MAJOR ARTICLE

Replication, Pathogenicity, Shedding, and Transmission of *Zaire ebolavirus* in Pigs

Gary P. Kobinger,[1,2] Anders Leung,[1] James Neufeld,[3] Jason S. Richardson,[1] Darryl Falzarano,[1,2] Greg Smith,[3] Kevin Tierney,[3] Ami Patel,[1,2] and Hana M. Weingartl[2,3]

[1]Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada; [2]Department of Medical Microbiology, University of Manitoba; and [3]National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, Manitoba

(See the editorial commentary by Bausch, on pages 179–81.)

Background. *Reston ebolavirus* was recently detected in pigs in the Philippines. Specific antibodies were found in pig farmers, indicating exposure to the virus. This important observation raises the possibility that pigs may be susceptible to Ebola virus infection, including from other species, such as *Zaire ebolavirus* (ZEBOV), and can transmit to other susceptible hosts.

Methods. This study investigated whether ZEBOV, a species commonly reemerging in central Africa, can replicate and induce disease in pigs and can be transmitted to naive animals. Domesticated Landrace pigs were challenged through mucosal exposure with a total of 1×10^6 plaque-forming units of ZEBOV and monitored for virus replication, shedding, and pathogenesis. Using similar conditions, virus transmission from infected to naive animals was evaluated in a second set of pigs.

Results. Following mucosal exposure, pigs replicated ZEBOV to high titers (reaching 10^7 median tissue culture infective doses/mL), mainly in the respiratory tract, and developed severe lung pathology. Shedding from the oronasal mucosa was detected for up to 14 days after infection, and transmission was confirmed in all naive pigs cohabiting with inoculated animals.

Conclusions. These results shed light on the susceptibility of pigs to ZEBOV infection and identify an unexpected site of virus amplification and shedding linked to transmission of infectious virus.

Outbreaks of Ebola hemorrhagic fever in endemic areas, as well as the introductions of single cases into nonendemic countries, are unpredictable and always a matter of considerable concern to public health authorities. Ebola viruses are prime examples of "emerging/reemerging" pathogens causing the most severe hemorrhagic fever found in human and nonhuman primates [1–4]. In fatal cases, the immune response is insufficient to provide the rapid protection required to control fulminant Ebola virus replication. The species *Reston ebolavirus* (REBOV) has never been associated with human disease, despite multiple documented exposures [5, 6]. Alternatively, *Zaire ebolavirus* (ZEBOV) is associated with case fatality rates as high as 90% in humans [1, 7].

Currently, we are just beginning to understand the pathogenic mechanisms that lead to severe disease and death [1–4, 8–10]. The most reliable animal model for studying *ebolavirus* replication and its associated disease is the nonhuman primate [11, 12]. The difficulties of working with nonhuman primates in a high-containment laboratory have been limiting the speed at which researchers can fully elucidate the complex physiopathology and fulminating shock induced by *ebolavirus*. Although the nonhuman primates are the only species known to succumb to strains of *ebolavirus* isolated from human cases, sequential infection of mice or guinea pigs has generated variants that are also lethal to these 2 animal species [13, 14].

Accumulating evidence is identifying fruit bats as the natural reservoir of *ebolavirus* [15, 16]. One

Received 9 July 2010; accepted 28 September 2010.

Potential conflicts of interest: none reported.

Correspondence: Gary P. Kobinger, PhD, National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington St, Winnipeg, Manitoba R3E 3R2, Canada (gary_kobinger@phac-aspc.gc.ca).

The Journal of Infectious Diseases 2011;204:200–8

0022-1899 (print)/1537-6613 (online)/2011/2042-0007$14.00
DOI: 10.1093/infdis/jir077

current hypothesis is that bats transmit *ebolavirus* to an amplifying host, animal or human, and that high virus load reached in the first amplifying host favors transmission to and between humans mainly through contact with body fluids. Several human cases of *ebolavirus* infection have been associated with the butchering of infected bush meat derived from nonhuman primates [17, 18]. Recently, REBOV was detected in pigs in the Philippines and antibody to the virus was found in several pig farmers, supporting the concept of *ebolavirus* replication in pigs and zoonotic transmission between pigs and humans [19–21].

The identification of animal species that can replicate *ebolavirus* and transmit the virus to other animals and/or to humans is critical to the development of preventative measures to avert outbreaks in humans. The present work investigated the susceptibility of domestic pigs to highly pathogenic ZEBOV. Virus replication, pathogenicity, shedding, and transmission to naive animals were evaluated in 2 independent studies. The first study focused on virus replication, pathogenicity, and shedding, whereas the second study was designed to evaluate shedding and transmission of the virus from inoculated to contact pigs.

MATERIALS AND METHODS

Virus and Challenge

ZEBOV strain Kikwit 95 was produced on Vero E6 cells in minimal essential medium supplemented with 2% fetal bovine serum and antibiotics (penicillin/streptomycin 1% final, generating complete minimal essential medium [cMEM]). All virus titers were determined by standard median tissue culture infective dose ($TCID_{50}$) and/or immunoplaque assays on Vero E6 cells. Procedures for the production and propagation of ZEBOV and all subsequent experiments involving infectious materials were performed in the Containment Level (CL) 4 facilities of the Canadian Science Center for Human and Animal Health.

Animal Experiments

Pigs were obtained from a high health status herd operated by a recognized commercial supplier in Manitoba, Canada, and tested negative upon arrival to the research facility for porcine reproductive and respiratory syndrome virus (PRRSV), circovirus, and cytomegalovirus infection by means of routine diagnostic assays. The animals were all negative for Ebola antibodies when prebleed samples were drawn and at day 0, on the basis of ELISA and virus neutralization. For the first experiment (susceptibility), 8 pigs aged 4–5 weeks were acclimatized for 7 days prior to inoculation. Six piglets were inoculated under inhalation anesthesia with a combination of intranasal (0.5 mL dripped in each nostril), intraocular (0.25 mL in the conjunctival fornices of each eye), and oral (1 mL) routes with a suspension of ZEBOV containing 4×10^5 plaque-forming units (PFU)/mL, for a total infectious dose by all routes of 1×10^6 PFU per animal. Two control animals were mock inoculated with phosphate-buffered saline (PBS) and housed separately from the 6 experimental animals housed in the CL4 cubicle. For the second experiment (transmission), 3 pigs aged 3–4 weeks were inoculated as described above. The next day, 4 contact animals of the same age were transferred into the CL4 cubicle. Two control animals were housed separately from the 7 experimental animals housed in the CL4 cubicle. All manipulations on inoculated or contact animals were performed under CL4 conditions and followed approved animal use documents and guidelines of the Canadian Council on Animal Care. All animal procedures and scoring sheets were first approved by the Animal Care Committee at the Canadian Science Centre for Human and Animal Health, according to the guidelines set by the Canadian Council on Animal Care.

