RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Bacterial Infection
細菌感染分野

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*Continuing research abroad

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

1. Interaction of CagA with Crk plays an important role in *Helicobacter pylori*-induced loss of gastric epithelial cell adhesion.

Masato Suzuki, Hitomi Mimuro, Toshihiko Suzuki, Morag Park[1], Tadashi Yamamoto[2] and Chihiro Sasakawa: [1]Department of Cancer Biology, IMSUT, [2]Molecular Oncology Group, McGill University, Montreal, Canada

CagA protein is a major virulence factor of *Helicobacter pylori*, which is delivered into gastric epithelial cells and elicits growth factor-like responses. Once within the cells, CagA is tyrosine phosphorylated by Src family kinases and targets host proteins required to induce the cell responses. We show that the phosphorylated CagA binds Crk adaptor proteins (Crk-II, Crk-I, and Crk-L) and that the interaction is important for the CagA-mediated host responses during *H. pylori* infection. *H. pylori*-induced scattering of gastric epithelial cells in culture was blocked by overexpression of dominant-negative Crk and by RNA interference-mediated knockdown of endogenous Crk. *H. pylori* infection of the gastric epithelium induced disruption of E-cadherin/catenin-containing adherens junctions, which was also dependent on CagA/Crk signaling. Furthermore, inhibition of the SoS1/H-Ras/Raf1, C3G /Rap1/B-Raf, or Dock180/Rac1/Wiskott-Aldrich syndrome protein family verprolin homologous protein pathway, all of which are involved downstream of Crk adaptors, greatly diminished the CagA-associated host responses. Thus, CagA targeting of Crk plays a central role in inducing the pleiotropic cell responses to *H. pylori* infection that cause several gastric diseases, including gastric cancer.

2. *Shigella* Spa33 is an essential C-ring component of type III secretion machinery.

Tomoko Morita-Ishihara, Michinaga Ogawa, Hiroshi Sagara[1], Mitsutaka Yoshida[2], Eisaku Katayama[3] and Chihiro Sasakawa: [1]Department of Fine Morphology and [3]Depatrment of Basic medical Sciences, IMSUT, [2]Division of Ultrastructural Research, BioMedical Research

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Type III secretion machinery (TTSM), composed of a needle, a basal body and a C-ring compartment, delivers a subset of effectors into host cells. Here, we show that *Shigella* Spa33 is an essential component of the C-ring compartment involved in mediating the transit of various TTSM-associated translocated proteins. Electron microscopic analysis and pull-down assay revealed Spa33 to be localized beneath the TTSM via interaction with MxiG and MxiJ (basal body components). Spa33 is also capable of interacting with Spa47 (TTSM ATPase), MxiK, MxiN (required for the transit of MxiH, the needle component), Spa32 (required for determining needle length) and several effectors. Genetic and functional analyses of the Spa33 C-terminal region, which is highly conserved in the SpaO-YscQ-HrcQB-FliN family, indicate that some of the conserved residues are crucial for needle formation via interactions with MxiN. Thus, Spa33 plays a central role as the C-ring component in recruiting/exporting TTSM-associated proteins.

3. IpgB1 is a novel *Shigella* effector protein involved in bacterial invasion of host cells: ITS ACTIVITY TO PROMOTE MEMBRANE RUFFLING VIA RAC1 AND CDC42 ACTIVATION.

Kenji Ohya, Yutaka Handa, Michinaga Ogawa, Masato Suzuki and Chihiro Sasakawa

Shigella, the causative agent of bacillary dysentery, is capable of inducing the large scale membrane ruffling required for the bacterial invasion of host cells. *Shigella* secrete a subset of effectors via the type III secretion system (TTSS) into the host cells to induce membrane ruffling. Here, we show that IpgB1 is secreted via the TTSS into epithelial cells and plays a major role in producing membrane ruffles via stimulation of Rac1 and Cdc42 activities, thus promoting bacterial invasion of epithelial cells. The invasiveness of the *ipgB1* mutant was decreased to less than 50% of the wild-type level (100%) in a gentamicin protection or plaque forming assay. HeLa cells infected with the wild-type or a IpgB 1-hyperproducing strain developed membrane ruffles, with the invasiveness and the scale of membrane ruffles being comparable with the level of IpgB1 production in bacteria. Upon expression of EGFP-IpgB1 in HeLa cells, large membrane ruffles are extended, where the EGFP -IpgB1 was predominantly associated with the cytoplasmic membrane. The IpgB1-mediated formation of ruffles was significantly diminished by expressing Rac1 small interfering RNA and Cdc42 small interfering RNA or by treatment with GGTI-298, an inhibitor of the geranylgeranylation of Rho GTPases. When IpgB1 was expressed in host cells or wild-type *Shigella*-infected host cells, Rac1 and Cdc42 were activated. The results thus indicate that IpgB1 is a novel *Shigella* effector involved in bacterial invasion of epithelial cells via the activation of Rho GTPases.

4. *Shigella* effector IpaH9.8 binds to a splicing factor U2AF35 to modulate host immune responses.

Jun Okuda, Takahito Toyotome[1], Naoyuki Kataoka[1], Mutsuhito Ohno, Hiroyuki Abe[2], Yoshiro Shimura[3], Arefeh Seyedarabi[3], Richard Pickersgill and Chihiro Sasakawa: [1]Laboratory of Biochemistry, Institute for Virus Research, Kyoto University, [2]National Institutes of Natural Sciences, [3]School of Biological Sciences, Queen Mary, University of London, UK

Shigella effectors injected into the host cell via the type III secretion system are involved in various aspects of infection. Here, we show that one of the effectors, IpaH9.8, plays a role in modulating inflammatory responses to *Shigella* infection. In murine lung infection model, DeltaipaH9.8 mutant caused more severe inflammatory responses with increased pro-inflammatory cytokine production levels than did wild-type *Shigella*, which resulted in a 30-fold decrease in bacterial colonization. Binding assays revealed that IpaH9.8 has a specific affinity to U2AF (35), a mammalian splicing factor, which interferes with U2AF (35)-dependent splicing as assayed for IgM pre-mRNA. Reducing the U2AF (35) level in HeLa cells and infecting HeLa cells with wild-type caused a decrease in the expression of the il-8, RANTES, GM-CSF, and il-1beta genes as examined by RT-PCR. The results indicate that IpaH9.8 plays a role in *Shigella* infection to optimize the host inflammatory responses, thus facilitating bacterial colonization within the host epithelial cells.

