RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Virology
ウイルス感染分野

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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. Selection of antigenically advanced variants of seasonal influenza viruses

Li C[1], Hatta M[1], Burke DF[2], Ping J[1], Zhang Y[1], Ozawa M[3], Taft AS[1], Das SC[1], Hanson AP[1], Song J[1], Imai M, Wilker PR[1], Watanabe T, Watanabe S[4], Ito M, Iwatsuki-Horimoto K, Russell CA[5], James SL[2], Skepner E[2], Maher EA[1], Neumann G[1], Klimov AI[6], Kelso A[7], McCauley J[8], Wang D[9], Shu Y[9], Odagiri T[10], Tashiro M[10], Xu X[6], Wentworth DE[6], Katz JM[6], Cox NJ[6], Smith DJ[2], Kawaoka Y: [1]Department of Pathobiological Sciences, Influenza Research Institute, School of Veterinary Medicine, University of Wisconsin-Madison, USA. [2]Department of Zoology, University of Cambridge, UK. [3]Laboratory of Animal Hygiene; Transboundary Animal Distance Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Japan, [4]ERATO Infection-Induced Host Responses Project, Japan. [5]World Health Organization Collaborating Centre for Modelling, Evolution, and Control of Emerging Infectious Diseases, UK. [6]Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, USA. [7]WHO Collaborating Centre for Reference and Research on Influenza (VIDRL) at the Peter Doherty Institute for Infection and Immunity, Australia. [8]Division of Virology, MRC National Institute for Medical Research, UK. [9]Chinese National Influenza Center, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, China. [10]Influenza Virus Research Center, National Institute of Infectious Diseases, Japan.

Influenza viruses mutate frequently, necessitating constant updates of vaccine viruses. To establish experimental approaches that may complement the current vaccine strain selection process, we selected

antigenic variants from human H1N1 and H3N2 influenza virus libraries possessing random mutations in the globular head of the haemagglutinin protein (which includes the antigenic sites) by incubating them with human and/or ferret convalescent sera to human H1N1 and H3N2 viruses. We also selected antigenic escape variants from human viruses treated with convalescent sera and from mice that had been previously immunized against human influenza viruses. Our pilot studies with past influenza viruses identified escape mutants that were antigenically similar to variants that emerged in nature, establishing the feasibility of our approach. Our studies with contemporary human influenza viruses identified escape mutants before they caused an epidemic in 2014-2015. This approach may aid in the prediction of potential antigenic escape variants and the selection of future vaccine candidates before they become widespread in nature.

2. The host protein CLUH participates in the subnuclear transport of influenza virus ribonucleoprotein complexes

Ando T, Yamayoshi S, Tomita Y, Watanabe S[4], Watanabe T, Kawaoka Y

The nucleus is highly compartmentalized yet dynamic. Subnuclear functions are regulated by controlling the subnuclear localization of the nuclear proteins. Influenza viral ribonucleoprotein (vRNP) is replicated in the nucleus and then exported to the cytoplasm. However, the precise subnuclear localization and transport of vRNPs remain unclear. Here, we show that CLUH, a host protein whose cellular function is not well established, plays a key role in the subnuclear transport of vRNP. Viral PB2 and M1 induced CLUH translocation to the nucleoplasm and SC35-positive speckles, respectively, even though CLUH is usually cytoplasmic. CLUH depletion inhibited the translocation of M1 to SC35-positive speckles, but did not interfere with PB2 localization to the nucleoplasm and disrupted the subnuclear transport of vRNP, abolishing vRNP nuclear export without affecting viral RNA or protein expression. Our findings suggest that CLUH plays a role in the subnuclear transport of progeny vRNP.

3. Development of high-yield influenza B virus vaccine viruses

Ping J[1], Lopes TJ, Neumann G[1], Kawaoka Y

The burden of human infections with influenza A and B viruses is substantial, and the impact of influenza B virus infections can exceed that of influenza A virus infections in some seasons. Over the past few decades, viruses of two influenza B virus lineages (Victoria and Yamagata) have circulated in humans, and both lineages are now represented in influenza vaccines, as recommended by the World Health Organization. Influenza B virus vaccines for humans have been available for more than half a century, yet no systematic efforts have been undertaken to develop high-yield candidates. Therefore, we screened virus libraries possessing random mutations in the six "internal" influenza B viral RNA segments [i.e., those not encoding the major viral antigens, hemagglutinin (HA) and neuraminidase NA]] for mutants that confer efficient replication. Candidate viruses that supported high yield in cell culture were tested with the HA and NA genes of eight different viruses of the Victoria and Yamagata lineages. We identified combinations of mutations that increased the titers of candidate vaccine viruses in mammalian cells used for human influenza vaccine virus propagation and in embryonated chicken eggs, the most common propagation system for influenza viruses. These influenza B virus vaccine backbones can be used for improved vaccine virus production.