Virus Titration

Tissues were harvested during necropsies and homogenized in cMEM using a bead mill homogenizer according to the manufacturer's protocol (Tissue Lyser, Qiagen). Samples were inoculated in 10-fold serial dilutions of supernatant on Vero E6 cells with 6 replicates per dilution. At 72–96 hours postinfection, the plates were scored for cytopathic effect or positive fluorescence by immunostaining and $TCID_{50}$ virus titers were calculated using the Reed and Muench method [22]. Swabs were collected into 1 mL of cMEM, and virus titers were similarly determined in nasal washes and swab suspensions by direct titration on Vero E6 cells. Total RNA was extracted from tissues preserved in RNAlater. RNA was isolated from nasal washes and swabs using the QIAamp Viral RNA Mini Kit (QIAGEN, GmbH) and from tissues using the RNeasy Mini Kit (QIAGEN). ZEBOV was detected by quantitative reverse transcription polymerase chain reaction (q-RT-PCR) using the LightCycler 480 RNA Master Hydrolysis Probes (Roche, GmbH) assay targeting the glycoprotein (nt 7720-7783, AF086833). Reaction conditions were the following: 63°C– for 3 minutes, 95°C– for 30 seconds, and cycling of 95°C– for 15 seconds, 60°C– for 30 seconds for 45 cycles using a StepOne Plus (Applied Biosystems). The lower detection limit for this assay is 1 PFU/mL. The primer sequences are as follows: ZebovGPF (GGCCAACGAGACGACTCAA), ZebovGPR (AAAGGTGCGTAGCTCAGT TG TG), and ZebovGPP (6FAM-CTCTTCAACTGTTCCTGAGAG–MGBNFQ).

Histological Analysis

Tissues were fixed in 10% neutral phosphate-buffered formalin, routinely processed, sectioned at 5 μm, and stained with hematoxylin and eosin for histopathologic examination. For immunohistochemical analysis, paraffin tissue sections were processed as described elsewhere [23]. A 1:2000 dilution of rabbit polyclonal anti-ZEBOV VP40 antibody was applied to the sections for 1 hour. Staining was revealed with the horseradish peroxidase (HRP) Envision+ system (anti-rabbit) (Dako) and reacted with

the chromogen diaminobenzidine (DAB). Finally, the sections were counterstained with Gill's hematoxylin.

Immune Responses

Cytokine mRNA expression changes over time were assessed by relative computed tomographic qPCR using β-Actin as the housekeeping gene [24]. cDNA was generated using 10 ng of mRNA isolated from lung tissue with the High Capacity cDNA Reverse Transcription Kit (ABI). Gene Expressions was measured using the TaqMan Gene Expression Master Mix (ABI) along with porcine primers and probe from the TaqMan Gene Expression Assay library. Each sample was extracted and inactivated with AVL (QIAGEN), analyzed in duplicate, and repeated twice or more if the variation was >10%. Each value represents the average of 2 infected animals and 2 control animals analyzed in duplicate. Serum samples collected from pigs were inactivated at 56°C for 45 minutes, and serial dilutions of each sample (1:10, 1:20, 1:40, and so forth) were evaluated for neutralizing antibody using ZEBOV expressing the EGFP reporter gene (ZEBOV-EGFP) as described elsewhere [25, 26]. The total number of EGFP-positive cells were counted in each well and sample dilutions, which showed >50% reduction in the number of green cells, compared with controls scored positive for neutralizing antibody. Gamma-irradiated, safety-tested, sucrose gradient-purified ZEBOV was used as an antigen in an enzyme-linked immunosorbent assay (ELISA) using conditions previously described [27]. Anti-Ebola antibodies were detected with a goat antiswine immunoglobulin G (IgG)