5. Escape of Intracellular *Shigella* from Autophagy.

Michinaga Ogawa, Toshihiko Suzuki and Chihiro Sasakawa

Autophagy is an intracellular bulk degradation system in which cytoplasmic macro molecules or organelles are directed to the lysosomes. *Shigella* and *Listeria* are able to disrupt phagocytic vacuole of macrophage and epithe-

lial cells and escape into the cytoplasm. It implies that invaded bacteria in cytoplasm can be target for autophagy, and that cytoplasmic bacteria can exert some function to evade autophagy. we investigated the possibility that *Shigella* could be entrapped by autophagosomes. MDCK expressing GFP-LC3 (a marker of autophagosomes) were infected with *Shigella flexneri* wild type or *icsB* mutant and the colocalization of bacterium and GFP-LC3 was observed. Analysis by confocal laser microscopy, thin-sections electron microscopy (EM) and immunogold EM revealed that *Shigella* can escape autophagy by secreting IcsB via the type III secretion system. Mutant bacteria lacking IcsB were trapped by autophagy during multiplication within the host cells. IcsB did not directly inhibit autophagy. Rather, *Shigella* VirG, a protein required for intracellular actin-based motility, induced autophagy by binding to the autophagy protein, Atg 5. In non-mutant *Shigella*, this binding is competitively inhibited by IcsB binding to VirG. Based in these results, we are performing the experiments to elucidate the mechanism of autophagy caused by *Shigella* in detail.

Publications

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Department of Microbiology and Immunology

Division of Immunology
免疫調節分野

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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.

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Mature B-2 cells expressing sIgM and sIgD proliferate upon stimulation by CD38, CD40 or lipopolysaccharide (LPS) and differentiate into IgG1-producing plasma cells in the presence of cytokines. 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, a well-known IgG1-inducing factor, does not induce either μ to γ1 CSR or Blimp-1 expression in CD38-activated B-2 cells, while IL-4 does induce μ to γ1 CSR and IgG1 production in CD 40-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 stimula-

tion 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 53 BP1 may be required for CSR.

It is not known how AID expression is regulated. 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.

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). However, relationship between IL-7 withdrawal and activation of pre-BCR signaling is still unknown.

To examine roles of IL-7 and pre-BCR signaling in the pro-B to pre-B stage transition, we cultured pro-B cells from multi-potent hematopoietic progenitors in the presence of IL-7 and induced their differentiation by IL-7 withdrawal or by cross-linking of CD79b. Withdrawal of IL-7 from pro-B cell culture induced up-regulation of CD25 and CD2 and down-regulation of CD43 expression, which are characteristics of pre-B cells. These changes were not observed in the pro-B cultures of RAG-2-/- mice, indicating that formation of the pre-BCR is indispensable for differentiation signaling. On the other hand, addition of anti-CD79b antibody to the pre-B culture of RAG2-/- mice induced pro-B to pre-B transition. This transition was observed even in the presence of IL-7, indicating that IL-7 is not interfering pre-BCR-mediated differentiation signaling. It was also found that in the presence of IL-7, pro-B cells with cytoplasmic heavy chain accumulate in culture. Interestingly, heavy chains in these pro-B cells did not form pre-BCR even though surrogate light chains were co-expressed. From these observations, we argue that 1) pro-B to pre-B differentiation signal is generated by pre-BCR and 2) local abundance of IL-7 may act as a selection mechanism for pre-BCR expressing cells.

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. Although little is known about B-1 cell differentiation, existence of progenitors for B-1 cells is speculated, because the progenitors of B-1 cells are abundant in the fetal omentum and liver but is missing in the bone morrow of adult animals.

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. To examine role of IL-5 in the early determination of B-1 cell differentiation, we employed *in vitro* bone marrow culture system by which B-1 cell progenitors could expand and became mature B-1 cells after transferring into SCID mice. During the bone marrow culture, IL-5Rα expression was observed in the B-1 cells in the absence of IL-5, suggesting that transcription of IL-5Rα is induced in the early stage of B-1 cell differentiation. RT-PCR analysis of *il-5ra* gene transcription reveled that weak *il-5ra* gene expression was detected in pro-B cells in fetal liver as well as in pro-B and pre-B cells in adult bone marrow, but was not detected in lineage-marker negative progenitors. Addition of IL-5 into the bone marrow culture could in-

duce preferential differentiation of B-1 cells that appeared to migrate into intestine and become IgA-producing cells in the transferred SCID mice.

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

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

IL-5 is secreted from helper T 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 cDNA and Neo cassette were inserted into the first exon of *IL-5* gene to obtain targeting vector. After gene transfection, 1700 drug-selected ES colonies were examined and only a single colony was positive for homologous recombination. Using this IL-$5^{GFP/+}$ ES clone, we have obtained 12 chimeric mice with 5-40% chimerism, assessed by coat color. We are now constructing modified targeting vectors with different 5′ to 3′ length of the *IL-5* gene to obtain better homologous recombination efficiency. We are generating lymphocyte chimeric mice by transplanting ES cell-derived hematopoietic progenitors sorted from chimeric mice into RAG-2 deficient mice, in order to examine the IL-5 expression in non-lymphoid cells.

c. B-1 cells in elicitation of contact sensitivity

Atsuko Itakura, Yuji kikuchi[3], Taku Kouro, Masashi Ikutani, Satohi Takaki, and Kiyoshi Takatsu: [3]Laboratory of Immunoregulation, Department of Infection Control and Immunology, Kitasato Institute for Life Sciences, Kitasato University

Elicitation of contact sensitivity; a classic example of T cell-mediated immunity, requires antigen-specific IgM antibodies, which are produced by B-1 cells within 1 day after skin immunization. Due to IL-5 being important for the maintenance of B-1 cells, and the promotion of antibody production, we examined whether IL-5 is involved in elicitation of contact sensitivity. IL-5R$\alpha^{-/-}$ and C57BL/6 mice were immunized by painting oxazolone on the chest, abdomen and feet. On day 4, mice were challenged by topical application of same antigen on the ears, and ear thickness was measured at 24-hr post-challenge. It was found that ear swelling responses were impaired in IL-5R$\alpha^{-/-}$ mice. Histological examination of C57BL/6 ears showed edema of the connective tissue with massive accumulation of inflammatory cells including eosinophils. In IL-5 R$\alpha^{-/-}$ mice, edema and cell infiltration were much milder, and no eosinophils were observed. These results suggest that IL-5 is required for full elicitation of contact sensitivity.

