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Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus

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The innate immune system limits viral replication via type I interferon and also induces the presentation of viral antigens to cells of the adaptive immune response. Using infection of mice with vesicular stomatitis virus, we analyzed how the innate immune system inhibits viral propagation but still allows the presentation of antigen to cells of the adaptive immune response. We found that expression of the gene encoding the inhibitory protein Usp18 in metallophilic macrophages led to lower type I interferon responsiveness, thereby allowing locally restricted replication of virus. This was essential for the induction of adaptive antiviral immune responses and, therefore, for preventing the fatal outcome of infection. In conclusion, we found that enforced viral replication in marginal zone macrophages was an immunological mechanism that ensured the production of sufficient antigen for effective activation of the adaptive immune response.

The innate immune system limits viral replication during systemic infection via the production of type I interferon. Lack of the receptor for type I interferon promotes viral replication and leads to viral persistence and the death of virus-infected animals[1,2]. Macrophages are key participants during type I interferon–mediated suppression of virus[3–6]. With their potent phagocytic ability, macrophages act as a first line of defense against pathogens entering tissues. Splenic red pulp, marginal zone macrophages and Kupffer cells are associated with the endothelium and can capture antigens from the blood vessel lumen[5,7,8]. They clear the blood of immune complexes and complex particles of high molecular mass, including virus particles[7–9]. Phagocytosis of the virus is followed by suppression of viral replication in a type I interferon–dependent manner[4,5]. This mechanism suppresses the spread of virus but limits the amount of antigen available for priming of the adaptive immune system.

Binding of type I interferon leads to dimerization of type I interferon receptors, which phosphorylates and activates the tyrosine kinases Tyk2 and Jak1. These kinases recruit the transcription factors STAT1 and STAT2. The STAT proteins form homodimers and heterodimers that can translocate to the nucleus and initiate the transcription of type I interferon–stimulated genes[10]. More than 300 genes are regulated by interferon-α (IFN-α) or IFN-β[11]. Examples of genes that exert antiviral activity are *Isg15* (interferon-stimulated gene 15), *Mx1* (myxovirus resistance 1), *Oas1* (2′5′-oligoadenylate synthetase 1), *Eif2ak2* (protein kinase R) and *Rnasel* (ref. 2). A potent inhibitor of this signaling cascade is Usp18 (UBP43)[12], which binds to the Jak1-binding site of the receptor for IFN-α and IFN-β and inhibits its phosphorylation[12,13]. Accordingly, Usp18 deficiency in mice leads to high type I interferon sensitivity and limited viral replication after viral infection[12]. However, it remains unclear how Usp18 expression in various cell types influences the overall immune response.

In addition to controlling viral replication, cells of the innate immune response initiate the priming of cells of the adaptive immune response. Priming usually occurs in secondary lymphoid organs, such as lymph nodes and spleen. Antigens from the lymph can be captured by cells in the subcapsular zone of the lymph node, whereas antigens from the blood are filtered in the spleen by macrophages from the red pulp and the marginal zone[14]. Metallophilic macrophages in the marginal zone are characterized by the expression of the C-type lectin CD169 (siglec-1)[15]. $CD169^+$ macrophages present captured virus antigen directly to B cells[16]. Fibroblastic reticular cells form conduits together with type I and type III collagen[17]. These channels

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reach into the marginal zone of the spleen and can distribute antigens from metallophilic macrophages through the splenic white pulp[18]. Resident dendritic cells (DCs) take up these antigens and present them to T cells[18].

The amount of presented antigen limits the adaptive immune response[19–23]. *In vitro* studies have shown that at least ten complexes of peptide and major histocompatibility complex are required for the formation of an immunological synapse between DCs and T cells[24]. *In vivo* induction of T cell proliferation in the lymph node requires DCs with around 2×10^4 complexes of peptide and major histocompatibility complex[25]. Low-affinity T cell antigen receptors require a larger antigen dose than do high-affinity T cell antigen receptors[26]. Such findings suggest that a larger antigen dose improves T cell immunity. Consistently, application of a low dose of inactivated, replication-incompetent virus leads to limited induction of neutralizing antibodies, whereas replicating virus leads to a strong antibody response[27]. However, the existence of a specific compartment that would promote viral replication and increase the presented antigen to improve the adaptive immune response remains unknown.

In this study, using mouse vesicular stomatitis virus (VSV), we found that CD169$^+$ macrophages in the marginal zone of the spleen formed a compartment of enhanced viral replication. Early after infection, red pulp macrophages in the spleen and Kupffer cells in the liver captured virus and effectively suppressed viral replication in a type I interferon–dependent manner. In contrast, CD169$^+$ macrophages captured virus but did not respond to type I interferon and thus allowed anatomically restricted viral replication in the splenic marginal zone. Enhanced replication in CD169$^+$ macrophages was linked to overexpression of *Usp18*. *Usp18*$^{-/-}$ mice had little viral replication in CD169$^+$ macrophages. The lack of either CD169$^+$ cells or *Usp18* led to impaired and delayed adaptive immunity to VSV. The delayed induction of antibodies in *Usp18*$^{-/-}$ mice led to the distribution of virus to neuronal tissue and to the death of mice. Together our findings suggest that enforced viral replication in CD169$^+$ macrophages promotes the adaptive immune response and guarantees survival after infection with a prototypic cytopathic virus.

RESULTS

CD169$^+$ metallophilic macrophages allow viral replication

To determine how the reticuloendothelial system inhibits the spread of systemic virus while presenting sufficient amounts of antigen to cells of the adaptive immune response, we first analyzed the virus-capturing ability of macrophages after intravenous infection. We treated mice with clodronate liposomes to deplete them of macrophages before VSV infection[16]. Replicating virus was detectable in the blood for more than 60 min without macrophages (**Fig. 1a**). This suggested that macrophages have a crucial role in virus uptake. In mice lacking spleen and lymph nodes (alymphoplasia (aly/aly) mice given splenectomy)[28], virus inoculate was taken up efficiently (**Fig. 1a**), which suggested that without macrophages in the spleen, macrophages from other tissues, such as liver Kupffer cells, sufficiently took up virus. This indicated that macrophages in lymphoid and nonlymphoid compartments participated in systemic virus clearance.

