RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Bacterial Infection
細菌感染分野

Professor	Chihiro Sasakawa, Ph.D.	教授	医学博士	笹川千尋
Lecturer	Hitomi Mimuro, D. Ph.D.	講師	医学博士	三室仁美
Assistant Professor	Michinaga Ogawa, Ph.D.	助教	医学博士	小川道永
Assistant Professor	Masato Suzuki, Ph.D.	助教	医学博士	鈴木仁人
Assistant Professor	Hiroshi Ashida, Ph.D.	助教	医学博士	芦田浩

Research in this division is directed toward understanding the complex interactions that occur between pathogenic bacteria and the gastrointestinal epithelium and the process of infectious diseases. Our special interest is focused upon the molecular pathogenicity of enteropathogenic bacteria, such as Shigella, Helicobacter pylori, enteropathogenic E. coli and enterohemorrhagic E. coli. We are also searching for effective methods to protect or regulate bacterial infection by using knowledge accumulated, and interested in developing animal model for studying the bacterial pathogens.

1. A bacterial E3 ubiquitin ligase IpaH9.8 targets NEMO/IKKγ to dampen the host NF-κB-mediated inflammatory response.

Hiroshi Ashida, Minsoo Kim, Mare Schmidt-Supprian[1], Averil Ma[2], Michinaga Ogawa and Chihiro Sasakawa: [1]Max Planck Institute of Biochemistry, Martinsried, Germany, [2]Department of Medicine, University of California at San Francisco, San Francisco, USA.

NF-κB (nuclear factor κB) has a pivotal role in many cellular processes, including the inflammatory and immune responses and, therefore, its activation is tightly regulated by the IKK (IκB kinase) complex and by IκBα degradation. When *Shigella* bacteria multiply within epithelial cells they release peptidoglycans, which are recognized by Nod1 and stimulate the NF-κB pathway, thus leading to a severe inflammatory response. Here, we show that IpaH9.8, a *Shigella* effector possessing E3 ligase activity, dampens the NF-κB-mediated inflammatory response to the bacterial infection in a unique way. IpaH9.8 interacts with NEMO/IKKγ and ABIN-1, a ubiquitin-binding adaptor protein, promoting ABIN-1-dependent polyubiquitylation of NEMO. Consequently, polyubiquitylated NEMO undergoes proteasome-dependent degradation, which perturbs NF-κB activation. As NEMO is essential for NF-κB activation, we propose that the polyubiquitylation and degradation of NEMO during *Shigella* infection is a new bacterial strategy to modulate host inflammatory responses.

2. Bacterial interactions with the host epithelium.

Minsoo Kim, Hiroshi Ashida, Michinaga Ogawa, Yuko Yoshikawa, Hitomi Mimuro and Chihiro Sasakawa

The gastrointestinal epithelium deploys multiple innate defense mechanisms to fight microbial intruders, including epithelial integrity, rapid epithelial cell turnover, quick expulsion of infected cells, autophagy, and innate immune responses. Nevertheless, many bacterial patho-

gens are equipped with highly evolved infectious stratagems that circumvent these defense systems and use the epithelium as a replicative foothold. During replication on and within the gastrointestinal epithelium, gastrointestinal bacterial pathogens secrete various components, toxins, and effectors that can subvert, usurp, and exploit host cellular functions to benefit bacterial survival. In addition, bacterial pathogens use a variety of mechanisms that balance breaching the epithelial barrier with maintaining the epithelium in order to promote bacterial colonization. These complex strategies represent a new paradigm of bacterial pathogenesis.

3. The bacterial effector Cif interferes with SCF ubiquitin ligase function by inhibiting deneddylation of Cullin1.

Morikawa, Hanako, Minsoo Kim, Hitomi Mimuro, Claire Punginelli, Tomohiro Koyama, Shinya Nagai, Atsushi Miyawaki, Kazuhiro Iwai and Chihiro Sasakawa: [1]Nippon Institute for Biological Science, Ome, Tokyo, Japan, [2]Laboratory for Cell Function and Dynamics, Advanced Technology Development Group, Brain Science Institute, RIKEN, Wako-city, Saitama, Japan, [3]Department of Biophysics and Biochemistry, Graduate School of Frontier Biosciences/Graduate School of Medicine Osaka University, Suita, Osaka, Japan

Cycle inhibiting factor (Cif) is one of the effectors delivered into epithelial cells by enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) via the type III secretion system (TTSS). Cif family proteins, which inhibit host cell-cycle progression via mechanisms not yet precisely understood, are highly conserved among EPEC, EHEC, *Yersinia pseudotuberculosis, Photorhabdus luminescens* and *Burkholderia pseudomallei*. Levels of several proteins relevant to cell-cycle progression are modulated by Cullin-RING ligases (CRLs), which in turn are activated by conjugation and deconjugation of NEDD8 to Cullins. Here we show that Cif interacts with NEDD8 and interferes with SCF (Skp1-Cullin1-F-box protein) complex ubiquitin ligase function. We found that neddylated Cullin family proteins accumulated and ubiquitination of p27 decreased in cells infected with EPEC. Consequently, Cif stabilized SCF substrates such as CyclinD1, Cdt1, and p27, and caused G1 cell-cycle arrest. Using time-lapse-imaging of fluorescent ubiquitination-based cell-cycle indicator (Fucci)-expressing cells, we were able to monitor cell-cycle progression during EPEC infection and confirmed the arrest of infected cells at G1. Our in vitro and in vivo data show that Cif-NEDD8 interaction inhibits deneddylation of Cullins, suppresses CRL activity and induces G1 arrest. We thus conclude that the bacterial effector Cif interferes with neddylation-mediated cell-cycle control.

Publications

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Department of Microbiology and Immunology

Division of Host-Parasite Interaction
宿主寄生体学

Professor	Hideo Iba, Ph.D.	教　授	理学博士	伊　庭　英　夫
Assistant Professor	Taketoshi Mizutani, Ph.D.	助　教	医学博士	水　谷　壮　利
Assistant Professor	Takeshi Haraguchi, Ph.D.	助　教	理学博士	原　口　　健

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 a chromatine remodeling factor, SWI/SNF complex and also analyzing regulatory networks formed among microRNAs, SWI/SNF complex and such important transcriptional factors as AP-1 and NF-kappaB. Using these results, we also develop new retrovirus/lentivirus vectors that express proteins, short hairpin RNAs, miRNAs and efficient inhibitory RNAs targeting specific miRNAs (designated as Tough Decoy RNAs) for tools of human gene therapy and basic researches.

1. SWI/SNF complex as a pivotal regulator of NF-κB

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 been previously reported.