3. Regulatory functions of adapter proteins in lympho-hematopoietic system

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

Satoshi Takaki, Hideo Ema[4], Jun Seita[4], Kazuhiro Sudo[4], Azusa Matsubara[4], Yohei Morita[4], Mitsujiro Osawa[4], Atsuko Yoshikawa, Hiromitsu Nakauchi[4] and Kiyoshi Takatsu: [4]Laboratory of Stem Cell Therapy, Center for Experimental Medicine, IMSUT

Lnk, a recently identified intracellular adaptor protein, negatively regulates B-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. Competitive repopulation assays in irradiated host animals have demonstrated that the ability of hematopoietic progenitors to generate various blood cells is greatly enhanced by the absence of Lnk. We further investigated the effect of *lnk*-deficiency on the compartment size and ability of hematopoietic stem cells (HSCs) to proliferate, which is strictly regulated in normal conditions. Comparisons between wild-type mice and mutant mice deficient in the signal adaptor molecule Lnk showed that not only HSC numbers but also the self-renewal capacity of some HSCs are markedly increased when Lnk function is lost. Lnk appears to control HSC numbers by negatively regulating HSC self-renewal signaling.

b. Enhanced engraftment of hematopoietic stem cells and progenitor cells by the transient inhibition of an adaptor protein, Lnk.

Hitoshi Takizawa, Chiyomi Kubo-Akashi, Ikuo Nobuhisa[5], Sang-Mo Kwon, Masanori Iseki, Tetsuya Taga[5], Kiyoshi Takatsu and Satoshi Takaki: [5]Department of Cell Fate Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University.

Enhancement of the engrafting potential and expansion capabilities of HSCs as well as hematopoietic progenitor cells (HPCs), 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/HPCs and, thereby, potentiates the HSCs /HPCs for engraftment. Importantly, even transient expression of DN-Lnk in HSCs/HPCs facilitated their engraftment under nonmyeloablative conditions and fully reconstituted the lymphoid compartments of immunodeficient host animals. HPCs 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/HPCs without obvious side effects.

c. APS, an adaptor molecule containing PH and SH2 domains, has a negative regulatory role in B cell proliferation.

Masanori Iseki, Chiyomi Kubo-Akashi, Sang-Mo Kwon, Kiyoshi Takatsu and Satoshi Takaki

To further understand functions of the Lnk family adaptor proteins further, we tried to identify other members of the family, mouse APS was isolated. We generated $APS^{-/-}$ mice, and found that $APS^{-/-}$ mice had more B-1 cells in the peritoneal cavity and showed enhanced humoral immune responses against thymus-independent type-2 antigens than wild type mice. We further investigated the functions of APS in B cell development and activation by generating APS-transgenic (APS-Tg) mice that overexpressed APS in lymphocytes. The number of B-1 cells was reduced in APS-Tg mice, as were B-2 cells in the spleen. B cell development in the bone marrow was partially impaired at the transition stage from proliferating large pre-B to small pre-B cells. B cell proliferation induced by B cell receptor (BCR) crosslinking but not by other B cell mitogens was also impaired in APS-Tg mice. APS co-localized with BCR complexes and filamentous actin in activated APS-Tg B cells. Thus, APS appears to play novel negative regulatory roles in BCR signaling, actin reorganization pathways, and control of compartment sizes of B-lineage cells.

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

Koji Eto[4], Hitoshi Takizawa, Chiyomi Kubo-Akashi, Yuji Watanabe, Yukiko Iwasaki, Kiyoshi Takatsu, Hiromitsu Nakauchi[4] 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. Defective *lnk*-deficient platelet functions were also demonstrated by the reduced three-dimensional growth (height) of platelet thrombi on the collagen surface at high wall shear rate, while the two-dimensional surface coverage was not impaired. In addition, we found that adhesion behaviors of splenic B cells were also altered by the absence of Lnk. Thus, Lnk is a "dual-functioning" adapter molecule, which facilitates integrin-mediated signaling while suppressing cytokine-mediated signaling.

4. Mechanisms of preferential induction of Th 1 response upon immunization with Mycobacteria peptide

The Ag85B of *Mycobacterium (M.) tuberculosis* is immunogenic in C57BL/6 mice and induces the expansion of TCRVβ11$^+$ CD4$^+$ Th1 cells in conjunction with antigen-presenting cells (APC) in an I-A^b-restricted manner. Peptide-25 is the major antigenic epitope for Ag85B-specific Vβ 11$^+$ T cells.

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

Yoko Shimohakamada, Makiyo Nakada, Takeshi Tokunaga, Ai Kariyone, Toshiki Tamura and Kiyoshi Takatsu

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 CD 4^+ T cells into Th1 cells. However, it is unclear whether the TCR signaling events exert a direct influence on Th1 differentiation.

To elucidate cellular and molecular mechanisms of the induction of Th1 cells by Peptide-25, transgenic mice (P25 TCR-Tg) that express the TCR-Vα5-Vβ11 for recognition of Peptide-25, in conjunction with I-A^b molecules were generated. Naive $CD4^+$ T cells from P25 TCR-Tg mice preferentially differentiated into Th1 cells upon Peptide-25 stimulation in the presence of T and NK cells depleted I-A^b splenic APC under neutral condition. In contrast, a mutant of Peptide-25 could induce solely Th2 differentiation. Peptide-25-induced Th1 differentiation was observed even when the cells were stimulated with Peptide-25-loaded I-A^b-transfected Chinese hamster ovary cells (Peptide-25-I-A^b-CHO) in the absence of IFN-γ and IL-12. The TCR stimulation with P25-I-A^b-CHO induced transient T-bet up-regulation and down-regulation of GATA-3 in IFN-γ and IL-12 independent manner. Intriguingly, a significant Th1 differentiation was observed even when T-bet deficient naive $CD4^+$ T cells from P25 TCR-Tg were stimulated with P25-I-A^b-CHO. These results imply that direct interaction between TCR and Peptide -25/I-A^b primarily influences determination of the fate of naive $CD4^+$ T cells in differentiation toward the Th1 subset in the absence of T-bet expression and co-stimulatory signals.