We next analyzed viral production after uptake by macrophages. We detected replicating virus 1 h and 7 h after infection in the spleen, whereas we detected no virus in other tissues (**Fig. 1b**). To analyze viral replication without macrophages after intravenous infection, we depleted mice of macrophages by injection of clodronate liposomes and noticed enhanced replication of VSV in several organs (**Fig. 1c**). Accordingly, mice depleted of macrophages were highly susceptible to VSV infection (**Fig. 1d**). These observations indicated that macrophages were crucial for the control of VSV replication in nonlymphoid tissue. To test the hypothesis that type I interferon suppresses VSV replication in macrophages, we infected mice lacking the IFN-α receptor (*Ifnar1*$^{-/-}$ mice; called '*Ifnar*$^{-/-}$ mice' here) with VSV intravenously. *Ifnar*$^{-/-}$ mice had excessive VSV replication in the liver, lung, kidney, spleen, thymus and brain, whereas wild-type mice had infectious VSV only in the spleen (**Fig. 1e**). These results showed that macrophages in nonlymphoid tissues captured the virus and suppressed its replication in a type I interferon–dependent manner. In 'macrophage-competent' wild-type mice, we detected infectious virus particles only in the spleen (**Fig. 1b**). This suggested that type I interferon–dependent suppression of viral replication in macrophages was limited in the spleen.

To investigate the mechanism of the enhanced viral replication in the spleen, we next assessed viral protein expression by immunohistological analysis of spleen and liver tissue 7 h after VSV infection. We detected VSV glycoprotein in the spleen but not in the liver (**Fig. 1f**). Immunohistological analysis showed that CD169$^+$ metallophilic macrophages allowed expression of VSV proteins (**Fig. 1g**). After administration of ultraviolet (UV) light–inactivated VSV, we did not detect glycoprotein expression in CD169$^+$ cells (**Fig. 1h**), which supported the hypothesis that VSV actively replicates in these cells. To address the contribution of CD169$^+$ macrophages to VSV replication, we used mice that express the diphtheria toxin receptor under control of the *Cd169* promoter (CD169-DTR mice)[29]. Treatment with diphtheria toxin depletes these mice of CD169$^+$ macrophages. We generated bone marrow chimeras by transferring bone marrow from control wild-type or CD169-DTR mice into irradiated wild-type recipients. After reconstitution, we treated chimeric mice with diphtheria toxin and infected them with VSV intravenously. Administration of diphtheria toxin depleted mice of CD169$^+$ macrophages in the spleen (**Fig. 1i**). Immunohistological staining of VSV in the marginal zone was not detectable in mice depleted of CD169$^+$ macrophage but was detectable in diphtheria-treated wild-type chimeric mice (**Fig. 1i**), which suggested that CD169$^+$ macrophages in the marginal zone enforced viral replication, whereas conventional macrophages in the red pulp suppressed virus propagation. Spleen sections from *Ifnar*$^{-/-}$ mice showed VSV replication in F4/80$^+$ red pulp macrophages and in CD169$^+$ macrophages (**Fig. 1j**). Spleen sections from wild-type mice showed VSV replication only in CD169$^+$ macrophages (**Fig. 1j**). In agreement with that result, *Ifnar*$^{-/-}$ Kupffer cells expressed VSV glycoprotein in the liver, but wild-type Kupffer cells did not (**Fig. 1j**). These findings suggested that in wild-type mice, Kupffer cells and red pulp macrophages suppressed viral replication in a type I interferon–dependent manner, whereas CD169$^+$ macrophages were resistant to the effects of type I interferon. We conclude that early uptake of virus by macrophages followed by type I interferon–mediated suppression of replication is essential for the control of VSV infection and the survival of mice. CD169$^+$ macrophages, however, allow viral replication in the presence of type I interferon.

Usp18 in CD169$^+$ macrophages enhances viral replication

We next investigated the mechanism underlying the enforced viral replication in CD169$^+$ macrophages. Because Usp18 inhibits the type I interferon signaling pathway, competing with Jak1 (refs. 12,13), we hypothesized that *Usp18* expression might allow VSV replication in CD169$^+$ macrophages. We did *in situ* hybridization of *Usp18* during VSV infection. *Usp18* was upregulated during infection in the lymph follicle and the marginal zone but not in the red pulp (**Supplementary Fig. 1**). To directly compare the expression of *Usp18* in CD169$^+$ and F4/80$^+$ macrophages, we sorted F4/80$^+$ and CD169$^+$ macrophages by magnetic-activated cell sorting and flow cytometry

