RESEARCH ACTIVITIES

Department of Microbiology and Immunology

Division of Bacterial Infection
細菌感染分野

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

1. Escape of Intracellular *Shigella* from Autophagy.

Michinaga Ogawa, Tamotsu Yoshimori[1], Toshihiko Suzuki, Hiroshi Sagara, Noboru Mizushima[2] and Chihiro Sasakawa: [1]National Institute of Genetics, [2]Tokyo Metropolitan Institute of Medical Science

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 epithelial 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, Atg5. In non-mutant *Shigella*, this binding is competitively inhibited by IcsB binding to VirG.

2. Targeting of enteropathogenic *Escherichia coli* EspF to host mitochondria is essential for the bacterial pathogenesis: critical role of the 16th leucine residue in EspF.

Takeshi Nagai, Akio Abe[1] and Chihiro Sasakawa: [1]Kitasato Institute for Life Sciences, Kitasato University

The attachment of enteropathogenic *Escherichia coli* (EPEC) to host cells and the induction of attaching and effacing (A/E) lesions are

prominent pathogenic features. EPEC infection also leads to host cell death and damage to the intestinal mucosa, which is partly dependent upon EspF, one of the effectors. In this study, we demonstrate that EspF is a mitochondrial import protein with a functional mitochondrial targeting signal (MTS), since the EspF activity to import into mitochondria was abrogated by MTS deletion mutants. Substitution of the 16th leucine with glutamic acid (EspF(L16E)) completely abolished the EspF activity. Infection of HeLa cells with wild type but not the espF mutant (ΔespF) decreased mitochondrial membrane potential (ΔYm), leading to cell death. The ΔYm decrease and cell death were restored in cells infected with ΔespF/pEspF but not ΔespF/pEspF (L16E), suggesting the 16th leucine in the MTS to be a critical amino acid for EspF function. To demonstrate the impact of EspF in vivo, we exploited *Citrobacter rodentium* by infecting C3H/HeJ mice with ΔespFCR, ΔespFCR/pEspFCR or ΔespFCR/pEspF(L16E)CR. The results indicate that EspF activity contributes to bacterial pathogenesis, as judged by murine lethality and intestinal histopathology, and promotion of bacterial colonization of the intestinal mucosa.

3. A novel caspase-1/Toll-like receptor 4-independent pathway of cell death induced by cytosolic *Shigella* in infected macrophages.

Toshihiko Suzuki, Kenji Nakanishi[1], Hiroko Tsutsui[1], Hiroki Iwai, Shizuo Akira[2], Naohiro Inohara[3], Mathias Chamaillard[3,4], Gabriel Nuñez[3,4], and Chihiro Sasakawa: [1]Department of Immunology and Medical Zoology, Hyogo College of Medicine, [2]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, [3]Department of Pathology and [4]Comprehensive Cancer Center, The University of Michigan Medical School

Shigella-induced macrophage cell death is an important step in the induction of acute inflammatory responses that ultimately lead to bacillary dysentery. Cell death was previously reported to be dependent upon the activation of caspase-1 via interaction with IpaB secreted by intracellular *Shigella*, but in this study, we show that *Shigella* infection of macrophages can also induce cell death independent of caspase-1 or IpaB activity. Time-lapse imaging and electron microscopic analyses indicated that caspase-1-dependent and -independent cell death is morphologically indistinguishable, and that both resemble necrosis. Analyses of *Shigella* mutants or Escherichia coli using co-infection with *Listeria* suggested that a component common to Gram-negative bacteria is involved in inducing caspase-1-independent cell death. Further studies revealed that translocation of bacterial lipid A into the cytosol of macrophages potentially mediates cell death. Notably, cell death induced by cytosolic bacteria was TLR4-independent. These results identify a novel cell death pathway induced by intracellular Gram-negative bacteria that may play a role in microbial-host interactions and inflammatory responses.

4. Enteropathogenic *Escherichia coli* activates the RhoA signaling pathway via the stimulation of GEF-H1.

Takeshi Matsuzawa[1], Asaomi Kuwae[1], Sei Yoshida, Chihiro Sasakawa and Akio Abe[1]: [1]Kitasato Institute for Life Sciences, Kitasato University

Enteropathogenic *Escherichia coli* delivers a subset of effectors into host cells via a type III secretion system, and this step is required for the progression of disease. Here, we show that the type III effectors, EspG and its homolog Orf 3, trigger actin stress fiber formation and the destruction of the microtubule networks beneath adherent bacteria. Both effectors were shown to possess the ability to interact with tubulins, and to stimulate microtubule destabilization in vitro. A recent study showed that microtubule-bound GEF-H1, a RhoA-specific guanine nucleotide exchange factor, was converted to its active form by microtubule destabilization, and this sequence of events resulted in RhoA stimulation. Indeed, EspG- and Orf3-induced stress fiber formation was inhibited by the expression of dominant-negative forms of GEF-H1 and RhoA, but not of Rac1 and Cdc42, and by treatment with a ROCK inhibitor. These results indicate that the impact of EspG/Orf3 on microtubule networks triggers the activation of the RhoA-ROCK signaling pathway via GEF-H1 activity. This report reveals for the first time that a pathogen can exploit the host factor GEF-H1.

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, cytokines and adaptor proteins. We are also interested in elucidating cellular mechanisms of preferential induction of Th1 cells upon immunization with Mycobacterium-derived, Peptide-25 and its derivatives.

In 2004, Professor Dr. Fritz Melchers, Basel University, who were invited by the University of Tokyo as the Eminent Scientist of JSPS, has been joining us for our research projects. Dr. Melchers always encouraged us and suggested important issues for each project. We would like to appreciate his enormous contributions.

1. Roles of IL-5 in the B cell differentiation

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

Yumiko Tsukamoto, Atsushi Sato, Kiyoshi Takatsu

Class switch recombination (CSR) is the process that changes physiological activities of antibodies without changing their specificities to antigens. Murine B cells differentiate into IgG1- or IgE- secreting cells when activated by anti-CD40 and IL-4. However, IL-4 cannot induce CSR in anti-CD38-activated B cells, although IL-5 can induce CSR in both anti-CD40- and anti-CD38-activated B cells. Anti-CD38- and IL-4-activated B cells differentiate into IgG1-secreting cells when stimulated with 8-mercaptugoanosine (8-SGuo). 8-SGuo is a guanosine analogue, and it induces proliferation and differentiation of B cells. Activation-induced cytidine deaminase (AID), which is necessary for CSR, is expressed in anti-CD38, IL-4, and 8-SGuo-activated B cells, and isn't expressed in anti-CD38- and IL-4-activated B cells. It suggests that 8-SGuo induces expression of AID in B cells, and thus induces CSR.