Overall, the SWI/SNF complex interacts with various proteins, including transcriptional regulator, through many specific and varied associations with its several subunits. We previously demonstrated unique interaction between SWI/SNF complex and an important transcription factor, AP-1, which is composed of dimmers formed between Fos family proteins and Jun family proteins. Each Fos/Jun heterodimer had been shown to have distinctive transactivating potential for regulating cellular growth, differentiation, and development via AP-1 binding sites, but the molecular mechanism underlying dimer specificity and the molecules that facilitate transactivation remained undefined. In 2001, we showed that BAF60a, a subunit of the SWI/SNF chromatin remodeling complex, is a determinant of the transactivation potential of Fos/Jun dimers. BAF60a binds to a specific subset of Fos/Jun heterodimers using two different interfaces for c-Fos and c-Jun, respectively, explaining why a specific subset of Fos/Jun dimers recruits SWI/SNF complex to AP-1 binding sites present in many gene promoters via BAF60a to drastically induce their transcription simultaneously.

This year, we show that another very important transcription factor NFκB requires SWI/SNF complex to activate certain subsets of genes.

Like AP-1, NFκB plays crucial roles in such physiological processes as development, cell proliferation, apoptosis and innate and adaptive immune functions. The NFκB family proteins are composed of five different proteins; RelA (p65), RelB, c-Rel, p50 (which is processed from its precursor p105) and p52 (which is processed from its precursor p100). These proteins form active transcription factors as homodimers or heterodimers. Among various NFκB-activating signals, two different NFκB pathways have been well studied. One is the canonical NFκB pathway, which is activated by TNFα and IL-1 stimulation. Upon their stimuli, proteosomal degradation of cytosolic IκBα allows nuclear translocation of RelA/p50 dimer, which induces expression of *IL-8* and *IκBα*. The other is the non-canonical NFκB pathway, which is activated by lymphotoxin and CD40 ligand. Upon their treatment, activated NIK and IKKα induce the processing of p100 to p52, which triggers gene activation of *BLC* and *ELC* by RelB/p52 dimer. Aberrant regulation of NFκB is known to be involved with cancer development and progression. Importantly, some NFκB target genes stimulated by these cytokines or growth factors were often shown to require SWI/SNF complexes for their optimum induction. However, the underlying molecular mechanisms and factors involved in this process are largely unknown.

This year, we present evidence that Requiem (REQ/DPF2) protein, which was originally identified as an apoptosis inducing protein in mouse myeloid cells and belongs to the d4-family (DPF family) 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. We further show evidence that REQ is required for oncogenesis of several human tumor cell lines in which the non-canonical NFκB pathway is aberrantly regulated.

Requiem (DPF2) protein links RelB/p52 and the Brm-type SWI/SNF complex in a non-canonical NF-κB pathway

Toshio Tando, Aya Ishizaka, Hirotaka Watanabe, Taiji Ito, Shun Iida, Takeshi Haraguchi, Taketoshi Mizutani, Tomonori Izumi[1], Toshiaki Isobe[1], Taishin Akiyama[2], Jun-ichiro Inoue[1], and Hideo Iba: [1]Division of Proteomics Research and [2]Division of Cellular and Molecular Biology. Institute of Medical Science, University of Tokyo

The SWI/SNF chromatin remodeling complex plays pivotal roles in mammalian transcriptional regulation. In our current study, we identify the human requiem protein (REQ/DPF2) as an adaptor molecule that links NF-κB and SWI/SNF chromatin remodeling factor. Through *in vitro* binding experiments, REQ was found to bind to several SWI/SNF complex subunits and also to the p52 NF-κB subunit through its NLS-containing N-terminus region. REQ, together with Brm, a catalytic subunit of the SWI/SNF complex, enhances the NF-κB-dependent transcriptional activation that principally involves the RelB/p52 dimer. Both REQ and Brm were further found to be required for the induction of the endogenous *BLC (CXCL13)* gene in response to lymphotoxin stimulation, an inducer of the non-canonical NF-κB pathway. Upon lymphotoxin treatment, REQ and Brm form a larger complex with RelB/p52 and are recruited to the *BLC* promoter in a ligand-dependent manner. Moreover, a REQ knockdown efficiently suppresses anchorage-independent growth in several cell lines in which the non-canonical NF-κB pathway is constitutively activated. From these results, we conclude that REQ functions as an efficient adaptor protein between the SWI/SNF complex and RelB/p52 and plays important roles in non-canonical NF-κB transcriptional activation and its associated oncogenic activity.

2. Robust regulatory networks formed among Brm type-SWI/SNF complex, transcriptional factors and miRNAs separate most of the human tumor cell lines into two groups.

Accumulating evidence now indicates that alterations to SWI/SNF subunits contribute to tumorigenesis. In terms of the Brm catalytic subunit, we and others have reported that it is frequently deficient in various cancer cell lines such as SW13(vim-), AZ521and C33A, and also in primary tumors of the lung, stomach, and prostate. As described in the previous chapter, expression of retro/lenti virus vectors were stochastically and promptly silenced in all of these Brm-deficient cell lines. We further found however that a functional *Brm* gene is present and transcribed actively in all of the Brm-deficient cell lines in nuclear run-on transcription assays. This indicated that post-transcriptional gene silencing suppresses *Brm* in these human cancer cells. When we exogenously expressed Brm in such deficient cell lines, this resulted in a reduced oncogenic potential, suggesting that Brm has tumor suppressive properties. This possibil-

ity is consistent with our observations that a frequent loss of Brm expression in gastric cancers correlates with a less differentiated state. However, the molecular mechanisms underlying the post-transcriptional suppression of Brm remained to be elucidated.

MiRNAs are potent regulators of coding genes at the post-transcriptional level, including human genes. These molecules are ~22nt non-coding RNAs that mediate the repression of target transcripts by suppressing their translation or promoting degradation. More than 1000 species of miRNAs have now been identified in humans. Each miRNA has a number of target genes whereas individual mRNAs can be targeted by several different miRNAs. We here demonstrate 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. We further show from our analysis that the expression patterns of mature miR-199a-5p and -3p, and of the Brm protein, are mutually exclusive in many human cancer cell lines as well as in some human tumor cells. We finally present evidences that a double-negative feedback mechanism underlies this regulatory network and separates many cancer cell lines into two distinct groups (a). In 2008-2009, we also observed that miR-21 and its targets, PDCD4 and NFIB form also robust double-negative feedback loops and indicated that miR-21 RNA (highly expressed in cancer lesion) and PDCD4 protein (highly expressed in normal mucosa) show mutually exclusive expression patterns in human colorectal cancer specimen. So we believe miRNAs can generally function as molecular switches for epigenetical regulation by utilizing double-negative feedback regulation. In these processes, we also developed techniques for selective expression or suppression of miR-5p and miR-3p strands by using chemically-modified miRNA duplex (b) and by vectors expressing TuD-5p and TuD-3p, respectively.

