RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Host-Parasite Interaction
宿主寄生体学

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The goal of our Department is to elucidate the cellular defense system and the counteracting viral strategy at the level of gene regulation and to establish new approaches for suppressing cancer and pathogenic viruses and also for modulating human immunological response. We have been studying epigenetical regulation of human and virus genomes by analyzing regulatory networks formed among microRNAs, chromatine remodeling factor, SWI/SNF complex and such important transcriptional factors as AP-1 and NF-kappaB to explore the cause of human diseases. We have developed and are continuously improving retrovirus/lentivirus vectors that express efficient inhibitory RNAs targeting specific miRNAs (designated as Tough Decoy RNAs) and low molecular inhibitors of miRNA (designated synthetic TuD; S-TuD) for tools of human gene therapy and basic researches.

1. Gene regulatory networks formed in epithelial tumor cell lines.

The SWI/SNF chromatin remodeling complex plays important roles in the epigenetic regulation of many organisms and regulates a wide variety of genes. In human, this complex is an assembly of about nine polypeptides, and each complex contains a single molecule of either Brm or BRG1, but not both. These two proteins are the catalytic subunits and drive the remodeling of nucleosomes via their ATP-dependent helicase activity. Evidence has now accumulated that Brm and BRG1 regulate a set of target promoters that is not fully overlapping. Indeed, Brm and BRG1 show clear differences in their biological activities; Brm, but not BRG1, is essential for the maintenance of gene expression driven by LTRs of murine leukemia virus (MLV) and HIV as we have previously reported. This year, we described detailed mechanisms involved in the elongation steps of short HIV transcripts (a).

NFκB plays crucial roles in such physiological processes as development, cell proliferation viral replication, apoptosis and innate and adaptive immune functions. The NFκB family is composed of five different proteins; RelA (p65), RelB, c-Rel, p50 (which is processed its precursor p105) and p52 (which is processed its precursor p100). These proteins form active transcription factors as homodimers or heterodimers. Importantly, some NFκB target genes stimulated by these cytokines or growth factors were known to often require SWI/SNF complexes for their optimum induction. However, the underlying molecular mechanisms and factors involved in this process are largely unknown, mainly because no direct interaction between NFκB submits and SWI/SNF components has been reported. In 2011, we present evidence that DPF2 (Requiem) protein, which belongs to the d4-family of proteins, is a specific adaptor protein that links RelB/p52 with Brm-type SWI/SNF complexes and thereby plays pivotal roles in the most downstream non-canonical NFκB pathway. Using highly sensitive assay cell lines that harbor two NF-κB

binding sites and a minimal promoter just upstream of the reporter gene, we further found that DPF3a, DPF3b, DPF2 as well as DPF1 and PHF10 potentially activates three representative NF-κB dimmers, RelA/p50, RelB/p52 and c-Rel/p50 transactivation significantly, when they are exogenously expressed at very high levels. We finally showed that among them, DPF3a and DPF3b are most critical component for NF-κB RelA/p50 heterodimer transactivation induced by TNF-α stimulation (canonical pathway).

We previously found that a functional *Brm* gene is present and transcribed actively in all of the Brm-deficient human cancer cell lines in nuclear run-on transcription assays. This indicated that post-transcriptional gene silencing suppresses *Brm* in these human cancer cells. In 2011, we demonstrated that *Brm* mRNA is a target of miR-199a-5p and miR-199a-3p, both of which are processed from pre-miR-199a. By promoter analysis of the *miR-199a-2* gene, which was found to be the main contributor to the production of pri-miR-199a in these cell lines, Egr1 was identified as a major transcriptional activator at this gene locus. Our analysis further showed that the expression patterns of mature miR-199a-5p and -3p, and of the Brm protein, are mutually exclusive in many human tumor cell lines originated from epithelial cells. We final showed that these miRNAs, miR-199a-5p/-3p, Brm and Egr1 form double-negative feedback loops in a wide variety of human cancer cell lines, and each cell lines are fallen into either of the two steady states, [Brm (+)/Egr1(−)1/miR-199 (−)] cells and [Brm (−)/Egr1(+)/miR-199a (+)] cells, which are denoted hereafter as Type 1 and Type 2 cells, respectively. This year, we have analyzed basic biological properties that discuminate these two cell types and revealed that the robust regulatory network formed by Brm/miR-199a axis is very important to understand molecular mechanisms involved in either cancer development (b) or virus replication (c).

(a) 7SK small nuclear ribonucleoprotein complex is recruited to the HIV-1 promoter via short viral transcripts

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In this study, we demonstrate that the 7SK small nuclear ribonucleoprotein (snRNP) complex is recruited to the HIV-1 promoter via newly-synthesized HIV-1 nascent transcripts (short transcripts) in an hnRNP A1-dependent manner and negatively regulates vital transcript elongation. Our deep-sequence analysis showed these short transcripts were mainly arrested at approximately +50 to +70 nucleotides from the transcriptional start site in the U1 cells, an HIV-1 latent model. TNF-α treatment promptly disrupted the 7SK snRNP complex on the nascent transcripts and viral elongated transcripts were increased. This report provides insight into how 7SK snRNP complex is recruited to HIV-1 promoter in the absence of Tat.

(b) The miR-199a/Brm/EGR1 axis is a determinant of anchorage-independent growth in epithelial tumor cell lines

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In epithelial cells, miRNA-199a-5p/-3p and Brm, a catalytic subunit of the SWI/SNF complex were previously shown to form a double-negative feedback loop through EGR1, by which human cancer cell lines tend to fall into either of the steady states, types 1 [miR-199a(−)/Brm(+)/EGR1(−)] and 2 [miR-199a(+)/Brm(−)/EGR1(+)]. We show here, that type 2 cells, unlike type 1, failed to form colonies in soft agar, and that CD44, MET, CAV1 and CAV2 (miR-199a targets), all of which function as plasma membrane sensors and can co-localize in caveolae, are expressed specifically in type 1 cells. Single knockdown of any of them suppressed anchorage-independent growth of type 1 cells, indicating that the miR-199a/Brm/EGR1 axis is a determinant of anchorage-independent growth. Importantly, two coherent feedforward loops are integrated into this axis, supporting the robustness of type 1-specific gene expression and exemplifying how the miRNA-target gene relationship can be stably sustained in a variety of epithelial tumors.

(c) miR-199a target that affect host cell competency for virus replication.

Kyosuke Kobayashi, Shinya Nakamura, Fumiko Suemasa, Kazuyoshi Kobayashi, Hiroaki Hiramatsu, Takeshi Haraguchi, and Hideo Iba

It was previously reported that using global miRNA expression analysis, the miR-199a/miR-214

cluster manifests antiviral properties in mouse and human cells, and further that these molecules confer broad inhibitory potential against multiple viruses. Since in human cancer cell lines originated from epitherial cells, miR-199a/miR-214 expressing cells lacks Brm expression (Type 2 cells) as described above, their report is consistent with our previous report that Type 2 cells (expressing miR-199a/miR-214 at high levels) do not stably express lenti/retrovirus. Using human cancer cell lines infected with HSV-1, as a model system, we have confirmed that high level exogenous expression of miR-199a-3p in A549 cells (Type 1) suppresses HSV-1 replication, whereas suppression of miR-199a-3p in Type 2 cells activated HSV-1 replication. Since in A549 cellsthat are exogenously expressing miR-199a, HSV-1 produces almost equivalent virus proteins to those in parental A549, we expect virus morphogenesis step is affective by miR-199a. We have now identified several target proteins of miR-199a which are important for membrane transports and remodeling of cytoskeleton.

2. New regulatable expression vectors for decoy RNAs (TuD), which is useful to obtain proof of concept (POC).

To achieve the long-term suppression of a specific miRNA, we previously developed specialized plasmid- and virus- vectors carrying expression units producing inhibitory RNA molecules, which we have termed Tough Decoy (TuD) RNA. Last year, we have developed several regulatable RNA polymerase III promoters that can drive TuD production in the presence of doxocyclin. Using this TET-ON system, we have analyzed the process of epithelial-messenchymal transition (EMT) by transiently expressing S-TuD-200c (S-TuD targeting miR-200c) (d)

(d) Development of regulatable vectors for TuD RNA expression.

Takeshi Haraguchi, Masayuki Kondo, Hideo Iba

After comparing the miRNA inhibition activities of TuD RNA expression units with various Pol III promoters, we first developed an optimal Pol III promoter for TuD RNA expression. We next modified the promoter to express TuD RNA in a Tetracycline (Tet)-inducible manner and selected optimal number and position of Tet response elements in the promoter. We finally established Tet-inducible TuD RNA expression unit carried by lentivirus vectors. In the cancer cell lines transduced with the vectors, TuD RNA levels were very low in the absence of Doxycycline (Dox: a Tetracycline derivative), but the expression levels were drastically elevated in the presence of Dox.

It is well known that miR-200c has the activity of suppressing epithelial - mesenchymal transition (EMT) and we have previously reported that the inhibition of miR-200c by TuD RNA expression lentivirus vectors induces EMT. However, it remains unsolved whether maintenance of mesenchymal properties require constitutive miR-200c inhibition or not. Therefore, we are now analyzing the plasticity and the molecular mechanisms involved in EMT using this Tet-inducible TuD RNA expression lentivirus vector established here.

Publications

Siddesha J.M., Valente, A.J., Yoshida, T., Sakamuri, S.S.V.P., Delafontaine, P., Iba, H., Noda, M., Chandrasekar, B. Docosahexaenoic acid reverses angiotensin II-induced RECK suppression and cardiac fibroblast migration. *Cellular Signalling*, 26: 933-941 (2014)

Mizutani, T., Ishizaka, A., Suzuki, Y., and Iba, H. 7 SK small nuclear ribonucleoprotein complex is recruited to the HIV-1 promoter via short viral transcripts. *FEBS Letters*, 588: 1630-1636 (2014)

Kobayashi, K., Sakurai, K., Hiramatsu, H., Inada, K., Shiogama, K., Nakamura, S., Suemasa, F., Kobayashi, K., Imoto, S., Haraguchi, T., Ito, H., Ishizaka, A., Tsutsumi, Y., & Iba H. The miR-199a/Brm/EGR1 axis is a determinant of anchorage-independent growth in epithelial tumor cell lines, *Scientific Reports*, 5: 8428 (2015)

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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, 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 theses viral infections.

1. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses

Yamayoshi S, Yamada S, Fukuyama S, Murakami S[1], Zhao D, Uraki R, Watanabe T, Tomita Y, Macken C[2], Neumann G[3], Kawaoka Y: [1]Department of Veterinary Microbiology, Graduate School of Agriculture and Life Science, University of Tokyo, Japan, [2]Theoretical Division, Los Alamos National Laboratory, USA, [3]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, USA.

Novel avian-origin influenza A(H7N9) viruses were first reported to infect humans in March 2013. To date, 143 human cases, including 45 deaths, have been recorded. By using sequence comparisons and phylogenetic and ancestral inference analyses, we identified several distinct amino acids in the A(H7N9) polymerase PA protein, some of which may be mammalian adapting. Mutant viruses possessing some of these amino acid changes, singly or in combination, were assessed for their polymerase activities and growth kinetics in mammalian and avian cells and for their virulence in mice. We identified several mutants that were slightly more virulent in mice than the wild-type A (H7N9) virus, A/Anhui/1/2013. These mutants also exhibited increased polymerase activity in human cells but not in avian cells. Our findings indicate that the PA protein of A(H7N9) viruses has several amino acid substitutions that are attenuating in mammals.

2. Circulating avian influenza viruses closely related to the 1918 virus have pandemic potential.

Watanabe T, Zhong G[3], Russell CA[4], Nakajima N[5], Hatta M[3], Hanson A[3], McBride R[6], Burke DF[7],

Takahashi K[5], Fukuyama S, Tomita Y, Maher A[3], Watanabe S[8], Imai M[9], Neumann G[3], Hasegawa H[5], Paulson JC[6], Smith DJ[7], Kawaoka Y: [4]Department of Veterinary Medicine, University of Cambridge, UK, [5]Department of Pathology, National Institute of Infectious Diseases, Japan, [6]Department of Cell and Molecular Biology, The Scripps Research Institute, USA, [7]Department of Zoology, University of Cambridge, UK, [8]Laboratory of Veterinary Microbiology, Department of Veterinary Sciences, University of Miyazaki, Japan, [9]Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Japan.

Wild birds harbor a large gene pool of influenza A viruses that have the potential to cause influenza pandemics. Foreseeing and understanding this potential is important for effective surveillance. Our phylogenetic and geographic analyses revealed the global prevalence of avian influenza virus genes whose proteins differ only a few amino acids from the 1918 pandemic influenza virus, suggesting that 1918-like pandemic viruses may emerge in the future. To assess this risk, we generated and characterized a virus composed of avian influenza viral segments with high homology to the 1918 virus. This virus exhibited pathogenicity in mice and ferrets higher than that in an authentic avian influenza virus. Further, acquisition of seven amino acid substitutions in the viral polymerases and the hemagglutinin surface glycoprotein conferred respiratory droplet transmission to the 1918-like avian virus in ferrets, demonstrating that contemporary avian influenza viruses with 1918 virus-like proteins may have pandemic potential.

3. Disease severity is associated with differential gene expression at the early and late phases of infection in nonhuman primates infected with different H5N1 highly pathogenic avian influenza viruses.

Muramoto Y, Shoemaker JE, Le MQ[10], Itoh Y[11], Tamura D, Sakai-Tagawa Y, Imai H, Uraki R, Takano R, Kawakami E, Ito M, Okamoto K[11], Ishigaki H[11], Mimuro H[12], Sasakawa C[13], Matsuoka Y[14], Noda T, Fukuyama S, Ogasawara K[11], Kitano H[14], Kawaoka Y: [10]National Institute of Hygiene and Epidemiology, Vietnam, [11]Department of Pathology, Shiga University of Medical Science, Japan, [12]Division of Bacteriology, Department of Infectious Diseases Control, International Research Center for Infectious Diseases, Institute of Medical Science, The University of Tokyo, Japan, [13]Division of Bacterial Infection Biology, Institute of Medical Science, The University of Tokyo, Japan; Nippon Institute for Biological Science, Japan; Medical Mycology Research Center, Chiba University, Japan, [14]The Systems Biology Institute, Japan.

Occasional transmission of highly pathogenic avian H5N1 influenza viruses to humans causes severe pneumonia with high mortality. To better understand the mechanisms via which H5N1 viruses induce severe disease in humans, we infected cynomolgus macaques with six different H5N1 strains isolated from human patients and compared their pathogenicity and the global host responses to the virus infection. Although all H5N1 viruses replicated in the respiratory tract, there was substantial heterogeneity in their replicative ability and in the disease severity induced, which ranged from asymptomatic to fatal. A comparison of global gene expression between severe and mild disease cases indicated that interferon-induced upregulation of genes related to innate immunity, apoptosis, and antigen processing/presentation in the early phase of infection was limited in severe disease cases, although interferon expression was upregulated in both severe and mild cases. Furthermore, coexpression analysis of microarray data, which reveals the dynamics of host responses during the infection, demonstrated that the limited expression of these genes early in infection led to a failure to suppress virus replication and to the hyperinduction of genes related to immunity, inflammation, coagulation, and homeostasis in the late phase of infection, resulting in a more severe disease. Our data suggest that the attenuated interferon-induced activation of innate immunity, apoptosis, and antigen presentation in the early phase of H5N1 virus infection leads to subsequent severe disease outcome.

4. Detection sensitivity of influenza rapid diagnostic tests.

Sakai-Tagawa Y, Ozawa M[15], Yamada S, Uchida Y[16], Saito T[16], Takahashi K[17], Sugaya N[18], Tashiro M[19], Kawaoka Y: [15]Laboratory of Animal Hygiene; Transboundary Animal Distance Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Japan, [16]Influenza and Prion Disease Research Center, National Institute of Animal Health, Japan, [17]Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Japan, [18]Department of Pediatrics, Keiyu Hospital, Japan, [19]Influenza Virus Research Center, National Institute of Infectious Diseases, Japan.

The sensitivity of influenza rapid diagnostic tests (IRDTs) currently available in Japan for various influenza virus strains, including human H7N9 and H5N1 isolates, were compared and it was found that all of the IRDTs examined detected these viruses; however, their detection sensitivities differed.

5. Hemozoin as a novel adjuvant for inactivated whole virion influenza vaccine.

Uraki R, Das SC[3], Hatta M[3], Kiso M, Iwatsuki-Horimoto K, Ozawa M[15], Coban C[20], Ishii KJ[21], Kawaoka Y: [20]Laboratory of Malaria Immunology, Immunology Frontier Research Center (IFReC), Osaka University, Japan, [21]Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Japan; Laboratory of Vaccine Science, IFReC, Osaka University, Japan.

Because vaccination is an effective means to protect humans from influenza viruses, extensive efforts have been made to develop not only new vaccines, but also for new adjuvants to enhance the efficacy of existing inactivated vaccines. Here, we examined the adjuvanticity of synthetic hemozoin, a synthetic version of the malarial by-product hemozoin, on the vaccine efficacy of inactivated whole influenza viruses in a mouse model. We found that mice immunized twice with hemozoin-adjuvanted inactivated A/California/04/2009 (H1N1pdm09) or A/Vietnam/1203/2004 (H5N1) virus elicited higher virus-specific antibody responses than did mice immunized with non-adjuvanted counterparts. Furthermore, mice immunized with hemozoin-adjuvanted inactivated viruses were better protected from lethal challenge with influenza viruses than were mice immunized with non-adjuvanted inactivated vaccines. Our results show that hemozoin improves the immunogenicity of inactivated influenza viruses, and is thus a promising adjuvant for inactivated whole virion influenza vaccines.

6. Influenza virus-host interactome screen as a platform for antiviral drug development.

Watanabe T, Kawakami E, Shoemaker JE, Lopes TJS, Matsuoka Y[14], Tomita Y, Kozuka-Hata H[22], Gorai T, Kuwahara T, Takeda E, Nagata A, Takano R, Kiso M, Yamashita M, Sakai-Tagawa Y, Katsura H, Nonaka N, Fujii H, Fujii K, Sugita Y, Noda T, Goto H, Fukuyama S, Watanabe S, Neumann G[3], Oyama M[22], Kitano H[14], Kawaoka Y: [22]Medical Proteomics Laboratory, Institute of Medical Science, University of Tokyo, Japan.

Host factors required for viral replication are ideal drug targets because they are less likely than viral proteins to mutate under drug-mediated selective pressure. Although genome-wide screens have identified host proteins involved in influenza virus replication, limited mechanistic understanding of how these factors affect influenza has hindered potential drug development. We conducted a systematic analysis to identify and validate host factors that associate with influenza virus proteins and affect viral replication. After identifying over 1,000 host factors that coimmunoprecipitate with specific viral proteins, we generated a network of virus-host protein interactions based on the stage of the viral life cycle affected upon host factor downregulation. Using compounds that inhibit these host factors, we validated several proteins, notably Golgi-specific brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1) and JAK1, as potential antiviral drug targets. Thus, virus-host interactome screens are powerful strategies to identify targetable host factors and guide antiviral drug development.

7. A Bivalent Vaccine Based on a Replication-Incompetent Influenza Virus Protects against Streptococcus pneumoniae and Influenza Virus Infection.

