RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Host-Parasite Interaction
宿主寄生体学分野

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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. Regulatory networks formed among miRNA, transcription factors and Brm type-SWI/SNF complex.

The SWI/SNF chromatin remodeling complex plays important roles in the epigenetic regulation of many organisms and regulates a wide variety of genes. In mammals, 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.

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 often suggested to 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 up-

stream of the reporter gene, we further found that DPF2, DPF3a, DPF3b, 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 high levels. We finally showed that among them, DPF3a, DPF3b, DPF1 are most critical component for NF-κB RelA/p50 heterodimer transactivation induced by TNF-α stimulation (canonical pathway).

We further found however 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-199 a-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, allowing them to be categolized into two cancer cell types: [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 discriminate 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 (a) or virus replication (b).

(a) 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.

(b) miR-199a plays important roles in host cell competency for virus replication.

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

miR-199a-5p and -3p have been reported to suppress replication of wide-variety of viruses including herpes viruses (α, β, γ), HBV, HCV, MuLV and HIV and in this work, we have analyzed function of miR-199a-5p and -3p (miR-199a-5p/-3p) in human epithelial cells. By examining a panel of human cell lines that were derived from many kinds of epithelial tumors, we found that they tend to fall into either of the steady states, miR-199(−)/Brm(+)/EGR1(−) cells and miR-199a(+)/Brm(−)/EGR1(+) cells, denoted as type 1 and type 2, respectively. These two cell types showed clearly distinct gene expression profiles respectively, which can be mechanistically explained by a double-negative feedback loop (miR-199a/Brm/EGR1 axis) which is integrated with multiple feedforward loops. Using Human Simplex Virus-1 (HSV-1) infected to A549, a Type1 cell line, as a model system, we identified secondary envelopment of HSV-1 on intracellular membrane compartments as a major target point where exogenous miR-199a-5p/-3p shows inhibitory effects on virus replication. These miRNAs activated Cdc42 on Golgi membranes through the inhibition of ARHGAP21, a target of both miR-199a-5p and -3p to suppress virus envelopment. Consistent with these results, observation by transmission electron microscopy finally showed type-1 cell lines have generally higher efficiency of secondary envelopment than type-2 cell lines did.

2. Development new regulatable expression vectors for decoy RNAs (TuD) which strongly inhibit function of specific miRNAs and their application for the molecular analysis of epithelial-mesenchymal transition

The development of reagents that strongly suppress specific miRNAs will be important for both basic miRNA research and also as a possible therapeutic strategy. To achieve the long-term suppression of a specific miRNA, we developed specialized plasmid- and virus- vectors carrying expression units for inhibitory RNA molecules, which we have termed Tough Decoy (TuD) RNA. This year, we have developed several strictly regulatable pol III promoters designed for TuD production (Tet-On system) (c). By applying this system, we were able to analyze the kinetics of the epithelial-mesenchymal transition (EMT) induced by the simultaneous suppression of miR-200c and miR-141 in a human colorectal cell line (c).

(c) Dynamics and plasticity of epithelial to mesenchymal transition induced by miR-200 family inhibition

Takeshi Haraguchi, Masayuki Kondo, Ryo Uchikawa, Kazuyoshi Kobayashi, Hiroaki Hiramatsu, Kyousuke Kobayashi, Ung Weng Chit, Takanobu Shimizu & Hideo Iba

Whereas miR-200 family has been known to be involved in the epithelial-to-mesenchymal transition (EMT), a crucial biological process observed in normal and pathological contexts, it was largely unknown how far the functional levels of these tiny RNAs alone can propagate molecular events to accomplish this process in several days. By developing a potent inhibitor of miR-200 family members (TuD-141/200c), expression of which is strictly regulatable by Tet (tetracycline)-On system, we found that, in a human colorectal cell line, HCT116, several direct gene target mRNAs (*Zeb1*/*Zeb2, ESRP1, FN1* and *FHOD1*) of the miR-200 family were elevated in distinct kinetics. Prompt induction of transcriptional suppressors, Zeb1/Zeb2 in turn reduced expression levels of *miR-200c/-141* locus, *EpCAM, ESRP1* and *E-Cad.* The loss of ESRP1 subsequently switched the splicing isoforms of *CD44* and *p120 catenin* mRNAs to mesenchymal type. Importantly, within 9 days after the release from the inhibition of miR-200 family, all the expressional changes in 14 genes we observed here, returned to their original levels in the epithelial cells, indicating this inherent epithelial plasticity is supported by the fact that the key regulatory genes are not strongly retained in either the epithelial or mesenchymal states through epigenetic regulation in our experimental conditions using HCT116.