a. microRNAs miR-199a-5p and -3p target the Brm subunit of SWI/SNF to generate a double-negative feedback loop in a variety of human cancers

Kouhei Sakurai, Chihiro Furukawa, Takeshi Haraguchi, Ken-ichi Inada[3], Kazuya Shiogama[3], Takanobu Tagawa, Shuji Fujita, Yoshihito Ueno[4], Aya Ogata[4], Mai Ito[3], Yutaka Tsutsumi[3], and Hideo Iba: [3]First Department of Pathology, Faculty of Medicine, Fujita Health University, Aichi, Japan, [4]Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu, Japan,

The chromatin remodeling complex SWI/SNF is an important epigenetic regulator that includes one Brm or BRG1 molecule as catalytic subunit. Brm and BRG1 do not function identically so this complex can regulate gene expression either positively or negatively depending on the promoter to which it is recruited. Notably, Brm attenuation due to post-transcription suppression occurs often in human tumor cells, where this event contributes to their oncogenic potential. Here we report that the 3′UTR of *Brm* mRNA has two sites that are efficiently targeted by the microRNAs miR-199a-5p and -3p, revealing a novel mechanism for modulation of Brm-type SWI/SNF activity. Computational mapping of the putative promoter region of *miR-199a-2* (miPPR-199a-2) has been defined it as the major contributing genetic locus for miR-199a-5p and-3 p production in these tumor cell lines. We validated this predicted region by direct promoter analysis to confirm that Egr1 is a strong positive regulator of the *miR-199a-2* gene. Importantly, we also showed that Egr1, miR-199a-5p and -3p are expressed at high levels in Brm-deficient tumor cell lines, but only marginally in Brm-expressing tumor cells. Lastly, we also obtained evidence that Brm negatively regulates *Egr1*. Together, our results reveal that miR-199a and Brm form a double-negative feedback loop through Egr1, leading to the generation of these two distinct cell types during carcinogenesis. This mechanism may offer a partial explanation for why miR-199a-5p and -3p have been reported to be either up-regulated or down-regulated in a variety of tumors.

b. Biaryl modification of the 5′-terminus of one strand of a microRNA duplex induces strand specificity

Aya Ogata[*4], Chihiro Furukawa[*], Kouhei Sakurai[*], Hideo Iba, Yukio Kitade[4], and Yoshihito Ueno[4]: [*]These authors were contributed equally to this work.

MicroRNAs (miRNAs) are single-stranded non-coding RNAs composed of 20-23 nucleotides. They are initially transcribed in the nucleus as pri-miRNAs. After processing, one strand from the miRNA duplex (miR-5p/miR-3 p duplex) is loaded onto the RNA-induced silencing complex (RISC) to produce a functional, mature miRNA that inhibits the expression of multiple target genes. In the case of some miRNAs, both strands can be equally incorporated

into the RISC as single strands, and both strands can function as mature miRNAs. Thus, a technique for selective expression of miR-5p and miR-3p strands is required to identify distinct targets of miRNAs. In this paper, we report the synthesis and properties of miRNA duplexes carrying biaryl units at the 5′-terminus of one strand. We found that incorporation of biaryl units at the 5′-terminus of one strand of miRNA duplexes induced strand specificity in these duplexes. Further, we succeeded in identifying endogenous mRNA targets for each strand of the duplex by using the biaryl-modified miRNA duplexes.

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原口健，櫻井浩平，伊庭英夫：特集miRNA研究の最近の進歩―TuD　RNA発現ベクターを用いたmiRNAの活性阻害による機能解析　生体の科学　61(4)：326–331，2010.

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Department of Microbiology and Immunology

Division of Virology
ウイルス感染分野

Professor	Yoshihiro Kawaoka, D.V.M., Ph.D.	教　授	獣医学博士	河　岡　義　裕
Associate Professor	Taisuke Horimoto, D.V.M., Ph.D.	准教授	農学博士	堀　本　泰　介
				(2010年4月まで)
Assistant Professor	Hideo Goto, D.V.M., Ph.D.	助　教	獣医学博士	五　藤　秀　男
Assistant Professor	Masayuki Shimojima, D.V.M., Ph.D.	助　教	獣医学博士	下　島　昌　幸
				(2010年3月まで)
Research Associate	Yuko Sakai-Tagawa, Ph.D.	助　手	医学博士	坂井(田川)優子
Project Assistant Professor	Kiyoko Iwatsuki-Horimoto, D V M , Ph D	特任助教	獣医学博士	岩附(堀本)研子

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. T-705 (favipiravir) activity against lethal H5 N1 influenza A viruses

Kiso M, Takahashi K[1], Sakai-Tagawa Y, Shinya K[2], Sakabe S, Le QM[3], Ozawa M, Furuta Y[1], Kawaoka Y: [1]Research Laboratories, Toyama Chemical Co., Ltd., [2]Department of Microbiology and Infectious Diseases, Kobe University, [3]National Institute of Hygiene and Epidemiology, Vietnam.

The neuraminidase inhibitors oseltamivir and zanamivi are used to treat H5N1 influenza. However, oseltamivir-resistant H5N1 viruses have been isolated from oseltamivir-treated patients. Moreover, reassortment between H5N1 viruses and oseltamvir-resistant human H1N1 viruses currently circulating could create oseltamivir-resistant H5N1 viruses, rendering the oseltamivir stockpile obsolete. Therefore, there is a need for unique and effective antivirals to combat H5N1 influenza viruses. The investigational drug T-705 (favipiravir; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) has antiviral activity against seasonal influenza viruses and a mouseadapted H5N1 influenza virus derived from a benign duck virus. However, its efficacy against highly pathogenic H5N1 viruses, which are substantially more virulent, remains unclear. Here, we demonstrate that T-705 effectively protects mice from lethal infection with oseltamivir-sensitive or -resistant highly pathogenic H5N1 viruses. Furthermore, our biochemical analysis suggests that T-705 ribofuranosyl triphosphate, an active form of T-705, acts like purines or purine nucleosides in human cells and does not inhibit human DNA synthesis. We conclude that T-705 shows promise as a therapeutic agent for the treatment of highly pathogenic H5N1 influenza patients.

2. Characterization of the Ebola virus nucleoprotein -RNA complex

Noda T[4], Hagiwara K[5], Sagara H[6], Kawaoka Y: [4]International Research Center for Infectioous Diseases, IMSUT, [5]Viral Infectious Disease Unit, RIKEN, [6]Medical Proteomics Laboratory, IMSUT.

When Ebola virus nucleoprotein (NP) is expressed in mammalian cells, it assembles into helical structures. Here, the recombinant NP helix purified from cells expressing NP was characterized biochemically and morphologically. We found that the recombinant NP helix is associated with non-viral RNA, which is not protected from RNase digestion and that the morphology of the helix changes depending on the environmental salt concentration. The N-terminal 450 aa residues of NP are sufficient for these properties. However, digestion of the NP-associated RNA eliminates the plasticity of the helix, suggesting that this RNA is an essential structural component of the helix, binding to individual NP molecules via the N-terminal 450 aa. These findings enhance our knowledge of Ebola virus assembly and understanding of the Ebola virus life cycle.

