RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Bacterial Infection
細菌感染分野

Professor	Chihiro Sasakawa, D.M.Sc.	教　授　医学博士	笹　川　千　尋
Research Associate	Michinaga Ogawa, D.M.Sc.	助　手　医学博士	小　川　道　永
Research Associate	Hitomi Mimuro, D.M.Sc.	助　手　医学博士	三　室　仁　美
Research Associate	Masato Suzuki, D.M.Sc.	助　手　医学博士	鈴　木　仁　人

Research in this division is directed toward understanding the complex interactions that occur between pathogenic bacteria and their human hosts at very early stage of bacterial infectious processes. Our special interest is focused upon the molecular pathogenicity of enteropathogenic bacteria, such as Shigella, Helicobacter pylori, enteropathogenic E. coli and enterohemorrhagic E. coli. We are also searching for effective methods to protect or regulate bacterial infection by using knowledge accumulated.

1. Microtubule-Severing Activity of *Shigella* is Pivotal for Intercellular Spreading

Sei Yoshida, Yutaka Handa, Toshihiko Suzuki, Michinaga Ogawa, Masato Suzuki, Asuka Tamai[1], Akio Abe[1], Eisaku Katayama[2], and Chihiro Sasakawa: [1]Laboratory of Bacterial Infection, Kitasato Institute for Life Science, Kitasato University, [2]Department of Basic Medical sciences, IMSUT

Some pathogenic bacteria actually invade the cytoplasm of their target host cells. Invasive bacteria acquire the propulsive force to move by recruiting actin and its polymerization. Here we show that *Shigella* movement within the cytoplasm was severely hindered by microtubules and that the bacteria destroyed surrounding microtubules by secreting VirA via the type III secretion system. Degradation of microtubules by VirA was dependent on its alpha-tubulin-specific cysteine protease-like activity. *virA* mutants did not move within the host cytoplasm and failed to move into adjacent cells.

2. *Shigella* IpgB1 promotes bacterial entry through the ELMO-Dock180 machinery

Yutaka Handa, Masato Suzuki, Kenji Ohya, Hiroki Iwai, Nozomi Ishijima, Anthony J. Koleske[1], Yoshinori Fukui[2] and Chihiro Sasakawa: [1]Department of Molecular Biophysics and Biochemistry, Department of Neurobiology, and Interdepartmental Neuroscience Program, Yale University School of Medicine, [2]Division of Immunogenetics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University

Shigella use a special mechanism to invade epithelial cells called 'the trigger mechanism of entry'1-3, which allows epithelial cells to trap several bacteria simultaneously. On contact, *Shigella* deliver effectors into epithelial cells through the type III secretion system4-6. Here, we show that one of the effectors, IpgB1, has a pivotal role in producing membrane ruffles by exploiting the RhoG-ELMO-Dock180 pathway to stimulate Rac1 activity. Using pulldown assays, we identified engulfment and cell motility (ELMO) protein as the IpgB1 binding partner.

IpgB1 colocalized with ELMO and Dock180 in membrane ruffles induced by *Shigella*. *Shigella* invasiveness and IpgB1-induced ruffles were less in ELMO- and Dock180-knockdown cells compared with wild-type cells. Membrane association of ELMO-Dock180 with ruffles were promoted when cells expressed an IpgB1-ELMO chimera, establishing that IpgB1 mimics the role of RhoG in producing membrane ruffles. Taken together, our findings show that IpgB1 mimicry is the key to invasion by *Shigella*.

3. *Shigella* chromosomal IpaH proteins are secreted via the typeIII secretion system and act as effectors

Hiroshi Ashida, Takahito Toyotome, Takeshi Nagai and Chihiro Sasakawa

Shigella possess 220-kb plasmid, and the major virulence determinants, called effectors, and the type III secretion system (TTSS) are exclusively encoded by the plasmid. The genome sequences of *S. flexneri* strains indicate that several *ipaH* family genes are located on both the plasmid and the chromosome, but whether their chromosomal IpaH cognates can be secreted from *Shigella* remains unknown. Here we report that *S. flexneri* strain, YSH6000 encodes seven *ipaH* cognate genes on the chromosome and that the IpaH proteins are secreted via the TTSS. The secretion kinetics of IpaH proteins by bacteria, however, showed delay compared with those of IpaB, IpaC, and IpaD. Expression of the each mRNA of *ipaH* in *Shigella* was increased after bacterial entry into epithelial cells, and the IpaH proteins were secreted by intracellular bacteria. Although individual chromosomal *ipaH* deletion mutants showed no appreciable changes in the pathogenesis in a mouse pulmonary infection model, the Δ*ipaH-null* mutant, whose chromosome lacks all *ipaH* genes, was attenuated to mice lethality. Indeed, the histological examination for mouse lungs infected with the Δ*ipaH-null* showed a greater inflammatory response than induced by wild-type *Shigella*, suggesting that the chromosomal IpaH proteins act synergistically as effectors to modulate the host inflammatory responses.

4. High Vaccine Efficacy against Shigellosis of Recombinant Noninvasive *Shigella* Mutant That Expresses Yersinia Invasin

Toshihiko Suzuki, Yuko Yoshikawa, Hiroshi Ashida, Hiroki Iwai, Takahito Toyotome, Hidenori Matsui[1] and Chihiro Sasakawa: [1]Department of Infection Control and Immunology, Kitasato Institute for Life Sciences, Kitasato University

Live attenuated *Shigella* vaccines elicit protective immune responses, but involve a potential risk of inducing a strong inflammatory reaction. The bacterial invasiveness that is crucial for Ag delivery causes inflammatory destruction of infected epithelial cells and proinflammatory cell death of infected macrophages. In this study, the noninvasive *Shigella* mutant Δ*ipaB* was equipped with *Yersinia* invasin protein, which has been shown to mediate bacterial invasion and targeting to M cells located in follicle-associated epithelium. Invasin-expressing Δ*ipaB* (Δ*ipaB/inv*) was internalized into epithelial cells and retained in the intraphagosomal space. Δ*ipaB/inv* did not induce necrotic cell death of infected macrophages nor cause symptomatic damage after intranasal vaccination of mice. Δ*ipaB/inv* was safer and more effective than the conventional live vaccine, Δ*virG*. Infection by Δ*ipaB/inv* caused polymorphonuclear neutrophil infiltration in the lung, but did not induce production of large amounts of proinflammatory cytokines. We concluded that the low experimental morbidity and high vaccine efficacy of Δ*ipaB/inv* are primarily based on high protective immune responses, which may be enhanced by the polymorphonuclear neutrophil infiltration unaccompanied by tissue injury.

Publications

Yoshida, S., Handa, Y., Suzuki, T., Ogawa, M. Suzuki, M., Tamai, A., Abe, A., Katayama, E., and Sasakawa, C. Microtubule-Severing Activity of *Shigella* Is Pivotal for Intercellular Spreading. Science 314: 985-989, 2006

Handa, Y., Suzuki, M., Ohya, K., Iwai, H., Ishijima, N., Koleske, A.J., Fukui Y., and Sasakawa, C. *Shigella* IpgB1 promotes bacterial entry through the ELMO-Dock180 machinery. Nat. Cell Biol. Published online: 17 December 2006

Ashida, H., Toyotome, T., Nagai, T. and Sasakawa, C. *Shigella* chromosomal IpaH proteins are secreted via the type III secretion system and act as effectors. Mol. Microbiol. Accepted article online: 6-Dec-2006

Suzuki, T., Yoshikawa, Y., Ashida, H., Iwai, H., Toyotome, T., Matsui, H. and Sasakawa, C. High vaccine efficacy against shigellosis of recombinant noninvasive *Shigella* mutant that

expresses Yersinia invasin. J. Immunol. 177: 4709-4717, 2006

Ogawa, M. and Sasakawa, C. *Shigella* and Autophagy. Autophagy 2: 171-174, 2006

Department of Microbiology and Immunology

Division of Immunology
免疫調節分野

Professor	Kiyoshi Takatsu, Ph.D.	教　授	医学博士	高　津　聖　志
Associate Professor	Satoshi Takaki, M.D., Ph.D.*	助教授	医学博士	高　木　　智*
Research Associate	Taku Kouro, M.D., Ph.D.	助　手	医学博士	紅　露　　拓
Research Associate	Yoshinori Nagai, M.D., Ph.D.	助　手	医学博士	長　井　良　憲
Research Associate	Ai Kariyone, Ph.D.	助　手	医学博士	刈　米　ア　イ

Self-defense against invaded pathogenic microorganisms and foreign antigenic molecules is strictly controlled by the immune system and inflammation. Our major research interests are to elucidate cells and effector molecules in innate and acquired immunity and inflammation. In particular, we are focused on cellular and molecular mechanisms of development and activation of B cells and IgH class switch recombination under the influence of T cells, IL-5, and Lnk family adaptor proteins. We are also interested in elucidating cellular mechanisms of preferential induction of Th1 cells and enhanced cross-priming upon immunization with Mycobacterium-derived, Ag85B, Peptide-25, and their derivatives.

1. Molecular basis of B cell development and differentiation

a. Molecular mechanisms of class switch recombination in CD38-activated B cells.

Yumiko Tsukamoto, Yoshinori Nagai, Atsushi Sato, Shizuo Akira[1], Tsuneyasu Kaisho[2], and Kiyoshi Takatsu: [1]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, and [2]RIKEN Center for Allergy and Immunology, Yokohama

The process of class switch recombination (CSR) from IgM to other isotypes is highly regulated by cytokines and activation-induced cytidine deaminase (AID). Blimp-1 and XBP-1 play an essential role in the terminal differentiation of switched B-2 cells to Ig-producing plasma cells. As we reported, class switch recombination (CSR) is the process that changes physiological activities of antibodies without changing their antigen specificities. As we reported, IL-5 stimulation of anti-CD38-stimulated murine splenic B cells induces μ to γ1 CSR leading to a high level of lgG1 production. Further addition of IL-4 to this system, causes enhanced IL-5-dependent μ to γ1 CSR and IgG1 production. Stat5 activation is indispensable for IL-5-dependent μ to γ1 CSR in CD38activated B cells.

IL-4 does not induce either μ to γ1 CSR or Blimp-1 expression in CD38-activated B-2 cells. Interestingly, the addition of 8-mercaptoguanosine (8-SGuo) with IL-4 to cultured CD38-activated B cells can induce μ to γ1 CSR and IgG1 production. Intriguingly, 8-SGuo by itself induced AID expression in CD38-activated B cells. Furthermore, 8-SGuo stimulation also induced the mRNA expression of UNG, Bach2, and 53BP1 that are necessary for CSR. However, 8-SGuo did not induce μ to γ1 CSR in CD38-activated B cells. These results imply that the mode of B cell activation for extracellular stimulation affects the outcome of cytokine stimulation with respect to the efficiency and direction of CSR. Additional molecule (s) or factor (s)

other than AID, UNG, Bach2, and 53BP1 may be required for CSR.

We found that 8-SGuo stimulation induces AID expression and double-strand (ds) DNA cleavage in CD38-activated B cells through a TLR7 and MyD88 dependent pathway. Loxoribine (7-allyl-8-oxoguanosine), a ligand for toll-like receptor (TLR) 7 exerted a similar activity to 8-SGuo. These results indicate that TLR7 plays a critical role in AID expression and dsDNA cleavage in CD38-activated B cells.