Publications

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)

Dynamics and plasticity of epithelial to mesenchymal transition induced by miR-200 family inhibition. Takeshi Haraguchi, Masayuki Kondo, Ryo Uchikawa, Kazuyoshi Kobayashi, Hiroaki Hiramatsu, Kyousuke Kobayashi, Ung Weng Chit, Takanobu Shimizu & Hideo Iba *Scientific Reports, in press*

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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. Molecular determinants of virulence and stability of a reporter-expressing H5N1 influenza A virus

Zhao D, Fukuyama S, Yamada S, Lopes TJ, Maemura T, Katsura H, Ozawa M[1], Watanabe S[2], Neumann G[3], Kawaoka Y: [1]Laboratory of Animal Hygiene; Transboundary Animal Distance Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Japan, [2]Laboratory of Veterinary Microbiology, Department of Veterinary Sciences, University of Miyazaki, Japan, [3]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, USA.

We previously reported that an H5N1 virus carrying the Venus reporter gene, which was inserted into the NS gene segment from the A/Puerto Rico/8/1934(H1N1) virus (Venus-H5N1 virus), became more lethal to mice compared with the wild-type Venus-H5N1 (WT-Venus-H5N1) virus, and the reporter gene was stably maintained after mouse adaptation. However, the basis for this difference in virulence and Venus stability was unclear. Here, we investigated the molecular determinants behind this virulence and reporter stability by comparing WT-Venus-H5N1 virus with a mouse-adapted Venus-H5N1 (MA-Venus-H5N1) virus. To determine the genetic basis for these differences, we used reverse genetics to generate a series of reassortants of these two viruses. We found that reassortants with PB2 from MA-Venus-H5N1 (MA-PB2), MA-PA, or MA-NS expressed Venus more stably than did WT-Venus-H5N1 virus. We also found that a single mutation in PB2 (V25A) or in PA (R443K) increased the virulence of the WT-Venus-H5N1 virus in mice and that the presence of both of these mutations substantially enhanced the pathogenicity of the virus. Our results suggest roles for PB2 and PA in the stable maintenance of a foreign protein as an NS1

fusion protein in influenza A virus.

2. Development of high-yield influenza A virus vaccine viruses

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Vaccination is one of the most cost-effective ways to prevent infection. Influenza vaccines propagated in cultured cells are approved for use in humans, but their yields are often suboptimal. Here, we screened A/Puerto Rico/8/34 (PR8) virus mutant libraries to develop vaccine backbones (defined here as the six viral RNA segments not encoding hemagglutinin and neuraminidase) that support high yield in cell culture. We also tested mutations in the coding and regulatory regions of the virus, as well as chimeric hemagglutinin and neuraminidase genes. A combination of high-yield mutations from these screens led to a PR8 backbone that improved the titers of H1N1, H3N2, H5N1, and H7N9 vaccine viruses in African green monkey kidney and Madin-Darby canine kidney cells. This PR8 backbone also improved titers in embryonated chicken eggs, a common propagation system for influenza viruses. This PR8 vaccine backbone thus represents an advance in seasonal and pandemic influenza vaccine development.

3. Mapping of a region of the PA-X protein of influenza A virus that is important for its shut-off activity

Oishi K, Yamayoshi S, Kawaoka Y

The influenza A virus PA-X protein comprises an N-terminal PA endonuclease domain and a C-terminal PA-X-specific domain. PA-X reduces host and viral mRNA accumulation via its endonuclease function. Here, we found that the N-terminal 15 amino acids, particularly six basic amino acids, in the C-terminal PA-X-specific region are important for PA-X shut-off activity. These six basic amino acids enabled a PA deletion mutant to suppress protein expression at a level comparable to that of wild-type PA-X.

4. An ultrasensitive mechanism regulates influenza virus-induced inflammation

Shoemaker JE, Fukuyama S, Eisfeld AJ[3], Zhao D, Kawakami E, Sakabe S, Maemura T, Gorai T[3], Katsura H, Muramoto Y, Watanabe S[2], Watanabe T, Fuji K, Matsuoka Y[8], Kitano H[8], Kawaoka Y: [8]The Systems Biology Institute, Japan.

