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. Bacteria hijack integrin-linked kinase to stabilize focal adhesions and block cell detachment

Minsoo Kim, Michinaga Ogawa, Yukihiro Fujita, Yuko Yoshikawa, Takeshi Nagai, Tomohiro Koyama[1], Shinya Nagai[1], Anika Lange[2], Reinhard Fassler[2] and Chihiro Sasakawa: [1]Nippon Institute for Biological Science, Tokyo, Japan. [2]Department of Molecular Medicine, Max-Planck Institute of Biochemistry, Martinsried, Germany.

The rapid turnover and exfoliation of mucosal epithelial cells provides an innate defence system against bacterial infection. Nevertheless, many pathogenic bacteria, including *Shigella*, are able to surmount exfoliation and colonize the epithelium efficiently. Here we show that the *Shigella flexneri* effector OspE (consisting of OspE1 and OspE2 proteins), which is highly conserved among enteropathogenic *Escherichia coli*, enterohaemorrhagic *E. coli*, *Citrobacter rodentium* and *Salmonella* strains, reinforces host cell adherence to the basement membrane by interacting with integrin-linked kinase (ILK). The number of focal adhesions was augmented along with membrane fraction ILK by ILK-OspE binding. The interaction between ILK and OspE increased cell surface levels of β1 integrin and suppressed phosphorylation of focal adhesion kinase and paxillin, which are required for rapid turnover of focal adhesion in cell motility. Nocodazole-washout-induced focal adhesion disassembly was blocked by expression of OspE. Polarized epithelial cells infected with a *Shigella* mutant lacking the *ospE* gene underwent more rapid cell detachment than cells infected with wild-type *Shigella*. Infection of guinea pig colons with *Shigella* corroborated the pivotal role of the OspE-ILK interaction in suppressing epithelial detachment, increasing bacterial cell-to-cell spreading, and promoting bacterial colonization. These results indicate that *Shigella* sustain their infectious foothold by using special tactics to prevent detachment of infected cells.

2. *Listeria monocytogenes* ActA-mediated escape from autophagic recognition

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Autophagy degrades unnecessary organelles and misfolded protein aggregates, as well as cytoplasm-invading bacteria. Nevertheless, the bacteria *Listeria monocytogenes* efficiently escapes autophagy. We show here that recruitment of the Arp2/3 complex and Ena/VASP, via the bacterial ActA protein, to the bacterial surface disguises the bacteria from autophagic recognition, an activity that is independent of the ability to mediate bacterial motility. *L. monocytogenes* expressing ActA mutants that lack the ability to recruit the host proteins initially underwent ubiquitylation, followed by recruitment of p62/SQSTM1 and LC3, before finally undergoing autophagy. The ability of ActA to mediate protection from ubiquitylation was further demonstrated by generating aggregate-prone GFP-ActA-Q79C and GFP-ActA-170* chimaeras, consisting of GFP, the ActA protein and segments of polyQ or Golgi membrane protein GCP170. GFP-ActA-Q79C and GFP-ActA-170* formed aggregates in the host cell cytoplasm, however, these ActA-containing aggregates were not targeted for association with ubiquitin and p62. Our findings indicate that ActA-mediated host protein recruitment is a unique bacterial disguise tactic to escape from autophagy.

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

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

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Department of Microbiology and Immunology

Division of Host-Parasite Interaction
宿主寄生体学

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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 HIV genomes by a chromatine remodeling factor, SWI/SNF complex and also analyzing regulatory 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 (designated as 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. This year, we report a very similar observation in lentivirus; loss of the Brm-type SWI/SNF chromatin remodeling complex is a strong barrier to the Tat-independent transcription drived from HIV LTR. Therefore the basic requirement of Brm-type SWI/SNF complex for stable virus expression can be now extended from retrovirus to lentivirus. Interestingly our detailed analysis showed that in even apparently silenced HIV integrants, proviral gene transcription actually initiates to accumulate short transcripts. Elongation step, however, is strongly inhibited by the failure of disruption of nuc-1, which locates just downsteam of transcriptional initiation site, by the complex. We finally present evidences that Brm-type SWI/SNF complex is expressed at marginal levels in resting $CD4^+$T cells and further that this complex play crucial roles in the early Tat-independent phase of HIV transcription of the latenly infected cells (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.