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Streptococcus pneumoniae is a major causative pathogen in community-acquired pneumonia; together with influenza virus, it represents an important public health burden. Although vaccination is the most effective prophylaxis against these infectious agents, no single vaccine simultaneously provides protective immunity against both S. pneumoniae and influenza virus. Previously, we demonstrated that several replication-incompetent influenza viruses efficiently elicit IgG in serum and IgA in the upper and lower respiratory tracts. Here, we generated a replication-incompetent hemagglutinin knockout (HA-KO) influenza virus possessing the sequence for the antigenic region of pneumococcal surface protein A (PspA). Although this virus (HA-KO/PspA virus) could replicate only in an HA-expressing cell line, it infected wild-type cells and expressed both viral proteins and PspA. PspA- and influenza virus-specific antibodies were detected in nasal wash and bronchoalveolar lavage fluids and in sera from mice intranasally inoculated with HA-KO/PspA virus, and mice inoculated with HA-KO/PspA virus were completely protected from lethal challenge with either S. pneumoniae or influenza virus. Further, bacterial colonization of the nasopharynx was prevented in mice immunized with HA-KO/PspA virus. These results indicate that HA-KO/PspA virus is a promising bivalent vaccine candidate that simultaneously confers protective immunity against both S. pneumoniae and influenza virus. We believe that this strategy offers a platform for the development of bivalent vaccines, based on replication-incompetent influenza virus,

against pathogens that cause respiratory infectious diseases.

Publications

de Vries RP, Zhu X, McBride R, Rigter A, Hanson A, Zhong G, Hatta M, Xu R, Yu W, Kawaoka Y, de Haan CA, Wilson IA, Paulson JC. Hemagglutinin receptor specificity and structural analyses of respiratory droplet transmissible H5N1 viruses. J Virol 88: 768-773, 2014.

Fonseca W, Ozawa M, Hatta M, Orozco E, Martínez MB, Kawaoka Y. A recombinant influenza virus vaccine expressing the F protein of respiratory syncytial virus. Arch Virol 159: 1067-1077, 2014.

Horimoto T, Gen F, Murakami S, Iwatsuki-Horimoto K, Kato K, Akashi H, Hisasue M, Sakaguchi M, Kawaoka Y, Maeda K. Serological evidence of infection of dogs with human influenza viruses in Japan. Vet Rec 174: 96, 2014.

Yamayoshi S, Yamada S, Fukuyama S, Murakami S, Zhao D, Uraki R, Watanabe T, Tomita Y, Macken C, Neumann G, Kawaoka Y. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses. J Virol 88: 3127-3134, 2014.

Castrucci MR, Facchini M, Di Mario G, Garulli B, Sciaraffia E, Meola M, Fabiani C, De Marco MA, Cordioli P, Siccardi A, Kawaoka Y, Donatelli I. Modified vaccinia virus Ankara expressing the hemagglutinin of pandemic (H1N1) 2009 virus induces cross-protective immunity against Eurasian 'avian-like' H1N1 swine viruses in mice. Influenza Other Respir Viruses 8: 367-375, 2014.

Wang G, Deng G, Shi J, Luo W, Zhang G, Zhang Q, Liu L, Jiang Y, Li C, Sriwilaijaroen N, Hiramatsu H, Suzuki Y, Kawaoka Y, Chen H. H6 influenza viruses pose a potential threat to human health. J Virol 88: 3953-3964, 2014.

Liu Z, Kato A, Shindo K, Noda T, Sagara H, Kawaoka Y, Arii J, Kawaguchi Y. Herpes simplex virus 1 UL47 interacts with viral nuclear egress factors UL31, UL34 and Us3, and regulates viral nuclear egress. J Virol 88: 4657-4667, 2014.

Neumann G, Macken CA, Kawaoka Y. Identification of amino acid changes that may have been critical for the genesis of A(H7N9) influenza viruses. J Virol 88: 4877-4896, 2014.

Sakai K, Ami Y, Tahara M, Kubota T, Anraku M, Abe M, Nakajima N, Sekizuka T, Shirato K, Suzaki Y, Ainai A, Nakatsu Y, Kanou K, Nakamura K, Suzuki T, Komase K, Nobusawa E, Maenaka K, Kuroda M, Hasegawa H, Kawaoka Y, Tashiro M, Takeda M. The Host Protease TMPRSS2 Plays a Major Role in In Vivo Replication of Emerging H7N9 and Seasonal Influenza Viruses. J Virol 88: 5608-5616, 2014.

Teijaro JR, Walsh KB, Long JP, Tordoff KP, Stark GV, Eisfeld AJ, Kawaoka Y, Rosen H, Oldstone MB. Protection of ferrets from pulmonary injury due to H1N1 2009 influenza virus infection: immunopathology tractable by sphingosine-1-phosphate 1 receptor agonist therapy. Virology 452-453: 152-157, 2014.

Garulli B, Di Mario G, Stillitano MG, Kawaoka Y, Castrucci MR. Exploring mucosal immunization with a recombinant influenza virus carrying an HIV-polyepitope in mice with pre-existing immunity to influenza. Vaccine 32: 2501-2506, 2014.

Tani H, Iha K, Shimojima M, Fukushi S, Taniguchi S, Yoshikawa T, Kawaoka Y, Nakasone N, Ninomiya H, Saijo M, Morikawa S. Analysis of Lujo virus cell entry using pseudotype vesicular stomatitis virus. J Virol 88: 7317-7330, 2014.

Kurebayashi Y, Takahashi T, Otsubo T, Ikeda K, Takahashi S, Takano M, Agarikuchi T, Sato T, Matsuda Y, Minami A, Kanazawa H, Uchida Y, Saito T, Kawaoka Y, Yamada T, Kawamori F, Thomson R, von Itzstein M, Suzuki T. Imaging of influenza virus sialidase activity in living cells. Sci Rep 4: 4877, 2014.

Watanabe T, Zhong G, Russell CA, Nakajima N, Hatta M, Hanson A, McBride R, Burke DF, Takahashi K, Fukuyama S, Tomita Y, Maher A, Watanabe S, Imai M, Neumann G, Hasegawa H, Paulson JC, Smith DJ, Kawaoka Y. Circulating avian influenza viruses closely related to the 1918 virus have pandemic potential. Cell Host&Microbe 15: 692-705, 2014.

Sugaya N, Sakai-Tagawa Y, Bamba M, Yasuhara R, Yamazaki M, Kawakami C, Yamaguchi Y, Ide Y, Ichikawa M, Mitamura K, Kawaoka Y. Comparison between virus shedding and fever duration after treating children with pandemic A H1N1/09 and children with A H3N2 with a neuraminidase inhibitor. Antivir Ther (in press).

Menachery VD, Eisfeld AJ, Schäfer A, Josset L, Sims AC, Proll S, Fan S, Li C, Neumann G, Tilton SC, Chang J, Gralinski LE, Long C, Green R, Williams CM, Weiss J, Matzke MM, Webb-Robertson BJ, Schepmoes AA, Shukla AK, Metz TO, Smith RD, Waters KM, Katze MG, Kawaoka Y, Baric RS. Pathogenic influenza viruses and coronaviruses utilize similar and contrasting approaches to control interferon-stimulated gene responses. MBio 5: e01174-14, 2014.

Muramoto Y, Shoemaker JE, Le MQ, Itoh Y, Tamura D, Sakai-Tagawa Y, Imai H, Uraki R, Takano R, Kawakami E, Ito M, Okamoto K, Ishigaki H, Mimuro H, Sasakawa C, Matsuoka Y, Noda T, Fukuyama S, Ogasawara K, Kitano H, Kawaoka Y. Disease Severity Is Associated with Differential Gene Expression at the Early and Late Phases of Infection in Nonhuman Primates

Infected with Different H5N1 Highly Pathogenic Avian Influenza Viruses. J Virol 88: 8981-8997, 2014.

Kitano M, Itoh Y, Ishigaki H, Nakayama M, Ishida H, Pham VL, Arikata M, Shichinohe S, Tsuchiya H, Kitagawa N, Kobayashi M, Yoshida R, Sato A, Le QM, Kawaoka Y, Ogasawara K. Efficacy of Repeated Intravenous Administration of Peramivir against Highly Pathogenic Avian Influenza A (H5N1) Virus in Cynomolgus Macaques. Antimicrob Agents Chemother 58: 4795-4803, 2014.

Josset L, Tchitchek N, Gralinski LE, Ferris MT, Eisfeld AJ, Green RR, Thomas MJ, Tisoncik-Go J, Schroth GP, Kawaoka Y, Manuel de Villena FP, Baric RS, Heise MT, Peng X, Katze MG. Annotation of long non-coding RNAs expressed in Collaborative Cross founder mice in response to respiratory virus infection reveals a new class of interferon-stimulated transcripts. RNA Biol 11(7), 2014.

Itoh Y, Yoshida R, Shichinohe S, Higuchi M, Ishigaki H, Nakayama M, Pham VL, Ishida H, Kitano M, Arikata M, Kitagawa N, Mitsuishi Y, Ogasawara K, Tsuchiya H, Hiono T, Okamatsu M, Sakoda Y, Kida H, Ito M, Quynh Mai L, Kawaoka Y, Miyamoto H, Ishijima M, Igarashi M, Suzuki Y, Takada A. Protective efficacy of passive immunization with monoclonal antibodies in animal models of H5N1 highly pathogenic avian influenza virus infection. PLoS Pathog. 10: e1004192, 2014.

Garulli B, Di Mario G, Stillitano MG, Compagnoni D, Titti F, Cafaro A, Ensoli B, Kawaoka Y, Castrucci MR. Induction of antibodies and T cell responses by a recombinant influenza virus carrying an HIV-1 TatΔ51-59 protein in mice. Biomed Res Int 2014: 904038, 2014.

Fan S, Hatta M, Kim JH, Halfmann P, Imai M, Macken CA, Le MQ, Nguyen T, Neumann G, Kawaoka Y. Novel residues in avian influenza virus PB2 protein affect virulence in mammalian hosts. Nat Commun 5: 5021, 2014.