We asked whether these TLR7 ligands have some actions in innate immune cells such as DCs and macrophages. We found that loxoribine activated cDCs/macrophages and induced TNF-α production, whereas 8-SGuo did not have such actions in these cell types. These results suggest that 8-SGuo is uniquely important for CSR in B cells, and those two TLR7 ligands have different actions in different cell types. Theses indicate that more needs to be learned about roles of virus-mediated activation via TLR7 in induction of CSR, and further investigations might have some hints how different ligands act on TLR7 by itself.

b. Molecular mechanisms of Pre-B cell differentiation

Masashi Ikutani, Taku Kouro, Satoshi Takaki and Kiyoshi Takatsu

B cells are developed from hematopoietic progenitors in the bone marrow. This can be reproduced *in vitro* by culturing B cell progenitors in the bone marrow on stromal cell layer in the presence of IL-7. In such cultures, B-cell development stops at pro-B stage and withdrawal of IL-7 from the culture is required for inducing differentiation to the next differentiation stage, pre-B. At pro-B to pre-B transition, immunoglobulin heavy chain and surrogate light chain composed of VpreB and λ5 associate each other to form pre-B cell receptor (pre-BCR). It is speculated that pro-B to pre-B differentiation signal is generated by pre-BCR and that local abundance of IL-7 may act as a selection mechanism for pre-BCR-expressing cells because withdrawal of IL-7 from pro-B cultures of RAG-2 deficient mice does not induce pre-B cell differentiation while addition of anti-CD79b antibody to these cultures induces pre-B cell differentiation even in the presence of IL-7. Further, this signal is Btk-dependent because administration of anti-CD79b mAb to RAG-2 deficient mice results in emergence of pre-B-like cells in Btk-dependent manner.

To reveal pre-BCR-mediated, Btk-dependent differentiation signal, we employed gene chip analysis to screen genes specifically up- or down-regulated in bone marrow $B220^+$ cells upon administration of anti-CD79b in RAG-2 deficient mice, but not in RAG-2/Btk double knockout mice. We selected 10 candidate genes and examined their effects on pre-B cell differentiation by enforced expression in hematopoietic progenitors followed by transplantation into irradiated recipient mice. One of the genes (tentatively called Gene A) showed activity for pre-B cell development of the transfected progenitors. Gene A induced down-regulation of CD43 and up-regulation of CD2 and CD25 expression in RAG-2 deficient pro-B cell lines, indicating that Gene A induces pre-B cell differentiation. Suppression of Gene A by siRNA resulted in inhibition of pre-B cell differentiation. These results imply that Gene A may be a key molecule in pro-B to pre-B transition in the physiological situation.

2. Role of interleukin-5 (IL-5) and B-1 cells in mucosal immunity and elicitation of contact sensitivity

a. Origin and differentiation of B-1 cells

Taku Kouro, Masashi Ikutani, and Kiyoshi Takatsu

B cells are subdivided into conventional B (B-2) cells, marginal zone B cells and B-1 cells. B-1 cells, distinguishable from conventional B-2 cells by their cell surface marker, anatomical location, and self-replenishing activity, play an important role in innate immune responses. B-1 cells constitutively express three different markers, namely Mac-1 (CD11b/CD18), FcεR (CD23), and the IL-5 receptor α-chain (IL-5Rα). Mature B-1 cells are most abundantly found in peritoneal and pleural cavities and responsible for natural antibody formation. Studies of gene-targeted and transgenic mice have revealed that B cell receptor (BCR) signaling is critical for B-1 cell development or maintenance. Recently, B-1 cell progenitor was finally identified as $CD19^+$ $B220^-$ bone marrow (BM) cell, yet regulatory mechanism of its differentiation is still unknown.

As we reported the IL-5/IL-5R system plays an important role in maintaining the number and the cell size as well as the functions of mature B-1 cells. Thus we thought IL-5/IL-5R system as a candidate for regulator of early B-1 cell differentiation as well. Interestingly, IL-5Rα was expressed on $CD19^+$ $B220^-$ BM cells but not on pro-B cells or pre-B cells. To our surprise, this expression of IL-5Rα was not detected on $CD19^+$ $B220^-$ cells in fetal liver (FL). Instead IL-5Rα was expressed on a small subset of lineage

marker negative FL cells. Functional assay for these cells revealed that regardless of IL-5Rα expression, $CD19^+$ $B220^-$ cells are B-1 progenitors. Importantly, Btk deficient mice were found to lack these B-1 progenitors, implying involvement of this kinase not only in BCR signaling but also in early B-1 cell differentiation signaling.

b. Identification of IL-5-secreting cells *in vivo*

Masashi Ikutani, Taku Kouro, Satoshi Takaki, and Kiyoshi Takatsu

IL-5 is secreted from Th2 cells and induces antibody secretion from activated B cells. In addition, many other cell types such as mast cells, basophils, γδT cells, and non-hematopoietic cells have been reported to produce IL-5. B-1 cells depend on IL-5 secreted by non-lymphoid cells for their homeostatic proliferation and survival. To visually identify IL-5-producing cells in various tissues, we are attempting to generate GFP/IL-5 knock-in mouse. GFP cDNAs and Neo cassette were inserted into the first exon of *IL-5* gene to obtain targeting vector. After gene transfections, among 1700 mutant ES cells we selected a single colony with homologous recombination. After injecting this IL-$5^{GFP/+}$ ES clone into blastcysts, we have obtained 12 chimeric mice with 5-40% chimerism, assessed by coat color. These chimeric mice were crossed with C57BL/6 mice and we obtained one IL-$5^{GFP/+}$ mouse among～1000 litters. We will analyze IL-5-secreting cells with the use of IL-$5^{GFP/+}$, IL-$5^{GFP/GFP}$ and neo-deleted strains of mice. As IL-5 is involved in contact sensitivity by activating B-1 cells and eosinophils, we would also analyze IL-5-producing cells after chemical sensitization.

3. Homeostatic regulation by Lnk-family adapter proteins in the immune system and the non-immune systems

a. Control of hematopoietic stem cells and progenitor cells by Lnk adaptor protein.

Satoshi Takaki, Hitoshi Takizawa, Yukiko Iwasaki, Hiroko Katayama, Chiyomi Kubo-Akashi, Atsuko Yoshikawa, Yuji Watanabe and Kiyoshi Takatsu

Lnk, recently designated as SH2B adaptor protein 3 (SH2B3), belongs to the "SH2 adaptor family" composed of APS (adaptor protein with PH and SH2 domains; SH2B2) and SH2-B (SH2B1). These adaptor proteins share an N-terminal domain that mediates homodimerization, a pleckstrin homology (PH) domain, an SH2 domain and a tyrosine phosphorylation site within COOH-terminus.

Lnk negatively regulates lymphopoiesis and early hematopoiesis. The *lnk*-deficient mice show enhanced B cell production due to the hypersensitivity of B cell precursors to stem cell factor, SCF. In addition, not only numbers but also the self-renewal capacity of hematopoietic stem cells (HSCs) is markedly increased when Lnk function is lost. Enhancement of the engrafting potential and expansion capabilities of HSCs has been a long-time desire, as a means of reducing the risks and difficulties accompanying with BM transplantation. We have identified the functional domains of Lnk, and developed a dominant-negative (DN) Lnk mutant that inhibits the functions of Lnk endogenously expressed in the HSCs and, thereby, potentiates the HSCs for engraftment. Importantly, even transient expression of DN-Lnk in HSCs facilitated their engraftment under nonmyeloablative conditions and fully reconstituted the lymphoid compartments of immunodeficient host animals. Hematopoietic progenitor cells expressing DN-Lnk were efficiently trapped by immobilized vascular cell adhesion molecule-1 (VCAM-1) in a transwell migration assay, suggesting involvement of Lnk in the regulation of cell mobility or cellular interaction in microenvironments. Transient inhibition of Lnk or Lnk-mediated pathways could be a potent approach to augment engraftment of HSCs and progenitor cells without obvious side effects.

We also analyzed Lnk expression in various cells in the lympho-hematopoietic system, and found that dendritic cells (DCs) also expressed significant amounts of Lnk protein. The numbers of DCs in spleen and lymph nodes were increased in the $lnk^{-/-}$ mice. Production of DCs from bone marrow precursor cells upon stimulation of GM-CSF was enhanced in the absence of Lnk.

b. Lnk is a dual-functioning adapter molecule controlling integrin signaling as well as cytokine receptor signaling

Hitoshi Takizawa, Koji Eto[4], Hiromitsu Nakauchi[4], Chiyomi Kubo-Akashi, Kiyoshi Takatsu and Satoshi Takaki: [4]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, IMSUT

The *lnk*-deficient mice show thrombocytosis in addition to B cell overproduction and HSC expansion. Megakaryocyte number in the bone marrow was increased in *lnk*-deficient mice. Progenitor cells that responded to thrombopoietin (TPO) in culture was increased, and

their sensitivity to TPO was also increased in the absence of Lnk. Lnk expression is maintained in platelets, however, the functions of Lnk in mature platelets remains unknown. We found that *lnk*-deficient platelets showed filopodia-dominant shapes upon spreading on fibrinogen, indicating a defect in lamellipodia formation and impaired outside-in signaling from αIIbβ3 integrin. In platelets, Lnk forms a complex with c-Src and ADAP, Fyn-binding adaptor, in a manner dependent on αIIbβ3 ligation and Src activation. These results demonstrate for the first time that Lnk adaptor plays a pivotal role in the adhesion responses of platelets through regulation of integrin signaling. We also found that adhesion behaviors of splenic B cells were altered by the absence of Lnk. Thus, Lnk is a "dual-functioning" adapter molecule, which facilitates integrin-mediated signaling while suppressing cytokine-mediated signaling.

c. Differential role of SH2-B and APS in regulating energy and glucose homeostasis

Minghua Li[5], Decheng Ren[5], Masanori Iseki, Satoshi Takaki, and Liangyou Rui[5]: [5]Department of Molecular & Integrative Physiology, University of Michigan Medical School

Previous studies have shown that two other members of Lnk-family proteins, SH2-B and APS promote both insulin and leptin signaling in a similar fashion in cultured cells. We characterized genetically modified mice lacking SH2-B, APS, or both to determine the physiological roles of these two proteins in animals. Disruption of the SH2-B gene resulted in obesity, hyperglycemia, hyper insulinemia, and glucose intolerance. Conversely, deletion of the APS gene did not alter adiposity, energy balance, and glucose metabolism. Energy intake, energy expenditure, fat content, body weight, and plasma insulin, leptin, glucose, and lipid levels were similar between $APS^{-/-}$ and WT littermates fed either normal chow or a high-fat diet. Moreover, deletion of APS failed to alter insulin and glucose tolerance. $APS^{-/-}/SH2\text{-}B^{-/-}$ double knockout mice also developed energy imbalance, obesity, hyper leptinemia, hyper insulinemia, hyperglycemia, and glucose intolerance; however, plasma leptin and insulin levels were significantly lower in $APS^{-/-}/SH2\text{-}B^{-/-}$ than in $SH2\text{-}B^{-/-}$ mice. These results suggest that SH2-B, but not APS, is a key positive regulator of energy and glucose metabolism in mice.