Influenza viruses present major challenges to public health, as demonstrated by the 2009 influenza pandemic. Highly pathogenic influenza virus infections generally coincide with early, high levels of inflammatory cytokines that some studies have suggested may be regulated in a strain-dependent manner. However, a comprehensive characterization of the complex dynamics of the inflammatory response induced by virulent influenza strains has been lacking. Here, we applied gene co-expression and nonlinear regression analysis to time-course, microarray data developed from influenza-infected mouse lung to create mathematical models of the host inflammatory response. We found that the dynamics of inflammation-associated gene expression are regulated by an ultrasensitive-like mechanism in which low levels of virus induce minimal gene expression, but when a threshold virus titer is exceeded, expression is strongly induced. Cytokine assays confirmed that the production of several key inflammatory cytokines, such as interleukin 6 and monocyte chemotactic protein 1, exhibit ultrasensitive behavior. A systematic exploration of the pathways regulating the inflammatory-associated gene response suggests that the molecular origins of this ultrasensitive response mechanism lie within the branch of the Toll-like receptor pathway that regulates STAT1 phosphorylation. This study provides the first evidence of an ultrasensitive mechanism regulating influenza virus-induced inflammation in whole lung and provides insight into how different virus strains can induce distinct temporal inflammation response profiles. The approach developed here could be applied to the construction of gene regulatory models for other infectious diseases.

5. An Ebola whole-virus vaccine is protective in nonhuman primates

Marzi A[9], Halfmann P[3], Hill-Batorski L[3], Feldmann F[10], Shupert WL[9], Neumann G[3], Feldmann H[9], Kawaoka Y: [9]Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA, [10]Rocky Mountain Veterinary Branch, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA.

Zaire ebolavirus is the causative agent of the recent outbreak of hemorrhagic fever disease in West Africa. Previously, we showed that a whole Ebola virus (EBOV) vaccine based on a replication-defective EBOV (EBOVΔVP30) protects immunized mice

and guinea pigs against lethal challenge with rodent-adapted EBOV. Here, we demonstrate that EBOVΔVP30 protects nonhuman primates against lethal infection with EBOV. Although EBOVΔVP30 is replication-incompetent, we further inactivated the vaccine with hydrogen peroxide; the chemically inactivated vaccine remained antigenic and protective in nonhuman primates. EBOVΔVP30 thus represents a safe, efficacious, whole-EBOV vaccine candidate that differs from other EBOV vaccine platforms in that it presents all viral proteins and the viral RNA to the host immune system, which might contribute to protective immune responses.

6. Multi-spectral fluorescent reporter influenza viruses (Color-flu) as powerful tools for in vivo studies

Fukuyama S, Katsura H, Zhao D, Ozawa M[1], Ando T, Shoemaker JE, Ishikawa I, Yamada S, Neumann G[3], Watanabe S[2], Kitano H[8], Kawaoka Y

Seasonal influenza A viruses cause annual epidemics of respiratory disease; highly pathogenic avian H5N1 and the recently emerged H7N9 viruses cause severe infections in humans, often with fatal outcomes. Although there have been numerous studies of the pathogenicity of influenza viruses, influenza pathogenesis remains incompletely understood. Here, we generated influenza viruses expressing fluorescent proteins of different colors ('Color-flu' viruses) to facilitate the study of viral infection in in vivo models. On adaptation to mice, these viruses stably express the fluorescent proteins in infected animals and can be detected with different types of microscopy and by flow cytometry. We use this system to analyze the progression of viral spread in mouse lungs, for live imaging of virus-infected cells, and for differential gene expression studies in virus antigen-positive and virus antigen-negative live cells in the lungs of Color-flu-infected mice. Collectively, Color-flu viruses are powerful tools to analyze virus infections at the cellular level in vivo to better understand influenza pathogenesis.

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Department of Microbiology and Immunology

Division of Infectious Genetics
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. 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. Anterograde lysosomal trafficking regulates TLR7 dependent IFN-α production

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TLR7, an innate immune sensor for microbial nucleic acids (NAs), responds to self NA, activates dendritic cells (DCs) and B cells, and mounts antinuclear autoantibody production in murine models of systemic lupus erythematosus (SLE), an autoimmune disease where type I interferons (IFN-I) have a causative role. As the shared role in autoantibody production, RNA-sensing TLR7 promotes disease. TLR7 resides in the endoplasmic reticulum (ER), and is associated with Unc93B1, an ER-resident multiple transmembrane protein. Unc93B1 enable TLR7 to sense NA by transporting it to the endolysosomes, a site for NA-sensing, upon activation. TLR7 is double-edged sword and should be tightly controlled. However, specific regulatory mechanisms on TLR7 response is unknown. We identified a G protein specifically associated with TLR7 and regulates TLR7 response. The G protein was required for TLR7-dependent IFN-α production by plasmacytoid DCs (pDCs). The G protein specifically localizes on lysosome and regulates anterograde lysosomal trafficking. We are studying how the G protein regulates TLR7-dependent IFN-α production.