In anti-CD38- and 8-SGuo-activated B cells, AID is expressed; however these B cells don't differentiate into IgG1-secreting B cells. This suggests that IL-4 also induces some factor(s) necessary for CSR in anti-CD38, IL-4, and 8-SGuo-activated B cells. Cell division, transcription of germline γ1, and looping out of γ1-μ reciprocal DNA are necessary for CSR. In anti-CD

38- and 8-SGuo-activated B cells and anti-CD38, IL-4, and 8-SGuo-activated B cells, cell division and transcription of germline γ1 were induced. However, γ1-μ reciprocal DNA didn't exist in anti-CD38- and 8-SGuo-activated B cells. These results show that anti-CD38 and 8-SGuo cannot induce CSR because of lacking some factor(s) which are involved in CSR other than cell division, transcription of germline γ1, and AID expression. This factor(s) (Factor X) may be induced by IL-4 in anti-CD38, IL-4, and 8-SGuo-activated B cells. We examined the expression of UNG, Bach2, and 53BP1 genes which are necessary for CSR and found that they were all expressed in anti-CD38 and 8-SGuo-activated B cells. Hence Factor X is not UNG, Bach2, and 53 BP1. Identification of Factor X may reveal some new aspects of CSR.

b. IL-5 induces IgG1 isotype switch recombination in CD38-stimulated murine B cells

Atsushi Sato, Yumiko Tsukamoto, Keisuke Honiara, Kiyoshi Takatsu

As we reported, IL-5 stimulation of anti-CD38 -stimulated murine splenic B (B-2) cells induces μ-γ1 class switch recombination (CSR) leading to a high level of lgG1 production. Further addition of IL-4 in the system enhances IL-5-dependent μ-γ1 CSR. Stat5a- and Stat5b-deficient B-2 cells could not show μ-γ1 CSR and lgG1 production, despite of the intact induction of γ1 germline transcripts. Cell division cycle analyses of Stat5b deficient B-2 cells using CFSE revealed that Stat5b deficient B cells were normally divided to 5 or 6 times, but they could not express surface IgG1. These results implied that Stat5 plays pivotal roles in the μ-γ1 CSR induction. RT -PCR analysis revealed equivalent levels of *AID* expression in wild type and Stat5b deficient B cells, while expression of the *Blimp-1* gene was impaired in Stat5b deficient B-2 cells.

We examined the involvement of Stat5a and Stat5b in anti-CD38- and 8-SGuo-activated B cells. Cell division and transcription of germline γ1 were induced in anti-CD38- and 8-SGuo-activated B cells, but those B cells did not undergo CSR similar to anti-CD38- and IL-5-stimulated Stat5a deficient B cells. Hence we examined the phosphorylation of Stat5a and Stat5b in anti-CD38- and 8-SGuo-activated B cells. Stat5a and Stat5b were not phosphorylated. Downstream molecules of Stat5a and Stat5b may be involved in the process of CSR induction by anti -CD38, IL-4, and 8-SGuo.

c. Molecular mechanisms of nuclear factor (NF)-κB and the germline γ1 transcript expression in CD38-stimulated B cells

Hiroaki Kaku and Kiyoshi Takatsu

CD38 is an ectoenzyme with both ADP ribosyl cyclase and cADP ribosyl hydrolase activity. Ligation of CD38 on B-2 cells by anti-CD38 mAb induces B cell proliferation, IL-5Rα expression, and the germline γ1 mRNA expression. IL-5 promotes μ-γ1 CSR and IgM and IgG1 production from anti-CD38-stimulated B-2 cells in an IL-4 independent manner. We reported that NF-κB complexes play critical roles in the B cell activation in response to CD38 ligation, and that Btk, PKC, PI3-kinase, BASH/BLNK, PLC-γ2 and extracellular Ca^{2+} influx are involved in NF-κB activation induced by anti-CD38 and IgM and IgG_1 production induced by anti-CD38 and IL-5 co-stimulation. By analyzing the upstream molecules of CD38 signaling pathway, we found that anti-CD38-mediated NF-κB can be induced via activation of Syk and GTP-binding protein. Moreover, GTP-binding protein but not Syk was co-immunoprecipitated with CD38 in cell lysates prepared using Brij-58 detergent. These data suggest that CD38 directly associates with GTP-binding protein or that CD38 constitutively localizes in GTP-binding protein- or GPCR-associated membrane micro-domain. Therefore, we speculate that GTP-binding protein may link and modulate CD38-Syk signaling pathway or that anti-CD38-stimulation induce colocalization of GTP-binding protein into BCR-associated micro-domain.

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-1 cells form distinctive subset of B lymphocyte characterized by preferential distribution to peritoneal cavity and contribution to serum natural antibodies and intestinal IgA secretion. Although several lines of evidence showed that constitutive B cell receptor signaling causes expression of B-1 phenotype, it is still unknown how generation and differentiation of B-1 cells are controlled. IL-5 is indispensable for normal development of B-1 cells because IL-$5R^{-/-}$ mice show reduced number and cell size of peritoneal B-1 cells. To investigate roles of IL-5 in B-1 cell development, we first checked expression of IL-5R on early lymphoid progenitors in the fetal liver. IL-5Rα is not detected on lineage marker

negative (Lin^-) fetal liver cells but weakly detected on $B220^+$ proB cells. Its expression was also induced when Lin^- fetal liver cells were placed in the stroma-free, serum-free culture with B cell differentiating condition. Then Lin^- cells were stimulated or not stimulated with IL-5 in such cultures and transferred to lymphocyte -deficient SCID mice. Progenitors from either condition differentiated to B-1 cells in the SCID mice but more B-1b cells were observed in the mice received IL-5-stimulated progenitors. Interestingly, feces IgA was only restored in the mice received IL-5-stimulated progenitors. B-1b cells differentiated from IL-5-treated progenitors are likely responsible for intestinal IgA, because SCID mice transferred with B-1b cells secreted more IgA in the feces than mice received B-1a cells did. From these results, we speculate that IL-5 induces B-1b cell differentiation in the early stage of gestation, which eventually results in production of IgA in the gut.