4. Mechanisms of preferential induction of Th1 and cytotoxic T cell (CTL) response upon immunization with Mycobacteria peptide

The Ag85B of *Mycobacterium (M.) tuberculosis* is immunogenic in C57BL/6 mice and induces the expansion of TCRVα5Vβ11 $CD4^+$ Th1 cells in conjunction with antigen-presenting cells (APC) in an $I\text{-}A^b$-restricted manner. Peptide-25 is the major antigenic epitope of Ag85B, recognized by TCRVα5Vβ11 $CD4^+$ Th1 cells.

a. Role of MHC/peptide-TCR interaction in the Peptide-25-dependent Th1 differentiation

Yoko Shimohakamada, Takeshi Tokunaga, Ai Kariyone, Toshiki Tamura[6] and Kiyoshi Takatsu: [6]Research Center of Hansen Disease, Institute of Infectious Diseases

Activated $CD4^+$ Th cells can be classified into two subsets, Th1 and Th2, on the basis of cytokine production profiles. Th1 cells play a critical role in the induction of the cell-mediated immune responses that are important for the eradication of intracellular. In addition to the T cell antigen receptor (TCR) activation signals, the cytokine environment, type of APC, genetic background and co-stimulatory molecules expressed by activated APC may also be involved in the determination of the differentiation of naive $CD4^+$ T cells into Th1 cells. However, it is unclear whether the TCR signaling events exert a direct influence on Th1 differentiation. We generated transgenic mice (P25 TCR-Tg) that express the TCR-Vα5-Vβ11 for recognition of Peptide-25, in conjunction with $I\text{-}A^b$ molecules and reported that naive $CD4^+$ T cells from P25 TCR-Tg mice preferentially differentiate into Th 1 cells upon Peptide-25 stimulation in the presence of $I\text{-}A^b$ splenic APC under non-skewing condition. In contrast, an altered peptide ligand of Peptide-25 could induce solely Th2 differentiation.

Using P25 TCR-Tg mice, we demonstrate that TCR stimulation of naive $CD4^+$ T cells induces transient T-bet expression, interleukin (IL)-12 receptor β2 up-regulation, and GATA-3 down-regulation, which leads to T helper (Th) 1 differentiation even when the cells are stimulated with peptide-loaded $I\text{-}A^b$-transfected Chinese hamster ovary cells in the absence of interferon (IFN)-γ and IL-12. Sustained IFN-γ and IL-12 stimulation augments naive T-cell differentiation into Th1 cells. Intriguingly, a significant Th1 response is observed even when $T\text{-}bet^{-/-}$ naive $CD4^+$ T cells are stimulated through TCR in the absence of IFN-γ or IL-12. Stimulation of naive

$CD4^+$ T cells in the absence of IFN-γ or IL-12 with altered peptide ligand, whose avidity to the TCR is lower than that of original peptide, fails to up-regulate transient T-bet expression, sustains GATA-3 expression, and induces differentiation into Th2 cells. These results support the notion that direct interaction between TCR and peptide-loaded antigen presenting cells, even in the absence of T-bet expression and costimulatory signals, primarily determine the fate of naive $CD4^+$ T cells to Th1 cells.

b. Adjuvant activity of Peptide-25 for enhancing anti-tumor immune response

Takeshi Kikuchi, Ai kariyone, Toshiki Tamura and Kiyoshi Takatsu

$CD8^+$ cytotoxic T cells (CTL) play an important role in the protection against tumor growth. It remains unclear whether $CD4^+$ helper T cells together with CTLs mediate efficient immune responses leading to tumor rejection. As the immunization of C57BL/6 mice with Peptide-25 emulsified in incomplete Freund adjuvant (IFA) induces Th1 response to Peptide-25, we examined adjuvant activity of Peptide-25 for CTL generation to ovalbumin (OVA) as a model tumor antigen. Co-immunization of C57BL/6 mice with OVA and Peptide-25 or Peptide-25 and B16 melanoma peptide (tyrosinase-related protein (TRP)-2) for MHC class I led to a profound increase in $CD8^+$ T cells specific for OVA and TRP -2 peptides, respectively. This heightened response depended on Peptide-25 specific $CD4^+$ T cells and interferon-γ-producing T cells. In tumor protection assays, immunization with Peptide-25 and OVA resulted in the enhancement of $CD8^+$ cytotoxic cell generation specific for OVA and the growth inhibition of EL-4 thymoma expressing OVA peptide leading to the tumor rejection.

To elucidate the mechanisms of this adjuvant activity of Peptide-25, we examined the dendritic cell (DC) activation by P25 TCR Th1 cells in the presence of Peptide-25. When we cocultured DCs with P25 TCR $CD4^+$ T cells together with Peptide-25, expression of MHC class I, CD40, CD86, and ICAM-1 was enhanced and led to induce IL-12p40 production. Such activated DCs showed more effective OVA presentation to OVA specific $CD8^+$ T cells and enhanced proliferation of the cells. IFN-γ is indispensable for the enhanced cross-presentation by P25 TCR $CD4^+$ T cells. We infer from these results that Peptide-25-reactive $CD4^+$ T cells directly activate DC through Peptide-25/MHC class II complex leading to enhanced cross-presentation of antigen to $CD8^+$ T cells.

6. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment

Yoshinori Nagai, Karla P. Garrett[1], Shoichiro Ohta[2], Uleng Bahrun[2], Taku Kouro, Shizuo Akira[3], Kiyoshi Takatsu, Paul W. Kincade[1]: [1]Immunobiology and Cancer Research Program, Oklahoma Medical Research Foundation, OK 73104, [2]Division of Immnunology, Saga Medical School, Saga, Japan, [3]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Toll-like receptors (TLRs) are critical for recognition of microbial components and to the induction of innate immune responses. Activation via TLRs couples innate immunity with the adaptive immunity provided by lymphocytes. Cells responsible for both innate and adaptive immunity have finite lifespans and must be constantly replenished from hematopoietic stem cells (HSCs) and progenitors in bone marrow. Although TLRs on mature immune cells have been well studied, little is known about when maturing cells in bone marrow acquire functional TLRs and whether those receptors influence hematopoietic development.

We report that functional TLRs and their coreceptors are expressed by HSCs and certain other early hematopoietic progenitors. TLR ligation of $Flk\text{-}2^-$ long-term repopulating HSCs *in vitro* induced them to enter the cell cycle. TLR signals bypassed some normal growth and differentiation requirements and drove myeloid differentiation of stem/progenitors. The MyD88 adapter protein was required and TLR signaling caused lymphoid progenitors to become dendritic cells at the expense of B-lymphopoiesis. Finally, lipopolysaccharide (LPS) interacted directly with the TLR4/MD-2 complex on progenitors in bone marrow. Thus, the preferential pathogen-mediated stimulation of myeloid differentiation pathways may provide a means for rapid replenishment of the innate immune system during infection.

Publications

Iwasaki, H., S. Mizuno, Y. Arinobu, H. Shigematsu, K. Takatsu, D.G. Tenen, and K. Akashi. The order of expression of transcription factors directs hierarchical specification of he-

matopoietic lineages. *Genes Dev*. 20: 3010-3021, 2006.

Wakabayashi, Y., M. Kobayashi, S. Akashi-Takamura, N. Tanimura, K. Konno, K. Takahashi, T. Ishii, T. Mizutani, H. Iba, T. Kouro, S. Takaki, K. Takatsu, Y. Oda, Y. Ishihama, S. Saitoh, and K. Miyake. A Protein Associated with Toll-Like Receptor 4 (PRAT4A) Regulates Cell Surface Expression of TLR4. *J. Immunol*. 177: 1772-1779, 2006.

Horikawa, K., and K. Takatsu. Interleukin-5 regulates genes involved in B cell terminal maturation. *Immunology*, 118: 497-508, 2006.

Nagai, Y., K.P. Garrett, S. Ohta, U. Bahrun, T. Kouro, S. Akira, K. Takatsu, and P.W. Kincade. Toll-like receptors on hematopoietic ptogenitor cells stimulate innate immune system replenishment. *Immunity*, 24: 801-812, 2006.

Takizawa, H., C. Kubo-Akashi, I. Nobuhisa, S.-M. Kwon, M. Iseki, T. Taga, K. Takatsu, and S. Takaki. Enhanced engraftment of hematopoietic stem/progenitor cells by the transient inhibition of an adaptor protein, Lnk. *Blood*. 107: 2968-2975, 2006.

Itakura, A., Y. Kikuchi, T. Kouro, M. Ikutani, S. Takaki, P.W. Askenase, and K. Takatsu. Interleukin 5 plays an essential role in elicitation of contact sensitivity through dual effects on eosinophils and B-1 cells. *Int. Arch Allergy Immunol*. 140 (Suppl. 1): 8-16, 2006.

Kikuchi, T., S. Uehara, H. Ariga, T. Tokunaga, A. Kariyone, T. Tamura, and K. Takatsu. Augmented induction of $CD8^+$ cytotoxic T cell response and antitumor resistance by Th1-inducing peptide. *Immunology*, 117: 47-58, 2006.

Buza-Vidas, N., J. Antonchuk, H. Qian, R. Mansson, S. Luc, S. Zandi, K. Anderson, S. Takaki, J.M. Nygren, C.T. Jensen, and S.E. Jacobsen. Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. *Genes Dev*. 20: 2018-2023, 2006.

Li, M., D. Ren, M. Iseki, S. Takaki, and L. Rui. Differential role of SH2-B and APS in regulating energy and glucose homeostasis. *Endocrinology*. 147: 2163-2170, 2006.

Yokota, T., Y. Kawakami, Y. Nagai, Jian-xing Ma, Jen-Yue Tsai, P.W. Kincade, and S. Sato. Bone marrow lacks a transplantable progenitor for smooth muscle type alpha-actin expressing cells. *Stem Cells*, 24: 13-22, 2006.

Pelayo, R., R.S. Welner, Y. Nagai, P.W. Kincade. Life before the pre-B cell receptor checkpoint: Specification and commitment of primitive lymphoid progenitors in adult bone marrow. *Semin. Immunol*., 18: 2-11, 2006.

Igarashi, H., Y. Baba, Y. Nagai, S. Ghosh, P.W. Kincade. NF-κB is dispensable for lymphocyte development in steady bone marrow but required for protection of progenitors from TNFα. *Int. Immunol*., 18: 653-659, 2006.

Yokota, T., J. Huang, M. Tavian, Y. Nagai, J. Hirose, J-C Zuniga-Pflucker, B. Peault, P.W. Kincade. Tracing the first waves of lymphopoiesis in mice. *Development*, 133: 2041-2051, 2006.

Perry, S.S., R.S. Welner, T. Kouro, P.W. Kincade, and X.H. Sun. Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. *J. Immunol*., 177: 2880-2887, 2006

Department of Microbiology and Immunology

Division of Host-Parasite Interaction
宿主寄生体学

Professor	Hideo Iba, Ph.D.	教　授　理学博士	伊　庭　英　夫
Research Associate	Taiji Ito, Ph.D.	助　手　理学博士	伊　藤　太　二
Research Associate	Shigeru Minoguchi, Ph.D.	助　手　医学博士	箕　口　　滋
Research Associate	Taketoshi Mizutani, Ph.D.	助　手　医学博士	水　谷　壮　利

Cellular mechanisms for the surveillance and transcriptional suppression of DNA parasites such as retoroviruses and transposons are now being recognized as an important host cell defense system through epigenetical regulation of chromosome. On the other hand, transcripts of some retrotransposons of plant and yeast are suppressed at the post-transcriptional level by a mechanism designated RNA silencing (or RNA interference), which is sometimes interconnected with transcriptional gene silencing. But it is not clear whether similar mechanisms are operating in human retroviral gene silencing. Our goal is to elucidate the entire cellular defence system and the counteracting viral strategy. These studies would give us new ideas for preventing pathological effects of many human viruses including HIV and HTLV and also for the design of unique retroviral vectors that would achieve long-term transgene expression providing strong tools for human gene therapy and regeneration medicine.