4. Complete and Incomplete Genome Packaging of Influenza A and B Viruses

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The genomes of influenza A and B viruses comprise eight segmented, single-stranded, negative-sense viral RNAs (vRNAs). Although segmentation of the virus genome complicates the packaging of infectious progeny into virions, it provides an evolutionary benefit in that it allows viruses to exchange vRNAs with other strains. Influenza A viruses are believed to package their eight different vRNAs in a specific manner. However, several studies have shown that many viruses are noninfectious and fail to package at least one vRNA. Therefore, the genome-packaging mechanism is not fully understood. In this study, we used electron microscopy to count the number of ribonucleoproteins (RNPs) inside the virions of different influenza A and B virus strains. All eight strains examined displayed eight RNPs arranged in a "7 + 1" configuration in which a central RNP was surrounded by seven RNPs. Three-dimensional analysis of the virions showed that at least 80% of the virions packaged all eight RNPs; however, some virions packaged only five to seven RNPs, with the exact proportion depending on the strain examined. These results directly demonstrate that most viruses package eight RNPs, but some do indeed package

fewer. Our findings support the selective genome-packaging model and demonstrate the variability in the number of RNPs incorporated by virions, suggesting that the genome-packaging mechanism of influenza viruses is more flexible than previously thought.

5. Microminipigs as an animal model for influenza A virus infection

Iwatsuki-Horimoto K, Nakajima N[13], Shibata M[14], Takahashi K[13], Sato Y[13], Kiso M, Yamayoshi S, Ito M, Enya S[14], Otake M[14], Kangawa A[14], Lopes T, Ito H[15], Hasegawa H[13], Kawaoka Y: [13]Department of Pathology, National Institute of Infectious Diseases, Japan. [14]Swine and Poultry Research Center, Shizuoka Prefectural Research Institute of Animal Industry, Japan. [15]Ito business planning, Japan.

Pigs are considered a mixing vessel for the generation of novel pandemic influenza A viruses through reassortment because of their susceptibility to both avian and human influenza viruses. However, experiments to understand reassortment in pigs in detail have been limited because experiments with regular-sized pigs are difficult to do. Miniature pigs have been used as an experimental animal model, but, they are still large and require relatively large cages for housing. The microminipig is one of the smallest miniature pigs used for experiments. Introduced in 2010, microminipigs weight around 10kg at an early stage of maturity (6- to 7-months old), and are easy to handling. To evaluate the microminipig as an animal model for influenza A virus infection, we compared the receptor distribution of ten-week-old male pigs (Yorkshire Large White) and microminipigs. We found that both animals have SAα 2,3Gal and SAα2,6Gal in their respiratory tract, with similar distribution of both receptor types. We further found that the sensitivity of microminipigs to influenza A viruses was the same as that of larger miniature pigs. Our findings indicate that the microminipig could serve as a novel model animal for influenza A virus infection.

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感染遺伝学分野

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Immune cells express multiple Toll-like receptors (TLRs) that are concomitantly activated by a variety of pathogen products derived from microbes and viruses. TLRs also sense host derived products such as RNAs and DNAs. Recent reports have indicated that losing the balance of TLRs responses result in autoimmune diseases. Hence, there must exist regulatory mechanisms coordinating the expression, the localization and the function of TLRs to avoid excessive immune responses for endogenous ligands. We found recently a candidate for endogenous ligand. Our research focuses on regulatory mechanisms controlling pathogenic ligand recognition by TLRs.

1. The protective effect of the anti-Toll-like receptor 9 antibody against acute cytokine storm caused by immunostimulatory DNA

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Toll-like Receptor 9 (TLR9) is an innate immune receptor recognizing microbial DNA. TLR9 is also activated by self-derived DNA, such as mitochondrial DNA, in a variety of inflammatory diseases. We show that TLR9 activation *in vivo* is controlled by an anti-TLR9 monoclonal Ab (mAb). A newly established mAb, named NaR9, clearly detects endogenous TLR9 expressed in primary immune cells. The mAb inhibited TLR9-dependent cytokine production *in vitro* by bone marrow-derived macrophages and conventional dendritic cells. Furthermore, NaR9 treatment rescued mice from fulminant hepatitis caused by administering the TLR9 ligand CpGB and D-(+)-galactosamine. The production of proinflammatory cytokines induced by CpGB and D-(+)-galactosamine was significantly impaired by the mAb. These results suggest that a mAb is a promising tool for therapeutic intervention in TLR 9-dependent inflammatory diseases.