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

Masashi Ikutani, Byoung-Gon Moon, Taku Kouro and Kiyoshi Takatsu

B-1 cells differ from B-2 cells in surface markers, anatomical location and developmental pattern, and are known to be involved in natural immunity by producing natural antibodies. B-1 cells form distinctive subset of B lymphocytes characterized by preferential distribution to peritoneal and pleural cavities as well as unique antigen reactivity. Unlike conventional B-2 cells, B-1 cells are rather maintained by self-renewal than de novo differentiation and are activated independent of helper T cells though details in the proliferation and activation of B-1 cells are unknown. We are focusing on IL-5 as a candidate of soluble factor that controls B-1 cell development and activation. Indeed we have shown that IL-5 is indispensable for survival and homeostatic proliferation of peritoneal B-1 cells and mucosal IgA responses. IL-5 is originally found as a soluble factor secreted from helper T lymphocytes but recently significant amount of IL-5 is shown to be secreted from non-lymphoid tissues such as lung. B-1 cells also depend on IL-5 of non-lymphoid origin because neutralizing antibody against IL-5 still takes effect on peritoneal B-1 cells in the host mice lacking lymphocytes. However, technical difficulties have been preventing detection of IL-5 secreting cells that support B-1 cells. To overcome this problem, we are now generating mouse model in which green fluorescent protein gene is inserted in the IL-5 gene locus (IL-5/GFP knock-in mice).

c. Role of IL-5 and B-1 cells in mucosal immunity

Atsuko Itakura, Yuji kikuchi[1], Taku Kouro, Masashi Ikutani, Kiyoshi Takatsu: [1]Laboratory of Immunoregulation, Department of Infection Control and Immunology, Kitasato Institute for Life Sciences, Kitasato University

About a half of IgA is derived from B-1 cells in intestinal and respiratory mucosa, which plays an important role in primary host defense mechanism against pathogens that invade through mucosal tissues. B-1 cell numbers and the size of B-1 cells are decreased in IL-5 receptor α chain-deficient (IL-5Rα$^{-/-}$) mice, and their serum IgM and intestinal IgA levels were lower than those of C57BL/6 mice. Therefore, we tried to determine if lack of IL-5/IL-5R signaling enhances sensitivity to these pathogens.

IL-5Rα$^{-/-}$ and wild type C57BL/6 mice were orally or intranasally inoculated with *Salmonella typhimurium* or influenza A PR/8 virus, respectively. Results revealed that IL-5Rα$^{-/-}$ and wild type mice died in a similar manner following inoculation with *S. typhimurium*, and bacterial loads of Peyer's patches, spleens and mesenteric lymph nodes in IL-5Rα$^{-/-}$ mice were comparable to those in wild type mice. We found no difference in lung virus titers between IL-5Rα$^{-/-}$ and wild type mice inoculated with PR/8. ELISA for IgM, IgG and IgA titers in bronchoalveolar lavage and serum showed that PR/8-specific antibodies were induced in both IL-5 Rα$^{-/-}$ and wild type mice upon inoculation, and antibody titers in IL-5Rα$^{-/-}$ mice were roughly equivalent to those in wild type mice. These results indicate that IL-5/IL-5R signaling does not seem to be involved in host defense against *S. typhimurium* and influenza virus, and antibody responses to these pathogens.

d. B-1 cells in elicitation of contact sensitivity

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. Because IL-5 is important for maintenance of B-1 cells, and promotes antibody production, we examined if IL-5 is involved in elicitation of contact sensitivity. IL-5Rα$^{-/-}$ and wild type C57 BL/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.

We found that ear swelling responses were impaired in IL-5R$\alpha^{-/-}$ mice. Histological examination of wild type mice ears showed edema of the connective tissue with massive accumulation of inflammatory cells including eosinophils. In IL-5R$\alpha^{-/-}$ 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 adaptor proteins in the immune system

a. Control of hematopoietic stem/progenitor cell functions by Lnk adaptor protein, which functions as a molecular scaffold linking RTKs with cytoskeletal regulatory components

Satoshi Takaki, Sang-Mo Kwon, Hitoshi Takizawa, Masanori Iseki, Kazuhiro Sudo[2], Hideo Ema[2], Jun Seita[2], Mitsujiro Osawa[2], Hiromitsu Nakauchi[2] and Kiyoshi Takatsu: [2]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 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), which is strictly regulated in normal conditions. Measurement of competitive repopulation unit (CRU) as well as flow cytometric analysis revealed that there exist nearly 15-fold more functional HSCs in the bone marrow of adult $lnk^{-/-}$ mice compared to normal mice. Clonal analysis by single cell HSC transplantation indicated that a part of $lnk^{-/-}$ HSCs had highly repopulating capability.

Molecular mechanisms underlying Lnk-mediated regulation are not fully understood. We revealed that Lnk could control actin reorganization activated by receptor tyrosine kinases (RTKs), and thereby regulate cell migration. Lnk expressing fibroblasts showed flattened, spreading shapes, prominent actin polymerization accompanied by augmented Rac activation, and impaired migration in a wound-healing assay. Lnk co-immunoprecipitated with Rac, Vav, PAK and filamin A, indicating a complex formation of cytoskeletal regulatory proteins supported by Lnk. Adhesion and migration behavior of $lnk^{-/-}$ progenitor cells are now under investigation.

b. Enhancing repopulation ability of hematopoietic stem/progenitor cells by a targeted inhibition of Lnk

Hitoshi Takizawa, Chiyomi Kubo-Akashi, Ikuo Nobuhisa[3], Sang-Mo Kwon, Masanori Iseki, Tetsuya Taga[3], Kiyoshi Takatsu and Satoshi Takaki: [3]Laboratory of Gene Expression and Regulation, Center for Experimental Medicine, IMSUT

Repopulating ability of HSC/Ps in irradiated host animals is greatly enhanced by the absence of Lnk. In *lnk*-deficient mice, however, neither malignant transformation nor functional defect of blood cells was observed. We identified functional domains of Lnk, generated dominant-negative (DN) Lnk mutants and tested whether DN Lnk mutants could block functions of Lnk endogenously expressed in HSC/Ps and augment repopulation ability of HSC/Ps. Lnk consists of an N-terminal proline-rich region, SH2-, PH-domains and a conserved tyrosine phosphorylation site. Various Lnk mutants were generated and transducted into MC9 mast cells using retroviral vector, and their SCF-dependent growth was evaluated by monitoring $eGFP^{+}$ cells. While the wild-type Lnk efficiently inhibited growth of MC9 cells, a point mutation in the SH2 domain completely abolished the inhibitory effect. The SH2 mutants acted as DN mutants since they cancelled growth inhibition of MC9 transfectants overexpressing Lnk. The SH2 mutant with deletion of PH domain and C-terminal region was the most effective DN Lnk mutant. We, next, evaluated consequences of the DN Lnk expression in HSC/Ps by competitive repopulation assay. HSC/Ps expressing the DN Lnk was transferred into lethally irradiated host animals and percentage of $eGFP^{+}$ cells in peripheral blood was monitored. Cells expressing DN Lnk repopulated at much higher percentage in lymphoid and myeloid lineages than cells treated with control vectors. Inhibition of Lnk by the DN mutant could become a potent approach for expansion and regulation of HSC/Ps.

c. Control of actin reorganization and B-1 cell compartment size by adaptor molecule containing PH and SH2 domains, APS

Masanori Iseki, Chiyomi Kubo-Akashi, Sang-Mo Kwon, Nobuaki Yoshida[3], Kiyoshi Takatsu and Satoshi Takaki

