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 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. The *Shigella flexneri* effector OspI 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. A Tecpr1-dependent selective autophagy pathway targets bacterial pathogens.

Ogawa M, Yoshikawa Y, Kobayashi T, Mimuro H, Fukumatsu M, Kiga K, Piao Z, Ashida H, Yoshida M[1], Kakuta S[2], Koyama T[3],

Goto Y[4], Nagatake T[4], Nagai S[3], Kiyono H[4], Kawalec M[5], Reichhart JM[5], and Sasakawa, C.: [1]Division of Ultrastructural Research, BioMedical Research Center, Graduate School of Medicine, Juntendo University, Tokyo, Japan, [2]Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [3]Nippon Institute for Biological Science, Ome, Tokyo, Japan, [4]Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [5]Institute de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique Unité Propre de Recherche, Université de Strasbourg, Strasbourg Cedex, France.

Selective autophagy of bacterial pathogens represents a host innate immune mechanism. Selective autophagy has been characterized on the basis of distinct cargo receptors but the mechanisms by which different cargo receptors are targeted for autophagic degradation remain unclear. We identified a highly conserved Tectonin domain-containing protein, Tecpr1, as an Atg5 binding partner that colocalized with Atg5 at *Shigella*-containing phagophores. Tecpr1 activity is necessary for efficient autophagic targeting of bacteria, but has no effect on rapamycin- or starvation-induced canonical autophagy. Tecpr1 interacts with WIPI-2, a yeast Atg18 homolog and PI(3)P-interacting protein required for phagophore formation, and they colocalize to phagophores. Although Tecpr1-deficient mice appear normal, Tecpr1-deficient MEFs were defective for selective autophagy and supported increased intracellular multiplication of *Shigella*. Further, depolarized mitochondria and misfolded protein aggregates accumulated in the Tecpr1-knockout MEFs. Thus, we identify a Tecpr1-dependent pathway as important in targeting bacterial pathogens for selective autophagy.

3. *Shigella* Targets Epithelial Tricellular Junctions to Spread Between Cells via a Noncanonical Clathrin-dependent Endocytic Pathway

Fukumatsu M, Ogawa M, Arakawa S[1], Suzuki M, Furuse M[2], Nakayama K[3], Shimizu S[1], Kim M, Mimuro H, and Sasakawa C.: [1]Department of Pathological Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan, [2]Division of Cell Biology, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe, Japan, [3]Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.

Intercellular bacterial movement is a sequential pseudopodium-mediated process. Although bacterial and host factors are likely involved, the mechanisms underlying intercellular trafficking of bacteria-containing pseudopodia remain unclear. We found that *Shigella*-containing pseudopodia target epithelial tricellular junctions during cell-cell movement. These pseudopodia were engulfed by neighboring cells only in the presence of tricellulin, an essential protein for tricellular junction integrity. *Shigella* cell-cell spreading, 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. Knocking down tricellulin, Epsin-1, clathrin, or Dynamin-2 expression reduced *Shigella* cell-cell spreading, whereas AP-2, Dab2, and Eps15 were not critical for this process. Our findings highlight an important mechanism for disseminating *Shigella* into neighboring cells via tricellular junctions and a noncanonical clathrin-dependent endocytic pathway.

Publications

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Department of Microbiology and Immunology

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

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

The SWI/SNF chromatin remodeling complex plays important roles in the epigenetic regulation of many organisms and regulates a wide variety of genes. In mammals, this complex is an assembly of about nine polypeptides, and each complex contains a single molecule of either Brm or BRG1, but not both. These two proteins are the catalytic subunits and drive the remodeling of nucleosomes via their ATP-dependent helicase activity. Evidence has now accumulated that Brm and BRG1 regulate a set of target promoters that is not fully overlapping. Indeed, Brm and BRG1 show clear differences in their biological activities; Brm, but not BRG1, is essential for the maintenance of gene expression driven by LTRs of murine leukemia virus (MLV) and HIV-1 as we have previously reported.

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

cally 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. Among various NFκB-activating signals, two different NFκB pathways have been well studied. One is the canonical NFκB pathway, which is activated by TNFα and IL-1 stimulation. Upon their stimuli, proteosomal degradation of cytosolic IκBα allows nuclear translocation of RelA/p50 dimer, which induces expression of *IL-8* and *IκBα*. The other is the non-canonical NFκB pathway, which is activated by lymphotoxin and CD40 ligand. Upon their treatment, activated NIK and IKKα induce the processing of p100 to p52, which triggers gene activation of *BLC* and *ELC* by RelB/p52 dimer. Aberrant regulation of NFκB is known to be involved with cancer development and progression. Importantly, some NFκB target genes stimulated by these cytokines or growth factors have been 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.

