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Wiskott-Aldrich Syndrome Protein Is Needed for Vaccinia Virus Pathogenesis

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Smallpox, caused by variola virus, was a devastating disease in humans, but how the virus evolved a strategy to spread to tissue remains unknown. Through the use of microarrays, we identified the gene encoding the Wiskott-Aldrich syndrome protein (WASP), one of the five known WASP family members, which has been induced in the course of infection of human cells with different strains of vaccinia virus (VV) (S. Guerra, L. A. López-Fernández, A. Pascual-Montano, M. Muñoz, K. Harshman, and M. Esteban, J. Virol. 77:6493-6506, 2003; S. Guerra, L. A. López-Fernández, R. Conde, A. Pascual-Montano, K. Harshman, and M. Esteban, J. Virol. 78:5820-5834, 2004). In a mouse model, we evaluated the role of WASP in infection with VV, a close relative of variola virus. WASP$^{-/-}$ (KO) mice infected intranasally and intraperitoneally with VV showed reduced weight loss and mortality compared to wild-type (WT) mice. WASP expression correlated with VV replication in the ovaries but not in the liver or spleen. WT mouse macrophages express WASP but not N-WASP; after VV infection, WASP levels increase threefold. KO macrophages lack N-WASP expression and, when VV infected, are incapable of inducing actin tails and producing extracellular virus. These functions were rescued in KO macrophages after ectopic WASP expression. Overall, our findings demonstrate that WASP has a role in orthopoxvirus infections. Use of WASP proteins for virus spread via the actin tail provides a selective advantage for VV, and probably variola virus, dissemination to distant tissues.

Although smallpox has been eradicated, little is known of the mechanisms responsible for the high mortality rate due to this disease (9, 21). Since vaccination has been discontinued (9), recent acts of bioterrorism and the possible use of variola virus as a biological weapon raise major concerns about the safety of the human population. Second-generation vaccines are needed, as although the current vaccine has proved effective, adverse effects have been reported (21). Drugs must also be developed that can be used safely to resist a potential bioterrorist attack with variola virus. Further understanding of viral spreading mechanisms in the human host is thus needed. Using DNA microarrays of the Western Reserve (WR) and modified Ankara strains (14, 15), we identified a set of human genes that are induced selectively during the course of vaccinia virus (VV) infection. One of these genes encodes the Wiskott-Aldrich syndrome protein (WASP), a member of the structurally related protein family termed the WASP family. These proteins are implicated in numerous actin-regulated processes, including cell motility, epithelial cell adhesion, and pathogen F-actin tail formation (20, 34–36, 38). There are five known WASP family members, termed WASP, N-WASP, and three isoforms of WAVE (WASP family verprolin-homologous protein, also called Scar) (6).

VV is the only virus known to use actin-based motility to facilitate spreading between cells (7). Actin assembly on VV is mediated by the viral protein A36R (11, 12, 27, 28). VV replicates in the infected host cell cytoplasm in a complex process that leads to production of two distinct infectious forms (21), the intracellular mature virion (IMV) and the extracellular enveloped virion (EEV). The EEV is the form involved in virus dissemination through the host, contributing to distant virus spread, whereas the IMV is probably more efficient in local cell-to-cell transmission by cell fusion (3, 4, 30).

As infection of human cells with VV induces WASP (14, 15), and N-WASP is essential to the VV exit pathway in infected HeLa cells via actin tail formation (11, 12), we analyzed the role of WASP in in vivo infection. In WASP knockout (KO) mice, we found that VV requires WASP for virus spreading, with a correlation between loss of actin tail formation and a decrease in virulence. This in vivo study revealed a role for a cellular protein with a function in actin-regulated processes in VV infection.

MATERIALS AND METHODS

Cells, viruses, and infection conditions. VV wild-type (WT) WR was cultured in spinner HeLa cells, purified by banding on sucrose gradients as previously described (24), and titrated in BSC-40 African green monkey kidney cells. BSC-40 cells were maintained in Dulbecco's modified Eagle medium.

Viral inoculation of mice and sample collection. The origin of WASP$^{-/-}$ mice has been described previously (40). WASP$^{-/-}$ and control WT C57/BL-6 mice (8 to 12 weeks old) were immunized intraperitoneally (i.p.) with VV (10^8 PFU) in 200 μl of sterile phosphate-buffered saline (PBS) or intranasally (i.n.) with VV (10^6 PFU) in 20 μl of PBS. Animals were sacrificed at various times postinoculation, and the spleen, liver, ovaries, and lungs were removed, washed with sterile PBS, and stored at −70°C. Serum was obtained by retro-orbital bleeding before the inoculation and 3 to 10 days after the i.n. inoculation and was allowed to clot for 30 min at 37°C; after samples were left at 4°C overnight, they ere spun in a microcentrifuge and serum samples were removed and stored at −20°C.

Quantitative real-time RT-PCR. RNA (1 μg) was reverse transcribed with the Superscript first-strand synthesis system for reverse transcription (RT)-PCR (Invitrogen). A 1:40 dilution of the reaction mixture was used for quantitative PCR. The primers, probe set, and protocols (Assay-on-Demand; Applied Biosystems) were used for WASP and N-WASP amplification in accordance with the

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manufacturer's instructions. The mouse housekeeping gene for hypoxanthine ribosyltransferase was used for internal calibration. Thermal cycler conditions were 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C, followed by 1 min at 60°C. cDNA was amplified in a 96-well reaction plate. All samples were assayed in duplicate. Cycle threshold values were used to plot a standard curve in which the cycle threshold decreased in linear proportion to the log of the template copy number. Correlation values of standard curves were always >99%.