To understand functions of the Lnk family

adaptor proteins further, we tried to identify other members of the family, and isolated mouse APS (adaptor molecule containing PH and SH2 domains). APS is expressed in various tissues including spleen, bone marrow, brain and muscle, and in mature B but not in T or immature B cells. In cell lines, APS is tyrosine phosphorylated upon stimulation with IL-5, IL-3 or anti-IgM. To investigate roles of APS *in vivo*, we generated *APS*$^{-/-}$ mice. *APS*$^{-/-}$ mice were viable, fertile and show no anomalies or growth retardation. Lymphocyte or myeloid cell developments in bone marrow, thymus, spleen and lymph nodes were not perturbed. However, *APS*$^{-/-}$ mice had more B-1 cells in peritoneal cavity, and showed enhanced humoral immune responses against thymus-independent type-2 (TI-2) antigens, while *APS*$^{-/-}$ B-2 cells exhibited normal proliferative responses and tyrosine phosphorylation of intracellular proteins upon BCR crosslinking. On the other hand, in transgenic mice overexpressing APS in lymphocytes, the numbers of peritoneal B-1 and splenic B cells were reduced, and proliferation induced by anti-IgM stimulation was impaired. APS colocalized with filamentous actin (F-actin) accumulated during capping of BCR in *APS*-transgenic B cells. F-actin contents after BCR stimulation was decreased in *APS*$^{-/-}$ B-1 cells compared to wild-type B-1 cells. Our results indicated that APS may have a novel regulatory role in actin reorganization and control of B-lineage cell compartment size.

d. Roles of Lnk-family adaptor proteins, Lnk, SH2-B and APS in growth and functions of mast cells: APS-deficiency causes augmented degranulation and reduced actin assembly

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

Lnk, SH2-B and APS form an adaptor protein family conserved from drosophila. SH2-B is originally identified as a protein associated with immunoreceptor tyrosine-based activation motifs (ITAMs) of FcεRI γ-chain. APS is identified as a potential substrate of c-Kit. Since Lnk, SH2-B and APS are all expressed in bone marrow-derived mast cells (BMMCs), and may play roles in signaling mediated through c-Kit or FcεRI, we investigated consequences of the deficiency either of Lnk, SH2-B or APS in mast cell functions. We established IL-3-dependent BMMCs from *lnk*$^{-/-}$, *SH2-B*$^{-/-}$ and *APS*$^{-/-}$ mice. IL-3-dependent growth of those cells was comparable. Proliferation or adhesion mediated by c-Kit activation as well as degranulation induced by cross-linking FcεRI were normal in the absence of Lnk or SH2-B. In contrast, *APS*$^{-/-}$ BMMCs showed augmented degranulation after cross-linking FcεRI compared to wild type cells, while c-Kit-mediated proliferation and adhesion were kept unaffected. The enhanced degranulation from *APS*$^{-/-}$ BMMCs was due to augmented degranulation from each mast cell but not to increased proportion of cells that underwent degranulation. Calcium influx and tyrosine phosphorylation of various cellular proteins induced by cross-linking FcεRI were normal in the absence of APS, and cell survival mediated by binding of monomeric IgE to FcεRI was also normal. We found, however, that *APS*$^{-/-}$ BMMCs showed reduced F-actin assembly at steady state. *APS*$^{-/-}$ cells were resistant to latrunculin, an inhibitor disrupting F-actin microfilaments in FcεRI-mediated degranulation responses. Our results suggest potential roles of APS in controlling actin cytoskeleton and magnitude of degranulation in mast cells.

4. Mechanisms of preferential induction of Th_1 response upon immunization with Mycobacteria peptide

The Ag85 of *Mycobacterium* (*M.*) *tuberculosis* and *M. Bovis* BCG is immunogenic in C57BL/6 mice. Immunigation of C57BL/6 mice with Ag85B expands TCRVβ11$^+$ CD4$^+$ Th1 cells in conjunction with APCs in an I-A^b-restricted manner. We identified the major antigenic epitope (Peptide-25) for Ag85B-specific Vβ11$^+$ T cells as the 15-mer peptide, covering amino acid residues 240-254 of Ag85B.

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

Haruyuki Ariga, Makiyo Nakada, Takeshi Tokunaga, Yoko Shimohakamada, 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 pathogens and the development of organ-specific autoimmune diseases. In addition to the T cell antigen receptor (TCR) activation signals, other factors such as 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, and the differential activities of naive $CD4^+$ T cells from P25 TCR-Tg were examined.

Naive $CD4^+$ T cells from P25 TCR-Tg preferentially differentiated into Th1 cells upon Peptide-25 stimulation in the presence of T and NK cell-depleted I-A^b splenic APC under neutral conditions. In contrast, a mutant of Peptide-25 could induce solely Th2 differentiation. Peptide-25-induced Th1 differentiation was observed even in the presence of neutralizing monoclonal antibodies to IFN-γ and IL-12. Furthermore, naive $CD4^+$ T cells from STAT1 deficient P25 TCR-Tg also differentiated into Th1 cells upon Peptide-25 stimulation. Moreover, Peptide-25-loaded I-A^b-transfected Chinese hamster ovary cells (Peptide-25-I-A^b-CHO) that do not express co-stimulatory molecules, such as CD40/80/86 and ICAM-1 on their surface, and not produce any IFN-γ and IL-12 effectively, induced Th1 differentiation of naive $CD4^+$ T cells from P25 TCR-Tg. Within 3 hr after the TCR stimulation with Peptide-25-I-A^b-CHO, IFN-γ- and IL-12-independent transient *T-bet* up-regulation and suppression of *GATA-3* expression were observed. These results imply that direct interaction between Peptide-25/I-A^b and TCR primarily influences determination of the fate of naive CD 4^+ T cells in differentiation toward the Th1 subsets.

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. Tumor cells are thought to express an array of antigens recognizable by CTLs that principally contribute to tumor rejection. It still remains unclear, however, 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. Results revealed that co-immunization of C57BL/6 mice with OVA and Peptide-25 could induce higher OVA specific-IgG2a and IFN-γ production than OVA immunization. Intriguingly, a robust OVA-specific CTL generation was also induced when mice were co-immunized with OVA plus Peptide-25. The adjuvant effect of Peptide-25 was not observed in CD4 deficient or IFN-γ deficient mice. Co-immunization of OVA and Peptide-25 could prevent *in vivo* growth of EG.7 cell (EL4 thymoma transfected with cDNA encoding chicken OVA) leading to the tumor rejection. Furthermore, the enhancement of CTL generation by Peptide-25 was also observed when class I-binding OVA peptide (SIINFEKL) was used in place of intact OVA. Moreover, CTL generation specific for class I-binding B16 melanoma peptide (SVDFFVWL) was enhanced by co-immunization with Peptide-25. 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 surface markers on DC. When we co-cultured DC with $CD4^+$ T cells from P25 TCR-Tg mice together with Peptide-25, expressions of MHC class I and ICAM-1 were enhanced and led to the inducation of IL-12p40 production. Such DC showed more effective OVA presentation to OVA specific $CD8^+$ T cells and enhanced cell divisions.