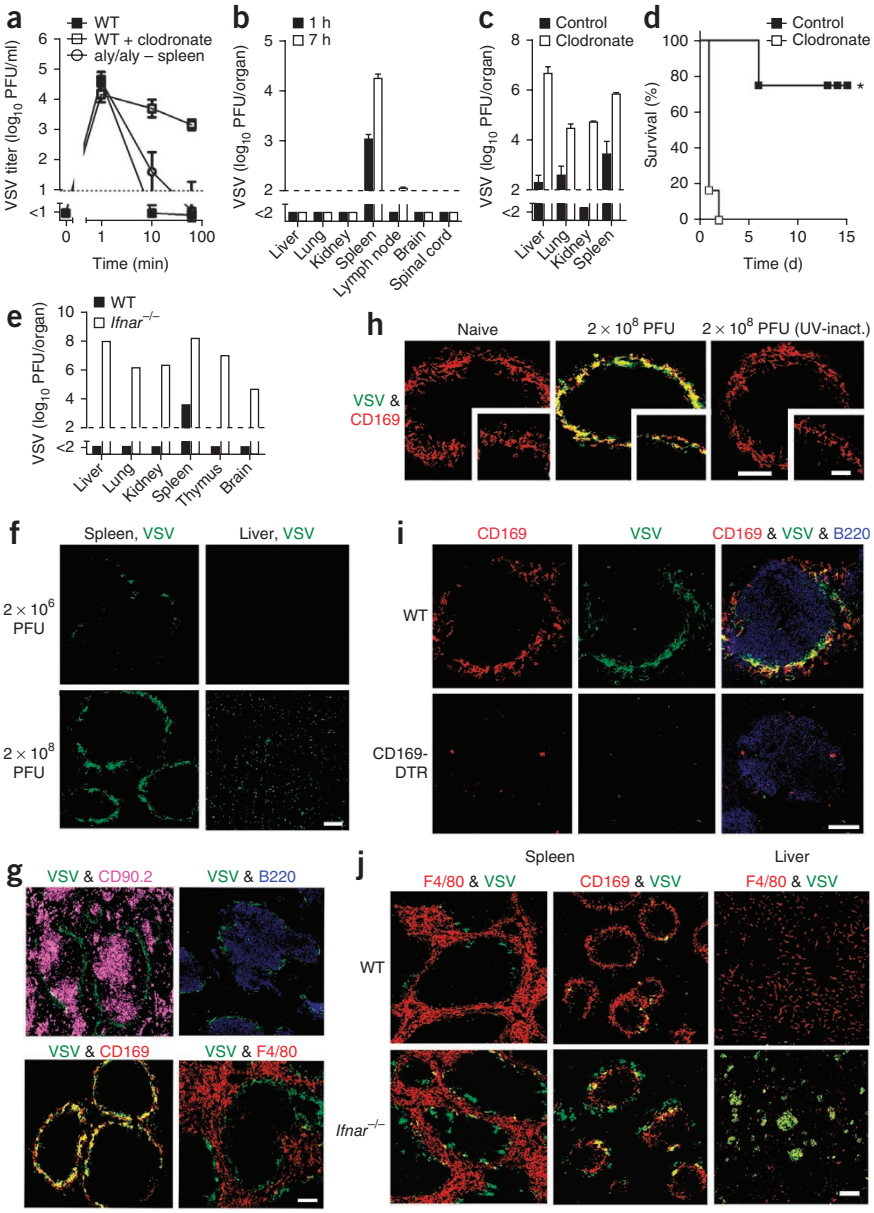

Figure 1 CD169$^+$ metallophilic macrophages allow viral replication in the spleen, but red pulp macrophages and Kupffer cells do not. (**a**) Blood titers of VSV in alymphoplasia mice given splenectomy (aly/aly – spleen), C57BL/6 mice depleted of macrophages (WT + clodronate), and control C57BL/6 wild-type mice (WT; $n = 4$ per group) injected intravenously with 2×10^6 PFU of VSV. (**b**) VSV titers of C57BL/6 mice ($n = 5$) infected intravenously with 2×10^6 PFU of VSV. (**c**,**d**) Viral replication, measured after 16 h (**c**), and survival (**d**) of mice depleted of macrophages (clodronate) or injected with empty liposome (control) and infected intravenously 1 d later with 2×10^7 PFU of VSV ($n = 5$ (**c**) or 4–6 (**d**) mice per group). (**e**) VSV titers of wild-type and *Ifnar*$^{-/-}$ mice ($n = 2$ per group) 16 h after intravenous infection with 2×10^6 PFU of VSV. (**f**) Immunofluorescence in spleen and liver sections from C57BL/6 mice ($n = 3$) 7 h after intravenous infection with 2×10^6 PFU or 2×10^8 PFU, stained for VSV glycoprotein (green). (**g**) Immunofluorescence of spleen sections from C57BL/6 mice ($n = 3$) 7 h after intravenous infection with 2×10^8 PFU of VSV, stained for VSV glycoprotein (green), B220 (B cells; blue), CD90.2 (T cells; magenta), F4/80 (macrophages; red) and CD169 (metallophilic macrophages; red). (**h**) Immunofluorescence of spleen sections from C57BL/6 mice ($n = 3$ per group) 7 h after intravenous infection with 2×10^8 PFU of active VSV or 2×10^8 PFU of UV light–inactivated VSV, stained for VSV glycoprotein (green) and CD169 (red). (**i**) Immunofluorescence of spleen sections from C57BL/6 bone marrow chimeras ($n = 4$ per group) reconstituted with CD169-DTR or wild-type bone marrow cells as control, treated intraperitoneally with diphtheria toxin and infected intravenously 3 d later with 2×10^8 PFU of VSV, stained for VSV glycoprotein (green) and CD169 (red) 7 h after infection. (**j**) Immunofluorescence of spleen and liver sections from wild-type and *Ifnar*$^{-/-}$ mice ($n = 3$ per genotype) 16 h after intravenous infection with 2×10^6 PFU of VSV, stained for F4/80 (red), CD169 (red) and VSV glycoprotein (green). Scale bars, 100 μm (main images) or 20 μm (insets). *$P < 0.01$ (Student's *t*-test). Data are representative of two (**a**–**d**,**i**) or one of two (**e**–**h**, **j**) experiments (mean ± s.e.m. (**a**) or mean and s.e.m. (**b**,**c**)).

(**Supplementary Fig. 2**) and measured *Usp18* mRNA expression by quantitative RT-PCR. The expression of *Usp18* was significantly higher in CD169$^+$ cells than in CD169$^-$F4/80$^+$ macrophages (**Fig. 2a**). To further confirm enhanced *Usp18* expression in CD169$^+$ macrophages, we did laser-capture microdissection of cells from the marginal zone and red pulp (**Supplementary Fig. 3**). Cells isolated from the marginal zone had higher expression of *Usp18* than did cells from the red pulp (**Fig. 2b**). To determine whether differences in *Usp18* expression can directly affect interferon-dependent VSV suppression, we transfected HeLa human cervical cancer cells with increasing concentrations of *Usp18* expression plasmids[13]. In addition we used treatment with IFN-α2 because Hela cells respond well to this interferon subtype. In the presence of IFN-α2, *Usp18* expression enhanced VSV replication in a dose-dependent manner (**Fig. 2c**). To further determine whether the expression of *Usp18* in CD169$^+$ macrophages was responsible for enhanced VSV replication *in vivo*, we infected *Usp18*$^{-/-}$ mice with VSV intravenously. Immunohistological analysis showed that the distribution of CD169$^+$ macrophages was similar in *Usp18*$^{-/-}$ mice and wild-type mice. However, we detected little expression of viral proteins in CD169$^+$ macrophages from *Usp18*$^{-/-}$ mice (**Fig. 2d**). VSV replication in the spleen of *Usp18*$^{-/-}$ mice was significantly lower than that in wild-type mice (**Fig. 2e**). Together these results indicated that expression of *Usp18* in CD169$^+$ macrophages in the lymph follicle was essential for the enforced replication of VSV.