Hatakeyama D, Shoji M, Yamayoshi S, Hirota T, Nagae M, Yanagisawa S, Nakano M, Ohmi N, Noda T, Kawaoka Y, Kuzuhara T. A novel functional site in the PB2 subunit of influenza A virus essential for acetyl-CoA interaction, RNA polymerase activity, and viral replication. J Biol Chem 289: 24980-24994, 2014.

Sakai-Tagawa Y, Ozawa M, Yamada S, Uchida Y, Saito T, Takahashi K, Sugaya N, Tashiro M, Kawaoka Y. Detection sensitivity of influenza rapid diagnostic tests. Microbiol Immunol 58: 600-606, 2014.

Nagaoka M, Hatta Y, Kawaoka Y, Malherbe LP. Antigen aignal strength during priming determines effector CD4 T cell function and antigen sensitivity during influenza virus challenge. J Immunol 193: 2812-2820, 2014.

Uraki R, Das SC, Hatta M, Kiso M, Iwatsuki-Horimoto K, Ozawa M, Coban C, Ishii KJ, Kawaoka Y. Hemozoin as a novel adjuvant for inactivated whole virion influenza vaccine. Vaccine 32: 5295-300, 2014.

Watanabe T, Kawakami E, Shoemaker JE, Lopes TJS, Matsuoka Y, Tomita Y, Kozuka-Hata H, Gorai T, Kuwahara T, Takeda E, Nagata A, Takano R, Kiso M, Yamashita M, Sakai-Tagawa Y, Katsura H, Nonaka N, Fujii H, Fujii K, Sugita Y, Noda T, Goto H, Fukuyama S, Watanabe S, Neumann G, Oyama M, Kitano H, Kawaoka Y. Influenza virus-host interactome screen as a platform for antiviral drug development. Cell Host& Microbe 16: 795-805, 2014.

Katsura H, Piao Z, Iwatsuki-Horimoto K, Akeda Y, Watanabe S, Horimoto T, Oishi K, Kawaoka Y. A Bivalent Vaccine Based on a Replication-Incompetent Influenza Virus Protects against Streptococcus pneumoniae and Influenza Virus Infection. J Virol 88: 13410-13417, 2014.

Florek NW, Weinfurter JT, Jegaskanda S, Brewoo JN, Powell TD, Young GR, Das SC, Hatta M, Broman KW, Hungnes O, Dudman SG, Kawaoka Y, Kent SJ, Stinchcomb DT, Osorio JE, Friedrich TC. Modified vaccinia virus ankara encoding influenza virus hemagglutinin induces heterosubtypic immunity in macaques. J Virol 88: 13418-13428, 2014.

Fan S, Hatta M, Kim JH, Le MQ, Neumann G, Kawaoka Y. Amino Acid Changes in the Influenza A Virus PA Protein That Attenuate Avian H5N1 Viruses in Mammals. J Virol 88: 13737-13746, 2014.

Peng X, Alföldi J, Gori K, Eisfeld AJ, Tyler SR, Tisoncik-Go J, Brawand D, Law GL, Skunca N, Hatta M, Gasper DJ, Kelly SM, Chang J, Thomas MJ, Johnson J, Berlin AM, Lara M, Russell P, Swofford R, Turner-Maier J, Young S, Hourlier T, Aken B, Searle S, Sun X, Yi Y, Suresh M, Tumpey TM, Siepel A, Wisely SM, Dessimoz C, Kawaoka Y, Birren BW, Lindblad-Toh K, Di Palma F, Engelhardt JF, Palermo RE, Katze MG. The draft genome sequence of the ferret (Mustela putorius furo) facilitates study of human respiratory disease. Nat Biotechnol 32: 1250-1255, 2014.

Li X, Shi J, Guo J, Deng G, Zhang Q, Wang J, He X, Wang K, Chen J, Li Y, Fan J, Kong H, Gu C, Guan Y, Suzuki Y, Kawaoka Y, Liu L, Jiang Y, Tian G, Li Y, Bu Z, Chen H. Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 avian influenza viruses. PLoS Pathog 10: e1004508, 2014.

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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 and the function of TLRs to avoid excessive immune responses for endogenous ligands. Our research focuses on molecular mechanisms controlling pathogenic ligand recognition by TLRs.

1. 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 homolog 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 CD8α$^-$ 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.

2. G protein specifically associates with TLR7 and regulates onset of Systemic Lupus Erythematosus

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TLR7 and 9, innate immune sensors for microbial nucleic acids (NAs), respond to self NA, activate dendritic cells (DCs) and B cells, and mount anti-nuclear autoantibody production in murine models of systemic lupus erythematosus (SLE), an autoimmune disease where type I interferons (IFN-I) have a causative role. Despite the shared role in autoantibody production, RNA-sensing TLR7 promotes disease, whereas DNA-sensing TLR9 regulates disease progression by counteracting TLR7. TLR7 and 9 reside in the endoplasmic reticulum (ER), and are associated with Unc93B1, an ER-resident multiple transmembrane protein. Unc93B1 enable TLR7 and 9 to sense NA by transporting them to the endolysosomes, a site for NA-sensing, upon activation. TLR9 antagonizes TLR7 by competing association with Unc93B1. Unc93B1 restricts TLR7 activation by giving preference to TLR9-association over TLR7. In contrast to TLR9 antagonism, little is known about a mechanism underlying disease promotion by TLR7. In addition, specific regulatory mechanisms on TLR7 response is also unknown. We identified a G protein specifically associated with TLR7 and regulates TLR7 responses. Dendritic cells (DCs) and B cells harbouring an insertional mutation in the G protein showed augmented TLR7 responses in proinflammatory cytokine production and proliferation, respectively. The G protein was required for TLR7-dependent IFN-I production by plasmacytoid DCs (pDCs). The G protein specifically localizes on lysosome and regulates Lysosomal trafficking. We are studying that TLR7 drives IFN-I-mediated autoimmunity such as systemic lupus erythematosus (SLE) by using model mice trough TLR-trafficking dependent on Unc93B1 complexed with G protein required for lysosomal trafficking. We analyze the relationship between this G protein and IFN-I dependent SLE like autoimmune disease.

3. The linkage of TLR4 activation and antigen presentation, in the light of molecular trafficking

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The bacterial compounds, such as lipopolysaccharide (LPS), lipoproteins, DNAs and RNAs, have played critical roles in the vaccinations as immunomodulators since early times. Considering clinical applications, the vaccine adjuvant must be reliable and safe. The bacterial compounds are now well-identified ligands to activate the innate immune system through Toll-like receptors (TLRs). This activation results in both inflammation and the boosts of acquired immune responses. For instance, TLR4 can be activated via lipid A, the active center of LPS, which is well known as a robust immunostimulator. Despite our knowledge of the TLR activation mechanism, we are still unable to harness the excessive inflammations and the effective boosts of acquired immunity using TLR-ligands. Recent studies on innate immune cell biology figured out the activation platforms of TLR4 where the respective responses occur; plasma membrane for inflammation, endosome for interferon production and mitochondria for reactive oxygen species (ROS). To address this issue, we have focused on the dynamics of signaling molecules in the downstream of TLR4 and ligand transfer mechanisms before TLR4 initiation. The signaling pathways are modulated by the fine structure of the ligands. We have found one interesting derivative, which induces low inflammations and enough antigen presentations, showing unique molecular rearrangements and extraordinary TLR4 initiation means. Currently we are analyzing these TLR4-activation/initiation events as a key of the antigen presentations triggering.

4. Unc93B1 dependent TLR5 function.

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Toll-like receptor 5 (TLR5), a sensor for bacterial flagellin, mounts both innate and adaptive immune responses, and has been implicated in infectious diseases, such as colitis and metabolic syndromes. Although TLR5 is believed to belong to cell surface TLRs, cell surface expression has never been verified. Moreover, it has remained unclear which types of immune cells express TLR5 and contribute to flagellin-dependent responses. For this reason, we established an anti-mouse/human TLR5 monoclonal antibody and studied cell surface expression of TLR5 on various immune cells. A macrophage cell line J774 expressed endogenous TLR5 on the cell surface and produced IL-6 and G-CSF in response to flagellin. In *in vivo*, cell surface TLR5 was mainly found on neutrophils and $CD11b^{hi}Ly6C^{hi}$ classical monocytes in the bone marrow, circulation,

spleen and an inflammatory lesion. Ly6C^{hi} classical monocytes, but not neutrophils, produced cytokines in response to flagellin. Splenic CD8^{-}CD4^{+} conventional dendritic cells and CD11c^{hi}CD11b^{hi} lamina propria DCs, also clearly expressed cell surface TLR5.

Additionally, we also clarified which molecules controlled cell surface expression of TLR5 and flagellin-induced responses. We demonstrated that cell surface TLR5 was completely abolished by silencing not only ER chaperon "PRotein Associated with TLR4 A (PRAT4A)" but also "Unc93B1" which had been believed to regulate the trafficking of nucleic acid sensing intracellular TLRs. Those molecules directly bound TLR5 and regulated its trafficking from ER to cell surface. Our findings revealed previously unexpected role of UNC93B1 in the regulation of cell surface TLR function.

5. The meaning of intracellular localization of TLRs.

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TLRs induce complex inflammatory responses that functions to protect the host from invading pathogens. But, in excessive or persistent inflammatory responses, hosts suffer the disadvantage such as septic shock, delayed tissue repair and autoimmune diseases. For this reason, hosts must strictly control TLR response to restrict dilated inflammation.

