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
Assistant Professor	Michinaga Ogawa, Ph.D.	助　教	医学博士	小　川　道　永
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 effector deamidates Ubc13 to dampen the inflammatory response.

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

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

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

Makoto Fukumatsu, Michinaga Ogawa, Satoko Arakawa[1], Masato Suzuki, Kazuhisa Nakayama[2], Shigeomi Shimizu[1], Minsoo Kim, Hitomi Mimuro, and Chihiro Sasakawa: [1]Department of Pathological Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan, [2]Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto Univer-

sity, 46-29 Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

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

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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	Takeshi Haraguchi, Ph.D.	助 教	理学博士	原 口 健
Assistant Professor	Kyousuke Kobayashi, D.V.M., Ph.D.	助 教	獣医学博士	小 林 郷 介
Assistant Professor	Hiroaki Ito, Ph.D.	助 教	理学博士	伊 藤 寛 明

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 also develop new retrovirus/lentivirus vectors that express efficient inhibitory RNAs targeting specific miRNAs (designated as Tough Decoy RNAs) as well as low molecular inhibitors of miRNA (designated synthetic TuD; S-TuD) 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 previously reported.

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. 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.

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 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. In the canonical NFκB pathway, upon stimulation by TNFα or IL-1, proteosomal degrada-

tion of cytosolic IκBα allows nuclear translocation of RelA/p50 dimer, which induces many NF-Kβtarget genes expression. In the non-canonical NFκB pathway, which is activated by lymphotoxin and CD40 ligand, activated NIK and IKKα induce the processing of p100 to p52. 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. 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. We further show evidence that DPF2 is required for oncogenesis of several human tumor cell lines in which the non-canonical NFκB pathway is aberrantly regulated.

Considering these previous findings, we speculate that the five proteins DPF1, DPF2, DPF3a, DPF3b, and PHF10, are candidate coactivators of the typical NF-κB heterodimer, RelA/p50, as well as two other NF-κB dimers, RelB/p52, and c-Rel/p50. This year, we show that each of these proteins can enhance the different NF-κB heterodimers to transactivate their targets efficiently.

Double PHD finger proteins DPF3a and 3b are required as transcriptional coactivators in the SWI/SNF complex-dependent activation of the NF-κB RelA/p50 heterodimer

Aya Ishizaka, Taketoshi Mizutani, Kazuyoshi Kobayashi, Toshio Tando, Kouhei Sakurai, Toshinobu Fujiwara[1], and Hideo Iba.: [1]Institute of Microbial Chemistry Laboratory of Disease Biology, Tokyo

We have previously shown that DPF2 (requiem/REQ) functions as a linker protein between the SWI/SNF complex and RelB/p52 NF-κB heterodimer and plays important roles in NF-κB transactivation via its non-canonical pathway. Using sensitive 293FT reporter cell clones that had integrated a SWI/SNF-dependent NF-κB reporter gene, we find in our current study that the overexpression of DPF1, DPF2, DPF3a, DPF3b and PHF10 significantly potentiates the transactivating activity of typical NF-κB dimers. Knockdown analysis using 293FT reporter cells that endogenously express these five proteins at low levels clearly showed that DPF3a and DPF3b, which are produced from the *DPF3* gene by alternative splicing, are the most critical for the RelA/p50 NF-κB heterodimer transactivation induced by TNF-α stimulation. Our data further show that this transactivation requires the SWI/SNF chromatin remodeling complex. DPF3a and DPF3b are additionally shown to interact directly with RelA, p50 and several subunits of the SWI/SNF complex *in vitro* and to be co-immunoprecipitated with RelA/p50 and the SWI/SNF complex from the nuclear fractions of cells treated with TNF-α. In chromatin immunoprecipitation experiments using a cell line harboring a native HIV-1 LTR reporter provirus, we further found that endogenous DPF3a/b and the SWI/SNF complex are continuously present at the LTR, whereas the kinetics of RelA/p50 recruitment to the LTR after TNF-α treatment correlate well with the viral transcriptional activation levels. In conclusion, our present data indicate that by linking RelA/p50 to the SWI/SNF complex, DPF3a/b induces the transactivation of NF-κB target gene promoters that exist in relatively inactive chromatin contexts.

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

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 possibility is consistent with our observations that a frequent loss of Brm expression in gastric cancers correlates with a less differentiated state. In 2011, we demonstrated that *Brm* mRNA is a target of miR-199a-5p and miR-199a-3p, both of which are processed from pre-miR-199a. By promoter analysis of the *miR-199a-2* gene, which was found to be the main contributor to the production of pri-miR-199a in these cell lines, Egr1 was identified as a major transcriptional activator at this gene locus. 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 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(−)/miR-199(−) cells and Brm(−)/Egr1(+)/miR-199a(+) cells, which are denoted hereafter as Type 1 and Type 2, respectively. This year, we have analyzed basic biological properties that discuminate these two cell types.

Characterization of two cancer cell types that utilize distinct regulatory loops formed by miR-199a and a catalytic subunit of the SWI/SNF chromatin remodeling factor, Brm.