This year, 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 gastric cancer (b).

a. Loss of the Brm-type SWI/SNF chromatin remodeling complex is a strong barrier to the Tat-independent transcriptional elongation of HIV-1 transcripts

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To elucidate the epigenetic regulation of Tat-independent HIV transcription following proviral integration, we constructed a HIV-1-based replication-defective viral vector that expresses a reporter *GFP* product from its intact LTR. We transduced this construct into human tumor cell lines that were either deficient or competent for the Brm-type SWI/SNF complex. One day after transduction, single cells that expressed GFP were sorted and the GFP expression profiles originating from each of these clones were analyzed. Unlike clones of the SWI/SNF competent cell line, which exhibited clear unimodal expression patterns in all cases, many clones originating from Brm deficient cell lines showed either a broad range distribution of GFP expression or were fully silenced. Resorting of GFP(-) populations of these isolated clones showed that GFP silencing is either reversible or irreversible depending upon the proviral integration sites. We further observed that even in these silenced clones, proviral gene transcription initiates to accumulate short transcripts of around 60 bases in length but that no elongation occurs. We found that this termination is caused by tightly closed nucleosome-1 (nuc-1) at 5'LTR. And the nuc-1 is remodeled by exogenous Brm in some integrants. From these results, we propose that Brm is required for occasional transcriptional elongation of the HIV-1 provirus in the absence of Tat. Since the Brm-type SWI/SNF complex is expressed at marginal levels in resting $CD4^+$ T cells and is drastically induced upon $CD4^+$ T cell activation, we speculate that it plays crucial roles in the early Tat-independent phase of HIV-transcription in affected patients.

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 Cdx2 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.

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, in 2008, we developed an algorithm that predicts promoter region of human *miRNA* genes that are involved in important regulatory systems evolutionarily conserved among vertebrates. 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 double-negative feedback mechanism to substain the *miR-21* expression.

This year, we identified the *PDCD4* genes as another target of miR-21. Its gene product, PDCD4 is a strong tumor suppressor and is known to function as an inhibitor of protein synthesis in cytoplasm, and also as a potent inhibitor of several transcription factors such as AP-1, SP1 and p53 in the cellular nuclei. By pathological analysis of surgically excised colorectal tumors and endoscopically resected colorectal polyps, we show that miR-21 expression is much higher in colorectal cancers than in normal mucosa and further that expression of miR-21 is frequently elevated in precancerous adenomas in polyps. Interestingly expression patterns of *miR-21* RNA and its target, PDCD4 protein were mutually exclusive. In these analyses, we observed that PDCD4 is exclusively present in cellular nuclei. Since high level expression of miR-21 is expected to downregulate PDCD4, which would in turn, release suppression of AP-1 and SP1 leading to further activation of *miR-21* promoter. Therefore like NFIB, PDCD4 would also be involved in double-negative feedback regulation of miR-21, which will explain the mutually exclusive expression patterns between miR-21 RNA and PDCD4 protein observed in cancer lesions. (a).

Since mir-21 suppress expression of several tumor suppressor genes simultaneously, and establish self-enforcing expression patterns in cancers using double-negative feedback mechanisms, miR-21 could be a potent target of tumor suppression. In this respect, it is 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* when expressed by lentivirus vectors as well as by plasmid vectors. (b).

a. LNA *in situ* hybridization analysis of miR-21 expression during colorectal cancer development.

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To better understand microRNA miR-21 function in carcinogenesis, we analyzed miR-21 expression patterns in different stages of colorectal cancer development using *in situ* hybridization (ISH). LNA (locked nucleic acids)/DNA probes and a biotin-free tyramide signal amplification system were used in ISH analyses of miRNA expression. Expression was determined in 39 surgically excised colorectal tumors and 34 endoscopically resected colorectal polyps. In the surgical samples, miR-21 expression was much higher in colorectal cancers than in normal mucosa. Strong miR-21 expression was also observed in cancer-associated stromal fibroblasts, suggesting miR-21 induction by cancer-secreted cytokines. Protein expression of PDCD4, a miR-21 target, was inversely correlated with miR-21 expression, confirming that miR-21 is indeed a negative regulator of PDCD4 *in vivo*. In the endoscopic samples, miR-21 expression was very high in malignant adenocarcinomas but

was not elevated in non-tumorigenic polyps. Precancerous adenomas also frequently showed miR-21 upregulation.

Using the LNA-ISH system for miRNA detection, miR-21 was detectable in precancerous adenomas. The frequency and extent of miR-21 expression increased during the transition from precancerous colorectal adenoma to advanced carcinoma. Expression patterns of miR-21 RNA and its target, tumor suppressor protein PDCD4, were mutually exclusive. This pattern may have clinical application as a biomarker for colorectal cancer development and might be emphasized by self-reinforcing regulatory systems integrated with the *miR-21* gene, which has been previously demonstrated in cell culture.

b. 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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櫻井浩平，古川千尋，原口健，伊庭英夫．miRNAの活性抑制法とその利用によるヒトがん治療「miRNAが変える創薬・がん・発生研究―次世代高感度アレイによる統合解析が明らかにするmiRNAの生命機能」．分子細胞治療　8：350–357，2009

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ウイルス感染分野

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Viruses can cause devastating diseases. The long-term goal of our research is to understand the molecular pathogenesis of viral diseases, using influenza and Ebola virus infections as models. Infections 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)

• Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003-2007.

