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, enteropathogenic E. coli, enterohemorrhagic E.coli and Helicobacter pylori. We are also searching for effective methods to protect or regulate bacterial infection by using knowledge accumulated.

1. IcsB, secreted via the type III secretion system, is chaperoned by IpgA and required at post invasion stage of *Shigella* pathogenicity

Michinaga Ogawa, Toshihiko Suzuki, Ichiro Tatsuno, Hiroyuki Abe and Chihiro Sasakawa

Shigella deliver a subset of effector proteins such as IpaA, IpaB and IpaC via the type III secretion system (TTSS) into host cells during the infection of colonic epithelial cells. Many bacterial effectors including some from *Shigella* require specific chaperones for protection from degradation and targeting to the TTSS. In this study, we have investigated the role of the *icsB* gene located upstream of the *ipaBCDA* operon in *Shigella* infection, since the role of IcsB as a virulence factor remains unknown. Here, we found that the IcsB protein is secreted via the TTSS of *Shigella in vitro* and *in vivo*. We show that IpgA protein encoded by *ipgA*, the gene immediately downstream of *icsB*, serves as the chaperone required for the stabilization and secretion of IcsB. We showed that IcsB was bound to IpgA in bacterial cytosol, and the binding site was in the middle of the IcsB protein. Furthermore, the contribution of IcsB to the pathogenicity of *Shigella* was demonstrated by the Sereny test and plaque-forming assay. When guinea pig eyes were infected with a non-polar *icsB* mutant, the bacteria failed to provoke keratoconjunctivitis. Furthermore, the ability of the *icsB* mutant to form plaques was greatly reduced as compared with that of the wild type in MDCK cell monolayers. By electron microscopy, accumulation of the multi-lamellar bodies, as are often seen in autophagy, was observed around *icsB* mutant in the cytosol of MDCK cells. Indeed, when MDCK cells stably expressing GFP-LC3 were infected with *Shigella* strains, the accumulation of GFP-LC3, as is known as a marker of autophagosome, around bacteria was obseved more frequently in *icsB* mutant than in wild type. Furthermore, the accumulation of GFP-LC3 in MDCK cells infected with *icsB* mutant was diminished by treatment with inhibitor of autophagy, *ex* wortmannin, 3-MA and BafA1. These suggest that *icsB* mutant could be arrested by autophagosome. These results suggest

that IcsB is secreted via the TTSS, chaperoned by IpgA, and required at the post-invasion stage of *Shigella* pathogenicity.

2. Exploiting host microtubule dynamics: a new aspect of bacterial invasion

Sei Yoshida and Chihiro Sasakawa

During infection, many pathogenic bacteria modulate the actin cytoskeleton of eukaryotic host cells to facilitate various infectious processes such as the attachment to or invasion of epithelial cells. Additionally, some pathogenic bacteria are capable of modulating the dynamics of host microtubule (MTs). Although the molecular basis for this is still poorly understood, a recent study of the *Shigella* VirA effector protein, which is delivered via a type III secretion system, suggests that MT destabilization plays an important role in *Shigella* infection.

3. Structural definition on the surface of *Helicobacter pylori* type IV secretion apparatus

Jiro Tanaka, Toshihiko Suzuki, Hitomi Mimuro and Chihiro Sasakawa

Genetic and functional studies have indicated that the type IV secretion system (TFSS) of *Helicobacter pylori* forms a secretion complex in the cell envelope that protrudes towards the outside in order to inject CagA protein into gastric epithelial cells. However, the proposed structural model is based on partial amino acid homology with the components of the *Agrobacterium tumefaciens* TFSS. Therefore, we undertook the identification of the structural features of the TFSS exposed on the surface of *H. pylori* and found that filamentous structures present on the bacterial surface are related to the secretion apparatus. Using immunofluorescence microscopy with antibodies directed to tyrosine-phosphorylated CagA (pY-CagA) and Hp0532 (VirB7) in the infection assay, pY-CagA signals were detected just below the host cell-attached bacteria, where Hp0532 (VirB7) signals were detected as colocalized, suggesting that the CagA injected into the host cell through the TFSS apparatus is still mostly confined to the areas just below the attached bacteria after being phosphorylated. Furthermore, the filamentous structures on bacterium were found to be associated with Hp0532 (VirB7) or Hp0528 (VirB9), the major components of TFSS, by immunogold electron microscopy. These results strongly suggest that the *H. pylori* TFSS apparatus is a filamentous macromolecular structure protruding from the bacterial envelope.