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. Guanosine and its modified derivatives are endogenous ligands for TLR7

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Toll-like receptor (TLR) 7 and 8 were considered to recognize single-strand RNA (ssRNA) from viruses. Although these receptors also respond to synthetic small chemical ligands, such as CL075 and R848, it remains to be determined whether these receptors sense natural small molecules or not. In the structure of human TLR8 (huTLR8) with ssRNA, there are two ligand-binding sites: one binds a uridine and the other binds an oligoribonucleotide (ORN). This finding demonstrates that huTLR8 recognizes degradation products of ssRNA, suggesting the presence of natural small ligands. We here show that TLR7 works as the sensor for guanosine (G)/2′-deoxyguanosine (dG) in the presence of ORN where ORN strengthens TLR7 interaction with G/dG. In addition, modified nucleosides such as 7-methylguanosine, 8-hydroxyguanosine (8-OHG) and 8-hydroxydeoxyguanosine (8-OHdG) activated TLR7 with ORNs. Importantly, 8-OHdG -a well-known oxidative DNA damage marker with unknown function- induced strong cytokine production comparable to G and dG both in mouse and human immune cells. Although 8-OHdG bound TLR7/ORN with lower affinity than dG did in isothermal titration calorimetry, administered 8-OHdG was metabolically more stable than dG in the serum, indicating that 8-OHdG acts on TLR7 as an endogenous ligand in vivo. To address a role of G analogs in the disease state, we also examined

macrophages from Unc93b1 D34A/D34A mice, which suffer from TLR7-dependent systemic inflammation, and found that Unc93b1 D34A/D34A macrophages showed significantly enhanced response to G alone or 8-OHdG with ORN. In conclusion, our results provide evidence that G, dG, 8-OHG and 8-OHdG are novel endogenous ligands for TLR7.

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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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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. To this end, we established specifications and methods for the master seed bank (MSB) of MucoRice-CTB, which was previously generated as a selection marker-free line, evaluated its propagation, and given that the stored seeds must be renewed periodically. The production of MucoRice-CTB incorporated a closed hydroponic system for cultivating the transgenic plants, to minimize variations in expression and quality during vaccine manufacture. This type of molecular farming factory can be operated year-round, generating three harvests annually, and is cost- and production-effective. Rice was polished to a ratio of 95% and then powdered to produce the MucoRice-CTB drug substance, and the identity, potency, and safety of the MucoRice-CTB product met pre-established release requirements. The formulation of MucoRice-CTB made by fine-powdering of drug substance and packaged in an aluminum pouch is being evaluated in a physician-initiated phase I study.

2. New Generation Nasal Vaccine development

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Cholera toxin (CT) induces severe diarrhea in humans but acts as an adjuvant to enhance immune responses to vaccines when administered orally. Nasally administered CT also acts as an adjuvant, but CT and CT derivatives including its B subunit (CTB) are taken up from the olfactory epithelium and transported to the olfactory bulbs and therefore may be toxic to the central nervous system. To assess the toxicity, we investigated whether nasally administrated CT or CT derivatives impair the olfactory system. In mice, nasal administration of CT, but not CTB or a non-toxic CT derivative, reduced the expression of olfactory marker protein (OMP) in the olfactory epithelium and olfactory bulbs and impaired odor responses, as determined with behavioral tests and optical imaging. Thus, nasally administered CT, like orally administered CT, is toxic and damages the olfactory system in mice. However, CTB and a non-toxic CT derivative, do not damage the olfactory system. The optical imaging we used here will be useful for assessing the safety of nasal vaccines and adjuvants during their development for human use and CT can be used as a positive control in this test.