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

Takeshi Kikuchi, Ai kariyone, Wen Xu, 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 Peptide-25. Results revealed that Peptide-25 stimulation alone did not enhance the expression of activation markers on DC. When we co-cultured DC with $CD4^+$ T cells from P25 TCR-Tg mice together with Peptide-25, expression of both MHC class I and ICAM-1 were enhanced and led to induce IL-12 p40 production. Such activated DC showed more effective OVA presentation to OVA specific $CD8^+$ T cells and enhanced proliferation of the cells, suggesting 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.

5. Effect of CpG ODN on prevention from antigen-induced anaphylaxis

Wen Xu, Toshiki Tamura and Kiyoshi Takatsu

Allergy is characterized by a predominant Th 2 response to allergens and the subsequent production of IL-4, IL-5, and IL-13 that mediate the clinical features of allergy and production of IgE by B cells. Anaphylaxis is a life-threatening allergic hypersensitivity response in which IgE/FcεRI-mediated systemic mast cell degranulation occurs. Bacterial DNA sequences with adjuvant properties, known as CpG DNA, have been found to suppress Th2 responses by stimulating the innate immune system, and consequently to cause the release of abundant Th1-skewing cytokines, and thus are of therapeutic significance for allergic diseases. It has been reported that CpG ODN seems to be capable of inhibiting allergic diseases such as asthma and allergic conjunctivitis. We attempted to investigate whether CpG ODN displayed Th1-fostering activities in mouse anaphylaxis model. It was found that CpG ODN pretreatment of mice before ovalbumin (OVA) sensitization was capable of inducing IL-12 and IFN-γ production in spleen cells, down-regulating IgE synthesis, ameliorating body temperature, inhibiting plasma histamine release, and inducing anaphylactic shock. Intriguingly, preventive effect of CpG ODN on anaphylaxis was observed in IFN-$\gamma^{-/-}$ mice, but was not observed in MyD88$^{-/-}$ mice, indicating

that CpG ODN-dependent IFN-γ-producing T cells are not essential in its protective effect. As B cells express TLR9 as a sensor for CpG ODN, we examined whether CpG ODN could influence class switch recombination (CSR) in B cells from IgM to IgE. CpG ODN inhibited IL-4-mediated CSR from IgM to IgG1 and IgE *in vitro*. These results imply that CpG ODN may directly act on antigen-activated B cells and suppress IgE and IgG1 production by inhibiting CSR leading to the protection against antigen-induced anaphylaxis.

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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. In Drosophila and plants, transcripts of retrotransposon are post-transcriptionally suppressed by a mechanism designated RNA silencing (or RNA interference), but it is not clear whether a similar mechanism is operating in human retroviral gene silencing. Our goal is to elucidate the entire cellular defence system and counteracting viral strategy. These studies would give us new ideas for latent infection observed in many human viruses including HIV or 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. Epigenetical regulation and SWI/SNF chromatin remodeling complex

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 regulation mechanisms is therefore essential to understand both host and viral strategies in this post-genome era. In cytoplasm, DNA methylation, histone acetylation and chromatin remodeling play major roles in epigenetical regulations. On the other hand, 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). And it has been suggested that chromosomal regulation and RNA silencing are interconnected through a putative pathway designated as RNA-directed transcriptional gene silencing (RdTS).

For the understanding of epigenetics, we have been concentrated on a major chromatin remodeling factor, SWI/SNF complex, which is composed of 10 protein subunits in human. 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. We previously 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 BAF 60a is the major determinant of AP-1 transactivating activity.

In 2002, we showed that MuLV-based retrovirus vector transgene expression is rapidly silenced in human tumor cell lines lacking expression of Brm, 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) and a complex including YY1, EZH2, EED and HDACs (Polycomb-G) counteract each other to maintain transcription of exogenously introduced genes.

Since the host protein, Brm plays pivotal roles in epigenetical regulation of retrovirus expression, we were next interested in the biogenesis of Brm and started initial studies using a human cell line, SW13, which lacks expression of both Brm and BRG-1. This year, we extended these earlier studies by analysing seven human cell lines deficient in Brm (designated as Brm-deficient cell lines thereafter) (a). We showed that in each cell line examined, the functional *Brm* gene is present and actively transcribed but is not expressed as mRNA, indicating that an unique molecular mechanism of post-transcriptional gene silencing (PTGS) is operating in these cell lines.

In the process of these studies, we have noticed that SWI/SNF complex does not necessarily function as a positive regulator of transcription; this complex strongly suppresses several neuron-specific genes in some non-neuronal cells. Therefore 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 (b).

(a) The *Brm* gene suppressed at the post-transcriptional level in various human cell lines is inducible by transient HDAC inhibitor treatment, which exhibits anti-oncogenic potential.

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To understand the biological function and biogenesis of Brm protein, we examined seven cell lines derived from various human tumors that do not produce Brm protein. We show that these Brm-deficient cell lines transcribe the *Brm* genes efficiently as detected by nuclear run-on transcription assay, whereas *Brm* primary transcript and *Brm* mRNA was undetectable by reverse transcription-polymerase chain reaction analysis. These results indicate that expression of *Brm* is strongly suppressed at the post-transcriptional level through processing and transport of the primary transcript as well as through stability of mature *Brm* mRNA. This suppression was strongly attenuated by transient treatment of these cell lines with HDAC inhibitors such as FK228 probably through indirect mechanism. Importantly, all of the treated cells showed prolonged induction of *Brm* expression after the removal of HDAC inhibitors, and acquired the ability to maintain retroviral gene expression. Since we previously reported that loss of Brm but not of BRG1 causes transcriptional gene silencing of murine leukemia virus-based retrovirus vectors, these results indicate that these Brm-deficient human tumor cell lines carry a functional *Brm* gene. Treatment with HDAC inhibitors or introduction of exogenous *Brm* into Brm-deficient cell lines significantly reduced the oncogenic potential as assessed by colony-forming activity in soft agar or invasion into collagen gel, indicating that, like *BRG1, Brm* is involved in tumor suppression.

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

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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.

2. 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 maintenance and establishment of CpG DNA methylation in mouse embryonic stem (ES) cells, we used a MSCV based-retroviral vector carrying *GFP* as a probe. Unlike its parents, 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.

