RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Virology
ウイルス感染分野

Professor	Yoshihiro Kawaoka, D.V.M., Ph.D.	教　授	獣医学博士	河　岡　義　裕
Project Professor	Makoto Yamashita, Ph.D.	特任教授	薬学博士	山　下　　誠
Associate Professor	Masaki Imai, D.V.M., Ph.D.	准教授	博士(獣医学)	今　井　正　樹
Project Associate Professor	Satoshi Fukuyama, M.D., Ph.D.	特任准教授	博士(医学)	福　山　　聡
Project Associate Professor	Tokiko Watanabe, D.V.M., Ph.D.	特任准教授	博士(獣医学)	渡　邉　登喜子
Project Associate Professor	Seiya Yamayoshi, D.V.M., Ph.D.	特任准教授	博士(医学)	山　吉　誠　也
Assistant Professor	Kiyoko Iwatsuki-Horimoto, D.V.M., Ph.D.	助　教	博士(獣医学)	岩附(堀本)研子
Assistant Professor	Shinya Yamada, Ph.D.	助　教	博士(医学)	山　田　晋　弥
Project Assistant Professor	Maki Kiso, D.V.M., Ph.D.	特任助教	博士(医学)	木　曽　真　紀
Research Associate	Yuko Sakai-Tagawa, Ph.D.	助　手	博士(医学)	坂井(田川)優子

Viruses can cause devastating diseases. The long-term goal of our research is to understand the molecular pathogenesis of viral diseases by using influenza and Ebola virus infections as models. Interactions between viral and host gene products during viral replication cycles determine the consequences of infection (i.e., the characteristics of disease manifestation, whether limited or widespread); hence, our research has centered on such interactions in these viral infections.

1. Characterization of a Feline Influenza A(H7N2) Virus.

Hatta M[1], Zhong G[1], Gao Y[1], Nakajima N[2], Fan S[1], Chiba S[1], Deering KM[1], Ito M, Imai M, Kiso M, Nakatsu S, Lopes TJ[1], Thompson AJ[3], McBride R[3], Suarez DL[4], Macken CA[5], Sugita S[6], Neumann G[1], Hasegawa H[2], Paulson JC[3], Toohey-Kurth KL[1], Kawaoka Y: [1]Department of Pathobiological Sciences, School of Veterinary Sciences, University of Wisconsin-Madison, USA. [2]Department of Pathology, National Institute of Infectious Diseases, Japan. [3]Departments of Molecular Medicine & Immunology and Microbiology, The Scripps Research Institute, USA. [4]US Department of Agriculture, USA. [5]The University of Auckland, New Zealand. [6]Japan Racing Association, Japan.

During December 2016-February 2017, influenza A viruses of the H7N2 subtype infected ≈500 cats in animal shelters in New York, NY, USA, indicating virus transmission among cats. A veterinarian who treated the animals also became infected with feline influenza A(H7N2) virus and experienced respiratory symptoms. To understand the pathogenicity and transmissibility of these feline H7N2 viruses in mammals, we characterized them in vitro and in vivo. Feline H7N2 subtype viruses replicated in the respiratory organs of mice, ferrets, and cats without causing severe lesions. Direct contact transmission of feline H7N2 subtype viruses was detected in ferrets and cats; in cats, exposed animals were also infected via respiratory droplet transmission. These results suggest that the feline H7N2 subtype viruses could spread among cats and also infect humans. Outbreaks of the feline H7N2 viruses could, therefore, pose a risk to public health.

2. Importance of the 1 + 7 configuration of ribonucleoprotein complexes for influenza A virus genome packaging.

Noda T[7], Murakami S[8], Nakatsu S, Imai H, Muramoto Y[7], Shindo K[7], Sagara H[9], Kawaoka Y: [7]Laboratory of Ultrastructural Virology, Institute for Frontier Life and Medical Sciences, Kyoto University, Japan. [8]Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, Japan. [9]Medical Proteomics Laboratory, Institute of Medical Science, University of Tokyo, Japan.