These results indicate that Peptide-25 exerts potent adjuvant activity and provides efficient help for CTL induction against neo-tumor antigen when concomitantly immunized.

5. Role of mast cells, eosinophils, and IL-5 in the development of asthma

Yoko Oe-Kikuchi and Kiyoshi Takatsu

Mast cells are though to contribute to the pathogenesis of allergic airway responses through IgE dependent mechanism. Eosinophilic inflammation is clearly a hallmark of both allergic and non-allergic asthma. Considerable evidence suggests that there is association between pulmonary eosinophil infiltration and AHR in human asthma. An immunopathogenic role for mast cells is suggested by the role of IL-4, IL-13, IL-5 in IgE synthesis and eosinophil differentiation and activation, however, the exact mechanism by which mast cells mediate eosinophilic inflammation and subsequent AHR are still not entirely clear.

We have studied the role of IL-5 on mast cell and eosinophil activation and role of activated mast cells in activation and survivability of eosinophils. First, we have studied IgE-dependent production of histamine and cytokine

(IL-5 and GM-CSF) by mast cells isolated from IL-5Ra KO and IL-5 KO mice to observe the function of IL-5 on releasability. We observed that mast cells, expressing detectable IL-5Ra, increased their steady state expression of IL-5 mRNA after cross-linking the IgE receptor. Although we have observed IL-5, GM-CSF, and histamine release from these mast cells, there were no significant difference on each releasability, however, we have found that IL-5 was able to reduce histamine content in mast cells in dose dependent manner. Second, we have established the system to isolate eosinophils from a long-term bone marrow culture supplemented with IL-5. Third, to understand the bioactivity of released product from activated mast cells, we have further investigated the effect of mast cell supernatants on activation (such as degranulation and expression of adhesion molecule) and survivability of eosinophils isolated from a culture of bone marrow cells.

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Cellular mechanisms for the surveillance and transcriptional suppression of introgenomic parasites such as retroviruses 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 as 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 human cellular defence system and counter-defence 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 inevitably uses epigenetical regulation systems to shut-off the virus gene expression. Therefore elucidation of epigenetical regulation mechanisms is now essential to understand both host and viral strategies in this post-genome era. In cellular nuclei, 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). There is a growing body of evidence indicating that chromosomal regulation and RNA silencing are interconnected.

For the understanding of epigenetics, we have been concentrated on the major chromatin remodeling factor in human, SWI/SNF complex, which is composed of 10 protein subunits. The 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 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.

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 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 this early study 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 also concentrated on the molecular mechanisms underlaying this phenomenon this year and found that SWI/SNF complex can supress of transcription in a specific context and plays rather broad biological function depending upon each promoter even in the same cell (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* 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 or through stability of mature *Brm* mRNA. This suppression was strongly attenuated by transient treatment of these cell lines with HDAC inhibitors 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 a key negative regulator of neuronal gene expression in human non-small cell lung carcinoma cell lines.

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In this year, we found 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 necessary 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 mSin 3A 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. Design and establishment of new retrovirus vectors that express shRNA efficiently.

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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 endogenous 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 directly or short hairpin (sh) RNA that functions as an artificial precursor of siRNA.

This year, 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 exogenous GFP gene). We finally established a MuLV-based retrovirus vector that can reduce the expression level of exogenous GFP up to 1%. To achieve such an efficient "kcock-down", following vector structures and transduction procedure were needed.

1. The MuLV based vector DNA was deleted in a large region of 3′-LTR U3, and to the deleted region, shRNA expression unit was inserted. Therefore after vector preparation by DNA transfection, the provirus lost functional Pol II promoter activity in both 5′-and 3′-LTR, and have a structure so called a "self-inactivating vector". The shRNA expression unit is composed of U6 promoter (drived by Pol III), the hairpin region which contains a loop sequence originated from mir-23a and subsequently oligoT (T_6) sequence (termination signal of Pol III). This unit is present at both LTRs of the provirus.
2. The vector should be prepared as VSV-G pseudotyped vector to ensure introduction of multi-copy proviruses into the human cell by a single transduction.
3. This shRNA expression vector shows dosage-dependent suppressing activity when introduced up to 3～7 copies per cell and can reduse the expression levels of the target gene to 10% of that of uninfected cell or empty vector transduced cell. But higher vector dosage of the same vector shows no additional suppression. When these cells were transduced with another vector that expresses a different shRNA of the same target gene, the expression levels of the target gene was reduced up to 1% of the original cell.

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 stem cells or non-dividing human cells. Therefore, we are now introducing the same shRNA expression unit into lentivirus vectors for broader application. We are also analyzing the molecular mechanisms of RNA silencing using the cell lines established above, in which the target GFP gene is efficiently suppressed.

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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. Production of novel Ebola virus-like particles from cDNAs: an alternative to Ebola virus generation by reverse genetics.

Watanabe S, Watanabe T, Noda T, Takada A, Feldmann H, Jasenosky LD, Kawaoka Y.

We established a plasmid-based system for generating infectious Ebola virus-like particles (VLPs), which contain an Ebola virus-like minigenome consisting of a negative-sense copy of the green fluorescent protein gene. This system produced nearly 10^3 infectious particles per ml of supernatant, equivalent to the titer of Ebola virus generated by a reverse genetics system. Interestingly, infectious Ebola VLPs were generated, even without expression of VP24. Transmission and scanning electron microscopic analyses showed that the morphology of the Ebola VLPs was indistinguishable from that of authentic Ebola virus. Thus, this system allows us to study Ebola virus entry, replication, and assembly without biosafety level 4 containment. Furthermore, it may be useful in vaccine production against this highly pathogenic agent.

2. A Human Macrophage C-Type Lectin Specific for Galactose and *N*-Acetylgalactosamine Promotes Filovirus Entry.

Takada A, Fujioka K, Tsuiji M, Morikawa A, Higashi N, Ebihara H, Kobasa D, Feldmann H, Irimura T, Kawaoka Y.

Filoviruses cause lethal hemorrhagic disease in humans and nonhuman primates. An initial target of filovirus infection is the mononuclear phagocytic cell. Calcium-dependent (C-type) lectins such as dendritic cell- or liver/lymph node-specific ICAM-3 grabbing nonintegrin (DC-SIGN or L-SIGN, respectively), as well as the hepatic asialoglycoprotein receptor, bind to Ebola or Marburg virus glycoprotein (GP) and enhance the infectivity of these viruses in vitro. Here, we demonstrate that a recently identified human macrophage galactose- and *N*-acetylga-

lactosamine-specific C-type lectin (hMGL), whose ligand specificity differs from DC-SIGN and L-SIGN, also enhances the infectivity of filoviruses. This enhancement was substantially weaker for the Reston and Marburg viruses than for the highly pathogenic Zaire virus. We also show that the heavily glycosylated, mucin-like domain on the filovirus GP is required for efficient interaction with this lectin. Furthermore, hMGL, like DC-SIGN and L-SIGN, is present on cells known to be major targets of filoviruses (i. e., macrophages and dendritic cells), suggesting a role for these C-type lectins in viral replication in vivo. We propose that filoviruses use different C-type lectins to gain cellular entry, depending on the cell type, and promote efficient viral replication.