3. Establishment of new retrovirus vectors that express shRNA efficiently.

Taketoshi Mizutani, Takeshi Haraguchi, Nobutake Yamamichi and Hideo Iba

In many organisms such as plant, nematoda, *Drosophila*, RNA silencing systems, which include such molecules as short interfering (si) RNA and micro (mi) RNA, are now known to regulate expression of specific endogenous genes, exogenously introduced viral genomes and intragenomic parasites such as exogenous retrovirus and retrotransposons. Whereas biological function of RNA silencing (or RNA interference) in human remain largely elusive, RNA silencing has recently emerged as a specific and efficient method to silence gene expression in human cells either by introducing siRNA or short hairpin (sh) RNA that function as an artificial precursor of siRNA.

1. We have designed several retrovirus vectors carrying expression units for shRNA. We have prepared all of them as VSV-G pseudotypes and selected the vectors that efficiently suppress the expression of a target gene, (we used the exogenously introduced GFP gene as the target). We finally established a MuLV -based retrovirus vector that can reduce the expression level of exogenous GFP up to 1%. These cell lines in which artificial RNA interference is operating efficiently are now used as an assay cellular system for screening low molecular reagents that would function as inhibitors on specific pathway on RNA interference.

   This procedure was applicable to most of the cell lines originated from human tumors, but we found that knock-down effects was significantly reduced in mouse embryonic cells or non-dividing human cells. Therefore, we also introduced the same shRNA expression unit into HIV-based lentivirus vectors

for broader application. These lentivirus vectors exhibited a similar strong suppression even in non-dividing cells. We are also analysing the molecular mechanisms of RNA silencing using both retro-and lenti-virus vectoirs and the cell lines established bytheir introduction.

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SWI/SNF complex is essential for NRSF-mediated suppression of neuronal genes in human nonsmall cell lung carcinoma cell lines. *Oncogene in press*

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」印刷中

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

1. The importance of both the coding and segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions

Fujii K, Fujii Y, Noda T, Muramoto Y, Watanabe T, Takada A, Goto H, Horimoto T, Kawaoka Y.

The genome of influenza A virus consists of eight single-strand negative-sense RNA segments, each comprised of a coding region and a noncoding region. The noncoding region of the NS segment is thought to provide the signal for packaging; however, we recently showed that the coding regions located at both ends of the hemagglutinin and neuraminidase segments were important for their incorporation into virions. In an effort to improve our understanding of the mechanism of influenza virus genome packaging, we sought to identify the regions of NS viral RNA (vRNA) that are required for its efficient incorporation into virions. Deletion analysis showed that the first 30 nucleotides of the 3′ coding region are critical for efficient NS vRNA incorporation and that deletion of the 3′ segment-specific noncoding region drastically reduces NS vRNA incorporation into virions. Furthermore, silent mutations in the first 30 nucleotides of the 3′ NS coding region reduced the incorporation efficiency of the NS segment and affected virus replication. These results suggested that segment-specific noncoding regions together with adjacent coding regions (especially at the 3′ end) form a structure that is required for efficient influenza A virus vRNA packaging.

2. Live bivalent vaccine for parainfluenza and influenza virus infections

Maeda Y, Hatta M, Takada A, Watanabe T, Goto H, Neumann G, Kawaoka Y.

Influenza and human parainfluenza virus infections are of both medical and economical importance. Currently, inactivated vaccines provide suboptimal protection against influenza, and vaccines for human parainfluenza virus in-

fection are not available, underscoring the need for new vaccines against these respiratory diseases. Furthermore, to reduce the burden of vaccination, the development of multivalent vaccines is highly desirable. Thus, to devise a single vaccine that would elicit immune responses against both influenza and parainfluenza viruses, we used reverse genetics to generate an influenza A virus that possesses the coding region for the hemagglutinin/neuraminidase ectodomain of parainfluenza virus instead of the influenza virus neuraminidase. The recombinant virus grew efficiently in eggs but was attenuated in mice. When intranasally immunized with the recombinant vaccine, all mice developed antibodies against both influenza and parainfluenza viruses and survived an otherwise lethal challenge with either of these viruses. This live bivalent vaccine has obvious advantages over combination vaccines, and its method of generation could, in principle, be applied in the development of a "cocktail" vaccine with efficacy against several different infectious diseases.

3. Characterization of a human H5N1 influenza A virus isolated in 2003

Shinya K, Hatta M, Yamada S, Takada A, Watanabe S, Halfmann P, Horimoto T, Neumann G, Lim W, Guan Y, Peiris M, Suzuki T, Suzuki Y, Kawaoka Y.

In 2003, H5N1 avian influenza virus infections were diagnosed in two Hong Kong residents who had visited the Fujian province in mainland China, affording us the opportunity to characterize one of the viral isolates, A/Hong Kong/213/03 (HK213; H5N1). In contrast to H5 N1 viruses isolated from humans during the 1997 outbreak in Hong Kong, HK213 retained several features of aquatic bird viruses, including the lack of a deletion in the neuraminidase stalk and the absence of additional oligosaccharide chains at the globular head of the hemagglutinin molecule. It demonstrated weak pathogenicity in mice and ferrets but caused lethal infection in chickens. The original isolate failed to produce disease in ducks but became more pathogenic after five passages. Taken together, these findings portray the HK213 isolate as an aquatic avian influenza A virus without the molecular changes associated with the replication of H5N1 avian viruses in land-based poultry such as chickens. This case challenges the view that adaptation to land-based poultry is a prerequisite for the replication of aquatic avian influenza A viruses in humans.

4. Enhanced expression of an α2,6-linked sialic acid on MDCK cells improves the isolation of human influenza viruses and the evaluation of their sensitivity to a neuraminidase inhibitor

Hatakeyama S, Sakai-Tagawa Y, Kiso M, Goto H, Kawakami C, Mitamura K, Sugaya N, Suzuki Y, Kawaoka Y.

The extensive use of neuraminidase (NA) inhibitors to treat influenza virus infections mandates close monitoring for resistant variants. Cultured cells do not provide a reliable means of evaluating the susceptibility of human influenza virus isolates to NA inhibitors. That is, the growth of such viruses in cell lines (e.g., Madin-Darby canine kidney [MDCK] cells) is not inhibited by these drugs, even though their sialidase activity is drug-sensitive. Matrosovich et al. (J. Virol. 77: 8418-8425, 2003) showed that an MDCK cell line overexpressing the human beta-galactoside alpha2,6-sialyltransferase I (ST6Gal I) gene has the potential to assess the sensitivity of human influenza virus isolates to NA inhibitors, based on studies with a limited number of viruses. Here, we asked whether clinical isolates of influenza virus are universally sensitive to an NA inhibitor (oseltamivir) in an MDCK cell line expressing the ST6Gal I gene. The sensitivity of viruses to oseltamivir correlated with the sensitivity of viral sialidase to the compound, demonstrating the potential utility of this modified cell line for detecting NA inhibitor-resistant viruses. Moreover, in ST6Gal I-overexpressing cells, the growth of human influenza viruses was up to 2 logs higher than in MDCK cells. We conclude that the human ST6Gal I-expressing MDCK cell line is useful not only for evaluating their sensitivity to NA inhibitors, but also for isolation of influenza viruses from clinical samples.