Takano R, Nidom CA, Kiso M, Muramoto Y, Yamada S, Sakai-Tagawa Y, Macken C, Kawaoka Y.

The wide distribution of H5N1 highly pathogenic avian influenza viruses is a global threat to human health. Indonesia has had the largest number of human infections and fatalities caused by these viruses. To understand the enzootic conditions of the viruses in Indonesia, twenty-four H5N1 viruses isolated from poultry from 2003 to 2007 were phylogenetically characterized. Although previous studies exclusively classified the Indonesian viruses into clades 2.1.1-2.1.3, our phylogenetic analyses showed a new sublineage that did not belong to any of the present clades. In addition, novel reassortant viruses were identified that emerged between this new sublineage and other clades in 2005-2006 on Java Island. H5N1 viruses were introduced from Java Island to Sulawesi, Kalimantan, and Sumatra Island on multiple occasions from 2003-2007, causing the geographical expansion of these viruses in Indonesia. These findings identify Java Island as the epicenter of the Indonesian H5N1 virus expansion.

• Growth determinants for H5N1 influenza vaccine seed viruses in MDCK cells.

Murakami S, Horimoto T, Mai le Q, Nidom CA, Chen H, Muramoto Y, Yamada S, Iwasa A, Iwatsuki-Horimoto K, Shimojima M, Iwata A, Kawaoka Y.

H5N1 influenza A viruses are exacting a growing human toll, with more than 240 fatal cases to date. In the event of an influenza pandemic caused by these viruses, embryonated chicken eggs, which are the approved substrate for human inactivated-vaccine production, will likely be in short supply because chickens will be killed by these viruses or culled to limit the worldwide spread of the infection. The Madin-

Darby canine kidney (MDCK) cell line is a promising alternative candidate substrate because it supports efficient growth of influenza viruses compared to other cell lines. Here, we addressed the molecular determinants for growth of an H5N1 vaccine seed virus in MDCK cells, revealing the critical responsibility of the Tyr residue at position 360 of PB2, the considerable requirement for functional balance between hemagglutinin (HA) and neuraminidase (NA), and the partial responsibility of the Glu residue at position 55 of NS1. Based on these findings, we produced a PR8/H5N1 reassortant, optimized for this cell line, that derives all of its genes for its internal proteins from the PR8 (UW) strain except for the NS gene, which derives from the PR8 (Cambridge) strain; its N1 NA gene, which has a long stalk and derives from an early H5N1 strain; and its HA gene, which has an avirulent-type cleavage site sequence and is derived from a circulating H5N1 virus. Our findings demonstrate the importance and feasibility of a cell culture-based approach to producing seed viruses for inactivated H5N1 vaccines that grow robustly and in a timely, cost-efficient manner as an alternative to egg-based vaccine production.

- **Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets.**

  Watanabe T, Watanabe S, Shinya K, Kim JH, Hatta M, Kawaoka Y.

The 1918 influenza pandemic was the most devastating outbreak of infectious disease in human history, accounting for about 50 million deaths worldwide. In addition to a significant number of cases of secondary bacterial pneumonia, this highly pathogenic strain of influenza A virus caused fatal primary viral pneumonia. To identify the viral gene(s) chiefly responsible for the high virulence of the 1918 virus, we generated a series of reassortants between the 1918 virus and a contemporary human H1N1 virus (A/Kawasaki/173/2001; K173) using reverse genetics. We then assessed their virulence properties in ferrets, a model closely resembling humans in terms of sensitivity to influenza virus infection and pattern of spread after intranasal inoculation. Substitution of single genes from the 1918 virus in the genetic background of K173 virus did not markedly alter the pattern of infection. That is, the reassortants grew well in nasal turbinates, but only sporadically (if at all) in the trachea and lungs. One exception was the 1918 PB1/K173 reassortant, which replicated efficiently in lung tissues as well as the upper respiratory tract. A reassortant virus expressing the 1918 viral RNA polymerase complex (PA, PB1, and PB2) and nucleoprotein showed virulence properties in the upper and lower respiratory tracts of ferrets that closely resembled those of wild-type 1918 virus. Our findings strongly implicate the viral RNA polymerase complex as a major determinant of the pathogenicity of the 1918 pandemic virus. This new insight may aid in identifying virulence factors in future pandemic viruses that could be targeted with antiviral compounds.