VSV replication promotes adaptive immunity

Next we analyzed the physiological role of VSV replication in metallophilic macrophages. Intravenous immunization with inactive (UV light–inactivated) virus leads to a limited adaptive immune response[27,30]. We hypothesized that enforced viral replication in CD169$^+$ macrophages could promote adaptive immunity. Inactivation of VSV replication by UV light limited the

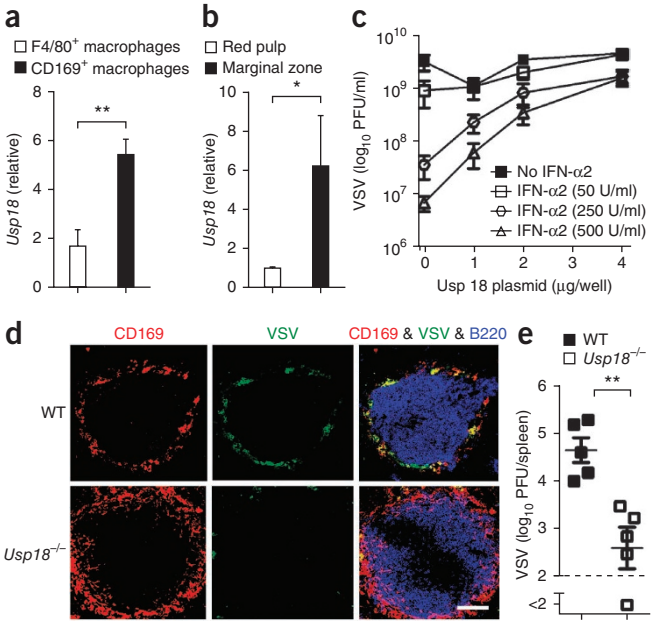

Figure 2 Expression of *Usp18* in CD169$^+$ cells is responsible for enhanced viral replication. (**a**) Expression of *Usp18* mRNA in CD169$^+$ macrophages and F4/80$^+$ macrophages sorted from naive C57BL/6 mice ($n = 4$–5), presented relative to the expression of *Gapdh* (encoding glyceraldehyde phosphate dehydrogenase). (**b**) Expression of *Usp18* mRNA in marginal zone tissue and red pulp tissue isolated by laser-capture microdissection from spleen sections from naive C57BL/6 mice ($n = 4$), presented relative to *Gapdh* expression. (**c**) VSV titers in supernatants of HeLa cells ($n = 6$ replicates) transfected with various concentrations of *Usp18*-expressing plasmid, infected with VSV (multiplicity of infection, 0.01) and treated 24 h later with various concentrations of recombinant IFN-α2 (key), assessed 24 h after infection. (**d**) Immunofluorescence of spleen sections from wild-type or *Usp18*$^{-/-}$ mice ($n = 3$ per genotype) 7 h after intravenous infection with 2×10^8 PFU of VSV, stained for VSV glycoprotein (green), CD169 (red) and B220 (blue). Scale bar, 100 μm. (**e**) Spleen VSV titers from wild-type or *Usp18*$^{-/-}$ mice ($n = 5$ per genotype) 7 h after intravenous infection with 2×10^6 PFU of VSV. $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test). Data are representative of two (**a**,**b**,**d**,**e**) or three (**c**) experiments (mean and s.e.m. (**a**,**b**) or mean ± s.e.m. (**c**,**e**)).

virus-specific CD8$^+$ T-cell response (**Fig. 3a**). Lack of replicating VSV blunted the induction of total neutralizing immunoglobulins (IgM and IgG) and of neutralizing IgG (**Fig. 3b**). Next we addressed the involvement of CD169$^+$ metallophilic macrophages during activation of the adaptive immune response. The antiviral B cell response in C57BL/6 mice that had received transplantation of CD169-DTR bone marrow and had been treated with diphtheria toxin before intravenous infection with VSV was delayed and diminished relative to that of C57BL/6 mice that had received wild-type bone marrow transplants and had been treated with diphtheria toxin (**Fig. 3c**). To explore the role of Usp18 in activation of the adaptive immune system, we analyzed adaptive immune responses in *Usp18*$^{-/-}$ mice after intravenous infection with VSV. *Usp18*-deficient mice had a very impaired VSV-specific CD8$^+$ T cell response relative to that of