The influenza A virus genome is composed of eight single-stranded negative-sense RNAs. Eight distinct viral RNA segments (vRNAs) are selectively packaged into progeny virions, with eight vRNAs in ribonucleoprotein complexes (RNPs) arranged in a specific "1 + 7" pattern, that is, one central RNP surrounded by seven RNPs. Here we report the genome packaging of an artificially generated seven-segment virus that lacks the hemagglutinin (HA) vRNA. Electron microscopy shows that, even in the presence of only seven vRNAs, the virions efficiently package eight RNPs arranged in the same "1 + 7" pattern as wild-type virions. Next-generation sequencing reveals that the virions specifically incorporate host-derived 18S and 28S ribosomal RNAs (rRNAs) seemingly as the eighth RNP in place of the HA vRNA. These findings highlight the importance of the assembly of eight RNPs into a specific "1 + 7" configuration for genome packagingin progeny virions and suggest a potential role for cellular RNAs in viral genome packaging.

3. Lung-derived exosomal miR-483-3p regulates the innate immune response to influenza virus infection.

Maemura T, Fukuyama S, Sugita Y[10], Lopes TJ[1], Nakao T, Noda T[7], Kawaoka Y: [10]Molecular Cryo-Electron Microscopy Unit, Okinawa Institute of Science and Technology Graduate University, Japan.

Exosomes regulate cell-cell communication by transferring functional proteins and RNAs between cells. Here, to clarify the function of exosomes during influenza virus infection, we characterized lung-derived exosomal microRNAs (miRNAs). Among the detected miRNAs, miR-483-3p was present at high levels in bronchoalveolar lavage fluid (BALF) exosomes during infection of mice with various strains of influenza virus, and miR-483-3p transfection potentiated gene expression of type I interferon and proinflammatory cytokine upon viral infection of MLE-12 cells. RNF5, a regulator of the RIG-I signaling pathway, was identified as a target gene of miR-483-3p. Moreover, we found that CD81, another miR-483-3p target, functions as a negative regulator of RIG-I signaling in MLE-12 cells. Taken together, this study indicates that BALF exosomal miRNAs may mediate the antiviral and inflammatory response to influenza virus infection.

4. In vivo imaging of the pathophysiological changes and neutrophil dynamics in influenza virus-infected mouse lungs.

Ueki H, Wang IH, Fukuyama S, Katsura H, Lopes TJ[1], Neumann G[1], Kawaoka Y.

The pathophysiological changes that occur in lungs infected with influenza viruses are poorly understood. Here we established an in vivo imaging system that combines two-photon excitation microscopy and fluorescent influenza viruses of different pathogenicity. This approach allowed us to monitor and correlate several parameters and physiological changes including the spread of infection, pulmonary permeability, pulmonary perfusion speed, number of recruited neutrophils in infected lungs, and neutrophil motion in the lungs of live mice. Several physiological changes were larger and occurred earlier in mice infected with a highly pathogenic H5N1 influenza virus compared with those infected with a mouse-adapted human strain. These findings demonstrate the potential of our in vivo imaging system to provide novel information about the pathophysiological consequences of virus infections.

5. N-terminal acetylation by NatB is required for the shutoff activity of influenza A virus PA-X.

Oishi K, Yamayoshi S, Kozuka-Hata H[11], Oyama M[11], Kawaoka Y: [11]Medical Proteomics Laboratory, Institution of Medical Science, University of Tokyo, Japan.

N-terminal acetylation is a major posttranslational modification in eukaryotes catalyzed by N-terminal acetyltransferases (NATs), NatA through NatF. Although N-terminal acetylation modulates diverse protein functions, little is known about its roles in virus replication. We found that NatB, which comprises NAA20 and NAA25, is involved in the shutoff activity of influenza virus PA-X. The shutoff activity of PA-X was suppressed in NatB-deficient cells, and PA-X mutants that are not acetylated by NatB showed reduced shutoff activities. We also evaluated the importance of N-terminal acetylation of PA, because PA-X shares its N-terminal sequence with PA. Viral polymerase activity was reduced in NatB-deficient cells. Moreover, mutant PAs that are not acetylated by NatB lost their function in the viral polymerase complex. Taken to-

gether, our findings demonstrate that N-terminal acetylation is required for the shutoff activity of PA-X and for viral polymerase activity.

6. Cryo-EM structure of the Ebola virus nucleoprotein-RNA complex at 3.6 Å resolution.

Sugita Y[10], Matsunami H[10], Kawaoka Y, Noda T[7], Wolf M[10].