- **Nucleotide sequence requirements at the 5' end of the influenza A virus M RNA segment for efficient virus replication.**

  Ozawa M, Maeda J, Iwatsuki-Horimoto K, Watanabe S, Goto H, Horimoto T, Kawaoka Y.

The mechanism by which the influenza A virus genome is packaged into virions is not fully understood. The coding and noncoding regions necessary for packaging of the viral RNA segments, except for the M segment, have been identified. Here, we delineate the M segment regions by incorporating a reporter viral RNA into virions and by generating viruses possessing mutations in the regions. We found that, like the other segments, the M segment coding regions are essential for virion incorporation and that the nucleotide length rather than the nucleotide sequence of the 5′ end of the coding region is important.

- **The Mitogen-activated protein kinase-activated kinase RSK2 plays a role in innate immune responses to influenza virus infection.**

  Kakugawa S, Shimojima M, Goto H, Horimoto T, Oshimori N, Neumann G, Yamamoto T, Kawaoka Y.

Viral infections induce signaling pathways in mammalian cells that stimulate innate immune responses and affect cellular processes, such as apoptosis, mitosis, and differentiation. Here, we report that the ribosomal protein S6 kinase alpha 3 (RSK2), which is activated through the "classical" mitogen-activated protein kinase pathway, plays a role in innate immune responses to influenza virus infection. RSK2 functions in the regulation of cell growth and differentiation but was not known to play a role in the cellular antiviral response. We have found that knockdown of RSK2 enhanced viral polymerase activity and growth of influenza viruses.

Influenza virus infection stimulates NK-kappaB- and beta interferon-dependent promoters. This stimulation was reduced in RSK2 knockdown cells, suggesting that RSK2 executes its effect through innate immune response pathways. Furthermore, RSK2 knockdown suppressed influenza virus-induced phosphorylation of the double-stranded RNA-activated protein kinase PKR, a known antiviral protein. These findings establish a role for RSK2 in the cellular antiviral response.

- **Mutational analysis of conserved amino acids in the influenza A virus nucleoprotein.**

Li Z, Watanabe T, Hatta M, Watanabe S, Nanbo A, Ozawa M, Kakugawa S, Shimojima M, Yamada S, Neumann G, Kawaoka Y.

The nucleoprotein (NP), which has multiple functions during the virus life cycle, possesses regions that are highly conserved among influenza A, B, and C viruses. To better understand the roles of highly conserved NP amino acids in viral replication, we conducted a comprehensive mutational analysis. Using reverse genetics, we attempted to generate 74 viruses possessing mutations at conserved amino acids of NP. Of these, 48 mutant viruses were successfully rescued; 26 mutants were not viable, suggesting a critical role of the respective NP amino acids in viral replication. To identify the step(s) in the viral life cycle that is impaired by these NP mutations, we examined viral-genome replication/transcription, NP localization, and incorporation of viral-RNA segments into progeny virions. We identified 15 amino acid substitutions in NP that inhibited viral-genome replication and/or transcription, resulting in significant growth defects of viruses possessing these substitutions. We also found several NP mutations that affected the efficient incorporation of multiple viral-RNA (vRNA) segments into progeny virions even though a single vRNA segment was incorporated efficiently. The respective conserved amino acids in NP may thus be critical for the assembly and/or incorporation of sets of eight vRNA segments.

- **Region required for protein expression from the stop-start pentanucleotide in the M gene of influenza B virus.**

Hatta M, Kohlmeier CK, Hatta Y, Ozawa M, Kawaoka Y.

Segment 7 of influenza B virus encodes two proteins, M1 and BM2. BM2 is expressed from a stop-start pentanucleotide, in which the BM2 initiation codon overlaps with the M1 stop codon. Here, we demonstrate that 45 nucleotides of the 3′ end of the M1 coding region, but not the 5′ end of the BM2 coding region, are sufficient for the efficient expression of the downstream protein. Placing these 45 nucleotides and the stop-start pentanucleotide in between the coding sequences induced the expression of at least three noninfluenza proteins, suggesting the utility of this system for expressing multiple proteins from one mRNA.

- **Influenza A virus lacking M2 protein as a live attenuated vaccine.**

Watanabe S, Watanabe T, Kawaoka Y

Mutant influenza virus that lacks the transmembrane and cytoplasmic tail domains of M2 (M2 knockout [M2KO]) is attenuated in both cell culture and mice. Here, we examined the potency of M2KO influenza virus as a live attenuated influenza vaccine. M2KO virus grew as efficiently as the wild-type virus in cells stably expressing the wild-type M2, indicating the feasibility of efficient vaccine production. Mice intranasally vaccinated with M2KO virus developed protective immune responses and survived a lethal challenge with the wild-type virus, suggesting that the M2KO virus has potential as a live attenuated.