Last year, 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. This year, considering these results of ours, 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. We show in our current analysis that each of these proteins can enhance the different NF-κB heterodimers to transactivate their targets efficiently when both they and the NF-κB components are exogenously expressed. We further show from our analysis that among these five proteins, DPF3a and DPF3b are the most effective cofactors for RelA/p50 activation in 293FT cells treated with TNF-α. Our current data further indicate that these two proteins directly bind to RelA, p50 and at least four subunits of the SWI/SNF complex in vitro. We additionally show that endogenous DPF3a/b and the SWI/SNF complex are continuously co-localized at the HIV-1 LTR throughout the period of TNF-α stimulation and that RelA/p50 is promptly recruited to the typical NF-κB binding sites within the HIV-1 LTR or endogenous IL-6 promoter upon stimulation.

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

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

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

This year, we demonstrate that *Brm* mRNA is a target of miR-199a-5p and miR-199a-3p, both of which are processed from pre-miR-199a. By promoter analysis of the *miR-199a-2* gene, which was found to be the main contributor to the production of pri-miR-199a in these cell lines, Egr1 was identified as a major transcriptional activator at this gene locus. We further show from our analysis that the expression patterns of mature miR-199a-5p and -3p, and of the Brm protein, are mutually exclusive in many human cancer cell lines as well as in some human tumor cells. We finally present evidences that a double-negative feedback mechanism underlies this regulatory network and separates many cancer cell lines into two distinct groups. In 2008-2009, we also observed that miR-21 and its targets, PDCD4 and NFIB form also robust double-negative feedback loops and indicated that miR-21 RNA (highly expressed in cancer lesion) and PDCD4 protein (highly expressed in normal mucosa) show mutually exclusive expression patterns in human colorectual cancer specimen. So we believe miRNAs can generally function as molecular switches for epigenetical regulation by utilizing double-negative feedback regulation.

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

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The SWI/SNF chromatin remodeling complex is an important epigenetic regulator in human cells and contains a single molecule of either Brm or BRG1, the functions of which are not fully overlapping, as the catalytic subunit. This complex can regulate the expression of many genes either positively or negatively depending on the promoter to which it is recruited. We have previously shown that the loss of Brm expression, which is often observed in human tumor cells and contributes to their oncogenic potential, is caused by post-transcriptional suppression. We show here that the 3′UTR of Brm mRNA has two sites that are efficiently targeted by miR-199a-5p and -3p, respectively, thus revealing the modulation of Brm-type SWI/SNF complex activity through these miRNAs. Previously, we computationally mapped the putative promoter region of miR-199a-2 (miPPR-199a-2), the major contributing gene loci for miR-199a-5p and-3p production in these tumor cell lines. We here validate this predicted region by direct promoter analysis and find that Egr1 is a strong positive regulator of the miR-199a-2 gene. Importantly, we also show that Egr1, miR-199a-5p and -3p are expressed at high levels in Brm-deficient tumor cell lines, but only marginally in Brm-expressing tumor cells. Furthermore, we provide evidence that Brm negatively regulates Egr1. These results reveal that miR-199a and Brm form a double-negative feedback loop through Egr1, leading to the generation of these two distinct cell types during carcinogenesis. This may partially explain why miR-199a-5p and -3p have been reported to be either up-regulated or down-regulated in a variety of tumors.

3. Development of decoy RNAs that strongly inhibit specific miRNA function.

The development of reagents that strongly suppress specific miRNAs will be important for both basic miRNA research and also as a possi-

ble therapeutic strategy. Most of the synthetic low-molecular weight inhibitors of specific miRNAs are usually based on anti-miRNA antisense oligonucleotides (AMO), where some or all of the ribonucleotides are modified to 2′-O-methylated RNA, locked nucleic acids (LNA)/DNA or 2′-methoxyethylated RNA to provide resistance to cellular nucleases and to increase affinity towards complementary miRNA sequences. However, since these reagents will be diluted by successive cell divisions, and in some cases metabolized in the cytoplasm, their effects are transient in many cases. To achieve the long-term suppression of a specific miRNA, specialized plasmid- and virus- vectors carrying expression units for inhibitory RNA molecules have also been developed. These inhibitory RNA molecules include "antagomir", "eraser" and "sponge". In 2009, we also reported on such plasmid- or lentivirus-based vectors expressing inhibitory RNA targeting specific miRNAs, 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.