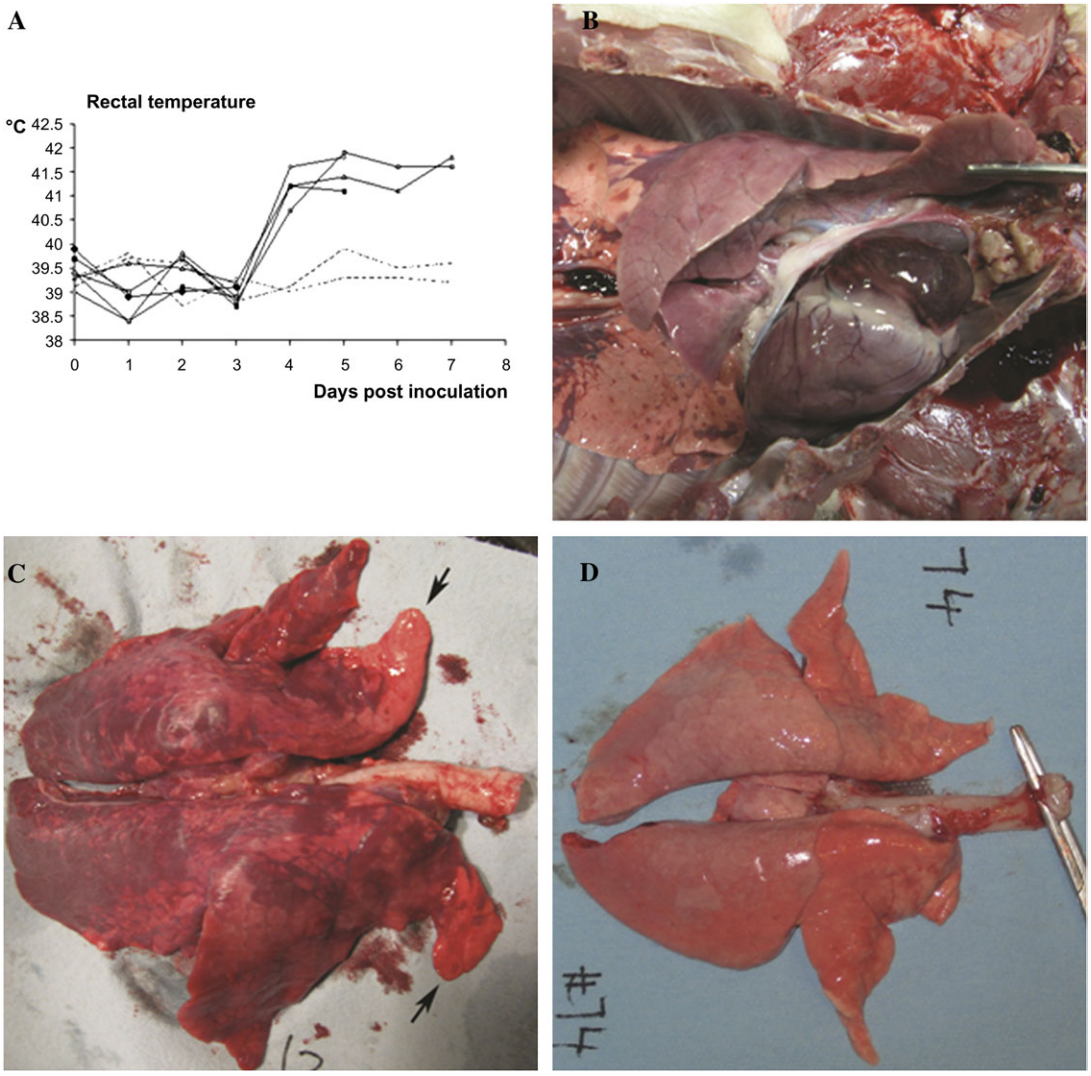

Figure 1. Body temperature and gross pathology. *A*, Body temperature and days postinfection are indicated on the *x*-axis; rectal temperature is plotted on the *y*-axis. Each curve represents 1 animal. Solid lines indicate piglets inoculated with ZEBOV; dashed lines indicate rectal temperatures of the 2 control animals. *B*, Lung and heart from pig infected with Ebola virus at 7 dpi. Note the dark hemorrhagic right atrium and consolidated lobes. *C*, Dorsal view of lungs from a pig infected with Ebola virus at 7 dpi. Note that only the top parts of the apical lobes remain relatively unaffected (pink areas; *arrows*), whereas the remaining lungs are consolidated with hemorrhages. *D*, Healthy lung from a control piglet.