5. The Ebola virus VP40 late domains are not essential for viral replication in cell culture

Neumann G, Ebihara H, Takada A, Noda T, Kobasa D, Jasenosky LD, Watanabe S, Kim JH, Feldmann H, Kawaoka Y.

Ebola virus particle formation and budding are mediated by the VP40 protein, which possesses overlapping PTAP and PPXY late domain motifs (7-PTAPPXY-13). These late domain motifs have also been found in the Gag proteins of retroviruses and the matrix proteins of rhabdo- and arenaviruses. While in vitro studies suggest a critical role for late domain motifs in the budding of these viruses, including Ebola virus, it

remains unclear as to whether the VP40 late domains play a role in Ebola virus replication. Alteration of both late domain motifs drastically reduced VP40 particle formation in vitro. However, using reverse genetics, we were able to generate recombinant Ebola virus containing mutations in either or both of the late domains. Viruses containing mutations in one or both of their late domain motifs were attenuated by one log unit. Transmission and scanning electron microscopy did not reveal appreciable differences between the mutant and wild-type viruses released from infected cells. These findings indicate that the Ebola VP40 late domain motifs enhance virus replication but are not absolutely required for virus replication in cell culture.

6. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production

Neumann G, Fujii K, Kino Y, Kawaoka Y.

The generation of vaccines for highly pathogenic avian influenza viruses, including those of the H5N1 subtype, relies on reverse genetics, which allows the production of influenza viruses from cloned cDNA. In the future, reverse genetics will likely be the method of choice for the generation of conventional influenza vaccine strains because gene reassortment by more traditional methods is cumbersome. Established systems for the artificial generation of influenza A viruses require transfection of cells with the eight to 12 plasmids that provide the eight influenza viral RNAs as well as the polymerase and nucleoproteins of the virus. However, cell lines appropriate for human vaccine production (e.g., Vero cells) cannot be transfected with high efficiencies. To overcome these problems, we established a reverse genetics system in which the eight RNA polymerase I transcription cassettes for viral RNA synthesis are combined on one plasmid. Similarly, two cassettes encoding the hemagglutinin and neuraminidase segments and six cassettes encoding the remaining proteins were combined. We also combined three RNA polymerase II transcription cassettes for the expression of the polymerase subunits. By combining these cassettes, we reduced the number of plasmids required for virus generation significantly and produced influenza A virus in Vero cells with higher efficiency than with the traditional 12 plasmid system. This new system is thus suitable for influenza virus vaccine production and may be applicable to other reverse genetics systems that rely on the introduction of several plasmids into eukaryotic cells.

7. Emergence of an oseltamivir-resistant H5N 1 influenza A virus

Le QM, Kiso M, Someya K, Sakai YT, NguyenTH, Nguyen KHL, Pham ND, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y.

The persistence of H5N1 avian influenza viruses in many Asian countries and their ability to cause fatal infections in humans have raised serious concerns over a global influenza pandemic. Given the difficulty of stockpiling a sufficient quantity of influenza vaccine to protect large populations, antiviral drugs such as the neuraminidase inhibitors oseltamivir and zanamivir are likely to be the main defense against a pandemic strain of H5N1 virus. Whether treatment of H5N1 viruses with a neuraminidase inhibitor can give rise to virulent drug-resistant mutants is presently unknown. Here we report the isolation of a highly oseltamivir-resistant virus from a 14-year-old Vietnamese girl who had been suspected to have acquired H5N1 infection and had received low-dose oseltamivir for 3 days. Sequencing of the neuraminidase gene indicated a tyrosine substitution at position 274 of the protein. When tested in ferrets, the virus was attenuated, but still replicated and was sensitive to zanamivir. Thus, H5N1 avian influenza viruses can acquire resistance to oseltamivir during replication in humans, a property that could alter strategies to control a global pandemic.

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Division of Infectious Genetics
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Our research main focuses on molecular mechanism underlying innate pathogen recognition by Toll-like receptors (TLRs). TLRs have been implicated in microbial recognition in the innate immune system but their microbial recognition mechanism remains enigmatic. We have proposed a concept that TLR requires a coreceptor, by discovering MD-2 that is associated with TLR4 and indispensable for endotoxin recognition by TLR4. Molecular cloning of MD-2 led us to a search for coreceptors of other TLRs. Such molecules would reveal molecular mechanisms underlying innate microbial recognition.

1. Agonistic Antibody to Toll-like receptor 4/MD-2 Protects Mice from Acute Lethal Hepatitis Induced by Tumor Necrosis Factor-α[1]

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LPS (lipopolysaccharide) is recognized by a heterodimer consisting of Toll-like receptor 4 (TLR4) and its coreceptor MD-2. LPS signal causes excessive inflammation and tissue damage. We here show that a mAb to TLR4/MD-2 protected mice from acute lethal hepatitis caused by LPS/D-galactosamine (D-GalN). The protective effect of the mAb was not due to inhibition of LPS response, because serum TNF-α, which was induced by LPS and caused lethal hepatitis, was 10 times up-regulated by this mAb pretreatment. Moreover, this mAb induced anti-apoptotic genes in liver in a TLR4/MD-2-dependent manner. In the present study, we demonstrated that a mAb to TLR4/MD-2 protected mice from acute lethal hepatitis induced by LPS/D-GalN not by inhibiting LPS signal but by delivering a protective signal through TLR4/MD-2.