Retro/lnetivirus vectors carrying TuD, however, have a potential disadvantage in therapeutic application, because it requires human gene therapy. Therefore we have wished to develop low-molecular weight reagents that can retain miRNA inhibitory activity even after several rounds of cell division by mimicking structural features of TuD RNA. This year, 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 miR-21, -200c, -16 and -106b retain potent inhibitory effects even when transfected at a low dose range of 1nM-30pM. We further show that a single transfection of S-TuD-miR200c with the appropriate MBSs can induce partial epithelial-mesenchymal transition, indicating that the inhibitory effects of S-TuD can tolerate dilution by several rounds of cell division.

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

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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.

Publications

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Division of Virology
ウイルス感染分野

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Viruses can cause devastating diseases. The long-term goal of our research is to understand the molecular pathogenesis of viral diseases, 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. Avian-type receptor-binding ability can increase influenza virus pathogenicity in macaques.

Watanabe T, Shinya K, Watanabe S, Imai M, Hatta M, Li C, Wolter BF, Neumann G, Hanson A, Ozawa M, Yamada S, Imai H, Sakabe S, Takano R, Iwatsuki-Horimoto K, Kiso M, Ito M, Fukuyama S, Kawakami E, Gorai T, Simmons HA, Schenkman D, Brunner K, Capuano SV 3rd, Weinfurter JT, Nishio W, Maniwa Y, Igarashi T, Makino A, Travanty EA, Wang J, Kilander A, Dudman SG, Suresh M, Mason RJ, Hungnes O, Friedrich TC, Kawaoka Y.

The first influenza pandemic of the 21st century was caused by novel H1N1 viruses that emerged in early 2009. An Asp-to-Gly change at position 222 of the receptor-binding protein hemagglutinin (HA) correlates with more-severe infections in humans. The amino acid at position 222 of HA contributes to receptor-binding specificity with Asp (typically found in human influenza viruses) and Gly (typically found in avian and classic H1N1 swine influenza viruses), conferring binding to human- and avian-type receptors, respectively. Here, we asked whether binding to avian-type receptors enhances influenza virus pathogenicity. We tested two 2009 pandemic H1N1 viruses possessing HA-222G (isolated from severe cases) and two viruses that possessed HA-222D. In glycan arrays, viruses possessing HA-222D preferentially bound to human-type receptors, while those encoding HA-222G bound to both avian- and human-type receptors. This difference in receptor binding correlated with efficient infection of viruses possessing HA-222G, compared to those possessing HA-222D, in human lung tissue, including alveolar type II pneumocytes, which express avian-type receptors. In a nonhuman primate model, infection with one of the viruses possessing HA-222G caused lung damage more severe than did infection with a virus encoding HA-222 D, although these pathological differences were

not observed for the other virus pair with either HA-222G or HA-222D. These data demonstrate that the acquisition of avian-type receptor-binding specificity may result in more-efficient infection of human alveolar type II pneumocytes and thus more-severe lung damage. Collectively, these findings suggest a new mechanism by which influenza viruses may become more pathogenic in mammals, including humans.

2. Host regulatory network response to infection with highly pathogenic H5N1 avian influenza virus.

Li C, Bankhead A 3rd, Eisfeld AJ, Hatta Y, Jeng S, Chang JH, Aicher LD, Proll S, Ellis AL, Law GL, Waters KM, Neumann G, Katze MG, McWeeney S, Kawaoka Y.

During the last decade, more than half of humans infected with highly pathogenic avian influenza (HPAI) H5N1 viruses have died, yet virus-induced host signaling has yet to be clearly elucidated. Airway epithelia are known to produce inflammatory mediators that contribute to HPAI H5N1-mediated pathogenicity, but a comprehensive analysis of the host response in this cell type is lacking. Here, we leveraged a system approach to identify and statistically validate signaling subnetworks that define the dynamic transcriptional response of human bronchial epithelial cells after infection with influenza A/Vietnam/1203/2004 (H5N1, VN 1203). Importantly, we validated a subset of transcripts from one subnetwork in both Calu-3 cells and mice. A more detailed examination of two subnetworks involved in the immune response and keratinization processes revealed potential novel mediators of HPAI H5N1 pathogenesis and host response signaling. Finally, we show how these results compare to those for a less virulent strain of influenza virus. Using emergent network properties, we provide fresh insight into the host response to HPAI H5N1 virus infection and identify novel avenues for perturbation studies and potential therapeutic interventions for fatal HPAI H5N1 disease.

