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Highly pathogenic viral agents causing emerging infectious diseases are of concern not only as public health problems but also as possible biological weapons. The ultimate goal of our research is to unlock the secrets of their pathogenicity in human and to develop effective vaccines and antiviral compounds against these pathogens. We have been investigating the molecular basis for replication cycle and extreme virulence of special pathogens, using Ebola, influenza, and Nipa viruses as models.

F1Fo-ATPase, F-type proton-translocating ATPase, at the plasma membrane is critical for efficient influenza virus budding.

Gorai T, Goto H, Noda T, Watanabe T, Kozuka-Hata H, Oyama M, Takano R, Neumann G, Watanabe S, Kawaoka Y.

The identification of host factors involved in virus replication is important to understand virus life cycles better. Accordingly, we sought host factors that interact with the influenza viral nonstructural protein 2 by using coimmunoprecipitation followed by mass spectrometry. Among proteins associating with nonstructural protein 2, we focused on the β subunit of the F1Fo-ATPase, which received a high probability score in our mass spectrometry analysis. The siRNA-mediated down-regulation of the β subunit of the F1Fo-ATPase reduced influenza virion formation and virus growth in cell culture. We further found that efficient influenza virion formation requires the ATPase activity of F1Fo-ATPase and that plasma membrane-associated, but not mitochondrial, F1Fo-ATPase is important for influenza virion formation and budding. Hence, our data identify plasma membrane-associated F1Fo-ATPase as a critical host factor for efficient influenza virus replication.

Enhanced growth of influenza vaccine seed viruses in Vero cells mediated by broadening the optimal pH range for virus membrane fusion.

Murakami S, Horimoto T, Ito M, Takano R, Katsura H, Shimojima M, Kawaoka Y.

Vaccination is one of the most effective preventive measures to combat influenza. Prospectively, cell culture-based influenza vaccines play an important role for robust vaccine production in both normal settings and urgent situations, such as during the 2009 pandemic. African green monkey Vero cells are recommended by the World Health Organization as a safe substrate for influenza vaccine production for human use. However, the growth of influenza vaccine seed viruses is occasionally suboptimal in Vero cells, which places limitations on their usefulness for enhanced vaccine produc-

tion. Here, we present a strategy for the development of vaccine seed viruses with enhanced growth in Vero cells by changing an amino acid residue in the stem region of the HA2 subunit of the hemagglutinin (HA) molecule. This mutation optimized the pH for HA-mediated membrane fusion in Vero cells and enhanced virus growth 100 to 1,000 times in the cell line, providing a promising strategy for cell culture-based influenza vaccines.

Phosphorylation of measles virus phosphoprotein at S86 and/or S151 downregulates viral transcriptional activity.

Sugai A, Sato H, Yoneda M, Kai C.

Measles virus phosphoprotein (P protein) is a cofactor of the viral RNA polymerase (L protein) that associates with the nucleoprotein-RNA complex to support viral transcription and replication. In this study, we showed a significant inverse correlation between the phosphorylation level of MV-P protein and viral transcriptional activity. Upregulation of P protein phosphorylation resulted in reduction of viral transcription. Additionally, we found that strong phosphorylation at S86 and S151 of P protein, which may be generally prevented by association with nucleoprotein, downregulates the viral transcriptional activity. These findings suggest that P protein is involved in regulation of viral transcription through changes in its phosphorylation status.

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Our special interest is focused upon searching for effective methods to protect or regulate bacterial infection by using accumulated knowledge based on molecular pathogenicity, and developing animal models for studying the bacterial pathogens and attenuated strains for novel vaccines. Our other research targets are HIV immune responses and viral tropism. In order to understand immunopathogenesis of HIV infection, we are focusing on T cell dysfunction during chronic HIV infection. The works have been conducted by close collaboration with Division of Bacterial Infection, Division of Infectious Diseases and Department of Infectious Diseases and Applied Immunology.

1. A bacterial effector deamidates Ubc13 to dampen the inflammatory response.

Sanada T, Kim M, Mimuro H, Suzuki M, Ogawa M, Oyama A, Ashida H, Kobyashi T, Koyama T[1], Nagai S[1], Shibata Y[2], Gohda J[2], Inoue J[2], Mizushima T[3], and Sasakawa C.: [1]Nippon Institute for Biological Science, Shinmachi, Ome, Tokyo, Japan, [2]Division of Cellular and Molecular Biology; Department of Cancer Biology; Institute of Medical Science, University of Tokyo, Tokyo, Japan, [3]Picobiology Institute; Department of Life Science; Graduate School of Life Science; University of Hyogo, Hyogo, Japan.