Immunohistochemistry. Organs were removed aseptically, frozen, and cryosectioned in accordance with standard procedures on a CM 1900 cryostat (Leica, Cambridge, United Kingdom). For immunostaining, 5-μm sections were hydrated, incubated with hyperimmune rabbit anti-VV serum (1/500 dilution), or anti-B5R (1/100 dilution), followed by ImmunoPure Elite ABC peroxidase staining (Pierce), and developed with 3′,3′-diaminobenzidine tetrahydrochloride (Sigma). Slices were visualized in a Leica DMRXA microscope, and images were captured with the DC100 imaging system (Leica).

Western blotting. Samples from individual mice were homogenized in lysis extraction buffer (50 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 10% NP-40, 1% sodium dodecyl sulfate [SDS]). Equal amounts of protein lysates were separated by SDS-polyacrylamide gel electrophoresis on 14 or 8% gels, transferred to nitrocellulose membranes, incubated with rabbit anti-VV antibody (1/1,000 dilution), and then incubated with peroxidase-conjugated secondary antibodies. Protein expression was detected by ECL (Amersham).

Anti-VV antibody measurement by enzyme-linked immunosorbent assay (ELISA). VV antigens used to coat 96-well flat-bottom plates at a concentration of 1 μg/ml consisted of viral proteins from extracts of chicken embryo fibroblast cells infected (1 PFU/cell) for 24 h with VV. Viral antigens were suspended in carbonate buffer (pH 9.6), plated at 50 μl/well, and incubated overnight at 4°C. Afterwards, the contents of the wells were removed and washed three times with PBS–0.05% Tween 20 (PBS-T), blocking buffer (PBS plus 10% fetal calf serum [FCS]) was added at 100 μl/well, and the plates were incubated for 1 h at 37°C. The plates were washed once with PBS-T, and samples diluted in blocking buffer were added in a volume of 100 μl/well and incubated for 1 h at 37°C. Plates were washed three times before the detection antibody was added. Peroxidase-conjugated goat anti-mouse immunoglobulin G antibodies were incubated at a dilution of 1:1,000 in blocking buffer for 1 h at 37°C. After the plates were washed three times with PBS-T, the wells were reacted with the peroxidase substrate o-phenylenediamine dihydrochloride (Sigma). After 10 to 15 min of incubation at room temperature, the reaction was stopped by adding 2 N H_2SO_4 and A_{492} was measured on a Multiskan Plus plate reader (Labsystems, Chicago, Ill.).

Isolation of WT and WASP$^{-/-}$ peritoneal macrophages. Resident peritoneal macrophages were isolated by peritoneal lavage with 10 ml of ice-cold PBS. Lavage fluid was centrifuged (500 × g, 5 min), and cells were cultured in petri dishes in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (3 h, 37°C, 5% CO_2). Nonadherent cells were removed by extensive washing with PBS.

Immunofluorescence analysis. HeLa cells and macrophages from WT and WASP$^{-/-}$ mice were cultured on coverslips and infected with VV (0.5 PFU/cell). At the indicated times postinfection (p.i.), cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized (when indicated) with 0.1% Triton X-100 in PBS (room temperature, 10 min). After washing, coverslips were blocked with PBS containing 20% bovine serum albumin. Cells were incubated (1 h, 37°C) with anti-A27L (mAbC3α14k) and anti-B5R viral protein (R19C2) antibodies. Coverslips were washed with PBS and incubated (1 h, 37°C) with fluorescein- or Texas red-conjugated, isotype-specific secondary antibodies. F-actin was stained with rhodamine-conjugated phalloidin (Molecular Probes). After washing with PBS, coverslips were mounted on microscope slides with Mowiol (Calbiochem). Images were captured with a Bio-Rad Radiance 2100 confocal laser microscope.

Ectopic WASP protein expression by retroviral transduction. WASP$^{-/-}$ macrophages were transduced with high-titer viral supernatants corresponding to the pRVWAS-ires-ΔNGFR and pRV-ires-ΔNGFR retroviral vectors obtained as previously described (1). High-titer viral supernatants were passed through a low-binding 0.22-μm-pore-size filter and supplemented with 20 to 25% complete Iscove's modified Dulbecco's medium (IMDM) plus 20% FCS. Protamine sulfate (5 μg/ml) was added, and cells were suspended in the supernatant and then divided among the wells of six-well plates, with a minimum of 1 ml of cell suspension with 5 × 10⁵ to 1 × 10⁶ cells/well. Plates were centrifuged (1,000 × g, 37°C, 1 h), and cells were collected, washed with fresh IMDM plus 5% FCS, and cultured (37°C, 95% humidity, 5% CO_2) for 3 to 4 days at 10⁶/ml in IMDM plus 5% FCS plus 10% conditioned medium (as a source of interleukin-3) plus 50 ng of stem cell factor per ml. Cells were collected on day 3 or 4, counted, stained, and sorted for ΔNGFR marker expression as previously described (1).