4. Hyper Adherence to Caco-2 Cells Caused by Disruption of *yhiE* and *yhiF* Genes in Enterohemorrhagic *Escherichia coli* O157: H7

Ichiro Tatsuno, Keiji Nagano[1], Kazuki Taguchi[1], Li Rong, Hiroshi Mori[1] and Chihiro Sasakawa: [1]Laboratory of Microbiology, Department of Public Health Pharmacy, Gifu Pharmaceutical University.

Adherence of enterohemorrhagic *Escherichia coli* (EHEC) to intestinal epithelium is essential for initiation of the infection including diarrhea, and for the adherence, expression of the genes of the locus for enterocyte effacement (LEE) is thought to be crucial. To identify genes involved in modulating the adherent capacity, a collection of an EHEC O157 : H7 strain (O157Sakai) mutagenized by mini-Tn*5*Km2 were screened for their ability to increase the number of microcolonies (MC) on Caco-2 cells, and eight hyper adherent mutants were isolated. Analysis of the mini-Tn*5*Km2-flanked DNA sequences indicated that one possessed the insertion within an O157 antigen gene cluster, the other within the *yhiF* gene, and the remaining 6 mutants had their insertions in the *yhiE* gene. *yhiE* and *yhiF* products share amino acid homology (23% identity) to each other and with the LuxR family known as transcriptional regulatory proteins. The mutant having the insertion within the O157 antigen gene cluster, but not the other seven mutants, did not express the O157 side chain as determined by agglutination test and immunoblotting with polyclonal O157-specific antiserum. Importantly, the other mutants showed enhanced type III secretion. Their related mRNAs of LEE, but not *ler* mRNA, were also increased as compared with those in the wild-type. Indeed, when we introduced an in-frame deletion into the *yhiE* or *yhiF* gene in O157Sakai, the capacity of the resultant mutants to adhere to Caco-2 cells was greatly increased. When one of the *yhiE* insertion mutants was orally inoculated into ICR mice, the number of bacteria shed into feces by day 14 was greater than that for wild-type. These results suggest that *yhiE* and *yhiF* are involved in the adherence of O157Sakai to epithelial cells as negative regulators for the expression of the genes required for the type III secretion system.

5. Distribution of the secondary type III secretion system locus found in enterohemorrhagic *Escherichia coli* O157:H7 isolates among Shiga toxinproducing *E. coli* strains

Sou-Ichi Makino[1 2], Toru Tobe[3], Hiroshi

Asakura[1], Masahisa Watarai[1], Tetsuya Ikeda[4], Koichi Takeshi[4], Chihiro Sasakawa: [1]Department of Applied Veterinary Science, Research Center for Animal Hygiene and Food Safety, [2]Obihiro University of Agriculture and Veterinary Medicine, [4]Hokkaido Institute of Public Health, [3]Division of Applied Bacteriology, Graduate School of Medicine, Osaka University

The ability of the complete genome sequence of enterohemorrhagic *Escherichia coli* O157 led to the identification of a 17-kb chromosomal region which contained a type III secretion system gene cluster at min 64.5. This locus contains open reading frames whose amino acid sequences show high degrees of similarity with those of proteins that make up the type III secretion apparatus, which is encoded by the *inv-spa-prg* locus on a *Salmonella* SPI-1 pathogenicity island. This locus was designated ETT2 (*E. coli* type III secretion 2) and consisted of the *epr*, *epa*, and *eiv* genes. ETT2 was found in enteropathogenic *E. coli* strains and also in some non-O157 Shiga toxin-producing *E. coli* (STEC) strains, but most of them contained a truncated portion of ETT2. Most O157 isolates had a complete collection of toxin-encoding genes *eae* and *hlyA* and the ETT2 locus, while most O26 strains had toxin-encoding genes *eae* and *hlyA* genes but an incomplete ETT2 locus. Thus, an intact copy of ETT2 might mark a pathogenic distinction for particular STEC strains. Therefore, the presence of the ETT2 locus can be used for identification of truly pathogenic STEC strains and for molecular fingerprinting of the epidemic strains in humans and animals.

