RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Bacterial Infection
細菌感染分野

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Research in this division is directed toward understanding the complex interactions that occur between pathogenic bacteria and their human hosts at very early stage of bacterial infectious processes. 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.

1. *Helicobacter pylori* CagA Phosphorylation-Independent Function in Epithelial Proliferation and Inflammation

Masato Suzuki, Hitomi Mimuro, Kotaro Kiga, Makoto Fukumatsu, Nozomi Ishijima, Hanako Morikawa, Shigenori Nagai[1], Shigeo Koyasu[1], Robert H. Gilman[2], Dangeruta Kersulyte[3], Douglas E. Berg[3] and Chihiro Sasakawa: [1]Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan, [2]Department of Microbiology, Facultad de Medecina, Universidad Peruana Cayetano Heredia, Lima 31, Peru, [3]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA

CagA, a major virulence factor of *Helicobacter pylori* (Hp), is delivered into gastric epithelial cells and exists in phosphorylated and nonphosphorylated forms. The biological activity of the phosphorylated form is well established; however, function(s) of the nonphosphorylated form remain elusive. Here, we report that a conserved motif in the C-terminal region of CagA, which is distinct from the EPIYA motifs used for phosphorylation and which we designate CRPIA (conserved repeat responsible for phosphorylation-independent activity), plays pivotal roles in Hp pathogenesis. The CRPIA motif in nonphosphorylated CagA was involved in interacting with activated Met, the hepatocyte growth factor receptor, leading to the sustained activation of phosphatidylinositol 3-kinase/Akt signaling in response to Hp infection. This in turn led to the activation of β-catenin and NF-κB signaling, which promote proliferation and inflammation, respectively. Thus, nonphosphorylated CagA activity contributes to the epithelial proliferative and proinflammatory responses associated with development of chronic gastritis and gastric cancer.

2. Transient Shielding of Intimin and the Type III Secretion System of Enterohemorrhagic and Enteropathogenic *Escherichia coli* by a Group 4 Capsule'

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Enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC, respectively) strains represent a major global health problem. Their virulence is mediated by the concerted activity of an array of virulence factors including toxins, a type III protein secretion system (TTSS), pili, and others. We previously showed that EPEC O127 forms a group 4 capsule (G4C), and in this report we show that EHEC O157 also produces a G4C, whose assembly is dependent on the etp, etk, and wzy genes. We further show that at early time points postinfection, these G4Cs appear to mask surface structures including intimin and the TTSS. This masking inhibited the attachment of EPEC and EHEC to tissue-cultured epithelial cells, diminished their capacity to induce the formation of actin pedestals, and attenuated TTSS-mediated protein translocation into host cells. Importantly, we found that Ler, a positive regulator of intimin and TTSS genes, represses the expression of the capsule-related genes, including etp and etk. Thus, the expression of TTSS and G4C is conversely regulated and capsule production is diminished upon TTSS expression. Indeed, at later time points postinfection, the diminishing capsule no longer interferes with the activities of intimin and the TTSS. Notably, by using the rabbit infant model, we found that the EHEC G4C is required for efficient colonization of the rabbit large intestine. Taken together, our results suggest that temporal expression of the capsule, which is coordinated with that of the TTSS, is required for optimal EHEC colonization of the host intestine.

3. Control of epithelial cell structure and developmental fate: Lessons from *Helicobacter pylori*

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Valuable insights into eukaryotic regulatory circuits can emerge from studying interactions of bacterial pathogens such as *Helicobacter pylori* with host tissues. *H. pylori* uses a type IV secretion system (T4SS) to deliver its CagA virulence protein to epithelial cells, where much of it becomes phosphorylated. CagA's phosphorylated and non-phosphorylated forms each interact with host regulatory proteins to alter cell structure and cell fate. Kwok and colleagues showed that CagA destined for phosphorylation is delivered using host integrin as receptor and *H. pylori*'s CagL protein as an integrin-specific adhesin, and that CagL-integrin-binding activates the kinase cascade responsible for CagA phosphorylation. This research contributes to understanding infectious disease and the control of cell fates.

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Department of Microbiology and Immunology

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

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The goal of our Department is to elucidate the cellular defense system and the counteracting viral strategy at the level of gene regulation and to establish new approaches for modulating human immunological response and for suppressing human pathogenic viruses. We have been studying epigenetical regulation of human and HIV genomes by a chromatine remodeling factor, SWI/SNF complex and also analyzing genome-wide networks formed between microRNAs and such important transcriptional factors as AP-1 and NF-kappaB to explore the cause of human diseases. Using these results, we also develop new retrovirus/lentivirus vectors that express proteins, short hairpin RNAs, miRNAs and efficient inhibitory RNAs targeting specific miRNAs (Tough Decoy RNAs) for human gene therapy and basic researches.

1. Epigenetical regulation of host and retroviral genes.

The SWI/SNF complex contributes to the regulation of gene expression by altering the chromatin structure, and plays many important roles in epigenetic regulation in many organisms. In mammals, this complex is composed of about 10 subunits, and each of the complex contains a single molecule of either Brm or BRG1, but not both. These two proteins are the catalytic subunits and have DNA-dependent ATPase activity that drives remodeling of nucleosomes. We previously reported that Brm and BRG1 have clear differences in their biological activities; Brm is essential for maintenance of murine leukemia virus (MuLV)-based retroviral gene expression, whereas BRG1 is not. Therefore, cell lines that do not express detectable levels of Brm protein undergo very rapid retroviral gene silencing that occurs stochastically and discontinuously.

Whereas SWI/SNF complex has been recognized as regulator at the transcriptional level, this year, we have demonstrated that this complex in conjugation with $p54^{nrb}$ can subsequently function as a splicing regulator for the transcript products. Considering that $p54^{nrb}$/PSF was previously reported to regulate HIV mRNA stability, this finding would be quite important to elucidate post-transcriptional regulation of HIV (a). In 2007, we showed a clear correlation between Brm-deficiency and undifferentiated status of gastric cancer, and further demonstrated that Brm-type, but not BRG1-type SWI/SNF complex is required for *villin* expression. By intensive analysis on the human *villin* promoter, we have shown that Cdx2 regulates intestinal *villin* expression through recruiting Brm-type SWI/SNF complex to the *villin* promoter. We are now convinced that Cdx2 is a key linker between the loss of Brm and undifferentiated status of gastic cancer (b). Like chromatin remodeling, DNA methylation plays crucial roles

in human epigenetical regulation. We here also present evidence that mouse ES (embryonic stem) cell clones that were transduced with MSCV (murine stem cell virus) do show variegated proviral expression. We found that the fidelity of DNA methylation among the genomic sequences that flank the proviral integration sites would be the determinant of this reversibility. These results would reflect a unique and interesting feature of ES cell epigenome regulation (c).