Upon infection of many bacterial pathogens, bacterial invasion is quickly sensed by the innate immune system and triggers acute inflammatory responses. However, it is still unclear how pathogens modulate host inflammatory responses. We found that a *Shigella* OspI effector delivered via the type III secretion system dampens acute inflammatory responses during bacterial invasion by targeting TNF receptor-associated factor 6 (TRAF6). OspI was a glutamine deamidase and selectively deamidated Gln100 to Glu100 in Ubc13. Consequently, the E2 ubiquitin-conjugating activity that is required for TRAF6 activation was inhibited, allowing *Shigella* OspI to modulate the diacylglycerol-CBM complex-TRAF6-NF-κB signaling pathway. We determined the 2.0 Å crystal structure of OspI, which contains a putative Cys-His-Asp catalytic triad. A mutational analysis showed that this catalytic triad was essential for deamidation activity. Our results suggest that *Shigella* inhibits acute inflammatory responses at the initial stage of infection by targeting the Ubc 13-TRAF6 complex.

2. Shigella Targets Epithelial Tricellular Junctionsand Uses a Noncanonical Clathrin-Dependent Endocytic Pathway to Spread Between Cells.

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Bacteria move between cells in the epithelium using a sequential pseudopodium-mediated process but the underlying mechanisms remain unclear. We show that during cell-to-cell movement, *Shigella*-containing pseudopodia target epithelial tricellular-junctions, the contact point where three epithelial cells meet. The bacteria-containing pseudopodia were engulfed by neighboring cells only in the presence of tricellulin, a protein essential for tricellular junction integrity. *Shigella* cell-to-cell spread, but not pseudopodium protrusion, also depended on phosphoinositide 3-kinase, clathrin, Epsin-1, and Dynamin-2, which localized beneath the plasma membrane of the engulfing cell. Depleting tricellulin, Epsin-1, clathrin, or Dynamin-2 expression reduced *Shigella* cell-to-cell spread, whereas AP-2, Dab2,and Eps15 were not critical for this process. Our findings highlight a mechanism for Shigella dissemination into neighboring cells via targeting of tricellular junctions and a noncanonical clathrin-dependent endocytic pathway.

3. DNA hypermethylation of a specific CpG site in IL-2 promoter region occurs in CD4+ T cells during chronic HIV-1 infection

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Although T cell functions are defective during chronic HIV-1 infection, the mechanism by which the persistent presence of HIV-1 damages immune cells is still not fully understood. To evaluate how HIV-1 affects disruption of T cell-mediated immune responses during chronic HIV-1 infection, we assessed capacity of T cells to express multiple cytokines in primary response to non-specific T cell stimulation in untreated chronic HIV-1-infected subjects. We found that only IL-2 expression was significantly lower in high viral load (HVL) than low viral load (LVL) subjects. As epigenetic modification is one of the important regulations of gene expression, we assessed DNA methylation status of IL-2 promoter/enhancer region in CD4+ and CD8+ T cells in HIV-1-infected subjects. We found one of the CpG sites (CpG site 1) was highly methylated in HVL subjects compared to LVL subjects only in CD4+ T cells, and the difference was significant. The DNA methylation status at CpG site 1 was inversely correlated to IL-2 mRNA expression in CD4+ T cells. These data suggest that the methylation status of CpG site 1 is critical for IL-2 transcription. Furthermore, in treated subjects, the DNA methylation status was almost comparable to that of LVL subjects, indicating hypermethylated status at CpG site 1, which associates with IL-2 expression level, was recovered after VL reduction by antiretroviral therapy. Notably, CD4+ T cells with CD57 expression, which is defined as a marker of replicative senescence, were highly methylated at CpG site 1 compare to CD57-negative CD4+ T cell. The IL-2 expression in CD57-positive CD4+ T cells was much lower than CD57-negative CD4+ T cells even after PMA/ionomycin stimulation. Our data suggest that cellular senescence following persistent immune activation and cell turnover during chronic HIV-1 infection leads to DNA hypermethylation at CpG site 1 within IL-2 promoter region in CD4+ T cells.