Publications

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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 2003, Professor Dr. Fritz Melchers, Basel University, who was invited by the University of Tokyo as the Eminent Scientist of JSPS, joined us for our research projects. Dr. Melchers always encouraged us and suggested important issues for each project. We appreciate his enormous contribution.

1. Roles of IL-5 and IL-5 receptor (IL-5R) system in B cell differentiation

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

Keisuke Horikawa, Atsushi Sato, Yumiko Tsukamoto, 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 did not show μ-γ1 CSR and lgG1 production, despite 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 normally divided from 5 to 6 times, but did not express surface IgG1. These results indicate 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.

As shown previously, IL-4 does not induce significant μ-γ1 CSR in anti-CD38-activated B-2 cells even though it induces the γ1 germline mRNA expression and cell division. After extensive screening of costimuli for inducing IgG1 responses in cooperation with IL-4, we found that 8-mercaptoguanosine was effective in anti-CD38 -activated B-2 cells. Stimulation of B-2 cells with 8-mercaptoguanosine and anti-CD38 induced

the AID expression. We are currently analyzing genes affected in B cells upon CD38 and 8-mercaptoguanosine stimulations.

b. Molecular cloning of genes induced by IL-5 in murine B cells

Keisuke Horikawa and Kiyoshi Takatsu

IL-5 exerts pleiotropic effects on B-1 and B-2 cells to enhance proliferation and differentiation into Ig-producing cells. IL-5, but not IL-4 induces μ-γ1 CSR and IgG1 secretion in anti-CD38 -activated B-2 cells. To elucidate the biochemical mechanism underlying IL-5-dependent CSR and B cell terminal maturation, we used DNA microarray to analyze gene expression change in anti-CD38-activated B-2 cells upon IL-5 stimulation. The genes inducible by IL-5 include Ig-related genes such as Cγ1, IgL and J-chain. Furthermore, genes encoding cytoskeletal components and molecules involved in DNA replication and cell cycle, cell metabolism, signal transduction, transcription, translation and transport were also enhanced by IL-5. Among IL-5-inducible genes, *Blimp-1* and *AID* genes were preferentially upregulated by IL-5. Retroviral introduction of *Blimp-1* gene and *AID* gene to anti -CD38- and IL-4-stimulated B cells enhanced the terminal maturation to plasma cells and induced μ-γ1 CSR, respectively, in those B cells.

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 anti-CD 38 stimulation of B-2 cells activated NF-κB complexes. The c-Rel and p50 deficient B-2 cells were impaired in the germline γ1 mRNA expression upon CD38 stimulation, suggesting that NF -κB complexes play pivotal roles in the B cell activation in response to CD38 ligation. Anti-CD38 -induced NF-κB activation and the germline γ1 mRNA expression were not observed in B-2 cells from Btk deficient, BLNK/BASH deficient, PLC-γ2 deficient or PI3 kinase deficient mice, while those B-2 cells showed NF-κB activation and the expression of germline γ1 transcripts upon CD40 stimulation. After extensive screening of costimuli for inhibiting NF-κB activation and germline γ1 transcripts upon anti-CD38 stimulation, we found that agonists and antagonists of b-adrenergic receptor were effective. These data suggest that not only Btk activation but also G-binding protein play a role in the NF -κB activation and germline γ1 transcripts in anti -CD38-stimulated B-2 cells.

d. Role of IL-5 for mature B-1 cells in homeostatic proliferation and cell survival

Byoung-Gon Moon, Satoshi Takaki, [3]Kensuke Miyake, and Kiyoshi Takatsu: [3]Division of Infectious Genetics, IMSUT