a. Brm transactivates the *telomerase reverse transcriptase (TERT)* gene and modulates the splicing patterns of its transcripts in concert with p54^{nrb}

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We report that a DBHS (for *Drosophila* behavior, human splicing) family protein, p54^{nrb}, binds both BRG1 and Brm, catalytic subunits of SWI/SNF chromatin remodeling complex, and also another core subunit of this complex, BAF60a. The N-terminal region of p54^{nrb} is sufficient to pull-down other core subunits of the SWI/SNF complex, suggesting that p54^{nrb} binds SWI/SNF-like complexes. Polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF), another DBHS family protein known to directly bind p54^{nrb}, was also found to associate with the SWI/SNF-like complex. When short hairpin (sh) RNAs targeting Brm were retrovirally expressed in a BRG1-deficient human cell line (NCI-H 1299), the resulting clones showed downregulation of the *telomerase reverse transcriptase (TERT)* gene and an enhancement of ratios of exon-7-and-8-excluded *TERT* mRNA that encodes an inactive protein. All of these clones display growth arrest within two months of the Brm-knockdown. In NCI-H1299 cells, Brm, p54^{nrb}, PSF, and RNA polymerase II phosphorylated on CTD serine 2, specifically co-localize at a region incorporating an alternative splicing acceptor site of *TERT* exon7. These findings suggest that at the *TERT* gene locus in human tumor cells containing functional SWI/SNF complex, Brm, and possibly BRG1, in concert with p54^{nrb} would initiate efficient transcription and could be involved in the subsequent splicing of *TERT* transcripts by accelerating exon-inclusion, which partly contributes to the maintenance of active telomerase.

b. Cdx2 and the Brm-type SWI/SNF complex cooperatively regulate *villin* expression in gastrointestinal cells.

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In our recent study showing a correlation between Brm-deficiency and undifferentiated status of gastric cancer, we found that the Brm-type SWI/SNF complex is required for *villin* expression. To elucidate intestinal *villin* regulation more precisely, this year, we have analyzed structure and function of the promoter of human *villin*. About 1.1kb upstream of the determined major TSS, we identified a highly conserved region (HCR-Cdx) among mammals, which contains two binding sites for Cdx. Expression analyses of 30 human gastrointestinal cell lines suggested that *villin* is regulated by Cdx2. Introduction of *Cdx* family genes into colorectal SW480 cells revealed that *villin* is strongly induced strongly by Cdx2, and the knockdown of Cdx2 in SW480 cells caused a clear downregulation of *villin*. Reporter assays showed that HCR-Cdx is crucial for Cdx2-dependent and Brm-dependent *villin* expression. Immunohistochemical analyses of gastric intestinal metaplasia and gastric cancer revealed that *villin* and Cdx2 expression are tightly coupled. GST pull-down assays demonstrated a direct interaction between Cdx2 and several SWI/SNF subunits . Chromatin immunoprecipitation analyses further showed the recruitment of Cdx 2 and Brm around the HCR-Cdx. From these results, we concluded that Cdx2 regulates intestinal *villin* expression through recruiting Brm-type SWI/SNF complex to the *villin* promoter.

c. Instability of retroviral DNA methylation in embryonic stem cells

Shigeru Minoguchi and Hideo Iba

The epigenetic status of pluripotent stem cells has been demonstrated to be extremely unstable. In our current study, we have attempted to further investigate the epigenetic dynamics of the stem cell genome by monitoring the expression of the murine stem cell virus (MSCV) retroviral

vector in embryonic stem (ES) cells. Whilst MSCV is progressively silenced by proviral DNA methylation in ES cells, a substantial number of MSCV-transduced ES cell clones do show variegated proviral expression. This expression profile is due in part to the transient and reversible properties of MSCV silencing. However, the spontaneous reactivation rates of the silenced proviruses differ significantly between these variegated clones, indicating that the reversibility of silencing is dependent on the proviral integration site. Our current data suggest that the fidelity of DNA methylation among the genomic sequences that flank the proviral integration sites may be the determinant of this reversibility of MSCV silencing. Given that the adjoining epigenome environment affects the epigenetic regulation of proviral DNA, the reversible MSCV silencing effect is thus likely to reflect a unique and interesting feature of ES cell epigenome regulation that has not previously been revealed.

2. Regulatory networks formed between transcriptional factors and miRNAs and development of new powerful tools for network analysis and for human therapy.

Recently new layers of molecular mechanisms are shown to be involved in the gene regulation; many RNA transcripts of exogenous as well as endogenous genes are regulated at post-transcriptional level by a mechanism designated as RNA interference (RNA silencing). In human cells, major players of RNA interference are endogenous micro (mi)RNAs as well as short interfering (si)RNAs that are produced from either host or viral aberrant RNAs rich in dsRNA regions.

MicroRNAs (miRNAs) are endogenous 20-24 nt RNAs known to mediate the repression of target mRNAs by suppressing translation or promoting mRNA decay in animal. More than 700 species of miRNAs have now been identified in human, and also predicted to target roughly 30% of the total coding genes in human. A significant body of evidence has accumulated that they are involved in cellular development, differentiation, innate and acquired immunity induction, and anti-viral responses by post-transcriptional regulation of important target mRNAs. In spite of the strong impact of miRNAs on regulatory network comparable to transcription factors, it still remains largely unknown how human miRNA expression itself is regulated at the transcriptional level, although the vertebrate miRNA genes are thought to be generally transcribed by RNA polymerase II (pol II) to produce a pri-miRNA containing a 5′-cap structure and polyA tail. To elucidate genome-wide interplay between miRNAs and transcriptional factors, this year, we developed an algorithm that predicts promoter region of human *miRNA* genes that are involved in important regulatory systems evolutionarily conserved among vertebrates (a). We have further scrutinized an important regulatory system involving, miR-21, which has been reported to be expressed at high levels in almost all the human cancers and further to be involved in differentiation of several kinds of stem cells. By detailed analysis on the promoter of the *miR-21* gene, we found that an evolutionarily conserved double-negative feedback regulation, which involves miR-21, NFIB protein and *miR-21* promoter, would be operating as a mechanism to substain *miR-21* expression (b). It is also quite important to develop efficient methods for the stable suppression of specific microRNA activity. We finally show that unique RNA decoys developed here (designated Tough Decoy: TuD) achieve the efficient and long-term-suppression of specific miRNA (c).

a. Putative promoter regions of miRNA genes involved in evolutionarily conserved regulatory systems among vertebrates.