1. Molecular mechanisms of retroviral gene silencing

Since retroviruses that are once integrated into host chromosomes cannot be excised, host cells use epigenetical regulation systems to shut-off virus gene expression. Elucidation of epigenetical suppression mechanisms is therefore essential to understand strategies of both host and virus in this post-genome era. In cytoplasm, DNA methylation, histone acetylation and chromatin remodeling are the major molecular basis for epigenetical regulation. In 2002, we showed that Murine leukemia virus (MLV)-based retrovirus vector transgene expression is rapidly silenced in human tumor cell lines lacking expression of Brm, a catalytic submit of SWI/SNF chromatin remodeling complex, even though these vectors can successfully enter, integrate, and initiate transcription. We detected this gene silencing as a reduction in the ratio of cells expressing the exogenous gene rather than a reduction in the average expression level, indicating that down-regulation occurs in an all-or-none manner. Retroviral gene expression was protected from silencing and maintained in Brm-deficient host cells by exogenous expression of Brm but not BRG1, an alternative ATPase subunit in the SWI/SNF complex. Introduction of exogenous Brm to these cells suppressed recruitment of protein complexes containing YY1 and histone deacetylase (HDAC) 1 and 2 to the 5′-LTR region of the integrated provirus, leading to the enhancement of acetylation of specific lysine residues (Lys-5 and Lys-8) in histone H4 located in this region. These results suggest that the Brm-containing SWI/SNF complex subfamily (trithorax-G) is essential for preventing the recruitment of a complex including YY1, EZH2, EED and HDACs (Polycomb-G) to the 5′-LTR

region to maintain transcription of exogenously introduced genes.

On the other hand, DNA methylation has been shown to mainly contribute to retroviral gene silencing in embryonic stem cells, which has been a serious barrier for retroviral vector to be used as a tools for constructing transgenic animals. Although MLV-LTR can not even start transcription efficiently in mouse ES cells, MSCV, a derivative of MLV, has overcome this barrier by several mutations in LTR as well as the tRNA binding site. This year, we have shown that expression status of MSCV after a long term ES cell culture, is reflecting dynamics of DNA methylation status around the provirus (a).

It is noteworthy that other layers of molecular mechanisms are recently shown to be involved in the gene silencing; some RNA transcripts of exogenous as well as endogenous genes are regulated at post-transcriptional level by a mechanism designated as RNA silencing (RNA interference). Importantly, it has been further suggested that in plant and yeast, chromosomal regulation and RNA silencing are interconnected through a putative pathway designated as RNA -directed transcriptional gene silencing (RdTS). In plants, for example, double-stranded RNA that targets the promoter regions of coding genes causes their transcriptional silencing, and this is associated with the methylation of the proximal DNA sequences. It is known also that double-stranded RNA generated from yeast centromeric transcripts contributes to the formation of heterochromatin within this region. Furthermore, such RNA-dependent transcriptional silencing (RdTS) has been reported to operate in human cell lines transiently transfected with siRNAs.

Since retroviral transcripts have a significant characteristic in that they inevitably contain a sequence corresponding to its own promoter in its 3′-UTR (U3 region), we can speculate enhanced frequencies of dsRNA formation in this region, which could trigger RNA interference. Therefore this year we tested whether retroviral gene silencing could be occurred through RdTS (b).

a) Stochastic and reversible switching of MSCV proviral gene expression reflects dynamics of DNA methylation in ES cells.

Shigeru Minoguchi, Hirotaka Watanabe, Taketoshi Mizutani and Hideo Iba

To examine molecular mechanisms of retroviral gene silencing in mouse embryonic stem (ES) cells, we used a MSCV based-retroviral vector carrying *GFP* as a probe. Unlike its parent, MLV, MSCV has been reported to escape from immediate and strong block of proviral expression in ES cells. Three days after the MSCV-transduction, we sorted each GFP-positive ES cell and clonally propagated them. Most of the cellular clones displayed variegated expression, indicating viral gene expression has suffered stochastic gene silencing in an all-or-none manner during the cloning procedure. We scrutinized three such clones and found that resorted GFP-positive cellular fraction always shows stochastic gene silencing. However dependent upon the clones, GFP-negative fraction can either retain entirely GFP-negative population or can be stochastically reactivated. This indicates that MSCV gene silencing is transient and reversible in at least some integrants. This reactivation was associated with loss of CpG methylation around the proviral 5′-LTR and required effective cell division. From these observations, we conclude that frequent and stochastic failure of maintenance DNA methylation and restoration by *de novo* methylation are dynamically operating in ES cells and further that the balance between them causes stochastic MSCV proviral gene switching in an integration site dependent manner.

The DNA methyltransferase (Dnmt) family proteins are responsible for establishing and maintaining patterns of genomic CpG methylation. Dnmt3L, which is structurally related to the *de novo* DNA methyltransferases, Dnmt3a and Dnmt3b, lacks the catalytic motifs that are characteristic of the DNA methyltransferases, and is enzymatically inactive on its own. To date, Dnmt3L has been proposed to function as a regulator of *de novo* methylation through the direct interaction with Dnmt3a and Dnmt3b. We have very recently found an alternative function of Dnmt3L as a regulator of maintenance methylation in murine ES cells.

b) RNA-dependent transcriptional silencing does not contribute to retroviral gene silencing in human cells

Takeshi Haraguchi, Taketoshi Mizutani and Hideo Iba

It is tempting to speculate that RdTS plays a role in the retroniral gene silencing since all retroviral RNA transcripts harbour a U3 promoter sequence that could potentially provide a good source for double-stranded RNA embedding the promoter sequence. To test this possibility, we constructed several model HeLa cell lines expressing GFP driven by the MLV-LTR. These cells were transduced with HIV-1 based

lentivirus vectors expressing a series of shRNA molecules that target the U3 region of the MLV-LTR. In most instances, transcriptional gene silencing was not induced at all, whereas these shRNAs induced RNA interference on RNA transcripts harbouring the target sequence. These results indicate that RdTS does not generally contribute to MLV gene silencing in host cells.

2. Broad and versatile functions of chromatin remodeling factor SWI/SNF complex in transcription of both coding and non-coding genes.

For the understanding of epigenetics, we have been concentrated on a major human chromatin remodeling factor, SWI/SNF complex, which is composed of 10 protein subunits. Its catalytic subunits, BRG1 and Brm, have ATPase activity with helicase motifs. Each SWI/SNF complex contains a single molecule of either BRG1 or Brm, but not both. In the previous chapter, we showed that Brm but not BRG1 is essential for maintaining retroviral gene expression.

We previously also showed mechanistic links between chromatin remodeling factor SWI/SNF complex and transcriptional factor AP-1, which is composed of heterodimers between Fos family proteins and Jun family proteins. AP-1 is known to play important roles in wide variety of biological function, such as host and viral immediate early responses, cellular growth, differentiation and tumor formation. Our results showed that a specific subset of Fos/Jun dimers (such as c-Fos/c-Jun dimmer) specifically bind to the BAF60a subunit of SWI/SNF complex and recruits the entire complex to the AP-1 DNA binding sites located in a relatively inactive context of chromatin. The recruited SWI/SNF complex remodels flanking nucleosomes to initiate the transcription. From these observations, we have concluded that BAF60a is the major determinant of AP-1 transactivating activity.

Last year, we find that several neuron-specific genes are activated in some non-neuronal cells, specifically when the cells do not express both Brm and BRG1. We have concentrated on the molecular mechanisms underlying this phenomenon this year and found that SWI/SNF complex can exhibit strong transcriptional suppression in a specific chromosomal context and plays rather broad biological function depending upon each promoter of coding genes (a).

A large body of evidence indicates that microRNA and siRNA are the major RNA molecules that are responsible for RNA interference. Unlike siRNA, expression of miRNA are known to be strictly regulated spatiotemporally during mammalian development, it is widely accepted that miRNA play important roles in cellular differentiation. But little is know about how miRNA gene expression itself is regulated, except that miRNA genes are transcribed by RNA polymerase II like the coding genes. This year, in the process of promoter analysis of microRNA genes, we came across SWI/SNF complex again and found that it plays essential roles in the expression of miR-21, which is inducible by PMA treatment (b).

(a) SWI/SNF complex is essential for NRSF-mediated suppression of neural genes in human non-small cell lung carcinoma cell lines.

Hirotaka Watanabe, Taketoshi Mizutani, Takeshi Haraguchi, Nobutake Yamamichi, Shigeru Minoguchi, Mitsue Yamamichi-Nishina, Nozomu Mori[1], and Hideo Iba: [1]Department of Molecular Anatomy and Neurobiology, Nagasaki University School of Medicine, Japan.

We came across unique obserbations that specific neuronal lineage marker genes such as *synaptophysin* and *SCG10* are expressed in several human non-small cell lung carcinoma cell lines deficient in both Brm and BRG1 expression. Exogenous expression of either Brm or BRG1 in these cell lines induced expression of *IL-6* but decreased expression of these neuron-specific genes, indicating that the SWI/SNF complex can function as either a positive or negative regulator in the same cell. In addition, retrovirus vectors expressing siRNAs designed to suppress expression of *Brm, BRG1*, or *Ini1*, which encodes another integral component of the SWI/SNF complex, induced expression of the neuronal genes even in SWI/SNF-competent lung carcinoma cell lines. These results reveal that the SWI/SNF complex is essential to suppress expression of these neuron-specific genes in lung epithelial cells. We present evidence that this suppression requires association of the SWI/SNF complex with a complex that includes neuron-restrictive silencer factor (NRSF), CoREST, mSin3A, and HDAC1/2. This larger complex induced efficient and specific deacetylation of histone H4 in the *synaptophysin* gene, when SWI/SNF complex was recruited to the NRSF binding site by mSin3A and CoREST. Patients with Brm/BRG1-deficient lung carcinoma are reported to have poor prognosis; epigenetic disturbance of these neuron-specific genes could enhance tumorigenicity and possibly provide selective markers for targeting these tumors.

(b) Prediction of miRNA promoter regions conserved among vertebrates: AP-1 triggers the transcription of *miR-21* during macrophage differentiation.

Shuji Fujita, Taiji Ito, Taketoshi Mizutani, Shigeru Minoguchi, Nobutake Yamamichi and Hideo Iba

Little is currently known about how *miRNA* gene expression itself is regulated. In this work, we speculated that if specific miRNA molecules are involved in evolutionally conserved regulatory systems in vertebrates, this would entail a high level of conservation of the miRNA molecule and its promoter. We have here developed an algorithm for predicting the promoter regions of miRNAs (miPPRs; Putative Promoter Regions of miRNAs) in vertebrates and have used this program to detect 49 human miPPRs for 68 miRNA species. In a few cases where the corresponding miRNA promoter information was available, our predicted miPPRs were found to be essentially consistent with previous reports. We further scrutinized the miPPR of miR-21 (miPPR-21) and showed that, unlike the previous report for the miR-21 promoter, miPPR-21 correctly predicted the transcription start site for the only one precursor RNA that embeds miR-21. Moreover, there are several binding sites within miPPR-21 for the AP-1 and PU. 1 transcription factors, which are shown to be responsible for miR-21 induction by PMA. The promoter analysis revealed that c-Fos and c-Jun, which are induced by PMA, bind to these AP-1 DNA binding sites as a dimmer and then recruite SWI/SNF chromatin remodeling complex to the promoter of the *miR-21* gene for the transcriptional initiation. In this process, C/EBP alpha which initially resides in the promoter, dissociates from the promoter. Therefore transcriptional regulatory mechanisms for this noncoding gene are shown to be very similar to those for the coding genes that are involved in the hemopoietic differentiation.