Kouhei Sakurai, Kazuyoshi Kobayashi, Hiroaki Hiramatsu, Jessica Chang, Ken-ichi Inada[2], Kazuya Shiogama[2], Aya Ishizaka, Kyosuke Kobayashi, Takeshi Haraguchi, Yutaka Tsutsumi[2], and Hideo Iba.: [2]First Department of Pathology, Faculty of Medicine, Fujita Health University, Aichi

We here evaluated the biological properties and gene expression patterns specific to these two cancer cell subtypes. We found that Type 1 cells specifically exhibited efficient anchorage-independent growth and expressed high levels of the miR-199a-3p target, *CD44*, as well as some inflammatory cytokine genes that are transactivated through NF-κB in a SWI/SNF complex-dependent manner. Furthermore, Fra-1 expression, which is known to be positively autoregulated via the *fra-1* promoter, was shown to be specifically highly expressed in Type 1 cells and also to be dependent upon SWI/SNF. These results indicate that regulatory loops formed by Brm/miR-199a axis function as molecular switches to establish the specific gene expression patterns in Type 1 and 2 cancer cells. Therefore, the typing according to expression status of Brm and miR-199a will be useful for cancer diagnosis and for guiding the development of distinct therapeutic strategies in each case.

3. A Novel dimention of miRNA function which regulates expression levels among family genes encoding transcription factors.

Caudal-type homeobox (Cdx) genes encode HOX domain-containing transcription factors that are conserved among vertebrates. In mouse and human, these genes are expressed during development in a tissue-specific manner and contribute to the formation of the anterior-posterior axis. In adults, two paralogues, Cdx1 and Cdx2 are expressed in the intestinal epithelium and aberrant expression is often associated with metaplasias or tumor formation. These factors have in fact been shown to be expressed in intestinal metaplasias, which are lesions that can progress to gastric adenocarcinoma. These two paralogous proteins are expected to play at least partially similar roles in the regulation of target gene expression by binding to identical recognition sequences. However, it has also been shown that there are several regulatory interactions between Cdx1 and Cdx2, suggesting that these two homologous transcription factors are not always functionally redundant.

Previous observations in Cdx1 transgenic mice and Cdx1 -/- mice have indicated that altered Cdx1 levels cause an inverse and dose-dependent modification of endogenous Cdx2 protein expression in the distal colon and jejunum. It has also been reported that the expression of endogenous Cdx2 protein and mRNA is drastically reduced by ectopic Cdx1 expression in the small intestinal villi and colon surface epithelium of mice. These results suggest that Cdx1 fine-tunes the expression of the *Cdx2* gene. Importantly, we have also shown that ectopic Cdx1 expression in the colon cancer cell line SW480, significantly reduces endogenous Cdx2 protein.

In our present study, we hypothesized that in colorectal cells, Cdx1 expression suppresses Cdx2 expression through the activity of some miRNAs and successfully identified a robust regulatory mechanism and some key factors involved in Cdx2 suppression by Cdx1, which would at least partly explain the mutually exclusive expression patterns for these proteins reported in the intestinal epithelia of mice. This regulatory network formed by miRNAs itself reveals the importance of homeostasis and the control of the transcriptional regulatory system through the limiting of transcription factors that share DNA recognition sequences.

Multiple microRNAs induced by Cdx1 suppress Cdx2 in human colorectal tumor cells

Takanobu Tagawa, Takeshi Haraguchi, Hiroaki Hiramatsu, Kazuyoshi Kobayashi, Kouhei Sakurai, Ken-Ichi Inada[2] and Hideo Iba

The mammalian transcriptional factors, Cdx1 and Cdx2 are paralogues and critical for the cellular differentiation of intestinal or colorectal epithelia. It was previously reported that in *cdx1* transgenic or knockout mice, endogenous Cdx2 levels are inversely correlated with Cdx1 levels. Recently, we found that exogenous Cdx1 expression can suppress Cdx2 in a human colorectal tumor cell line, SW480, although the underlying molecular mechanisms were unclear. Here, we show that several microRNAs induced by exogenous Cdx1 expression directly bind to the *Cdx2* mRNA 3'UTR to destabilize these transcripts finally leading to their degradation. Using microarray analysis, we found that several miRNAs that were computationally predicted to target *Cdx2* mRNAs are up-regulated by the exogenous Cdx1 expression in SW480 cells. Among these molecules, we identified miR-9, -16 and -22 as having the potential to suppress Cdx2 through the binding of the 3'UTR to its transcript. Importantly, simultaneous mutations of both the miR-9 and -16 binding sites in the *Cdx2* 3'UTR were shown to be sufficient to block Cdx2 suppression. These results suggest a unique feature of miRNAs in which they contribute to homeostasis by limiting the levels of transcription factors belonging to the

same gene family.

4. Development of decoy RNAs that strongly inhibit specific miRNA function (S-TuD).

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 have developed specialized plasmid- and virus- vectors carrying expression units for inhibitory RNA molecules, which we have termed Tough Decoy (TuD) RNA.

TuD RNAs have been already used for the basic research; the identification of miRNA targets and functional analysis of miRNA in cancer cells, in tumour formation in mice or in myoblast differentiation. TuD RNA is a single RNA molecule with a complex secondary structure composed of four elements: a stem of 18bp in length, two miRNA binding sites (MBSs) that have a sequence complementary to that of a mature miRNA of interest, a stem-loop structure which connects two MBSs, four linkers with three nucleotides connecting the two MBSs, and the flanking stems. These elements provide efficient nuclear export, binding to the target miRNA, resistance to cellular nucleases and enhancement of the MBS accessibility to the target miRNA, respectively. By screening several alternative MBS sequences to optimize the decoy activity, we have further identified a highly potent TuD, the MBS of which has a 4 nucleotide insertion between positions 10 and 11 from the 3' end of the perfectly complementary sequence to the entire mature miRNA of interest, where the Ago2-containing RISC cleaves target mRNAs. When these TuD RNAs were expressed by lentivirus vectors, they were shown to be efficiently transported to the cytoplasm and exhibit strong inhibitory effects for more than one month.