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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. TLRs also sense our internal molecules such as RNA and DNA. There is presumably a need to coordinate the expression and function of TLRs in individual cells. Recent reports also have indicated that losing the balance of TLRs responses is a mechanism to cause autoimmune diseases. Our research focuses on molecular regulatory mechanisms to coordinate pathogen and internal ligands recognition by TLRs.

1. MD-1 regulates the production of anti-phospholipid antibody

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LPS (Lipopolysaccharide) sensing is regulated by several molecules. LBP (Lipid Binding Protein) and CD14 binds to LPS, and transfer it to the TLR4/MD-2 receptor. In this case, LBP and CD14 work as an accelerater for LPS response. RP105/MD-1, of which the extracellular structure is similar to TLR4/MD-2, enhances mouse B cell response to LPS. We established anti-mouse MD-1 mAbs, and we could find MD-1 not only on the B cell-surface but also in the serum. We here found that MD-1 is able to bind to cardiolipin. Cardiolipin is a negatively charged phospholipid, and an autoantigen in APS (anti-phospholipid syndrome). Previous paper showed that the production of anti-phospholipid antibody was found in autoimmune-prone mice. To ask a role for MD-1 in autoantibody production against cardiolipin, we generated MD-1-/-B6/lpr mice. We found that MD-1 negatively regulates the production of anti-cardiolipin autoantibodies. We are currently studying molecular mechanism underlying MD-1-dependent negative regulation of anti-cardiolipin autoantibody production.

2. Human TLR4 polymorphisms influences cell surface expression and responses of TLR4

Natsuko Yamakawa, Sachiko Akashi-Takamura, Natsuko Tanimura, and Kensuke Miyake

Human TLR4 polymorphisms have been shown to influence susceptibility to the diseases. The Asp299Gly/Thr399Ile polymorphismis susceptible to infectious diseases such as Gram-

negative infections. On the other hand, this variant is associated with a decreased risk of atherosclerosis. Despite these association studies, little is known about a molecular mechanism how TLR4 polymorphism influences TLR4-signaling.

We here compared wild type TLR4, D299G TLR4, T399I TLR4, and D299G/T399I TLR4 in cell surface expression and their response to a variety of microbial as well as endogenous ligands. Previous reports revealed a discrepancy whether D299G/T399I TLR4 has a problem in cell surface expression. We addressed this issue with two distinct mAb to human TLR4. As reported previously, previous mAb bound to wt TLR4 but poorly to variant TLR4s. In contrast, newly established mAb detected variant TLR4s as much as wt TLR4, demonstsrating that TLR4 polymorophisms do not influence cell surface expression of TLR4. We are currently comparing wt and variant TLR4s in TLR4 response to microbial ligands.

3. Protective role of mast cell-derived TNF and VEGF in murine malaria

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Mast cells reside closely with blood vessels and secrete cytokines such as TNF and VEGF. It has been considered that TNF is a major protective cytokine in malaria. Our clinical study indicated that VEGF levels were increased in malaria patients. In this study, we examined protective role of mast cell-derived TNF and VEGF, and interaction of these cytokines. Mice were infected with *Plasmodium berghei* ANKA and protective activity was evaluated by parasitemia. Decreased protection was induced by injection of anti-TNF or anti-VEGF after infection. Higher parasitemia was found in mast cell-deficient Wsh/Wsh mice with lower TNF and VEGF levels compared with the control. Wsh/Wsh mice reconstituted with cultured mast cells from normal mice recovered protection and these cytokine levels. VEGF levels after infection in $TNF^{-/-}$ or anti-TNFR-treated mice were lower than those in the control. Wsh/Wsh mice reconstituted with cultured mast cells from $TNF^{-/-}$ mice had lower VEGF levels and protection after infection. In addition, mast cell-derived VEGF is likely to induce pathogenic molecule heme oxygenase-1. These results suggest that malarial infection stimulates mast cells to secrete TNF which subsequently induces VEGF, resulting in protection and pathogenesis.