2. Type I IFN contributes to the phenotype of *Unc93b1*$^{D34A/D34A}$ mice by regulating TLR7 expression in B cells and dendritic cells

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Toll-like receptor 7 (TLR7) recognizes pathogen-derived and self-derived RNA, and thus a regulatory system for control of the TLR7 response is required to avoid excessive activation. Unc93 ho-

molog B1 (Unc93B1) is a regulator of TLR7 that controls the TLR7 response by transporting TLR7 from the endoplasmic reticulum to endolysosomes. We have previously shown that a D34A mutation in Unc93B1 induces hyperactivation of TLR7, and that *Unc93b1*$^{D34A/D34A}$ mice (D34A mice) have systemic inflammation spontaneously. Here, we examined the roles of inflammatory cytokines such as IFN-γ, IL-17A and type I IFNs to understand the mechanism underlying the phenotype in D34A mice. mRNAs for IFN-γ and IL-I7A in $CD4^+$ T cells increased, but inflammatory phenotype manifesting as thrombocytopenia and splenomegaly was still observed in *Ifng*$^{-/-}$ or *Il17a*$^{-/-}$ D34A mice. In contrast to T cell-derived cytokines, *Ifnar1*$^{-/-}$ D34A mice showed an ameliorated phenotype with lower expression of TLR7 in B cells and cDCs. The amount of TLR7 decreased in B cells from *Ifnar1*$^{-/-}$ D34A mice, but the percentage of $TLR7^+$ cells decreased among $CD8\alpha^-$ conventional dendritic cells (cDCs). In conclusion, type I IFNs maintain expression of TLR7 in B cells and cDCs in different ways; total amount of TLR7 is kept in B cells and $TLR7^+$ population is retained among cDCs. Our results suggested that these TLR7-expressing cells are activated initially and influence TLR7-dependent systemic inflammation.

3. Licensing Toll-like receptor 7 to induce type I interferon by CD11a/CD18 Integrin

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Plasmacytoid dendritic cells (pDCs) sense viral RNA through Toll-like receptor (TLR) 7 and produce type I interferons (IFN-1) to initiate pDC responses against viral infection. pDCs form clusters upon virus infection and cell adhesion enhances IFN-1 responses. Little is known, however, about the molecular mechanism linking cell adhesion with IFN-1 expression. Here we show that cell adhesion licenses TLR7 to traffic for IFN-1 induction. Liganded TLR7 activated CD11a/CD18 integrin in MyD88-dependent manner to induce microtubule elongation. TLR7-containing lysosome was linked with microtubule through a GTPase Arl8b and its effector SKIP, resulting in peripheral TLR7 localization. An IFN-1 signaling molecule, TNF receptor associated factor 3 (TRAF3), was constitutively associated with downstream signaling molecules IκB kinase α and mTORC1. Liganded TLR7 trafficked to mTORC1 and induced association of TRAF6 with TRAF3 and interferon regulatory factor 7 (IRF7). IFN-1 was produced predominantly in pDCs in cell cluster rather than isolated pDCs. These results suggest that IFN-1 induction by TLR7 is limited to clustered pDCs through licensing by cell adhesion molecules.

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

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Toll-like receptor 7 (TLR7) and TLR8 are considered to recognize single-strand RNA (ssRNA) from viruses in endolysosomes and induce antiviral immune response. In addition, these receptors respond to synthetic small molecules, such as R848 and CL075. However, it remains unclear how those two different types of ligands can activate both TLR7 and TLR8. In the crystal structure of human TLR8 (huTLR8) complexed with ssRNA, there were two ligand-binding sites; the first site, a binding site for R848, bound a uridine and the second site bound an oligoribonucleotide (ORN). In reporter assay using HEK293T cells, uridine and ORNs synergistically activated huTLR8, suggesting that huTLR8 recognizes degradation products of ssRNA. We also show that TLR7 recognizes guanosine (G) in the presence of ORN. G and ORN synergistically activated TLR7 in plasmacytoid DCs and induced IFN-α production. By isothermal titration calorimetry experiment, specific binding of G to TLR7/ORN complex was detected and the affinity of G to TLR7/ORN complex was determined to be Kd = 1.5 μM. These results strongly suggest that TLR7 also recognizes degradation products of ssRNA, guanosine and ORN, but not ssRNA itself. In conclusion, endolysosomal nucleosides play a decisive role in TLR7 and TLR8 activation by ssRNA.