In our current study, We have developed a synthetic miRNA inhibitor composed of two strands of 2'-O-methylated RNA oligonucleotides, the structure of which is very similar to that of the corresponding TuD RNA and we have designated this as S-TuD (Synthetic TuD). We provide evidence that if appropriate MBSs are selected, S-TuDs targeting several miRNAs retain potent inhibitory effects even when transfected at a low dose range of 1nM- 30pM.

A potent 2'-O-methylated RNA based microRNA inhibitor with unique secondary structures.

Takeshi Haraguchi, Haruo Nakano[4], Takanobu Tagawa, Tokimitsu Ohki[3], Yoshihito Ueno[3], Tetsuo Yoshida[4] and Hideo Iba: [3]Department of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Gifu. [4]Inovative Drug Research Laboratories, Kyowa Hakko Kirin Co., Ltd., Tokyo

MicroRNAs (miRNAs) are involved in various biological processes and human diseases. The development of strong low-molecular weight inhibitors of specific miRNAs is thus expected to be useful in providing tools for basic research or in generating promising new therapeutic drugs. We have previously described the development of "Tough Decoy (TuD) RNA" molecules, which achieve the long-term suppression of specific miRNA activity in mammalian cells when expressed from a lentivirus vector. In our current study, we describe new synthetic miRNA inhibitors, designated as S-TuD (Synthetic TuD), which are composed of two fully 2'-O-methylated RNA strands. Each of these strands includes a miRNA binding site. Following the hybridization of paired strands, the resultant S-TuD forms a secondary structure with two stems, which resembles the corresponding TuD RNA molecule. By analyzing the effects of S-TuD against miR-21, miR-200c, miR-16 and miR-106b, we have elucidated the critical design features of S-TuD molecules that will provide optimum inhibitory effects following transfection into human cell lines. We further show that the inhibitory effects of a single transfection of S-TuD-miR200c are quite long-lasting ($>$ 7days) and induce partial EMT, the full establishment of which requires 11 days when using a lentivirus vector that expresses TuD-miR200c continuously.

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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	Takeshi Noda, D.V.M., Ph.D.	准教授	獣医学博士　野田岳志
Assistant Professor	Kiyoko Iwatsuki-Horimoto, D.V.M., Ph.D.	助　教	獣医学博士　岩附(堀本)研子
Project Assistant Professor	Shinya Yamada, Ph.D.	特任助教	医学博士　山田晋弥
Project Assistant Professor	Maki Kiso, D.V.M., Ph.D.	特任助教	医学博士　木曽真紀
Research Associate	Yuko Sakai-Tagawa, Ph.D.	助　手	医学博士　坂井(田川)優子

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. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets.

Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y.

Highly pathogenic avian H5N1 influenza A viruses occasionally infect humans, but currently do not transmit efficiently among humans. The viral haemagglutinin (HA) protein is a known host-range determinant as it mediates virus binding to host-specific cellular receptors. Here we assess the molecular changes in HA that would allow a virus possessing subtype H5 HA to be transmissible among mammals. We identified a reassortant H5 HA/H1N1 virus-comprising H5 HA (from an H5N1 virus) with four mutations and the remaining seven gene segments from a 2009 pandemic H1N1 virus-that was capable of droplet transmission in a ferret model. The transmissible H5 reassortant virus preferentially recognized human-type receptors, replicated efficiently in ferrets, caused lung lesions and weight loss, but was not highly pathogenic and did not cause mortality. These results indicate that H5 HA can convert to an HA that supports efficient viral transmission in mammals; however, we do not know whether the four mutations in the H5 HA identified here would render a wholly avian H5N1 virus transmissible. The genetic origin of the remaining seven viral gene segments may also critically contribute to transmissibility in mammals. Nevertheless, as H5N1 viruses continue to evolve and infect humans, receptor-binding variants of H5 N1 viruses with pandemic potential, including avian-human reassortant viruses as tested here, may emerge. Our findings emphasize the need to prepare for potential pandemics caused by influenza viruses possessing H5 HA, and will help individuals conducting surveillance in regions with circulating H5N1 viruses to recognize key residues that predict the pandemic potential of isolates, which will inform the development, production and distribution of effective countermeasures.

2. Three-dimensional analysis of ribonucleoprotein complexes in influenza A virus.

Noda T, Sugita Y, Aoyama K, Hirase A, Kawakami E, Miyazawa A, Sagara H, Kawaoka Y.

The influenza A virus genome consists of eight single-stranded negative-sense RNA (vRNA) segments. Although genome segmentation provides advantages such as genetic reassortment, which contributes to the emergence of novel strains with pandemic potential, it complicates the genome packaging of progeny virions. Here we elucidate, using electron tomography, the three-dimensional structure of ribonucleoprotein complexes (RNPs) within progeny virions. Each virion is packed with eight well-organized RNPs that possess rod-like structures of different lengths. Multiple interactions are found among the RNPs. The position of the eight RNPs is not consistent among virions, but a pattern suggests the existence of a specific mechanism for assembly of these RNPs. Analyses of budding progeny virions suggest two independent roles for the viral spike proteins: RNP association on the plasma membrane and the subsequent formation of the virion shell. Our data provide further insights into the mechanisms responsible for segmented-genome packaging into virions.