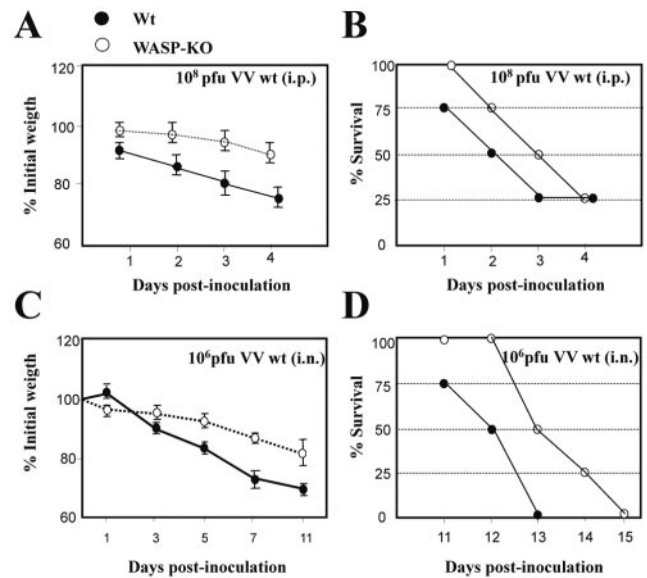FIG. 1. Virulence of VV in WT and WASP$^{-/-}$ mice. (A) Mice were inoculated i.p. with purified WT VV (10⁸ PFU per mouse). Mice were weighed individually daily, and the mean percent weight loss of each group (n = 9) was compared with the weight immediately prior to infection. (B) Survival rate after i.p. inoculation with VV (10⁸ PFU per mouse). Dead animals were scored daily and are represented as the percentage of surviving animals. (C) Mean percent weight loss of each group (n = 9) after WT VV administration (10⁶ PFU per mouse) by the i.n. route. (D) Survival rate after i.n. inoculation of WT VV (10⁶ PFU per mouse). $P \leq 0.01$ in all experiments.

RESULTS

VV virulence is reduced in WASP$^{-/-}$ mice. To analyze illness in WASP$^{-/-}$ mice, two groups of nine mice each were either not infected or infected i.p. (10⁸ PFU per mouse) with purified VV (24). Infected mice were scored for prominent indicators of viral pathogenesis (weight loss and death). After VV infection, most WT animals rapidly developed weight loss whereas WASP$^{-/-}$ mice did not (Fig. 1A). WT mice showed a poorer survival pattern than WASP$^{-/-}$ mice, although both groups died by day 5 (Fig. 1B). Since the upper respiratory tract is a natural route for variola virus entry and spread (10), we next inoculated mice i.n. with purified WT VV (10⁶ PFU per mouse). The WT mice show greater weight loss than the WASP$^{-/-}$ mice (Fig. 1C); similarly, WASP$^{-/-}$ mouse death was delayed (Fig. 1D). These findings indicate a role for WASP in VV virulence during in vivo infection when virus was administered by the systemic or mucosal route.

N-WASP expression does not compensate for the absence of WASP in KO mice. The expression of various WASP family members in the same organism suggests that each protein may perform similar functions but is differentially expressed in distinct organs. To explore the role of WASP and N-WASP in various organs, we used quantitative RT-PCR to define the expression levels of both genes in the ovaries, spleens, livers, and lungs of WT and WASP$^{-/-}$ mice (Fig. 2). WASP and N-WASP mRNA expression levels in the ovaries were used as a reference for the other organs. In WT mice, WASP expression was ~50-fold higher in the spleen and 8-fold higher in the liver than in the ovaries. WASP mRNA levels in the lungs were
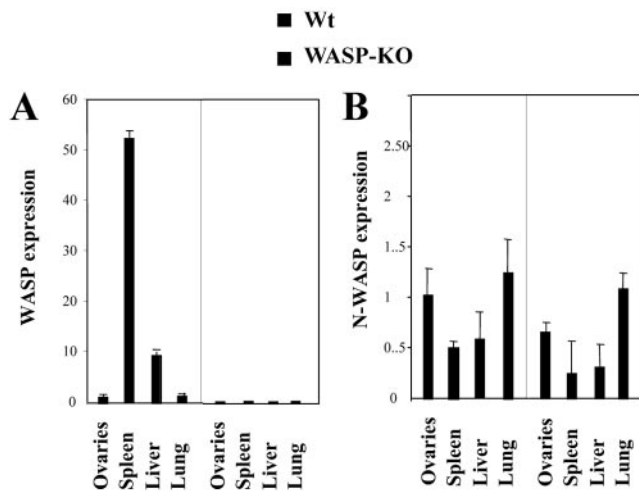
FIG. 2. Quantitative WASP and N-WASP mRNA expression in WT and WASP$^{-/-}$ mouse organs. RNA (1 µg) from the ovaries, spleen, liver, or lungs was reverse transcribed, and a 1:40 dilution of the RT reaction mixture was assayed in a quantitative RT-PCR for WASP (A) and N-WASP (B) mRNA expression. All samples were assayed in duplicate. The level of each gene in WT mouse ovaries was considered the baseline (= 1).

similar to those in the ovaries of WT mice (Fig. 2A). As predicted, no WASP mRNA was detected in WASP$^{-/-}$ mice. Our RT-PCR findings of liver WASP expression differ from those obtained by Northern blot analysis, probably because of the greater sensitivity of the RT-PCR technique (8).

In WT mice, N-WASP expression levels were 0.5-fold lower in the spleen and 0.6-fold lower in the liver, and the lungs showed N-WASP mRNA levels similar to those in the ovaries (Fig. 2B). In WASP$^{-/-}$ mice, N-WASP expression levels were slightly reduced in all of the organs analyzed compared to the levels in WT mouse ovaries. These results indicate that absence of WASP mRNA in WASP$^{-/-}$ mice is not compensated for by higher N-WASP gene expression levels.

