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Autophagy-Dependent Viral Recognition by Plasmacytoid Dendritic Cells

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Science **315**, 1398 (2007);
DOI: 10.1126/science.1136880

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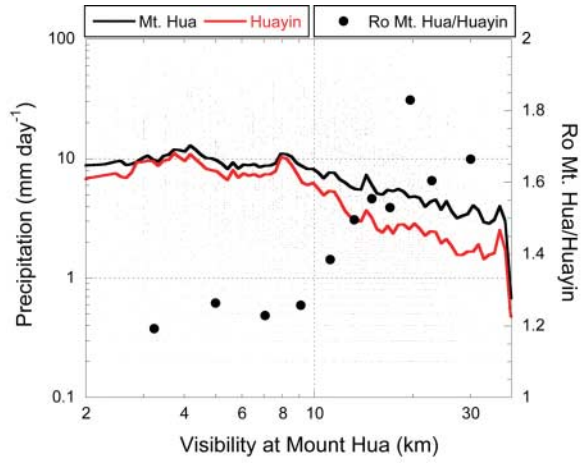

Fig. 4. The daily rainfall at Mt. Hua and Huayin as a function of the visibility at Mt. Hua for 1980 to 2004. The red and black lines are interpolated curve fits with a weight applied. The weight is applied to 20% of the data (–10% to +10% of the data around the current point). Ro, which is the distance between the lines along the logarithmic ordinate, is reduced for lower-visibility distances. Ro for discrete visibility intervals (between 2, 4, 6, 8, 10, 12, 14, 16, 18, 21, 25, and 42 km) is denoted by the large black circles with the values on the right ordinate. The suppression effect saturates at a visibility distance of ~8 km. See table S3, A and B, for data.

decrease in Ro of about 30% is consistent with the findings of the case study reported at the Rocky Mountain–top observatory, where a 50% reduction in the snow rate was related to aerosols that caused smaller cloud drops and reduced efficiency of their riming on snowflakes (*11*).

These observations show that a proxy to CCN concentrations in the free troposphere—the visibility at the top of Mt. Hua at 2060 m above sea level—is directly correlated with the decreasing trend of Ro on a climatologically meaningful scale. The observed suppression of precipitation at Mt. Hua is only one such observation in China, but given the extensive occurrence of this phenomenon elsewhere in the world, it is likely that this is a major cause for suppressing precipitation at the mountain ranges that provide much of the world's water resources.

The sensitivity of the precipitation from orographic clouds to the detrimental impact of pollution aerosols is not one-sided. This also means that seeding the same clouds with aerosols that are engineered to accelerate the conversion of cloud water into precipitation can enhance the precipitation, especially under conditions where the water losses resulting from air pollution are the greatest. This has already been shown to be the case in Israel (*12*).

Quantifying the response of precipitation to aerosol amounts addresses the recommendation of the U.S. National Research Council (*13*) that the concept of radiative forcing should be expanded to metrics of nonradiative forcing, such as those caused by the effects of aerosols on clouds and precipitation, which affect Earth's water and energy cycles.

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14. This research was supported by the National Natural Science Foundation of China (grant 40575004), the Chinese Ministry of Science and Technology (grant 2005DIB3J099), the Foreign Expert Bureau of Shaanxi Province, and the Meteorological Bureau of Shaanxi Province. We are grateful to the Meteorological Information Center of Shaanxi Province for providing all data in this research.

Supporting Online Material

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27 November 2006; accepted 30 January 2007
10.1126/science.1137949

Autophagy-Dependent Viral Recognition by Plasmacytoid Dendritic Cells

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Plasmacytoid dendritic cells (pDCs) detect viruses in the acidified endosomes by means of Toll-like receptors (TLRs). Yet, pDC responses to certain single-stranded RNA (ssRNA) viruses occur only after live viral infection. We present evidence here that the recognition of such viruses by TLR7 requires transport of cytosolic viral replication intermediates into the lysosome by the process of autophagy. In addition, autophagy was found to be required for the production of interferon-α by pDCs. These results support a key role for autophagy in mediating ssRNA virus detection and interferon-α secretion by pDCs and suggest that cytosolic replication intermediates of viruses serve as pathogen signatures recognized by TLR7.