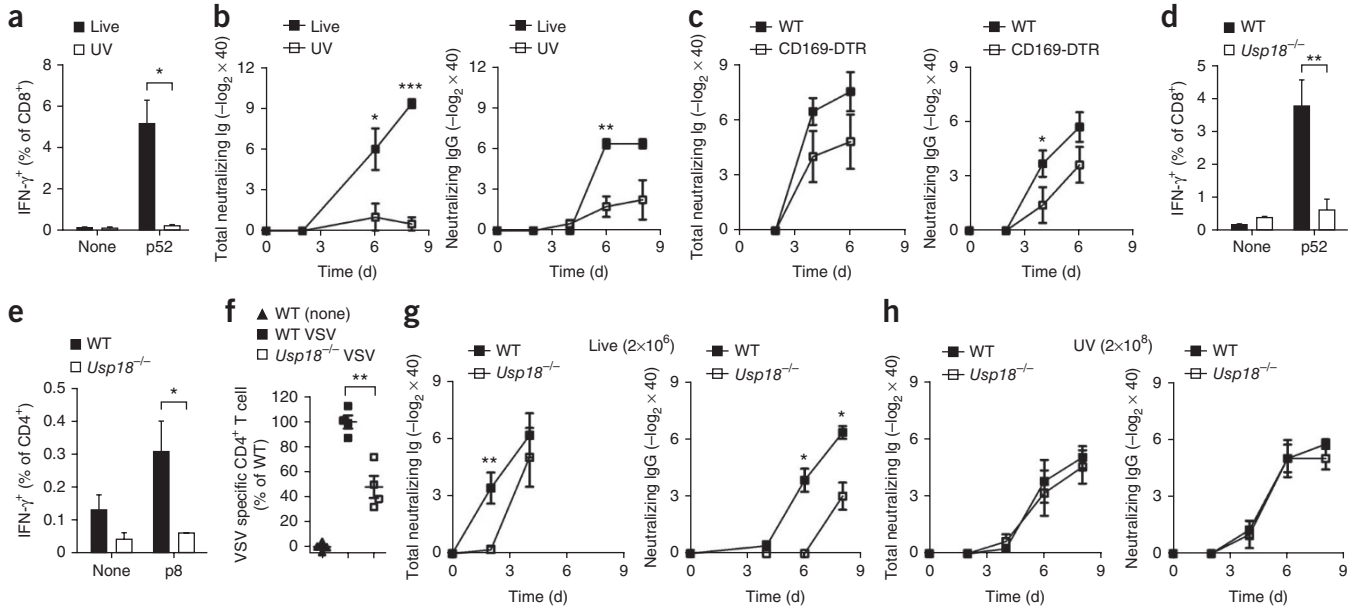

Figure 3 VSV replication in the spleen is required for efficient T cell and B cell responses. (**a**) Intracellular staining of IFN-γ in slenocytes obtained from C57BL/6 mice ($n = 3$) 7 d after intravenous immunization with replicating VSV (Live; 2×10^6 PFU) or UV light–inactivated VSV (UV; 2×10^6 PFU) and 6 h after no restimulation (none) or restimulation with VSV-derived major histocompatibility complex class I–restricted p52 peptide (p52). (**b**) VSV-neutralizing antibodies from C57BL/6 mice ($n = 3$–4) after intravenous immunization as in **a**. (**c**) VSV-neutralizing antibodies from C57BL/6 bone marrow chimeras ($n = 5$–6 per group) reconstituted with CD169-DTR cells or wild-type cells (control), treated intraperitoneally with diphtheria toxin and infected intravenously 3 d later with 2×10^6 PFU of VSV. (**d**,**e**) Intracellular IFN-γ staining of splenocytes derived from wild-type or *Usp18*$^{-/-}$ mice ($n = 5$ (**d**) or 3 (**e**) mice per group) infected intravenously with 2×10^6 PFU VSV and not restimulated or restimulated with p52 peptide (**d**) or with the VSV-derived major histocompatibility complex class II–restricted p8 peptide (**e**). (**f**) Population expansion of CD4$^+$ T cells with transgenic expression of a VSV-specific T cell antigen receptor (L7 cells) after transfer into wild-type and *Usp18*$^{-/-}$ mice ($n = 4$ per genotype), followed by no infection (none) or intravenous infection with 2×10^6 PFU of VSV 1 d later, assessed 3 d after infection and presented relative to expansion in VSV-infected wild-type mice, set as 100%. (**g**,**h**) VSV-neutralizing antibodies from wild-type and *Usp18*$^{-/-}$ mice ($n = 3$–6 (**g**) or 7–8 (**h**) mice per genotype) infected intravenously with 2×10^6 PFU of VSV (**g**) or immunized with 2×10^8 PFU of UV light–inactivated VSV (**h**). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test). Data are representative of one of two (**a**,**b**) or two (**c**–**f**) or three (**g**,**h**) experiments (mean and s.e.m. (**a**,**d**,**e**) or mean ± s.e.m. (**b**,**c**,**f**–**h**)).

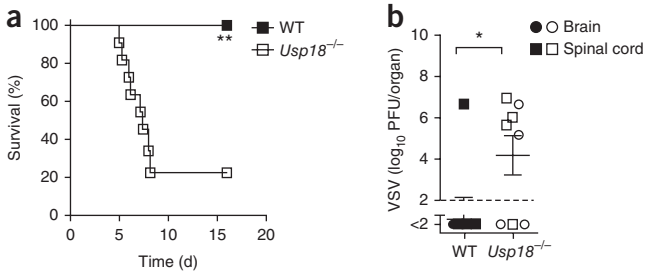

Figure 4 Defective induction of the adaptive immune response leads to the spread of VSV into the brain. Survival (**a**) and VSV titers in the brain and/or spinal cord (**b**) of wild-type and *Usp18*$^{-/-}$ mice (n = 7–11 (**a**) or 4–5 (**b**) mice per genotype) after intravenous infection with 2×10^6 PFU VSV, assessed 7–8 d after infection in **b**. $^*P < 0.05$ and $^{**}P < 0.01$ (Student's t-test). Data are representative of three experiments (mean ± s.e.m. in **b**).

wild-type mice (**Fig. 3d**). Additionally, the CD4$^+$ T cell response was lower in *Usp18*$^{-/-}$ mice than in wild-type mice (**Fig. 3e**).

Next we analyzed whether the observed fewer VSV-specific CD4$^+$ T cells depended on defective antigen presentation early after infection. We transferred splenocytes from mice with transgenic expression of a VSV-specific T cell antigen receptor (L7 mice) into VSV-infected wild-type and *Usp18*$^{-/-}$ mice[31]. The population expansion of adoptively transferred CD4$^+$ T cells was significantly lower in *Usp18*$^{-/-}$ mice than in wild-type mice (**Fig. 3f**). This suggested that *Usp18* deficiency in antigen-presenting cells was responsible for the lower population expansion of VSV-specific CD4$^+$ T cells in *Usp18*$^{-/-}$ mice *in vivo*, but *Usp18* deficiency in T cells was not.

Next we analyzed B cell responses. *Usp18*$^{-/-}$ mice had a delayed VSV-neutralizing IgM response and delayed formation of VSV-neutralizing IgG antibodies after infection with live VSV (**Fig. 3g**). To assess whether the delayed antibody induction in *Usp18*$^{-/-}$ mice was due to less replication of VSV, we immunized wild-type and *Usp18*$^{-/-}$ mice with nonreplicating UV light–inactivated VSV. To induce measurable antibody titers, we used 100× the dose of UV light–inactivated VSV. Immunization with replication-deficient UV light–inactivated VSV did not lead to any difference between wild-type and *Usp18*$^{-/-}$ mice in the induction of VSV-specific B cell responses (**Fig. 3h**). This suggested that the differences in VSV replication activity were responsible for the differences in antibody induction. Together these findings indicated that the replication of VSV in metallophilic macrophages was essential for the induction of an efficient adaptive immune response.

Usp18 in DCs allows live virus–derived antigen presentation

DCs are professional antigen-presenting cells and therefore probably contribute to the activation of the adaptive immune system during VSV infection. Some DCs expressed VSV glycoprotein at later time points of infection (**Supplementary Fig. 4**). We investigated whether replication of VSV in DCs might contribute to activation of the adaptive immune system. Active viral replication in DCs is correlated with T cell priming[32]. We infected *in vitro*–generated wild-type and *Usp18*$^{-/-}$ DCs with VSV and added transgenic CD4$^+$ T cells 2 h later. We observed less T cell proliferation in cocultures with virus-infected *Usp18*$^{-/-}$ DCs than in those with wild-type DCs (**Supplementary Fig. 4**). Next we analyzed the activation capacity of *Usp18*$^{-/-}$ DCs in the presence of nonreplicating virus. Coculture of DCs with 100× more UV light–inactivated VSV particles showed greater activation of T cells without *Usp18*. Peptide-labeled DCs induced proliferation in peptide-specific CD8$^+$ T cells independently of *Usp18* expression (**Supplementary Fig. 4**). Together these findings indicated that *Usp18* expression in DCs enhanced the presentation of replicating antigen.