TLR-dependent responses are greatly influenced by a site of pathogen sensing. TLR heterodimers TLR1/TLR2 and TLR2/TLR6 recognize tri- or di-acylated microbial lipopeptides, respectively. Although TLR1, 2, and 6 are believed to localize on the cell surface of immune cells, little is known about where lipopeptides are signaled. To clarify this point, we have established monoclonal antibodies to TLR1, 2, and 6. TLR1, 2, and 6 were expressed on the surface of B cells, monocytes, and dendritic cells in a manner dependent on a TLR-specific chaperone PRAT4A (protein associated with TLR4 A). However, cell surface localization of TLR1 or TLR6 was not necessarily required for TLR2 response. Furthermore, a dynamin inhibitor 'Dynasore' abolished the lipopeptide responses by preventing lipopeptide internalization into LAMP-1 and LAMP-2 positive compartment. Our findings strongly suggest that lipopeptides elicit TLR1/2 and TLR2/6 signaling in the endolysosomes, but not on the cell surface.

Publications

Huh JW, Shibata T, Hwang M, Kwon EH, Jang MS, Fukui R, Kanno K, Jung DJ, Jang MH, Miyake K, and Kim YM. UNC93B1 is essential for the plasma membrane localization and signaling of Toll-like receptor 5. *Proc. Natl. Acad. Sci. USA* 111: 7072-7077, 2014.

Kanno A, Tanimura N, Ishizaki M, Ohko K, Motoi Y, Onji M, Fukui R, Shimozato T, Yamamoto K, Shibata T, Sano S, Sugahara-Tobinai A, Takai T, Ohto U, Shimizu T, Saitoh SI, and Miyake K. Targeting cell surface TLR7 for therapeutic intervention in autoimmune diseases. *Nat. Commun.* (in press)

Kurashima Y, Amiya T, Fujisawa K, Shibata N, Suzuki Y, Kogure Y, Hashimoto E, Otsuka A, Kabashima K, Sato S, Sato T, Kubo M, Akira S, Miyake K, Kunisawa J, and Kiyono H. The enzyme cyp26b1 mediates inhibition of mast cell activation by fibroblasts to maintain skin-barrier homeostasis. *Immunity*. 40: 530-541, 2014.

Li X, Saitoh SI, Shibata T, Tanimura N, Fukui R, Miyake K. Mucolipin 1 positively regulates TLR7 responses in dendritic cells by facilitating RNA transportation to lysosomes. *Int Immunol.* (in press)

Chan MP, Onji M, Fukui R, Kawane K, Shibata T, Saitoh SI, Ohto U, Shimizu T, Barber GN, and Miyake K. DNAse II-dependent DNA digestion is required for DNA sensing by TLR9. *Nat. Commun.* (in press)

Miyake K and Kaisho T. Homeostatic inflammation in innate immunity. *Curr Opin Immunol.* 30C: 85-90, 2014.

Motoi Y, Shibata T, Takahashi K, Kanno A, Murakami Y, Xiaobing L, Kasahara T, and Miyake K. Lipopeptides are signaled by Toll-like receptor 1, 2, and 6 in endolysosomes. *Int Immunol*. 26: 563-573, 2014.

Murakami Y, Fukui R, Motoi Y, Kanno A, Shibata T, Tanimura N, Saitoh SI, and Miyake K. Roles of the Cleaved N-Terminal TLR3 Fragment and Cell Surface TLR3 in Double-Stranded RNA Sensing. *J Immunol.* 193: 5208-5217, 2014.

Tanimura N, Saitoh S, Ohto U, Akashi-Takamura A, Fujimoto Y, Fukase K, Shimizu T, and Miyake K. The attenuated inflammation of MPL is due to the lack of CD14-dependent tight dimerization of TLR4/MD2 complex at the plasma membrane. *Int. Immunol.* 26: 307-314, 2014.

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Division of Mucosal Immunology
炎症免疫学分野

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The mucosal immune system not only plays an important role as the first line of immunological defense for preventing the host from invasion of harmful microorganisms, but also contributes to the establishment and maintenance of mucosal homeostasis. Our major focus is the elucidation and understanding of molecular and cellular nature of the mucosal immune system for the development of mucosal vaccine against infectious diseases and mucosal immune therapy for mucosa-associated diseases, such as food allergy and inflammatory bowel diseases.

1. MucoRice for New Generation of Oral Vaccine

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We previously produced hygromycin phosphotransferase (HPT) selection marker-free MucoRice-CTB/Q line 51A for human use, and determined the location and structure of the transgenes in the genome of the MucoRice-CTB line. To establish a seed bank of MucoRice-CTB, we confirmed the complete sequence of CTB in the MucoRice-CTB line and oral immunogenicity with protective immunity in mice. We also determined Specification and Test Methods for MucoRice-CTB. For clinical study, marker-free MucoRice-CTB line was cultured in a closed hydroponic GMP (Good Manufacturing Practices) factory at the Institute of Medical Science, the University of Tokyo (IMS-UT) to produce MucoRice-CTB preparation for the stability and safety studies. Most of pre-clinical studies including single and repeated oral toxicity study in dogs and rat have been done in this time. In addition, we had a couple of pre-meeting with Pharmaceuticals and Medical Devices Agency (PMDA). After approving Institutional Review Board (IRB) of IMS-UT and Investigational New Drug Application (IND) of PMDA, we will start physician based-phase I study at IMS-UT hospital from July, 2015.

2. New Generation Nasal Vaccine development

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We previously established a nanosized nasal vaccine delivery system by using a cationic cholesteryl group-bearing pullulan nanogel (cCHP nanogel), which is a protein-based antigen-delivery vehicle for adjuvant-free nasal vaccination. In the present study, we examined the central nervous system safety and efficacy of nasal vaccination with our developed cCHP nanogel containing pneumococcal surface protein A (PspA-nanogel) against pneumococcal infection in macaques. When [^{18}F]-labeled PspA-nanogel was nasally administered to a rhesus macaque (Macaca mulatta), longer-term retention of PspA was noted in the nasal cavity when compared with administration of PspA alone. Of importance, no deposition of [^{18}F]-PspA was seen in the olfactory bulbs or brain. Nasal PspA-nanogel vaccination effectively induced PspA-specific serum IgG with protective activity and mucosal secretory IgA (SIgA) Ab responses in cynomolgus macaques (Macaca fascicularis). These results demonstrate that nasal PspA-nanogel vaccination is a safe and effective strategy for the development of a nasal vaccine for the prevention of pneumonia in humans.

3. Antibody Therapy for Arthritis and Infectious Disease

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We previously developed a rice transgenic system for the production of a llama variable domain of a heavy-chain antibody fragment (VHH) specific for rotavirus in rice seed (which we termed 'MucoRice-ARP1) to control gut infectious disease. In this study, we developed anti-TNF VHH antibody-producing rice (MucoRice-mTNF-VHH). Because anti-TNF antibody therapy is now commonly used to treat patients suffering from these inflammatory conditions, but the cost of treatment continues to be a concern. MucoRice-mTNF-VHH was produced at high levels in the rice seeds when we used our most recent transgene-overexpression system with RNA interference technology that suppresses the production of major rice endogenous storage proteins while enhancing the expression of the transgene-derived protein. Production levels of VHH reached an average of 218 μg per rice seed. Further, approximately 91% of VHH was released easily when the powder form of MucoRice-TNF-VHH was mixed with PBS. MucoRice-mTNF-VHH purified by means of single-step gel filtration from rice PBS extract showed high neutralizing activity in an in vitro TNF cytotoxicity assay using WEHI164 cells. In addition, purified MucoRice-mTNF-VHH suppressed progression of collagen-induced arthritis in mice. These results show that this rice-expression system is useful for the production of neutralizing VHH antibody specific for mTNF.

4. MicroRNA biomarkers engaged in MucoRice-CTB-induced protective humoral immunity

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We are aiming in understanding the underlying molecular mechanisms to generate orally-vaccinated MucoRice-CTB-specific secretory IgA response and identifying novel and applicable microRNA (miRNA) biomarkers. We have performed miRNA microarray using serum samples from the mice orally immunized with MucoRice-CTB or WT rice and obtained a large number of miRNA biomarker candidates. Among them, we found the certain miRNAs were highly expressed in the small intestines of the mice orally immunized with MucoRice-CTB compared to WT rice. In addition, the expression of thymic stromal lymphopoietin (TSLP), one of the strong candidates of mucosal adjuvant to activate antigen-presenting cells such as B and dendritic cells, was remarkably increased on the intestinal epithelium of MucoRice-CTB-immunized mice. To demonstrate the precise role of TSLP-TSLPR interaction in the protective humoral immunity induced by oral immunizations with MucoRice-CTB, we utilized and analyzed TSLPR-KO mice and found TSLP was critical for producing oral antigen-specific secretory IgA antibodies and further protecting intestinal diarrhea against cholera-toxin challenge after oral immunizations with MucoRice-CTB. We are currently investigating of the molecular mechanisms, in which miRNAs, their putative targets, and TSLP play an important role in propagating oral antigen-induced protective humoral immunity. On the other hand, we face up to conducting the clinical study (Phase I) for oral vaccine MucoRice-CTB towards realization of its clinical appli-

cation this year. Our current study will provide information beneficial for the clinical study and further for developing novel oral vaccines or adjuvants with safety and efficacy.

5. Critical role of TSLP-TSLPR interaction in inducing secretory IgA responses after nasal immunizations.

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There are currently great interest and demand in developing mucosal vaccine and adjuvant to prevent life-threatening microbial infections. The adaptive humoral immune defense at mucosal surfaces is dominantly mediated by secretory IgA (SIgA) antibodies. Strategy to elicit antigen (Ag)-specific SIgA responses at nasal respiratory tissues, will be crucial for designing efficacious nasal vaccines. However, molecular pathways underlying the induction and maintenance of IgA antibody responses by nasal immunizations are largely unknown. Thymic stromal lymphopoietin (TSLP) is an interleukin-7-like cytokine and predominantly expressed by epithelial cells of thymus, skin, lung, intestine, and tonsils, as well as stromal cells, mast cells, and dendritic cells (DCs). TSLP plays an important role in activating DCs to drive T- helper 2 differentiation and is increased in atopic dermatitis or asthma. On the other hand, TSLP has been reported to promote antibody class switch recombination (CSR) in humans. However, there is no evidence to date for TSLP-mediated regulation of IgA CSR in the context of nasal immunizations. In current study, we found that TSLP expression was up-regulated in the nasal respiratory tissues of the mice nasally immunized, suggesting of the TSLP-TSLPR interaction as a potential cue of mediating nasal respiratory immunity. To demonstrate whether TSLP-TSLPR interaction mediates humoral immunity upon nasal immunizations, we first examined Ag-specific antibody production in WT or TSLPR-KO mice nasally immunized. Interestingly, Ag-specific IgA, but not IgG, responses in both serum and mucosal secretion were significantly reduced in the TSLPR-KO mice compared to WT mice immunized, although there was no difference in total IgG or IgA titers in the steady state between both mice. Our study is beneficial for better understandings of the precise roles of TSLP signaling in protective local immunity upon nasal immunizations and will contribute to developing more efficacious nasal vaccines.