3. Efficacy of the New Neuraminidase Inhibitor CS-8958 against H5N1 Influenza Viruses

Kiso M, Kubo S[7], Ozawa M, Le QM[2], Nidom CA[8], Yamashita M[7], Kawaoka Y: [7]Biological Research Laboratories, Daiichi Sankyo Co. Ltd., [8]Tropical Disease Centre, Airlangga University, Indonesia.

Currently, two neuraminidase (NA) inhibitors, oseltamivir and zanamivir, which must be administrated twice daily for 5 days for maximum therapeutic effect, are licensed for the treatment of influenza. However, oseltamivir-resistant mutants of seasonal H1N1 and highly pathogenic H 5N1 avian influenza A viruses have emerged. Therefore, alternative antiviral agents are needed. Recently, a new neuraminidase inhibitor, R-125489, and its prodrug, CS-8958, have been developed. CS-8958 functions as a long-acting NA inhibitor in vivo (mice) and is efficacious against seasonal influenza strains following a single intranasal dose. Here, we tested the efficacy of this compound against H5N1 influenza viruses, which have spread across several continents and caused epidemics with high morbidity and mortality. We demonstrated that R-125489 interferes with the NA activity of H5N1 viruses, including oseltamivir-resistant and different clade strains. A single dose of CS-8958 (1,500 mg/kg) given to mice 2 h post-infection with H5N1 influenza viruses produced a higher survival rate than did continuous five-day administration of oseltamivir (50 mg/kg twice daily). Virus titers in lungs and brain were substantially lower in infected mice treated with a single dose of CS-8958 than in those treated with the five-day course of oseltamivir. CS-8958 was also highly efficacious against highly pathogenic H5N1 influenza virus and oseltamivir-resistant variants. A single dose of CS-8958 given seven days prior to virus infection also protected mice against H5N1 virus lethal infection. To evaluate the improved efficacy of CS-8958 over oseltamivir, the binding stability of R-125489 to various subtypes of influenza virus was assessed and compared with that of other NA inhibitors. We found that R-125489 bound to NA more tightly than did any other NA inhibitor tested. Our results indicate that CS-8958 is highly effective for the treatment and prophylaxis of infection with H5N1 influenza viruses, including oseltamivir-resistant mutants.

4. Sensitivity of influenza rapid diagnostic tests to H5N1 and 2009 pandemic H1N1 viruses.

Sakai-Tagawa Y, Ozawa M, Tamura D, Le QM[3], Nidom CA[8], Sugaya N[9], Kawaoka Y: [9]Department of Pediatrics, Keiyu Hospital.

Simple and rapid diagnosis of influenza is useful to treatment decision-making in the clinical setting. Although many influenza rapid diagnostic tests (IRDTs) are available for the detection of seasonal influenza virus infections, their sensitivity for other viruses, such as H5N1 viruses and the recently emerged swine-origin pandemic (H1N1) 2009 virus, remains largely unknown. Here, we examined the sensitivity of 20 IRDTs to various influenza virus strains, including H5N1 and 2009 pandemic H1N1 viruses. Our results indicate that the detection sensitivity to swine-origin H1N1 viruses varies widely among IRDTs, with some tests lacking sufficient sensitivity to detect the early stages of infection when the virus load is low.

5. Biological and structural characterization of a host-adapting amino acid in influenza virus.

Yamada S, Hatta M[10], Staker B[11], Watanabe S, Imai M[10], Shinya K[2], Sakai-Tagawa Y, Ito M, Ozawa M, Watanabe T[10], Li C[10], Kim J-H[10], Myler PJ[11], Phan I[11], Raymond A[11], Smith E[11], Stacy R[11], Nidom CA[8], Lank SM[12], Wiseman RW[12], Bimber BN[12], O'Connor DH[12], Neumann G[10], Stewart LJ[11], Kawaoka Y: [10]Influenza Research Institute, Department of Pathobiological

Sciences, School of Veterynary Medicine, University of Wisconsin-Madison, USA, [11]Seattle Structural Genomic Center for Infectious Disease, USA, [12]Wisconsin National Primate Research Center, University of Wisconsin-Madison, USA.

Two amino acids (lysine at position 627 or asparagine at position 701) in the polymerase subunit PB2 protein are considered critical for the adaptation of avian influenza A viruses to mammals. However, the recently emerged pandemic H1N1 viruses lack these amino acids. Here, we report that a basic amino acid at position 591 of PB2 can compensate for the lack of lysine at position 627 and confers efficient viral replication to pandemic H1N1 viruses in mammals. Moreover, a basic amino acid at position 591 of PB2 substantially increased the lethality of an avian H5N1 virus in mice. We also present the X-ray crystallographic structure of the C-terminus of a pandemic H1N1 virus PB2 protein. Arginine at position 591 fills the cleft found in H5N1 PB2 proteins in this area, resulting in differences in surface shape and charge for H1N 1 PB2 proteins. These differences may affect the protein's interaction with viral and/or cellular factors, and hence its ability to support virus replication in mammals.

6. Characterization of oseltamivir-resistant 2009 H1N1 pandemic influenza A viruses.

Kiso M, Shinya K[2], Shimojima M, Takano R, Takahashi K, Katsura H, Kakugawa S, Le MQ[3], Yamashita M[7], Furuta Y[1], Ozawa M, Kawaoka Y.

Influenza viruses resistant to antiviral drugs emerge frequently. Not surprisingly, the widespread treatment in many countries of patients infected with 2009 pandemic influenza A (H1N 1) viruses with the neuraminidase (NA) inhibitors oseltamivir and zanamivir has led to the emergence of pandemic strains resistant to these drugs. Sporadic cases of pandemic influenza have been associated with mutant viruses possessing a histidine-to-tyrosine substitution at position 274 (H274Y) in the NA, a mutation known to be responsible for oseltamivir resistance. Here, we characterized in vitro and in vivo properties of two pairs of oseltaimivir-sensitive and -resistant (possessing the NA H274Y substitution) 2009 H1N1 pandemic viruses isolated in different parts of the world. An in vitro NA inhibition assay confirmed that the NA H274Y substitution confers oseltamivir resistance to 2009 H1N1 pandemic viruses. In mouse lungs, we found no significant difference in replication between oseltamivir-sensitive and -resistant viruses. In the lungs of mice treated with oseltamivir or even zanamivir, 2009 H1N1 pandemic viruses with the NA H274Y substitution replicated efficiently. Pathological analysis revealed that the pathogenicities of the oseltamivir -resistant viruses were comparable to those of their oseltamivir-sensitive counterparts in ferrets. Further, the oseltamivir-resistant viruses transmitted between ferrets as efficiently as their oseltamivir-sensitive counterparts. Collectively, these data indicate that oseltamivir-resistant 2009 H1N1 pandemic viruses with the NA H274 Y substitution were comparable to their oseltamivir-sensitive counterparts in their pathogenicity and transmissibility in animal models. Our findings highlight the possibility that NA H 274Y-possessing oseltamivir-resistant 2009 H1N1 pandemic viruses could supersede oseltamivir-sensitive viruses, as occurred with seasonal H1N 1 viruses.