Enforced VSV replication promotes survival

We found that replication of VSV in the spleen was necessary for neutralizing antibody production. Such neutralizing antibodies are essential for control of VSV[33]. Next we investigated whether Usp18-mediated viral replication in lymph follicles would be beneficial for survival during VSV infection. After intravenous infection with VSV, *Usp18*$^{-/-}$ mice showed typical VSV-mediated paralysis and died 1 week after infection, whereas wild-type mice survived VSV infection (**Fig. 4a**). The paralysis and death of *Usp18*$^{-/-}$ mice could have been explained by the spread of VSV into the spinal cord or the brain (**Fig. 4b**). We concluded that Usp18-mediated enforced VSV replication in CD169$^+$ macrophages promoted adaptive immunity to guarantee fast neutralization of infectious VSV and survival of the infected host.

Next we analyzed whether enforced viral replication in lymph follicles and spleen would be beneficial for the survival of VSV peripherally. Intranasal administration of VSV leads to fast spread of VSV to the central nervous system[34]. Given our data, we speculated that with this infection route, viral replication in lymphoid tissue is limited and that this would lower the adaptive immune response to VSV. To directly determine whether enforced replication of virus in the spleen could prevent spread of VSV from peripheral sites to central nervous system, we infected mice intranasally with VSV. In addition, we infected mice by intravenous injection with 2×10^6 plaque-forming units (PFUs) of replication-competent VSV or UV light–inactivated VSV. Mice that received live virus intravenously in addition to intranasal infection with VSV had higher titers of neutralizing antibody (**Fig. 5a**) and survived the infection (**Fig. 5b**). Mice that received UV light–inactivated VSV had lower antibody titers and died of intranasal VSV infection (**Fig. 5a**,**b**). Thus, intravenous infection with live VSV induced protective adaptive immunity and protected mice from lethal intranasal infection with VSV.

Lymphotoxin-β receptor mediates enforced viral replication

Lymphotoxins are important for the development and function of various cells of the innate immune response, including DCs and CD169$^+$ macrophages[35,36]. We speculated that lymphotoxins are involved in the process of enforced viral replication. VSV glycoprotein expression was considerably impaired in the spleen of mice

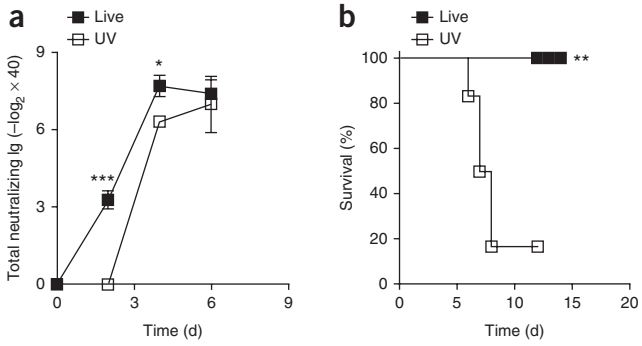

Figure 5 Replication of VSV in the spleen protects mice from lethal intranasal infection. VSV-neutralizing antibodies (**a**) and survival (**b**) of wild-type mice (n = 6–7 mice per genotype) infected intranasally with 5×10^5 PFU of live VSV and injected intravenously with 2×10^6 PFU of live VSV or 2×10^6 PFU of UV light–inactivated VSV. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t-test). Data are representative of two experiments (mean ± s.e.m. in **a**).

deficient in the receptor for lymphotoxin-b ($Ltbr^{-/-}$ mice), which correlated with the low number of $CD169^+$ metallophilic macrophages (**Supplementary Fig. 5**). Accordingly, early viral titers in the spleen were much lower in $Ltbr^{-/-}$ mice than in wild type mice after VSV infection (**Supplementary Fig. 5**), which suggested that lymphotoxins were involved in the process of enforced viral replication. $CD8^+$ T cell responses were impaired in $Ltbr^{-/-}$ mice (**Supplementary Fig. 5**). In agreement with those results, the VSV-specific B cell response was delayed in $Ltbr^{-/-}$ mice (**Supplementary Fig. 5**). $Ltbr^{-/-}$ mice developed typical VSV-mediated paralysis and died 7 d after infection (**Supplementary Fig. 5**). The death of $Ltbr^{-/-}$ mice could have been explained by VSV propagation in the brain or spinal cord or both (**Supplementary Fig. 5**). These data showed that lymphotoxins influenced early viral replication and this might have partially contributed to the immunodeficiency in these mice.

DISCUSSION

In this study, we found that $CD169^+$ metallophilic macrophages allowed viral replication for the promotion of adaptive immunity. Macrophages of the spleen and the liver captured viral particles after systemic virus infection. Red pulp macrophages and Kupffer cells suppressed viral replication in a type I interferon–dependent manner. $CD169^+$ macrophages had higher expression of *Usp18*, and the subsequent type I interferon resistance enforced viral replication. Either a lack of $CD169^+$ macrophages or deficiency in *Usp18* led to limited viral replication in the spleen. Low viral titers in the spleen led to impaired priming of the adaptive immune system. Lack of neutralizing antibodies in $Usp18^{-/-}$ mice allowed the spread of virus from low replication at peripheral sites to neuronal tissue, thus leading to paralysis and death of the mice.

The innate immune response is a double-edged sword. Type I interferon production inhibits viral replication, and this inhibition is crucial to the prevention of virus distribution[1]. In addition, type I interferon enhances proteasomal degradation and cross-priming[37,38], upregulates costimulatory molecules on DCs[39] and increases the proliferative ability of activated T cells[40] and B cells[41]. Our findings have confirmed published studies indicating that macrophages capture virus particles and suppress viral replication in the red pulp of the spleen and the liver[5]. However, the inhibition of viral replication decreases the amount of antigen that can be presented to the adaptive immune system. The amount of antigen is positively correlated with the degree of stimulation of the adaptive immune system[25,26]. Our findings suggest that viral replication is required for the promotion of T cell priming and the production of neutralizing antibodies.