6. Regulation of Mast Cells in the Allergic and non-Allergic Diseases

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Mast cells (MCs) mature locally, thus possessing tissue-dependent phenotypes for their critical roles in both protective immunity against pathogens and the development of allergic or inflammatory responses. We previously reported that MCs highly express P2X7, a receptor for extracellular ATP, in the colon but not in the skin. An ATP-P2X7 pathway induced activation of MCs and accelerates inflammatory symptom. In addition, we recently identified the unique mechanisms by which P2X7 expression on MCs is reduced by fibroblasts in the skin but not in other tissues. The retinoic-acid-degrading enzyme Cyp26b1 is highly expressed in skin fibroblasts, and its inhibition resulted in upregulation of P2X7 on MCs. We also noted increased expression of P2X7 on skin MCs and consequent P2X7- and MC-dependent dermatitis (so-called retinoid dermatitis) in the presence of excessive amounts of retinoic acid.

In addition to the down regulation of P2X7, multiple receptors which regulated MC functions are tightly modulated by skin fibroblasts. We thus now aim to elucidate a unique skin-barrier homeostatic network operating through MC-fibroblast pathways.

We also aim to elucidate immunological mechanisms of oral immune therapy (OIT) which is recent approach for the treatment of food allergy. For analyzing the underlined mechanisms of OIT, we successfully generate novel OIT mice model. In those mice, the symptom of allergic diarrhea is cured by the protocol of OIT with heated antigen inoculation.

7. Functional roles of Marcks-like protein expressed by murine Peyer's patch M cells

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The mucosal immune response is triggered by antigen uptake from the lumen across the mucous and epithelial cell layers to organized mucosa-associated lymphoid tissues such as Peyer's patches (PPs) in the small intestine. This antigen uptake is mediated mainly by microfold cells (M cells), which are located in follicle-associated epithelium (FAE). To investigate the molecular biology of PP M cells, we previously identified MARCKS-like protein (MLP) and GP2 as M-cell-specific molecules. Further study revealed that GP2 acts as the scaffold receptor for the fimbrial protein of bacteria such as *E. coli.* and *Salmonella* Typhimurium on M cells. Although MLP is known as a substrate for protein kinase C (PKC), the biological role of MLP in M cells is still unknown.

To address this issue, we generated intestinal epithelial cell (IEC)-specific MLP-deficient ($Mlp^{IEC\Delta/\Delta}$) mice. The expression of MLP was completely abolished in the FAE of PPs in these mice. Matured M cells, defined by the GP2 expression and the unique ultra-architectures of shorter microvilli and pocket-like formation, could be found in $Mlp^{IEC\Delta/\Delta}$ mice, indicating that MLP is not involved in the development and differentiation of M cells. When the sampling ability of M cells in $Mlp^{IEC\Delta/\Delta}$ mice were examined, orally-administered fluorescence-nanoparticles were equally taken up into PPs. However, uptake of *Yersinia enterocolitica*, but not *S*. Typhimurium was markedly lower in $Mlp^{IEC\Delta/\Delta}$ mice than in control mice.

Since *Yersinia* outer proteins such as YadA and invasin have been known to interact with integrin β1 on M-cell surfaces, we further examined whether MLP regulates integrin molecules by *in vitro* assay. MLP-introduced MODE-K cells enhanced the cellular response to fibronectin, which is the ligand for integrin α5β1. On the other hand, mutated MLP, which lacked PKC phosphorylation sites, had no effect to enhance the response to fibronectin. In addition, phosphatase inhibitor treatment canceled the responsiveness to fibronectin of MLP-expressing cells, suggesting that movement of MLP between the plasma membrane and cytoplasm, depending on the phosphorylation status of MLP, is required to activate integrin molecules. Taken together, our findings suggest that MLP regulates the activity of integrin α5β1 in M cells, depending on its phosphorylation status, and contributes to the uptake of *Y. enterocolitica*.

8. Innate lymphoid cells govern intestinal epithelial fucosylation

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Fucosylation of intestinal epithelial cells, catalyzed by fucosyltransferase 2 (Fut2), represents a major mechanism of host-microbiota symbiosis. Commensal bacteria induce epithelial fucosylation and epithelial fucose is utilized as a dietary carbohydrate by many of the bacteria. However, the molecular and cellular mechanisms of the induction of epithelial fucosylation remain unknown. Here, we show that type 3 innate lymphoid cells (RORγt + ILC3) are critical inducers of intestinal epithelial Fut2 expression and fucosylation and that this induction is mediated by the production of interleukin 22 and Lymphotoxin in a commensal bacteria-dependent and -independent manner, respectively. Disruption of intestinal fucosylation leads to susceptibility to infection by Salmonella typhimurium. Our data unveil a novel role of ILC3 in creating the appropriate gut microenvironment through regulating the epithelial glycosylation.

Publications

Journals (Refereed)

1. Fukuyama, Y., Yuki, Y., Katakai, Y., Harada, N., Takahashi, H., Takeda, S., Mejima, M., Joo, S., Kurokawa, S., Sawada, S., Shibata, H., Park, EJ., Fujihashi., K, Briles, D., Yasutomi, Y., Tsukada, H., Akiyoshi, K., Kiyono, H.: Nanogel-based pneumococcal surface protein A nasal vaccine induces microRNA-associated Th17 cell responses with neutralizing antibodies against *Streptococcus pneumoniae* in macaques. Mucosal Immunol. 2015 (in press)
2. Kashima, K., Mejima, M., Kuroda, M., Kiyono, H., Yuki, Y. Comparative whole-genome analyses of selection marker-free rie-based choleva toxin B-Subunit vaccine lines and wild-type lines BMC genomics 16: 48, 2015.
3. Kurokawa, S., Kuroda, M., Mejima, M., Nakamura, R., Takahashi, Y., Sagara, H., Takeyama, N., Satoh. S., Kiyono. H., Teshima. R., Masumura. T., and Yuki, Y. RNAi-mediated suppression of endogenous storage proteins leads to a change in localization of overexpressed cholera toxin B-subunit and the allergen protein RAG2 in rice seeds. Plant Cell Rep. 33: 75-87, 2014.
4. Nakajima-Adachi, H., Kikuchi, A., Fujimura, Y., Shibahara, K., Makino, T., Goseki-Sone, M., Kihara-Fujioka, M., Nochi, T., Kurashima, Y., Igarashi, O., Yamamoto, M., Kunisawa, J., Toda, M., Kaminogawa, S., Sato, R., Kiyono, H., and Hachimura, S. Peyer's patches and mesenteric lymph nodes cooperatively promote enteropathy in a mouse model of food allergy. PLoS One. 9: e107492, 2014.
5. Abe, M., Yuki, Y., Kurokawa, S., Mejima, M., Kuroda, M., Park, E.J., Scheller, J. Nakanishi, U. and Kiyono, H. A rice-based soluble form of a murine TNF -specific llama variable domain of heavy-chain antibody suppresses collagen -induced arthritis in mice. J. Biotechnol. 10: 175: 45-52, 2014.
6. Kotani Y., Kunisawa J., Suzuki Y., Sato I., Saito T., Toba M., Kohda N., and Kiyono H. Role of *Lactobacillus pentosus* strain b240 and the Toll-like receptor 2 axis in Peyer's patch dendritic cell-mediated immunoglobulin A enhancement, PLoS One 9: e91857, 2014.
7. Takemura, N., Kawasaki, T., Kunisawa, J., Sato, S., Lamichhane, A., Kobiyama, K., Aoshi, T., Ito, J., Mizuguchi, K., Karuppuchamy, T., Matsunaga, K., Miyatake, S., Mori, N., Tsujimura, T., Satoh, T., Kumagai, Y., Kawai, T., Standle, D.M., Ishii, K.J., Kiyono, H., Akira, S., and Uematsu, S. Blockade of TLR3 protects mice from lethal radiation-induced gastrointestinal syndrome. Nat Commun. 5: 3492, 2014.
8. Hiyama, S., Iijima, H., Shinzaki, S., Inoue, T., Shiraishi, E., Kawai, S., Araki, M., Kato, M., Hayashi, Y., Nishida, T., Fujii, H., Mukai, A., Shibata, N., Sato, S., Kiyono, H., Gotoh, K., Motooka, D., Nakamura, S., Iida, T., Tsujii, M., and Takehara, T. Peyer's patches play a protective role in nonsteroidal anti-inflammatory drug-induced enteropathy in mice. Inflamm Bowel Dis. 20: 790-799, 2014.
9. Masahata, K., Umemoto, E., Kayama, H., Kotani, M., Nakamura, S., Kurakawa, T., Kikuta, J., Gotoh, K., Motooka, D., Sato, S., Higuchi, T., Baba, Y., Kurosaki, T., Kinoshita, M., Shimada, Y., Kimura, T., Okumura, R., Takeda, A., Tajima, M., Yoshie, O., Fukuzawa, M., Kiyono, H., Fagarasan, S., Iida, T., Ishii, M., and Takeda, K. Generation of colonic IgA-secreting cells in the cecal patch. Nat Commun. 5: 3704, 2014.
10. Kurashima, Y., Amiya, T., Fujisawa, K., Shibata, N., Suzuki, Y., Kogure. Y., Hashimoto, E., Otsuka, A., Kabashima, K., Sato, S., Sato, T., Kubo, M., Akira, S., Miyake, K., Kunisawa, J., and Kiyono, H. The enzyme cyp26B1 mediates inhibition of mast cell activation by fibroblasts to maintain skin-barrier homeostasis. Immunity 40: 530-541, 2014.
11. Tokuhara, D., Nochi, T., Matsumura, A., Mejima, M., Takahashi, Y., Kurokawa, S., Kiyono, H. and Yuki Y. Specific expression of apolipoprotein A-IV in the follicle-associated epithelium of the small intestine. Dig. Dis. Sci. 59: 2682-2692, 2014.
12. Goto, Y., Obata, T., Kunisawa, J., Sato, S., Ivanov, I., Lamichhane, A., Takeyama, N., Kamioka, M., Sakamoto, M., Matsuki, T., Setoyama, H., Imaoka, A., Uematsu, S., Akira, S., Domino, S., Kulig, P., Becher, B., Renauld, J-C., Sasakawa, C., Umesaki, Y., Benno, Y. and Kiyono, H. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. Science 345 (6202): 1254009, 2014.
13. Kunisawa, J., Hashimoto, E., Inoue, A., Nagasawa R., Suzuki, Y., Ishikawa, I., Shikata, S., Arita, M., Aoki, J. and Kiyono, H. Regulation of intestinal IgA responses by dietary palmitic acid and its metabolism. J. Immunol. 193: 1666-1671, 2014.
14. Sato, A., Suwanto, A., Okabe, M., Sato, S., Nochi, T., Imai, T., Koyanagi, N., Kunisawa, J., Kawaguchi, Y. and Kiyono, H. Vaginal memory T cells induced by intranasal vaccination are critical for protective T cell recruitment and prevention of genital HSV-2 disease. J Virol. 88 (23): 13699-13708, 2014.
15. Mejima, M., Kashima, K., Kuroda, M., Takeyama, N., Kurokawa S., Fukuyama Y., Kiyono H., Itoh, K., Mitsui, T., and Yuki, Y. Determination of genomic location and structure of the transgenes in marker-free rice-based chol-