Shuji Fujita and Hideo Iba

Just as transcription factors, miRNA genes modulate global patterns of gene expression during differentiation, metabolic activation, stimulus response and also carcinogenesis. However, little is currently known how the miRNA gene expression itself is regulated owing to lack of basic information of their gene structure. Global prediction of promoter regions of miRNA genes would allow us to explore the mechanisms underlying gene-regulatory mechanisms involving these miRNAs. We speculate that if specific miRNA molecules are involved in evolutionarily conserved regulatory systems in vertebrates, this would entail a high level of conservation of the promoter of miRNAgene as well as the miRNA molecule. By our current screening of putative promoter regions of miRNA genes (miPPRs) on this base, we identified 59 miPPRs that would direct production of 79 miRNAs. We present both biochemical and bioinformatical verifications of these putative promoters.

b. *miR-21* gene expression triggered by AP-1 is sustained through a double negative feedback mechanism.

Shuji Fujita, Taiji Ito, Taketoshi Mizutani,

Shigeru Minoguchi, Nobutake Yamamichi, Kouhei Sakurai and Hideo Iba

miR-21 has been reported to be highly expressed in various cancers and also to be inducible in a human promyelocytic cell line, HL-60, after PMA treatment. To examine molecular mechanisms involved in miR-21 expression, we analyzed structure of the *miR-21* gene by determining its promoter and primary transcripts. We show that AP-1 activates the *miR-21* transcription in conjugation with SWI/SNF complex, after PMA stimulation, through the conserved AP-1 and PU.1 binding sites in the promoter identified here. The previous findings of enhanced miR-21 expression in several cancers may therefore reflect the elevated AP-1 activity in these carcinomas. A single precursor RNA containing miR-21 was transcribed just downstream from the TATA box in this promoter, which is located in an intron of a coding gene *TMEM49*. Importantly, expression of this overlapping gene is completely PMA-independent and all its transcripts are polyadenylated before reaching the miR-21 hairpin embedding region, indicating that *miRNAs* could have their own promoter even if overlapped with other genes. By available algorithms that predict miRNA target using a conservation of sequence complementary to the miRNA seed sequence, we next predicted and confirmed that the *NFIB* mRNA is a target of miR-21. NFIB protein usually binds the *miR-21* promoter in HL-60 cells as a negative regulator and is swept off from the *miR-21* promoter during PMA-induced macrophage differentiation of HL-60. The translational repression of *NFIB* mRNA by miR-21 accelerates clearance of NFIB in parallel with the simultaneous miR-21-independent transcriptional repression of *NFIB* after PMA stimulation. Since exogenous miR-21 expression moderately induced endogenous miR-21, an evolutionarily conserved double negative feedback regulation would be operating as a mechanism to sustain miR-21 expression.

c. Vectors expressing efficient RNA decoys achieve the long-term suppression of specific microRNA activity in mammalian cells.

Takeshi Haraguchi, Yuka Ozaki and Hideo Iba

Whereas the strong and stable suppression of specific microRNA activity would be essential for the functional analysis of these molecules, and also for the development of therapeutic applications, effective inhibitory methods to achieve this have not yet been fully established. In our current study, we tested various RNA decoys which were designed to efficiently expose indigestible complementary RNAs to a specific miRNA molecule. These inhibitory RNAs were at the same time designed to be expressed in lentiviral vectors and to be transported into the cytoplasm after transcription by RNA polymerase III. We report the optimal conditions that we have established for the design of such RNA decoys (we term these molecules TuD RNAs; tough decoy RNAs). We demonstrate that TuD RNAs induce specific and strong biological effects and also show that TuD RNAs achieve the efficient and long-term-suppression of specific miRNAs for over one month in mammalian cells.

Publications

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Viruses can cause devastating diseases. The long-term goal of our research is to understand the molecular pathogenesis of viral diseases, using influenza and Ebola virus infections as models. Interactions between viral and host gene products during viral replication cycles determine the consequences of infection (i.e., the characteristics of disease manifestation, whether limited or widespread); hence, our research has centered on such interactions in theses viral infections.

1. A naturally occurring deletion in its NS gene contributes to the attenuation of an H5N1 swine influenza virus in chickens

Zhu Q, Yang H, Chen W, Cao W, Zhong G, Jiao P, Deng G, Yu K, Yang C, Bu Z, Kawaoka Y, Chen H.

In 2001 and 2003, we isolated two H5N1 viruses, A/swine/Fujian/1/01 (SW/FJ/01) and A/swine/Fujian/1/03 (SW/FJ/03), from pigs in Fujian Province, southern China. Genetically, these two viruses are similar, although the NS gene of the SW/FJ/03 virus has a 15-nucleotide deletion at coding positions 612 to 626. The SW/FJ/01 virus is highly lethal for chickens, whereas the SW/FJ/03 virus is nonpathogenic for chickens when administrated intravenously or intranasally. To understand the molecular basis for the difference in virulence, we used reverse genetics to create a series of single-gene recombinants of both viruses. We found that a recombinant virus containing the mutated NS gene from the SW/FJ/03 virus in the SW/FJ/01 virus background was completely attenuated in chickens. We also found that viruses expressing the mutant NS1 protein of SW/FJ/03 did not antagonize the induction of interferon (IFN) protein. Conversely, only the recombinant virus containing the wild-type SW/FJ/01 NS gene in the SW/FJ/03 background was lethal in chickens and antagonized IFN protein levels. Further, we proved that the NS1 genes of the two viruses differ in their stabilities in the host cells and in their abilities to interact with the chicken cleavage and polyadenylation specificity factor. These results indicate that the deletion of amino acids 191 to 195 of the NS1 protein is critical for the attenuation of the SW/FJ/03 virus in chickens and that this deletion affects the ability of the virus to antagonize IFN induction in host cells.

2. A novel approach to the development of effective H5N1 influenza A virus vaccines: the use of M2 cytoplasmic tail mutants

Watanabe T, Watanabe S, Kim JH, Hatta M,

Kawaoka Y.

Outbreaks of highly pathogenic H5N1 influenza viruses in avian species began in Asia and have since spread to other continents. Concern regarding the pandemic potential of these viruses in humans is clearly warranted, and there is an urgent need to develop effective vaccines against them. Previously, we and others demonstrated that deletions of the M2 cytoplasmic tail caused a growth defect in A/WSN/33 (H1N1) influenza A virus in vitro. We therefore tested the feasibility of using M2 tail mutants as live attenuated vaccines against H5N1 virus. First we generated a series of highly pathogenic H5N 1 (A/Vietnam/1203/04 [VN1203]) M2 cytoplasmic tail deletion mutants and examined their growth properties in vitro and in vivo. We found that one mutant, which contains an 11-amino-acid deletion from the C terminus (M2del 11 virus), grew as well as the wild-type virus but replicated in mice less efficiently. We then generated a recombinant VN1203M2del11 virus whose hemagglutinin (HA) gene was modified by replacing sequences at the cleavage site with those of an avirulent type of HA (M2del11-HAavir virus). This M2del11-HAavir virus protected mice against challenge with lethal doses of homologous (VN1203; clade 1) and antigenically distinct heterologous (A/Indonesia/7/2005; clade 2) H5N1 viruses. Our results suggest that M2 cytoplasmic tail mutants have potential as live attenuated vaccines against H5N1 influenza viruses.