Ebola virus causes haemorrhagic fever with a high fatality rate in humans and non-human primates. It belongs to the family Filoviridae in the order Mononegavirales, which are viruses that contain linear, non-segmented, negative-sense, single-stranded genomic RNA. The enveloped, filamentous virion contains the nucleocapsid, consisting of the helical nucleoprotein-RNA complex, VP24, VP30, VP35 and viral polymerase. The nucleoprotein-RNA complex acts as a scaffold for nucleocapsid formation and as a template for RNA replication and transcription by condensing RNA into the virion. RNA binding and nucleoprotein oligomerization are synergistic and do not readily occur independently. Although recent cryo-electron tomography studies have revealed the overall architecture of the nucleocapsid core, there has been no high-resolution reconstruction of the nucleocapsid. Here we report the structure of a recombinant Ebola virusnucleoprotein-RNA complex expressed in mammalian cells without chemical fixation, at near-atomic resolution using single-particle cryo-electron microscopy. Our structure reveals how the Ebola virus nucleocapsid core encapsidates its viral genome, its sequence-independent coordination with RNA by nucleoprotein, and the dynamic transition between the RNA-free and RNA-bound states. It provides direct structural evidence for the role of the N terminus of nucleoprotein in subunit oligomerization, and for the hydrophobic and electrostatic interactions that lead to the formation of the helical assembly. The structure is validated as representative of the native biological assembly of the nucleocapsid core by consistent dimensions and symmetry with the full virion. The atomic model provides a detailed mechanistic basis for understanding nucleocapsid assembly and highlights key structural features that may serve as targets for anti-viral drug development.

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Department of Microbiology and Immunology

Division of Infectious Genetics
感染遺伝学分野

Professor	Kensuke Miyake, M.D., Ph.D.		教　授	医学博士	三　宅　健　介
Associate Professor	Shin-Ichiroh Saitoh, Ph.D.		准教授	博士(医学)	齋　藤　伸一郎
Assistant Professor	Ryutaro Fukui, Ph.D.		助　教	博士(医学)	福　井　竜太郎
Assistant Professor	Takuma Shibata, Ph.D.		助　教	博士(医学)	柴　田　琢　磨
Project Assistant Professor	Ryota Sato, Ph.D.		特任助教	博士(医学)	佐　藤　亮　太

Immune cells express multiple Toll-like receptors (TLRs) that are concomitantly activated by a variety of pathogen products derived from microbes and viruses. Recent reports have indicated that losing the balance of TLRs responses result in autoimmune diseases. Nucleic acid-sensing (NA-sensing) TLRs sense bacterial and viral NAs, but also host-derived NAs. To avoid excessive immune responses for host-derived NAs, there must exist regulatory mechanisms coordinating the expression, the localization and the function of TLRs. Our research focuses on the regulatory mechanisms controlling pathogenic ligand recognition by TLRs and identification of their endogenous ligands.

1. Cleavage of Toll-like receptor 9 ectodomain is required for in vivo responses to single strand DNA

Ryutaro Fukui[1], Chikako Yamamoto[1], Fumi Matsumoto-Fukui[1], Masahiro Onji[1,2], Takuma Shibata[1], Yusuke Murakami[1,3], Atsuo Kanno[1], Takuto Hayashi[1], Natsuko Tanimura[1], Nobuaki Yoshida[4], and Kensuke Miyake[1,5]: [1]Division of Innate Immunity, Department of Microbiology and Immunology, [4]Laboratory of Developmental Genetics, [5]Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo. [2]Institute of Molecular Biotechnology of the Austrian Academy of Sciences, [3]Department of Pharmacotherapy, Research Institute of Pharmaceutical Sciences, Musashino University

Mouse Toll-like receptor 9 (TLR9) is an endosomal sensor for single-stranded DNA (ssDNA). TLR9 is transported from the ER to endolysosomes by a multiple transmembrane protein Unc93 homolog B1, and proteolytically cleaved at its ectodomain. The structure of TLR9 and its biochemical analyses have shown that the proteolytic cleavage of TLR9 ectodomain enables TLR9-dimerization and TLR9 activation. However, the requirement of TLR9 cleavage *in vivo* has not been studied. We here show that the 13 amino acids deletion at the cleavage site made TLR9 resistant to proteolytic cleavage. The deletion mutation in the *Tlr9* gene impaired TLR9-dependent cytokine production in conventional dendritic cells from the mutant mice. Not only *in vitro*, *in vivo* production of inflammatory cytokines induced by administration of TLR9 ligand was also impaired. These results demonstrate that the TLR9 cleavage is required for TLR9 responses *in vivo*.