- **In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses.**

Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, Muramoto Y, Tamura D, Sakai-Tagawa Y, Noda T, Sakabe S, Imai M, Hatta Y, Watanabe S, Li C, Yamada S, Fujii K, Murakami S, Imai H, Kakugawa S, Ito M, Takano R, Iwatsuki-Horimoto K, Shimojima M, Horimoto T, Goto H, Takahashi K, Makino A, Ishigaki H, Nakayama M, Okamatsu M, Takahashi K, Warshauer D, Shult PA, Saito R, Suzuki H, Furuta Y, Yamashita M, Mitamura K, Nakano K, Nakamura M, Brockman-Schneider R, Mitamura H, Yamazaki M, Sugaya N, Suresh M, Ozawa M, Neumann G, Gern J, Kida H, Ogasawara K, Kawaoka Y.

Influenza A viruses cause recurrent outbreaks at local or global scale with potentially severe consequences for human health and the global economy. Recently, a new strain of influenza A virus was detected that causes disease in and transmits among humans, probably owing to little or no pre-existing immunity to the new strain. On 11 June 2009 the World Health Organization declared that the infections caused by

the new strain had reached pandemic proportion. Characterized as an influenza A virus of the H1N1 subtype, the genomic segments of the new strain were most closely related to swine viruses. Most human infections with swine-origin H1N1 influenza viruses (S-OIVs) seem to be mild; however, a substantial number of hospitalized individuals do not have underlying health issues, attesting to the pathogenic potential of S-OIVs. To achieve a better assessment of the risk posed by the new virus, we characterized one of the first US S-OIV isolates, A/California/04/09 (H1N1; hereafter referred to as CA 04), as well as several other S-OIV isolates, in vitro and in vivo. In mice and ferrets, CA04 and other S-OIV isolates tested replicate more efficiently than a currently circulating human H1N1 virus. In addition, CA04 replicates efficiently in non-human primates, causes more severe pathological lesions in the lungs of infected mice, ferrets and non-human primates than a currently circulating human H1N1 virus, and transmits among ferrets. In specific-pathogen-free miniature pigs, CA04 replicates without clinical symptoms. The assessment of human sera from different age groups suggests that infection with human H1N1 viruses antigenically closely related to viruses circulating in 1918 confers neutralizing antibody activity to CA04. Finally, we show that CA04 is sensitive to approved and experimental antiviral drugs, suggesting that these compounds could function as a first line of defence against the recently declared S-OIV pandemic.

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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. Soluble MD-1 detection using novel monoclonal antibodies

Richard Jennings, Sachiko Akashi-Takamura, Takuma Shibata, Toshihiko Kobayashi, Natsuko Yamakawa, And Kensuke Miyake

RP105 is a lymphocyte or myeloid derived cell-surface molecule which has LRR (leucine rich-repeat) motief like TLRs (Toll-like receptors). In the absence of MD-1, cells are unable to express RP105 on their surface, and thusly RP 105 loses function. RP105/MD-1 complex induces lymphocyte B-cell proliferate signal, and this signal inhibits B-cell apoptosis which was induced by radiation. This result has shown RP105/MD-1 to positively regulate TLR stimulation. On the other hand, current data indicates RP105/MD-1 has an inhibitory role in myeloid derived cells. This apparent functional dichotomy between cell types is, as of yet, unexplained. There is the possibility that this dichotomy is explained not through the RP105/MD-1 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, because there is no suitable anti-MD-1 mAb.

Recently we established novel anti-MD-1 mAbs (JR clones). 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. Later 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 anti-RP105 mAb 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. We established the ELISA system of MD-1 by these mAbs. Now we are studying about MD-1 concentration in the serum, urine, or others. Further experiment on extra-cellular MD-1 may lead to a more exhaustive understanding of it's immunobiology.

2. C4BP associates with TLR molecules

Ikuko Yamai, Sachiko Akashi-Takamura, Koichiro Takahashi, Takashi Ishii, Takuma Shibata, Natsuko Yamakawa, and Kensuke Miyake