$CD169^+$ macrophages are situated to promote such viral replication. They reach into the marginal sinus and filter pathogens from the bloodstream[6]. Their anatomical location in the spleen allows the presentation of antigen to B cells[14,16,35]. Additionally, $CD169^+$ cells connect to fibroblastic reticular cell conduits, which can transport antigen to DCs in the T cell zone[18]. $CD169^+$ cells are important for the presentation of virus to plasmacytoid DCs and therefore are essential for activation of the innate immune system[42]. For subcutaneous infection with VSV, the induction of protective type I interferon has been reported only in the presence of VSV replication in the lymph node[42]. This observation could suggest that enforced viral replication is important not only for activation of the adaptive immune system but also for activation of the innate immune system.

Several signaling molecules influence the responsiveness of cells to type I interferon[43]. Therefore, enhanced expression of suppressor of cytokine signaling (SOCS) proteins could contribute to the type I interferon unresponsiveness of $CD169^+$ macrophages[2,10,43]. Indeed, we found enhanced basal expression of *Socs1* and *Socs3*, but not of *Oas1* or *Isg15*, in $CD169^+$ macrophages (data not shown). Therefore, in addition to Usp18, SOCS1 and SOCS3 may act synergistically to allow VSV replication in $CD169^+$ macrophages. In this study, we focused on the role of Usp18 and its influence on VSV replication.

Lack of Usp18 led to VSV replication in the brain and spinal cord, early paralysis and death after systemic infection with 2×10^6 PFU of VSV. Usp18 deficiency prevents death after intracranial infection with 10 PFU of VSV[12]. Control of viral replication during infection via an intracranial route depends only on the innate immune response. In contrast, the adaptive immune response is essential for the prevention of lethal VSV infection during systemic intravenous infection with 2×10^6 PFU of VSV[33,44]. Therefore, the expression of Usp18, although it may locally augment VSV replication in the spleen, is of overall benefit for the host during systemic infection.

It remains to be investigated how the mechanism of enforced viral replication contributes to adaptive immune responses to other viruses and to vaccination strategies for human pathogenic viruses. Through the use of the noncytopathic lymphocytic choriomeningitis virus (LCMV), we found that enforced viral replication also contributed to the adaptive immune response to this virus (data not shown). Published studies have indicated that in the case of human pathogenic viruses, replicating viruses elicit a stronger neutralizing antibody response than do inactivated virus particles[45,46]. Several live attenuated vaccines provide sufficient protection, whereas inactivated vaccines do not induce a protective antibody response (for example, vaccines asgainst rubella, measles, mumps, yellow fever, varicella and chicken pox). Vaccination with active rubella virus or poliovirus induces long-term protective IgG responses and also induces the production of IgA[45,46]. IgA can very efficiently neutralize poliovirus in the gut; therefore, immunization with active virus would not only provide longer protection but also be beneficial in disrupting the chain of infection. We speculate that Usp18 expression in the splenic marginal zone may enforce polioviral replication and induce rapid protective neutralizing antibodies. Indeed, although type I interferon suppresses poliovirus in several cell types, in type I interferon–competent mice, virus replicates in the marginal zone[47]. It remains to be determined whether this replication depends on Usp18 expression and whether it is essential for the induction of IgA.

We found that the absence of Usp18 in DCs was important for the activation of $CD4^+$ T cells during immunization with live virus. That finding suggested that immune activation at later stages of VSV infection depended on Usp18 expression and viral replication in DCs. During influenza virus infection, active viral replication in DCs leads to greater antiviral T cell responses[32]. It remains to be addressed whether enforced viral replication contributes to the strong costimulatory ability of DCs.

$CD169^+$ macrophages may enhance the amount of foreign virus antigen through enforced viral replication, so that immunological ignorance is converted into immune activation. In the RIP-GP model of virus-induced autoimmune diabetes, limited viral replication or soluble antigen cannot induce diabetes[48–50]. That finding is explained mainly by limited costimulation and limited inflammatory signals in beta-islet cells[49,50]. Given our findings, we propose that the lack of antigen amplification in $CD169^+$ macrophages may be related to the lack of diabetes during immunization with soluble nonreplicating antigen. In conclusion, we found here that enforced viral replication was triggered by Usp18 expression in $CD169^+$ metallophilic macrophages and led to anatomically restricted viral replication and to rapid production of virus-neutralizing antibodies, thereby preventing fatal disease.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

N.H. planned and did most experiments; N.S. planned and did several experiments; G.C. and S.E.B. did laser-capture dissection; U.R.S. contributed to *Ltbr*$^{-/-}$ mouse experiments; D.-E.Z. contributed to experiments on *Usp18*; M.T. and C.B. contributed to transfection experiments; K.K., M.S. and R.K. did and interpreted *in situ* hybridization; N.G. did *in vitro* experiments; N.v.R. contributed to macrophage depletion experiments; M.G. did *in vitro* stimulation of DCs; M.L., H.H., K.P., M.T., D.H. and M.R. discussed and interpreted data and helped to write the manuscript; and P.A.L. and K.S.L. initiated and designed the study and wrote most of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *Usp18*$^{-/-}$ mice were bred heterozygously onto a Sv129 × C57BL/6 background F_4. *Usp18*$^{-/-}$ mice were directly compared with littermate controls. All other mice used in this study were maintained on a C57BL/6 background. During survival experiments, the health status of the mice was checked twice daily. After the appearance of clinical signs of VSV replication in the central nervous system, such as paralysis, mice were removed from the experiment and considered dead. Animal experiments were done with the authorization of Veterinäramt Nordrhein-Westfalen (Düsseldorf, Germany) and in accordance with the German law for animal protection, the institutional guidelines of the Ontario Cancer Institute, or both.

Bone marrow chimeras. For the generation of bone marrow chimeras, C57BL/6 mice were irradiated with 1,050 rads. After 24 h, mice were reconstituted intravenously with 1×10^7 bone marrow cells. At 15 d later, all mice were treated with 200 µl clodronate liposomes to guarantee donor-derived origin of marginal zone macrophages. At 35 d after reconstitution, mice were used for experiments.