2. ADP-ribosylation factor-like 8b is required for the development of mouse models of systemic lupus erythematosus

Shin-Ichiroh Saitoh[1], Yoshiko Mori Saitoh[1], Kenji Kontani[2], Katsuaki Sato[3], and Kensuke Miyake[1,4]:

[1]Division of Innate Immunity, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, Tokyo 108-8639, Japan. [2]Department of Biochemistry, Meiji Pharmaceutical University, Kiyose, Tokyo 204-8588, Japan. [3]Division of Immunology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. [4]Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan.

Toll-like receptor (TLR) 7 and type I interferon (IFN-1) are essential for the development of systemic lupus erythematosus (SLE) models such as BXSB.*Yaa* and 2,6,10,14-tetramethyl-pentadecane (TMPD)-induced experimental lupus. However, the mechanism underlying the development of SLE remains undefined. We report a requirement for ADP-ribosylation factor-like 8b (Arl8b) for TLR7-dependent IFN-1 production in plasmacytoid dendritic cells (pDCs). We analyzed whether Arl8b plays a role in two SLE models by comparing wild-type and Arl8b-deficient Arl8b GeneTrap (*Arl8b*$^{Gt/Gt}$) mice. We found that BXSB.*Yaa* *Arl8b*$^{Gt/Gt}$ mice showed none of the abnormalities characterized in BXSB.*Yaa* mice. TMPD treatment of *Arl8b*$^{Gt/Gt}$ mice significantly inhibited the development of SLE. pDCs were required for TMPD-induced peritonitis. Our data demonstrate that Arl8b contributes to disease pathogenesis in two SLE models via IFN-1 dependent and independent mechanisms and suggest that Arl8b is an attractive new target for therapeutic intervention in SLE.

3. Cytidine deaminase enables Toll-like receptor 8 activation by cytidine or its analogs

Katsuhiro Furusho[1,2], Takuma Shibata[1], Ryota Sato[1], Ryutaro Fukui[1], Yuji Motoi[1], Yun Zhang[1], Shin-ichiroh Saitoh[1], Takeshi Ichinohe[3], Masafumi Moriyama[2], Seiji Nakamura[2] and Kensuke Miyake[1]: [1]Division of Innate Immunity, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [2]Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, Fukuoka 812-8582, Japan. [3]Division of Viral Infection, Department of Infectious Disease Control, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan.

Toll-like receptor 8 (TLR8), a sensor for pathogen-derived single-stranded RNA (ssRNA), binds to uridine (Uri) and ssRNA to induce defense responses. We here show that cytidine (Cyd) with ssRNA also activated TLR8 in peripheral blood leukocytes (PBLs) and a myeloid cell line U937, but not in an embryonic kidney cell line 293T. Cyd deaminase (CDA), an enzyme highly expressed in leukocytes, deaminates Cyd to Uri. CDA expression enabled TLR8 response to Cyd and ssRNA in 293T cells. CDA deficiency and a CDA inhibitor both reduced TLR8 responses to Cyd and ssRNA in U937. The CDA inhibitor also reduced PBL response to Cyd and ssRNA. A Cyd analogue, azacytidine, is used for the therapy of myelodysplastic syndrome and acute myeloid leukemia. Azacytidine with ssRNA induced tumor necrosis factor-α expression in U937 and PBLs in a manner dependent on CDA and TLR8. These results suggest that CDA enables TLR8 activation by Cyd or its analogues with ssRNA through deaminating activity. Nucleoside metabolism might impact TLR8 responses in a variety of situations such as the treatment with nucleoside analogues.

4. Guanosine and its modified derivatives are endogenous ligands for TLR7

Takuma Shibata[1], Yuji Motoi[1], Kensuke Miyake[1]: [1]Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, TOKYO 1208-8639, Japan.