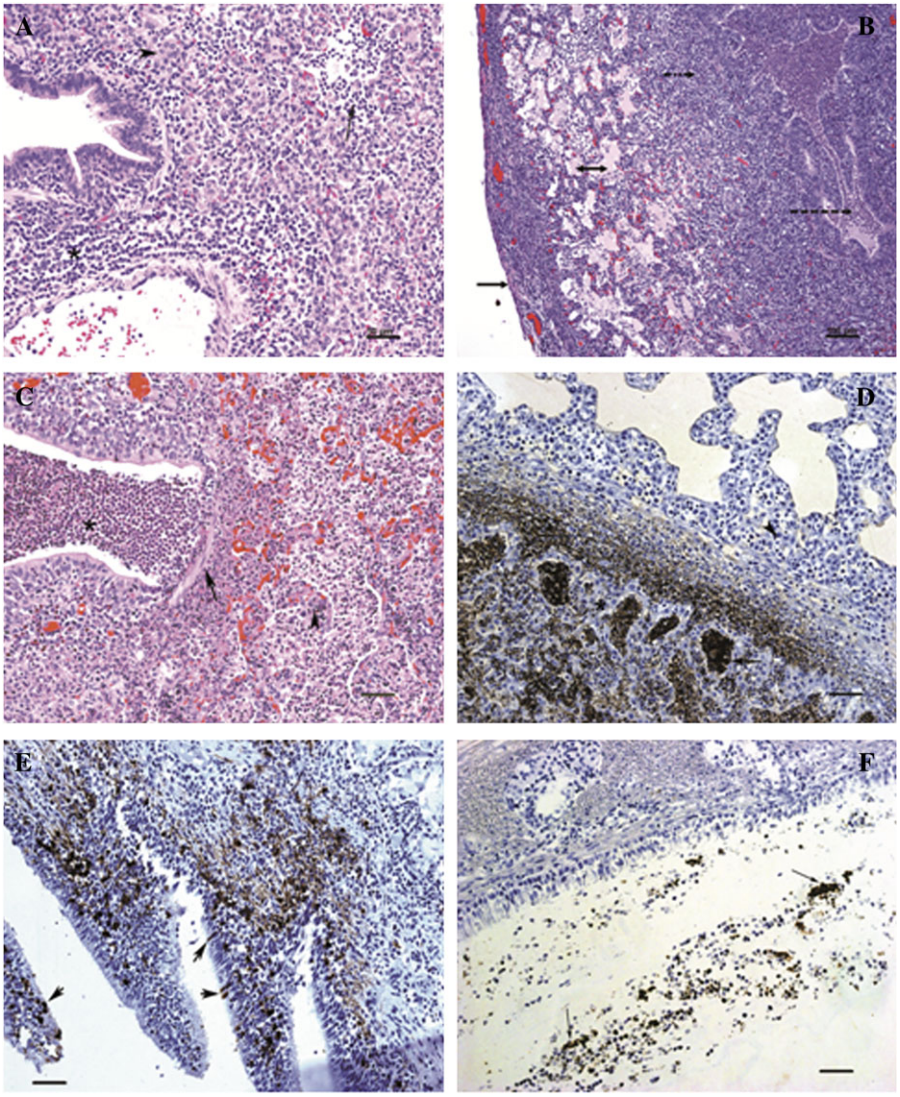

Figure 2. Histopathological examination of ZEBOV-infected lungs. Hematoxylin and eosin or immunohistochemistry stained slides from animals euthanized at days 3–7 were examined for pathological changes. *A,* At 3 dpi, consolidation is pronounced, with evidence of lymphomononuclear perivascular infiltrate (*asterisk*). Alveolar septae are thickened, pneumocytes are hyperplastic (*arrowhead*), and alveoli contain inflammatory cells (*arrow*). Hematoxylin and eosin (H&E). Bar = 50 μm. *B,* At 5 dpi, infiltration of inflammatory cells into an affected lung lobule is shown. Double arrow indicates alveoli filled with edema fluid. Long jointed arrow indicates a bronchiole with luminal inflammatory exudates. Short jointed arrow indicates alveoli filled with inflammatory cells. Solid arrow indicates expansion of pleura by fibrin and inflammatory cells. H&E. Bar = 100 μm. *C,* Bronchiolar epithelium (*arrow*) and alveolar septae (*arrowhead*) are necrotic at 7 dpi, and the lumen of the bronchiole, as well as nearby alveolar spaces, is filled with inflammatory cells (*asterisk*). H&E. Bar = 50 μm. *D,* Positive immunohistochemical staining of lung for Ebola virus antigen at 7 dpi. The staining is heavy throughout the affected lobule (*asterisk*), whereas an adjacent lobule remains negative (*arrowhead*). Note that proteinaceous edema and cellular infiltrates in the alveoli are heavily stained for *ebolavirus* antigen (*arrow*). Bar = 50 μm. *E,* Immunohistochemistry is positive for Ebola virus antigen in bronchiolar epithelial cells (*arrowheads*) at 5 dpi, as well as in macrophages and endothelial cells extending into the lung parenchyma. Intensity decreases with distance from the bronchiole. Bar = 50 μm. *F,* Positive immunohistochemistry for Ebola virus antigen at 7 dpi in the inflammatory cell exudate within the tracheal lumen (*arrows*). Bar = 50 μm.

HRP-conjugated antibody (Kikefaard and Perry Laboratories). Values for the optical density at 405 nm of >0.350 were considered positive for the presence of anti-ZEBOV antibodies, on the basis of cutoff values established with serum samples from 17 uninfected pigs. All manipulations with infectious Ebola virus were performed under CL4 conditions and followed approved standard operation procedures.

Coagulation

Blood from infected animals was collected into citrated vaccutainers (BD BioSciences) on the indicated day by venous bleed. Blood was centrifuged at 2500 g for 15 minutes at room temperature and the citrated plasma removed for immediate analysis of prothrombin (STA-Neoplastine Cl Plus), activated partial thromboplastin time (aPTT) (PTT Automate), and fibrinogen

(Fibri-Prest Automate) (all reagents: Stago) or frozen at –80°C for later analysis of D-dimer levels (Imubind D-Dimer ELISA kit) (American Diagnostica). All coagulation assays were run on the StART4 coagulation analyzer (Stago) according to the manufacturer's instructions under CL4 conditions and followed approved standard operation procedures. D-dimer ELISA was performed according to the manufacturer's instructions also under CL4 conditions.

RESULTS

Virus Replication, Pathogenicity, and Shedding of ZEBOV in Pigs

All ZEBOV-infected pigs developed a fever at day 4 after inoculation that lasted until the last time point at 7 days postinfection (dpi) (Figure 1A). The most prominent and progressive clinical signs were respiratory, with a rate increasing from around 35 breaths per minute to >80 breaths per minute by 7 dpi. At this time point, breathing was laborious and involved a strong abdominal component. Coughing was not observed; however, animals stopped eating, lost interest in human presence, stopped playing activities with cage mates, and were reluctant to stand up and move. Macroscopic examination of internal organs revealed obvious pathological changes in lungs (Figure 1B, 1C). The lungs demonstrated progressive consolidation from 3 to 7 dpi, starting with the cardiac lobes and spreading to the diaphragmatic lobes. Lung-associated lymph nodes were enlarged and occasionally mildly hemorrhagic. Interestingly, the right atrium of the heart was hemorrhagic in both pigs euthanized at 7 dpi; however, it is not clear what the basis is for this pathological observation (Figure 1B). At 3 dpi, histological analysis revealed capsular inflammation in bronchial lymph nodes and patchy alveolitis and bronchiolitis in the lung with accumulation of neutrophils and necrotic debris in the lumen of alveoli of both animals (Figure 2A). At 5 dpi, lymphadenitis was pronounced and in the lung there was acute and severe alveolitis characterized by edematous alveoli filled with fibrin, hemorrhage, and extensive infiltrates consisting of neutrophils and macrophages, as well as thickening of alveolar walls by inflammatory infiltrates (Figure 2B). Many bronchioles were damaged at 7 dpi, with evidence of extensive necrosis and neutrophil infiltration. Large consolidated areas with complete loss of alveolar spaces and severely damaged septae containing areas of necrosis and fibroblast proliferation were observed (Figure 2C). Viral antigen was detected in abundance in some lobules, whereas an adjacent lobule might remain negative (Figure 2D). Interestingly, there was early staining of airway epithelial cells surrounding bronchioles, which then seemed to spread internally to alveoli with a decreasing intensity gradient. This was observed in several lung sections of both pigs at 5 dpi (Figure 2E). Inflammatory exudates positive by immunocytochemical staining for viral antigen were also detected in the trachea at 7 dpi (Figure 2F). Several cell types, including macrophages, type I and II pneumocytes, and epithelial cells were positive by immunocytochemical analysis in affected lobules; however, none of the infiltrating lymphocytes showed ZEBOV antigen–positive staining (Figure 3).

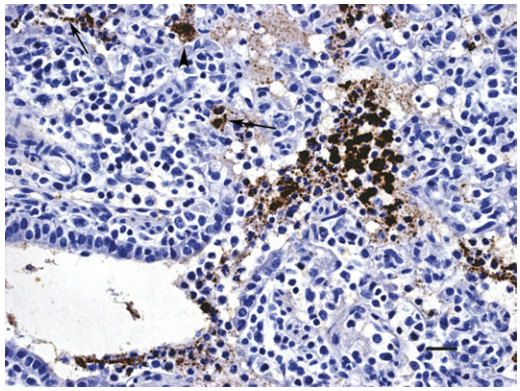

Figure 3. Histopathological examination of ZEBOV-infected lung cells. Positive immunocytochemistry for Ebola virus antigen at 7 dpi in epithelial cells and type I and II pneumocytes (*arrows and arrowhead, respectively*). Note that adjacent lymphomononuclear infiltrating cells are not stained. Bar = 20 μm.