Publications

Fukui R, Kanno A, Miyake K. Type I IFN Contributes to the Phenotype of Unc93b1$^{D34A/D34A}$ Mice by Regulating TLR7 Expression in B Cells and Dendritic Cells. J Immunol. 196: 416-27. 2016

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Department of Microbiology and Immunology

Division of Mucosal Immunology
炎症免疫学分野

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Mucosal surfaces are the first line of host defense against foreign substances such as pathogenic microorganisms and allergens. In addition, the mucosal immune system not only senses harmful foreign antigens, but also establishes a tolerance that does not react excessively to antigens such as food-derived proteins and commensal bacteria. Our mission is the understanding molecular and cellular aspects of the mucosal immune system, providing mucosal vaccines to prevent infectious diseases, and establishing mucosal immune therapy to control food allergy and autoimmune diseases such as inflammatory bowel diseases.

1. Phase I Clinical Trial of MucoRice-CTB, a Rice-Based Oral Vaccine

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Despite our knowledge of their advantages, plant-based vaccines remain unavailable for human use in both developing and industrialized countries. A leading, practical obstacle to their widespread use is producing plant-based vaccines that meet governmental regulatory requirements. Here we report the first production according to current Good Manufacturing Practices of a rice-based vaccine, the cholera vaccine MucoRice-CTB, at an academic institution. We conducted a doctor-led Phase I clinical trial using MucoRice-CTB at the Hospital of Institute of Medical Science, the University of Tokyo since 2015 to 2016.

The main endpoint was to confirm the safety and tolerability to the human, and the immunogenicity was also evaluated as a secondary endpoint. In a double-blind clinical trial with placebo-controlled study drug, a trial cohort study group was set up, and 10 real drugs and 10 placebo were administrated in each group. 1 g, 3 g, or 6 g of MucoRice-CTB formulated and pulverized was administered. Safety to the human body which is the main endpoint was confirmed by the safety evaluation committee.

We are currently searching miRNA as biomarkers in serum that may correlate with mucosal IgA immune responses in humans vaccinated with MucoRice-CTB. We are also investigating the antigen specific antibodies with neutralizing activity in se-

rum and stool samples collected from the subjects and metagenome analysis using the stools. From now on, it is expected that new knowledge on the relationship between intestinal bacterial flora and immunity can be obtained along with the development of a plant-based oral vaccine.

2. Development of Nanogel-based Nasal Vaccination System against *S. Pneumoniae*

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Streptococcus pneumoniae cause bacterial pneumonia deaths in the worldwide and its victim is primarily young children and elderly adults. Although there are several types of vaccines comprising several pneumococcal surface polysaccharides, they cannot cover the broad protections. Recently we established the nanogel-based vaccine against *S. pneumoniae* using recombinant protein of pneumococcal surface protein A (PspA) which is well-known as a highly immunogenicity. We chose a couple of PspA proteins including alpha helical regions and proline-rich domains. To examine the PspA-specific immune responses, mice were nasally administered with PspA-nanogel, and then PspA-specific serum IgG antibodies were measured and compared with s.c. injection of PspA precipitated in alum. Nasal administration was performed three times with 1-week interval and then mice were boosted with same antigens in 6-7 weeks after the final immunization. PspA-specific IgG antibodies were appeared around 1-2 weeks after the final immunization of each PspA proteins and levels were comparable to those in systemic immunization. Memory responses after boosting were also equivalent in both immunization methods. To assess the protective immunity, we investigated the efficacy of the vaccination in murine pneumococcal airway infections by five strains of the different clades of *S. pneumoniae*. To perform this, mice were divided into two groups, nasal administration of PspA-nanogel and systemically immunization of PspA in alum. When the PspA antigens were combined and immunized in mice, specific antibodies against all antigens were induced. Finally intranasal vaccination with PspA provided protections against lethal challenges with each S. pneumoniae strain as much as systemic vaccination. Thus, our findings illustrate the nasally administered PspA-nanogel effectively elicit a protective immunity and provide a new approach of the mucosal vaccine system.