VV multiplication is restricted in WASP$^{-/-}$ mouse spleens and livers but not in their ovaries. To determine the extent of virus multiplication, we used plaque assays to follow VV replication in target tissues of infected mice. WT and WASP$^{-/-}$ mice were inoculated with VV (10^8 PFU) by the i.p. route, and the extent of virus replication in various organs was measured by plaque assay. Similar viral titer generation kinetics were found in the ovaries and lungs of both groups (Fig. 3). In contrast, viral titers in the liver and spleen were markedly reduced in WASP$^{-/-}$ mice compared to those in WT mice at all of the times analyzed p.i. The results indicated that VV replication is restricted in the livers and spleens of WASP$^{-/-}$ mice.

To confirm the restriction of VV replication in different WASP$^{-/-}$ mouse organs, WASP$^{-/-}$ and WT mice were analyzed for the ability of VV to infect mouse tissues. Liver, spleen, lung, and ovary cryosections were stained immunohistochemically at 24 h p.i., and VV antigens were detected with a rabbit anti-VV or anti-B5R antibody on fixed sections. The virus-infected region in WT mouse livers and spleens was visualized by characteristic antigen staining. Staining for VV antigens was markedly reduced in WASP$^{-/-}$ mice, with few positive cells per section in the liver and spleen, reflecting differences

in virus propagation compared to that in WT mouse tissue. We found no differences in viral antigen staining of ovary and lung tissue samples from WT and WASP$^{-/-}$ mice. Analyses at different times p.i. gave similar results (data not shown).

This apparent decrease in virus production in some tissues of infected WASP$^{-/-}$ mice prompted us to study viral proteins synthesized in different organs. Viral proteins in liver, ovary, and spleen extracts were evaluated by Western blotting with rabbit anti-VV antibody. In tissue extracts obtained at various times after i.p. inoculation of VV, protein levels in WT mouse ovaries were similar to those in WASP$^{-/-}$ mouse ovaries (up to 120 h p.i.); viral proteins in the spleen and liver were markedly reduced in WASP$^{-/-}$ mice compared to those in WT mice (Fig. 4). The reduction in viral proteins in some tissues of infected WASP$^{-/-}$ mice is consistent with the lower virus yields in plaque assays and the reduced infection revealed by histological staining of tissue sections. These findings demonstrate that VV multiplication is restricted in the spleens and livers, but not in the ovaries, of infected WASP$^{-/-}$ mice.

To provide further evidence for a reduction of VV multiplication in WASP$^{-/-}$ mice, we inoculated four mice per group with two doses of VV (10^4 and 10^5 PFU) by the i.n. route and determined the viral titers in serum samples from individual animals at 3 and 7 days postinoculation. As shown in Fig. 5A more-than-sevenfold virus titer reduction was observed in WASP$^{-/-}$ versus WT mice. At later times (7 days) p.i., there was clearance of the virus in serum samples from both groups of animals, as no virus plaques were detected. As expected, the reduction in virus multiplication observed in KO mice was followed by a decrease in specific antibodies to VV, as confirmed by ELISA. Serum samples obtained at 25 days p.i. from the same WASP$^{-/-}$ mice inoculated as described above had three- to fourfold lower anti-VV antibody levels than did serum samples from WT mice (Fig. 5B).

EEV formation is inhibited in WASP$^{-/-}$ macrophages. The restricted VV multiplication in the WASP$^{-/-}$ mouse liver and spleen suggests that infected cells have active mechanisms to diminish virus replication. Since mouse peritoneal macrophages can be infected by VV after several days in culture (22), we infected macrophages isolated from WT mice with VV and
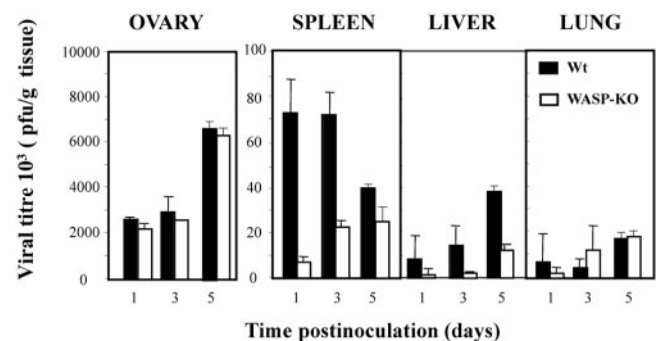