Toll-like receptor 7 (TLR7) in the endolysosome is a sensor for single-stranded RNA (ssRNA) from viruses and it induces antiviral immune response. In addition, this receptor also responds to synthetic small molecules such as R848 and Imiquimod. However, it remains unclear how and why TLR7 can sense these two distinct ligands. We have found that TLR7 recognizes guanosine (G) and its analogue, deoxyguanosine (dG), in the presence of uridine-containing oligoribonucleotide (U-ORN). With U-ORN, G/dG synergistically activated TLR7 and strongly induced cytokine production in macrophages, cDCs and pDCs. In consistent with this finding, specific binding between G/dG, but not other nucleosides, and TLR7/U-ORN complex was detectable by isothermal titration calorimetry. Furthermore, there were two ligand-binding sites in the crystal structure of TLR7: the first site bound to a G/dG, and the second site bound to an U-ORN. These results strongly suggest that TLR7 recognizes degradation products of ssRNA, G and U-ORN, but not ssRNA itself, and those also raise the possibility that TLR7 sense degradation products of genomic DNA. Additionally, we have also found the evidence that abnormal accumulation of endolysosomal G/dG in macrophages elicit an autoinflamma-

tory disease with hepatosplenomegaly and histiocytosis in TLR7-dependent manner. Our findings reveal that TLR7 is a Guanosine sensor and suggest its important roles in inflammatory disorders.

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Department of Microbiology and Immunology

Division of Molecular Virology
ウイルス病態制御分野

Professor	Yasushi Kawaguchi, D.V.M., Ph.D.	教　授	博士(獣医学)	川　口　　寧
Assistant professor	Akihisa Kato, Ph.D.	助　教	博士(医学)	加　藤　哲　久
Assistant professor	Jun Arii, Ph.D.	助　教	博士(獣医学)	有　井　　潤
Assistant professor	Naoto Koyanagi, Ph.D.	助　教	博士(生命科学)	小　柳　直　人

To date, approximately 250 herpesviruses have been identified, affecting most animal species. These viruses are associated with a variety of diseases such as encephalitis, malignancy and mucocutaneous diseases in human and animals. The objective of our research is to understand the mechanisms by which herpesviruses replicate in cells and manifest diseases in their hosts. Our goal is to apply our fundamental findings for the development of anti-herpetic drugs and vaccines for the control of these viral infections.

1. ESCRT-III mediates budding across the inner nuclear membrane and regulates its integrity

Jun Arii, Mizuki Watanabe, Fumio Maeda, Noriko Tokai-Nishizumi[1], Takahiro Chihara[2,3], Masayuki Miura[2], Yuhei Maruzuru, Naoto Koyanagi, Akihisa Kato, and Yasushi Kawaguchi: [1]Microscope Core Laboratory, The Institute of Medical Science, The University of Tokyo [2]Department of Genetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo [3]Present address: Graduate School of Science, Hiroshima University

Vesicle-mediated nucleocytoplasmic transport is a nuclear pore-independent mechanism for the nuclear export of macromolecular complexes, but the molecular basis for this transport remains largely unknown. Here we show that endosomal sorting complex required for transport-III (ESCRT-III) is recruited to the inner nuclear membrane (INM) during the nuclear export of herpes simplex virus 1 (HSV-1). Scission during HSV-1 budding through the INM is prevented by depletion of ESCRT-III proteins. Interestingly, in uninfected human cells, the depletion of ESCRT-III proteins induces aberrant INM proliferation. Our results show that HSV-1 expropriates the ESCRT-III machinery in infected cells for scission of the INM to produce vesicles containing progeny virus nucleocapsids. In uninfected cells, ESCRT-III regulates INM integrity by downregulating excess INM.

2. Roles of the Phosphorylation of Herpes Simplex Virus 1 UL51 at a Specific Site in Viral Replication and Pathogenicity

Akihisa Kato, Shinya Oda, Mizuki Watanabe, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Naoto Koyanagi, Yuhei Maruzuru, Jun Arii, Yasushi Kawaguchi: [1]Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo

Herpes simplex virus 1 (HSV-1) UL51 is a phosphoprotein that functions in the final envelopment in the cytoplasm and viral cell-cell spread, leading to efficient viral replication in cell cultures. To clarify the mechanism by which UL51 is regulated in HSV-1-infected cells, we focused on the phosphorylation of UL51. Mass spectrometry analysis of purified UL51 identified five phosphorylation sites in UL51. Alanine replacement of one of the identified

phosphorylation sites in UL51, serine 184 (Ser-184), but not the other identified phosphorylation sites, significantly reduced viral replication and cell-cell spread in HaCaT cells. This mutation induced membranous invaginations adjacent to the nuclear membrane, the accumulation of primary enveloped virions in the invaginations and perinuclear space, and mislocalized UL34 and UL31 in punctate structures at the nuclear membrane; however, it had no effect on final envelopment in the cytoplasm of HaCaT cells. Of note, the alanine mutation in UL51 Ser-184 significantly reduced the mortality of mice following ocular infection. Phosphomimetic mutation in UL51 Ser-184 partly restored the wild- type phenotype in cell cultures and in mice. Based on these results, we concluded that some UL51 functions are specifically regulated by phosphorylation at Ser-184 and that this regulation is critical for HSV-1 replication in cell cultures and pathogenicity *in vivo*.