Detection of viral infection by the innate arm of the mammalian immune system relies on several distinct pathways. The classical viral recognition pathway, used by most cell types, involves cytoplasmic pattern recognition molecules to detect viral signatures such as double-stranded RNA (dsRNA) structures (*1*) and 5′-triphosphate RNA (*2*, *3*). In contrast, the plasmacytoid dendritic cells (pDCs) are thought to detect viruses without being directly infected and achieve this through the use of Toll-like receptors (TLRs) in the endosomes (*4*). Viral particles containing dsDNA genomes, such as herpes simplex viruses (HSV) and murine cytomegalovirus, are recognized by TLR9 (*5*, *6*), whereas those containing single-stranded RNA (ssRNA) genomes, such as influenza virus and vesicular stomatitis virus (VSV), are detected by TLR7 (*7*–*9*). When experimentally targeted to the endosome by liposomal agents, purified dsDNA and ssRNA can trigger TLR-dependent expression of type I interferons (IFNs) (*7*, *9*, *10*). Moreover, ultraviolet (UV)–irradiated HSV (*6*, *11*, *12*) and heat-inactivated (*7*) or formaldehyde-fixed (*13*) influenza virus can induce IFN-α responses comparable to their live counterparts in pDCs. pDC recognition of HIV-1 occurs independently of fusion or replication but requires attachment and endocytosis showing that infection by live virus is not required (*14*). These observations have led to the idea that the TLR-mediated recognition of viruses in pDCs occurs without direct infec-

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tion and that the presence of viral genomic nucleic acids within the endosomal/lysosomal compartments is sufficient to activate antiviral pathways (*15*). However, this paradigm does not explain why UV irradiation renders particular ssRNA viruses, such as respiratory syncytial virus (*16*) and VSV (*17*), incapable of inducing IFN-α secretion from pDCs. In these cases, the mechanism of recognition of ssRNA viruses by pDCs remains unclear and suggests that a separate pathway may be operating to ensure recognition of such viruses in pDCs.

To explore the intracellular mechanisms of ssRNA viral recognition in pDCs, we examined the requirement of different stages in viral infection. In contrast to the situation with HSV recognition (*6*, *11*, *12*), robust IFN-α production by purified pDCs (*18*) in response to the ssRNA viruses, Sendai virus (SV), and VSV, required live virus infection (Fig. 1). Thus, UV-irradiated SV and VSV induced comparatively lower levels of IFN-α production in pDCs relative to live viruses (Fig. 1A). This difference in IFN-α levels could not be accounted for by increased virion production with live viruses, because pDCs failed to produce a significant number of virions during 18 hours of incubation (fig. S1). In contrast to SV and VSV, both UV-irradiated and live influenza virus particles induced comparable levels of IFN-α (Fig. 1A). In addition, recognition of all of these ssRNA viruses depended entirely on TLR7 in pDCs (Fig. 1B). Thus, robust IFN-α production in pDCs after stimulation with VSV and SV, but not influenza (*7*), requires a recognition event coupled to viral replication.

We reasoned that if viral replication intermediates were being detected in pDCs following VSV and SV infection, then fusion and entry of viruses must precede recognition. To examine this possibility, we used a VSV mutant with a single amino acid deletion (methionine 51) in the matrix (M) protein (VSVΔM51) (*19*). In wild-type (WT) VSV, the M protein blocks transport of mRNA from the nucleus to the cytosol (*20*), thus preventing translation of host genes, including IFN-α. We reasoned that if fusion and viral protein synthesis were a prerequisite for viral recognition, the VSV mutant lacking a functional M protein would be expected to result in higher levels of IFN-α synthesis. Indeed, VSVΔM51 induced significantly higher levels of IFN-α than did WT VSV, at the same multiplicity of infection (MOI) (Fig. 1C) in the absence of productive virion synthesis (fig. S1). These results supported the hypothesis that viral replication intermediates generated after viral fusion and delivery of the viral nucleoprotein particles to the cytosol provide a major stimulus for IFN-α production by pDCs upon VSV infection.