FIG. 3. Viral replication in target organs. Viral titers in target organs are shown. Mice were inoculated i.p. with WT VV (10^8 PFU per mouse). At the indicated times p.i., infectious virus in the ovaries, spleens, livers, and lungs of WT (black bars) and WASP$^{-/-}$ mice (white bars) was evaluated by plaque assay in BSC-40 cells. Results represent the mean ± the standard deviation for individual samples from four mice per day per group.
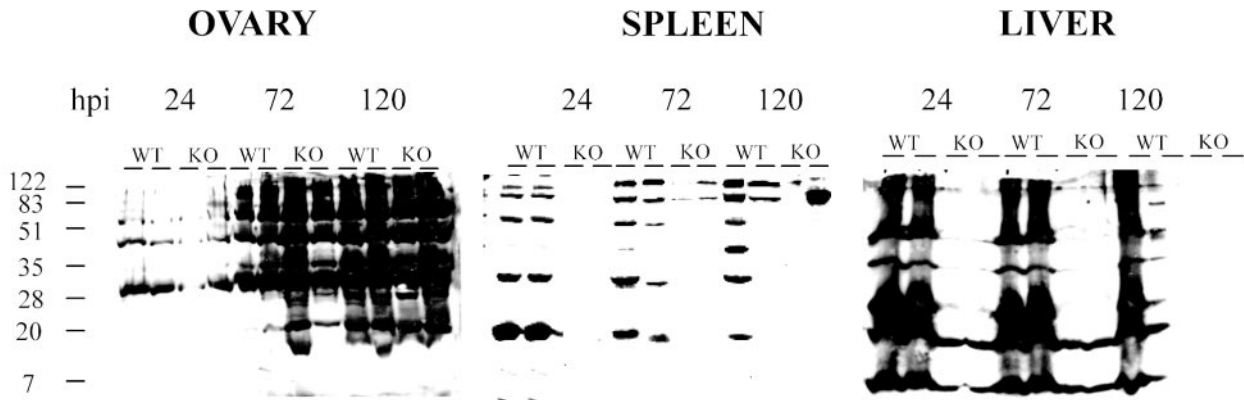
OVARY   SPLEEN   LIVER

FIG. 4. Viral protein synthesis in the ovaries, spleens, and livers of infected WT and WASP$^{-/-}$ mice. Samples from individual mice were homogenized in lysis extraction buffer (see Materials and Methods). Samples of protein lysates (10 µg) from two mice per group were separated by SDS–14% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Viral protein was determined by Western blotting with a rabbit anti-VV antibody. The values on the left are molecular sizes in kilodaltons.

analyzed WASP mRNA expression by real-time RT-PCR; WASP expression was threefold higher than in uninfected cells (data not shown). Macrophages do not express N-WASP (34). These data agree with those previously obtained with VV-infected HeLa cells (15), indicating that VV infection of macrophages increases WASP mRNA expression.

To further analyze the role of WASP in the VV cycle, we infected peritoneal macrophages from WT and WASP$^{-/-}$ mice with VV and analyzed the production of the two infections VV forms, IMVs and EEVs, by confocal microscopy. Under permeable and nonpermeable conditions, we monitored the presence of two viral proteins, one encoded by the *A27L* gene and located in the IMV membrane (25) and the other encoded by *B5R* and found in the outer membrane of EEVs (37). As predicted, under permeable conditions, intracellular staining for both proteins was observed as well-defined spots corresponding to the IMV and EEV forms, some of which colocalized (Fig. 6A). Under nonpermeable conditions, we visualized B5R protein on the surface of VV-infected WT macrophages but not on WASP$^{-/-}$ macrophages (Fig. 6A); again, some colocalization of IMVs and EEVs was found. Since EEVs should not react with the A27L antibody under nonpermeable conditions because of the extra membrane present in EEVs, the few spots observed are probably due to EEV membrane disruption or to residual input virions. Confocal microscopy analysis showed normal IMV production in the absence of WASP protein, whereas EEVs are not produced. The A27L monoclonal antibody, which neutralizes the IMV form (26), was used in virus titration from macrophage supernatants. Yields of extracellular virus were severely impaired in WASP$^{-/-}$ mice compared to those in WT mice (Fig. 6B). These experiments showed that the VV life cycle is blocked at the level of extracellular virus production in WASP$^{-/-}$ macrophages.

VV actin tail formation is impaired in WASP$^{-/-}$ macrophages. To analyze the effect of WASP deficiency in cells, we characterized VV actin tail formation. WASP$^{-/-}$ and WT macrophages were infected with VV (5 PFU/cell), and the actin cytoskeleton was developed by phalloidin staining at 24 h p.i. As a control, we used HeLa cells, in which actin tail formation

after VV infection has been well analyzed. VV-infected WT macrophages had characteristic actin tails (92% of the infected cells had tails; Fig. 7A); in contrast, no actin tails were detected in WASP$^{-/-}$ cells. The presence of VV at the tip of the actin tails was monitored with the A27L-specific antibody. Confocal images (Z series) of infected macrophages from WT mice showed viral particle localization in the cytoplasm and in actin tails. In WASP$^{-/-}$ mouse macrophages, however, only IMV forms were observed in the cytoplasm (Fig. 7A).

WASP expression is required for VV actin tail formation in macrophages. To determine whether WASP expression could reverse the defect in VV actin tail formation in WASP$^{-/-}$
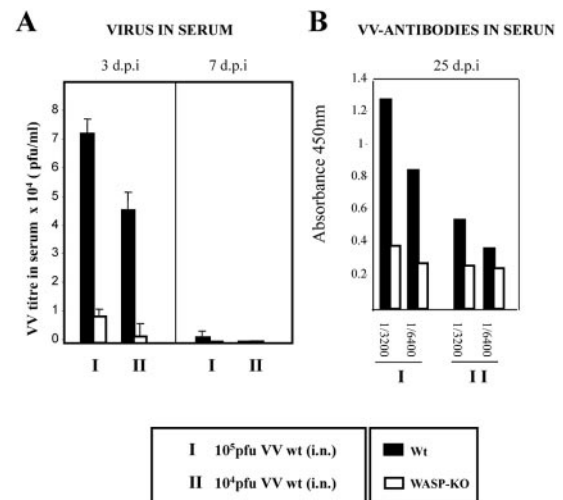

FIG. 5. Viral titers and antibody levels in serum samples from VV-infected WT and WASP$^{-/-}$ mice. WT and KO mice were inoculated with WT VV by the i.n. route at 10^5 (I) or 10^4 (II) PFU per mouse. (A) Viral titers in serum samples collected at 3 and 7 days p.i. (d.p.i.) were determined by plaque assay in BSC-40 cells. Results represent the mean ± the standard deviation of individual serum samples from four mice per day per group. (B) Viral antibodies detected by ELISA from serum samples collected at 25 days p.i. Results represent the mean ± the standard deviation of pooled samples from four mice. The serum sample dilutions are indicated.