3. The Development of Nasal Anti-obesity Vaccine

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Obesity is associated with multiple comorbidities such as cardiovascular diseases and has a huge economic impact on the healthcare system. However, the treatment of obesity remains insufficient in terms of efficacy, tolerability, and safety. We aimed to develop a new vaccine against obesity because vaccination provides advantages in terms of low frequency of administration and prolonged therapeutic effect. To avoid the injectable administration-caused pain and skin-related adverse event, we chose the intranasal route of antigen-delivery. We developed a vaccine-antigen (ghrelin-PspA), which is a recombinant fusion protein incorporating ghrelin, a hormone that stimulates food intake and decreases energy expenditure, and pneumococcal surface protein A (PspA), a candidate of pneumococcal vaccine as a carrier protein. The ghrelin-PspA antigen was mixed with cyclic di-GMP adjuvant to enhance the immunogenicity and incorporated within a nanometer-sized hydrogel for the effective antigen-delivery. Intranasal immunization with the ghrelin-PspA vaccine elicited serum IgG antibodies against ghrelin and attenuated body-weight gain in diet-induced obesity mice. This obesity-attenuating effect was caused by a decrease in fat accumulation and an increase in energy expenditure that was partially due to an increase in the expression of mitochondrial uncoupling protein 1 in brown adipose tissue. The development of this nasal vaccine provides a new strategy for the prevention and treatment of obesity.

4. Mucosal cytokine and chemokine regulation by nasal vaccination

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Mucosal vaccines, especially nasal vaccination, is currently recognized as a promising strategy for the induction of both systemic and mucosal immunity contributing the prevention of infectious disease. In addition to the remarkable ability of mucosal vaccinations to elicit mucosal immune responses over systemic vaccination, nasal vaccination can effectively induce antigen-specific immune responses in both respiratory and vaginal mucosal tissues. To establish fundamental platform and achieve better approaches for developing nasal vaccines, we should understand the molecular and cellular mechanisms underlying the induction of antigen-specific immunity in mucosal surfaces including airway and reproductive tissues following nasal immunization and its associated crosstalk between different mucosal surfaces (e.g., nasal and reproductive mucosa). This research is concerned with specific mucosal cytokine and chemokine signaling pathway after nasal vaccination and their effects on antigen-specific antibody responses. In order to address these objectives, firstly a new immunological role of TSLP (thymic stromal lymphopoietin), an epithelial cytokine, following nasal immunization was investigated. Using pneumococcal surface protein A (PspA) plus cholera toxin (CT) nasal immunization model and TSLPR-KO mice, we found that TSLP-TSLPR signaling cascade is critical for the induction of pneumococcal vaccine antigen-specific IgA response, but not IgG responses, following nasal immunization. After nasal immunization with PspA plus CT, TSLP expression is enhanced in mucosal tissue. It is further demonstrated that mucosal dendritic cells (DCs), but not peripheral DCs, are responsive to TSLP for the induction of IgA production in an IL-6 dependent manner. We anticipate that this investigation on mucosal cytokine / chemokine regulation by nasal immunization could provide a better understanding of unknown molecular mechanism in mucosal immunity. This study highlights the importance of new strategies that activate the specific imprinting and homing pathway for the design of intranasal vaccines against pathogenic respiratory and sexually transmitted diseases.

5. Critical role of Bacteria-type 3 innate lymphoid cells (ILC3)-IL-22 axis for the induction and regulation of Paneth cells

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Intestinal epithelial cells (IECs) have α(1,2)fucosylation which is one of glycosylation pattern for the creation of cohabitation and protective niches. Our previous research showed that α(1,2)fucosylation of Peyer's patch M cells and columnar ECs was distinctly regulated by two forms of α(1,2)fucosyltransferase: Fut1 and Fut2, respectively. Paneth cells are subsets of IECs locating at the crypt parts of intestinal villi, where they secrete antimicrobials. We revealed that Paneth cells possess α(1,2)fucose regulated by Fut1 and Fut2. Analysis of α(1,2)fucose of Paneth cells revealed that there are two types of Paneth cells; $Fut2^{+}$ and $Fut2^{-}$ Paneth cells. We also showed that $Fut2^{+}$ Paneth cells are maintained under Bacteria-ILC3s-IL-22 axis. Moreover, the expression of Reg-III family, which is a pivotal player of the immunosurveillance in the intestine, is associated with Fut2 expression of Paneth cells. Taken together, our findings suggest that Bacteria-ILC3s-IL-22 axis plays critical roles for induction and regulation of Fut2- and Reg-III-positive Paneth cells. Our current study is aiming for the molecular and cellular understanding of the Bacteria-ILC3s-IL-22 axis dependent Paneth cells for their contributions in the creation of healthy intestinal environments.