4. Unc93B1 restricts systemic lethal inflammation by orchestrating Toll-like Receptor 7 and 9-trafficking

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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). TLR7 hyper-response is thought as a factor of autoimmune disease and several mice models were reported, thus we thought that this TLR7/TLR9 balance is important for avoiding TLR7 hyper-response.

To investigate the significance of reciprocal TLR7/TLR9 balance *in vivo*, we generated *Unc93b1*$^{D34A/D34A}$ mice and analysed their phenotypes. *Unc93b1*$^{D34A/D34A}$ mice were born according to Mendelian rule, however, they develop various symptoms spontaneously and over half of mice died within 1 year (Fukui et al., *Immunity* 2011). For example, splenomegaly, hepatitis, thrombocytopenia and glomerulonephritis were observed in *Unc93b1*$^{D34A/D34A}$ mice and it was thought that severe acute hepatitis is a factor of death. Dendritic cells, macrophages, or B cells derived from *Unc93b1*$^{D34A/D34A}$ mice were TLR7 hyper-responsive as observed *in vitro*, and TLR7 deficiency recovered *Unc93b1*$^{D34A/D34A}$ mice from these phenotypes.

Not only innate immune cells, T cells and B cells were activated in *Unc93b1*$^{D34A/D34A}$ mice because *Rag2*$^{-/-}$ *Unc93b1*$^{D34A/D34A}$ mice did not develop phenotypes. Especially, T cells were activated and expanded Th1 or Th17 were detected,

but T cells express little TLR7. We hypothesized that B cells (TLR7 expressing) were dominantly activated and contributed to T cell activation, generated *Ighm*$^{-/-}$ *Unc93b1*$^{D34A/D34A}$ mice to limit B cell maturation. As result, T cells activation in *Ighm*$^{-/-}$ *Unc93b1*$^{D34A/D34A}$ mice were attenuated and most phenotypes were not observed. From these results, it is suggested that endogenous TLR7 ligands activate myeloid cells or B cells, and these cells (especially, B cells) activate T cells in *Unc93b1*$^{D34A/D34A}$ mice.

In conclusion, Unc93B1 keeps TLR9-skewed balance to restrict TLR7 hyper-response and avoid lethal homeostatic inflammation. This function is essential for keeping homeostasis in vivo, thus we are going to search new molecules and mechanisms contributing to reciprocal TLR7/TLR9 balance.

5. 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 disease progression by counteracting TLR7. TLR7 and 9 reside in the endoplasmic reticulum (ER), and are associated with Unc93B1, an ER-resident multiple transmembrane protein. Unc93B1 enable TLR7 and 9 to sense NA by transporting them to the endolysosomes, a site for NA-sensing, upon activation. TLR9 antagonizes TLR7 by competing association with Unc93B1. Unc93B1 restricts TLR7 activation by giving preference to TLR9-association over TLR7. In contrast to TLR9 antagonism, little is known about a mechanism underlying disease promotion by TLR7. 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.

6. The linkage of TLR4 activation and antigen presentation, in the light of molecular trafficing

Natsuko Tanimura, Yuji Motoi 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 dissect this issue in details, we focused on the dynamics of TRAM, an adaptor protein at the downstream of TLR4 signaling. The signaling pathways are modulated by the fine structure of the ligands. We have found one intriguing derivative, which induced low inflammations and enough antigen presentations, showing a unique TRAM dynamics. Currently we are analyzing the molecular mechanisms of this dynamics as a key of activation platform for antigen presentations.

Publications

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

1. MucoRice for New Generation of Oral Vaccine

Yoshikazu Yuki[1], Daisuke Tokuhara[1], Mio Mejima[1], Shiho Kurokawa[1], Yuko Takahashi[1], Eun Jeong Park[1], Koji Kashima[1], Natsumi Takeyama, Michiyo Abe, Tomoko Hiroiwa[1], Yingju Chen[1], Tomonori Nochi[1], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo.

We have developed MucoRice-CTB as a cold-chain-free oral vaccine aganst Cholera and enterotoxigenic *Escherichia col*i -induced diarrhea. In the current transgenic system to produce MucoRice-CTB, a selectable marker gene, usually the HPT (hygromycin phosphotransferase) gene, is co-delivered with the vaccine antigen gene. Because the safety of mHPT gene within the rice genome has not yet been tested for human use, the selectable marker genes must be removed from Mucorcie-CTB. We established the selectable-marker-free MucoRice-CTB and then its seed bank by co-transfection with two kinds of Agrobacterium, one carrying a selection marker (HPT gene) cassette alone and the other carrying vaccine cassette; this will be followed by PCR selection for the CTB gene and self-pollination for the next generation.