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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	Shin-Ichiroh Saitoh, Ph.D.	助　教　医学博士	齋　藤　伸一郎
Assistant Professor	Ryutaro Fukui, Ph.D.	助　教　医学博士	福　井　竜太郎

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

1. Human TLR4 polymorphism D299G/T399I alters TLR4/MD-2 conformation and response to a weak ligand monophosphoryl lipid A

Natsuko Yamakawa[1], Umeharu Ohto[2], Sachiko Akashi-Takamura[1], Koichiro Takahashi[1], Shin-Ichiroh Saitoh[1], Natsuko Tanimura[1], Takayoshi Suganami[3], Yoshihiro Ogawa[3,4], Takuma Shibata[1,5], Toshiyuki Shimizu[2], and Kensuke Miyake[1,5]: [1]Division of Innate Immunity, The Institute of Medical Science, The University of Tokyo, [2]Graduate School of Pharmaceutical Sciences, The University of Tokyo, [3]Department of Molecular Medicine and Metabolism, Medical Research Institute, Tokyo Medical and Dental University, [4]Department of Molecular Endocrinology and Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, [5]Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo

A cell surface heterodimeric Toll-like receptor 4 (TLR4)/MD-2 senses lipopolysaccharide (LPS), a principal membrane component of Gram-negative bacteria. LPS binds to MD-2 and induces dimerization of TLR4/MD-2. Dimerized TLR4 activates downstream signaling. TLR4 polymorphism replacing Asp299 with Gly and Thr399 with Ile (D299G/T399I) causes LPS-hyporesponsiveness, and is associated with a variety of infectious and noninfectious diseases. However, a molecular mechanism underlying the LPS-hyporesponsiveness remains controversial. We here asked whether the TLR4 polymorphism influenced cell surface expression of TLR4/MD-2, ligand-dependent TLR4/MD-2-dimerization, or TLR4/MD-2 responses to a weak agonist monophosphoryl lipid A (MPL). Newly established anti-TLR4 detected D299G/T399I TLR4/MD-2 on Ba/F3 cells, whereas previous anti-TLR4 did not, suggesting that the D299G/T399I polymorphism caused a conformational change in TLR4. Hyporesponsiveness of D299G/T399I TLR4/MD-2 was much more apparent when cells were stimulated with MPL than with lipid A. MPL-dependent TLR4/MD-2 dimerization was impaired by the D299G/T399I polymorphism. The D299G/T399I polymorphism did not alter LPS-binding to soluble TLR4/MD-2, but impaired its dimerization. These results suggest that the D299G/T399I TLR4 polymorphism impairs

TLR4/MD-2 responses by altering ligand-dependent dimerization.

2. Influence on the immune response by lipid associated molecules

Sachiko Akashi-Takamura[1], Natsuko Yamakawa[1], Takuma Shibata[1], Umeharu Ohto[2], Hiroki Nakanishi[3], Toshiyuki Shimizu[2], and Kensuke Miyake[1]: [1]The Institute of Medical Science, The University of Tokyo, [2]Graduate School of Pharmaceutical Science, The University of Tokyo, [3]Research Center for Biosignal, Akita University

TLR is not only indispensable sensor as an infection protective mechanism, but it is a regulator for disease development of symptoms. RP105 is a molecule which makes the mature B cell avoid from the radiation or steroid induced apoptosis, and induces a strong proliferative reaction. RP105 is also one of the important TLR molecules from follower reason. 1) RP105 resembles TLR4 structurally. 2) As TLR4 associates with secretion protein called MD-2, RP105 associates with MD-1. 3) RP105/MD-1 reinforces the B cell activation and the antibody production through TLR4/MD-2.

Recently the structural analysis of MD molecules is reported that MD-2 binds to LPS (Lipopolysaccharide), and MD-1 binds to Phosphatidyl choline (PC) or Phosphatidyl ethanolamine (PE). We recognized that MD-1 also binds to the negative-charged phospholipids by using purified MD-1 or immune-precipitation technique.

In order to examine a meaning in the living body about MD-1, we established MD-1-deficient SLE model mice. Compared with the control mice, splenomegaly and lymph nodes swelling was reinforced in MD-1 deficient SLE model mice. Furthermore, the serum antibody titer against phospholipids was higher in MD-1 deficient SLE model mice than control mice. It was possible that MD-1 is a molecule which has on the survival extinction and the immune response against lipid of B cells.

3. Collapse of TLR7/TLR9 balance develops strain-dependent lethal phenotypes.

Ryutaro Fukui[1], Shin-Ichiroh Saitoh[1], Atsuo Kanno[1], Masahiro Onji[1], Takuma Shibata[1,2], Mitsuru Matsumoto[4], Nobuaki Yoshida[3], and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, [3]Laboratory of Developmental Genetics, Center for Experimental Medicine and Systems Biology, [4]Division of Molecular Immunology, Institute for Enzyme Research, University of Tokushima

We found that the responses of TLR7 (Toll-like receptor 7) and TLR9 are controlled reciprocally by Unc93B1 (Unc93 homolog B1). This function depends on 34th aspartic acid from N-terminal of Unc93B1 (D34), and alanine mutant of the aspartic acid (D34A) up-regulates TLR7 response and down-regulates TLR9 response (Fukui et al., *J. Exp. Med.* 2009). To investigate the significance of reciprocal TLR7/TLR9 balance *in vivo*, we generated *Unc93b1*$^{D34A/D34A}$ mice and observed their phenotypes. As results, over half of *Unc93b1*$^{D34A/D34A}$ mice died within one year by various phenotypes, and not only inntate immune cells, but also B cells and T cells were activated (Fukui et al, *Immunity* 2011).