3. Limited compatibility between the RNA polymerase components of influenza virus type A and B

Iwatsuki-Horimoto K, Hatta Y, Hatta M, Muramoto Y, Chen H, Kawaoka Y, Horimoto T.

Reassortants between type A and B influenza viruses have not been detected in nature, although both viruses co-circulate in human populations. One explanation for this may be functional incompatibility of RNA transcription and replication between type A and B viruses. To test this possibility, we constructed type A/B mosaic polymerase machinery, containing PB2, PB1, PA and nucleoprotein from each of the two virus types, and assessed their polymerase activities with a type A promoter in a reporter assay. Type B polymerase machinery containing homologous components was functional with the type A promoter albeit to various extents depending on the segments from which the regions downstream of the promoter sequence were derived, indicating functional compatibility between the type A promoter and B polymerase machinery. However, all of the A/B mosaic polymerase machinery, except that containing PA from a type A and the others from a type B virus strain, did not function with the type A promoter, indicating limited compatibility among polymerase components of both types. Taken together, these data suggest that incompatibility among components of the polymerase machinery for RNA transcription and replication alone is not responsible for the lack of heterotypic reassortants.

4. A vaccine prepared from a non-pathogenic H7N7 virus isolated from natural reservoir conferred protective immunity against the challenge with lethal dose of highly pathogenic avian influenza virus in chickens

Sakabe S, Sakoda Y, Haraguchi Y, Isoda N, Soda K, Takakuwa H, Saijo K, Sawata A, Kume K, Hagiwara J, Tuchiya K, Lin Z, Sakamoto R, Imamura T, Sasaki T, Kokumai N, Kawaoka Y, Kida H.

During 2001-2004, 41 H7 influenza viruses (2 H7N1 and 39 H7N7 strains) were isolated from fecal samples of migratory ducks that flew from Siberia in the autumn of each year to Japan and Mongolia. A phylogenetic analysis of the hemagglutinin (HA) genes of the nine representative isolates revealed that they belonged to the Eurasian lineage and the deduced amino acid sequence at the cleavage site of the HAs represented apathogenic profiles. One of the H7 isolates A/duck/Mongolia/736/02 (H7N7) was chosen from these H7 isolates for the preparation of the test vaccine. To improve the growth potential of A/duck/Mongolia/736/02 (H7N7) in chicken embryos, A/duck/Hokkaido/Vac-2/04 (H7N7) was generated by genetic reassortment between A/duck/Mongolia/736/02 (H7N 7) as the donor of the PB2, PB1, PA, HA, NA, and NS genes and A/duck/Hokkaido/49/98 (H 9N2) as that of NP and M genes. The test vaccine was prepared as follows; A/duck/Hokkaido/Vac-2/04 (H7N7) was propagated in chicken embryos and the virus in the allantoic fluid was inactivated and adjuvanted to form an oil-in-water emulsion. The test vaccine conferred immunity to chickens, completely protecting the manifestation of clinical signs against the challenge with lethal dose of H7 highly pathogenic avian influenza virus. These results indicate that influenza viruses isolated from natural reservoirs are useful for vaccine strains.

5. Comparison of the clinical effectiveness of oseltamivir and zanamivir against influenza virus infection in children

Sugaya N, Tamura D, Yamazaki M, Ichikawa M, Kawakami C, Kawaoka Y, Mitamura K.

We compared the clinical effectiveness of oseltamivir and zanamivir in children with influenza A (H1N1) virus, influenza A (H3N2) virus, and influenza B virus infections. Total febrile period and the duration of fever after the start of treatment were compared between an oseltamivir-treated group (mean age, 8.9 years; range, 4.0-15.9 years) and a zanamivir-treated group (mean age, 10.0 years; range, 4.0-15.7 years) in the pediatric outpatient clinics of our hospitals. Oseltamivir was used to treat 91 children with influenza A (H3N2) infection and 24 children with influenza A (H1N1) infection. Zanamivir was used to treat 35 children with influenza A (H3N2) infection and 12 children with influenza A (H1N1) infection. Oseltamivir was also used to treat 128 children with influenza B virus infection, and zanamivir was used to treat 59 with influenza B virus infection. There was no statistically significant difference in total febrile period or duration of fever after the start of treatment between the oseltamivir-treated group and the zanamivir-treated group of children with influenza A (H3N2) infection (mean duration of febrile period, 2.40 days vs. 2.39 days; mean duration of fever after the start of treatment, 1.35 days vs. 1.40 days), influenza A (H1 N1) (mean duration of febrile period, 2.60 days vs. 2.46 days; mean duration of fever after the start of treatment, 1.79 days vs, 1.54 days), or influenza B (mean duration of febrile period, 2.95 days vs. 2.84 days; mean duration of fever after the start of treatment, 1.86 days vs. 1.67 days). Oseltamivir was more effective against influenza A (H3N2) than against influenza A (H1N1) or influenza B. Oseltamivir and zanamivir were equally effective in reducing the febrile period of children with influenza A (H1N1), influenza A (H3N2), and influenza B virus infection.

6. Drosophila RNAi screen identifies host genes important for influenza virus replication

Hao L, Sakurai A, Watanabe T, Sorensen E, Nidom CA, Newton MA, Ahlquist P, Kawaoka Y.

All viruses rely on host cell proteins and their associated mechanisms to complete the viral life cycle. Identifying the host molecules that participate in each step of virus replication could provide valuable new targets for antiviral therapy, but this goal may take several decades to achieve with conventional forward genetic screening methods and mammalian cell cultures. Here we describe a novel genome-wide RNA interference (RNAi) screen in Drosophila1 that can be used to identify host genes important for influenza virus replication. After modifying influenza virus to allow infection of Drosophila cells and detection of influenza virus gene expression, we tested an RNAi library against 13,071 genes (90% of the Drosophila genome), identifying over 100 for which suppression in Drosophila cells significantly inhibited or stimulated reporter gene (Renilla luciferase) expression from an influenza-virus-derived vector. The relevance of these findings to influenza virus infection of mammalian cells is illustrated for a subset of the Drosophila genes identified; that is, for three implicated Drosophila genes, the corresponding human homologues ATP6V0D1, COX6 A1 and NXF1 are shown to have key functions in the replication of H5N1 and H1N1 influenza A viruses, but not vesicular stomatitis virus or vaccinia virus, in human HEK 293 cells. Thus, we have demonstrated the feasibility of using genome-wide RNAi screens in Drosophila to identify previously unrecognized host proteins that are required for influenza virus replication. This could accelerate the development of new classes of antiviral drugs for chemoprophylaxis and treatment, which are urgently needed given the obstacles to rapid development of an effective vaccine against pandemic influenza and the probable emergence of strains resistant to available drugs.