era vaccine by using whole genome resequencing approach. Plant Cell Tiss. Organ Cult. 120: 35-48, 2015
16. Okura, H., Sato, S., Kishikawa, S., Kaneto, S., Nakashima, T., Yoshida, N., Takayanagi, H. and Kiyono, H. Runx2-I isoform contributes to fetal bone formation even in the absence of specific N-terminal amino acids. PLoS One. 9(9): e108294, 2014.
17. Suenaga, F., Ueha, S., Abe, J., Kosugi-Kanaya, M., Wang, Y., Yokoyama, A., Shono, Y., Shand, F.H., Morishita, Y., Kunisawa, J., Sato, S., Kiyono, H. and Matsushima, K. Loss of lymph node fibroblastic reticular cells and high endothelial cells is associated with humoral immunodeficiency in mouse graft-versus-host disease. J Immunol. 194(1): 398-406, 2014.

Reviews (Refereed)

18. Sato, S., Kiyono, H., Fujihashi K. Mucosal Immunosenescence in the Gastrointestinal Tract: A Mini-Review. Gerontology. 2014 Dec 20. [Epub ahead of print] PubMed PMID: 25531743.
19. Kurashima, Y., Kiyono, H., and Kunisawa, J. Pathophysiological role of extracellular purinergic mediators in the control of intestinal inflammation, Mediators of Inflammation 2014 (in press)
20. Fujihashi, K., Sato S., and Kiyono, H. Mucosal adjuvants for vaccines to control upper respiratory infections in the elderly. Exp. Gerontol. 54: 21-6, 2014.
21. Kurashima, Y. and Kiyono, H. New era for mucosal mast cells: their roles in inflammation, allergic immune responses and adjuvant development. Exp. Mol. Med. 46: e83, 2014.
22. Azegami, T., Yuki, Y., and Kiyono, H. Challenge in mucosal vaccines for the control of infectious diseases. Intern. Immunol. 26: 517-528, 2014.
23. Azegami, T., Itoh, H., Kiyono, H. and Yuki, Y. Novel transgenic rice-based vaccines. Arch. Immunol. Ther. Exp. (in press)
24. Lamichhane, A., Azegami, T. and Kiyono, H. The mucosal immune system for vaccine development. Vaccine 32(49): 6711-6723, 2014.
25. Kunisawa J, Kiyono H. Vitamins mediate immunological homeostasis and diseases at the surface of the body. Endocr. Metab. Immune Disord. Drug Targets (in press)

Chapters in Books

26. Mason, H., Thuenemann, E., Kiyono, H., Kessans, S., Matoba, N. and Mor, T. Mucosal vaccines from plant biotechnology In *Mucosal Immunology,* Mestecky, J. et al (eds), pp. 1-13, 2014.

JAPANESE JOURNALS, REVIEWES AND CHAPTERS

27. Kurashima, Y., Kiyono, H., Kunisawa, J., Purinergic signaling mediates mast cell activation in intestinal inflammation Seikagaku, The Japanese Biochemical Society, (in press)
28. Kurashima, Y., Sato, T., Kiyono., H, Kunisawa, J., DAMPs-mediated mast cell activation in the development of immune disease. Clinical Immunology and Allergology, Kagaku hyoronsya, (in press)
29. Yuki, Y. New drug device development except injectable and oral preparation: Molecular imaging of nasal vaccine in mice and macaques. Gijyutu-Joho- Kyokai. 145-149, 2014.
30. Fukuyama, Y., and Kiyono, H. Development for the new generation of mucosal vaccine. The Medical Frontline (SAISHIN-IGAKU), Saishinigaku-sha, 69(4): 62-69, 2014.
31. Yamanishi, K., Hirayama, K. and Kiyono, H. Expectation of New Generation Vaccine for the Control of Infectious Disease. The Medical Frontline (SAISHIN-IGAKU), Saishinigaku-sha, 69(4): 6-17, 2014.
32. Azegami, T. and Kiyono, H. Mucosal immunology. Journal of Clinical and Experimental Medicine (IGAKU NO AYUMI), Ishiyaku Publishers, Inc. 249(5): 396, 2014.
33. Kashima, K., Yuki, Y. and Kiyono, H. Challenge on developing novel oral vaccines. BIO INDUSTRY, CMC Publishing CO., LTD. 31(6): 4-10, 2014.
34. Takasato, Y., Kurashima, Y., Kiyono, H. and Kunisawa, J. Current status in the development of new generation of anti-allergic mucosal vaccine. BIO INDUSTRY, CMC Publishing CO., LTD. 31(6): 55-60, 2014.
35. Goto, Y. and Kiyono, H. Regulation of intestinal epithelial cell glycosylation by innate lymphoid cells. Molecular Gastrointestinal Medicine 11(4): 86-88 (Sentan-igaku-sha), 2014.

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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. Herpes Simplex Virus 1 UL47 Interacts with Viral Nuclear Egress factors UL31, UL34 and Us3, and Regulates Viral Nuclear Egress

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Herpesviruses have evolved a unique mechanism for nuclear egress of nascent progeny nucleocapsids: the nucleocapsids bud through the inner nuclear membrane into the perinuclear space between the inner and outer nuclear membranes (primary envelopment) and enveloped nucleocapsids then fuse with the outer nuclear membrane to release nucleocapsids into the cytoplasm (de-envelopment). We have shown that the herpes simplex virus 1 (HSV-1) major virion structural protein UL47 (or VP13/VP14) is a novel regulator for HSV-1 nuclear egress. In particular: (i) UL47 formed a complex(es) with HSV-1 proteins UL34, UL31, and/or Us3, which have all been reported to be critical for viral nuclear egress, and these viral proteins co-localized at the nuclear membrane in HSV-1-infected cells; (ii) the UL47-null mutation considerably reduced primary enveloped virions in the perinuclear space, although capsids accumulated in the nucleus; and (iii) UL47 was detected in primary enveloped virions in the perinuclear space by immunoelectron microscopy. These results suggested that UL47 promoted HSV-1 primary envelopment, probably, by interacting with the critical HSV-1 regulators for viral nuclear egress and by modulating their functions.

Like other herpesviruses, HSV-1 has evolved a vesicle - mediated nucleocytoplasmic transport mechanism for nuclear egress of nascent progeny nucleocapsids. Although previous reports identified and characterized several HSV-1 and cellular proteins involved in viral nuclear egress, complete details of HSV-1 nuclear egress remain to be elucidated. In this study, we have presented data suggesting that (i) the major HSV-1 virion structural protein UL47 (or VP13/14) formed a complex with known viral regulatory proteins critical for viral nuclear egress, and (ii) UL47 played a regulatory

role in HSV-1 primary envelopment. Thus, we identified UL47 as a novel regulator for HSV-1 nuclear egress.

