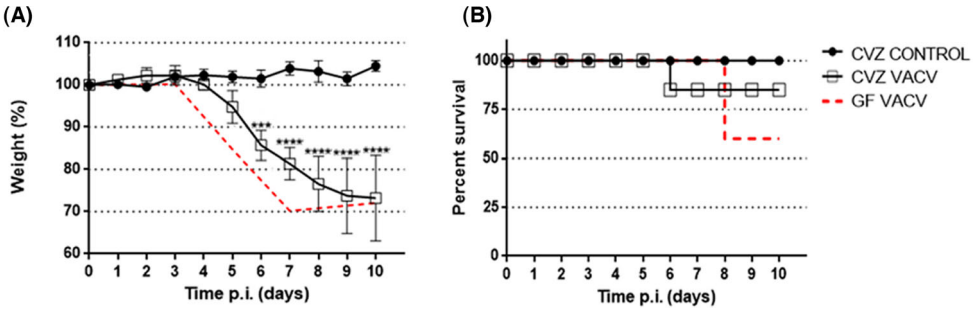

Figure 4. Intranasal infection of conventionalized mice with *Vaccinia virus*. Relative mean weights (**A**) and survival (**B**) of experimental and control groups ($n = 5$–10) of CVZ mice inoculated with 10^6 pfu of VACV-WR or PBS, respectively, via an intranasal route. In red, data from Fig. 1 for GF mice intranasally infected with VACV are shown. CVZ: conventionalized; GF: germ-free; PBS: phosphate-buffered saline; VACV: *Vaccinia virus*. Asterisks indicate a statistically significant difference: ***$P < 0.001$ and ****$P < 0.0001$. There were no significant differences in the survival curves in (B).

1972; Isaak, Bartizal and Caulfield 1988; Kane *et al.* 2011; Kuss *et al.* 2011; Ichinohe *et al.* 2011; Abt *et al.* 2012; Wilks and Golovkina 2012; Wilks *et al.* 2013; Kernbauer, Ding and Cadwell 2014). However, such correlations between commensal bacteria and many viral families such as the *Poxviridae* remain unknown. For this reason, the effect of the indigenous microbiota on VACV infection in this study was evaluated by using and comparing CV and GF mouse models. Additionally, the influence of two different routes of VACV administration (intranasal inoculation and tail scarification) was evaluated, bearing in mind that the intranasal route is considered the primary route of smallpox transmission in humans and the secondary route of transmission for monkeypox (Damon 2013). It has been already described that the intranasal inoculation of VACV causes systemic infections and lethality, which vary according to the dose and mouse lineage (Turner 1967; Hayasaka, Ennis and Terajima 2007; Ferreira *et al.* 2008). In this study, CV NIH Swiss mice were totally resistant to VACV administration, whereas this viral infection has been demonstrated to be lethal for Balb/c mice in another report (Turner 1967). No clinical signals or viral titers were observed in organs of these mice. On the other hand, all GF mice displayed expressive clinical symptoms and only 60% survived. Moreover, high viral titers were found in the lungs and spleen (10^4 pfu/g) of these animals. These results suggest an influence of the indigenous microbiota on VACV infection in NIH Swiss mice. Although NIH Swiss is not the standard breed used for OPV infections, this refractory model made clear the differences caused by absence of the microbiota. This role of the microbiota on the susceptibility of mice to VACV infection might occur by direct interactions between components of the microbiota and the virus or by an indirect effect via the immune system; indeed, it is well known that GF animals have immature secondary lymphoid tissues (Wilks *et al.* 2013). The intestinal mucosa of GF animals is characterized by fewer intra-epithelial lymphocytes (IELs) and lamina propria lymphocytes. Furthermore, Peyer's patches are less developed and lack a fully developed germinal center. The indigenous microbiota has also been shown to influence the development of $CD4^+CD8^+\alpha^+$ IELs by an extra-thymic pathway. Moreover, both α–β and γ–δ IELs from GF mice showed a lower proliferation rate than that of CV animals (Kamada and Núñes 2014).

Since GF and CV mice exhibited a different response via the intranasal route, tail scarification was used to evaluate the effects of VACV infection by another route. Infection via tail scarification in mice provides a useful model of VACV inoculation, since it simulates the immunological and virological parameters of human smallpox vaccination (Melamed *et al.* 2007; Mota *et al.* 2011). In the present study, the cutaneous lesions showed differences in appearance and inflammation level between GF and CV mice, which can be associated with a microbiota-dependent regulation of inflammatory responses (Souza *et al.* 2004). However, PRNT and viral titers in the lesions showed similarities between GF and CV mice. Furthermore, these results showed that contrary to intranasal inoculation, CV mice are not refractory to VACV infection through the tail scarification route.