7. Cross-clade protective immunity of H5N1 influenza vaccines in a mouse model

Murakami S, Iwasa A, Iwatsuki-Horimoto K, Ito M, Kiso M, Kida H, Takada A, Nidom CA, Le QM, Yamada S, Imai H, Sakai-Tagawa Y, Kawaoka Y, Horimoto T.

H5N1 highly pathogenic avian influenza viruses evolved into several clades, leading to appreciably distinct antigenicities of their hemagglutinins. As such, candidate H5N1 prepandemic vaccines for human use should be sought. Here, to evaluate fundamental immunogenic variations between H5N1 vaccines, we prepared four inactivated H5N1 test vaccines from different phylogenetic clades (clade 1, 2.1, 2.2, and 2.3.4) in accordance with the WHO recommendation, and tested their cross-clade immunity in a mouse model by vaccination followed by challenge with heterologous virulent viruses. All H5N1 vaccines tested provided full

or partial cross-clade protective immunity, except one clade 2.2-based vaccine, which did not protect mice from clade 2.3.4 virus challenge. Among the test vaccines, a clade 2.1-based vaccine possessed the broadest-spectrum cross-immunity. These results suggest that currently stockpiled pre-pandemic vaccines, especially clade 2.1-based vaccines, will likely be useful as backup vaccines in a pandemic situation, even one involving antigenic-drifted viruses.

8. Local not systemic modulation of dendritic cell S1P receptors in lung blunts virus-specific immune responses to influenza

Marsolais D, Hahm B, Edelmann KH, Walsh KB, Guerrero M, Hatta Y, Kawaoka Y, Roberts E, Oldstone MB, Rosen H.

The mechanism by which locally delivered sphingosine analogs regulate host response to localized viral infection has never been addressed. In this report, we show that intratracheal delivery of the chiral sphingosine analog (*R*) -2-amino-4- (4-heptyloxyphenyl) -2-methylbutanol (AAL-R) or its phosphate ester inhibits the T-cell response to influenza virus infection. In contrast, neither intraperitoneal delivery of AAL-R nor intratracheal instillation of the nonphosphorylatable stereoisomer AAL-S suppressed virus-specific T-cell response, indicating that in vivo phosphorylation of AAL-R and sphingosine 1-phosphate (S1P) receptor modulation in lungs is essential for immunomodulation. Intratracheal delivery of water-soluble S1P1 receptor agonist at doses sufficient to induce systemic lymphopenia did not inhibit virus-specific T-cell response, indicating that S1P1 is not involved in the immunosuppressive activities of AAL-R and that immunosuppression acts independently of naive lymphocyte recirculation. Accumulation of dendritic cells (DCs) in draining lymph nodes was inhibited by intratracheal but not intraperitoneal delivery of AAL-R. Direct modulation of DCs is demonstrated by the impaired ability of virus-infected bone marrow-derived DCs treated in vitro with AAL-R to trigger in vivo T-cell response after adoptive transfer to the airways. Thus, our results suggest that locally delivered sphingosine analogs induce immunosuppression by modulating S1P receptors other than S1P1 or S1P2 on dendritic cells in the lungs after influenza virus infection.

9. Compatibility among polymerase subunit proteins is a restricting factor in reassortment between equine H7N7 and human H3 N2 influenza viruses

Li C, Hatta M, Watanabe S, Neumann G, Kawaoka Y.

Reassortment is an important driving force for influenza virus evolution, and a better understanding of the factors that affect this process could improve our ability to respond to future influenza pandemics and epidemics. To identify factors that restrict the generation of reassortant viruses, we cotransfected human embryonic kidney cells with plasmids for the synthesis of viral RNAs of both A/equine/Prague/1/56 (Prague; H7N7) and A/Yokohama/2017/03 (Yokohama; H3N2) viruses together with the supporting protein expression plasmids. Of the possible 256 genotypes, we identified 29 genotypes in 120 randomly plaque-picked reassortants examined. Analyses of these reassortants suggested that the formation of functional ribonucleoprotein (RNP) complexes was a restricting factor, a finding that correlated with the activities of RNP complexes composed of different combinations of the proteins from the two viruses, as measured in a minigenome assay. For at least one nonfunctional RNP complex (i.e., Prague PB2, Prague PB1, Yokohama PA, and Prague NP), the lack of activity was due to the inability of the three polymerase subunit proteins to form a heterotrimer. Adaptation of viruses possessing a gene encoding a chimera of the PA proteins of the two viruses and the remaining genes from Prague virus resulted in compensatory mutations in the PB2 and/or PA protein. These results indicate substantial incompatibility among the gene products of the two test viruses, a critical role for the RNP complex in the generation of reassortant viruses, and a functional interaction of PB2 and PA.

10. Establishment of Canine RNA Polymerase I-Driven Reverse Genetics for Influenza A Virus: Its Application for H5N1 Vaccine Production

Murakami S, Horimoto T, Yamada S, Kakugawa S, Goto H, Kawaoka Y.

In the event of a new influenza pandemic, vaccines whose antigenicities match those of circulating strains must be rapidly produced. Here, we established an alternative reverse genetics system for influenza virus using the canine polymerase I (PolI) promoter sequence that works efficiently in the Madin-Darby canine kidney cell line, a cell line approved for human vaccine production. Using this system, we were able to generate H5N1 vaccine seed viruses more efficiently than can be achieved with the current system that uses the human PolI promoter in

African green monkey Vero cells, thus improving pandemic vaccine production.

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Immune cells express multiple Toll-like receptors (TLRs) that are concomitantly activated by a variety of pathogen products during microbial and viral infection. There is presumably a need to coordinate the expression function of TLRs in individual cells. Recent reports also have indicated that the balance of TLRs responses has an important role in inducing autoimmune diseases. Our research main focuses on molecular regulatory mechanism to coordinate pathogen recognition by TLRs.