Publications

Watanabe H., Mizutani T., Haraguchi T., Yamamichi N., Minoguchi S., Yamamichi-Nishina M., Mori N., Kameda T., Sugiyama T., and Iba H. SWI/SNF complex is essential for NRSF-mediated suppression of neuronal genes in human nonsmall cell lung carcinoma cell lines. *Oncogene* 25: 470-479 (2006)

Wakabayashi, Y., Kobayashi, M., Akashi-Takamura, S., Tanimura, N., Konno, K., Takahashi, K., Ishii, T., Mizutani, T., Iba, H., Kouro, T., Takaki, S., Takatsu, K., Oda, Y., Ishihama, Y., Saitoh, S-I., Miyake, K.A Protein Associated with Toll-like Receptor 4 (PRAT4 A) Regulates Cell Surface Expression of TLR4. *J. Immunol*, 177: 1772-1779 (2006)

Hasegawa, N., Kawaguchi, H., Hirachi, A., Takeda, K., Mizuno, N., Nishimura, M., Koike, C., Tsuji, K., Iba, H., Kato, Y., and Kurihara, H. Behavior of transplanted bone marrow derived mesenchymal stem cells in periodontal defects. *Journal of Periodontology*, 77: 1003-7 (2006)

水谷壮利，原口健，伊庭英夫「shRNA発現ユニットを搭載したウイルスベクター」遺伝子医学MOOK「RNAと創薬」pp 158−164，2006

Department of Microbiology and Immunology

Division of Virology
ウイルス感染分野

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

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

1. Tyro3 family-mediated cell entry of Ebola and Marburg viruses

Shimojima M, Takada A, Ebihara H, Neumann G, Fujioka K, Irimura T, Jones S, Feldmann H, Kawaoka Y.

Filoviruses, represented by the genera *Ebolavirus* and *Marburgvirus*, cause a lethal hemorrhagic fever in humans and in nonhuman primates. Although filovirus can replicate in various tissues or cell types in these animals, the molecular mechanisms of its broad tropism remain poorly understood. Here we show the involvement of members of the Tyro3 receptor tyrosine kinase family-Axl, Dtk, and Mer-in cell entry of filoviruses. Ectopic expression of these family members in lymphoid cells, which otherwise are highly resistant to filovirus infection, enhanced infection by pseudotype viruses carrying filovirus glycoproteins on their envelopes. This enhancement was reduced by antibodies to Tyro3 family members, Gas6 ligand, or soluble ectodomains of the members. Live Ebola viruses infected both Axl-and Dtk-expressing cells more efficiently than control cells. Antibody to Axl inhibited infection of pseudotype viruses in a number of Axl-positive cell lines. These results implicate each Tyro3 family member as a cell entry factor in filovirus infection.

2. The development and characterization of H 5 influenza virus vaccines derived from a 2003 human isolate

Horimoto T, Takada A, Fujii K, Goto H, Hatta M, Watanabe S, Iwatsuki-Horimoto K, Ito M, Tagawa-Sakai Y, Yamada S, Ito H, Ito T, Imai M, Itamura S, Odagiri T, Tashiro M, Lim W, Guan Y, Peiris M, Kawaoka Y.

The pandemic threat posed by highly pathogenic H5N1 influenza A viruses has created an urgent need for vaccines to protect against H5 virus infection. Because pathogenic viruses grow poorly in chicken eggs and their virulence poses a biohazard to vaccine producers, avirulent viruses produced by reverse genetics have become

the preferred basis for vaccine production. Here, we investigated two key characteristics of potential H5 vaccine candidates: the hemaggutinin (HA) cleavage site sequence and its modification to attenuate virulence and the choice of background virus to provide a high-growth rate. We produced recombinant (6:2 reassortant) viruses that possessed a series of modified avirulent-type HA and neuraminidase genes, both of which were derived from an H5N1 human isolate. The other genes of these recombinant viruses were derived from donor virus strains known to grow well in eggs: the human strain A/Puerto Rico/8/34 (PR8) or an avian strain. All of the recombinant viruses grew well in eggs, were avirulent in chicks, and protected animals against infection with a wild-type virus. However, one of the recombinant viruses with an avian virus background acquired a mutation in the HA cleavage site sequence that conferred virulence potential to this virus. Moreover, vaccine candidates with the avian virus background were more virulent than those with the human virus background. We conclude that 6:2 recombinant viruses with a PR8 background are more suitable than those with an avian virus background for vaccine development and that the HA cleavage site sequence must be modified to minimize the potential for a vaccine virus to convert to a virulent form.

3. Hierarchy among vRNA segments in their role in vRNA incorporation into influenza A virions

Muramoto Y, Takada A, Fujii K, Noda T, Iwatsuki-Horimoto K, Watanabe S, Horimoto T, Kida H, Kawaoka Y.

The genome of influenza A viruses comprises eight negative-strand RNA segments. Although all eight segments must be present in cells for efficient viral replication, the mechanism (s) by which these viral RNA (vRNA) segments are incorporated into virions is not fully understood. We recently found that sequences at both ends of the coding regions of the HA, NA, and NS vRNA segments of A/WSN/33 play important roles in the incorporation of these vRNAs into virions. In order to similarly identify the regions of the PB2, PB1, and PA vRNAs of this strain that are critical for their incorporation, we generated a series of mutant vRNAs that possessed the green fluorescent protein gene flanked by portions of the coding and noncoding regions of the respective segments. For all three polymerase segments, deletions at the ends of their coding regions decreased their virion incorporation efficiencies. More importantly, these regions not only affected the incorporation of the segment in which they reside, but were also important for the incorporation of other segments. This effect was most prominent with the PB2 vRNA. These findings suggest a hierarchy among vRNA segments for virion incorporation and may imply intersegment association of vRNAs during virus assembly.

4. The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly

Iwatsuki-Horimoto K, Horimoto T, Noda T, Kiso M, Maeda J, Watanabe S, Muramoto Y, Fujii K, Kawaoka Y.

The viral replication cycle concludes with the assembly of viral components to form progeny virions. For influenza A viruses, the matrix M1 protein and two membrane integral glycoproteins, hemagglutinin and neuraminidase, function cooperatively in this process. Here, we asked whether another membrane protein, the M2 protein, plays a role in virus assembly. The M2 protein, comprising 97 amino acids, possesses the longest cytoplasmic tail (54 residues) of the three transmembrane proteins of influenza A viruses. We therefore generated a series of deletion mutants of the M2 cytoplasmic tail by reverse genetics. We found that mutants in which more than 22 amino acids were deleted from the carboxyl terminus of the M2 tail were viable but grew less efficiently than did the wild -type virus. An analysis of the virions suggested that viruses with M2 tail deletions of more than 22 carboxy-terminal residues apparently contained less viral ribonucleoprotein complex than did the wild-type virus. These M2 tail mutants also differ from the wild-type virus in their morphology: while the wild-type virus is spherical, some of the mutants were filamentous. Alanine-scanning experiments further indicated that amino acids at positions 74 to 79 of the M2 tail play a role in virion morphogenesis and affect viral infectivity. We conclude that the M2 cytoplasmic domain of influenza A viruses plays an important role in viral assembly and morphogenesis.

5. Molecular determinants of Ebola virus virulence in mice

Ebihara H, Takada A, Kobasa D, Jones S, Neumann G, Theriault S, Bray M, Feldmann H, Kawaoka Y.

Zaire ebolavirus (ZEBOV) causes severe hemorrhagic fever in humans and nonhuman pri-

mates, with fatality rates in humans of up to 90%. The molecular basis for the extreme virulence of ZEBOV remains elusive. While adult mice resist ZEBOV infection, the Mayinga strain of the virus has been adapted to cause lethal infection in these animals. To understand the pathogenesis underlying the extreme virulence of Ebola virus (EBOV), here we identified the mutations responsible for the acquisition of the high virulence of the adapted Mayinga strain in mice, by using reverse genetics. We found that mutations in viral protein 24 and in the nucleoprotein were primarily responsible for the acquisition of high virulence. Moreover, the role of these proteins in virulence correlated with their ability to evade type I interferon-stimulated antiviral responses. These findings suggest a critical role for overcoming the interferon-induced antiviral state in the pathogenicity of EBOV and offer new insights into the pathogenesis of EBOV infection.

6. Assembly and budding of Ebolavirus

Noda T, Ebihara H, Muramoto Y, Fujii K, Takada A, Sagara H, Kim JH, Kida H, Feldmann H, Kawaoka Y.

Ebolavirus is responsible for highly lethal hemorrhagic fever. Like all viruses, it must reproduce its various components and assemble them in cells in order to reproduce infectious virions and perpetuate itself. To generate infectious Ebolavirus, a viral genome-protein complex called the nucleocapsid (NC) must be produced and transported to the cell surface, incorporated into virions, and then released from cells. To further our understanding of the Ebolavirus life cycle, we expressed the various viral proteins in mammalian cells and examined them ultrastructurally and biochemically. Expression of nucleoprotein alone led to the formation of helical tubes, which likely serve as a core for the NC. The matrix protein VP40 was found to be critical for transport of NCs to the cell surface and for the incorporation of NCs into virions, where interaction between nucleoprotein and the matrix protein VP40 is likely essential for these processes. Examination of virus-infected cells revealed that virions containing NCs mainly emerge horizontally from the cell surface, whereas empty virions mainly bud vertically, suggesting that horizontal budding is the major mode of Ebolavirus budding. These data form a foundation for the identification and development of potential antiviral agents to combat the devastating disease caused by this virus.

7. Architecture of ribonucleoprotein complexes in influenza A virus particles

Noda T, Sagara H, Yen A, Takada A, Kida A, Cheng RH, Kawaoka Y.

In viruses, as in eukaryotes, elaborate mechanisms have evolved to protect the genome and to ensure its timely replication and reliable transmission to progeny. Influenza A viruses are enveloped, spherical or filamentous structures, ranging from 80 to 120 nm in diameter. Inside each envelope is a viral genome consisting of eight single-stranded negative-sense RNA segments of 890 to 2,341 nucleotides each. These segments are associated with nucleoprotein and three polymerase subunits, designated PA, PB1 and PB2; the resultant ribonucleoprotein complexes (RNPs) resemble a twisted rod (10-15 nm in width and 30-120 nm in length) that is folded back and coiled on itself. Late in viral infection, newly synthesized RNPs are transported from the nucleus to the plasma membrane, where they are incorporated into progeny virions capable of infecting other cells. Here we show, by transmission electron microscopy of serially sectioned virions, that the RNPs of influenza A virus are organized in a distinct pattern (seven segments of different lengths surrounding a central segment). The individual RNPs are suspended from the interior of the viral envelope at the distal end of the budding virion and are oriented perpendicular to the budding tip. This finding argues against random incorporation of RNPs into virions, supporting instead a model in which each segment contains specific incorporation signals that enable the RNPs to be recruited and packaged as a complete set. A selective mechanism of RNP incorporation into virions and the unique organization of the eight RNP segments may be crucial to maintaining the integrity of the viral genome during repeated cycles of replication.