3. A protective immune response in mice to viral components other than hemagglutinin in a live influenza A virus vaccine model.

Horimoto T, Takada A, Iwatsuki-Horimoto K, Kawaoka Y.

Previously, we generated influenza A viruses that possess chimeric type (A/B) hemagglutinins (HA), in which immunogenic regions of type A HA were replaced with those of type B HA, and showed that these viruses were attenuated in mice. Here we intranasally immunized mice with these viruses and then challenged them with a wild-type A virus to assess a protective immune response to viral components other than HA in the form of a live virus. All immunized mice survived challenge with a lethal dose of wild-type virus; none or a limited amount of virus, if any, was recovered from nasal turbinates or lungs of the mice 3 days post-challenge. These results provide direct evidence that immune responses to viral components other than HA confer protection against influenza A virus infection in a mouse model, suggesting the usefulness of live vaccines for viruses that have undergone antigenic drift with respect to HA, or for viruses with heterosubtypic HAs.

4. Influenza B Virus Requires BM2 Protein for Replication.

Hatta M, Goto H, Kawaoka Y.

The BM2 protein of influenza B virus functions as an ion channel, which is suggested to be important for virus uncoating in endosomes of virus-infected cells. Because direct support for this function is lacking, whether BM2 plays an essential role in the viral life cycle remains unknown. We therefore attempted to generate BM 2 knockout viruses by reverse genetics. Mutant viruses possessing M segments with the mutated initiation codon of BM2 protein at the stop -start pentanucleotide were viable and still expressed BM2. The introduction of multiple stop codons and a one-nucleotide deletion downstream of the stop-start pentanucleotide, in addition to disablement of the BM2 initiation codon, failed to generate viable mutant viruses, but the mutant M segments still expressed proteins that reacted with the BM2 peptide antiserum. To completely abolish BM2 expression, we generated a mutant M gene whose BM2 open reading frame was deleted. Although this mutant was not able to replicate in normal MDCK cells, it did replicate in a cell line that we established which constitutively expresses BM2. Furthermore, a virus possessing the mutant M gene lacking the BM2 open reading frame and a mutant NA gene containing the BM2 open reading frame instead of the NA open reading frame underwent multiple cycles of replication in MDCK cells, with exogenous sialidase used to supplement the deleted viral sialidase activity. These findings demonstrate that the BM2 protein is essential for influenza B virus replication.

5. Characterization of a neuraminidase-deficient influenza A virus as a potential gene delivery vector and a live vaccine.

Shinya K, Fujii Y, Ito H, Ito T, Kawaoka Y.

We recently identified a packaging signal in the neuraminidase (NA) viral RNA (vRNA) segment of an influenza A virus, allowing us to produce a mutant virus [GFP(NA)-Flu] that lacks most of the NA open reading frame but contains instead the gene encoding green fluorescent protein (GFP). To exploit the expanding knowledge of vRNA packaging signals to establish influenza virus vectors for the expression of foreign genes, we studied the replicative properties of this virus in cell culture and mice. Compared to wild-type virus, GFP(NA)-Flu was highly attenuated in normal cultured cells but was able to grow to a titer of $>10^6$ PFU/ml in a mutant cell line expressing reduced levels of sialic acid on the cell surface. GFP expression from this virus was stable even after five passages in the latter cells. In intranasally infected mice, GFP was detected in the epithelial cells of nasal mucosa, bronchioles, and alveoli for up to 4 days postinfection. We attribute the attenuated growth of GFP(NA)-Flu to virion aggregation at the surface of bronchiolar epithelia. In studies to test the potential of this mutant as a live attenu-

ated influenza vaccine, all mice vaccinated with $\geq 10^5$ PFU of GFP(NA)-Flu survived when challenged with lethal doses of the parent virus. These results suggest that influenza virus could be a useful vector for expressing foreign genes and that a sialidase-deficient virus may offer an alternative to the live influenza vaccines recently approved for human use.

6. Generation of influenza A virus NS2(NEP) mutants with an altered nuclear export signal sequence.

Iwatsuki-Horimoto K, Horimoto T, Fujii Y, Kawaoka Y.

The NS2 (NEP) protein of influenza A virus contains a highly conserved nuclear export signal (NES) motif in its amino-terminal region ($_{12}$ ILMRMSKMQL$_{21}$, A/WSN/33), which is thought to be required for nuclear export of viral ribonucleoprotein complexes (vRNPs) mediated by a cellular export factor, CRM1. However, simultaneous replacement of three hydrophobic residues in the NES with alanine does not affect NS 2 (NEP) binding to CRM1, although the virus with these mutations is not viable. To determine the extent of sequence conservation required by the NS2 (NEP) NES for its export function during viral replication, we randomly introduced mutations by degenerative mutagenesis into the region of NS cDNA encoding the NS2 (NEP) NES and then attempted to generate mutant viruses containing these alterations by reverse genetics. Sequence analysis of the recovered viruses showed that although some of the mutants possessed amino acids other than those conserved in the NES, hydrophobicity within this motif was maintained. Nuclear export of vRNPs representing all of the mutant viruses was completely inhibited in the presence of a CRM1 inhibitor, leptomycin B, as was the transport of wild-type virus, indicating that the CRM1-mediated pathway is responsible for the nuclear export of both wild-type and mutant vRNPs. The vRNPs of some of the mutant viruses were exported in a delayed manner, resulting in limited viral growth in cell culture and in mice. These results suggest that the NES motif may be an attractive target for the introduction of attenuating mutations in the production of live vaccine viruses.

7. Resistant influenza A viruses in children treated with oseltamivir: descriptive study.

Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden FG, Sugaya N, Kawaoka Y.