4. Phenotypic determination of HIV-1 envelope tropism using Dual Split Protein (DSP) mediated cell fusion assay system.

Noriaki Hosoya, Phairote Teeranaipong[1], Stucki Heinz[1], Yoshimi Kashi[1], Ai Kawana-Tachikawa[1], Naoyuki Kondo[2], Hiroo Hoshino[3], Zene Matsuda[2], Aikichi Iwamoto: [1]Division of Infectious Diseases, Advanced Clinical Research Center, [2]Research Center for Asian Infectious Diseases, [3]Gunma University Graduate School of Medicine, Gunma, Japan

Human Immunodeficiency virus type 1 (HIV-1) can be divided into three different classes based on its ability to utilize the CCR5 and CXCR4 co-receptors: viruses using CCR5 but not CXCR4 (R5 virus), those using CXCR4 but not CCR5 (X4 virus), and those that can use either co-receptor (dualtropic virus, R5/X4 virus). Co-receptor usage is one of the most fundamental steps in HIV-1 replication. Since the first CCR5 inhibitor, maraviroc, was commercialized, tropism assays have become a leading topic in HIV-1 research. We developed a novel HIV-1 phenotypic tropism assay based on the cell fusion. The procedure does not include the infectious viruses in the procedure and very rapid to get the results. We employed dual split protein (DSP) composed of split green fluorescent protein (GFP) and split renilla luciferase (RL) as a marker for cell

fusion. DSP_{1-7} and DSP_{8-11} are fusion proteins of GFP and RL. Although expression of either of them does not express the activities, both activities can be recovered by after cell fusion events. N terminus of GFP and RL (DSP_{1-7}) were stably expressed in NP2 cells expressing CD4/CXCR4 (N4X4-DSP_{1-7}) or CD4/CCR5 (N4R5-DSP_{1-7}). The HIV-1 envelope genes from HIV-1 reference strains or clinical samples were ligated into an expression vector containing the C terminus of GFP and RL (DSP_{8-11}), so called pRE11-env. pRE11-env was transfected into 293FT cells. Two days post-transfection, the 293FT cells were overspread on to N4X4-DSP_{1-7} or N4R5-DSP_{1-7}. After 6 hours of co-cultivation the tropism could be determined by detection of either GFP signal (by Fluorescent Microscope, In Cell Analyzer) or luciferase activity (by Luminometer and Enduren) that resulted from re-association of DSP_{1-7} and DSP_{8-11} among fused cells. We could determine the tropism of clinical samples using DSP-Pheno assay and compared the results with those of a pesudovirus assay. The assay can be used for basic research, epidemiologic study, diagnostic tests, drug development, etc, in both resource-rich and -limited settings.

Publications

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International Research Center for Infectious Diseases

Department of Infectious Disease Control Division of Viral Infection
感染制御部門・ウイルス学分野

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We focus on understanding how viruses are recognized by NLRP3 inflammasome and how the innate recognition receptor controls antigen-specific adaptive immune responses. We study immune responses to influenza viruses in the lung. Our recent focus also includes the study of how microbiota regulates adaptive immune responses to these pathogens. Our ultimate goal is to utilize the knowledge we gain through these areas of research in the rational design of effective vaccines for the prevention of infectious diseases.

1. Encephalomyocarditis virus viroporin 2B activates NLRP3 inflammasome.

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Nod-like receptors (NLRs) comprise a large family of intracellular pattern- recognition receptors. Members of the NLR family assemble into large multiprotein complexes, termed the inflammasomes. The NLR family, pyrin domain-containing 3 (NLRP3) is triggered by a diverse set of molecules and signals, and forms the NLRP3 inflammasome. Recent studies have indicated that both DNA and RNA viruses stimulate the NLRP3 inflammasome, leading to the secretion of interleukin 1 beta (IL-1β) and IL-18 following the activation of caspase-1. We previously demonstrated that the proton-selective ion channel M2 protein of influenza virus activates the NLRP3 inflammasome. However, the precise mechanism by which NLRP3 recognizes viral infections remains to be defined. Here, we demonstrate that encephalomyocarditis virus (EMCV), a positive strand RNA virus of the family Picornaviridae, activates the NLRP3 inflammasome in mouse dendritic cells and macrophages. Although transfection with RNA from EMCV virions or EMCV-infected cells induced robust expression of type I interferons in macrophages, it failed to stimulate secretion of IL-1β. Instead, the EMCV viroporin 2B was sufficient to cause inflammasome activation in lipopolysaccharide-primed macrophages. While cells untransfected or transfected with the gene encoding the EMCV non-structural protein 2A or 2C expressed NLRP3 uniformly throughout the cytoplasm, NLRP3 was redistributed to the perinuclear space in cells transfected with the gene encoding the EMCV 2B or influenza virus M2 protein. 2B proteins of other picornaviruses, poliovirus and enterovirus 71, also caused the NLRP3 redistribution. Elevation of the intracellular $Ca(2+)$ level, but not mitochondrial reactive oxygen species and lysosomal cathepsin B, was important in EMCV-induced NLRP3 inflammasome activation. Chelation of extracellular $Ca(2+)$ did not reduce virus-induced IL-1β secretion. These results indicate that EMCV activates the NLRP3 inflammasome by stimulating $Ca(2+)$ flux from intracellular storages to the cytosol, and highlight the importance of viroporins, transmembrane pore-forming viral proteins, in virus-induced NLRP3 inflammasome activation.