7. Significance of seasonal influenza viruses in the stool of pediatric patients.

Tamura D, Fujino M[13], Ozawa M, Iwatsuki-Horimoto K, Goto H, Sakai-Tagawa Y, Horimoto T, Nirasawa M[13], Kawaoka Y: [13]Department of Pediatrics, Tokyo Saiseikai Central Hospital.

Seasonal influenza patients occasionally exhibit gastrointestinal symptoms. Although seasonal influenza viral RNA has been detected in stool specimens from influenza patients, the virological significance is unknown. To detect influenza viral genes, 28 stool specimens were collected from 18 pediatric influenza patients and subjected to one step RT-PCR. To isolate infectious virus from stool samples, the stool specimens were inoculated into Madin-Darby canine kidney cells and embryonated chicken eggs. Although more than 60% of the stool specimens tested positive by RT-PCR, 46% of patients with viral gene-positive stools exhibit no gastrointestinal symptoms. An infectious virus was recovered from only one sample. These results indicate that fecal excretion of seasonal influenza viruses does not appear to be a public health concern.

8. High genetic compatibility between swine-origin H1N1 and highly pathogenic avian H 5N1 influenza viruses.

Octaviani CP, Ozawa M, Yamada S, Goto H, Kawaoka Y.

Reassortment is an important mechanism for

the evolution of influenza viruses. Here, we co-infected cultured cells with the pandemic swine-origin influenza virus (S-OIV) and a contemporary H5N1 virus and found that these two viruses have high genetic compatibility. Studies of human lung cell lines indicated that some reassortants had better growth kinetics than their parental viruses. We conclude that reassortment between these two viruses can occur and could create pandemic H5N1 viruses.

9. The HA and NS genes of human H5N1 influenza A virus contribute to high virulence in ferrets.

Imai H, Shinya K[2], Takano R, Kiso M, Muramoto Y, Sakabe S, Murakami S, Ito M, Yamada S, Le MQ[3], Nidom CA[8], Sakai-Tagawa Y, Takahashi K, Omori Y, NodaT, Shimojima M, Kakugawa S, Goto H, Iwatsuki-Horimoto K, Horimoto T, Kawaoka Y.

Highly pathogenic H5N1 influenza A viruses have spread across Asia, Europe, and Africa. More than 500 cases of H5N1 virus infection in humans, with a high lethality rate, have been reported. To understand the molecular basis for the high virulence of H5N1 viruses in mammals, we tested the virulence in ferrets of several H5N 1 viruses isolated from humans and found A/Vietnam/UT3062/04 (UT3062) to be the most virulent and A/Vietnam/UT3028/03 (UT3028) to be avirulent in this animal model. We then generated a series of reassortant viruses between the two viruses and assessed their virulence in ferrets. All of the viruses that possessed both the UT3062 hemagglutinin (HA) and nonstructural protein (NS) genes were highly virulent. By contrast, all those possessing the UT3028 HA or NS genes were attenuated in ferrets. These results demonstrate that the HA and NS genes are responsible for the difference in virulence in ferrets between the two viruses. Amino acid differences were identified at position 134 of HA, at positions 200 and 205 of NS1, and at positions 47 and 51 of NS2. We found that the residue at position 134 of HA alters the receptor-binding property of the virus, as measured by viral elution from erythrocytes. Further, both of the residues at positions 200 and 205 of NS1 contributed to enhanced type I interferon (IFN) antagonistic activity. These findings further our understanding of the determinants of pathogenicity of H5N1 viruses in mammals.

10. A cross-reactive neutralizing monoclonal antibody protects mice from H5N1 and pandemic (H1N1) 2009 virus infection.

Sakabe S, Iwastuki-Horimoto K, Horimoto T, Nidom CA[8], Le QM[3], Takano R, Kubota-Koketsud R[14], Okuno Y[15], Ozawa M, Kawaoka Y: [14]Research Center for Infectious Disease Control, Research Institute for Microbial Diseases, Osaka University, [15]Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University.

A novel influenza (H1N1) virus caused an influenza pandemic in 2009, while highly pathogenic H5N1 avian influenza viruses have continued to infect humans since 1997. Influenza, therefore, remains a serious health threat. Currently, neuraminidase (NA) inhibitors are the mainstay for influenza therapy; however, drug-resistant mutants of seasonal H1N1 and H5N1 viruses have emerged highlighting the need for alternative therapeutic approaches. One such approach is antibody immunotherapy. Here, we show that the monoclonal antibody C179, which recognizes a neutralizing epitope common among H1, H2, H5, and H6 hemagglutinins (HAs), protected mice from a lethal challenge with various H5N1 and pandemic (H1N1) 2009 viruses when administered either intraperitoneally or intranasally. The protective efficacy of intranasally inoculated C179 was comparable to that of intraperitoneal administration. Our results suggest that direct administration of this anti-influenza antibody to viral replication sites is an effective strategy for prophylaxis and therapy.

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Department of Microbiology and Immunology

Division of Infectious Genetics
感染遺伝学分野

Professor	Kensuke Miyake, M.D., Ph.D.	教　授	医学博士　三　宅　健　介
Assistant Professor	Sachiko Akashi-Takamura, M.D., Ph.D.	助　教	医学博士　高村(赤司)祥子
Assistant Professor	Takahisa Furuta, D.V.M., Ph.D.	助　教	農学博士　古　田　隆　久
Assistant Professor	Shin-Ichiroh Saitoh, Ph.D.	助　教	医学博士　齋　藤　伸一郎

Immune cells express multiple Toll-like receptors (TLRs) that are concomitantly activated by a variety of pathogen products during microbial and viral infection. TLRs also sense our internal molecules such as RNA and DNA. There is presumably a need to coordinate the expression and function of TLRs in individual cells. Recent reports also have indicated that losing the balance of TLRs responses is a mechanism to cause autoimmune diseases. Our research focuses on molecular regulatory mechanisms to coordinate pathogen and internal ligands recognition by TLRs.