8. Influenza virus receptors in the human airway

Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y.

Although more than 100 people have been infected by H5N1 influenza A viruses, human-to-human transmission is rare. What are the molecular barriers limiting human-to-human transmission? Here we demonstrate an anatomical difference in the distribution in the human airway of the different binding molecules preferred by the avian and human influenza viruses. The respective molecules are sialic acid linked to ga-

lactose by an alpha-2,3 linkage (SAalpha2,3Gal) and by an alpha-2, 6 linkage (SAalpha2, 6Gal). Our findings may provide a rational explanation for why H5N1 viruses at present rarely infect and spread between humans although they can replicate efficiently in the lungs.

9. Hemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors

Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso K, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y.

H5N1 influenza A viruses have spread to numerous countries in Asia, Europe and Africa, infecting not only large numbers of poultry, but also an increasing number of humans, often with lethal effects. Human and avian influenza A viruses differ in their recognition of host cell receptors: the former preferentially recognize receptors with saccharides terminating in sialic acid-alpha2,6-galactose (SAalpha2,6Gal), whereas the latter prefer those ending in SAalpha2,3 Gal. A conversion from SAalpha2,3Gal to SAalpha2,6Ga recognition is thought to be one of the changes that must occur before avian influenza viruses can replicate efficiently in humans and acquire the potential to cause a pandemic. By identifying mutations in the receptor-binding haemagglutinin (HA) molecule that would enable avian H5N1 viruses to recognize human-type host cell receptors, it may be possible to predict (and thus to increase preparedness for) the emergence of pandemic viruses. Here we show that some H5N1 viruses isolated from humans can bind to both human and avian receptors, in contrast to those isolated from chickens and ducks, which recognize the avian receptors exclusively. Mutations at positions 182 and 192 independently convert the HAs of H5N1 viruses known to recognize the avian receptor to ones that recognize the human receptor. Analysis of the crystal structure of the HA from an H5N1 virus used in our genetic experiments shows that the locations of these amino acids in the HA molecule are compatible with an effect on receptor binding. The amino acid changes that we identify might serve as molecular markers for assessing the pandemic potential of H5N1 field isolates.

Publications

Original papers

Shimojima M, Takada A, Ebihara H, Neumann G, Fujioka K, Irimura T, Jones S, Feldmann H, Kawaoka Y. Tyro3 family-mediated cell entry of Ebola and Marburg viruses. J Virol 80: 10109-10116, 2006.

Horimoto T, Takada A, Fujii K, Goto H, Hatta M, Watanabe S, Iwatsuki-Horimoto K, Ito M, Tagawa-Sakai Y, Yamada S, Ito H, Ito T, Imai M, Itamura S, Odagiri T, Tashiro M, Lim W, Guan Y, Peiris M, Kawaoka Y. The development and characterization of H5 influenza virus vaccines derived from a 2003 human isolate. Vaccine 24: 3669-3676, 2006.

Muramoto Y, Ozaki H, Takada A, Park CH, Sunden Y, Umemura T, Kawaoka Y, Matsuda H, Kida H. Highly pathogenic H5N1 influenza virus causes coagulopathy in chickens. Microbiol Immunol 50: 73-81, 2006.

Urata S, Noda T, Kawaoka Y, Yokosawa H, Yasuda J. Cellular factors required for Lassa virus budding. J Virol 80: 4191-4195, 2006.

Watanabe S, Noda T, Kawaoka Y. Functional mapping of the nucleoprotein of Ebola virus. J Virol 80: 3743-3751, 2006.

Muramoto Y, Takada A, Fujii K, Noda T, Iwatsuki-Horimoto K, Watanabe S, Horimoto T, Kida H, Kawaoka Y. Hierarchy among vRNA segments in their role in vRNA incorporation into influenza A virions. J Virol 80: 2318-25, 2006.

Bär S, Takada A, Kawaoka Y, Alizon M. Detection of cell-cell fusion mediated by the Ebola virus glycoproteins. J Virol 80: 2815-22, 2006.

Iwatsuki-Horimoto K, Horimoto T, Noda T, Kiso M, Maeda J, Watanabe S, Muramoto Y, Fujii K, Kawaoka Y. The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. J Virol 80: 5233-5240, 2006.

Chen H, Li Y, Li Z, Shi J, Shinya K, Deng G, Qi Q, Tian G, Fan S, Zhao H, Sun Y, Kawaoka Y. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J Virol 80: 5976-5983, 2006.

Muramoto Y, Le TQ, Phuong LS, Nguyen T, Nguyen TH, Sakai-Tagawa Y, Iwatsuki-Horimoto K, Horimoto T, Kida H, Kawaoka Y. Molecular characterization of the hemagglutinin and neuraminidase genes of H5N1 influenza A viruses isolated from poultry in Vietnam from 2004 to 2005. J Vet Med Sci 68: 527-531, 2006.

Kogure T, Suzuki T, Takahashi T, Miyamoto D,

Hidari KI, Guo CT, Ito T, Kawaoka Y, Suzuki Y. Human trachea primary epithelial cells express both sialyl (alpha2-3) Gal receptor for human parainfluenza virus type 1 and avian influenza viruses, and sialyl (α2-6) Gal receptor for human influenza viruses. Glycoconj J 23: 101-106, 2006.

Co DO, Hogan LH, Karman J, Heninger E, Vang S, Wells K, Kawaoka Y, Sandor M. Interactions between T cells responding to concurrent mycobacterial and influenza infections. J Immunol 177: 8456-8465, 2006.

Ebihara H, Takada A, Kobasa D, Jones S, Neumann G, Theriault S, Bray M, Feldmann H, Kawaoka Y. Molecular determinants of Ebola virus virulence in mice. PLoS Pathog 2: 705-711, 2006.

Noda T, Ebihara H, Muramoto Y, Fujii K, Takada A, Sagara H, Kim JH, Kida H, Feldmann H, Kawaoka Y. Assembly and budding of Ebolavirus. PLoS Pathog 2: 864-872, 2006.

Noda T, Sagara H, Yen A, Takada A, Kida A, Cheng RH, Kawaoka Y. Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature 439: 490-492, 2006.

Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Influenza virus receptors in the human airway. Nature 440: 435-436, 2006.

Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso K, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y. Hemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 444: 378-382, 2006.

Reviews

Neumann G, Kawaoka Y. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg Infect Dis 12: 881-886, 2006.

Horimoto T, Kawaoka Y. Strategies for developing vaccines against H5N1 influenza A viruses. Trends Mol Med 12: 506-154, 2006.

Department of Microbiology and Immunology

Division of Infectious Genetics
感染遺伝学分野

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

Our research main focuses on molecular mechanism underlying innate pathogen recognition by the Toll family of receptors, a family of pathogen sensor. Previously, MD-2, a coreceptor for endotoxin recognition, was discovered. We, this year, reported another molecule, PRAT4A, that regulates the subcellular distribution and resonsiveness of multiple TLRs. Further study on this molecule would reveal how immune cells use pathogen sensors to sense a pathogen and to determine a type of defense responses.

1. A Protein Associated with Toll-like Receptor 4 (PRAT4A) Regulates Cell Surface Expression of TLR4[1]

Yasutaka Wakabayashi, Makiko Kobayashi, Sachiko Akashi-Takamura, Natsuko Tanimura, Kazunori Konno, Koichiro Takahashi, Takashi Ishii, Taketoshi Mizutani[†], Hideo Iba[†], Taku Kouro[†], Satoshi Takaki[‡], Kiyoshi Takatsu[‡], Yoshiya Oda, § Yasushi Ishihama[§], Shin-ichiroh Saitoh, and Kensuke Miyake¶[3] [†]Division of Host-Parasite Interaction, and [‡]Division of Immunology, at The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [§]Laboratory of Seeds Finding Technology, Eisai Co. Ltd., Tsukuba, Japan; [¶]CREST, Japan Science and Technology Corporation, Tokyo, Japan

TLRs recognize microbial products. Their subcellular distribution is optimized for microbial recognition. Little is known, however, about mechanisms regulating the subcellular distribution of TLRs. Lipopolysaccharide (LPS) is recognized by the receptor complex consisting of TLR 4 and MD-2. Although MD-2, a coreceptor for TLR4, enhances cell surface expression of TLR4, an additional mechanism regulating TLR4 distribution has been suggested. We here show that a novel protein associated with TLR4 (PRAT4A) regulates cell surface expression of TLR4. PRAT 4A is associated with the immature form of TLR 4 but not with MD-2 or TLR2. PRAT4A knockdown abolished LPS responsiveness in a cell line expressing TLR4/MD-2 probably due to the lack of cell surface TLR4. PRAT4A knockdown downregulated cell surface TLR4/MD-2 on dendritic cells. These results demonstrate a novel mechanism regulating TLR4/MD-2 expression on the cell surface.

2. A protein associated with Toll-like receptor 4 (PRAT4A) coordinately regulates responsiveness of multiple Toll-like receptors

Koichiro Takahashi, Sachiko Akashi-Takamura, Takuma Shibata, Yasutaka Wakabayashi, Natsuko Tanimura, Toshihiko Kobayashi, Fumi Matsumoto, Ryutaroh Fukui, Taku Kouro[2], Kiyoshi Takatsu[2], Shin-ichiroh Saitoh, and Kensuke Miyake[1,3] [2]Division of Immunology, at The Institute of Medical Science, The

University of Tokyo, Tokyo, Japan; [3]CREST, Japan Science and Technology Corporation, Tokyo, Japan

Toll-like receptors (TLRs) serve as pathogen sensors in the immune system. TLRs sensing microbial membrane reside on the cell surface, whereas nucleic acid-sensing TLR9 reside in ER. Multiple TLRs work in concert to sense a pathogen and to mount defense responses. Little is known, however, about a mechanism coordinately regulating responsiveness of multiple TLRs. We here show that a protein associated with TLR4 (PRAT4A) is also associated with TLR1 and TLR9. PRAT4A gene silencing impaired cell surface expression of TLR1 and TLR 4, and ligand-induced TLR9 trafficking from ER, leading to hyporesponsiveness to all their ligands. PRAT4A suggests a mechanism coordinating responsiveness of cell surface TLRs and intracellular TLR9 through controlling their subcellular distribution.

3. Regulatory Roles for MD-2 and Toll-like receptor 4 (TLR4) in Ligand-induced Receptor Clustering

Makiko Kobayashi[1], Shin-ichiroh Saitoh[1], Natsuko Tanimura[1], Koh-ichiroh Takahashi[1], Kiyoshi Kawasaki[2], Masahiro Nishijima[2],Yukari Fujimoto[3], Koichi Fukase[3], Sachiko Akashi-Takamura[1], and Kensuke Miyake[1,4]: [1]Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [2]Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan; [3]Department of Chemistry, Graduate School of Science, Osaka University; [4]CREST, Japan Science and Technology Corporation, Tokyo, Japan

Lipopolysaccharide (LPS), a principal membrane component in Gram-negative bacteria, is recognized by a receptor complex consisting of Toll-like receptor 4 and MD-2. MD-2 is an extracellular molecule that is associated with the extracellular domain of TLR4 and has a critical role in LPS recognition. MD-2 directly interacts with LPS and the region from Phe119 to Lys132 (Arg132 in mice) has been shown to be important for interaction between LPS and TLR4/MD-2. The other LPS association molecules CD14 and LPS binding protein (LBP) are reqired for LPS transfer to TLR4/MD-2. After LPS association with TLR4/MD-2, TLR4 induces activation of two important signaling pathways which are MyD88 dependent and MyD88 independent. Two TIR-domain containing adaptor molecules TRAM and TRIF play a essential role for MyD88 independent pathway which induces type I interferon production. It has been reported these days that the type I interferon production depend on CD14 and LBP. LPS-signaling is thought to be triggered by ligand-induced TLR4 -clustering, which is also regulated by MD-2. Little is known, however, about mechanism of CD14 dependent type I interferon production and ligand-induced receptor clustering. MD-2 mutants substituting alanine for Phe126 or Gly 129 impaired LPS-induced TLR4-clustering but not LPS binding to TLR4/MD-2, demonstrating that ligand-induced receptor clustering is differentially regulated by MD-2 from ligand-binding. These results support a principal role for MD-2 in LPS recognition and TLR4clustering. Wetry to find out how TLR4 controls type I interferon production using MD−2 mutants.