2. IL-1R signaling in dendritic cells replaces pattern-recognition receptors in promoting CD8 + T cell responses to influenza A virus.

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Immune responses to vaccines require direct recognition of pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) on dendritic cells (DCs). Unlike vaccination, infection by a live pathogen often impairs DC function and inflicts additional damage on the host. Here we found that after infection with live influenza A virus, signaling through the interleukin 1 receptor (IL-1R) was required for productive priming of CD8 + T cells, but signaling through the PRRs TLR7 and RIG-I was not. DCs activated by IL-1 in *trans* were both required and sufficient for the generation of virus-specific CD8 + T cell immunity. Our data demonstrate a critical role for a bystander cytokine in the priming of CD8 + T cells during infection with a live virus.

Publications

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International Research Center for Infectious Diseases

Department of Infectious Disease Control Division of Bacteriology
感染制御系　細菌学分野

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Bacteria-gut interplay and the host immune response are the most critical issues in determining the fate of bacterial infection and severity of the diseases. Our group has been studying pathogenesis of gastrointestinal bacteria, such as Helicobacter pylori, enteropathogenic E. coli, and Shigella, by defining the molecular and cellular mechanisms of infection and the roles of factors of pathogens and host in infection. The expected output of our research will not only shed further light into understanding bacterial pathogenesis, but also provide new paradigm in microbiology, cell biology, immunity, and pathology, and strengthen the molecular basis in developing diagnostic products, vaccines, animal models, and therapeutic agents.

1. The *Shigella flexneri* effector OspI deamidates UBC13 to dampen the inflammatory response.

Sanada T, Kim M[1], Mimuro H, Suzuki M[2], Ogawa M[2], Oyama A[2], Ashida H[1], Kobayashi T, Koyama T[3], Nagai S[3], Shibata Y[4], Gohda J[4], Inoue J[4], Mizushima T[5], Sasakawa C[3].: [1]Division of Bacterial Infection Biology, Institute of Medical Science, University of Tokyo, [2]Division of Bacterial Infection, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, [3]Nippon Institute for Biological Science, [4]Division of Cellular and Molecular Biology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, [5]Picobiology Institute, Department of Life Science, Graduate School of Life Science, University of Hyogo.

Many bacterial pathogens can enter various host cells and then survive intracellularly, transiently evade humoral immunity, and further disseminate to other cells and tissues. When bacteria enter host cells and replicate intracellularly, the host cells sense the invad- ing bacteria as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) by way of various pattern recognition receptors. As a result, the host cells induce alarm signals that activate the innate immune system. Therefore, bacteria must modulate host inflammatory signalling and dampen these alarm signals. How pathogens do this after invading epithelial cells remains unclear, however. Here we show that OspI, a *Shigella flexneri* effector encoded by ORF169b on the large plasmid and delivered by the type III secretion system, dampens acute inflammatory responses during bacterial invasion by suppressing the tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6)-mediated signalling pathway. OspI is a glutamine deamidase that selectively deamidates the glutamine residue at position 100 in UBC13 to a glutamic acid residue. Consequently, the E2 ubiquitin-conjugating activity required for TRAF6 activation is inhibited, allowing *S. flexneri* OspI to modulate the diacylglycerol- CBM (CARD-BCL10-MALT1)

complex-TRAF6-nuclear-factor- κB signalling pathway. We determined the 2.0 Å crystal structure of OspI, which contains a putative cysteine-histidine-aspartic acid catalytic triad. A mutational analysis showed this catalytic triad to be essential for the deamidation of UBC13. Our results suggest that *S. flexneri* inhibits acute inflammatory responses in the initial stage of infection by targeting the UBC13-TRAF6 complex.

2. *Shigella* targets epithelial tricellular junctions and uses a noncanonical clathrin-dependent endocytic pathway to spread between cells.