1. The search for internal ligand of MD-1.

Sachiko Akashi-Takamura, Natsuko Yamakawa, Takuma Shibata, And Kensuke Miyake

MD-1 is a glycoprotein which associates with RP105 protein. RP105 is a lymphocyte or myeloid derived cell-surface molecule which has LRR (leucine rich-repeat) motif like TLRs (Toll-like receptors). In the absence of MD-1, cells are unable to express RP105 on their surface, and thusly RP105 loses function. RP105/MD-1 complex induces lymphocyte B-cell proliferate signal, and this signal inhibits B-cell apoptosis which was induced by radiation.

$RP105^{-/-}$ or $MD\text{-}1^{-/-}$ mice-derived B cells are hyporesponsive to TLR2 or TLR4 ligands. These results show that RP105/MD-1 posititively regulate TLR2/4 stimulation. Recently several other groups showed that MD-1 interacts with lipopolysaccharide (LPS, ecdotoxin) or lipidIVa, which are also TLR4 ligands. These results show that RP105/MD-1 closely affects the TLR2/4 function.

Previously our laboratory member reported that B cells are constitutively activated by TLR2/4 and RP105 (Kobayashi T., International Immunology., 2008). Such B cell activation was revealed by the γ3 germ line transcript and serum IgG3 production, both of which were impaired by the lack of RP105 or TLR2/4. Serum IgG3 was not altered in germ-free or antibiotics-treated mice, suggesting that the microbial flora hardly contributes to the continuous activation of B cells.

We supposed this tonic activity was induced from some internal ligand from host. Recently we established novel anti-MD-1 mAbs (JR clones). One of antibody is able to recognize the conformation change of MD-1. To identify the internal ligand, now I check the conformation change of MD-1 on B cell surface after incubation with several internal ligand.

The lack of RP105-dependent B cell activation ameliorated disease progression in lupus-prone MRL/lpr mice (Kobayashi T., International Immunology., 2008). $RP105^{-/-}$MRL/lpr mice showed less lymphoadenopathy/splenomegaly and longer survival than MRL/lpr mice. These results suggest that RP105-dependent tonic B

cell activation has a pathogenic role in MRL/lpr mice. Now we check the pathogenic role of MD-1 in MRL/lpr mice.

2. Roles for Unc93 homolog B1-dependent TLR7/9 balance in vivo

Ryutaro Fukui[1], Shin-Ichiroh Saitoh[1], Atsuo Kanno[1], Masahiro Onji[1], Takuma Shibata[1,2], Akihiko Itoh[3,4], Nobuaki Yoshida[2], & Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, [2]Center for Experimental Medicine and Systems Biology, [3]Division of Molecular Pathology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, TOKYO1208-8639, Japan. [4]Present address: Department of Pathology, Kinki University Faculty of Medicine, 377-2 Ohno-Higashi, Sayama City, Osaka 589-8511, Japan

Toll-like receptor 7 (TLR7) and TLR9 recognize microbial RNA and DNA, respectively. These nucleic acid sensing TLRs potentially recognize self-derived nucleic acid and have been shown to have a role in autoimmune diseases. It is important to keep the responsiveness of the nucleic acid-sensing TLRs under the tight control.

Unc93 homolog B1 (Unc93B1) is reported to be indispensable for TLR7/9 response. We showed that the alanine substitution for the 34th aspartic acid (D34A) of Unc93B1 enhanced TLR7 response but downregulated TLR9 response. These results suggest that TLR7 and TLR9 are reciprocally linked by Unc93B1, and the TLR7/TLR9 balance is biased towards TLR9 in the steady state.

To clarify a role for Unc93B1-dependent TLR7/TLR9 balance *in vivo*, we generated knock-in mice harboring the D34A mutation in the Unc93B1 gene. D34A knock-in mice were born normally. Ex vivo analyses revealed TLR7 hyper-responsiveness and TLR9 hypo-responsiveness in conventional dendritic cells (cDC), which is consistent with preceding in vitro studies. Further study is underway to see TLR7/9 responses in other types of immune cells and in vivo consequences of TLR7 skewing of the TLR7/9 balance.

3. Participation of mast cell-derived VEGF and proteases in severe Dengue virus infection.

Takahisa Furuta[1*], Lyre Anni Murao[9], Nguyen Thi Phuong Lan[2], Nguyen Tien Huy[6], Cao Thi Phi Nga[5], Vo Dinh Tham[5], Vu Thi Que Huong[3], Vo Dinh Tham[5], Tran Van Dat[6], Tran Thi Ngoc Ha[6], Yasukazu Ohmoto[7], Mihoko Kikuchi[6,8], Kouichi Morita[9,10], MichioYasunami[6], Kenji Hirayama[6,10], and Naohiro Watanabe[11]: [1]Division of Infectious Genetics, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [2]Laboratory of Bacteriology, Pasteur Institute, Ho Chi Minh City, Viet Nam, [3]Arbovirus Laboratory, Pasteur Institute, Ho Chi Minh City, Viet Nam, [4]Children's Hospital No. 2, Ho Chi Minh City, Viet Nam, [5]Center for Preventive Medicine, Vinh Long Province, Viet Nam, [6]Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan, [7]Free Radical Research Institute, Ostuka Pharmaceutical CO., Ltd., Tokushima, Japan, [8]Center of International Collaborative Research, Nagasaki University, Nagasaki, Japan, [9]Department of Virology, NEKKEN, Nagasaki University, Nagasaki, Japan, [10]Global COE Program, Nagasaki University, Nagasaki, Nagasaki, Japan, [11]Department of Tropical Medicine, Jikei University School of Medicine, Tokyo, Japan.

Recent in vitro study has suggested the involvement of mast cells in Dengue virus infection, however, the role of mast cells in this infection has not yet been elucidated. To clarify these, plasma level of mast cell-derived mediators, vascular endothelial cell growth factor (VEGF), tryptase and chymase was compared between Dengue patients and control subjects. Plasma samples were obtained from children in Southern Vietnam. The levels of these mediators were significantly increased in Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) patients compared with Dengue fever (DF) and the control (febrile illness and healthy subjects), and soluble form of VEGF receptor (sVEGFR-1 and -2) levels was also significantly changed in these patients. After the treatment of Dengue patients, these levels were returned to similar levels with DF and the control. Furthermore, since IL-9 is a T cell-derived mast cell activator and secreted from Th17 cells, plasma levels of IL-9 and IL-17 were also measured in Dengue patients and the control. IL-9 and IL-17 levels were increased in DHF and DSS, and in vitro production of VEGF in human mast cells was significantly enhanced in the presence of IL-9 when these cells were inoculated with Dengue virus and human Dengue immune serum. Since mast cells are known as a main source of VEGF, and tryptase and chymase are considered as a specific marker for mast cell activation, mast cells and mast cells-derived mediators might participate in the development of Dengue hemorrhagic fever.