These study was performed by using 129 background mice, so we generated C57BL/6 (B6), BALB/c (BALB), C3H/HeN, DBA1/J, and NOD background *Unc93b1*$^{D34A/D34A}$ mice to analyze the effect of genetical background on the phenotypes of *Unc93b1*$^{D34A/D34A}$ mice. The difference of phenotypes among the background was observed, for example, over half of B6 background of *Unc93b1*$^{D34A/D34A}$ mice died within one year but few of BALB background of *Unc93b1*$^{D34A/D34A}$ mice died. As a phenotype of B6 *Unc93b1*$^{D34A/D34A}$ mice, most all of mice developed thrombocytopenia at four month old but no BALB *Unc93b1*$^{D34A/D34A}$ mice developed at same age.

Whereas the phenotypes were different between B6 and BALB background, collapse of TLR7/TLR9 balance were strain-independent. In the cells from both of strain, TLR7 was hyper-response and TLR9 was hypo-resonse. From these data, we hypothesized that some modifier genes link TLR7 inducing-inflammation to phenotypes, and started to search the factor by cytokine analysis, cell analysis and linkage analysis.

4. G protein regulates TLR7 and TLR9 responses

Shin-Ichiroh Saitoh[1], Kenji Kontani[3], Ryutaro Fukui[1], Atsuo Kanno[1], Mabel Chan[1], Fumiko Abe[3], Masahiro Onji[1], Yuji Motoi[1], Takuma Shibata[1,2], Toshiaki Katada[3], and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, The Institute of Medical Science, The University of Tokyo, [3]Department of physiological Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo

TLR7 and 9, innate immune sensors for microbial nucleic acids (NAs), respond to self NA, activate dendritic cells (DCs) and B cells, and mount anti-nuclear autoantibody production in murine models of systemic lupus erythematosus (SLE), an autoimmune disease where type I interferons (IFN-I) have a causative role. Despite the shared role in autoantibody production, RNA-sensing TLR7 promotes disease, whereas DNA-sensing TLR9 regulates dis-

ease progression by counteracting TLR7. TLR7 and 9 reside in the endoplasmic reticulum (ER), and are associated with Unc93B1, an ER-resident multiple transmembrane protein. Unc93B1 enable TLR7 and 9 to sense NA by transporting them to the endolysosomes, a site for NA-sensing, upon activation. TLR9 antagonizes TLR7 by competing association with Unc93B1. Unc93B1 restricts TLR7 activation by giving preference to TLR9-association over TLR7. In contrast to TLR9 antagonism, little is known about a mechanism underlying disease promotion by TLR7. We are studying that TLR7 drives IFN-I-mediated autoimmunity through TLR-trafficking dependent on Unc93B1 complexed with G protein required for lysosomal trafficking. Dendritic cells (DCs) and B cells harbouring an insertional mutation in the G protein protein showed augmented TLR7 responses in proinflammatory cytokine production and proliferation, respectively. The G protein was required for TLR7-dependent IFN-I production by plasmacytoid DCs (pDCs). The G protein was also required for TLR9-dependent IFN-I production by pDCs stimulated with A type CpG oligonucleotide. We analyze the relationship between this G protein and IFN-I dependent autoimmune disease.

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

Natsuko Tanimura and Kensuke Miyake

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.

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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
Associate Professor	Jun Kunisawa, Ph.D.	准教授	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
Project Assistant Professor	Eun Jeong Park, Ph.D.	特任助教	医学博士	朴	恩　正

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 the molecular and cellular nature of the mucosal immune system for the development of a mucosal vaccine against infectious diseases and mucosal immune therapy for mucosa-associated diseases, such as food allergies and inflammatory diseases.

1. MucoRice for New Generation of Oral Vaccine

Yoshikazu Yuki[1], Mio Mejima[1], Shiho Kurokawa[1], Koji Kashima[1],Tomoko Hiroiwa[1], Natsumi Takeyama[1], Yoshiko Fukuyama[1], Michiyo Abe[1], Yuko Takahashi[1], Daisuke Tokuhara[1], Yingju Chen[1], Sunny Joo[1], Yuko Katakai[2], Hiroaki Shibata[2], Eun Jeong Park[1], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo. [2]Tsukuba Primate Research Center, National Institute of Biomedical Innovation.

We have developed MucoRice-CTB as a cold-chain-free oral vaccine against Cholera and enterotoxigenic *Escherichia col*i -induced diarrhea. In order to obtain POC of MucoRice-CTB in humans, we submitted a Test Substance Summary Report and a Clinical Protocol of MucoRice-CTB to the Institutional Review Board (IRB) of Institute of Medical Science, The University of Tokyo (IMSUT) in October, 2012. The Test Substance Summary report contains (1) Preparation of selectable maker free MucoRice (MF-MucoRice) and establishment of MF-MucoRice seed banking (2) Quality characterization of MF-MucoRice-CTB (3) GMP preparation of bulk substance and finished product of MF-MucoRice-CTB (4) Specification of bulk substance and of finished product of MF-MucoRice-CTB (5) Accelerated and/or stress stability test for MF-MucoRice-CTB (6) GLP single dose and repeated dose toxicity studies, etc. The Clinical Protocol of MucoRice-CTB consists of (1) Selection of Subject (2) Test Design (3) Test Method (4) Clinical Evaluation (primary/secondary endpoints and safety issues) (5) Informed Consent (6) Record (Case Report Form) (7) Monitoring (8) Clinical Committee, etc. After approval from the IRB, Dr. H. Ohono as in charge of doctor's team enters a first-in-man human clinical study for MucoRice-CTB at the IMSUT-hospital in January, 2013. Although mucosal Abs immune responses on diversity of bacteria in the mammalian intestine have recently attracted a great deal of attention, we will more specifically analyze bacteria's metagenomes and antigen-specific IgA Ab immune responses in fecal samples of subjects before and after MucoRice-CTB oral administration. We expect that vaccine studies from bench to clinical feeds back from clinical to bench.