3. Oral Antibody Therapy for Infectious Disease

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To develop oral antibody therapy against rotavirus infection, we previously produced a recombinant fragment of llama heavy-chain antibody to rotavirus (ARP1) in rice seeds (MucoRice-ARP1). We intend to use a purification-free rice powder for clinical application but needed to check whether MucoRice-ARP1 had increased levels of known allergen proteins. To address this concern, we used two-dimensional fluorescence difference gel electrophoresis to compare the rice allergen protein levels in MucoRice-ARP1 and wild-type rice. We detected no notable differences, except in the levels of α-amylase/trypsin inhibitor-like family proteins. Because by this approach we could not completely separate ARP1 from the proteins of this family, we confirmed the absence of changes in the levels of these allergens by using shotgun mass spectrometry as well as immunoblot. By using immunoelectron microscopy, we also showed that RAG2, a member of the α-amylase/trypsin inhibitor-like protein family, was relocated from protein bodies II to the plasma membrane or cell wall in MucoRice-ARP1 seed. The relocation did not affect the level of RAG2. We demonstrated that most known rice allergens were not considerably upregulated by the genetic modification in MucoRice-ARP1, suggesting that MucoRice-ARP1 is a potentially safe oral antibody for clinical application.

4. Critical role of TSLP-responsive mucosal dendritic cells in the induction of nasal antigen-specific IgA response

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Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-7 like cytokine involved in T helper 2 type immune responses. The primary target of TSLP is myeloid dendritic cells (DCs), however, little is known about the mechanism by which TSLP elicits respiratory IgA immune responses upon mucosal immunization. Here, we found that the levels of TSLP and TSLPR were up-regulated in the mucosal DCs of mice nasally-immunized with pneumococcal surface protein A (PspA) plus cholera toxin (CT) compared with those immunized with PspA alone. PspA-specific IgA responses, but not IgG Ab responses were significantly reduced in both serum and mucosal secretions of TSLPR knockout mice compared with wild-type mice after nasal immunization with PspA plus CT. Furthermore, $CD11c^+$ mucosal DCs isolated TSLPR knockout mice nasally immunized with PspA plus CT were less activated and exhibited markedly reduced

expression of IgA-enhancing cytokines (e.g., APRIL, BAFF and IL-6) compared with those from equivalently immunized wild-type mice. Finally, exogenous TSLP promoted IgA productions in an in vitro DC-B cell co-culture system as exhibited by enhanced IL-6 production. These results suggest that TSLP-TSLPR signaling is pivotal in the induction of nasal respiratory immunity against pathogenic pneumococcal infection.

5. Critical role of commensal flora-dependent type 3 innate lymphoid cells (ILC3) for the induction and regulation of Paneth cells

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Intestinal epithelial cells (ECs) have fucosylation which is one of glycosylation pattern for the creation of cohabitation and protective niches. Our previous research showed that 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. However, further analysis using Fut1- and Fut2-deficient mice revealed that fucosylation of Paneth cells is regulated by both Fut1 and Fut2, and Paneth cells can be at least divided into two subsets, Fut1 only, and Fut2 expressed double positive cells. We also revealed that Fut2 expressing Paneth cells is induced and regulated by ILC3 in commensal bacteria-dependent manner. Moreover, the expression of Reg-III family, which is a pivotal player of the immunosurveillance in the intestine, is associated with Fut2-expressing Paneth cells by the commensal flora-ILC3 axis dependent manner. Taken together, our findings suggest that the commensal flora-ILC3 axis plays critical roles for induction and regulation of Fut2- and Reg-III-positive Paneth cells. Our current study is aiming at the molecular and cellular understanding of the commensal flora-ILC3 axis dependent Paneth cells for their contributions in the creation of healthy intestinal environments.

6. Profiles of microRNA networks in intestinal epithelial cells in a mouse model of colitis

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Inflammatory bowel diseases (IBDs) accompany a critical loss of the frontline barrier function that is achieved primarily by intestinal epithelial cells (IECs). Although the gene-regulation pathways underlying these host-defense roles of IECs presumably are deranged during IBD pathogenesis, the quantitative and qualitative alterations of posttranscriptional regulators such as microRNAs (miRNAs) within the cells largely remain to be defined. We aimed to uncover the regulatory miRNA-target gene relationships that arise differentially in inflamed small- compared with large-IECs. Whereas IBD significantly increased the expression of only a few miRNA candidates in small-IECs, numerous miRNAs were upregulated in inflamed large-IECs. These marked alterations might explain why the large, as compared with small, intestine is more sensitive to colitis and shows more severe pathology in this experimental model of IBD. Our in-depth assessment of the miRNA-mRNA expression profiles and the resulting networks prompts us to suggest that miRNAs such as miR-1224, miR-3473a, and miR-5128 represent biomarkers that appear in large-IECs upon IBD development and co-operatively repress the expression of key anti-inflammatory factors. The current study provides insight into gene-regulatory networks in IECs through which dynamic rearrangement of the involved miRNAs modulates the gene expression-regulation machinery between maintaining and disrupting gastrointestinal homeostasis.