4. Identification of regulatory molecules for UNC93b1, TLR7, and TLR9

Shin-ichiroh Saitoh, He Zhao, Mabel Chan, Masahiro Onji, Atsuo Kanno, Yusuke Murakami, Ryutaroh Fukui, and Kensuke Miyake

Toll-like receptors (TLR) 7 and 9 recognize microbial nucleic acids in endolysosomes, and initiate innate and adaptive immune responses. TLR7/9 in dendritic cells (DC) also responds to self-derived RNA/DNA, respectively, and drive autoantibody production. However, little is known about mechanism of RNA/DNA recognition by TLR7/9. Our goal is to understand the mechanism. Unc93 homolog B1 (Unc93B1) is reported to be indispensable for TLR7/9 response. Unc93B1 associates with and delivers TLR7/9 from the ER to endolysosomes. We try to identify regulatory molecules associated with UNC 93b1 as a positive and negative regulator by using LC-Ms/Ms analysis. We also try complementation cloning with a TLR7/9-unresponsive cell line.

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Department of Microbiology and Immunology

Division of Mucosal Immunology
炎症免疫学分野

Professor	Hiroshi Kiyono, D.D.S., Ph.D.	教　授	医学博士	清　野　　宏
Assistant Professor	Jun Kunisawa, Ph.D.	講　師	薬学博士	國　澤　　純
Assistant Professor	Yoshikazu Yuki, M.B.A., Ph.D.	助　教	医学博士	幸　　義　和
Assistant Professor	Shintaro Sato, Ph.D.	助　教	医学博士	佐　藤　慎太郎

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 diseases.

1. MucoRice for New Generation of Oral Vaccine

Yoshikazu Yuki[1], Daisuke Tokuhara, Mio Mejima[1], Shiho Kurokawa[1], Yuko Takahashi[1], Tomonori Nochi[1], Aya Chubachi[1], Yingju Chen[1], Ushio Nakanishi[1], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo,

In general, protein-based drugs, including vaccines, are not stable for long periods at room temperature (RT). However, considering the field conditions under which vaccination is performed in developing countries, vaccine preparations should be able to tolerate even higher temperatures than RT. We therefore developed the rice-based oral delivery system as a cold-chain-free vaccine. Our studies have proven that rice-seed-expressed vaccine (or MucoRice) (1) is stable at RT for 3 years without loss of quantity or quality (including immunogenicity), (2) have long-lasting protection and boosting effects of MucoRice-CTB vaccination against CT-induced diarrhea, (3) induces protection against Vibrio cholerae- or ETEC- induced diarrhea, (4) induces CTB-specific SIgA against toxin-induced diarrhea. In terms of last point, blockade of the formation and transepithelial transport of SIgA in MucoRice-CTB-immunized pIgR (polyimmunoglobulin receptor)-deficient mice resulted in a marked reduction in antigen-specific mucosal IgA levels in the intestinal secretions. In contrast, lack of CTB-specific SIgA transport caused a noticeable increase in serum CTB-specific IgA levels in oral MucoRice-CTB-immunized pIgR-deficient mice, whereas the antigen-specific serum IgG titer was comparable to that in wild-type (WT) mice orally immunized with MucoRice-CTB. When these two groups of MucoRice-CTB-vaccinated mice were orally challenged with native CT, WT mice showed protection against CT-induced diarrhea, whereas pIgR-deficient mice developed severe diarrhea despite the presence of high titers of antigen-specific serum IgG and IgA. These findings directly demonstrate the essential role of CTB-specific intestinal SIgA in providing protection against the toxin. In light of this important feature of the MucoRice system, our efforts are directed toward creating the optimum human-

applicable MucoRice preparations by growing the rice hydroponically in a fully enclosed molecular farming production system.

2. New Generation Nasal Vaccine

Yoshikazu Yuki[1], Il Gyu Kong[1], Tomonori Nochi[1], Mio Mejima[1], Ayuko Sato[1], Daisuke Tokuhara[1], Shiho Kurokawa[1], Yuko Takahashi[1], Tomoko Hiroiwa[1], Haruko Takahashi[2], Norihiro Harada[3], Shinichi Sawada[2], Tomoko Kohda[4], Yuko Katakai[5], Hiroaki Shibata[5], Shunji Kozaki[3], Hideo Tsukada[3], Kazunari Akiyoshi[2], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]Department of Organic Materials, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, [3]PET Center, Central Research Laboratory, Hamamatsu Photonics K.K., [4]Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, [5]Tsukuba Primate Research Center, National Institute of Biomedical Innovation

Because mucosal administration of vaccines can induce both systemic and mucosal immune responses, the mucosal vaccination might provide a basis for preventing a range of infectious diseases. However, most of inactivated or subunit type vaccines are poor immunogens to both systemic and mucosal compartments when given nasally. Thus, mucosal adjuvants were needed, however, most of potent mucosal adjuvants are toxin-based one and potencial redirection of adjuvant itself and/or co-administered antigen to central nerve system (CNS) have caused concerns of safety issue, even when deleted toxin activity. To overcome these critical concerns, adjuvant-free and safe nasal delivery system must be developed. We recently developed an effective intracellular vaccine delivery system with self-assembled cationic nanosize hyrogels (Nanogel), which composed of an amphiphilic polysaccharide: ethylendiamine group functionalization of cholesteryl group-bearing pullulan (cCHP). Nasally administered $CHPNH_2$ holding subunit type of botulinum vaccine (BoHc-$CHPNH_2$) or Tetanus Toxoid (TT) or Pneumococal surface protein A (PspA) induced brisk levels of antigen-specific systemic and mucosal immune responses even in the absence of mucosal adjuvant. We also developed real-time quantitative tracking method for nasal vaccine by using [^{18}F]-labeled BoHc/A-positron emission tomography (PET), a newly established *in vivo* molecular imaging system for the estimation of safety. Now, we are focusing studies for the precise mechanism and safety issues of the nasal vaccine.

3. Characterization of Mucosal Antigen-sampling System: M Cells

Yoshikazu Yuki[1], Tomonori Nochi[1], Kazutaka Terahara[2], Yoshikyuki Goto[1], Yuko Takahashi[1], Yoshida Masato[1], Shiho Kurokawa[1], Mio Mejima[1], Gemilson S. Pontes[1], Tomoko Hiroiwa[1], Hirotsugu Hatai[1], Steven E. Domino[3] and Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]Department of Immunology, National Institute of Infectious Diseases, [3]Department of Obstetrics and Gynecology, The University of Michigan Medical School

A single layer of intestinal epithelium containing columnar epithelial cells (ECs) and M cells covers the lymphoid tissues (for example, Peyer's patches; PPs), and fucosylation of the apical surface of ECs (F-ECs) and M cells is involved in distinguishing the two populations and in their response to commensal flora in mice. Interestingly, fucosylation of PP M cells and F-ECs is distinctly regulated by $\alpha(1,2)$ fucosyltransferase (FUT) 1 and FUT2, respectively. We recently showed that Fut2-mediated F-ECs share M cell-related fucosylated molecules but maintain distinctive EC characteristics, Fut1 is, therefore, a reliable marker for M cells. We previously developed the M cell-targeted mucosal vaccine with newly established monoclonal antibody (NKM 16-2-4) specific for both M cells in PPs (PP M cells) and F-ECs (or villous M cells) in mice. Because high levels of Tetanus toxioid (TT)-specific serum IgG antibody responses were induced in PP-lacking when orally immunized TT-conjugated NKM 16-2-4, antigen-delivery system to not only PP M cells but also F-ECs may be important to induce of brisk immune responses in mucosal and systemic compartments. Our current studies are focusing on the development of PP and villous M cell-targeted mucosal vaccine in non-human primates.