2. New Generation Nasal Vaccine

Yoshikazu Yuki[1], Il Gyu Kong[1], Ayuko, Sato[1], Yoshiko Fukuyama[1], Mio Mejima[1], Shiho Kurokawa[1], Tomonori Nochi[1], Haruko Takahashi[2], Shinichi Sawada[2], 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

Nasal vaccination has been considered a potent and practical immunization route for the effective mucosal vaccine to infectious diseases. One essential point is targeting efficient delivery of antigens to nasopharynx-associated lymphoid tissues (NALT) and/or NALT-independent nasal tissues in the turbinate covered with single-layer epithelium, where antigen-specific immune responses are initiated. To accomplish this point, we have developed an efficient antigen delivery system with self-assembled nanosize hydrogels (nanogel), which is composed of a cationic type of cholesteryl group-bearing pullulan. It should be emphasized that when prototype BoHc antigen of *Clostridium botulinum* and PspA antigen of *Streptococcus pneumonia* were nasally given with cCHP nanogel, high titers of antigen-specific protective immunity were induced, indicating that the cCHP nanogel can be universally used as a novel delivery vehicle for nasal vaccine in mice. For the development of a nanogel-based nasal vaccine for human use, we need to show POC and demonstrate the induction of systemic and mucosal immune responses with protective immunity after nasal immunization, with nanogel holding recombinant forms of vaccine such as PspA in macaques. We will also examine the metabolic fate of nasally administered nanogel holding PspA in macaques as well as ADME (Absorption, Distribution, Metabolism, Excretion). We will especially Locus on the olfactory bulb and brain by using ^{18}F-PspA-PET (positron emission tomography) system, which we recently established using the *in vivo* molecular imaging method.

3. Homeostasis of Gut Microenvironment

Yoshiyuki Goto[1,2], Jun Kunisawa[1-5], Shintaro Sato[1,2], Naoko Shibata[1,3], Aayam Lamichhane[1-3], Mariko Kamioka[1-3], Hideki Iijima[6], Yoshinori Umesaki[7], Yoshimi Benno[8], and Hiroshi Kiyono[1-4]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, [4]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo. [2]CREST, JST. [3]Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo. [5]Laboratory of Vaccine Materials, National Institute of Biomedical Innovation. [6]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine. [7]Yakult Central Institute for Microbiological Research. [8]Microbe Division/ Japan Collection of Microorganisms, RIKEN BioResource Center.

A wide variety of commensal microbes peacefully colonize in our gastrointestinal (GI) tract and contribute to the establishment of the host mucosal immune system. Accumulating evidence suggests that indigenous gut microbiota modulate host mucosal immune systems: maturation of secondary lymphoid tissues such as Peyer's patches (PPs), development of T cells, and induction of IgA and antimicrobial molecules. Mutually, the host mucosal immune system provides a favorable environment for resident microorganisms. Perturbations of the dynamic equilibrium between gut microbiota and the host mucosal immune system may lead to undesired enteric diseases such as inflammatory bowel disease (IBD) and colon cancer. We showed that the opportunistic bacteria, *Alcaligenes* species, were predominantly observed in PPs, with the associated preferential induction of anti-*Alcaligenes* mucosal IgA antibodies (Abs) (Obata T. *Proc Natl Acad Sci USA*. 2010; 107: 7419-7424). Preferential colonization 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 the local antigen-specific Ab production; this may create an optimal symbiotic environment on the interior of the PPs. On the other hand, we performed collaborative research with Dr. David Artis's group at the University of Pennsylvania and found that in the absence of IL-22 producing innate lymphoid cells, *Alcaligenes* spread out into peripheral tissues from inside of PPs and consequently induced systemic inflammation, which was also associated with some chronic diseases such as Crohn's disease and the progressive hepatitis C virus infection. Therefore, our current work is now focusing on the role of *Alcaligenes* and anti-*Alcaligenes* IgA antibodies in the establishment of inflammatory bowel diseases.

In our GI tract, crosstalk between host and microbiota occurs across a single layer of intestinal epithelial cells (IECs). For example, *Bacteroides thetaiotaomicron* induce and utilize fucose expressed on apical surface of IECs as an energy source. Indeed, epithelial fucose is observed in conventionally raised mice and induced by commensal microbes. However, previous reports suggested the role of *Bacteroides*, but not resident commensal microbiota. Using metagenomic approaches and gnotobiotic mouse models, we discovered that segmented fila-

mentous bacteria (SFB) specifically induce epithelial fucose under physiological conditions. We also found that defects of epithelial fucose, which is regulated by fucosyltransferase 2 (Fut2), leads to the colonization of aberrant gut microbiota. Recent genome-wide and candidate gene association studies have shown that *Fut2* represents susceptible genes in IBD. From these observations, our current interest is the role of epithelial fucose in the induction of colitis. We are investigating the mechanism of how epithelial fucose regulates intestinal homeostasis.