It was unclear how cytosolic viral replication intermediates might be recognized by TLR7 in the lysosome of pDCs. A well-known pathway by which cytosolic materials are introduced to the lysosome involves autophagy (*21*). To determine whether autophagy is involved in the transport of cytosolic viral replication intermediates to the lysosome for TLR7-mediated detection of viruses in pDCs, we first examined the formation of autophagic vesicles in pDCs after virus stimulation. Historically, autophagosomes have been detected by electron microscopy (EM). However, it is difficult to distinguish and quantitate true autophagic vacuoles from other structures by morphology solely based on EM. Therefore, we used transgenic mice expressing the LC3 protein fused with the green fluorescent protein (GFP), which allowed us to visualize autophagosome formation in real time in live cells (*22*). During autophagosome formation, LC3 undergoes transition from the cytosolic form to a membrane-associated form, which is scored as cytoplasmic puncta by fluorescence microscopy (*23*). The LC3-GFP transgene expression had no effect on the biological functions of pDCs (fig. S2). To obtain statistically significant quantification of autophagosomes, we used multispectral imaging flow cytometry (MIFC), which allows characterization of single cells within a population by assessing a combination of morphology and immunofluorescence patterns. Autophagosomes in pDCs were quantitated by enumerating $CD11c^+$ $PDCA\text{-}1^+$ cells displaying cytosolic punctate GFP pattern (Fig. 2). These analyses revealed that autophagosome formation occurs constitutively in pDCs and that infection with VSV did not result in a significant increase in the percentage of pDCs containing autophagosomes (table S1).

We next examined the requirement for autophagy in innate viral recognition by pDCs. To this end, we stimulated bone marrow cells with VSV in the presence of different doses of pharmacological inhibitors of autophagy, 3-methyladenine (3-MA) (*24*) and Wortmannin (*25*). At doses of 3-MA (1 to 10 mM) and Wortmannin (10 to 100 nM) that are known to specifically inhibit autophagy (*24*, *25*), IFN-α production was completely abolished in response to VSV (Fig. 3). Further, in purified pDCs, lower doses of these inhibitors still resulted in selective partial inhibition of responses to VSV, but not influenza, without affecting cell survival (fig. S3 and supporting online material text). One potential complication in the use of Wortmannin is that it can also inhibit endocytosis (*26*). To rule out this possibility, we examined the extent of infection by VSV, which depends on endocytosis, at varying doses of 3-MA and Wortmannin. The inhib-

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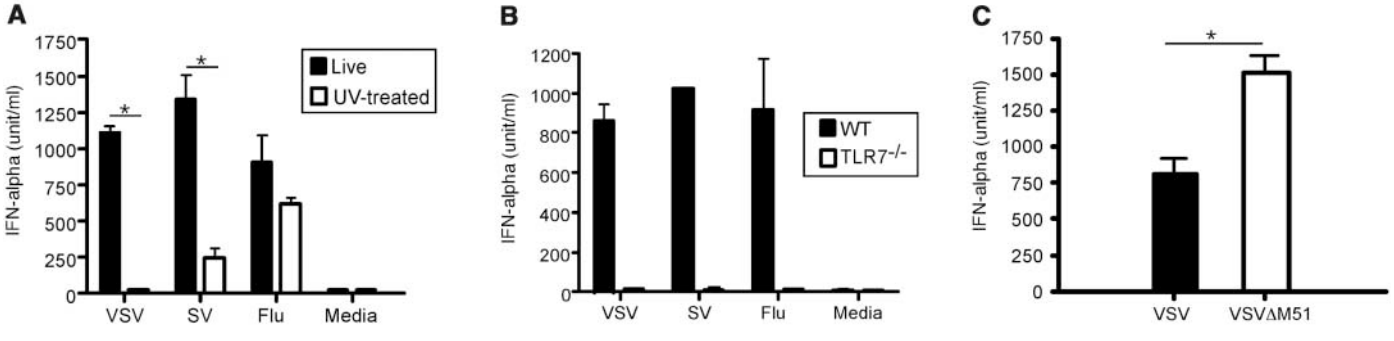

Fig. 1. Differential recognition of ssRNA viruses by pDCs. (**A** to **C**) WT or (B) $TLR7^{-/-}$ mouse bone marrow pDCs (2×10^5) were sorted by fluorescence-activated cell sorting (FACS) and stimulated for 18 hours with [(A) and (B)] live or UV-inactivated VSV (MOI = 10), SV (2.8×10^5 chicken embryo ID_{50}/ml), or influenza A/PR8 (MOI = 5); or (C) VSVΔM51 or VSV (both at MOI = 10). IFN-α levels were detected in culture supernatants by enzyme-linked immunosorbent assay. Asterisk, $P < 0.05$. The data are representative of three similar experiments.