Toll-like receptor (TLR) plays an essential role in the innate recognition of microbial products. Cell-surface TLR, including TLR4, TLR1/TLR2, and TLR6/TLR2, is able to recognize microbial membrane lipids, whereas TLRs which reside in intracellular organelles, TLR3, TLR7, TLR8, and TLR9, recognize microbial nucleic acids. Microbial recognition, signaling, and modulation of TLR responses are known to require the presence of co-receptors/accessory molecules. To identify the accessory molecules with TLR2 on cell-surface, we established anti-TLR2 mAb (CB225 clone). A rat was immunized with RAW cells (mouse macrophage cell-line). CB225 clone was selected by functional assay which inhibited TLR2 ligand-induced NF-κB activation on Ba/F3TLR2/κB-luciferase expressing transfectant. By immuno-precipitation assay using CB225-coupled beads on B cell-line, we found that bovine C4BP which was contained with culture serum, associated with mouse TLR2. To clearly show the association between mouse C4BP and mouse TLR2, next we made the stable transfectant of C4BP-HA on RAW cell. By immuno-precipitation assay, we were able to show the C4BP-HA association with not only TLR2 but also other TLRs. This result suggests that C4BP affects to immunological response to TLR ligands. Now we are studying about C4BP function in vivo by using C4BP knockout mice.

3. Elevated levels of vascular endothelial growth factor (VEGF) and soluble vascular endothelial growth factor receptor (VEGFR)-2 in Human Malaria

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In cerebral malaria, the binding of parasitized erythrocytes to the cerebral endothelium and the consequent angiogenic dysregulation play a key role in pathogenesis. Because vascular endothelial growth factor (VEGF) is widely regarded as a potent stimulator of angiogenesis, edema, inflammation, and vascular remodeling, the plasma levels of VEGF and the soluble form of the VEGF receptor (sVEGFR)-1 and -2 in uncomplicated malaria patients and healthy adults were measured by enzyme-linked immunosorbent assay (ELISA) to examine their roles in malaria. The results showed that VEGF and sVEGFR-2 levels were significantly elevated in malaria patients compared with healthy adults. Moreover, it was confirmed that malarial parasite antigens induced VEGF secretion from the human mast cell lines HMC-1 or KU812 cell. This is the first report to suggest that the interaction of VEGF and sVEGFR-2 is involved in the host immune response to malarial infection and that malarial parasites induce VEGF secretion from human mast cells.

4. Cathepsins are required for Toll-like receptor 9 responses

Shin-ichiroh Saitoh, Natsuko Tanimura, Ryutaroh Fukui, and Kensuke Miyake

Toll-like receptors (TLR) recognize a variety of microbial products and activate defense responses. Pathogen sensing by TLR2/4 requires accessory molecules, whereas little is known about a molecule required for DNA recognition by TLR9. After endocytosis of microbes, microbial DNA is exposed and recognized by TLR9 in lysosome. We here show that cathepsins, lysosomal cysteine proteases, are required for TLR9 responses. A cell line Ba/F3 was found to be defective in TLR9 responses despite TLR9 expression. Complementation cloning with Ba/F3 identified cathepsin B/L as a molecule required for TLR9 responses. The protease activity was essential for the complementing effect. TLR9 responses were also recovered by cathepsin S or F, but not by cathepsin H. TLR7/9-dependent B cell proliferation and CD86 upregulation were apparently downregulated by cathepsin B/L inhibitors. Cathepsin B inhibitor downregulated interaction of CpG-BCpG-B interaction with TLR9. These results suggest roles for cathepsins in DNA recognition by TLR9. It has been reported that the ectodomain of TLR9 is cleaved to generate a functional receptor. We found the cleaved region in the ectodomain of TLR9 to do detailed experimant.

5. Unc 93 B 1 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. For a next step, we established D34A Unc93B1 Knock-in mice to do in vivo experiments. In addition, we are now trying to identify the regulatory molecules associated with Unc93B1 by using yeast two hybrid screening and LC-Ms/Ms analysis of molecules co-immunoprecipitated with UNC93B1 to unveil the mechanism.

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炎症免疫学分野

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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, Tomonori Nochi[1], Mio Mejima[1], Shiho Kurokawa[1], Yuko Takahashi[1], Hirotsugu Hatai[1], Aya Chubachi[1], Yingju Chen[1], Ushio Nakanishi[1], Yuko Katakai[2], Hiroaki Shibata[3], Kenji Terao[3], Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]The Corporation for Production and Research of Laboratory Primates, Ibaraki., [3]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Ibaraki