6. Epithelial fucosylation in the upper airway regions

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Fucosylated carbohydrates are expressed on intestinal epithelial cells. They are involved in the creation of an environmental niche for commensal bacteria. Fucosyltransferase 2 (Fut2) is a key enzyme regulating intestinal epithelial α(1,2)-fucosylation. Although polymorphism of the *FUT2* gene is reported to be associated with various diseases, its role in the upper airway region is still unknown. In current study, we examined α(1,2)-fucosylation in the nasal passage and salivary gland of mice. Fucosylated nasal epithelial cells and salivary gland cells were observed in wild type mice, while those were not found in Fut2 deficient mice. In these regions, α (1,2)-fucosylation was Fut2-dependent. Next we evaluated the extent of α(1,2)-fucosylation in IL-10-deficient mice, the model of spontaneous enterocolitis. Interestingly, α(1,2)-fucosylation in the nasal passage and salivary gland was increased in the IL-10-deficient mice, compared to those in the wild type mice. From these results, we propose that the existence of enterocolitis may have some impact on the fucosylation of extraintestinal regions.

7. Allograft inflammatory factor 1 is a regulator of transcytosis in M cells

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Microfold (M) cells in follicle-associated epithelium (FAE) are specialised antigen-sampling cells that take up intestinal luminal antigens. Transcription factor Spi-B regulates M-cell maturation, but the molecules that promote transcytosis within M cells are not fully identified. Here, we show that mouse allograft inflammatory factor 1 (Aif1) is expressed by M cells and contributes to M-cell transcytosis. FAE in *Aif1*$^{-/-}$ mice shows suppressed uptake of particles and commensal bacteria compared with that in wild-type mice. Translocation of *Yersinia enterocolitica*, but not of *Salmonella enterica* serovar Typhimurium, leading to the generation of antigen-specific IgA antibodies, is also diminished in Aif1-deficient mice. Although β1 integrin, which acts as a receptor for *Y. enterocolitica* via invasin protein, is expressed on the apical surface membranes of M cells, its active form is rarely found in *Aif1*$^{-/-}$ mice. These findings show that Aif1 is important for bacterial and particle transcytosis in M cells.

8. Functional analysis of the tissue-specific molecules expressed in tissue mast cells

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Mast cells (MCs) are located at the tissues associated with body surface such as skin and mucosa. These tissues are continuously exposed to physical and chemical stimuli leading to the unexpected activation of MCs. Once MC activation occurs locally, various inflammatory mediators are released and excessive immune reactions such as allergic and inflammatory responses are subsequently induced. To avoid unnecessary activation of MCs and maintain appropriate immunological homeostasis, there exists a unique suppressive pathway in MCs, mediated by fibroblasts.

In this study, a novel regulatory pathway mediated by skin fibroblasts via the usage of a novel molecule X in skin MCs was newly identified. Molecule X was specifically and highly expressed by skin MCs (unpublished data). In addition, *in vitro* co-culture of bone marrow-derived MCs and skin fibroblasts that the expression of molecule X on MCs was induced by skin fibroblasts. Molecule X deficient and WT mice were subjected to hapten-induced contact dermatitis and it was demonstrated that deficiency of Molecule X enhanced ear swelling response and neutrophil infiltration in comparison to WT mice. These results revealed that the skin

MCs and fibroblasts form anti-inflammatory pathway by cell to cell interaction using molecule X.

9. Generation of oral therapeutic murine model for food allergy for analyzed underlying mechanisms of immune tolerance

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Food allergy is estimated to affect about 5% of children and adults, and can occasionally lead to life-threatening reactions in affected patients. Upon contact with allergen, food allergies frequently lead to gastrointestinal symptoms, including diarrhea, vomiting, and abdominal pain in infants and children. To cure the food allergy, allergen inoculation from oral route to the patients called oral immunotherapy, OIT, is potentially effective and a newly developed therapy; however, most patients experience allergic reactions during the therapy due to the lack of basic understandings of immunological events at intestinal mucosa. In this study, we analyzed OIT murine model and successfully generated a therapeutic model for allergic diarrhea. We further investigated the effects of OIT in the mucosal compartment and found OIT effectively suppressed degranulation of local mast cells. In addition, local increase of regulatory T cells was found. We are now working on effectively modifying the OIT protocol as well as uncovering the immunological events of induction of tolerance in OIT.