Because MucoRice-CTB is a form of medical drug, the quantity and quality of the vaccine expressed by rice should not be influenced by variations in natural rice-growing environments. To maintain consistent expression and quality, we established the closed rice culture system as a GMP facility, including growth-associated factors such as levels of light, water, temperature, chemicals, and fertilization, which carefully and stably controlled to minimize variations in vaccine production. As a result of our efforts, the development of MucoRice-CTB is approaching application in humans. Ideally, at the end of 2012, we will enter human clinical study for Mucorice-CTB in IMSUT-hospital after completing non-clinical study including safety and stability studies.

2. New Generation Nasal Vaccine

Yoshikazu Yuki[1], Il Gyu Kong[1], Yoshiko Fuku-

yama[1], Yuko Takahashi[1], Tomoko Hiroiwa[1], Ayuko Sato[1], Haruko Takahashi[2], Norihiro Harada[3], Shinichi Sawada[2], Yuko Katakai[4], Hiroaki Shibata[4], Tomonori Nochi[1], Hideo Tsukada[3], Kazunari Akiyoshi[2], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]Department of Organic Materials, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, [3]PET Center, Central Research Laboratory, Hamamatsu Photonics K.K., [4]Tsukuba Primate Research Center, National Institute of Biomedical Innovation

Nasal administration is an effective route for a needle-free vaccine. We have developed an effective intracellular vaccine delivery system with self-assembled nanosize cationic hydrogels (nanogel), which composed of a cationic type of cholesteryl group-bearing pullulan (cCHP). We have shown that mice nasally immunized with nanogel-vaccine including botulinum vaccine (BoHc), tetanus toxoid and Pneumococal surface protein A (PspA) induce brisk levels of antigen-specific systemic and mucosal protective immunity without mucosal adjuvant. We also directly showed that, in addition to delivering the vaccine antigen to the classical inductive site (i.e. NALT), cCHP nanogel effectively conveys the vaccine antigen to a wide area of the nasal cavity, thus activating the NALT-independent immune responses initiated by nasal DCs. In addition, we elucidated that nasal administration of mice with nanogel-BoHc or -PspA did not redirect to CNS such as olfactory bulb and brain. In this regards, we developed real-time quantitative tracking method for nasal vaccine by using [^{18}F]-labeled BoHc/A-positron emission tomography (PET), a newly established *in vivo* molecular imaging system for the estimation of safety. This method provides results that are consistent with direct counting of [^{18}F] radioactivity or the traditional [^{111}In]-radiolabel method in dissected tissues of mice and non-human primates. In addition to experiment in mice, we are focusing efficacy and safety studies of nasal vaccine in macaques.

3. Molecular and Cellular Analysis of the Interaction between Gut Microbiota and Host Mucosal Immune System

Yoshiyuki Goto[1], Jun Kunisawa[1], Shintaro Sato[1], Naoko Shibata[1], Aayam Lamichhane[1], Hideki Iijima[2], Yoshinori Umesaki[3], Yoshimi Benno[4] and Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, [3]Yakult Central Institute for Microbiological Research, [4]Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center

In our intestine, mucosal surface is exposed to a wide variety of environmental antigens including harmless food-derived materials and commensal bacteria. The indigenous gut microbiota modulate the 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. Our recent report has shown that 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 U S A*. 2010; 107: 7419-7424). This preferential presence of *Alcaligenes* inside PPs and the associated-induction of intestinal IgA Abs were also observed in both monkeys and humans. Thus, indigenous opportunistic bacteria uniquely inhabit PPs, leading to the local antigen-specific Ab production; this may create an optimal symbiotic environment on the interior of the PPs. Our current work is now focusing on the role of *Alcaligenes* and anti-*Alcaligenes* IgA antibodies in the establishment of inflammatory bowel diseases.