2. Analysis about a link between endocytosis of Toll-like receptor 4 and type 1 Interferon expression

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We have concentrated on one question about the mechanism of TLR4 recognition against their ligands. We reported about TLR4 oligomerization on cell surface after Lipopolysaccharide (LPS) stimulation last year. In this year we analyzed more detail about TLR4 after LPS stimulation using the confocal microscopy. TLR2 and TLR4 exist and are activetd by their ligands on cell surface. First of all, our question was what is the difference between TLR2 and TLR4, because TLR4 stimulation only induces type 1 interferon compared with TLR2. To observe the expression level of TLR2 and TLR4 on cell surface using Flow cytometry, BaF/3 with TLR4, MD-2 and CD14 was established. The BaF/3 cell was stained with anti-TLR4/MD-2 antibody (Sa 15-21) or anti-TLR2 antibody after stimulation. The expression level of TLR4 on cell surface gradually decreased. Finally we were not able to detect TLR4 on the cell surface from 120min after stimulation. On the other hand, the expression level of TLR2 on cell surface did not change at all after stimulation. The same phenotype was observed on macrophage cell line, RAW 264.7 cell. To analyze more detail using confocal microscopy, BaF/3 cell with TLR2-GFP, RAW 264.7 cell with TLR2-GFP and BaF/3 with TLR4-GFP, MD-2 and CD14 were established. Confocal analysis showed that almost all of TLR 2 exists on the cell surface before and after stimulation with any TLR2 ligands. However TLR4 mainly exists intracellulary and a part of TLR4 exists on cell surface before stimulation. From confocal data, TLR4 internalized and redistributed after stimulation into endosome. Moreover co-localization between TLR4-GFP and LPS (406)-Alexia 568 was detected from 60 min after stimulation. Interestingly the activation of IRF3 which is a essential signaling molecule for type 1 Interferon expression was observed from 60 min after LPS stimulation. In this time TLR4 already internalized. As expected, IRF3 activation occurred at rate phase of TLR4 activation compared with MyD88 activation which occurred within 5 min after stimulation. From time course analysis, TLR4 recognizes LPS and activates MyD88 signaling pathway on cell surface, and then internalizes into endosome and activates the IRF3 pathway. Dr. Jiang reported about the importance of CD14 for the activation of IRF3 pathway. We could not observe the internalization of TLR4 without CD 14 by using BaF/3 cell expressing TLR4 and MD -2 with or without CD14 and peritoneal macrophage from CD14KO mice. TRAM and TRIF are important upstream molecules for IRF3 activation. We observed that TRAM exists on cell membrane before stimulation and internalizes after stimulation. TRAM internalization was also CD14 dependent. Colocalization of TRIF with TLR4 was detected from 60 min after LPS stimulation. To directly address TLR4 internalization into endosome really important for type 1 interferon, MD-2 mutants which severely delayed or inhibited on TLR4 internalization after LPS stimulation were analyzed. MD-2 with mutation and wild type MD-2 were expressed on bone marrow derived DC from MD-2 KO mice using retrovirus system. TNF-α and Interferon-β expression were detected after lipid A stimulation for 2 hours using real time PCR. TNF-α expression which is mainly regulated by MyD88 pathway was partially inhibited on MD-2 mutants. On the other hand, Interferon-β expression was severely inhibited on these MD-2 mutants. These data suggest that TLR4 internalization is important phenomenon for type 1 interferon expression after LPS stimulation.

3. Activation of innate immunity by peroxiredoxin derived from malaria parasite

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Peroxiredoxin (Prx) of malaria parasites constitutes about 0.5% of the total protein in stage of trophozoites, and the parasite likely uses Prx as one enzyme to reduce peroxides. Prx is expressed during infection and stimulates IgE antibody responses in malaria. In the present studies, we examined the possibility that malarial Prx induces innate immunity via Toll-like receptors (TLRs) in malaria. We first investigated whether Prx can induce cytokine production in macrophages from TLR-or MyD88-deficient mice. Peritoneal macrophages from those mice were cultured with Prx, and concentration of TNF-α in culture supernatant was measured by ELISA. The level of TNF-α in macrophages of $TLR4^{-/-}$ or $MyD88^{-/-}$ mice was significantly lower than those of $TLR2^{-/-}$ or wild type (wt) mice, and pretreatment of Prx with Polymyxin B did not affect TNF production on macrophages. Since activation of mast cells by pathogens is

mediated by TLRs, it is probable that Prx induces TNF production in mast cells via TLRs. We developed cultured bone marrow-derived mast cells (BMMCs) from TLRs$^{-/-}$ mice, and then BMMCs were stimulated with Prx. Since the level of TNF in BMMCs from TLR4$^{-/-}$ mice was significantly lower than those from TLR2$^{-/-}$ or wt mice, it was suggested that cytokine production by Prx in BMMCs is TLR4-dependant. Costimulatory molecule expression on macrophages induced by Prx was investigated by flow cytometric analysis, macrophages from TLR2$^{-/-}$ mice but not TLR4$^{-/-}$ showed increased expression of CD80 and CD86. Form the above the findings, the results suggested that malarial Prx activates innate immunity via TLR4 and that Prx induces not only acquired immunity but also innate immunity in malaria.

4. The search for the association molecules with mouse TLR2 to recognize the ligands and to regulate signaling.

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The existence of co-receptor or the association molecules for the TLR-ligand recognition or signaling were reported. From some evidences, it is speculated that TLR2 requires the molecule(s) to recognize the specific ligads and to induce the cell-responses. Indeed, TLR1, TLR6, CD16 and CD14 already reported as the functionally associated molecules with TLR2. In search of the other molecules involved in the function of TLR 2, we tried to establish the cell lines that are non -responders to synthetic TLR2 ligands, in spite of normal expression of TLR2, utilizing the random mutation method with ICR191, the frame shift-inducing agent. We established a cell line in which the responses to synthetic TLR2 ligands almost abolished. This cell line were transfected the cDNA library constructed in expression vector obtained from mouse macrophage cell line or normal mouse splenocytes. Twelve clones that can response to TLR2 ligand were selected by functional screening and the transefected gene were identified. One of the identified genes was TLR1. It was already reported that TLR1 augmented TLR2 signaling with the TLR1-TLR2 heterodimer formation. The other identified genes are analyzed as the candidates of the functionally associated molecules with TLR2. Especially, one of the candidates perfectly restored the responses to the synthetic TLR2 ligands. We are now analyzing the functions of this molecule on the ligand-recognition and signal transduction by TLR2.