4. Immunological Crosstalk with Dietary Materials in the Regulation of Gut Immunity

Jun Kunisawa[1-5], Yosuke Kurashima[1,2], Yoshiyuki Goto[1,2], Makoto Kinoshita[6], Takashi Kusu[6], Eri Hashimoto[1], Yuji Suzuki[1], Shiori Shikata[1], Risa Sumiya[1], Takeaki Amiya[1,2], Kumiko Fujisawa[1,2], Kiyoshi Takeda[6], and Hiroshi Kiyono[1-4]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, [3]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo. [2]CREST, JST. [4]Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo. [5]Laboratory of Vaccine Materials, National Institute of Biomedical Innovation. [6]Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University.

Accumulating evidence has revealed that immunological homeostasis, as well as immune diseases are mediated by crosstalk between the host immune system and gut environmental factors (e.g., commnesal bacteria and diets). Based on the fact that an increased number of immune disease patients are associated with an increased consumption of westernized diets, it has been considered that diets directly regulate the immune system, especially in the gut.

In this project, we have been interested in several dietary materials (e.g., vitamins, fatty acids, and nucleotides). For example, we show that vitamin B9 is a survival factor for regulatory T (Treg) cells expressing high levels of vitamin B9 receptor (folate receptor 4). Indeed, in a vitamin B9-reduced condition, Treg cells could be differentiated from naïve T cells, but failed to survive. Thus, the reduction of Treg cells in mice fed a vitamin B9-deficient diet subsequently led to the development of intestinal inflammation. These findings provide a novel strategy to establish mucosal homeostasis through the interaction with mucosal environmental factors.

5. Roles of Mast Cells in the Intestinal Allergic and non-Allergic Diseases

Yosuke Kurashima[1-3], Takeaki Amiya[1,2,4], Kumiko Fujisawa[1,2], Yuta Kogure[1], Naoko Shibata[1,2], Yuji Suzuki[1], Eri Hashimoto[1], Kenji Kabashima[5], Atsushi Otsuka[5,6], Masato Kubo[7,8], Yoshifumi Yokota[9], Shintaro Sato[1,2], Kensuke Miyake[3], Jun Kunisawa[1,4,10], and Hiroshi Kiyono[1,2,4]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, [3]Division of Infectious Genetics, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo. [2]CREST, JST. [4]Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo. [5]Department of Dermatology, [6]Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University Graduate School of Medicine. [7]Research Center for Allergy and Immunology, RIKEN. [8]Division of Molecular Pathology, Research Institute for Biological Sciences, Tokyo University of Sciences. [9]Department of Molecular Genetics, School of Medicine, University of Fukui. [10]Laboratory of Vaccine Materials, National Institute of Biomedical Innovation.

Mast cells (MCs) play a critical role in protective immunity against pathogenic microorganisms, whereas their dysregulated activation results in inflammatory responses. We found that activation of MCs in intestinal inflammation is mediated by ATP -reactive P2X7 purinoceptors. Increased numbers of MCs expressing P2X7 purinoceptors were noted in the colons of mice with colitis and patients with Crohn's disease. Treatment of mice with a P2X7 purinoceptor-specific antibody inhibited MC activation and subsequent intestinal inflammation. In addition, we found that expression levels of P2X7 in MCs were diverse among the tissues. Transfer or reconstitution of immature MCs into the MC-deficient mice showed the same patterns of P2X7 expression levels in transferred matured MCs as that of MCs in the wild type mice, indicating that tissue-specific P2X7 regulation systems in MCs existed. These results demonstrate the novel homeostatic network exhibited by the regulation of ATP-mediated MC activation by tissue-specific regulation of P2X7 expression.

6. Identification of key molecule for the development of Peyer's patch M cells

Shintaro Sato[1,2], Satoshi Kaneto[1,3], Naoko Shibata[1,4], Yuko Takahashi[1], Hideaki Okura[1,4], Yoshikazu Yuki[1], Jun Kunisawa[1,2,4,5,6], and Hiroshi Kiyono[1-5]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, [5]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo. [2]CREST, JST. [3]Graduate School of Medicine, The University of Tokyo. [4]Department

of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo. [6]Laboratory of Vaccine Materials, National Institute of Biomedical Innovation.

Although many of the biological features of microfold cells (M cells) have been known for many years, the molecular mechanisms of M-cell development and antigen recognition have remained unclear. Here, we report that *Umod* is a novel M cell-specific gene, the translation products of which might contribute to the uptake function of M cells. Transcription factor Spi-B was also specifically expressed in M cells among non-hematopoetic lineages. Spi-B-deficient mice showed reduced expression of most, but not all, other M-cell-specific genes and M-cell surface markers. Whereas uptake of *Salmonella* Typhimurium via M cells was obviously reduced in Spi-B-deficient mice, the abundance of intra-tissue cohabiting bacteria was comparable between wild-type and Spi-B-deficient mice. These data indicate that there is a small M-cell population with developmental regulation that is Spi-B independent; however, Spi-B is probably a candidate master regulator of M-cell functional maturation and development by another pathway.