To confirm the importance of the immune system in the NIH Swiss murine strain infected by VACV, CV animals were immunosuppressed using daily DEX doses. In these animals, the clinical symptoms, lethality rate and viral titers in the lungs were similar to those observed in GF mice, even if the spleen presented slightly lower viral titers (10^2 pfu/g and 10^3 pfu/g) than those of GF mice. However, considerable DEX-induced changes in lymphocytes from the spleen might explain this variation (Ahmed and Sriranganathan 1994). These results demonstrate a similarity between the immature and the suppressed immunological systems of GF and DEX-treated murine models, respectively, and reinforce the indirect influence of the indigenous microbiota on VACV infection in Swiss NIH animals.

To determine whether conventionalization of GF mice could restore resistance to VACV, probably by maturing the immune response, CVZ animals were intranasally inoculated with VACV. The CVZ animals showed a lower lethality rate (12.5%) than GF mice infected with VACV, but all showed clinical symptoms, with substantial variations among the infected animals in the group. This might be due to an incorrect maturation of the immune system due to a conventionalization that occurred at an adult stage instead of soon after birth, as occurs naturally. The results obtained from GF mice conventionalized in adulthood showed failures in immune maturation compared with animals colonized at birth. Therefore, a time window exists (soon after delivery) for an effective immune modulator effect due to microbiota colonization (El Aidy *et al.* 2013).

In conclusion, data from the present study demonstrate that the microbiota is essential to mice to instigate an effective immune response against VACV in a model of systemic infection (intranasal). This response includes controlling the virus at the initial site of infection (lung) and the generation of an immune memory. These results raise new questions concerning how variations in the microbiota between individuals or during episodes of dysbiosis might influence OPV infections.

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Conflict of interest. None declared.

REFERENCES

Abt MC, Osborne LC, Monticelli LA *et al.* Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 2012;**37**:158–70.

Ahmed SA, Sriranganathan N. Differential effects of dexamethasone on the thymus and spleen: alterations in programmed cell death, lymphocyte subsets and activation of T cells. *Immunopharmacology* 1994;**28**:55–66.

Campos MAS, Kroon EG. Critical period for irreversible block of Vaccinia virus replication. *Rev Microbiol* 1993;**24**: 104–10.

Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. *Nutrients* 2014;**7**:17–44.

Damon I. Poxviridae and their replication. *Fields Virology* 2013;**6**:2160–84.

de Souza Trindade G, da Fonseca FG, Marques JT *et al.* Araçatuba virus: a vaccinia like virus associated with infection in humans and cattle. *Emerg Infect Dis* 2003;**9**:155–60.

El Aidy S, Hooiveld G, Tremaroli V *et al.* The gut microbiota and mucosal homeostasis: colonized at birth or at adulthood, does it matter? *Gut Microbes* 2013;**4**:118–24.

Fenner F, Henderson DA, Arita I *et al. Smallpox and its Eradication*. Geneva: World Health Organization Press, 1988.

Ferreira JM, Drumond BP, Guedes MI *et al.* Virulence in murine model shows the existence of two distinct populations of Brazilian Vaccinia virus strains. *PLoS One* 2008;**3**:e3043.

Franco-Luiz AP, Fagundes-Pereira A, Costa GB *et al.* Spread of vaccinia virus to cattle herds, Argentina, 2011. *Emerg Infect Dis* 2014;**20**:1576–8.

Gouet P, Yvore P, Naciri M *et al.* Influence of digestive microflora on parasite development and the pathogenic effect of Eimeria ovinoidalis in the axenic, gnotoxenic and conventional lamb. *Res Vet Sci* 1984;**36**:21–3.

Harleman JH, Meyer RC. Life cycle of Isospora suis in gnotobiotic and conventionalized piglets. *Vet Parasitol* 1984;**17**:27–39.

Hayasaka D, Ennis FA, Terajima M. Pathogeneses of respiratory infections with virulent and attenuated vaccinia viruses. *Virol J* 2007;**4**:22.

Ichinohe T, Pang IK, Kumamoto Y *et al.* Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci U S A* 2011;**108**:5354–9.

Isaak DD, Bartizal KF, Caulfield MJ. Decreased pathogenicity of murine leukemia virus-Moloney in gnotobiotic mice. *Leukemia* 1988;**2**:540–4.

Johnson J, Reid WM. Ascaridia galli (Nematoda): development and survival in gnotobiotic chickens. *Exp Parasitol* 1973;**33**: 95–9.

Joklik WK. The purification of four strains of poxvirus. *Virology* 1962;**18**:9–18.

Kalthoff D, Bock WI, Hühn F *et al.* Fatal cowpox virus infection in cotton-top tamarins (*Saguinus oedipus*) in Germany. *Vector Borne Zoonotic Dis* 2014;**14**:303–5.

Kamada N, Núñez G. Regulation of the immune system by the resident intestinal bacteria. *Gastroenterology* 2014;**146**: 1477–88.

Kane M, Case LK, Kopaskie K *et al.* Successful transmission of a retrovirus depends on the commensal microbiota. *Science* 2011;**334**:245–9.

Kernbauer E, Ding Y, Cadwell K. An enteric virus can replace the beneficial function of commensal bacteria. *Nature* 2014;**516**:94–8.

Kouttab NM, Jutila JW. Friend leukemia virus infection in germfree mice following antigen stimulation. *J Immunol* 1972;**108**:591–5.