4. Characterization of TLR4-mediated signaling by malarial peroxiredoxin

Takahisa Furuta and Naohiro Watanabe[1]: Division of Infectious Genetics, Institute of medical Science, University of Tokyo, Department of Tropical Medicine, Jikei University School of Medicine, Tokyo 105-8461, Japan.

Malarial 2-cys peroxiredoxin (Prx) constitutes about 0.25% of the total protein in trophozoites and is likely used as one enzyme to reduce peroxides. So far we demonstrated that Prx is expressed during the infection and induces not only acquired immunity but also innate immunity via Toll-like receptors 4 (TLR4) in malaria. In the recognition of LPS by TLR4, TLR4 requires association with MD-2, and a complex of TLR4/MD-2 directly binds LPS. To examine the possibility that the association with MD-2 is involved in recognition of Prx by TLR4, we stimulated peritoneal macrophages from MD-$2^{-/-}$ or wild-type (WT) mice by Prx to compare TNF production. TNF production in macrophages from MD-$2^{-/-}$ mice was significantly lower than that in WT mice, suggesting that MD-2 was essential in the recognition of Prx by TLR4. MyD 88 is critical for the signaling from all TLRs except TLR3, and stimulation of TLR4 facilitates MyD88-depedant and MyD88-independent pathway. In the next study, we examined the activation of MyD88-dependent and MyD88-independent pathway by Prx. Peritoneal macrophages from C57BL/6 mice were stimulated in vitro by Prx, then degradation of IRAK-1 and phosphorylation of IRF3 were examined by immunoblot using cellular extracts. Since degradation of IRAK-1 and phosphorylation of IRF3 were observed, both signaling pathways occurred in murine malaria. To examine direct rec-

ognition of TLR4 with Prx, beads coupled with TLR4/MD-2 or TLR2 were immunoprecipitated with Prx, and then precipitated proteins were subjected to SDS-page and immunoblot. The precipitated band recognized by anti-Prx specific antibody was associated with TLR4/MD-2 but not TLR2. From the findings mentioned above, Prx is directly recognized with TLR4/MD-2 and then stimulates MyD88-dependent and MyD88-independent signaling pathways in murine malaria.

5. Regulatory role of CD14 in Ligand-Induced Receptor re-localization

Shin-Ichiroh Saitoh[1], Natsuko Tanimura[1], Yuji Motoi[1], Ryutaro Fukui[1], Fumi Matsumoto[1], CaiHua Xuan[1], Mason W Freeman[2], Kensuke Miyake[1,3]; [1]Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [2]Lipid Metabolism Unit, Massachusetts General Hospital, Harvard Medical School; [3]CREST, Japan Science and Technology Corporation, Tokyo, Japan

Toll-like receptor 4 (TLR4) and MD-2 are an important role for recognizing the bacterial endotoxin lipopolysaccharide (LPS) as a ligand. MD-2 is an extracellular molecule that is associated with the extracellular domain of TLR4 constitutively and binds LPS. After LPS association on MD-2, TLR4/MD-2 clusters and activates signaling molecules. TLR4/MD-2 localizes mainly Golgi and cell surface membrane before LPS stimulation. After stimulation of peritoneal macrophage with LPS, surface expression of TLR4/MD-2 stained with anti-TLR4/MD-2 antibody decreased. However surface expression of TLR4/MD-2 did not decrease on peritoneal macrophage from CD14KO mice after LPS stimulation. To confirm the endocytosis of TLR4/MD-2 by confocal imaging Ba/F3 cells expressing TLR4GFP and MD-2 with or without CD14 were stimulated with LPS. LPS stimulation induced TLR4 endocytosis into lysosome and promoted aggregation of TLR4 in patches on lysosome. However TLR4 re-localization after LPS stimulation was not observed on Ba/F3 cell without CD14. Alexa568 labeled LPS was also endocytosed via CD14 into lysosome and colocalized with TLR4. These results suggest that TLR4 re-localization and colocalization with LPS depend on CD14.

Publications

Akashi-Takamura S, Miyake K. Toll-like receptors (TLRs) and immune disorders. J Infect Chemother. 2006 Oct; 12 (5): 233-40. Epub 2006 Nov 6.

Wakabayashi Y, Kobayashi M, Akashi-Takamura S, Tanimura N, Konno K, Takahashi K, Ishii T, Mizutani T, Iba H, Kouro T, Takaki S, Takatsu K, Oda Y, Ishihama Y, Saitoh S, Miyake K.A protein associated with toll-like receptor 4 (PRAT4A) regulates cell surface expression of TLR4. J Immunol. 2006 Aug 1; 177 (3): 1772-9.

Kobayashi M, Saitoh S, Tanimura N, Takahashi K, Kawasaki K, Nishijima M, Fujimoto Y, Fukase K, Akashi-Takamura S, Miyake K. Regulatory roles for MD-2 and TLR4 in ligand-induced receptor clustering. J Immunol. 2006 May 15; 176 (10): 6211-8.

Akashi-Takamura S, Furuta T, Takahashi K, Tanimura N, Kusumoto Y, Kobayashi T, Saitoh S, Adachi Y, Doi T, Miyake K. Agonistic antibody to TLR4/MD-2 protects mice from acute lethal hepatitis induced by TNF-alpha. J Immunol. 2006 Apr 1; 176 (7): 4244-51.

Furuta, T., Kikuchi, T., Iwakura, Y., and Watanabe, N. (2006). Protective roles of mast cells and mast cell-derived TNF in murine malaria. J. Immunol., 177: 3294-3302.

Furuta, T., Takane Kikuchi, T., Shizuo Akira, S., Watanabe, N., and Yoshikawa, Y. (2006). Roles of the small intestine for induction of Toll-like receptor 4-mediated innate resistance in naturally acquired murine toxoplasmosis. Int. Immunol. 18: 1655-1662.

Youn HS, Lee JY, Saitoh SI, Miyake K, Hwang DH. Auranofin, as an anti-rheumatic gold compound, suppresses LPS-induced homodimerization of TLR4. Biochem Biophys Res Commun Dec 1; 350 (4): 866-71., 2006

Youn HS, Lee JY, Saitoh SI, Miyake K, Hwang DH. Suppression of MyD88-and TRIF-dependent signaling pathways of toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea. Biochem Pharmacol. 72 (7): 850-859, 2006

Youn HS, Saitoh SI, Miyake K, Hwang DH. Inhibition of homodimerization of Toll-like receptor 4 by curcumin. Biochem Pharmacol. 72 (1): 62-69, 2006

Tanimura N, Saitoh S, Kawano S, Kosugi A, Miyake K. Palmitoylation of LAT contributes to its subcellular localization and stability. Biochem Biophys Res Commun. 341 (4): 1177-1183, 2006

Konno K, Wakabayashi, Akashi-Takamura S, Ishii T, Kobayashi M, Takahashi K, Kusumoto

Y, Saitoh S, Yoshizawa Y, Miyake K.A molecule that is associated with Toll-like receptor 4 and regulates its cell surface expression. Biochem Biophys Res Commun. 339 (4): 1076-1082, 2006

Department of Microbiology and Immunology

Division of Mucosal Immunology
炎症免疫学分野

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

The mucosal immune system not only acts as a first line of defense against microbial infection, but also contributes to the maintenance of immunological homeostasis. The goal of our research is to understand the molecular and cellular aspects of the mucosal immune system and their contribution to the host defense and homeostasis against infectious diseases, inflammation and immunological disorders. Further, we also aim to apply these fundamental findings to the development of mucosal vaccine and mucosal immunotherapy.

1. M cell Biology

Osamu Igarashi, Kazutaka Terahara, Tomonori Nochi, Satoshi Fukuyama, Takaharu Taketomi, Yoshiyuki Gotoh, Takashi Obata, Masato Yoshida, Shiho Kurokawa, Naoko Takayama, Yoshikazu Yuki and Hiroshi Kiyono: Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo

M cells, specialized antigen-sampling cells, are located within follicle-associated epithelium (FAE) covering Peyer's patches (PP) and nasopharynx-associated lymphoid tissues (NALT), and villous epithelium covering small intestinal lamina propria. They can transport luminal antigens to proximal immunocompetent cells for the initiation of acquired immunity, and thus are ideal targets for vaccine delivery. For better understanding of M cell biology, we first identified 8 molecules as M cell-specific genes, two of which (peptideglycan-recognition protein-S: PGRP-S and MARCKS-like protein: MLP) were further confirmed to be M cell-specific at protein level. These molecules were intracellular proteins and therefore applied for the generation of KO mice to assess their functional relevance in M cells. Another success in the last year was establishment of a monoclonal antibody (mAb) NKM16-2-4 that bound specifically to and was taken up from the surfaces of FAE- and villous M cells. When coupled with model vaccine antigens such as influenza H5N1 or tetanus toxoid, and administered via oral or nasal route, this mAb dramatically reduced threshold doses of the antigens required for the antigen-specific antibody production, promising its usefulness as a vaccine delivery vehicle. Further, for practical application, we cloned and sequenced cDNA encoding the immunoglobulin heavy and light chains, and produced recombinant form of the single-chain variable fragment (scFv) antibody by bacterial expression system. This recombinant scFv antibody bound specifically to M cell surface. Thus, our current efforts are aiming at production of recombinant vaccine antigens genetically linked with the scFv antibody.

One of the characteristics of M cells is expression of a terminally fucosylated glycan, which is recognized by lectin UEA-I. We recently found

that this glyco-epitope was inducible in villous epithelial cells by treatment of mice with inflammatory agents such as sodium dextran sulfate and indomethacin, and a mucosal adjuvant cholera toxin. Unlike villous M cells we discovered previously, these fucosylated epithelial cells were morphologically enterocytes, but exhibited an enhanced antigen-uptake ability, and therefore we named them acquired villous M cells. These findings thus provide helpful information for our better understanding of M cell biology. Our efforts are currently aiming at elucidating the molecular bases by which acquired M cells develop.