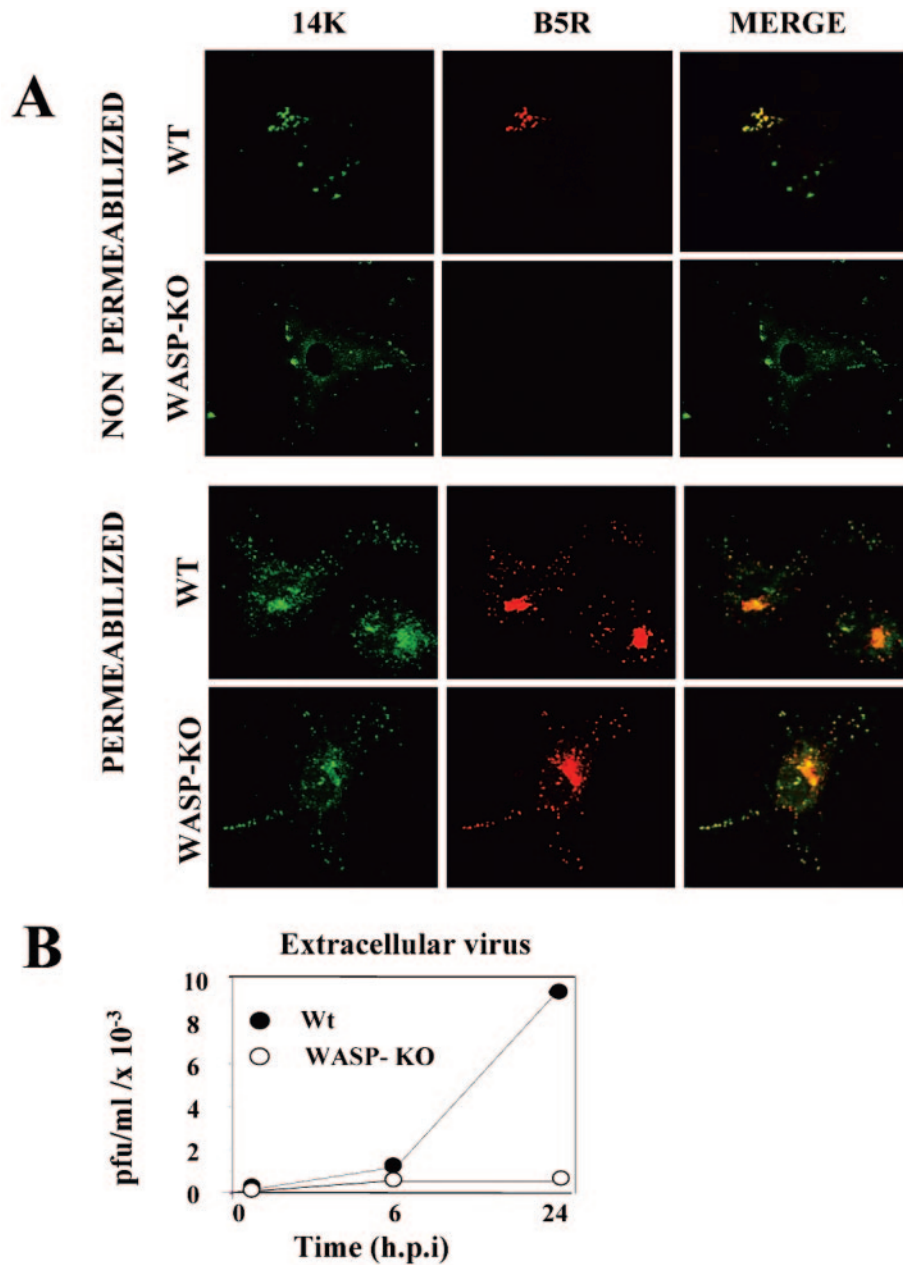FIG. 6. VV maturation is restricted in WASP$^{-/-}$ macrophages. (A) Immunofluorescence microscopy showing the IMV and EEV forms in VV-infected macrophages from WT and WASP$^{-/-}$ mice. Peritoneal macrophages from WT and WASP$^{-/-}$ mice were VV infected for 16 h and double labeled with anti-A27L (to detect EEVs) and anti-B5R (to detect EEVs) antibodies, followed by the appropriate fluorescent secondary antibody. Cells were visualized by confocal immunofluorescence microscopy. (B) Time course of IMV and EEV production in WT and WASP$^{-/-}$ macrophages. Cultures were inoculated at 1 PFU/cell, and release of infectious particles into the medium was determined by assay of plaque formation on BSC-40 cells. Samples were neutralized for EEVs with anti-A27L antibody (ascites; 1:1,000 dilution). Each point represents the average of two independent experiments.

macrophages, we tested whether ectopic WASP expression rescued tail formation with pRVWAS-ires-ΔNGFR, an optimized retroviral vector that expresses WASP. Confocal microscopy showed that retroviral transduction of the *WASP* gene in WASP$^{-/-}$ macrophages resulted in efficient rescue of VV actin tail formation, whereas WASP$^{-/-}$ macrophages infected with the pRV-ires-ΔNGFR control vector did not form VV actin tails (Fig. 7B). These findings demonstrate that WASP is essential for the actin-based movements of VV in macrophages.