1. A single base mutation in the PRAT4A gene reveals differential interaction of PRAT4A with Toll-like receptors

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Toll-like receptors (TLRs) play an essential role in defense responses. Immune cells express multiple TLRs which are simultaneously activated by microbial pathogens. PRAT4A (PRotein Associated with Tlr4 A) is a chaperone-like endoplasmic reticulum (ER)-resident protein required for the proper subcellular distribution of multiple TLRs. PRAT4A$^{-/-}$ mice show impaired expression of TLR2/4 on the cell surface and the lack of ligand-induced TLR9 relocation from the ER to endolysosome. Consequently, TLR responses to whole bacteria as well as to TLR2, 4, and 9 ligands are impaired. We here compare the interaction of these TLRs with PRAT4A. Association of endogenous PRAT4A was easily detected only with TLR4. The TLR4 region responsible for strong interaction with PRAT4A is very close to the site necessary for interaction with MD-2. By using transient expression, we were able to detect PRAT4A interaction with TLR2 and TLR9. The PRAT4A single-nucleotide mutant replacing methionine 145 with lysine (M145 K) associates with TLR9 but does not rescue ligand-dependent TLR9 trafficking. By contrast, the M145K mutant weakly, if at all, associates with TLR2 and TLR4. The M145K mutant appreciably rescues cell surface TLR2 expression and its responses in PRAT4A$^{-/-}$ bone marrow-derived dendritic cells, whereas little if any rescue of cell surface TLR4/MD-2 expression and its responses occurs. These results demonstrate that PRAT4A differentially interacts with each TLR, and suggest that a single-nucleotide change in the PRAT4A gene influences not only the strength of TLR responses but can also alter the relative activity of each TLR.

2. Detectin of soluble PRAT4A (PRotein Associated with Toll-like receptor 4) by novel anti-PRAT4A monoclonal antibodies

Takuma Shibata, Sachiko Akashi-Takamura, Richard Jennings, and Kensuke Miyake

Immunocompetent cells express multiple Toll-like receptors (TLRs) which play sentinel roles in detecting microbial products. Orchestrated TLR activation induces appropriate immune responses. It is known that TLR associating molecules, such as MD-2, CD36, UNC93B1, HMGB-1, gp96, play essential role in ligand recognition by TLRs. It is important to clarify how the TLR accessory molecules function in immune cells to correctly understand TLR-dependent immune responses.

We have previously reported that a novel protein 'PRotein Associated with TLR4 (PRAT4 A)' is required for multiple TLR responses by modulating TLR trafficking. And mRNA level of PRAT4A significantly decreased after various TLR stimulation. These results suggest that PRAT4A is essential for controlling multiple TLR responses. Furthermore, we have recognized by immunoprecipitation assay that Ba/F3 cells over-expressing PRAT4A secreted PRAT4A in the culture supernatant without any stimulation.

To delineate the undetected function of endogenous PRAT4A in immune cells, we have established several monoclonal anti-PRAT4A antibodies and studied about the dynamics of endogenous PRAT4A. We could detect secreted endogenous PRAT4A by immunoprecipitation assay in the normal mice serum and the culture supernatant of Bone Marrow derived Macrophages (BMMfs), but not of BM derived Dendritic cells (BMDCs).

In addition, we found by FACS analysis that PRAT4A was detectable on the cell surface of BMMfs, but not of BMDCs, after various TLR ligand stimulation, especially TLR4 and TLR9 ligand stimulation.

There is possibility that PRAT4A is not only a TLR associating molecule but also a modulator of immune responses induced by TLR activation. Further study is under way to reveal a role of soluble PRAT4A in immune responses.

3. Soluble MD-1 detection using novel monoclonal antibodies

Richard Jennings, Sachiko Akashi-Takamura, Shibata Takuma, Toshihiko Kobayashi, and Kensuke Miyake

In the absence of MD-1, cells are unable to express RP105 on their surface, and thusly RP105 loses function. Current data indicates that RP105/MD-1 has an inhibitory role in myeloid derived cells but not in B cells, where results have shown RP105/MD-1 to positively regulate TLR stimulation. This apparent functional dichotomy between cell types is, as of yet, unexplained. There is the possibility that this dichotomy is explained not through the complex itself, but either through associated molecules, or distinctive functions of RP105 and MD-1, outside of the RP105/MD-1 complex. Previous experimentation has been unable to distinguish the functions of MD-1 and RP105 as individual molecules. In this study, through the production of a novel series of antibody clones (JR clones), we are able to show, for the very first time, that under physiological conditions soluble MD-1 exists extra-cellularly.

MD-1KO BALB/c mice were immunized with Ba/F3 cells stably transfected to express myc-tagged murine MD-1 on their surface. Initial screening utilized the immunogen cell line. Lateer screening utilized splenic cells harvested from B6 background wild type, MD-1KO, and RP105KO mice. 7 antibody clones were obtained, these antibodies all show the ability to recognize MD-1 on the surface of the immunogen cell line, and vary in their ability to recognize cell surface MD-1 on splenic cells. Selective clones show mitogenic properties, although co-stimulation with RP14 results in the blunting of this mitogenic property. Using these antibodies, MD-1 was successfully immuno-precipitated from cell lysates, RAW cell supernatant, BALB/c WT serum, and BALB/c RP105KO serum. This clearly shows that MD-1 exists extra-cellularly, and that this property of MD-1 is not dependent on RP105 expression. Further experiment on extra-cellular MD-1 may lead to a more exhaustive understanding of it's immunobiology.

4. Involvement of mast cells and mast cell-derived TNF in the pathogenic mechanisms of superantigen-mediated diseases

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Roles of mast cells and mast cell-derived cytokines in induction of a lethal response in mice injected with a combined injection of staphylococcal enterotoxin A (SEA) and D-Galactosamine (GalN) were examined to further understand the pathogenic mechanisms of the superantigen-mediated diseases. All of mast cell-deficient W/W^v mice were survived upon injection of SEA and GalN, whereas the control littermate $+/+$ mice were died with high serum TNF level and degranulation of mast cells upon the lethal challenge. The results suggested that mast cell and mast cell-derived TNF were critically important in the induction of the SEA-induced lethal response. This was confirmed by reconstitution experiments with bone marrow-derived mast cells (BMMCs). W/W^v mice reconstituted with BMMCs of $+/+$ mice were recovered the susceptibility to the lethal challenge and died. However, W/W^v mice reconstituted with BMMCs of $TNF^{-/-}$ mice were not recovered the susceptibility to the lethal challenge and survived. In vitro studies showed that increased TNF level was observed when BMMCs from $+/+$ or $IFN^{-/-}$ but not $TNF^{-/-}$ were cultured with Antigen Presenting Cell and $CD4^+$ T cells. These findings propose a novel viewpoint that mast cells and mast cells-derived TNF play important roles in the pathogenic mechanism of the superantigen-mediated diseases.

5. Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling

Natsuko Tanimura, Shinichiroh Saitoh, Fumi Matsumoto, Sachiko Akashi-Takamura, and Kensuke Miyake

Toll-like receptor 4 (TLR4) activates two distinct signaling pathways including production of proinflammatory cytokines or type I interferons (IFNs), respectively. Two adaptor molecules, MyD88 and TIRAP/Mal, are essential for the former but not for the latter signaling pathway, which instead requires two adaptors, TRIF/TICAM-1 and TRAM/TICAM-2. TIRAP is a sorting adaptor molecule recruiting MyD88 to activated TLR4 in the plasma membrane. TRAM is thought to serve as a signaling molecule between TLR4 and TRIF by associating with these molecules. Little is known, however, how TRAM interacts with TLR4 or with TRIF during LPS responses. Here we show that LPS induces upregulation of TLR4-association with TRAM, TRIF recruitment to the plasma membrane, and their subsequent internalization into endosome/lysosome. The internalized signaling complex consisting of TLR4 and TRAM co-localizes with TRAF3, a signaling molecule downstream of TRIF, in endosome/lysosome. These results suggest that TLR4 activates TRIF-signaling pathway in endosome/lysosome.

6. Unc93B1 biases Toll-like receptor-mediated responses in dendritic cells towards DNA- and against RNA-sensing

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Toll-like receptors (TLR) 3, 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. Remarkably, TLR7 and TLR9 appear to have mutually opposing, pathogenic or protective, impacts on lupus nephritis in MRL/lpr mice. Little is known, however, about a contrasting relationship between TLR7 and TLR9. We here show that TLR7 and TLR9 are inversely linked by Unc93B1, a multiple membrane-spanning ER protein. Complementation cloning with a TLR7-unresponsive, but TLR9-responsive cell line revealed that D34 in Unc93B1 repressed TLR7-mediated responses. D34A mutation rendered Unc93B1-deficient DCs hyperresponsive to TLR7 ligand but hyporesponsive to TLR9 ligand with TLR3 responses unaltered. Unc93B1 associates with and delivers TLR7/9 from the ER to endolysosomes. D34A mutation biased Unc93B1 association against TLR9 and towards TLR7. Taken together, Toll-like receptor response to DNA and RNA in DCs is biased towards DNA-sensing by Unc93B1.

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The mucosal immune system not only plays an important role as the first line of immunological defense for preventing the host from invasion of 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. M Cell Biology

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Membranous or microfold cells (M cells) located in the follicle-associated epithelium (FAE) of Peyer's patches (PPs) play a pivotal role in up-taking the luminal antigens for initiation of antigen-specific immune responses in both systemic and mucosal compartments. We recently established the purification strategy of isolated M cells from PPs with our previously developed M cell-specific monoclonal antibody (NKM 16-2-4) and subsequently performed DNA microarray using NKM 16-2-4^{+} M cells to identify the responsible genes which involve in antigen-sampling and cell differentiation. As results, 1329 genes were raised up as M cell-specific gene candidates and our subsequent *in situ* hybridization analysis clearly showed that glycoprotein 2 (GP2) and MARCS-like protein (MLP) mRNAs were specifically expressed by PP M cells. In addition, immunohistochemical analysis with newly established GP2-specific monoclonal antibody (10F5-9-2) and MLP-specific polyclonal antibody also confirmed the specific expression in M cells at protein level. Since our current effort is aimed to elucidate the biological role of two M cell-specific molecules, the genome-wide assessment of gene expression would facilitate to understand the M cell immunobiology.

2. Mucosal vaccine development

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Because needle-free vaccine delivery has a potential to lead to significant advances, development of the vaccine is one of the defined as "grand challenges in global health". In order to develop effective and preventive needle-free vaccine, we chose a recombinant form of nontoxic fragment of C-terminal half of heavy chain (Hc) of botulinum neurotoxin type A (BoNT/A) and tested the potential as vaccine against botulism on two mucosal administration routes in cynomologus macaques. Nasal immunization of 0.5mg Hc without adjuvant induced brisk toxin-specific antibody immune responses with neutralizing activity in both systemic and mucosal compartment. In contrast, sublingual immunization of 1.0mg Hc without adjuvant induced certain toxin-specific systemic antibody with weak neutralizing activity and no mucosal immune responses. Furthermore, the nasal immunization with Hc protected these macaques from challenging with BoNT/A of 35,000 fold of lethal amount of monkey, without any clinical signs of disease for 30 days. The results show that nasal immunization with Hc is an attractive strategy for the protection of botulism, which is naturally occurred or stemmed from acts of bioterrorism.

3. Rice-based vaccine: MucoRice™

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Rice-expressed cholera toxin B subunit (MucoRice™-CTB) was previously constructed and proved to be an effective cold-chain free oral vaccine for the induction of enterotoxin neutralizing immunity. Our further investigations revealed that oral MucoRice™-CTB immunization protected against live *Vibrio cholera*- and Enterotoxigenic *Escherichia coli*-induced diarrhea in mice. This finding indicated that MucoRice™-CTB could use as a cold-chain free vaccine against cholera as well as travellers' diarrhea in humans. We also created a second generation of rice-based vaccine expressing nontoxic double mutant cholera toxin (dmCT). Oral immunization of MucoRice™-dmCT induced CTB-specific serum IgG and mucosal IgA antibodies with neutralizing activity but not any CTA-specific serum and mucosal antibodies. Although the potency of MucoRice-dmCT as a CT vaccine was almost equal to that of MucoRice-CTB, these results suggest that the MucoRice system has potential for the development of multicomponent vaccines.

4. Marine Vaccine Project

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Because many of fisheries stocks have declined in the worldwide, fish farming, especially shrimp and salmon farming has boomed. Thus, marine industry needs vaccines for these fishes to protect against infectious diseases instead of antibiotics due to food safety and ecological concerns from consumers. Based on a collaboration with Tokyo University of Marine Science and Technology, we started a marine project for the development of fish vaccines. In this study, we found that *in vivo* immunostimulatory effects of a recombinant Atlantic salmon (*Salmo salar*) interferon-α (rIFN-α). A minimal injection dose of 0.1 μg of rIFN-α per g fish significantly protected trout against a lethal dose of infectious haematopoietc necrosis virus (IHNV) and showed that a salmonid rIFN can modulate the innate immune response of salmon and mediate early antiviral protection from many of viruses' threat. We also showed that a potential oral vaccine against white spot syndrome virus (WSSV), a major threat to shrimp culture worldwide, to assess the efficacy of the oral administration of recombinant VP28, an envelope protein of WSSV, in providing protection in shrimp, *Penaeus japonicus* upon challenge with WSSV.