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itors at the ranges effective in suppressing IFN-α production did not affect endocytosis or subsequent steps involved in VSV infection in pDCs or in non-pDCs, as determined by the VSV G-GFP expression (Fig. 3). These results indicated that 3-MA and Wortmannin specifically inhibited autophagosome formation without affecting viral entry and infection, and inhibited VSV recognition in pDCs.

To definitively demonstrate the role of autophagy in viral recognition, we used a genetic approach by examining pDCs isolated from mice deficient in ATG5, a gene essential for autophagosome formation (*27*). Because ATG5$^{-/-}$ mice are incapable of survival beyond 1 day of life (*27*), mature ATG5$^{-/-}$ pDCs were obtained from lethally irradiated WT mice reconstituted with liver and spleen cells from the ATG5$^{-/-}$ neonates. Upon full reconstitution with ATG5$^{-/-}$ cells (fig. S4A), all hematopoietic cells lacked the ATG gene (fig. S4B). Such mice had no obvious defects in survival or growth, and reconstituted hematopoietic compartments, including pDCs in the spleen, lymph nodes, and bone marrow (Fig. 4A), indicating that ATG5 is not required for the development of pDCs in vivo. To examine the requirement for autophagy in innate viral recognition, pDCs purified from the ATG5$^{+/+}$ or ATG5$^{-/-}$ chimeras were infected with VSV. After VSV infection, secretion of both IFN-α and interleukin-12 (IL-12) was diminished in the absence of ATG5 (Fig. 4, B and C). The diminished cytokine secretion could not be accounted for by the difference in the rate of infection (Fig. 4D) or survival of pDCs (fig. S6). In contrast, HSV-1 or CpG stimulation of ATG5$^{-/-}$ pDCs resulted in similar secretion of IL-12 and decreased levels of IFN-α (fig. S5). These data indicated that, although innate recognition of HSV-1 and CpG remains intact in ATG5$^{-/-}$ pDCs, the IFN-α production pathway is altered in the absence of ATG5. Although the mechanism is unknown, the selective defect in IFN-α, but not IL-12, production by the ATG5$^{-/-}$ pDCs might reflect defective sorting of the TLR downstream signaling components to the lysosomes. Further, to investigate the role of autophagy in antiviral responses by pDCs in vivo, ATG5$^{+/+}$ and ATG5$^{-/-}$ chimeric mice were infected with VSV. Systemic infection with VSV is known to target splenic pDCs and induce peak IFN-α production in the serum at around 12 hours (*17*). Since IL-12 and other inflammatory cytokines are secreted by non-pDCs in response to VSV infection, we measured serum IFN-α levels. Infection of ATG5$^{-/-}$ chimeric mice resulted in significantly reduced IFN-α responses compared with the ATG5$^{+/+}$ chimeras (Fig. 4E). Collectively, our data indicated that autophagy occurs constitutively in pDCs and is required for recognition of VSV, but not HSV-1, infection in pDCs. In addition, autophagy is required for pDC production of type I IFNs in vitro and in vivo.