2. Development of Mucosal Vaccine

Yoshikazu Yuki[1], Tomonori Nochi[1], Hidenori Takagi[2], Lijun Yang[2], Takehiro Masumura[3], Mio Mejima[1], Ushio Nakanishi[1], Akiko Matsumura[1], Akihiro Uozumi[1], Takachika Hiroi[1], Shigeto Morita[3], Kunisuke Tanaka[3], Fumio Takaiwa[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]Plant Biotechnology Department, National Institute of Agrobiological Sciences, [3]Laboratory of Genetic Engineering, Graduate School of Agriculture, Kyoto Prefectural University

Mucosal vaccination can induce antigen specific immune responses in both systemic and mucosal compartments, and thus considered an ideal immunization for the control of infectious diseases. In this time, we developed a rice-based vaccine expressing cholera toxin B subunit (CTB) under the control of the endosperm specific expression promoter for expression in rice seed. In average, 30 μg of CTB per seed was accumulated into the protein body as a rice storage organelle. Orally immunized rice seed expressing CTB were taken up by M cells and induced toxin-specific serum IgG and mucosal IgA antibodies with neutralizing activity. CTB expressed in rice was protected from pepsin digestion *in vitro* and stable at room temperature for more than 18 months. Furthermore, a much lower dose of rice-based CTB than of purified recombinant CTB is required for the induction of antigen-specific mucosal immune responses. Although we have used CTB as an example antigen for demonstrating the usefulness and attractiveness of rice transgenic expression system for oral vaccine development, one can assume that rice-based CTB expression system can be used for the oral vaccine delivery vehicle. To this end, our current efforts are aimed at the development of rice-based vaccine expressing other vaccine candidate antigens.

3. NALT Organogenesis

Satoshi Fukuyama, Takahiro Nagatake, Kaoru Takamura, Dong-Young Kim, Aya Kawamura, and Hiroshi Kiyono: Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute for Medical Science, The University of Tokyo

NALT (nasopharynx-associated lymphoid tissue) is considered to be a key targeted lymphoid organ to induce mucosal and systemic immune responses. Therefore, in order to develop effective nasal vaccination, it is important to elucidate the characteristics of NALT immunity. Our findings demonstrated that NALT organogenesis is independent of lymphotoxin (LT) β receptor signaling pathway, which have been shown to play a pivotal role for the organogenesis program of secondary lymphoid tissue development. $CD3^{-}CD4^{+}CD45^{+}$ cells which are differentiated from fetal liver progenitors induce the organogenesis of secondary lymphoid tissues (e. g., NALT, PP, and lymph node). CXCL13 and its ligand CXCR5 are essential for the induction of PP organogenesis by $CD3^{-}CD4^{+}CD45^{+}$ cells. However, our study demonstrated that lymphoid chemokines (e.g., CXCL13, CCL19 and CCL21) were not involved in the accumulation of $CD3^{-}CD4^{+}CD45^{+}$ cells at NALT anlagen. Otherwise, the maturation of NALT structure required lymphoid chemokines. In spite of disorganized NALT microarchitecture, antigen-specific mucosal and systemic immune responses were not impaired in nasally-immunized $CXCL13^{-/-}$ mice. Further, we identified nasal passage M cells as new gateway in upper respiratory tract. These findings indicate the involvement of NALT independent mucosal immune system in $CXCL13^{-/-}$ mice. Thus, our current efforts are aimed at the identification of NALT specific signaling molecules for NALT-genesis and the analysis of characterization of nasal passage M cell.

4. Mucosal allergy

Naoko Takayama[1], Osamu Igarashi[1], Mi-Na Kweon[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]Mucosal Immunology Section, International Vaccine Institute, Seoul, Korea

Generally, intestinal allergic disease is characterized by severe diarrhea with elevated Th2 cy-

tokine and allergen-specific IgE responses. Our food allergic model revealed that systemically primed BALB/c mice develop severe diarrhea after repeated oral administration of high dose of ovalbumin (OVA). Since Peyer's patches (PP) have been shown to be a key site for the initiation and regulation of antigen-specific mucosal immune responses, we examined the role of PP for the development of OVA-induced intestinal allergy using anti-IL-7Rα treatment system. PP-intact and -null mice were primed by subcutaneous injection with 1mg OVA in 100μl of complete Freurd's adjuvant (CFA). One week after the priming, mice were orally challenged with 10mg of OVA at three times per week for several weeks. Lymphocytes were isolated from different mucosal tissues and then re-stimulated with 1mg of OVA for the characterization of cytokine synthesis and cell surface phenotype. When mice were orally challenged with 10mg of OVA after systemic priming, the symptom of allergic diarrhea was induced much faster in PP-null mice than PP-intact mice. More strikingly, we observed that the clinical symptoms of intestinal allergy including diarrhea were induced in PP-null mice but not PP-intact mice when low dose of OVA was used for oral challenge. When PP lymphocytes from the diseased mice were examined for cytokine synthesis, elevated IL-10 production was noted after OVA stimulation. Furthermore, the number of $CD4^+CD25^+$ T cells in the PP was also increased. These findings suggest that IL-10 producing regulatory T cells are induced in PP of mice with allergic diarrhea. Thus, the removal of PP may lead to the acceleration of allergic diarrhea due to the lack of $CD4^+CD25^+$ regulatory T cell induction.

5. Mucosal Trafficking

Jun Kunisawa, Yosuke Kurashima, Masashi Gohda, Morio Higuchi, Fumi Miura, Izumi Ishikawa, Ikuko Ogahara, and Hiroshi Kiyono: Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute for Medical Science, The University of Tokyo

It is generally considered that lymphocyte trafficking is regulated by chemokine and adhesion molecules. In addition to these molecules, recent studies have indicated that sphingosine 1-phosphate (S1P), a lipid mediator, is also involved in the regulation of lymphocyte trafficking, but its role in the regulation of mucosal immune system remains obscure. In this project, we have revealed that intraepithelial lymphocytes (IEL) is principally regulated by S1P for the migration into the intestine, especially large intestine. In addition, we also found that S1P regulates peritoneal B cell trafficking into the intestine and subsequent intestinal IgA production. Furthermore, it was revealed that pathogenic T cells causing allergic diarrhea was regulated in their trafficking from the systemic compartment into the large intestine by S1P. Thus, disruption of S1P-mediated pathway resulted in the inhibition of allergic diarrhea. Taken together, our current findings reveal a critical role of S1P in the regulation of lymphocyte trafficking in the mucosal compartments.

6. Mucosal Antigen Processing and Presentation

Jun Kunisawa[1], Morio Higuchi[1], Fumi Miura[1], Yosuke Kurashima[1], Masashi Gohda[1], Ikuko Ogahara[1], Izumi Ishikawa[1], Nilabh Shastri[2], and Hiroshi Kiyono[1]: [1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute for Medical Science, The University of Tokyo, [2]Division of Immunology, Department of Molecular and Cellular Biology, University of California, Berkeley

To achieve immunological surveillance and homeostasis at mucosal sites, mucosal immune system establishes a unique antigen presentation pathway. For instance, epithelial cells (ECs) present non-classical MHC molecules as well as classical one, which play an important role in intraepithelial lymphocyte (IEL)- and/or natural killer (NK) cells-mediated innate and acquired mucosal immunity. Although several lines of evidence have suggested that microbial stimulation enhances both classical and non-classical MHC expressions on ECs, it is unclear how ECs regulate these two types of MHC expressions against microbial stimuli. In this study, we have investigated how ECs regulate both classical and non-classical MHC expression against microbial stimulation and revealed that viral stimulation increased both classical and non-classical MHC molecules, while their dependencies on IFNs were quite different. Additionally, using a novel method (KOVAK system) to detect antigenic intermediates generated in living cells, our additional efforts are aimed to clarify the antigen processing pathway in ECs regulated by viral stimulation. These will provide important information for the development of mucosal vaccine and immune therapy.

7. Analysis of T cell-specific TGF-β-activating kinase 1 (TAK1) deficient mice

Shintaro Sato[1,2] and Shizuo Akira[2,3]: [1]Division of Mucosal Immunology, Department of Micro-

biology and Immunology, Institute for Medical Science, The University of Tokyo, [2]Akira Innate Immunity Project, ERATO, JST, [3]Depertment of Host Defense, Research Institute for Microbial Diseases, Osaka University

TAK1 is critical for Toll-like receptor- and TNF-mediated cellular responses. We previously reported that, in B cells, TAK1 is also essential for the activation of MAP kinases, but not NF-κB, in antigen receptor signaling. In this study, we generate T cell-specific TAK1-deficient ($Lck^{Cre/+}Tak1^{flox/flox}$) mice and show that TAK1 is indispensable for the maintenance of peripheral CD4 and CD8 T cells. In thymocytes, TAK1 is essential for T cell receptor-mediated activation of both NF-κB and MAP kinases. Additionally, $Lck^{Cre/+}Tak1^{flox/flox}$ mice developed colitis as they aged. In these mice, accumulations of activated/memory T cells as well as B cells were observed. Development of regulatory T (Treg) cells in thymus was abrogated in $Lck^{Cre/+}Tak1^{flox/flox}$ mice, suggesting that the loss of Treg cells is the cause of the disease. Taken together, these results show that TAK1, by controlling the generation of central Treg cells, is important for preventing spontaneously developing colitis. Thus, our mouse model appears quite useful for investigating the mechanisms of autoimmune colitis.

Publications

1. Fukuyama, S,. Nagatake, T,. Kim, DY,. Takamura, K,. Park, EJ,. Kaisho, T,. Tanaka, N,. Kurono, Y. and Kiyono, H,. Cutting edge: Uniqueness of lymphoid chemokine requirement for the initiation and maturation of nasopharynx-associated lymphoid tissue organogenesis. J, Immunol. 177: 4276-80, 2006.
2. Jang, MH,. Sougawa, N,. Tanaka, T,. Hirata, T. Hiroi, T,. Tohya, K,. Guo, Z,. Umemoto, E,. Ebisuno, Y,. Yang, BG,. Seoh, JY,. Lipp, M,. Kiyono, H. and Miyasaka, M. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. J, Immunol. 176: 803-10, 2006
3. Duverger, A,. Jackson, RJ,. van Ginkel, FW,. Fischer, R,. Tafaro, A,. Leppla, SH,. Fujihashi, K,. Kiyono, H,. McGhee, JR. and Boyaka, PN. Bacillus anthracis edema toxin acts as an adjuvant for mucosal immune responses to nasally administered vaccine antigens. J, Immunol. 176: 1776-83, 2006
4. Hagiwara, Y,. Kawamura, YI,. Kataoka, K,. Rahima, B,. Jackson, RJ,. Komase, K,. Dohi, T,. Boyaka, PN,. Takeda, Y,. Kiyono, H,. McGhee, JR. and Fujihashi, K. A second generation of double mutant cholera toxin adjuvants: enhanced immunity without intracellular trafficking. J, Immunol. 177: 3045-54, 2006
5. Maddaloni, M,. Staats, HF,. Mierzejewska, D,. Hoyt, T,. Robinson, A,. Callis, G,. Kozaki, S,. Kiyono, H,. McGhee, JR,. Fujihashi, K. and Pascual, DW. Mucosal vaccine targeting improves onset of mucosal and systemic immunity to botulinum neurotoxin A. J, Immunol. 177: 5524-32, 2006
6. Nochi, T. and Kiyono, H. Innate immunity in the mucosal immune system. Curr, Pharm, Des. 12: 4203-13, 2006
7. Kunisawa, J. and Shastri, N. Hsp90α chaperones large C-terminally extended proteolytic intermediates in the MHC class I antigen processing pathway. Immunity 24: 523-34, 2006
8. Sato, S,. Sanjo, H,. Tsujimura, T,. Ninomiya-Tsuji, J,. Yamamoto, M,. Kawai, T,. Takeuchi, O. and Akira, S. TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells. Int. Immunol. 18: 1405-1411, 2006.