2. The Role of Herpes Simplex Virus 1 Immediate-Early Protein ICP22 in Viral Nuclear Egress

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In order to investigate the novel function(s) of the herpes simplex virus 1 (HSV-1) immediate-early protein, ICP22, we screened for ICP22-binding proteins in HSV-1-infected cells. Our results were as follows. (i) Tandem affinity purification of ICP22 from infected cells, coupled with mass spectrometry-based proteomics and subsesquent analyses demonstrates that ICP22 forms a complex(es) with the HSV-1 proteins UL31, UL34, UL47 (or VP13/14) and/or Us3. All these proteins have previously been reported to be important for viral egress through the nuclear membrane. (ii) ICP22 co-localizes with UL31 and UL34 at the nuclear membrane in wild-type HSV-1-infected cells. (iii) The UL31-null mutation prevents the targeting of ICP22 to the nuclear membrane. (iv) The ICP22-null mutation resulted in UL31 and UL34 mis-localized in the endoplasmic reticulum (in addition to the nuclear membrane) and significantly reduced numbers of primary enveloped virions in the perinuclear space, although capsids accumulated in the nuclei. Collectively, these results suggest that (i) ICP22 interacts with HSV-1 regulators of nuclear egress including UL31, UL34, UL47 and Us3 in HSV-1-infected cells, (ii) UL 31 mediates the recruitment and anchorage of ICP 22 at the nuclear membrane, and (iii) ICP22 plays a regulatory role in HSV-1 primary envelopment probably by interacting with and regulating UL31 and UL34. Here we report a previously unknown function for ICP22 in the regulation of HSV-1 nuclear egress.

HSV-1 ICP22 is primarily recognized as a regulator of viral gene expression. In this study, we show that ICP22 interacts with the HSV-1 proteins UL31 and UL34, that play crucial roles at the nuclear membrane in HSV-1 primary envelopment during viral nuclear egress. We also demonstrate that UL 31 is required for the targeting of ICP22 to the nuclear membrane and that ICP22 is required for correct localization of UL31 and/or UL34. Further, we confirm that ICP22 is required for efficient HSV-1 primary envelopment during viral nuclear egress. Thus we report, for the first time, that ICP22 plays a regulatory role in HSV-1 nuclear egress.

3. Role of the Nuclease Activities Encoded by Herpes Simplex Virus 1 UL12 in Viral Replication and Neurovirulence

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The herpes simplex virus 1 (HSV-1) UL12 gene encodes a nuclease with both endo- and exonuclease activities and an alkaline pH optimum in vitro. The UL12 amino acid sequence is well-conserved in all *Herpesviridae* subfamilies. Thus far, the exact role (s) of the UL12 nuclease activities in HSV-1 viral replication in cell cultures has not been fully determined. Here we showed that enzyme-dead mutations in the herpes simplex virus 1 UL12 gene that abolished its endo- and exonuclease activities only slightly reduced viral replication in cell cultures. However, the UL12 null-mutation significantly reduced viral replication. In contrast, the enzyme-dead mutations significantly reduced viral neurovirulence in mice. Most of the molecular mechanisms for UL12 functions proposed so far are based on the nuclease activities of UL12. However, we have presented data here showing that UL12 nuclease activities had only a minor or no significant role in viral replication in cell cultures, indicating that an undefined activity(ies) of UL12, unrelated to its nuclease activities, played a major role. Although the nuclease activities of UL12 appeared to play only a minor role in viral replication in cell cultures, we demonstrated here that the nuclease activities of UL12 played a major role in neurovirulence in mice following intracerebral inoculation. Therefore, we note that the previously proposed functions of UL12 based on its nuclease activities may be important for HSV-1 pathogenesis *in vivo*.

4. The UL12 Protein of Herpes Simplex Virus 1 Is Regulated by Tyrosine Phosphorylation

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The herpes simplex virus 1 UL12 protein (pUL12) is a nuclease that is critical for viral replication in vitro and neurovirulence in vivo. In this study, mass spectrometric analysis of pUL12 and phosphate-affinity SDS-polyacrylamide gel electrophoresis analysis identified tyrosine at pUL12 residue 371 (Tyr-371) as a pUL12 phosphorylation site: Tyr-371 is conserved in pUL12 homologs in herpesviruses in all *Herpesviridae* subfamilies. Replacement of Tyr-371 with phenylalanine (Y371F) in pUL12 (i) abol-

ished its exonuclease activity in HSV-1-infected Vero, HEL and A549 cells; (ii) reduced viral replication and cell-cell spread, and expression of pUL12 in infected cells in a cell type-dependent manner; (iii) led to aberrant subcellular localization of pUL 12 in infected cells in a cell type-dependent manner; and (iv) reduced HSV-1 neurovirulence in mice. The effects of the pUL12 Y371F mutation in cell cultures and mice were similar to those of a nuclease-dead double mutation in pUL12, although the Y371F mutation reduced viral replication several-fold more than the nuclease-dead double mutation in a cell type- and multiplicity of infection-dependent manner. Replacement of Tyr-371 with glutamic acid, which mimics constitutive phosphorylation, restored the wild-type phenotype in cell cultures and mice. These results suggested that phosphorylation of pUL12 Tyr-371 was essential for pUL12 to express its nuclease activity in HSV-1-infected cells, and that this phosphorylation promoted viral replication and cell-cell spread in cell cultures and nerurovirulence in mice mainly by up-regulating pUL12 nuclease activity and, in part, by regulating subcellular localization and expression of pUL12 in HSV-1-infected cells.

Herpesviruses encode a considerable number of enzymes for their replication. Like cellular enzymes, the viral enzymes need to be properly regulated in infected cells. Although the functional aspects of herpesvirus enzymes have gradually been clarified, there is a lack of information on how most of these enzymes are regulated in infected cells. In the present study, we have reported that the enzymatic activity of herpes simplex virus 1 alkaline nuclease pUL12 was regulated by phosphorylation of pUL12 Tyr-371 in infected cells, and that this phosphorylation promoted viral replication and cell-cell spread in cell cultures and nerurovirulence in mice, mainly by up-regulating pUL12 nuclease activity. Interestingly, pUL12 and tyrosine at pUL12 residue 371 appeared to be conserved in all herpesviruses in the family *Herpesviridae*, raising the possibility that the herpesvirus pUL12 homologs may also be regulated by phosphorylation of the conserved tyrosine residue.

Publications

Fujii, H., Kato, A., Mugitani, M., Kashima, Y., Oyama, M., Kozuka-Hata, H., Arii, J. and Kawaguchi, Y. The UL12 Protein of Herpes Simplex Virus 1 Is Regulated by Tyrosine Phosphorylation. J. Virol. 88: 10624-10634, 2014.

Kato, A., Hirohata, Y., Arii, J. and Kawaguchi, Y. Phosphorylation of Herpes Simplex Virus 1 dUTPase Up-regulated Viral dUTPase Activity to Compensate for Low Cellular dUTPase Activity for Efficient Viral Replication. J. Virol. 88: 7776-7785, 2014.

Maruzuru, Y., Shindo, K., Liu, Z., Oyama, M., Kozuka-Hata, H., Arii, J., Kato, A. and Kawaguchi, Y. The Role of Herpes Simplex Virus 1 Immediate-Early Protein ICP22 in Viral Nuclear Egress. J. Virol. 88: 7445-7454, 2014.

Liu, A., Kato, A., Shindo, K., Noda, T., Sagara, H., Kawaoka, Y., Arii, J. and Kawaguchi, Y. Herpes Simplex Virus 1 UL47 Interacts with Viral Nuclear Egress factors UL31, UL34 and Us3, and Regulates Viral Nuclear Egress. J. Virol. 88: 4657-4667, 2014.

Kato, A., Shindo, K., Maruzuru, Y. and Kawaguchi, Y. Phosphorylation of a herpes simplex virus 1 dUTPase by a viral protein kinase Us3 dictates viral pathogenicity in the central nervous system but not at the periphery. J. Virol. 88: 2775-2785, 2014.

Fujii, H., Mugitani, M, Koyanagi, N., Liu, Z., Tsuda, S., Arii, J., Kato, A. and Kawaguchi, Y. Role of the Nuclease Activities Encoded by Herpes Simplex Virus 1 UL12 in Viral Replication and Neurovirulence. J. Virol. 88: 2359-2364, 2014.

Kato, A., Tsuda, S., Liu, Z., Kozuka-Hata, H., Oyama, M. and Kawaguchi, Y. Herpes simplex virus 1 protein kinase Us3 phosphorylates viral dUTPase and regulates its catalytic activity in infected cells. J. Virol. 88: 655-666, 2014.

Fukuda, M. and Kawaguchi, Y. Role of the Immunoreceptor Tyrosine-Based Activation Motif of Latent Membrane Protein 2A (LMP2A) in Epstein-Barr Virus LMP2A-Induced Cell Transformation. J. Virol. 88: 5189-5194, 2014.

Koyanagi, N., Imai, T., Arii, J., Kato A. and Kawaguchi Y. Roles of herpes simplex virus 1 Us3 in viral neuroinvasiveness. Microbiol. Immunol. 58: 31-37, 2014.

Imamura, K., Imamachi, N., Akizuki, G., Kumakura, M, Kawaguchi, A., Nagata, K., Kato, A., Kawaguchi, Y., Sato, H., Yoneda, M., Kai, C., Yada, T., Suzuki, Y., Yamada, T., Ozawa, T., Kaneki, K., Inoue, T., Kobayashi, M., Kodama, T., Wada, Y., Sekimizu, K. and Akimitsu, N. Long Noncoding RNA NEAT1-Dependent SFPQ Relocation from Promoter Region to Paraspeckle Mediates IL8 Expression upon Immune Stimuli. Mol. Cell 53: 393-406, 2014.

Sato, A., Suwanto, A., Okabe, M., Sato, S., Nochi, T., Imai, T., Koyanagi, N., Kunisawa, J., Kawaguchi, Y. and Kiyono, H. Vaginal memory T cells induced by intranasal vaccination are critical for protective T cell recruitment and prevention of genital HSV-2 disease. J. Virol. 88: 13699-13708, 2014.

Arii, J., Hirohata, Y., Kato, A. and Kawaguchi, Y. Non-Muscle Myosin Heavy Chain IIB Mediates Herpes Simplex Virus 1 Entry. J. Virol. (in press)

Kato, A., Arii, J., Koyanagi, Y. and Kawaguchi, Y. Phosphorylation of Herpes Simplex Virus 1 dUTPase Regulates Viral Virulence and Genome Integrity by Compensating for Low Cellular dUTPase Activity in the Central Nervous System. J. Virol. (in press)