DISCUSSION

Natural poxvirus infections are generally characterized by virus dissemination to various tissues. Virus enters the host by several routes, including i.n., oral, or epidermal, in several discrete stages. After virus entry, multiplication takes place at or near the inoculation site. Primary viremia leads to infection of the liver and spleen, after which a second viremia results in involvement of other organs, including the skin, intestine, and
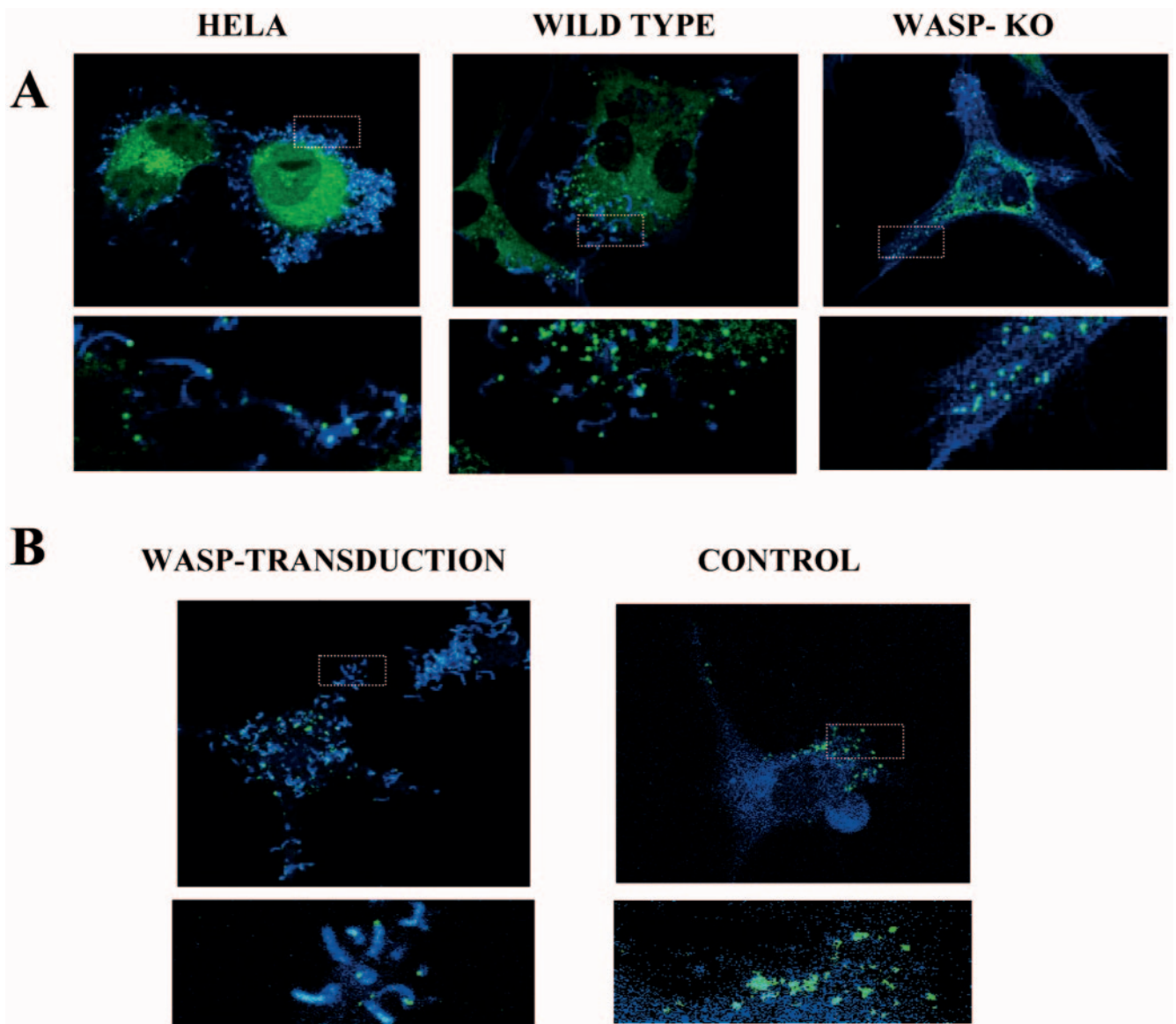
FIG. 7. WASP is necessary for actin tail formation. (A) Actin tail formation on VV in macrophages requires WASP expression. WT and WASP$^{-/-}$ peritoneal macrophages were VV infected (5 PFU/cell) and phalloidin stained after 24 h for actin tails (blue). Location of VV particles was developed by anti-A27L staining, followed by a fluorescent secondary antibody (green). Infection induced in HeLa cells was used as an internal control. The upper images show representative fields (×73 magnification); the lower images show the indicated areas at higher magnification. (B) Ectopic *WASP* expression in WASP$^{-/-}$ peritoneal macrophages rescues VV-dependent actin tail formation. WASP$^{-/-}$ peritoneal macrophages were transduced with pRVWASP-ires-ΔNGFR (for WASP expression) or control pRV-ires-ΔNGFR retroviral vectors (see Materials and Methods). After cell sorting for the ΔNGFR marker, cells were stained as for panel A, with phalloidin in blue and VV in red.

lungs, leading to severe illness. Variola major virus strain mortality during smallpox epidemics was reported as up to 30% (10).