B-1 cells, distinguishable from conventional B-2 cells by their cell surface marker, anatomical location and self-renewing activity, give rise to antibody-producing cells in response to IL-5 and lipopolysaccharide (LPS). B-1 cells constitutively express IL-5Rα, respond to IL-5 for their proliferation and differentiation, and play important roles in innate immune responses. In the present study, we analyzed roles of IL-5/IL-5R system in maintenance of number and cell size and functions of mature B-1 cells. Administration of anti-IL-5 mAb into wild type (WT) mice, T-cell depleted mice or mast cell-depleted mice can reproduce decrease in the total number and cell size of B-1 cells at an extent similar to IL-5Rα deficient (IL-5Rα$^{-/-}$) mice. B-1 cell survival and homeostatic proliferation, determined by cell transfer into WT mice and RAG-2$^{-/-}$ mice, respectively, are impaired in IL-5Rα$^{-/-}$ B-1 cells. IL -5 stimulation of WT B-1 cells, but not IL-5Rα$^{-/-}$ B-1 cells, enhances CD40 expression and augments IgM and IgG production following stimulation with anti-CD40 mAb. Furthermore, oral administration of LPS into WT mice induces the enhanced IgA production, while this is not observed in IL-5Rα$^{-/-}$ mice. Our results demonstrate that IL-5 plays critical roles in the homeostatic proliferation and cell survival of mature B-1 cells and regulates IgA production in the mucosal tissues in response to LPS.

e. Origin and differentiation of B-1 cells

Taku Kouro, Byoung-Gon Moon and Kiyoshi Takatsu

B-1 cells are distinguishable from conventional B-2 cells and give rise to antibody-producing cells in response to various stimuli. There are two hypotheses regarding the origin of B-1 cells, namely "selection theory" and "lineage theory". A significant proportion of B-1 cells recognize phosphatidylcholine, and B cells

forced to express such B cell receptors (BCR) are known to show B-1 cell like phenotype. These imply that B cells are selected to become B-1 cells when they are exposed to BCR gene rearrangement. This is called "selection theory". Existence of B-1 cell progenitors is also suggested by the fact that progenitors from fetal liver can reconstitute B-1 cells while these from adult bone marrow cannot. This supports "lineage theory". To identify B-1 cell progenitors in the fetal liver and examine whether these are different from B-2 cell progenitors, we put blood cell progenitors from Day 14 to Day 15 fetal livers into serum- and stromal cell-free culture in the presence of SCF and FL with or without IL-7 and IL-5. CD19-positive B lineage cells emerged only when IL-7 was added. IL-5 did not replace IL-7 activity for induction of CD19-positive B cells. We are currently pursuing cell transfer experiments into SCID mice.

2. Regulatory functions of adapter proteins in the immune system

a. Impaired B-lymphopoiesis and altered B-subpopulations in transgenic mice overexpressing Lnk adaptor protein

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

Lnk is a 68-kD adaptor protein expressed mainly in lymphocytes and hematopoietic progenitor cells. Together with APS and SH2-B, Lnk forms part of an adaptor protein family, whose members share the presence of a homologous N-terminal domain with putative proline-rich protein interaction motifs, followed by PH and SH2 domains, and a conserved C-terminal tyrosine phosphorylation site. Marked expansion of B-lineage cells occurs in *lnk*$^{-/-}$ mice, indicating that Lnk regulates B cell production by negatively controlling pro-B cell expansion.

We used a transgenic approach to define critical aspects of Lnk function in more detail, and showed that Lnk overexpression resulted in impaired expansion of lymphoid precursor cells and altered mature B cell subpopulations. The representation of both B-lineage and T-lineage cells was reduced in transgenic mice overexpressing Lnk under the control of a lymphocyte-specific expression vector. Whereas the overall number of B and T cells correlated with Lnk protein expression levels, marginal zone (MZ) B cells in spleen and B1 cells in the peritoneal cavity were relatively resistant to Lnk overexpression. The C-terminal tyrosine residue, conserved among Lnk family adaptor proteins, was dispensable for the negative regulatory roles of Lnk in lymphocyte development. Our results illuminate the novel negative regulatory mechanism mediated by the Lnk adaptor protein in controlling lymphocyte production and function.