Another interesting example of host-microbiota symbiosis is crosstalk between host intestinal epithelial cells (IECs) and commensal bacteria. Although it has been reported that *Bacteroides thetaiotaomicron* utilize fucose expressed on IECs as an energy source, the mechanism of the epithelial fucosylation is not fully understood. To investigate the mechanism of the induction of fucosylated epithelial cells (F-ECs), we focused on the role of commensal bacteria because the development of F-ECs was completely inhibited in germfree mice. To identify bacterial species responsible for the induction of F-ECs, we adopted metagenomic approaches, 16S rDNA clone library method, and gnotobiotic mouse model. We found that segmented filamentous bacteria (SFB) were colonized predominantly in the ileum, which has a large number of F-ECs, and SFB gnotobiotic mice have comparable number of F-ECs as wild-type mice, indicating that SFB have a potential to induce F-ECs. We further examined the role of epithelial fucose in the establishment of gut microbiota. Defect of epithelial fucose, which are regulated by fucosyltransferase 2 (Fut2), represented the coloniza-

tion of aberrant gut microbiota. In contrast to the reduction of lactic acid bacteria, comparable number of SFB was observed in Fut2-deficient mice. These results suggest that epithelial fucoses have distinct effects on indigenous bacteria and a three-way conversation between SFB, epithelail fucose and lactic acid bacteria contributes to maintain intestinal homeostasis. We are currently investigating the mechanism of how epithelial fucose regulate intestinal homeostatic environment including commensal bacteria and mucosal immune systems.

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

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

In this project, we have been interested in the several dietary materials (e.g., vitamins, fatty acids, secondary bile acid, and nucleotides). For example, we recently 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 vitamin B9-reduced condition, Treg cells could be differentiated from naïve T cells but failed to survive. Another example is vitamin B6, which is required for the metabolic pathway of sphingosine 1-phosphate (S1P), a lipid mediator that regulates cell trafficking. We recently found that vitamin B6-mediated S1P metabolism is a potential target to control mast cell trafficking and thus treatment of the intestinal allergy. 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

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Mast cells are widely existed under the intestinal mucosal surface. Mast cells are known effector cells in allergic and inflammatory diseases, but their precise roles in intestinal inflammation remain unknown. 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 of patients with Crohn's disease. Treatment of mice with a P2X7 purinoceptor-specific antibody inhibited MC activation and subsequent intestinal inflammation. Similarly, intestinal inflammation was ameliorated in MC-deficient $Kit^{W-sh/W-sh}$ mice, and reconstitution with wild-type, but not $P2x7^{-/-}$, MCs resulted in recovered susceptibility to inflammation. ATP-P2X 7 purinoceptor-mediated activation of MCs induced not only inflammatory cytokines, but also chemokines and leukotrienes, to recruit neutrophils and subsequently exacerbated intestinal inflammation. These findings reveal the pivotal role of P2X7 purinoceptor-mediated MC activation in both initiation and exacerbation of intestinal inflammation.

6. Identification and Functional Analysis of Natural Killer Cells in Nasal Passage

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Natural killer (NK) cells are important for host innate defense especially against viral infection and tumor suppression. Accumulating evidences show heterogeneity of NK cells in peripheral tissues, however, little is known about characteristics of NK cells in nasal passage. In this project, we have been investigated the characterization and functions of murine nasal NK cells using $Ncr1^{GFP/+}$ knock-in mice in which

NKp46-positive NK cells express GFP.

Flowcytometric analysis showed nasal NKp46$^+$ cells were of NK cell linage, by expression of NK1.1, CD122, CD94 and CD49b. The expression pattern of CD27/CD11b, CD62L, CD69, B220 and CD11c on nasal NK cells revealed that these cells showed more immature and activated phenotype when compared with splenic and pulmonary counterparts. Interestingly, by *in vitro* PMA/ionomycine stimulation, nasal NK cells showed lower responses on degranulation and IFN-γ production than NK cells from spleen and lung. However, it was suggested that nasal NK cells controlled *in vivo* influenza virus infection since the depletion of NK cells resulted in failure of preventing the proliferation of influenza virus in nasal passage. These our findings indicate that nasal NK cells have unique characteristics both immunologically and functionally, and play a critical role for the clearance on nasally infected influenza virus.

7. Crosstalk between Maxillofacial Immunity and Reproductive Immunity

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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 are investigating the molecular and cellular mechanisms how intranasal and ocular immunizations induce both humoral and cellular immune responses specific for the viral or bacterial antigens in the distant reproductive mucosa. Especially, elucidation of reproductive imprinting molecules will lead to the basic foundation for the development of STD vaccine. Our project will thus contribute scientifically for our understanding of the reproductive imprinting system, which leads to the development of effective vaccine against STDs for the improvement of public health.

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