3. Regulation of Herpes Simplex Virus 2 Protein Kinase UL13 by Phosphorylation and Its Role in Viral Pathogenesis

Naoto Koyanagi, Akihisa Kato, Kosuke Takeshima, Yuhei Maruzuru, Hiroko Kozuka-Hata, Masaaki Oyama, Jun Arii, Yasushi Kawaguchi

UL13 proteins are serine/threonine protein kinases encoded by herpes simplex virus 1 (HSV-1) and HSV-2. Although the downstream effects of the HSV protein kinases, mostly those of HSV-1 UL13, have been reported, there is a lack of information on how these viral protein kinases are regulated in HSV-infected cells. In this study, we used a large-scale phosphoproteomic analysis of HSV-2-infected cells to identify a physiological phosphorylation site in HSV-2 UL13 (i.e., Ser-18) and investigated the significance of phosphorylation of this site in HSV-2-infected cell cultures and mice. Our results were as follows. (i) An alanine substitution at UL13 Ser-18 (S18A) significantly reduced HSV-2 replication and cell-to-cell spread in U2OS cells to a level similar to those of the UL13-null and kinase-dead mutations. (ii) The UL13 S18A mutation significantly impaired phosphorylation of a cellular substrate of this viral protein kinase in HSV-2-infected U2OS cells. (iii) Following vaginal infection of mice, the UL13 S18A mutation significantly reduced mortality, HSV-2 replication in the vagina, and development of vaginal disease to levels similar to those of the UL13-null and the kinase-dead mutations. (iv) A phosphomimetic substitution at UL13 Ser-18 significantly restored the phenotype observed with the UL13 S18A mutation in U2OS cells and mice. Collectively, our results suggested that phosphorylation of UL13 Ser-18 regulated UL13 function in HSV-2-infected cells and that this regulation was critical for the functional activity of HSV-2 UL13 *in vitro* and *in vivo* and also for HSV-2 replication and pathogenesis.

4. Herpes Simplex Virus 1 VP22 Inhibits AIM2-Dependent Inflammasome Activation to Enable Efficient Viral Replication

Yuhei Maruzuru, Takeshi Ichinohe[1], Ryota Sato[2], Kensuke Miyake[2], Tokuju Okano[3], Toshihiko Suzuki[3], Takumi Koshiba[4], Naoto Koyanagi, Shumpei Tsuda, Mizuki Watanabe, Jun Arii, Akihisa Kato, and Yasushi Kawaguchi: [1]Division of Viral Infection, Department of Infectious Disease Control, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo [2]Division of Innate Immunity, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo [3]Department of Bacterial Pathogenesis, Infection and Host Response, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University [4]Department of Biology, Faculty of Science, Kyushu University

The AIM2 inflammasome is activated by DNA, leading to caspase-1 activation and release of pro-inflammatory cytokines interleukin 1β (IL-1β) and IL-18, which are critical mediators in host innate immune responses against various pathogens. Some viruses employ strategies to counteract inflammasome-mediated induction of pro-inflammatory cytokines, but their *in vivo* relevance is less well understood. Here we show that the herpes simplex virus 1 (HSV-1) tegument protein VP22 inhibits AIM2-dependent inflammasome activation. VP22 interacts with AIM2 and prevents its oligomerization, an initial step in AIM2 inflammasome activation. A mutant virus lacking VP22 (HSV-1ΔVP22) activates AIM2 and induces IL-1β and IL-18 secretion, but these responses are lost in the absence of AIM2. Additionally, HSV-1ΔVP22 infection results in diminished viral yields *in vivo*, but HSV-1ΔVP22 replication is largely restored in AIM2-deficient mice. Collectively, these findings reveal a mechanism of HSV-1 evasion of the host immune response that enables efficient viral replication *in vivo*.

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