b. Expansion and function of hematopoietic progenitor cells regulated by Lnk

Hitoshi Takizawa, Sang-Mo Kwon, Kiyoshi Takatsu and Satoshi Takaki

Lnk is also expressed in hematopoietic stem cells or progenitor cells (HSC/P), and the numbers and hematopoietic ability of HSC/Ps are significantly enhanced in the absence of Lnk without developing any malignant transformations. *lnk*$^{-/-}$ hematopoietic precursors have an advantage in repopulating the hematopoietic system of irradiated host animals in competitive environments. We identified functional domains of Lnk critical for growth inhibition mediated by Lnk. Various Lnk mutants carrying deletions or substitutional mutations were generated and retrovirally transduced into mast cell line MC9 cells. SCF-induced growth of MC9 cells expressing various Lnk mutants was then evaluated. The inhibitory effect of Lnk was completely abolished by a point mutation in the conserved phosphotyrosine binding site of the SH2 domain. SH2 Lnk mutants acted as dominant-negative (DN) mutants, since they cancelled growth inhibition of cells overexpressing wild-type Lnk. We further evaluated whether the DN Lnk mutants inhibit functions of Lnk endogenously expressed in HSC/Ps. Repopulation capacity of HSC/Ps was evaluated by competitive repopulation assay after retroviral transduction of DN Lnk mutants. HSC/Ps expressing DN Lnk showed facilitated repopulation in irradiated host animals. Lnk inhibitors such as DN Lnk mutants are a potentially useful tool for expanding HSC/Ps and controlling their function.

c. Lnk regulates actin cytoskeleton as a molecular scaffold linking receptor tyrosine kinases with Vav, Rac, PAK and filamin A

Sang-Mo Kwon, Hitoshi Takizawa, Masanori Iseki, Ikuo Nobuhisa[2], Tesuya Taga[2], Kiyoahi Takatsu and Satoshi Takaki: [2]Department of Cell Fate Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University

Lnk is phosphorylated by and associated with c-Kit, and inhibited c-Kit-mediated proliferation by attenuating phosphorylation of Gab2 and activation of MAPK cascade in MC9 mast cell line. However, analysis of *lnk*-deficient mice and *lnk*-

transgenic mice suggested negative regulatory roles of Lnk in c-Kit-independent signaling pathways in addition to c-Kit-dependent pathways.

We revealed that Lnk controlled actin reorganization activated by receptor tyrosine kinases (RTKs), thereby regulating cell division and migration. Lnk-expressing fibroblasts showed flattened, spreading morphology and prominent actin polymerization that resulted in multinuclear cell formation due to impaired cytokinesis. Actin reorganization by Lnk was accompanied by augmented Rac activation and recruitment to the cell membrane. Lnk was co-immunoprecipitated with Rac, Vav, PAK and filamin A, which suggests the formation of a cytoskeletal regulatory protein complex supported by Lnk. The PH domain of Lnk was indispensable for membrane sublocalization and association with Vav and filamin A, while the SH2 domain of Lnk was critical for Rac activation mediated via RTKs. Thus, Lnk functions as a novel scaffold molecule that links RTKs with cytoskeletal regulatory components.

d. Increased numbers of B-1 cells and enhanced responses against TI-2 antigen in mice lacking APS, an adaptor molecule containing PH and SH2 domains

Masanori Iseki, Chiyomi Kubo-Akashi, Sang-Mo Kwon, Akiko Yamaguchi, Yuki Kataoka[3], Nobuaki Yoshida[3], Kiyoshi Takatsu and Satoshi Takaki: [3]Laboratory of Gene Expression and Regulation, Center for Experimental Medicine, IMSUT

APS is an intracellular adaptor protein that forms an adaptor family along with Lnk and SH 2-B. While experiments using cultured cell lines have demonstrated that APS is phosphorylated in response to various stimuli, its *in vivo* functions remain unclear. We attempted to determine the physiological roles of APS by generating *APS*-deficient ($APS^{-/-}$) mice. $APS^{-/-}$ mice were viable, fertile, and showed no abnormalities or growth retardation. Immunologically, $APS^{-/-}$ mice showed normal development and distribution of lymphocytes and myeloid cells, except for increased numbers of B-1 cell in the peritoneal cavity. $APS^{-/-}$ mice exhibited an enhanced humoral immune response against TNP-Ficoll, a thymus-independent type-2 antigen, while $APS^{-/-}$ B-2 cells exhibited normal proliferative responses and tyrosine phosphorylation of intracellular proteins upon B cell receptor (BCR) crosslinking. We found that F-actin contents after BCR stimulation was decreased in $APS^{-/-}$ B-1 cells compared to wild-type cells. Our results indicate that APS has a novel regulatory role in actin reorganization and control of B-1 cell compartment size.