7. Host-commensal interaction in the upper respiratory tract

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Nasal mucosa is constitutively exposed to countless numbers of aerobic microorganisms including pathogenic, non-pathogenic, and opportunistic bacteria. It has been reported that these bacteria form biofilm in response to the stimuli of extracellular microenvironment and interact with host epithelial surfaces. However, the composition and the physiological roles of commensals colonized in the upper respiratory tract are still unknown. In current study, we examined microbiome reside in the nasal polyps and normal nasal tissues by using next generation sequencer. Interestingly, *Pseudomonas stutzeri*, which are not usually detected by laboratory culture of nasal lavage, were relatively abundant in the nasal polyps. There is a possibility that *Pseudomonas* species play an important role in the pathogenesis of nasal polyposis. Furthermore, *P. aeruginosa* preferentially colonized in the intestine and nasal passages after depletion of gut microbiota, suggesting that intestinal commensals have a role in the prevention of *P. aeruginosa* colonization both in the intestine and upper respiratory tract. We also examined salivary microbiome in order to evaluate the role of commensals in the pathogenesis of chronic rhinosinusitis. At the results, the relative abundance of the genus *Haemophilus* in the eosinophilic chronic rhinosinusitis patients was low compared to that in the non-eosinophilic chronic rhinosinusitis patients. From this data, we hypothesize that low abundance of the genus *Haemophilus* in the saliva may be associated with eosinophilic inflammation.

8. Specific expression of phagocytosis and membrane ruffling associated molecule Aif1 by M cells

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Microfold (M) cells are known as antigen uptake intestinal epithelial cells. It has been reported that transcription factor Spi-B acts as a pivotal transcription factor for the development of M cells with inducing M-cell-specific functional protein, Glycoprotein2 (GP2). The DNA microarray analysis of the follicle associated epithelium of Peyer's patches from *Spib*$^{+/+}$ and *Spib*$^{-/-}$ mice revealed M-cell specific expression of Aif1 associating with phagocytosis and formation of membrane ruffling in microglia and macrophages. Confocal microscopic analysis confirmed that Aif1 was specifically expressed in GP2-positive matured M cells. Therefore, we next investigated whether newly found Aif1 is involved in M-cell development and/or function. When the presence of M-cell was examined in Aif1-deficient mice, comparable numbers of M cells were found with wild-type (WT) mice suggesting that Aif1 is not involved in M-cell development. Contrary, however, Aif1-deficient mice showed significant lower particle antigen uptake compared with WT mice, suggesting that Aif1 has an essential role in M-cell transcytosis function. We are now investigating cellular and molecular mechanisms which might be involved in the Aif1-regulating antigen uptake by M cells.

9. The construction of the new oral immunotherapy mouse model and the analysis of the intestinal tract immunoresponse.

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There is the history that the oral immunotherapy was provided as a folk remedy for a long time and takes in an antigen causing a serious reaction to a body while increasing gradually (the acute phase) and is a cure to acquire the tolerance to an antigen by taking in a fixed quantity continuously for a long term afterwards (maintenance phase).A clinical trial of the oral immunotherapy is carried out as radical treatment of the food allergy much in our country, but yet the treatment has a problem with the safety, and the effectiveness. Development of better treatment is expected, however no detail is understood about the mechanism of immunotherapy.

In particular the local immunorespons at the intestinal mucosa in the acute phase reaction is still less understood. We tried the manufacture of the oral immunotherapy mouse model for the purpose of the analysis. First we sensitized mice using com-

plete Freund's aducant and chicken egg white albumin (OVA), and induce food allergy with the onset of the allergic diarrhea by oral administration of OVA afterwards.

Second, Oral immunotherapy for this allergic mouse in various protocols was taken. As the results, a digestive organ symptom onset rate was significantly improved in comparison with an untreated group in oral immunotherapy group. It was confirmed in the treatment group that the increase of regulatory T cell with the desensitization of mucosal mast cell in the large intestine. In conclusion, we made an effective oral immunotherapy model for a food allergy mouse. This model is expected as an effective tool analyzing oral immunization generosity in future.