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Bacteria move between cells in the epithelium using a sequential pseudopodium-mediated process but the underlying mechanisms remain unclear. We show that during cell-to-cell movement, *Shigella*-containing pseudopodia target epithelial tricellular junctions, the contact point where three epithelial cells meet. The bacteria-containing pseudopodia were engulfed by neighboring cells only in the presence of tricellulin, a protein essential for tricellular junction integrity. *Shigella* cell-to-cell spread, but not pseudopodium protrusion, also depended on phosphoinositide 3-kinase, clathrin, Epsin-1, and Dynamin-2, which localized beneath the plasma membrane of the engulfing cell. Depleting tricellulin, Epsin-1, clathrin, or Dynamin-2 expression reduced Shigella cell-to-cell spread, whereas AP-2, Dab2, and Eps15 were not critical for this process. Our findings highlight a mechanism for *Shigella* dissemination into neighboring cells via targeting of tricellular junctions and a noncanonical clathrin-dependent endocytic pathway.

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International Research Center for Infectious Diseases

Pathogenic Microbes Repository Unit
病原微生物資源室

Associate Professor Hitomi Mimuro, Ph.D. | 准教授 医学博士 三 室 仁 美

This unit is collecting standardized bacterial strains and distributing to research organizations, hospital laboratories, and medical educational institutions throughout the country. In addition, under cooperation with the Japanese Society for Bacteriology, we are distributing authorized bacterial strains for microbiology course for medical school.

Our society is always threatened by emerging and reemerging infectious diseases with various kinds of altitude pathogenic microbes owing to increased foreign tourism, import increase including food, food poisoning such as the O-157 epidemic, and bioterrorism. In addition, by advanced medical developments, the aging society, and increased HIV infection, the quick identification of and therapy for opportunistic infection causative agents and multiple drug resistance bacteria have become important in the medical field.

The need for researchers and clinical practitioners specialized in bacteriology and infectious diseases have risen remarkably, and the substantial study and education required is an emergent problem. For thorough study and education, knowledge of bacteriology, a system of collecting pathogenic microorganism strains of reliable origin, to maintain and save them appropriately, and to provide them to cutting-edge researchers or educational establishments is indispensable. However, in Japan, research into pathogenic microorganisms and infectious diseases is performed mainly in universities, where there is no system for conservation and supply. Therefore, valuable bacterial strains have faced disappearance. Furthermore, under the CARTAGENA PROTOCOL ON BIOSAFETY for conventions of biological diversity, the provision and purchase of pathogenic microorganisms from foreign countries has become difficult.

In such circumstances, we are collecting, saving, and analyzing the pathogenicity of microorganisms and distributing pathogenic bacteria to 1) offer type cultures as a positive control in research, education and examinations, 2) prepare pathogenic bacterial strains that have socially high importance, and 3) offer microbes to universities or public research organizations for training or research. We possess about 1,500 strains that almost cover the main pathogenic microbes, including strains valuable internationally such as pathogenic *E. coli* of Orskov's collection, which is stored only in our laboratory in Japan. Furthermore, it is important to secure their utility as type cultures by preparing genomic and genetic information about the pathogenicity of our bacterial collection based on the researches of the Division of Bacterial Infection. Thus, our laboratory is expected to contribute to countermeasures against infectious disease, and to the education and research of medical microbiology in our country.

Collection, preservation and data management of bacterial strains

It is necessary for us to collect representative type strains and the derivatives of pathogenic microbes corresponding to the following six items.

a) Comprehensive collection of genome sequencing

strains.

b) The causative agents of hospital-acquired (nosocomial) infection, such as opportunistic infectious bacteria and antibiotic-resistant bacteria.
c) Pathogenic *Escherichia coli* associated with the intestinal and urinary tract or meningeal infections, including *Shigella*, EPEC and EHEC O-157.
d) Intracellular bacterial pathogens such as *Mycobacterium avium* and obligate intracellular bacteria.
e) Zoonotic agents causing brucellosis (*Brucella*), leptospirosis (*Leptospira*), and so on.
f) Pathogens causing newly emerging infections and outbreaks, such as *Helicobacter pylori*, *Salmonella* spp. and *Clostridium* perfringens.

We dissect the biochemical properties of bacterial strains collected by deposition, and maintain them appropriately. We are also opening the database of our collection to the public.

Distribution of bacterial strains

We are distributing standardized bacterial strains to research organizations, hospital laboratories, and medical educational institutions throughout the country. In addition, under cooperation with the Japanese Society for Bacteriology, we are distributing authorized bacterial strains for microbiology course for medical school.

Value-added creation of a bacterial strain collection by pathogenic analysis

We are analyzing the pathogenicity of pathogenic microorganisms, especially pathogenic *E. coli*, the pathogenicity of new bacterial infection causative agents in cooperation with the Division of Bacterial Infection. Our collection has original added value by offering this information to users.