4. Molecular and Cellular Analysis of Host-Microflora Interaction

Yoshiyuki Goto[1], Jun Kunisawa[1], Shintaro Sato[1], Naoko Shibata[1], Yuko Akiyama[1], Hideki Iijim[2], Yoshinori Umesaki[3], Yoshimi Benno[4] and Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science,

The University of Tokyo, [2]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, [3]Yakult Central Institute for Microbiological Research, [4] Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center

The mucosal surface of the small intestine is constitutively exposed to a wide variety of environmental antigens including harmless food-derived antigens and gut microbiota. The indigenous gut microbiota play an important role in the development of the host mucosal immune systems including the maturation of secondary lymphoid tissues. Although the current understanding is host-commensal bacterial mutualism is established in the intestinal lumen, it is plausible that direct interaction between bacteria and mucosal immune cells is observed in the lymphoid organs such as Peyer's Patches (PPs). Based on this hypothesis, we analyzed the bacterial population in murine PPs by using 16S rDNA based approaches such as 16S rDNA clone libraries and fluorescent in situ hybridization (FISH). We found that opportunistic bacteria, Alcaligenes species, were dominantly observed in PPs with the associated preferential induction of anti-Alcaligenes mucosal IgA antibodies (Abs). This preferential presence of Alcaligenes inside PPs and the associated-induction of intestinal IgA Abs were also observed in both monkeys and humans. Thus, indigenous opportunistic bacteria uniquely inhabit PPs leading to DCs dependent, local antigen-specific Ab production; this may involve the creation of an optimal symbiotic environment on the interior of the PPs. Our current work is focusing on the role of PPs-Alcaligenes in the development of host diseases.

Another example of the crosstalk between host and gut microbiota was explored. It has been reported that specific commensal bacteria, Bacteroides thetaiotaomicron, induce epithelial fucoses and utilize them as an energy source. However, the mechanism of epithelial fucosylation induced by bacteria is not fully understood yet. To analyze the role of commensal bacteria in the induction of fucosylated epithelial cells (F-ECs), we firstly checked germfree mice. The development of F-ECs was completely inhibited in germfree mice, suggesting that gut microbiota induce epithelial fucosylation. Since a large number of F-ECs were observed in the ileum, we further analyzed the ileal bacterial population to identify bacteria responsible for the induction of F-ECs. 16S rDNA clone libraries revealed that segmented filamentous bacteria (SFB), which are clostridium related bacteria, were dominant in the ileum. Finally, we analyzed SFB gnotobiotic mice. F-ECs were observed in SFB gnotobiotic mice as wild-type mice, indicating that SFB physiologically induce F-ECs. We are now trying to identify the detail mechanism of the crosstalk between host and gut microbiota, especially SFB, for the development of F-ECs.

5. Mucosal Immunological Homeostasis and Diseases

Jun Kunisawa, Yosuke Kurashima, Takeaki Amiya, Taichi Wake, Yoshifumi Kotani, Eri Hashimoto, Yuji Suzuki, Shiori Shikata, and Hiroshi Kiyono: Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo

Mucosal tissues equip a unique mucosal immune system to maintain the immunological homeostasis. Accumulating evidence has revealed that disruption of the mucosal quiescent immune system results in the mucosal immune diseases such as food allergy, intestinal inflammation, rhinitis, and asthma. In this project, we aim to elucidate the immunological features of mucosal immune system in the regulation of immunological homeostasis. As host immune competent cells, we focus on the T cells (Th1/Th2/Th17, regulatory T cells), mast cells, IgA plasma cells, dendritic cells, and epithelial cells and found that immunological cross-talk among them played a key role in the maintenance of immunological homeostasis.

In addition to the host-host interaction, several lines of evidence have shown that immunological cross-talk between host immune system and environmental factors regulate the homeostasis of mucosal immunity. In this issue, we recently found that several environmental factors derived from diets and commensal bacteria (e.g., fatty acids, vitamins, herbal and fruit extracts, secondary bile acid, and nucleotides) are critically involved in the regulation of intestinal homeostasis. Therefore, improper cross-talk of host mucosal immune system with these environmental factors induced the mucosal immune diseases, and improvement of these pathways resulted in the amelioration of the diseases. These findings provide a novel strategy to establish mucosal homeostasis through the interaction with mucosal environmental factors.

6. The Airway Antigen Sampling System: Respiratory M cells as an Alternative Gateway for Inhaled Antigens

Ayuko Sato[1], Dong-Young Kim[1,2], Satoshi

Fukuyama[1], Hiroshi Sagara[3], Takahiro Nagatake[1], Il Gyu Kong[1,2], Kaoru Goda[1], Tomonori Nochi[1], Jun Kunisawa[1], Shintaro Sato[1], Yoshifumi Yokota[4], Chul Hee Lee[2], and Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, and [3]Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, [2]Department of Otorhinolaryngology, Seoul National University College of Medicine, [4]Department of Molecular Genetics, School of Medicine, University of Fukui

We demonstrated a new airway Ag sampling site by analyzing tissue sections of the murine nasal passages. We revealed the presence of respiratory M cells, which had the ability to take up OVA and rSalmonella-GFP, in the turbinates covered with single-layer epithelium. These M cells were also capable of taking up respiratory pathogen Group A Streptococcus (GAS) after nasal challenge. Inhibitor of DNA binding/differentiation 2 (Id2)-deficient mice, which are deficient in lymphoid tissues, including nasopharynx-associated lymphoid tissue (NALT), had a similar frequency of M cell clusters in their nasal epithelia to that of their littermates, $Id2^{+/-}$ mice. The titers of Ag-specific antibodies were as high in $Id2^{-/-}$ mice as in $Id2^{+/-}$ mice after nasal immunization with rSalmonella-ToxC or GAS, indicating that respiratory M cells were capable of sampling inhaled bacterial Ag to initiate an Ag-specific immune response. Taken together, these findings suggest that respiratory M cells act as a NALT-independent alternative gateway for Ag sampling and subsequent induction of Ag-specific immune responses in the upper respiratory tract.

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