Depletion of macrophages. For the depletion of CD169$^+$ macrophages, diphtheria toxin (30 µg per kg body weight) was injected into each mouse intraperitoneally (Sigma-Aldrich). For depletion of complete macrophages, 200 µl clodronate liposomes were injected intravenously. Control mice were injected with empty liposomes.

Generation of DCs. Bone marrow cells from wild-type and *Usp18*$^{-/-}$ mice were cultured for 9 d with mouse granulocyte-macrophage colony-stimulating factor (4 ng/ml; Pan Biotech). IFN-α4 was from PBL Interferonsource.

Virus. VSV (Indiana strain, Mudd-Summers isolate), was originally obtained from D. Kolakofsky. Virus was propagated on BHK-21 hamster kidney fibroblasts at a multiplicity of infection of 0.01 and was then 'plaqued' onto Vero cells. VSV was inactivated for 10 min with UV light. Peptides were from the Polypeptide Group.

Neutralizing antibody assay. Serum was prediluted (1:40). The complement system was inactivated for 30 min at 56 °C. For analysis of IgG kinetics, diluted samples were treated with β-mercaptoethanol (0.1 M) for removal of IgM. Serum was titrated 1:2 over 12 steps and incubated with 1×10^3 PFU of VSV. After 90 min of incubation, the virus-serum mixture was 'plaqued' on Vero cells. Overlay was added after 1 h. Plaques were counted 24 h later by crystal violet staining.

Histology. Histological analyses of snap-frozen tissue used monoclonal antibody to VSV glycoprotein (Vi10; made in-house). Anti-CD45R (B220; RA3-6B2) and anti-CD90.2 (53-2.1) were from eBioscience. Red pulp macrophages were stained with anti-F4/80 (BM8; eBiosciences) and DCs were stained with anti-CD11c (N418; eBiosciences). Anti-CD169 (MOMA-1) was from Abcam.

***In situ* hybridization.** *Usp18* mRNA was located through use of the clone pBK-CMV-mUBP43 containing full-length mouse *Usp18* cDNA. Tissue sections (5 µm in thickness) were dewaxed and hybridized as described[51]. Hybridization mixtures contained ^{35}S-labeled RNA antisense or sense control probes obtained from full-length mouse *Usp18* (500 ng/ml) in 10 mM Tris-HCl, pH 7.4, 50% (vol/vol) deionized formamide, 600 mM NaCl, 1 mM EDTA, 0.02% (wt/vol) polyvinylpyrrolidone, 0.02% (vol/vol) Ficoll, 0.05% (wt/vol) BSA, 10% (wt/vol) dextrane sulfate, 10 mM dithiothreitol, denatured sonicated salmon sperm DNA (200 µg/ml) and rabbit liver tRNA (100 µg/ml). Hybridization with RNA probes proceeded for 18 h at 42 °C. Slides were then washed as described followed by 1 h at 55 °C in 2× standard saline citrate. Unhybridized single-stranded RNA probes were digested for 30 min at 37 °C with RNase A (20 µg/ml) in 10 mM Tris-HCl, pH 8.0, and 0.5 M NaCl. Tissue slide preparations were exposed to autoradiography film for 3 weeks and stained with hematoxylin and eosin.

Laser-capture microdissection. Frozen tissue sections (10 µm in thickness) were cut under RNase-free conditions. On the day of microdissection, sections were either stained with biotin–anti-CD169 (MOMA-1; Abcam), and streptavidin-peroxidase or with a HistoGene Frozen Section Staining Kit according to the manufacturer's protocol (Applied Biosystems). For dehydration, slides were incubated for 30 s consecutively in 75%, 95% and 100% (vol/vol) ethanol and then for 5 min in xylene. After the dehydration procedure, sections were air-dried for 12 min. Samples of spleen tissue were captured from the stained slides on Capsure HS LCM caps by using a PixCell II laser capture microscope (Applied Biosystems) with the laser pulse power set at 75 mW and a threshold voltage of 200 mV.

Total RNA extraction, cDNA synthesis and quantitative real-time PCR. Total RNA was extracted from cells on CapSure HS LCM caps (Applied Biosystems) with a PicoPure RNA isolation kit according to the manufacturer's protocol (Arcturus; Applied Biosystems). RNA from sorted cells was isolated with an RNA Mini Kit (Qiagen). RNA was reverse-transcribed to cDNA with a Quantitect Reverse Transcription kit (Qiagen). Expression of *Gapdh* and *Usp18* was analyzed with primers from Applied Biosystems Mm00449455_m1 and Mm03302249_g1. Expression was normalized to that of *Gapdh* and compared for the study groups.

Flow cytometry. Lymphocytes were stained with anti-CD8 (53-6.7; BD Biosciences), anti-CD4 M1/70; eBiosciences and anti-IFN-γ (XMG1.2; eBiosciences) after 6 h of restimulation with VSV antigen p8 peptide or p52 peptide. For staining of CD169$^+$ macrophages, 36 µg biotin–anti-CD169 (MOMA-1; Abcam) was injected intravenously. After 15 min, spleens were digested with Liberase and DNAse. CD169$^+$ macrophages were stained with streptavidin-phycoerythrin (eBiosciences) in combination with anti-CD11b (RA3-6B2; eBiosciences) or anti-F4/80 (BM8; eBiosciences).

Sorting of macrophages and T cells. Biotin–anti-CD169 (36 µg; Abcam) was injected into C57BL/6 mice. After 10 min, splenocytes were digested with Liberase DNAse (Roche) and stained with anti-biotin microbeads (Miltenyi) and streptavidin-phycoerythrin (eBiosciences) or with phycoerythrin–anti-F4/80 (BM8; eBiosciences) and anti-PE microbeads (Miltenyi). After 30 min of incubation, splenocytes were sorted by magnetic-activated cell sorting. CD169$^+$ cells were additionally sorted by flow cytometry. T cells were sorted with a MACS (130-090-861; Miltenyi) kit.

Transfection with *Usp18* plasmid. For transcription of *Usp18*, the construct pcDNA3-hUSP18 was used (C.B. Fan, J.B. and D.-E.Z., unpublished data). HeLa cells (1×10^6) were transfected, through the use of a Superfect-Kit (Qiagen), with 4 µg DNA derived from *Usp18*-expressing plasmid and empty vector at various ratios. IFN-α2 was from PBL Interferonsource.

Statistical analysis. Student's *t*-test was used to detect significant differences between two groups. *P* values of less than 0.05 were considered significant.

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