Kroon EG, Mota BE, Abrahão JS *et al.* Zoonotic Brazilian Vaccinia virus: from field to therapy. *Antiviral Res* 2011;**92**:150–63.

Kuss SK, Best GT, Etheredge CA *et al.* Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science* 2011;**334**:249–52.

Marietta E, Rishi A, Taneja V. Immunogenetic control of the intestinal microbiota. *Immunology* 2015;**145**:313–22.

Martins WA, Melo AL, Nicoli JR *et al.* A method of decontaminating *Strongyloides venezuelensis* larvae for the study of strongyloidiasis in germ-free and conventional mice. *J Med Microbiol* 2000;**49**:387–90.

Melamed S, Sharon M, Paran N *et al.* Tail scarification with Vaccinia virus Lister as a model for evaluation of smallpox vaccine potency in mice. *Vaccine* 2007;**25**:7743–53.

Mota BE, Gallardo-Romero N, Trindade G *et al.* Adverse events post smallpox-vaccination: insights from tail scarification infection in mice with Vaccinia virus. *PLoS One* 2011;**6**: e18924.

Owen D. Eimeria falciformis (Eimer, 1870) in specific pathogen free and gnotobiotic mice. *Parasitology* 1975;**71**:293–303.

Phillips BP, Wolfe PA. The use of germfree guinea pigs in studies on the microbial interrelationships in amoebiasis. *Ann N Y Acad Sci* 1959;**78**:308–14.

Pleasants JR. Gnotobiotics. In Melby EC, Jr, Altman NH (eds). *Handbook of Laboratory Animal Science*, **vol. I**. Cleveland, OH: CRC Press 1974, 119–74.

Przyjalkowski ZW, Wescott RB. Trichinella spiralis: establishment in gnotobiotic mice affected by Bacillus mesentericus, B. subtilis, and Pseudomonas aeruginosa. *Exp Parasitol* 1969;**25**:8–12.

Reid WM, Botero H. Growth of the cestode Raillietina cesticillus in bacteria-free chickens. *Exp Parasitol* 1967;**21**:149–53.

Reynolds MG, Carroll DS, Karem KL. Factors affecting the likelihood of monkeypox's emergence and spread in the post-smallpox era. *Curr Opin Virol* 2012;**2**:335–43.

Rutter JM, Beer RJ. Synergism between Trichuris suis and the microbial flora of the large intestine causing dysentery in pigs. *Infect Immun* 1975;**11**:395–404.

Salkowski CA, Bartizal KF, Balish MJ *et al.* Colonization and pathogenesis of Cryptococcus neoformans in gnotobiotic mice. *Infect Immun* 1987;**55**:2000–5.

Sekirov I, Russell SL, Antunes LC *et al.* Gut microbiota in health and disease. *Physiol Rev* 2010;**90**:859–904.

Silva ME, Evangelista EA, Nicoli JR *et al.* American trypanosomiasis (Chagas' disease) in conventional and germfree rats and mice. *Rev Inst Med Trop Sao Paulo* 1987;**29**:284–8.

Singh RK, Hosamani M, Balamurugan V *et al.* Buffalopox: an emerging and re-emerging zoonosis. *Anim Health Res Rev* 2007;**8**:105–14.

Souza DG, Vieira AT, Soares AC *et al.* The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *J Immunol* 2004;**173**:4137–46.

Stecher B, Hardt WD. The role of microbiota in infectious disease. *Trends Microbiol* 2008;**16**:107–14.

Torres MF, Uetanabaro AP, Costa AF *et al*. Influence of bacteria from the duodenal microbiota of patients with symptomatic giardiasis on the pathogenicity of *Giardia duodenalis* in gnotoxenic mice. *J Med Microbiol* 2000;**49**:209–15.

Turner GS. Respiratory infection of mice with vaccinia virus. *J Gen Virol* 1967;**1**:399–402.

Vieira EC, Nicoli JR, Moraes-Santos T *et al*. Cutaneous leishmaniasis in germfree, gnotobiotic, and conventional mice. *Rev Inst Med Trop Sao Paulo* 1987;**29**:385–7.

Visco RJ, Burns WC. Eimeria tenella in bacteria-free and conventionalized chicks. *J Parasitol* 1972;**58**:323–31.

Wescott RB. Experimental Nematospiroides dubius infection in germfree and conventional mice. *Exp Parasitol* 1968;**22**:245–9.

Wescott RB, Todd AC. A comparison of the development of nippostrongylus brasiliensis in germ-free and conventional mice. *J Parasitol* 1964;**50**:138–43.

Wilks J, Beilinson H, Golovkina TV. Dual role of commensal bacteria in viral infections. *Immunol Rev* 2013;**255**:222–9.

Wilks J, Golovkina T. Influence of microbiota on viral infections. *PLoS Pathog* 2012;**8**:e1002681.

Wilson KH. Ecological concepts in the control of pathogens. In: Roth JA (ed.). *Virulence Mechanisms of Bacterial Pathogens*, Washington, DC: American Society for Microbiology 1995, 245–56.