5. A molecule that is associated with Toll-like receptor 4 and regulates its cell surface expression.

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Toll-like receptors (TLRs) recognize microbial products and induce immune responses. Their subcellular distribution is believed to be optimized for their pathogen recognition. Little is known, however, about molecular mechanisms regulating the subcellular distribution of TLR. Lipopolysaccharide (LPS), a principal membrane component of the Gram-negative bacteria, is recognized by the receptor complex consisting of Toll-like receptor 4 (TLR4) and MD-2. We here show that a novel molecule, PRAT4B (a PRotein Associated with Tlr4), regulates cell surface expression of TLR4. PRAT4B has a signal peptide followed by a mature peptide. PRAT4B is associated with the hypoglycosylated, immature form of TLR4 but not with MD-2 or TLR2. Downregulation of PRAT4B mRNA with small interfering RNA decreased cell surface TLR4 on HEK293 cells. These results suggest a novel mechanism regulating the subcellular distribution of TLR4.

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The mucosal surface provides a first line of defense for the host. The goal of our research is to understand the molecular and cellular aspects of the mucosal immune system and their contribution for the host defense against infectious diseases, inflammation and immunological disorders. Further, it is important to apply our fundamental findings of the mucosal immune system for the development of mucosal vaccine and mucosal immunotherapy with all haste.

1. Identification of M cell-specific molecules for the development of M-cell targeted mucosal vaccine.

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The epithelium covering lymphoid tissues such as Peyer's patch (PP) and nasopharynx-associated lymphoid tissue (NALT) possess a unique subset of antigen-sampling cells known as M cells. We previously discovered M cells within the villous epithelium and designated them as villous M cells. Since M cells can transport antigens to under lying antigen-presenting cells for the initiation of antigen-specific mucosal immune responses, it is thought to be one of the key players that bridge innate and acquired immunities at the mucosa. We have focused on the elucidation of molecular and cellular aspects of M cells to facilitate both the understanding of M cell biology and the design of effective mucosal vaccines. To this end, we recently succeeded in the generation of a monoclonal antibody (mAb) which reacted specifically with PP dome-associated M cells. Importantly, this mAb reacted with villous M cells and NALT-epithelial M cells as well. Thus, our current efforts are aiming at the determination of its epitope and adoptability as a vehicle for the development of M cell-targeted vaccine delivery system. In addition, we have identified a total of 8 genes including peptidoglycan-recognition protein-S (PGRP-S) as M cell-specific genes by using DNA array and *in situ* hybridization. In this regard, our efforts are also aiming at the generation of specific mAb to confirm the expression respective M cell specific genes at protein level and of the gene deficient mouse to examine their relevance in the development as well as function of M cells.

2. Development of Rice-based Edible Vaccine

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We have developed physically and chemically stable and immunologically effective transgenic rice expressing a vaccine antigen in seed. The use of transgenic rice for vaccine production has several potential benefits when compared with other plants. First, the rice-based vaccine produces sufficient quantities of inserted antigen for the immunization. Second, it preserves expressed antigen for long time at room temperature. Third, it induces protective immunity. Finally, it protects from enzymatic digestion in gastrointestinal tract and effectively delivers the inserted antigen to mucosal inductive tissues including M cells. Taking together, rice-based subunit vaccine is considered to be not only effective but also practical mucosal vaccine. To this end, our current efforts are focusing on the development of oral vaccine against human infectious diseases and bioterrorism as well as feed-based oral vaccine for fishes and shrimps.

3. NALT Target Mucosal Vaccine: Mechanism of NALT Organogenesis

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NALT (nasopharynx-associated lymphoid tissue) is thought to be a key targeted mucosa-associated lymphoid tissue 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. The mechanism of NALT organogenesis is quite different from that of PP in spite of the similarity of immunological function of these two inductive tissues. Lymphotoxin (LT) β receptor signals have been shown to play a pivotal role for the organogenesis program of secondary lymphoid tissue development. However, our findings demonstrated that NALT organogenesis is (independent of LTβ receptor signaling pathway. $CD3^{-}CD4^{+}CD45^{+}$ cells which are differentiated from fetal liver progenitors induce the organogenesis of secondary lymphoid tissues (e.g., NALT, PP, and lymph node). CXCL 13 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, CCL 19 and CCL21) were not involved in the accumulation of $CD3^{-}CD4^{+}CD45^{+}$ cells at NALT anlagen. Thus, our current efforts are aimed at the identification of NALT specific signaling molecules for NALT-genesis.

4. Mucosal immune surveillance at epithelial layer by mucosal intranet among T, B, and epithelial cells

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The host continuously interacts with harsh outer environment at mucosal epithelium where numerous kinds of immune competent cells contribute to create and maintain mucosal homeostasis and to provide protective immunity against infection caused by pathogenic microorganisms. They include conventional T and B cells as well as unique T and B cell subsets, known as intraepithelial lymphocyte (IEL) and B-1 B cell. In addition, epithelial cells play a pivotal role in not only physical barrier, but also immune surveillance by expressing immune-regulative and innate molecules, pattern recognition molecules such as classical and non-classical MHC molecules, co-stimulatory molecules, cytokines, and chemokines. We are currently investigating the role of mucosal intranets organized by these unique immuno-competent cells and immune surveillance molecules in the establishment and maintenance of mucosal homeostasis. Especially, our effort is focus on the regulation pathway of IEL migration and secretory IgA production by sphingosine-1-phosphate, a lipid mediator originated from plasma membrane. We also aim to clarify antigen processing and presentation by epithelial cells, subsequently leading to the IEL activation. Our studies will provide a novel strategy for prospective mucosal vaccine using this unique mucosal intranet.

5. Discovery and characterization of intestinal eosinophilic dendritic cells for the regulation of oral tolerance

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Recently, we found the novel type of F4/80$^+$ Gr-1$^+$CD11b$^+$CD11c$^+$ cells as segmented cells in intestinal lamina propria (i-LP). Since these cells possessed morphological characteristics of granulocytes like fraction of dendritic cells, it named eosinophilic dendritic cells (EDCs). Interestingly, newly identified EDCs were essential for the induction of oral tolerance (OT) independent on T regulatory (Treg) cells. Thus, adoptive transfer of *in vivo* OVA-primed EDCs into anti-CD25 treated Treg cells deficient mice resulted in the induction of OT. Transferred EDCs with i-LP origin migrated to spleen and expressed high levels of MHC class-II. Ag-specific CD4$^+$ T cell proliferation was also inhibited by CpG/Flt3-L stimulated EDCs with dendritic morphology. Taken together, Intestinal EDCs are a prime candidate for the immediate responder cells that can negatively regulate immune responses against orally administered antigens. Thus, our study has provided new avenue that EDCs may be key target cells as a therapeutic treatment for the control of allergy and autoimmune diseases.

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