10. Lymphoid tissue-resident *Alcaligenes* LPS induces IgA production without excessive inflammatory responses via weak TLR4 agonist activity

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Alcaligenes are opportunistic commensal bacteria that reside in gut-associated lymphoid tissues such as Peyer's patches; however, how they create and maintain their homeostatic environment, without inducing an excessive inflammatory response remained unclear. We show here that *Alcaligenes*-derived lipopolysaccharide (*Alcaligenes* LPS) acts as a weak agonist of toll-like receptor 4 and promotes IL-6 production from dendritic cells, which consequently enhances IgA production. The inflammatory activity of *Alcaligenes* LPS was weaker than that of *E. coli*-derived LPS and therefore no excessive inflammation was induced by *Alcaligenes* LPS *in vitro* or *in vivo*. These features create commensal bacteria-mediated homeostatic inflammatory conditions within Peyer's patches that produce optimal IgA induction without causing pathogenic inflammation. In addition, the weak activity of *Alcaligenes* LPS as a TLR4 agonist raises the possibility of its use as an adjuvant. We are doing additional study of *Alcaligenes* LPS-mediated signaling, such as its role in T cell differentiation and capacity to induce antigen-specific IgA *in vivo*, which can result in the development of a safe and effective adjuvant.

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ウイルス病態制御分野

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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. Multiple Roles of the Cytoplasmic Domain of Herpes Simplex Virus 1 Envelope Glycoprotein D in Infected Cells

Jun Arii, Keiko Shindo, Naoto Koyanagi, Akihisa Kato and Yasushi Kawaguchi

Herpes simplex virus 1 (HSV-1) envelope glycoprotein D (gD) plays an essential role in viral entry. The functional regions of gD responsible for viral entry have been mapped to its extracellular domain, whereas the gD cytoplasmic domain plays no obvious role in viral entry. Thus far, the role(s) of the gD cytoplasmic domain in HSV-1 replication remained to be elucidated. In this study, we showed that ectopic expression of gD induced microvilli-like tubular structures at the plasma membrane, which resembled the reported projection structures of the plasma membrane induced in HSV-1-infected cells. Mutations in the arginine cluster (residues 365 to 367) in the gD cytoplasmic domain greatly reduced gD-induced plasma membrane remodeling. In agreement with this, the mutations in the arginine cluster in the gD cytoplasmic domain reduced the number of microvilli-like tubular structures at the plasma membrane in HSV-1-infected cells. In addition, the mutations produced an accumulation of unenveloped nucleocapsids in the cytoplasm, and reduced viral replication and cell-cell spread. These results suggested that the arginine cluster in the gD cytoplasmic domain was required for the efficient induction of plasma membrane projections and viral final envelopment, and these functions of the gD domain may lead to efficient viral replication and cell-cell spread.

2. The Interaction between Herpes Simplex Virus 1 Tegument Proteins UL51 and UL14 and Its Role in Virion Morphogenesis

Shinya Oda, Jun Arii, Naoto Koyanagi, Akihisa Kato and Yasushi Kawaguchi

To investigate the molecular mechanism(s) by which herpes simplex virus 1 (HSV-1) tegument protein UL51 promotes viral replication, we screened for viral proteins that interact with UL51 in infected cells. Affinity purification of tagged UL51 in HSV-1-infected Vero cells was coupled with immunoblotting of the purified UL51 complexes with various antibodies to HSV-1 virion proteins. Subsequent analyses revealed that UL51 interacted with another tegument protein, UL14, in infected cells. Mutational analyses of UL51 showed that UL51 amino

acid residues Leu-111, Ile-119, and Tyr-123 were required for interaction with UL14 in HSV-1-infected cells. Alanine substitutions of these UL51 amino acid residues reduced viral replication and produced an accumulation of unenveloped and partially enveloped nucleocapsids in the cytoplasm at levels comparable to those of UL51-null, UL14-null, and UL51/UL14 double-null mutations. In addition, although UL51 and UL14 colocalized at juxtanuclear domains in HSV-1-infected cells, the amino acid substitutions in UL51 produced aberrant localization of UL51 and UL14. The effects of these substitutions on localization of UL51 and UL14 were similar to those of the UL51-null and UL14-null mutations on localization of UL14 and UL51, respectively. These results suggested that the interaction between UL51 and UL14 was required for proper localization of these viral proteins in infected cells and that the UL51-UL14 complex regulated final viral envelopment for efficient viral replication.

3. p53 is a Host Cell Regulator during Herpes Simplex Encephalitis

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p53 is a critical host cell factor in the cellular response to a broad range of stress factors. We recently reported that p53 is required for efficient herpes simplex virus 1 (HSV-1) replication in cell culture. However, a defined role for p53 in HSV-1 replication and pathogenesis in vivo remains elusive. In this study, we examined the effects of p53 on HSV-1 infection in vivo using p53-deficient mice. Following intracranial inoculation, p53 knockout reduced viral replication in the brains of mice and led to significantly reduced HSV-1 mortality due to encephalitis. These results suggest that p53 is an important host cell regulator for HSV-1 replication and pathogenesis in the central nervous system (CNS).