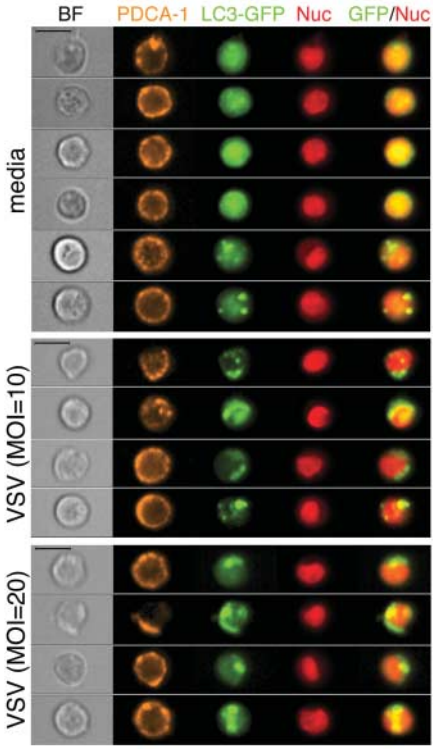

Fig. 2. Formation of autophagosomes in pDCs. PDCA-1^{+}CD11c^{+} cells obtained from LC3-GFP transgenic mice were analyzed for the presence of punctate GFP^{+} autophagosomes by MIFC 5 hours after incubation with VSV at the indicated MOIs or media control. The upper four cells in the media control represent cytosolic GFP^{+} cells, whereas the rest of the cells represent pDCs with GFP puncta. The columns represent bright-field (BF) imaging (gray) and PDCA-1 (orange), LC3-GFP (green), DRAQ5 nuclear (Nuc, red), and GFP/nuclear (overlay) stains. Scale bars, 10 μm. Each row is a composite of different micrographs of the same cells. These experiments have been repeated three times with similar results.

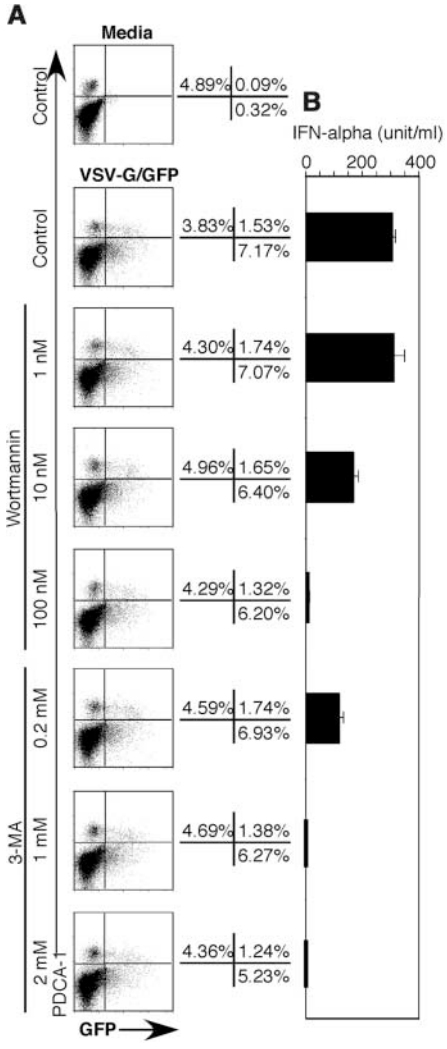

Fig. 3. Inhibitors of autophagy block VSV recognition. Bone marrow cells were stimulated with VSV-G/GFP (MOI = 10) in the presence of the indicated doses of inhibitors of autophagy, Wortmannin, and 3-MA, and (**A**) infection (GFP) and (**B**) IFN-α secretion were assessed. Neither infection of pDCs (upper right quadrant) nor infection of non-pDCs (lower right quadrant) was affected by these inhibitors at any doses examined, whereas IFN-α production was inhibited in a dose-dependent manner. The data are representative of three similar experiments.

Autophagy is an ancient pathway for homeostatic turnover of long-lived intracellular components and for nutrient acquisition during starvation. Recent studies indicated the importance of autophagosomes as an antimicrobial mechanism used to degrade and destroy cytosolic pathogens. Certain virus infections, such as HSV-1, are known to trigger autophagy (*28*) for the purposes of viral degradation (*29*), whereas poliovirus and rhinovirus usurp autophagosomal machinery for viral synthesis (*30*). Our study reveals the importance of autophagy in innate recognition of viral pathogens and IFN-α production in pDCs. The findings of this report highlight the differential requirement for recognition of diverse classes of viruses in pDCs. A broad spectrum of viruses and their infection cycles, from the virion-associated genomic nucleic acids in the endosomes to the replication of viral genomes in the cytosol, are detected by pDCs. Finally, immunological interventions designed to combat certain chronic viral infections might benefit from incorporating agents to promote autophagy for both viral degradation and innate recognition.