We have been investigating effectiveness of cold chain-and needle/syringe-free rice-based vaccine expressing cholera toxin B subunit (MucoRice™-CTB) against live *V. cholerae*-and enterotoxigenic *E. coli* (ETEC)-induced diarrhea in mice. Here, we examined whether the rice-based vaccine can induce CT-specific Ab responses in nonhuman primates. Orally administered MucoRice-CT-B induced high levels of CT-neutralizing serum IgG Abs in the 3 cynomolgus macaques we immunized. Although the Ab level gradually decreased, detectable levels were maintained for at least 6 months, and high titers were rapidly recovered after an oral booster dose of the rice-based vaccine. In contrast, no serum IgE Abs against rice storage protein was induced even after multiple immunizations. Interestingly, the macaques harbored naturally acquired intestinal secretory IgA (SIgA) Abs that were capable of reacting with CT and homologous heat-labile enterotoxin (LT) produced by ETEC and that had toxin-neutralizing activity. The SIgA Abs were present in macaques 1 month to 29 years old, and the level was not enhanced after oral vaccination with MucoRice-CT-B or after subsequent exposure to the native form of CT. These results show that oral MucoRice-CT-B can effectively induce CT-specific, neutralizing, serum IgG Ab responses even in the presence of pre-existing, naturally occurring CT-and LT-reactive SIgA Abs in nonhuman primates. Because of the traits of intestinal natural immunity against *V. cholerae* and ETEC, the efficiency of MucoRice™-CTB for induction of CT-specific intestinal SIgA Ab re-

sponses could not been precisely valued in non-human primates. We are now extending the research for the human clinical study by establishing production system of MucoRice using a full-close type hydroponic culture.

2. Nanogel-based Nasal Vaccine Development

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Mucosal vaccination has been considered to be an ideal strategy in departure from the current needle/syringe required vaccine, since it is now proven to induce comparable or improved levels of protective immunity against most of infectious diseases. Further, mucosal vaccine can effectively induce antigen (Ag)-specific immunity in the front line of aero-digestive and reproductive mucosa where most of pathogens invade the host. The current vaccine generally required adjuvant for the induction of maximum protective immunity, however it sometime causes exaggerated and undesired responses. An ideal mucosal vaccine thus should be adjuvant free form. To achieve the goal, we here developed novel delivery system of nasal vaccine with nanogel of cationic type of cholesteryl group-bearing pullulan (cCHP) as a nanocarrier. Because it forms nanosize hydrogel, so called nanogel, with the manner of self-assembly, and simultaneously holds the protein Ag, nasally administered cationic nanogel-botulinum vaccine antigen Hc complex (cCHP-Hc) effectively adhered nasal epithelial cells for subsequent induction of brisk levels of botulinum neurotoxin (BoNT)-specific serum IgG and secretory IgA (SIgA) antibody (Ab) responses with the toxin-neutralizing activity. These results indicate that cationic nanogel is universally feasible adjuvant-free and self-assemble mucosal antigen delivery system for nasal vaccine development. Now, we focus on studies for the precise mechanism and safety issues of nanogel-based nasal vaccine.

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

Gemilson S. Pontes[1], Yoshida Masato[1], Tomonori Nochi[1], Koji Hase[2], Kazutaka Terahara[1], Shiho Kurokawa[1], Mio Mejima[1], Yuko Takahashi[1], Anson W. Lowe[3], Yoshikazu Yuki[1], Hiroshi Ohno[2] and Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, [2]Laboratory for Epithelial Immunobiology, Research Center for Allergy and Immunology, RIKEN, [3]Department of Medicine, Stanford University

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. In order to address the antigen-sampling mechanisms, we previously performed the comprehensive gene expression analysis with highly purified M cells and identified that glycoprotein 2 (GP2) was specifically expressed by M cells. Our subsequent collaborative study with Dr. H. Ohno of RIKEN further revealed that, although M cells were normally developed, the impaired immune responses against *Salmonella typhimurium* or *Escherichia coli* were induced when those bacteria were orally administered in $GP2^{-/-}$ mice. Moreover, *in vitro* study showed that GP2 specifically bound to FimH, a component of bacterial type I pili. These results provide a first evidence of GP2-mediated antigen-sampling system for $FimH^{+}$ bacteria in M cells, leading to initiate the bacteria-specific mucosal immune responses.

In addition, we previously developed the M cell-targeted mucosal vaccine with monoclonal antibody (NKM 16-2-4) specific for both M cells in PPs (PP M cells) and intestinal villi (villous M cells). Orally administered vaccine antigen conjugated with NKM 16-2-4 induced high levels of antigen-specific mucosal and systemic immune responses. To understand the precise immunological mechanisms of inducing the immune response triggered by PP M cells and villous M cells, we performed the immunization study with PP-lacking mice. Interestingly, high levels of Tetanus toxioid (TT)-specific serum IgG antibody responses were induced in PP-lacking mice when TT-conjugated NKM 16-2-4 but not TT-conjugated control antibody was orally immunized. The antigen-specific intestinal IgA antibody responses were hardly induced in PP-lacking even if the antigen was delivered to villous M cells with NKM 16-2-4. These results

highlight the importance of antigen-delivery system to both PP and villous M cells to initiate the induction of distinct immune responses in mucosal and systemic compartments, respectively. Our current studies are focusing on the development of M cell-targeted mucosal vaccine can be used for practical application in human.