5. Mucosal Trafficking

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A lipid mediator, sphingosine 1-phosphate (S1 P), is known to regulate lymphocyte trafficking at systemic immune compartments (*e.g.*, thymus) and we have recently reported that unique gut-associated immunocompetent cells such as intraepithelial T lymphocytes and pathogenic T and mast cells causing food allergy also utilize S1P for their trafficking. In this study, we have identified the role of S1P in the regulation of innate and acquired gut IgA production. In Peyer's patches, major sites for acquired-type IgA production pathway, B cells change the S1P receptor expression during their differentiation to IgA^+ plasmablasts, which enable them to determine whether they stay in or emigrate out from Peyer's patches. Thus, inhibition of S1P-mediated pathway by FTY720, an $S1P_1$ modulator, resulted in the selective accumulation of S1P $receptor^+$ IgA^+ plasmablasts in the Peyer's patches and consequent impairment of intestinal IgA responses against orally immunized protein antigen. We also found that S1P played a key role in the innate intestinal IgA responses by showing that peritoneal B cells used S1P in their trafficking into the intestine. In this pathway, NF-κB-inducing kinase (NIK) expressed in peritoneal stromal cells is essential. Thus, although peritoneal B cells from NIK-mutated alymphoplasia (*aly*) mice expressed substantial levels of S1P receptor and normally migrated toward S 1P *in vitro* and *in vivo, aly* mice showed decreased sensitivity to FTY720. We found that NIK-mutated stromal cells showed aberrant expression of VCAM-1, ICAM-1, and CXCL13, leading to the impaired ability to support S1P-mediated peritoneal B cell emigration in *aly* mice. Thus, transfer of wild-type stromal cells into the peritoneum restored S1P-mediated trafficking of *aly* peritoneal B cells. We are now extending these studies by investigating the involvement of gut environmental factors (e.g., dietary materials and commensal microbiota) in the regulation of gut immune system because these factors affect the generation of lipid mediators.

6. Food Allergy

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To establish novel and effective strategies to regulate intestinal allergic diseases, we have elucidated molecular and cellular mechanisms of the development of food allergy using ovalbumin-specific food allergy murine model. In this year, we have revealed the involvement of regulatory-type T cells and a lipid mediator in the development of food allergy. In the former case, we found that Peyer's patches (PPs) possessed regulatory function in allergic diarrhea by showing that mice lacking PPs were more susceptible to the allergy development. Additional analyses revealed that PPs contained IL-10 producing regulatory $CD4^+CD25^+$ $Foxp3^+$ T cells (Treg) and inhibited the development of allergic diarrhea. Indeed, the treatment with anti-CD25 or anti-IL-10 antibody resulted in the rapid and severe development of allergic diarrhea. These studies demonstrate that PP is the site to induce IL-10-producing Treg cells for the control of intestinal allergic responses. In the latter study, we showed that sphingosine 1-phosphate (S1P), a lipid mediator, played important roles in the development of food allergy by regulating the trafficking of pathogenic T and mast cells. When mice were treated with FTY720, a S1P receptor modulator, the incidence of allergic diarrhea was markedly inhibited. In those mice, the number of mast cells and activated $CD4^+$ Th2 cells was decreased in the intestinal compartment. These findings suggest that S1P-mediated trafficking of mast cells and activated T cells could be an effective target for the prevention and treatment of food allergy. Based of these findings, we are now trying to elucidate the detailed molecular and cellular mechanisms of induction, activation, and trafficking of allergen-specific $CD4^+$ Th2 cells, mucosal mast cell and Treg cells to develop the immunotherapy against intestinal allergic diseases.

7. Non-canonical second lymphoid organogenesis

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Mucosa-associated lymphoid tissue plays a key role in the regulation of mucosal immune system. Nasopharynx-associated lymphoid tissue (NALT) and Peyer's patch (PP) develop at respiratory tract and intestine, respectively. We have recently identified that tear duct-associated lymphoid tissue (TALT) developed at murine lacrimal sac. TALT was shown to take up ocular antigens and generated antigen-specific T cell response as well as germinal center reaction and immunoglobulin-class switch recombination. $CD3^-CD4^+CD45^+$ lymphoid tissue inducer cells (LTi) initiate developmental program of lymphoid organs. Transcriptional regulators, inhibitor of DNA binding/differentiation (Id)2 and retinoic acid receptor-related orphan receptor (ROR)γt regulate the differentiation of LTi. We could reveal the presence of three distinct LTi subsets for mucosa-associated lymphoid tissue organogenesis. Based upon the expression level of CD4 and the requirement of Id2 and RORγt, PP inducer was determined as Id2- and RORγt-dependent $CD3^-CD4^{high}CD45^+$ cells, whereas NALT inducer was Id2-dependent, RORγt-independent $CD3^-CD4^{low}CD45^+$ cells. Newly identified TALT was induced by Id2- and RORγt-independent $CD3^-CD4^{low}CD45^+$ cells. Collectively, organogenesis of mucosa-associated lymphoid tissues (e.g., TALT, NALT, PP) is initiated by distinct inducer population.

8. Molecular and Cellular Analysis of Host-Microflora Interaction

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Our mucosa acts directly as an immunological interface between the external environment and the host. The surface area of the mucosa is over 200 times larger than that of the skin. Of these mucosal areas, the intestine is most frequently exposed to a huge number and a wide variety of environmental antigens including bacteria and food products. It has been demonstrated that gut microbiota are ingested into Peyer's patches (PPs) and induce host immune responses in the experiment with culurable *Enterobacter cloacae.* However, the behavior of intestinal bacteria is still well understood because more than 90% of the intestinal microbes have not been cultured. In this study, we revealed the bacterial composition in the various intestinal compartments by using 16S rRNA gene clone library analysis. For example, *Lactobacillius* and segmented filamentous bacteria (SFB) were dominated at the surface of PPs. In contrast, some kinds of opportunistic bacteria were predominantly existed inside PPs.

We also revealed that gut microflora in the ileal villous parts consists of unique bacterial population and is involved in the induction of intestinal epithelial fucosylation, which has shown to be utilized by some kinds of commensal bacteria as a nutrient. Germ-free (GF) mice had low numbers of fucosylated epithelial cells (F-ECs) compared with conventional mice and the F-ECs were restored when GF mice were conventionalized. These epithelial fucosylation is regulated by fucosyltransferase 2 (Fut2) induced by commensal bacteria. We also found *Fut2*-deficient mice, which are defective in F-ECs, have aberrant intestinal bacterial population in the ileum, in particular, the frequency of *Lactobacillus* decreased. These results suggest a novel symbiotic relationship between host and gut microbiota. We are now trying to reveal the contribution of site-specific indigenous gut microbiota to the host mucosal immune system.

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