4. Roles of Us8A and its Phosphorylation Mediated by Us3 in Herpes Simplex Virus 1 Pathogenesis

Akihisa Kato, Tomoko Ando, Shinya Oda, Mizuki Watanabe, Naoto Koyanagi, Jun Arii and Yasushi Kawaguchi

The herpes simplex virus 1 (HSV-1) Us8A gene overlaps the gene that encodes for glycoprotein E (gE). Previous studies have investigated the roles of Us8A in HSV-1 infection using null-mutations in Us8A and gE; therefore, the role of Us8A remains to be elucidated. In this study, we investigated the function of Us8A and its phosphorylation at serine 61 (Ser-61), which we recently identified as a phosphorylation site by mass spectrometry-based phosphoproteomic analysis of HSV-1-infected cells, in HSV-1 pathogenesis. We observed that (i) the phosphorylation of Us8A Ser-61 in infected cells was dependent on the activity of the virally encoded Us3 protein kinase; (ii) the Us8A null mutant virus exhibited a 10-fold increase in the 50% lethal dose for virulence in the central nervous system (CNS) of mice following intracranial infection compared with a repaired virus; (iii) replacement of Ser-61 with alanine (S61A) in Us8A had little effect on virulence in the CNS of mice following intracranial infection, whereas it significantly reduced the mortality of mice following ocular infection to levels similar to the Us8A null mutant virus; (iv) the Us8A S61A mutation also significantly reduced viral yields in mice following ocular infection, mainly in the trigeminal ganglia and brains; and (v) a phosphomimetic mutation at Us8A Ser-61 restored wild-type viral yields and virulence. Collectively, these results indicate that Us8A is a novel HSV-1 virulence factor and suggest that the Us3-mediated phosphorylation of Us8A Ser-61 regulates Us8A function for viral invasion into the CNS from peripheral sites.

5. Characterization of a herpes simplex virus 1 (HSV-1) chimera in which the Us3 protein kinase gene is replaced with the HSV-2 Us3 gene

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Us3 protein kinases encoded by herpes simplex virus 1 (HSV-1) and 2 (HSV-2) play important roles in viral replication and pathogenicity. To investigate type-specific differences between HSV-1 Us3 and HSV-2 Us3 in cells infected by viruses with all the same viral gene products except for their Us3

kinase, we constructed and characterized a recombinant HSV-1 in which its Us3 gene was replaced with the HSV-2 Us3 gene. Replacement of HSV-1 Us3 with HSV-2 Us3 had no apparent effect on viral growth in cell cultures or on the range of proteins phosphorylated by Us3. HSV-2 Us3 efficiently compensated for HSV-1 Us3 functions, including blocking apoptosis, controlling infected cell morphology, and down-regulating cell surface expression of viral envelope glycoprotein B. In contrast, replacement of HSV-1 Us3 by HSV-2 Us3 changed the phosphorylation status of UL31 and UL34, which are critical viral regulators of nuclear egress. It also caused aberrant localization of these viral proteins, aberrant accumulation of primary enveloped virions in membranous vesicle structures adjacent to the nuclear membrane, and reduced viral cell-cell spread in cell cultures and pathogenesis in mice. These results clearly demonstrated biological differences between HSV-1 Us3 and HSV-2 Us3, especially in regulation of viral nuclear egress and phosphorylation of viral regulators critical for this process. Our study also suggested that the regulatory role(s) of HSV-1 Us3, which were not carried out by HSV-2 Us3 was important for HSV-1 cell-cell spread and pathogenesis in vivo.

6. Ubiquitin-Specific Protease 9X in Host Cells Interacts with Herpes Simplex Virus 1 ICP0

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Herpes simplex virus 1 (HSV-1) expresses infected cell protein 0 (ICP0), a multi-functional protein with E3 ubiquitin ligase activity and a critical regulator of the viral life cycle. To obtain novel insights into the molecular mechanism by which ICP0 regulates HSV-1 replication, we analyzed HEp-2 cells infected with HSV-1 by tandem affinity purification and mass spectrometry-based proteomics. This screen identified 50 host-cell proteins that potentially interact with ICP0, including ubiquitin-specific protease 9X (USP9X). The interaction between ICP0 and USP9X was confirmed by co-immunoprecipitation. Notably, USP9X depletion increased the ICP0 abundance and promoted viral replication. These results suggest that USP9X-dependent regulation of ICP0 expression is part of a complex feedback mechanism that facilitates optimal HSV-1 replication.

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