Interestingly, ZEBOV replication in the airways induced the recruitment of large numbers of immune cells, possibly contributing to disease severity. To explore this hypothesis, the innate immune response was monitored by evaluating cytokine mRNA expression profiles by real-time RT-PCR in lung tissues of infected animals (right cardiac lobe). Early induction of interferon-γ (IFN-γ), interleukin (IL)-6, IL-8, and tumor necrosis factor was observed from infected pigs at 3 dpi, supporting the concept of virus-induced airway inflammation through the stimulation of proinflammatory cytokines (Figure 4A). Interestingly, IFN-α was substantially downregulated at 5 and 7 dpi. An adaptive antibody response against ZEBOV as measured by ELISA or neutralization assays was not detected at these early time points. Results of blood work (cell count and chemical analysis) and coagulation abnormalities measured by means of prothrombin time and aPTT assays and D-dimer levels from infected animals did not show statistical significance in comparison with prebleed or PBS-treated pigs at any time points (data not shown). An increase in fibrinogen levels was observed on days 5 and 7, suggestive of an inflammatory response. Virus was isolated from nasal washes and oral and rectal swabs at 3 and/or 5 dpi, although not from all animals and with relatively low infectious titers ranging from 1×10^2 to 1×10^3 $TCID_{50}$/mL per sampled site (Figure 4B). Infectious virus was also detected at low levels in blood and serum of 1 animal but only at 5 dpi. Moderate levels of infectious virus were detected in different organs, including the bladder and heart of that animal. In

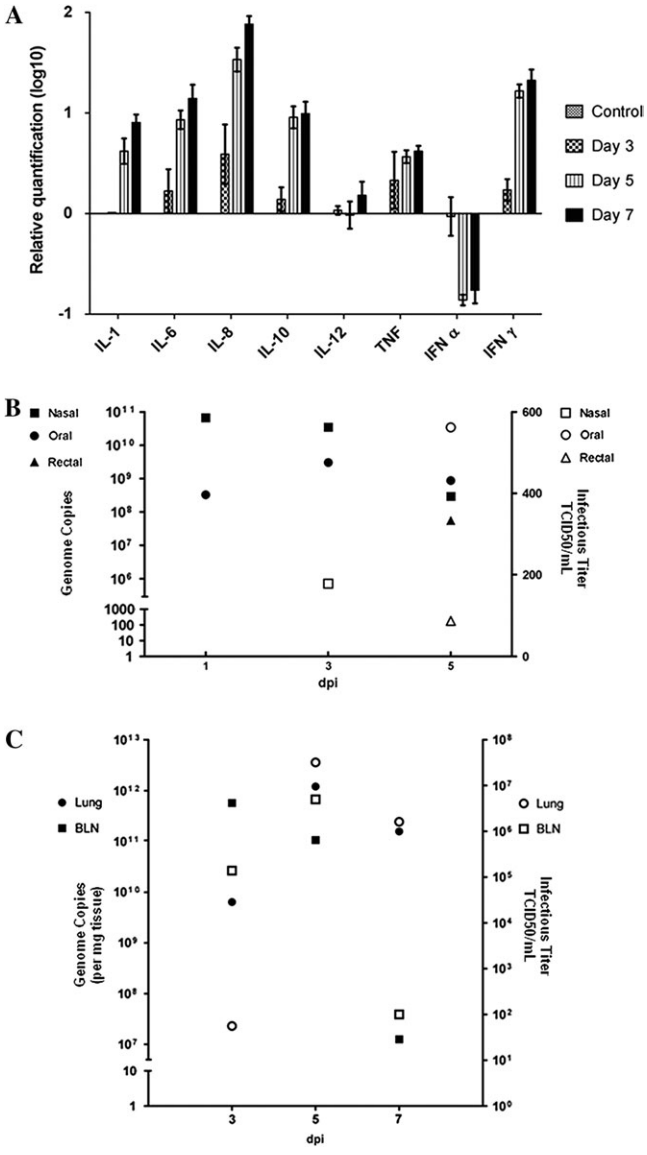

Figure 4. Innate immune responses detected through cytokine mRNA expression profile in lung tissue and viral loads over time. *A,* Lung tissue from infected animals was examined in ZEBOV-infected animals at 3, 5, and 7 dpi. Each tissue sample was compared with control lung tissue from noninfected animals and normalized with porcine β-Actin. The relative quantification mean value is shown on the *y*-axis in a $\log_{10}$ scale and the cytokine identification at each dpi is on the *x*-axis. Bars represent mean level of mRNA expression per 2 pigs per time point performed twice ± the standard deviation. *B,* Total ZEBOV genome copy or infectious particle detected from nasal, oral, or rectal swabs over time. Samples were analyzed on days 0, 1, 3, 5, and 7 by means of real-time reverse transcription polymerase chain reaction and direct infection. The *x*-axis represents days postinfection plotted against viral genome copies on the left *y*-axis and infectious viral particles on the right *y*-axis. *C,* Total ZEBOV genome copy or infectious particles detected in the lung and bronchial lymph nodes. Animals killed on days 3, 5, and 7 were analyzed by means of real-time RT-PCR or direct infection. The *x*-axis represents days postinfection plotted against viral genome copies on the left *y*-axis or infectious viral particles on the right *y*-axis. For *B* and *C,* each point represents the average genome copy per milligram of tissue or per 140 μL sampled from swabs or washes or infectious particle number from assays performed in duplicate or triplicate, respectively, for both animals at each time point. dpi, days postinfection; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

contrast, high levels of infectious virus were detected from lung tissues and bronchial lymph nodes from 3 to 7 dpi, with titers reaching up to 3.2×10^7 $TCID_{50}$/mL at 5 dpi (Figure 4C). ZEBOV particles quantified by real-time RT-PCR as genome copy numbers were highest on 1 and 3 dpi in the mucosa and remained elevated at all time points in the airway.