10. LPS from symbiotic bacteria, *Alcaligenes*, acts as a weak agonist to TLR4 and participate in the production of IgA in the GALT

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We previously demonstrated that commensal bacteria are present not only in the intestinal lumen and epithelium but also inside of Peyer's patches (PPs), a major gut-associated lymphoid tissue in the small intestine. In this issue, we identified *Alcaligenes*, which develop symbiosis inside the PPs. Since *Alcaligenes* grow in the PPs of germ-free (GF) mice 3 weeks after oral inoculation, it is likely that at least some portions of *Alcaligenes* are alive in the PPs. We also demonstrated that *Alcaligenes* participates in IgA production by inducing the production of IL-6, BAFF and TGF-β from DCs and consequently *Alcaligenes*-mono association in GF mice exhibited the increase of SIgA production in the feces. Although these findings reveal the involvement of *Alcaligenes* in the development of host immune system especially in the PPs, it remains to be investigated why *Alcaligenes* can establish symbiosis without excessive inflammatory responses in the PPs.

In this study, we focus on the lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria. It is known that LPS structure is different among bacteria, which determines the inflammatory activity. We show here that *Alcaligenes*-derived LPS acts as a weak agonist for toll-like receptor 4 and induces IL-6 production from dendritic cells and subsequent IgA responses. The inflammatory activity of *Alcaligenes*-derived LPS was lower than that of *E. coli*-derived LPS and thus no excessive inflammation was induced by *Alcaligenes*-derived LPS *in vitro* and *in vivo*. These features allow commensal bacteria-mediated homeostatic inflammatory condition inside of Peyer's patches.

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Department of Microbiology and Immunology

Division of Molecular Virology
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. Role of Host Cell p32 in Herpes Simplex Virus 1 De-envelopment During Viral Nuclear Egress

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To clarify the function(s) of the herpes simplex virus 1 (HSV-1) major virion structural protein UL47 (also designated VP13/14), we screened cells overexpressing UL47 for UL47-binding cellular proteins. Tandem affinity purification of transiently expressed UL47 coupled with mass spectrometry-based proteomics technology and subsequent analyses showed that UL47 interacted with cell protein p32 in HSV-1-infected cells. Unlike in mock-infected cells, p32 accumulated at the nuclear rim in HSV-1-infected cells and this p32 recruitment to the nuclear rim required UL47. p32 formed a complex(es) with HSV-1 proteins UL31, UL34, Us3, UL47 and/or ICP22 in HSV-1-infected cells. All these HSV-1 proteins were previously reported to be important for HSV-1 nuclear egress, in which nucleocapsids bud through the inner nuclear membrane (primary envelopment) and the enveloped nucleocapsids then fuse with the outer nuclear membrane (de-envelopment). Like viral proteins UL31, UL34, Us3 and UL47, p32 was detected in primary enveloped virions. p32 knock-down reduced viral replication and induced membranous invaginations adjacent to the nuclear rim containing primary enveloped virions and aberrant localization of UL31 and UL34 in punctate structures at the nuclear rim. These effects of p32 knock-down were reduced in the absence of UL47. Therefore, the effects of p32 knock-down in HSV-1 nuclear egress were similar to those of the previously reported mutation(s) in HSV-1 regulatory proteins for HSV-1 de-envelopment during viral nuclear egress. Collectively, these results suggested that p32 regulated HSV-1 de-envelopment and replication, in a UL47-dependent manner.

2. Herpes simplex virus 1 recruits CD98 heavy chain and β1 integrin to the nuclear membrane for viral de-envelopment

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Herpesviruses have evolved a unique mechanism for nucleocytoplasmic transport of nascent nucleocapsids: the nucleocapsids bud through the inner nuclear membrane (INM) (primary envelopment), and the enveloped nucleocapsids then fuse with the outer nuclear membrane (de-envelopment). Little is known about the molecular mechanism of herpesviral de-envelopment. Here we showed that knockdown of both CD98 heavy chain (CD98hc) and its binding partner β1 integrin induced membranous structures containing enveloped herpes simplex virus 1 (HSV-1) virions that are invaginations of the INM into the nucleoplasm, and induced aberrant accumulation of enveloped virions in the perinuclear space and in the invagination structures. These effects were similar to those of the previously reported mutation(s) in HSV-1 proteins gB, gH, UL31 and/or Us3, which were shown here to form a complex(es) with CD98hc in HSV-1-infected cells. These results suggested that cellular proteins CD98hc and β1 integrin synergistically or independently regulated HSV-1 de-envelopment, probably by interacting directly and/or indirectly with these HSV-1 proteins.