7. Crosstalk between Maxillofacial Immunity and Reproductive Immunity

Ayuko Sato[1,2], Manami Okabe[1], Il Gyu Kong[1,3], Kazunari Okada[1,2], Takahiko Imai[4], Naoto Koyanagi[4], Akihisa Kato[4], Yasushi Kawaguchi[4], Jun Kunisawa[1,2,5,6], Shintaro Sato[1,2], and Hiroshi Kiyono[1-3,5]: [1]Division of Mucosal Immunology, [4]Division of Molecular Virology, Department of Microbiology and Immunology, and [5]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, The University of Tokyo. [2]CREST, JST. [3]Graduate School of Medicine, The University of Tokyo. [6]Laboratory of Vaccine Materials, National Institute of Biomedical Innovation.

The vaccine against human papilloma virus, causing cervical carcinoma, has been established; however, the vaccine preventing other sexual transmitted diseases (STDs), caused by HIV, HSV, or Chlamydia, has not yet been developed. In this study, we have been investigating the molecular and cellular mechanisms by which intranasal and ocular immunizations induce both humoral and cellular immune responses specific for the viral or bacterial antigens in the distant reproductive mucosa. Using the genital HSV infection model, we found that the effector T cells induced by mucosal immunization, but not by systemic immunization, obtained the ability to localize and provide against the secondary infection in the mucosal compartment. We are now elucidating the reproductive imprinting molecules which are expressed on the effector T cells. Our project will thus contribute scientifically to our understanding of the reproductive imprinting system, which will lead to the development of effective vaccine against STDs, for the improvement of public health.

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Department of Microbiology and Immunology

Division of Molecular Virology
ウイルス病態制御分野

Professor	Yasushi Kawaguchi, D.V.M., Ph.D.	教　授	獣医学博士	川　口　　寧
Assistant Professor	Akihisa Kato, Ph.D.	助　教	医学博士	加　藤　哲　久

To date, approximately 250 herpesviruses have been identified, affecting most animal species. These viruses are associated with a variety of diseases such as encephalitis, malignancy and mucocutaneous diseases in human and animals. The objective of our research is to understand the mechanisms by which herpesviruses replicate in cells and manifest diseases in their hosts. Our goal is to apply our fundamental findings for the development of anti-herpetic drugs and vaccines for the control of these viral infections.

1. Herpes Simplex Virus 1 VP22 Regulates Translocation of Multiple Viral and Cellular Proteins and Promotes Neurovirulence

Michiko Tanaka[1], Akihisa Kato, Yuko Satoh, Takahiro Ide, Ken Sagou, Kayo Kimura, Hideki Hasegawa[1] and Yasushi Kawaguchi: [1]Department of Pathology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan

Herpes simplex virus 1 (HSV-1) protein VP22, encoded by the UL49 gene, is a major virion tegument protein. In the present study, we showed that VP22 was required for efficient redistribution of viral proteins VP16, VP26, ICP0, ICP4 and ICP27 and cellular protein Hsc-70 to the cytoplasm of infected cells. We found that two di-leucine motifs in VP22, at amino acids 235-236 and 251-252, were necessary for VP22 regulation of the proper cytoplasmic localization of these viral and cellular proteins. The di-leucine motifs were also required for proper cytoplasmic localization of VP22 itself and for optimal expression of viral proteins VP16, VP22, ICP0, UL41 and glycoprotein B. Interestingly, a recombinant mutant virus with alanine substitutions for the di-leucines at amino acids 251-252 had about a 10^3-fold lower LD_{50} for neurovirulence in mice following intracerebral inoculation than the LD_{50} of the repaired virus. Furthermore, replication and spread of this mutant virus in the brains of mice following intracerebral inoculation were significantly impaired compared to those of the repaired virus. The ability of VP22 to regulate localization and expression of various viral and cellular proteins, as shown in this study, was correlated with an increase in viral replication and neurovirulence in the experimental murine model. Thus, HSV-1 VP22 is a significant neurovirulence factor in vivo.

2. Evasion of $CD8^+$ T cells Mediated by a Kinase Encoded by Herpes Simplex Virus 1 Contributes to Viral Replication In Vivo

Takahiko Imai, Naoto Koyanagi, Tadahiro Suenaga[1,2], Ayuko Sato[3], Akihisa Kato, Hiroshi Kiyono[3,4], Hisashi Arase[1,2,4] and Yasushi Kawaguchi: [1]Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan, [2]WPI Immunology Frontier Research Center, Osaka University, Suita, Osaka, Japan, [3]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan, [4]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan

Detection and elimination of virus-infected cells by $CD8^+$ cytotoxic T lymphocytes (CTLs) depends on recognition of virus-derived peptides presented by major histocompatibility complex class I (MHC-I) molecules on the surface of infected cells. Although numerous reports have revealed the molecular mechanisms by which various herpesvirus proteins inhibit different steps of the MHC-I antigen presentation pathway in vitro, data on how these activities contribute to viral replication and/or pathogenesis in vivo is limited to some non-human, animal herpesviruses in the *Betaherpesvirinae* and *Gammaherpesvirinae*. In the present study, we showed that the activity of viral kinase Us3 encoded by herpes simplex virus 1 (HSV-1), the etiologic agent of several human diseases and the member of the *Alphaherpesvirinae*, inhibited cell surface expression of MHC-I, thereby preventing CTL recognition of infected cells in vitro and down-regulation of induction of HSV-1-specific $CD8^+$ T cells in mice. Interestingly, depletion of $CD8^+$ T cells in mice significantly increased replication of a recombinant virus encoding a kinase-dead mutant of Us3, but had no effect on replication of a recombinant virus in which the kinase-dead mutation was repaired. These results suggested that evasion of $CD8^+$ T cells by HSV-1 Us3-mediated inhibition of MHC-I antigen presentation contributed to viral replication in vivo.

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