e. Roles of a conserved family of adaptor proteins, Lnk, SH2-B and APS for mast cell development, growth and functions

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

Lnk, SH2-B, and APS are all expressed in mast cells and their possible functions in signaling through c-Kit or Fc ε RI are subject to speculation. To investigate roles of Lnk, SH2-B or APS in mast cells, we established IL-3-dependent mast cells from $lnk^{-/-}$, $SH2\text{-}B^{-/-}$ and $APS^{-/-}$ mice. IL-3-dependent growth of those cells was comparable. Proliferation or adhesion mediated by c -Kit as well as degranulation induced by cross-linking Fc ε RI were normal in the absence of Lnk or SH2-B. In contrast, *APS*-deficient mast cells showed augmented degranulation after cross-linking FcεRI compared to wild-type cells, while c-Kit-mediated proliferation and adhesion remained unaffected. *APS*-deficient mast cells showed reduced actin assembly at steady state, although their various intracellular responses induced by cross-linking FcεRI were indistinguishable compared to wild-type cells. Our results suggest potential roles of APS in controlling actin cytoskeleton and the magnitude of degranulation in mast cells.

f. Impaired B cell receptor-mediated proliferation and enhanced actin reorganization in mice overexpressing APS.

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

Analyses of $APS^{-/-}$ mice and bone marrow derived mast cells indicate that APS might regulate actin reorganization. We further investigated APS functions in actin reorganization or in other cellular responses by generating *APS*-transgenic (*APS*-Tg) mice that overexpressed APS under the control of the *lck* proximal promoter and the *Eμ* enhancer. In contrast to $APS^{-/-}$ mice, *APS*-Tg mice showed reduced numbers of B-1 cells in the peritoneal cavity. *APS*-Tg splenic B cells were decreased in number and showed impaired proliferation and survival following B cell receptor (BCR) crosslinking. Stimulated B cells from *APS*-Tg mice showed augmented F-actin contents and colocalization of APS with BCR complexes during

receptor capping. F-actin assembly was also augmented in APS overexpressed fibroblast cells, and APS colocalized with F-actin. Our results suggest that APS have a novel negative regulatory role in BCR signaling through modulating actin reorganization.

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

The Ag85B of *Mycobacterium* (*M.*) *tuberculosis* and *M. Bovis* BCG is immunogenic in C57BL/6 mice with Ag85B to expand TCRVβ11$^+$ CD4$^+$ Th 1 cells in conjunction with APCs in an I-A^b-restricted manner. We identified the major antigenic epitope (Peptide-25) for Ag85B-specific Vβ 11$^+$ T cells as the 15-mer peptide, covering amino acid residues 240-254 of Ag85B.

a. Role of IFN-γ in the Peptide-25 dependent Th1 development

Ai Kariyone, Toshiki Tamura, Makiyo Nakada, Kiyoshi Takeda[3], Shizuo Akir[3] and Kiyoshi Takatsu: [3]Institute of Microbial Diseases, Osaka University

We examined the roles of IFN-γ in the generation of Peptide-25-reactive CD4$^+$ Th1 cells. Peptide-25 was immunogenic in inducing the development of IFN-γ- and TNF-α-producing cells of CDT4$^+$ TCRVβ11$^+$ and CDT4$^+$ TCRVβ 11$^-$T cells. Compared with other I-A^b-binding peptides such as Peptide-9 of Ag85B, 50V of pigeon cytochrome c and OVA$_{265–280}$ of ovalbumin, only Peptide-25 was capable of inducing enormous expansion of TCRVβ11$^+$ IFN-γ-producing T cells. Treatment of C57BL/6 mice with anti-Vβ 11 antibody before Peptide-25 immunization reduced the development of CD4$^+$ IFN-γ-producing T cells. In Peptide-25-immunized IFN-γ deficient mice expansion of CD4$^+$ TCRVβ11$^+$ T cells upon Peptide-25 stimulation *in vitro* decreased as compared with WT mice. Interestingly, Peptide-25-primed cells from MyD88 deficient mice responded to Peptide-25 and differentiated into IFN-γ-producing cells to a similar extent to wild type mice, indicating Toll-like receptor-independent IFN-γ production. These results clearly demonstrate important roles of IFN-γ in the generation and expansion of CD4$^+$ TCRVβ 11$^+$ T cells in response to Peptide-25, and provides useful information for delineating the regulation of Th1 development and for inducing a Th1-dominant immune response.