Oseltamivir is an effective inhibitor of influenza virus neuraminidase. Although viruses resistant to oseltamivir emerge less frequently than those resistant to amantadine or rimantadine, information on oseltamivir-resistant viruses arising during clinical use of the drug in children is limited. Our aim was to investigate oseltamivir resistance in a group of children treated for influenza. We analysed influenza A viruses (H3N2) collected from 50 children before and during treatment with oseltamivir. We sequenced the genes for neuraminidase and haemagglutinin and studied the mutant neuraminidases for their sensitivity to oseltamivir carboxylate. We found neuraminidase mutations in viruses from nine patients (18%), six of whom had mutations at position 292 (Arg292Lys) and two at position 119 (Glu119Val), which are known to confer resistance to neuraminidase inhibitors. We also identified another mutation (Asn294Ser) in one patient. Sensitivity testing to oseltamivir carboxylate revealed that the neuraminidases of viruses that have an Arg292Lys, Glu119Val, or Asn294Ser mutation were about 10(4)-10(5)-fold, 500-fold, or 300-fold more resistant than their pretreatment neuraminidases, respectively. Oseltamivir-resistant viruses were first detected at day 4 of treatment and on each successive day of the study. More than 10(3) infectious units per mL of virus were detected in some of the patients who did not shed drug-resistant viruses, even after 5 days of treatment. Oseltamivir-resistant mutants in children being treated for influenza with oseltamivir arise more frequently than previously reported. Furthermore, children can be a source of viral transmission, even after 5 days of treatment with oseltamivir.

8. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus.

Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, Suzuki H, Nishimura H, Mitamura K, Sugaya N, Usui T, Murata T, Maeda Y, Watanabe S, Suresh M, Suzuki T, Suzuki Y, Feldmann H, Kawaoka Y.

The 'Spanish' influenza pandemic of 1918-19 was the most devastating outbreak of infectious disease in recorded history. At least 20 million people died from their illness, which was characterized by an unusually severe and rapid clinical course. The complete sequencing of several genes of the 1918 influenza virus has made it possible to study the functions of the proteins encoded by these genes in viruses generated by reverse genetics, a technique that permits the

generation of infectious viruses entirely from cloned complementary DNA. Thus, to identify properties of the 1918 pandemic influenza A strain that might be related to its extraordinary virulence, viruses were produced containing the viral haemagglutinin (HA) and neuraminidase (NA) genes of the 1918 strain. The HA of this strain supports the pathogenicity of a mouse-adapted virus in this animal. Here we demonstrate that the HA of the 1918 virus confers enhanced pathogenicity in mice to recent human viruses that are otherwise non-pathogenic in this host. Moreover, these highly virulent recombinant viruses expressing the 1918 viral HA could infect the entire lung and induce high levels of macrophage-derived chemokines and cytokines, which resulted in infiltration of inflammatory cells and severe haemorrhage, hallmarks of the illness produced during the original pandemic.

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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. Anti-Toll like receptor 4 /MD-2 Antibody Protects Mice from Acute Lethal Hepatitis induced by Tumor Necrosis Factor-α

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MD-2 is associated with the extracellular domain of TLR4, and TLR4/MD-2 complex recognizes LPS. However, the function of TLR4/MD-2 for recognition of LPS has not been fully elucidated yet. To investigate the function of TLR4/MD-2, we established two-type of mAbs to mouse TLR4/MD-2, Sa15-21 and MTS510.

Sa15-21, which is able to co-precipitate the LPS directly bound to TLR4/MD-2, prevents mice from endotoxin shock induced by LPS and D-galactosamine (D-GalN) completely. On the other hand, MTS510, which is not able to recognize the LPS/TLR4/MD-2 complex but it inhibits LPS signaling, does not prevent mice from LPS/(D-GalN) induced endotoxin shock.

In this article, we show that serum TNF-α induced by LPS/D-GalN is upregulated by the Sa 15-21 pretreatment. We also show that the Sa15-21 protects mice from lethal liver failure induced by TNF-α/D-GalN. Lastly, we show that Sa15-21 activates NF-κB activity as judged by a reporter cell line, and induces NF-κB-dependent anti-apoptotic genes in the liver, and these effect of Sa15-21 is dependent on TLR4-signaling. We conclude that Sa15-21 mAb inhibits mice hepatocyte apoptosis and liver damage caused by the agonistic signal through TLR4. These results suggest therapeutic application of an agonistic anti-TLR4/MD-2 mAb. (Japanese Ministry of Education and Science Research; the Naito Foundation)

2. Protective roles of tumor necrosis factor derived from mast cells in murine malaria

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In this study, we first demonstrated the importance of mast cells for host immunity in malaria. The key molecule responsible for protection in this malaria model is TNF from mast cells. Association between mast cell activation and elevation of serum TNF levels resulted in enhanced expression of protection. Critical evidences on the essential role of mast cell-derived TNF for protection against *P. berghei* ANKA infection were obtained by experiments using reconstitution of mast cell deficient mice with BMMCs of +/+ or TNF deficient mice. It has been well known that TNF is a major cytokine responsible for protection and pathogenesis in malaria. TNF controls both protective immunity and severity of malaria. TNF levels are used as an indicator of clinical severity in human malaria. The importance of TNF in protection was also confirmed in our studies with anti-TNF treatment. The sources of TNF have been considered to be macrophages and/or T cells with interaction of these cells. We proposed that mast cells are a critical source of TNF in addition to macrophages and T cells. Distinctive features of mast cells include their ability to storage and release large amounts of TNF, and their close association with blood vessels. It is noteworthy that presynthesized TNF in mast cells is a candidate for the immediate initiation of the host response to infection. Since the secretion of TNF by other cell types is greatly delayed by the time required to complete *de novo* synthesis of this cytokine, involvement of mast cell activation through innate immunity or direct stimulation is likely to function in the present model. Indeed, higher parasitemia was found in W/W^v mice in an early stage of infection when acquired immune responses might not operate. After the stage of innate immunity, mast cells are activated with IgE antibody and complement-fixing antibody, through FcεR and anaphylatoxin respectively, as an acquired immunity. These features play special importance during malaria.

3. Lipid A Antagonist Revealed a Role for MD -2 in a Link between LPS interaction and subsequent Toll-like receptor 4-oligomerization

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Lipopolysaccharide (LPS) is a membrane component of Gram negative bacteria with potent immuno-stimulating activity. Toll-like receptor 4 (TLR4) delivers a transmembrane activation signal upon LPS stimulation. MD-2, an extracellular molecule that is associated with the extracellular domain of TLR4, is indispensable for cell surface expression of and LPS recognition by TLR4. Despite identification of TLR4-MD-2 as the LPS recognition molecules, little is known about a molecular mechanism for LPS recognition. Recently we demonstrated the direct interaction between LPS and TLR4-MD-2 on the cell surface. We here show a subsequent event, LPS-dependent TLR4-MD-2 oligomerization. Epitope -tagged TLR4 was coprecipitated with TLR4 tagged with another epitope during 5-150 min after LPS stimulation. TLR4 oligomerization required membrane CD14, but not the cytoplasmic signaling portion of TLR4. TLR4 oligomerization was triggered by lipid A but not by a lipid A antagonist lipid IVa, although both bind similarly to TLR4-MD-2 (although lipid IVa binds to TLR4-MD2 even better than lipid A). To address a role for MD-2 in TLR4-oligomerization, we took an advantage that lipid IVa is agonistic on mouse TLR4-MD-2 but antagonistic when mouse MD-2 is replaced with human MD-2. In keeping with this, Lipid IVa-dependent mouse TLR4 oligomerization was observed with mouse MD-2 but not with human MD-2. Taken together, MD-2 plays an important role in LPS recognition by linking LPS interaction with TLR 4-MD-2 to TLR4 oligomerization. We also demonstrate the antagonistic mechanism of lipid IVa by the competitive inhibition of TLR4-oligomerization. More effective drug to treat septic shock will be designed from these results in near future.