Shedding and Transmission of ZEBOV in Infected and Naive Contact Pigs

To address transmission between animals, 3 pigs aged 4 weeks were infected as described above and 4 naive piglets were brought into the same living space 24 hour later. The inoculated animal analyzed at 7 dpi had macroscopic and microscopic lesions in the lungs consistent with the results of the first study, although less severe. However, this animal had detectable levels of viral RNA present in several organs, including lung, lymph nodes, tonsil nasal turbinate, heart, muscle, liver, and gut (Figure 5A). Virus was detected by real-time RT-PCR in inoculated pigs, peaking at 5 dpi from nasal washes and 3 and 7 dpi from oral swabs (Figure 5B and C). Viral RNA was occasionally found in blood, serum, and rectal swab samples from inoculated pigs by means of real-time RT-PCR. Infectious virus was also recovered by limiting dilution from mucosal samples of inoculated animals, also peaking at 5 dpi albeit at low levels. The lungs, trachea, and liver of the animal analyzed at 7 dpi also contained infectious virus (Figure 6). Viral RNA was detected from the mucosa of all contact animals, peaking at 10 dpi (Figure 5B and C). Three of 4 contact pigs analyzed at 21 dpi or later showed detectable levels of viral RNA by means of RT-PCR (Figure 5A). Low levels of infectious virus were recovered from mucosal samples of 2 of 4 contact pigs, interestingly also peaking around 10 dpi, which suggests a replication and transmission cycle of approximately 5 days with the possibility for another transmission cycle from contact pigs. Total IgG, as well as neutralizing antibodies against ZEBOV, developed after 3 weeks in both infected and contact pigs (Figure 5D). Hematology and coagulation parameters were similar to those of PBS-treated control pigs for all inoculated and contact pigs (data not shown). Overall, the clinical course of the disease was less severe in the transmission study, not requiring euthanasia of inoculated animals, and with a transient and/or delayed fever. Because the virus lot, the infection dose, and routes were identical, the severity of clinical disease in ZEBOV-infected pigs may be related to genotype, age, or immune status. It is difficult at this point to comment on the underlying mechanisms, as commercial pigs are outbred, and although the piglets in the individual experiments were from the same litter, they were from different sows. In