4. Mucosal Immunological Homeostasis and Diseases

Jun Kunisawa, Haruyo Adachi, Akihiro Uozumi, Yosuke Kurashima, Tomokazu Ishizuka, Yoshifumi Kotani, Atsuhiro Matsumoto, Takeaki Amiya, Izumi Ishikawa, Eri Hashimoto, and Hiroshi Kiyono: Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo

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

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

5. Molecular and Cellular Analysis of Host-Microflora Interaction at Mucosal Compartment

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The mucosal surface of the gastrointestinal (GI) tract is constitutively exposed to a huge number and a wide variety of environmental antigens including GI microbiota. The indigenous GI microbiota plays an important role in the development of the host mucosal immune system including the production of intestinal mucosal antibodies (Abs). The current understanding is that commensal bacteria create natural cohabitation niches together with mucosal Abs such as secretory IgA (SIgA) in the GI tract. However, the population of commensal bacteria colonized in the IgA inducible second lymphoid tissues such as Peyer's patches (PPs) is unknown yet. To analyze the bacterial population in PPs, we adopted 16S rDNA based approaches such as 16S rDNA clone library and fluorescent *in situ* hybridization (FISH) and found that opportunistic bacteria, largely *Alcaligenes* species, specifically inhabited host PPs with the associated preferential induction of antigen-specific mucosal IgA Abs in the GI tract. This preferential presence of *Alcaligenes* inside PPs and the associated-induction of intestinal SIgA Abs were also observed in both monkeys and humans. *Alcaligenes*-stimulated $CD11c^+$ dendritic cells (DCs) produced the Ab-enhancing cytokines TGF-β, BAFF and IL-6 in PPs. These $CD11c^+$ DCs did not migrate beyond the draining mesenteric lymph nodes. Thus, indigenous opportunistic bacteria uniquely inhabit PPs leading to PP-DCs dependent, local antigen-specific Ab production; this may involve the creation of an optimal symbiotic environment on the interior of the PPs.

The other mucosal immunity mediated symbiotic system was operated by epithelial fucose and some specific microorganisms such as *Bacteroides* species have ability to utilize it as an energy source. However, the molecular and cellular mechanisms of the induction of epithelial fucosylation are largely unknown. We have found that the development of epithelial fucose was completely blocked in germ-free mice, suggest-

ing that gut microbiota induces epithelial fucosylation. To analyze whether the host bacterial recognition systems mediate this process, we used *Myd88*$^{-/-}$ mice which lack signalling cascades for Toll-like receptors (TLRs). Fucosylated ECs (F-ECs) were markedly reduced in *Myd88*$^{-/-}$ mice compared with wild-type mice. This data suggest that F-ECs are induced by MyD88-mediated innate immune systems. We are now trying to identify the bacterial recognition receptors and cells responsible for the development of F-ECs.

6. Ocular and Upper Respiratory Mucosal Immune Systems

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Our group has suggested the presence of mucosal immune system consisted with lacrimal tear duct and nasal cavity. Thus, the presence of tear duct-associated lymphoid tissue (TALT) located in the lacrimal sac which bridges between ocular surface and nasal cavity was identified. The tissue developmental program of TALT was different from other secondary lymphoid tissue genesis. We also discovered a new airway-antigen-sampling-site in the upper respiratory tract. By immunohistochemistry and electron microscopy analysis, we were able to identify UEA-1^{+}NKM16-2-4^{+} M cells at away from organized lymphoid tissue, i.e. nasopharynx-associated lymphoid tissue (NALT). These "Respiratory M cells" were capable of taking-up inhaled antigens for the initiation of antigen-specific immune responses. Thus, NALT-independent immune system might exist in the upper respiratory tract. Indeed, we found that unique B1 cell population was distributed in the nasal mucosa but not in the NALT. Unlike peritoneal B1 cells, the migration of nasal B1 cells to nasal mucosa was shown to be independent of lymphoid chemokine, CXCL13. We found that the number of nasal B2 cells was reduced in *Cxcl13*$^{-/-}$ mice, while nasal B1 cells were normally present. Consistent with these findings, nasal B1 cells lacked the expression of CXCR5. Instead, nasal B1 cells were found to express CCR10 and showed chemotactic activity against CCL28, abundantly produced in the upper respiratory tract. Nasal immunization of T-independent antigen (e.g., phosphorylcholine; PC) induced comparable levels of PC-specific secretary IgA in respiratory tract of both *Cxcl13*$^{-/-}$ and wild type mice. These results collectively indicate the presence of NALT-independent respiratory immune system by which respiratory M cells and CXCR5^{-}CCR10^{+} nasal B1 cells play a central role for the antigen-specific immune response.

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