4. The search of the association molecules with toll-like receptors to recognize microbial components

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We research the mechanisms of recognition of bacterial components by toll-like receptors (TLRs). TLR4 and MD-2 make a complex and recognize lipopolysaccharide (LPS). It is considered that some cell surface proteins or serum proteins work in cooperated with TLR4/MD-2 complex or other TLRs to recognize the bacterial components. CD14 and LBP play the roles of the effective transfer of LPS to TLR4/MD-2 complex. Several findings indicate that other molecules associate with the binding of LPS to TLR 4/MD-2. Immunoprecipitation of TLR4/MD-2 in presence of LPS indicated that plasma gelsolin was one candidate as an associated molecule to recognize LPS by TLR4/MD-2. In regard to TLR 2, it was reported that this TLR make complex with other TLRs, TLR1 or TLR6, and recognized bacterial lipopeptide. However, splenocyte obtained from TLR1 or TLR6 deficient mice show us the existence of other molecules to recognize lipopeptide. We also search the associated molecules with TLR2 when it recognizes lipopeptide utilizing the artificially mutated cells in the responses to TLR2 ligands. We have continuously analyzed the function of gelsolin or other associating molecules to TLR4/MD-2 and also searched the associating molecules to TLR2.

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炎症免疫学分野

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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. Mucosal Vaccine:

I. Development of M-cell Target Mucosal Vaccine

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Peyer's patch (PP) and nasopharynx-associated lymphoid tissue (NALT) possess a unique subset of antigen-sampling cell called M-cell. Mucosally delivered antigens are generally taken up by M-cells and delivered to professional antigen presenting cells, including macrophages and dendritic cells located in the pocket of M-cell. In addition to PP associated M cells, we have identified the development of villous M cells, as a new and novel gateway for the outside environment. Elucidation of the molecular and cellular characteristics of PP associated and villous M-cells would not only greatly facilitate the fundamental aspect of M cell biology, but also lead to the design of effective mucosal vaccine and the discovery of novel targets for developing new mucosal antigen and drug delivery vehicle to combat pathogens and allergens. To this end, our efforts have been focusing on the identification of M-cell-specific gene(s) and corresponding proteins by using DNA micro array and proteomics approaches.

II. Development of M Cell Targeted Edible Vaccine

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The production of vaccine antigens in plants has many potential advantages for new generation of vaccine which will contribute further advancement of clinical medicine for public health. For example, the plant system more economically produces vaccine antigen than the industrial fermentation system. Second, the purifica-

tion requirement can be eliminated when plant tissue containing vaccine is used as food or given via the physiological route of ingestion. Third, the plant system can direct accumulation of vaccine antigen into the intracellular compartment where they are more stable. Finally the risk arising from contamination with potential human pathogens can be minimized. Taking together, plant-based subunit vaccine is considered to be not only effective but also practical mucosal vaccine. To this end, our efforts have been focusing on the development of new generation of mucosal vaccine, M cell targeted edible vaccine for combating infectious diseases and bioterrorism.

III. NALT Target Mucosal Vaccine: Mechanism of NALT Organogenesis

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NALT is thought to be a key targeted lymphoid tissue for the nasal vaccination. The mechanism of NALT organogenesis is quite different from that of Peyer's patch (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). CXCL13 and its ligand CXCR5 are essential for the induction of PP organogenesis by $CD3^{-}CD4^{+}CD45^{+}$ cells. Thus, our current efforts are aimed at the elucidation of CXCL13/CXCR5 interaction for the genesis of NALT.

2. Mucosal allergy: DNA Vaccine for The Control of Allergic Diarrhea

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To develop therapic vaccine against food allergy, we demonstrated that nasally administrated murine IL-12 naked DNA expression plasmids, containing both the p35 and p40 subunits (IL-12p70) resulted in the synthesis of co-responding cytokine in large intestine leading to the inhibition of antigen-specific Th2 type response. The development of the allergic diarrhea was prevented in mice nasally treated with IL-12p70 DNA with the inhibition of clinical symptom including OVA-specific IgE Ab synthesis. To clarify the distribution of co-responding protein treated by naked DNA nasally, the expression of nasally administrated naked GFP^{+}DNA was chronologically demonstrated by the using in vivo image analysis and confocal microscopy. Our further analysis revealed that nasally administrated DNA resulted in the preferential protein expression of IL12p70 by CD11c positive cells in NALT, spleen and intestine. In addition, IL-12p70 producing DCs were located near lymph vessel of large intestine. The nasal IL-12p 70 DNA treatment was also effective even after the establishment of allergic diarrhea. Our findings provide new possibility for the use of the clinical investigation of mucosal DNA vaccine and mucosal gene therapy for the control of mucosal immune disease.

3. Uniqueness of mucosal antigen presenting cells

I. Classical and non-classical MHC-mediated antigen presentation for the regulation of mucosal immune system

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Understanding of antigen processing and presentation pathway at mucosal sites will provide us important information for the development of efficient mucosal vaccine. However, very minimum information is currently available about the molecular aspect of antigen processing and presentation at mucosal compartment. To address this, we developed a novel enzymatic method (KOVAK system) to detect antigenic intermediates generated in living cells, which revealed that intracellular proteolysis pathway for the generation of antigenic intermediates and the function of molecular chaperones in the antigen-processing pathway. One of our

current efforts is aimed to clarify the molecular pathway for the antigen processing and presentation at the mucosal site using the KOVAK system, and to apply the novel information to the development of mucosal vaccine. Additionally, recent study has proposed that non-classical MHC molecules expressed on the mucosal epithelial cells also play an important role in the maintenance of mucosal homeostasis mediated by intraepithelial lymphocytes (IELs). Thus, an additional purpose of our study is to identify the resemblance and discrepancy between classical MHC- and non-classical MHC-mediated antigen-presentation pathway and to reveal their function for the regulation of mucosal immune system.

II. 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 $F4/80^+$ $Gr\text{-}1^+CD11b^+CD11c^+$ cells as segmented cells in intestinal lamina propria (i-LP). Since these cells possessed morphological characteristics of neutrophiles 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 the T regulatory by adoptive transfer of *in vivo* OVA-primed EDCs into anti-CD25 treated T regulatory deficient mice. 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.

Publications

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