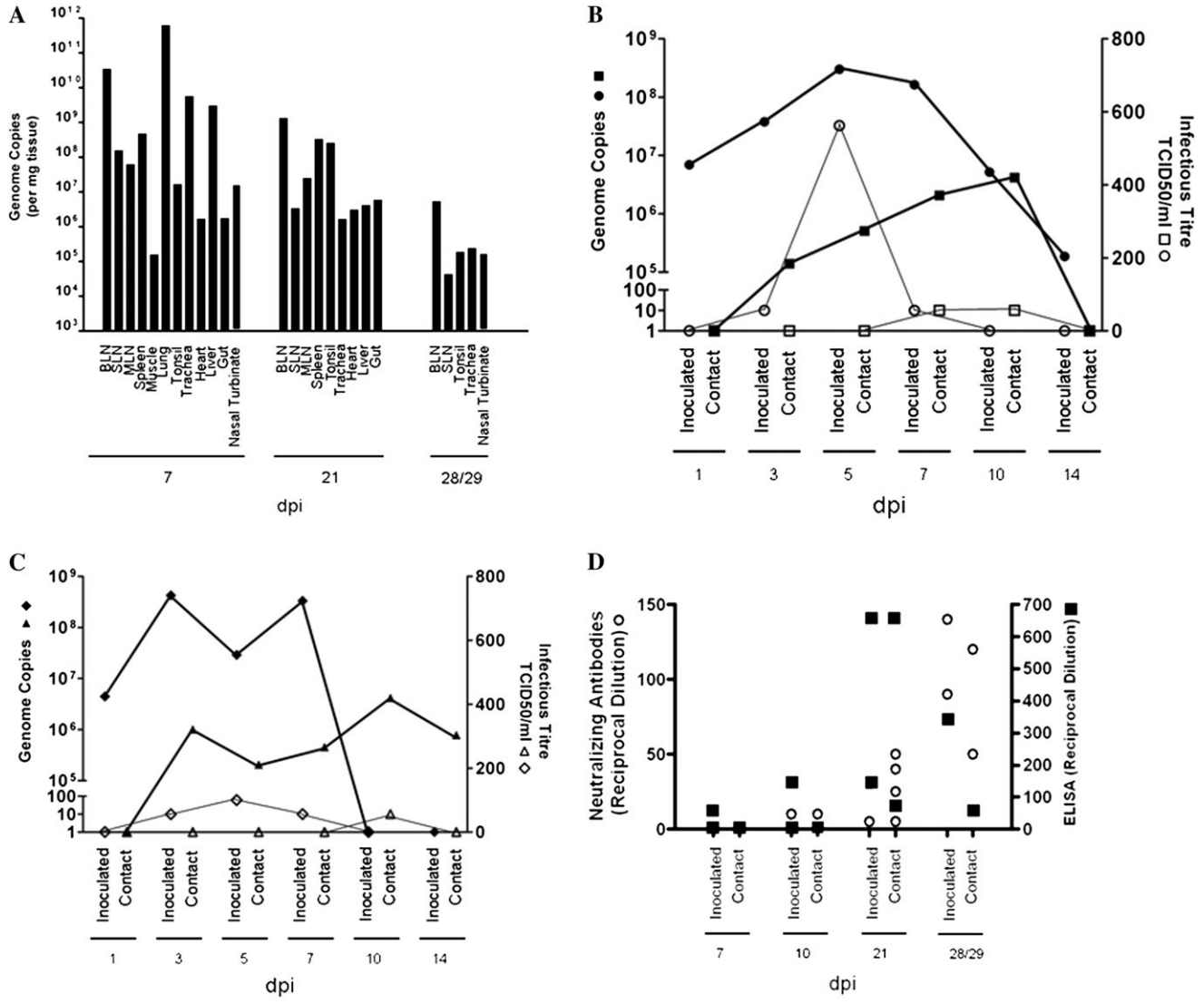

Figure 5. Total genome copies or infectious ZEBOV particles detected from inoculated and contact pigs over time. *A,* ZEBOV genome copy number in different organs of 1 inoculated pig (7 dpi) or contact pigs (21–29 dpi) over time. Each bar represents the titer per milligram of tissue from 1 organ. *B,* Viral loads from nasal or *C,* oral samples of inoculated or contact pigs at different time points. Each data point represents 1 site averaged for all animals per group expressed in genome copy per milligram of tissue or sample of 140 μL or median tissue culture infective dose ($TCID_{50}$)/mL for infectious particles. *D,* Neutralizing antibodies or total IgG were detected in serum samples from ZEBOV-infected animals and assayed on ZEBOV-expressing EGFP for neutralization or enzyme immunosorbent assay (ELISA) for total IgG. Days postinfection are indicated on the *x*-axis and reciprocal titer on the *y*-axis. Neutralizing and ELISA assays were repeated twice, and each data point represents the titer from 1 animal.

addition, at the age of about 3–6 weeks, the animal development is rapid, including the immune system [28].

DISCUSSION

The recent detection of REBOV in domesticated pigs coinfected with PRRSV, along with the detection of antibody against REBOV in 6 of 141 individuals who worked on pig farms or with swine products, strongly suggests that pigs are susceptible to *ebolavirus* replication and can transmit the virus [21]. The present study demonstrates that domesticated pigs are susceptible to ZEBOV infection following mucosal challenge and develop a respiratory disease with a clinical manifestation that can be severe. This observation raises the possibility that pigs are capable of shedding relatively high viral loads into the environment. Our data indicate that infection can be acquired following mucosal exposure to the virus and efficiently transmitted to naive animals cohabiting with infected mates. The presence of relatively high viral loads in the upper airway suggests that the respiratory mucosa is a participant in acquisition and subsequent transmission of infection. Although the involvement of the gastrointestinal tract cannot be completely ruled out, the presence of Ebola viral genomes was detected inconsistently from gut samples and infectious virus could not

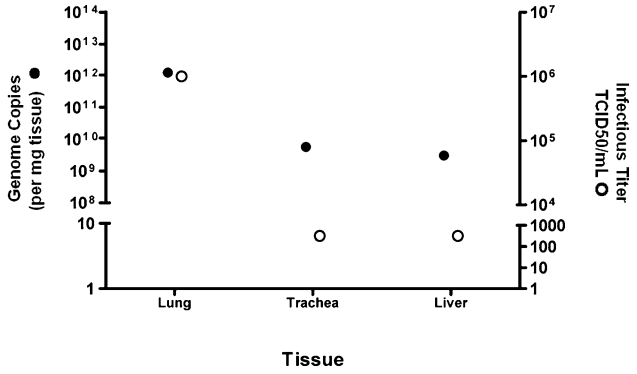

Figure 6. Total genome copy and infectious ZEBOV particles in lung, trachea, and liver tissues from 1 inoculated pig. The animal was analyzed at 7 dpi, and each data point represents infectious (*circles*) or genome copy per milligram (*square*) of ZEBOV per organ.

be recovered. In addition, despite the relatively uniform presence of Ebola virus in the respiratory mucosa, the detection of the virus systemically was sporadic, the opposite of what one could expect from an acquisition of infection involving the gastrointestinal tract.

Taken together, these data suggest that ZEBOV can infect pigs through the oronasal mucosa, entering the airway epithelia from the apical side and then replicating in macrophages, pneumocytes, and endothelial cells in the lung parenchyma to high concentrations, following which ZEBOV is released back into the airway in inflammatory exudates where it can be transmitted de novo to other naive pigs through mucosal exposure. Relatively few viruses are able to penetrate the airway epithelial barrier from the apical side, limiting both virus entry and subsequent replication [29]. There is increasing experimental evidence indicating that the Ebola virus glycoprotein can mediate entry from the apical side into intact airway epithelia of mouse, nonhuman primate, or human origin [28, 30–32]. The presence of Ebola antigens was also detected in the respiratory mucosa, alveoli, and pulmonary lymphatic tissue of nonhuman primates following aerosolized Ebola challenge, demonstrating that the virus can infect nonhuman primates through mucosal exposure with *ebolavirus* [33]. More recently, pigs infected with REBOV showed lung pathology, the presence of viral antigens in pulmonary tissues, and evidence of filamentous Ebola virus particles in lymph nodes [21]. Whether transmission in swine occurs through the aerosol route or from contamination of the environment with mucosal secretions remains to be determined in future experiments.

These studies underline some differences in the pathology induced by ZEBOV in pigs, compared with nonhuman primates and humans. In contrast to the severe systemic syndrome often leading to shock and death in primates, pigs developed a respiratory syndrome that could be mistaken for other porcine respiratory diseases. Further work is warranted to answer additional questions, such as the effect of age and breed on the susceptibility of pigs to Ebola virus, the precise role of the immune response, and the susceptibility of pigs to other filoviruses. Considering the specific downregulation of IFN-α mRNA, the role of the immune response is of particular interest, because a delayed and lower than expected antibody response correlated with immune suppression. Pigs usually mount a fast antibody response detectable by day 5, peaking above 1:1000 serum dilutions by day 14 [34]. One can speculate that the delayed antibody response in conjunction with increased proinflammatory cytokines may constitute the basis of Ebola virus–induced pathogenesis in pigs and, indirectly, transmission. Although downregulation of interferon has been well documented [35], the precise mechanism leading to death during *ebolavirus* infection remains to be fully elucidated. Pigs may offer new and/or complementary insights into *ebolavirus* pathogenesis. In this study, ZEBOV rather than REBOV was selected because of its well-documented impact on human life in past outbreaks. These data also have implications for the management of human outbreaks following accidental or hypothetically intentional exposure of pigs to Ebola virus.

Funding

This work was supported by the Public Health Agency of Canada and the Canadian Food Inspection Agency.

Acknowledgments

We thank Shane Jones, Gregory Schumer, Mike Gray, Estella Moffat, Shelley Ganske, Beata Stachowiak, and Brad Collignon for technical assistance with the animals and immunocytochemical analysis.