3. Non-Muscle Myosin Heavy Chain IIB Mediates Herpes Simplex Virus 1 Entry

Jun Arii, Yoshitaka Hirohata, Akihisa Kato and Yasushi Kawaguchi

Non-muscle myosin heavy chain IIA (NMHC-IIA) has been reported to function as a herpes simplex virus 1 (HSV-1) entry co-receptor by interacting with viral envelope glycoprotein B (gB). Vertebrates have three genetically distinct isoforms of the NMHC-II, designated NMHC-IIA, NMHC-IIB and NMHC-IIC. COS cells, which are readily infected by HSV-1, do not express NMHC-IIA but do express NMHC-IIB. This observation prompted us to investigate whether NMHC-IIB might associate with HSV-1 gB and be involved in an HSV-1 entry like NMHC-IIA. In these studies, we show that: (i) NMHC-IIB co-precipitated with gB in COS-1 cells upon HSV-1 entry; (ii) a specific inhibitor of myosin light chain kinase inhibited both cell-surface expression of NMHC-IIB in COS-1 cells upon HSV-1 entry as well as HSV-1 infection as reported with NMHC-IIA; (iii) overexpression of mouse NMHC-IIB in IC21 cells significantly increased their susceptibility to HSV-1 infection; (iv) knock-down of NMHC-IIB in COS-1 cells inhibited HSV-1 infection as well as cell-cell fusion mediated by HSV-1 envelope glycoproteins. These results supported the hypothesis that, like NMHC-IIA, NMHC-IIB associated with HSV-1 gB and mediated HSV-1 entry.

4. The Function of the Herpes Simplex Virus 1 Small Capsid Protein VP26 is Regulated by Phosphorylation at a Specific Site.

Ryosuke Kobayashi, Akihisa Kato, Shinya Oda, Naoto Koyanagi, Masaaki Oyama, Hiroko Kozuka-Hata, Jun Arii and Yasushi Kawaguchi

Replacement of the herpes simplex virus 1 small capsid protein VP26 phosphorylation site Thr-111 with alanine reduced viral replication and neurovirulence to levels observed with the VP26 null-mutation. This mutation reduced VP26 expression and mis-localized VP26 and its binding partner, the major capsid protein VP5, in the nucleus. VP5 mis-localization was also observed with the VP26 null-mutation. Thus we postulate that phosphorylation of VP26 at Thr-111 regulates VP26 function *in vitro* and *in vivo*.

5. 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

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A mutation in herpes simplex virus 1 dUTPase (vdUTPase), which precluded its phosphorylation at Ser-187, decreased viral neurovirulence and increased mutation frequency in progeny virus genomes in the brains of mice where endogenous cellular dUTPase activity was relatively low, and overexpression of cellular dUTPase restored viral neurovirulence and mutation frequency altered by the mutation. Thus, phosphorylation of vdUTPase appeared to regulate viral virulence and genome integrity by compensating for low cellular dUTPase activity *in vivo*.

6. Interactome Analysis of Herpes Simplex Virus 1 Envelope Glycoprotein H

Yoshitaka Hirohata, Akihisa Kato, Masaaki Oyama, Hiroko Kozuka-Hata, Naoto Koyanagi, Jun Arii and Yasushi Kawaguchi

Herpes simplex virus 1 (HSV-1) envelope glycoprotein H (gH) is important for viral entry into cells and nuclear egress of nucleocapsids. To clarify additional, novel roles of gH during HSV-1 replication, we screened host cell proteins interacting with gH by tandem affinity purification coupled with mass spectrometry-based proteomics in 293T cells

transiently expressing gH. This screen identified 123 host cell proteins as potential gH interactors. Of these proteins, general control nonderepressive-1 (GCN1), a *trans*-acting positive effector of GCN2 kinase, which regulates the phosphorylation of the α subunit of translation initiation factor 2 (eIF2α), was subsequently confirmed to interact with gH in HSV-1-infected cells. eIF2α phosphorylation is known to downregulate protein synthesis, and various viruses have evolved mechanisms to prevent the accumulation of phosphorylated eIF2α in infected cells. Here, we showed that GCN1 knockdown reduced the phosphorylation of eIF2α in HSV-1-infected cells and that the *gH*-null mutation increased eIF2α in HSV-1-infected cells, whereas gH overexpression in the absence of other HSV-1 proteins reduced eIF2α phosphorylation. These results suggest that GCN1 can regulate eIF2α phosphorylation in HSV-1-infected cells and that the GCN1-binding viral partner gH is necessary and sufficient to prevent the accumulation of phosphorylated eIF2α. Our database of 123 host cell proteins potentially interacting with gH will be useful for future studies to unveil further novel functions of gH and the roles of the cellular proteins in HSV-1-infected cells.

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