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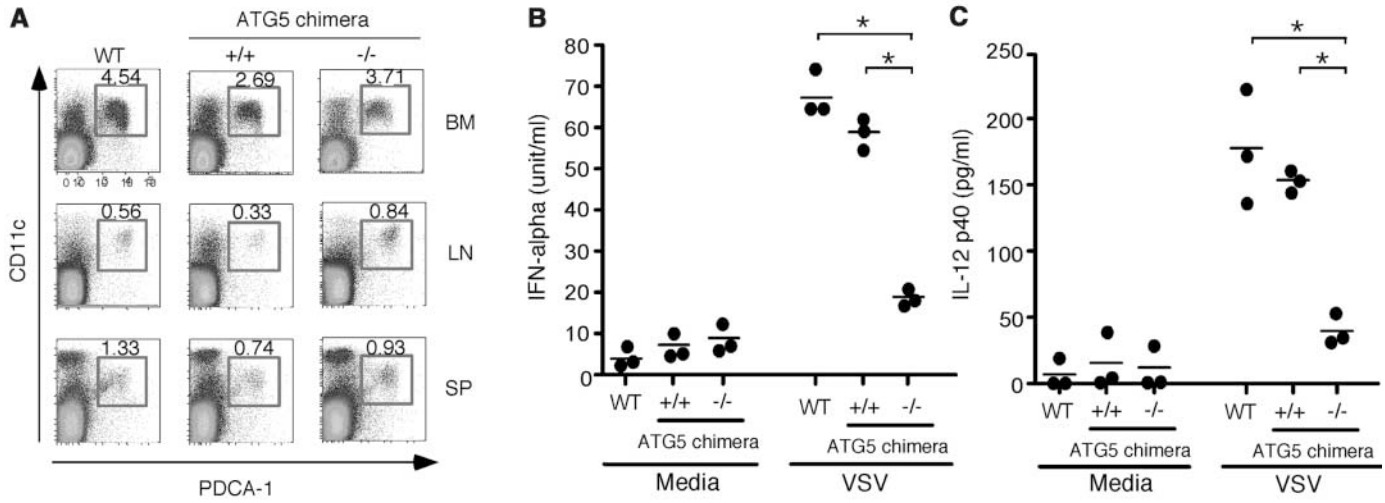

Fig. 4. Autophagy is required for VSV recognition. Liver and spleen cells from ATG5$^{+/-}$ × ATG5$^{+/-}$ pups were used to reconstitute lethally irradiated C57BL6 mice. (**A**) The presence of pDCs in the spleen (SP), lymph nodes (LN), and bone marrow (BM) of the reconstituted chimeric mice was assessed by flow cytometry. Secretion of (**B**) IFN-α and (**C**) IL-12 from 25,000 FACS-sorted pDCs from the indicated chimera was measured following VSV infection (MOI = 10). Each dot represents an individual mouse. (**D**) The extent of viral infection in the bone marrow cells obtained from the respective chimeric mice was assessed after infection with VSV-G/GFP at MOI = 5. (**E**) Serum IFN-α responses were measured from ATG5 chimeric mice after infection with 5×10^7 plaque-forming units of VSV intravenously at the indicated time points. Each dot represents an individual mouse. Asterisk, $P < 0.05$. The data are representative of five [(B) and (C)] or three [(A), (D), and (E)] similar experiments.

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31. We are grateful to J. Publicover and J. Rose for reagents and expertise, R. Medzhitov for critical review of the manuscript, W. Mothes for helpful discussions, and M. Zamora and G. Lyon for technical assistance. We thank P. J. Morrissey, B. Hall, and T. C. George of Amnis Corporation for analysis of samples using ImageStream technology. This work was supported by Public Health Service grants AI054359 and AI064705 (to A.I.) from NIH. J.M.L. was supported by the training grant AI07019 from NIH. H.K.L. was supported by the Ministry of Science and Technology of Korea. A.I. holds an Investigators in Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.

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30 October 2006; accepted 17 January 2007
Published online 1 February 2007;
10.1126/science.1136880
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