References

1. Feldmann H, Jones S, Klenk HD, Schnittler HJ. Ebola virus: from discovery to vaccine. Nat Rev Immunol **2003**; 3:677–85.
2. Pattyn SR. Ebola virus hemorrhagic fever. Amsterdam: Elsevier/North-Holland, 1978.
3. Peters CJ, LeDuc JW. An introduction to Ebola: the virus and the disease. J Infect Dis **1999**; 179(suppl 1);ix–xvi.
4. Sanchez A, Khan A, Zaki S, Nabel G, Ksiazek T, Peters CJ. *Filoviridae*—Marburg and Ebola viruses. In: Knipe DM, Howley PM eds. Field's Virology, 4th ed. Vol 1. Philadelphia: Lippincott Williams and Wilkins, **2001**; 1279–301.
5. Hayes CG, Burans JP, Ksiazek TG, et al. Outbreak of fatal illness among captive macaques in the Philippines caused by an Ebola-related filovirus. Am J Trop Med Hyg **1992**; 46:664–71.
6. Miranda ME, Ksiazek TG, Retuya TJ, et al. Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. J Infect Dis **1999**; 179(suppl 1):S115–9.
7. Muyembe T, Kipasa M. Ebola haemorrhagic fever in Kikwit, Zaire. Lancet **1995**; 345:1448.
8. Feldmann H, Sanchez A, Klenk HD. Filoviruses. In: Collier LH ed. Microbiology and microbial infections, 9th ed. London: Edward Arnold, 1998: 651–64.
9. Ryabchikova EI, Kolesnikova LV, Luchko SV. An analysis of features of pathogenesis in two animal models of Ebola virus infection. J Infect Dis **1999**; 179(suppl 1):S199–202.

10. Schnittler HJ, Feldmann H. Molecular pathogenesis of filovirus infections: role of macrophages and endothelial cells. Curr Top Microbiol Immunol **1999**; 235:175–204.
11. Bente D, Gren J, Strong JE, Feldmann H. Disease modeling for Ebola and Marburg viruses. Dis Model Mech **2009**; 2:12–7.
12. Bowen ET, Platt GS, Simpson DI, McArdell LB, Raymond RT. Ebola haemorrhagic fever: experimental infection of monkeys. Trans R Soc Trop Med Hyg **1978**; 72:188–91.
13. Bray M, Davis K, Geisbert T, Schmaljohn C, Huggins J. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J Infect Dis **1998**; 178:651–61.
14. Ryabchikova E, Kolesnikova L, Smolina M, et al. Ebola virus infection in guinea pigs: presumable role of granulomatous inflammation in pathogenesis. Arch Virol **1996**; 141:909–21.
15. Leroy EM, Kumulungui B, Pourrut X, et al. Fruit bats as reservoirs of Ebola virus. Nature **2005**; 438:575–6.
16. Leroy EM, Epelboin A, Mondonge V, et al. Human Ebola outbreak resulting from direct exposure to fruit bats in Luebo, Democratic Republic of Congo, 2007. Vector Borne Zoonotic Dis **2009**; 9:723–8.
17. Leroy EM, Rouquet P, Formenty P, et al. Multiple Ebola virus transmission events and rapid decline of central African wildlife. Science **2004**; 303:387–90.
18. Outbreak(s) of Ebola haemorrhagic fever, Congo and Gabon, October 2001-July 2002. Wkly Epidemiol Rec **2003**; 78:223–8.
19. Agence France-Presse. Farm worker has Ebola strain. Vol. 2009., 2009.
20. Agence France-Presse. Philippines pig farm worker infected with Ebola strain. WHO, 2009. http://newssurggection.blogspot.com/2008/11/new-guidelines-to-strengthen-anti.html. Last Accessed 15 March 2011.
21. Barrette RW, Metwally SA, Rowland JM, et al. Discovery of swine as a host for the *Reston ebolavirus*. Science **2009**; 325:204–6.
22. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Amer Jour Hyg **1938**; 27:493–7.
23. Kobasa D, Jones SM, Shinya K, et al. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature **2007**; 445:319–23.
24. Nygard AB, Jorgensen CB, Cirera S, Fredholm M. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Mol Biol **2007**; 8:67.
25. Richardson JS, Yao MK, Tran KN, et al. Enhanced protection against Ebola virus mediated by an improved adenovirus-based vaccine. PLoS One **2009**; 4:e5308.
26. Ebihara H, Theriault S, Neumann G, et al. In vitro and in vivo characterization of recombinant Ebola viruses expressing enhanced green fluorescent protein. J Infect Dis **2007**; 196(suppl 2);S313–22.
27. Sullivan NJ, Geisbert TW, Geisbert JB, et al. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature **2003**; 424:681–4.
28. Sinn PL, Hickey MA, Staber PD, et al. Lentivirus vectors pseudotyped with filoviral envelope glycoproteins transduce airway epithelia from the apical surface independently of folate receptor alpha. J Virol **2003**; 77:5902–10. Jones and Bartlett Publishers Sudbury, MA, USA.
29. Sompayrac L. How Pathogenic Viruses Work. Sudbury, MA: Jones and Bartlett Publishers, 2002: 8.
30. Kobinger GP, Weiner DJ, Yu QC, Wilson JM. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. Nat Biotechnol **2001**; 19:225–30.
31. Medina MF, Kobinger GP, Rux J, et al. Lentiviral vectors pseudotyped with minimal filovirus envelopes increased gene transfer in murine lung. Mol Ther **2003**; 8:777–89.
32. Limberis MP, Bell CL, Heath J, Wilson JM. Activation of transgene-specific T cells following lentivirus-mediated gene delivery to mouse lung. Mol Ther **2010**; 18:143–50.
33. Johnson E, Jaax N, White J, Jahrling P. Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. Int J Exp Pathol **1995**; 76:227–36.
34. Butler JE, Lager KM, Splichal I, et al. The piglet as a model for B cell and immune system development. Vet Immunol Immunopathol **2009**; 128:147–70.
35. Basler CF, Amarasinghe GK. Evasion of interferon responses by Ebola and Marburg viruses. J Interferon Cytokine Res **2009**; 29:511–20.