The specific host response, both humoral and cellular, is a major factor in the resolution of virus infection (2, 5, 21). Early in the infection process, interferon, the alternative pathway of complement activation, inflammatory cells, and natural killer cells contribute to slowing the spread of infection. Poxviruses have acquired a large number of genes with which to sabotage key immune response components, including production of interferon, chemokines, and inflammatory cytokines, as well as the activity of cytotoxic T lymphocytes, natural killer cells, and

complement (2). Poxviruses also manipulate a variety of intracellular signal transduction pathways to their advantage. In vitro, VV is reported to usurp the tyrosine kinase receptor signaling pathways that control actin polymerization, facilitating actin-based motility to spread virus between cells (11). The mechanism of VV actin tail formation has been studied intensively. Although the A33R, A34R, and A36R VV membrane proteins are all required for actin tail formation, only A36R has a direct role in this process (16, 27, 39). Phosphorylation of A36R Tyr 112 and Tyr 132 by an Scr family kinase that probably resides in the plasma membrane results in recruitment of the adaptor proteins Nck, WIP, N-WASP, and Grb2, leading

to activation of the Arp2/3 complex and nucleation of actin polymerization (11, 12, 29). A36R deletion leads to loss of actin tail formation, reduction in extracellular virus release, and decreased virulence (23).

In previous studies with HeLa cells, we described upregulation of the expression of the genes for different WASP family members after infection with the WR and modified Ankara VV strains (14, 15). As there is evidence of a role for N-WASP in actin tail formation and the use of actin tails for VV movement in cultured cells (11), it was important to establish whether the WASP family members are necessary for virus spread and pathogenesis in an animal model system. N-WASP$^{-/-}$ is an embryonic lethal mutation in mice, but analysis of cultured fibroblasts from N-WASP$^{-/-}$ embryos indicated impairment of actin tail formation (19, 32). WAVE$^{-/-}$ mice were recently generated and show sensorimotor defects (33).

To analyze the in vivo effect of WASP in VV replication, we infected WASP$^{-/-}$ mice and analyzed pathogenesis in this murine system. Human and mouse WASP-deficient T cells are unable to cap CD3 in response to T-cell receptor signaling (13, 31), whereas WASP null bone marrow macrophages show impaired phagocytosis (17, 18) and chemotaxis (41). Considering the anomalies in the immune response due to the absence of *WASP* genes, these mice would be expected to be more susceptible to VV infection. However, we found significantly reduced VV virulence in i.p. inoculated WASP$^{-/-}$ mice (Fig. 1). When inoculated i.n., WT mice showed more severe weight loss and mortality than WASP$^{-/-}$ mice (Fig. 1). Moreover, WASP$^{-/-}$ mice showed reduced virus production in the spleen and liver, but not in the ovaries and lungs, as confirmed by virus titration and Western blotting and immunohistochemical analyses of tissue sections.

The differences in virus replication between WASP$^{-/-}$ mouse tissues may be due to the presence or absence of other WASP family members. In a quantitative RT-PCR analysis of WT mice, we found that the WASP mRNA level was ~50-fold higher in the spleen and 8-fold higher in the liver than in the ovaries. N-WASP mRNA levels were similar in WASP$^{-/-}$ and WT mice, with lower expression in the liver and spleen than that in the ovaries. These data indicated that the lack of WASP in WASP$^{-/-}$ mice was not counterbalanced by an increase in N-WASP expression, possibly explaining the limited ability of VV to spread from the liver and spleen in these mice. WASP$^{-/-}$ mice eventually died after high-dose VV infection by the i.p. or i.n. route, an effect that may result from normal amounts of virus produced in other tissues, such as the ovaries and lungs. A decrease in virus production should lead to a reduction in antibody levels, as observed in VV-infected WASP$^{-/-}$ mice compared with WT mice. It should be noted that production of T-cell-dependent and -independent antibodies is normal in WASP$^{-/-}$ mice (31). Thus, a reduction of virus infection in the spleens and livers of WASP$^{-/-}$ mice will contribute to a decrease in the humoral immune response to VV antigens. WASP, but not N-WASP, is expressed in WT mouse peritoneal macrophages, and WASP expression is increased after VV infection. These results indicated the importance of WASP expression in macrophages for VV spreading and suggested that VV could enhance WASP expression to its own benefit. Neither WASP nor N-WASP mRNA is expressed in WASP$^{-/-}$ mouse macrophages, and extracellular virus for-

mation is impaired. We linked this blockade in extracellular virus production in VV-infected WASP$^{-/-}$ macrophages to the absence of actin tail formation. Although WASP deficiency leads to loss of actin tail formation, this phenotype was rescued when cells were infected with a retroviral vector expressing the *WASP* gene. We conclude that cellular WASP expression is essential for in vivo VV replication. VV has developed a dual mechanism with the WASP or N-WASP protein in the assembly of actin tails to ensure tissue-specific virus dissemination in the host.

There is >94% sequence conservation between VV A36R and its orthologue A39R in variola virus, the causative agent of smallpox. In addition, Tyr 112 and Tyr 132 are conserved and are essential for N-WASP recruitment, suggesting that variola virus also uses actin-based motility to spread between cells. This study is the first demonstration that VV requires WASP family members for virus spread and pathogenesis. Targeting in vivo actin tail formation with specific drugs would prevent VV, and possibly variola virus infection. This could be an effective strategy to control smallpox and other poxvirus infections.

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