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

Haruyuki Ariga, Takeshi Tokunaga, 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. Development of each Th subset is affected by cytokines, antigenpresenting cells (APCs), co-stimulatory molecules and genetic background of the recipient. Upon recognition of MHC/peptide complex, the T cell receptor (TCR) complex initiates a complex cascade of signaling events resulting in cytokine production, proliferation and differentiation. However, it is still unclear whether the TCR signaling events affect directly Th1 and Th2 differentiation.

As we demonstrated, Peptide-25 specific CD 4$^+$ T cells express TCRVα5-Vβ11. To examine whether Peptide-25 itself is able to determine the fate of Th subset development, we generated transgenic (P25-Tg) mice expressing TCR that recognize Peptide-25 in the context with I-A^b molecules. Splenic CD4$^+$ naïve T cells of P25-Tg mice, stimulated *in vitro* with Peptide-25 in the presence of I-A^b APCs, produced IFN-γ but not IL-4 upon re-stimulation with Peptide-25, indicating that CD4$^+$ naïve T cells of P25-Tg mice differentiate into solely Th1 subset in response to Peptide-25. This was also observed in naïve T cells from RAG-2 deficient P25-Tg mice. This Th1 development was induced in the presence of mAbs against IFN-γ, IL-12 and IL-18. Furthermore, Peptide-25-loaded I-A^b-transfected Chinese hamster ovary cells (Peptide-25-I-A^b-CHO) effectively trigger naïve T cells from RAG-2 deficient P25-Tg mice into Th1 cells. Within 3 hr after TCR stimulation with Peptide-25-I-A^b-CHO, IFN-γ independent transient induction of the *T-bet* gene expression and suppression of the *GATA-3* expression were observed. As Peptide-25-I-A^b-CHO does not express CD80/86 and ICAM-1 and induce Peptide-25-dependent Th1 development, MHC/peptide-TCR interaction may determine directly the fate of Th cell development to Th1.

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

Takeshi Kikuchi, Toshiki Tamura, Xu Wen, [3]Kiyoshi Takeda, [3]Shizuo Akira, and Kiyoshi Takatsu: [3]Institute of Microbial Diseases, Osaka University

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 when we immunized mice with Peptide-25 and OVA simultaneously. Results revealed that co-immunization of C57BL/6 mice with OVA and Peptide-25 induced higher OVA specific-IgG2a and IFN-γ production than OVA immunization. Intriguingly, the OVA-specific CTL generation was also enhanced when mice were co-immunized with OVA plus Peptide-25. The adjuvant effect of Peptide-25 was no observed in CD4 deficient or IFN-γ deficient mice. Co-immunization of OVA and Peptide-25 prevented *in vivo* growth of E. G7-OVA cell (EL4 thymoma transfected with cDNA encoding chicken OVA) leading to prolonged survival. Moreover, 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. Furthermore, CTL generation specific for class I-binding B16 melanoma peptide (SVDFFVWL) was enhanced by co-immunization with Peptide-25. These results indicate that Peptide-25 exerts potent adjuvant activity and provides efficient help for CTL induction against neo-tumor antigen when concomitantly immunized.

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

Yoko Oe-Kikuchi and Kiyoshi Takatsu

Mast cells are thought to contribute to the pathogenesis of allergic airway responses through an IgE dependent mechanism. Eosinophilic inflammation is clearly a hallmark of both allergic and nonallergic asthma. Considerable evidence suggests that there is an 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 is still not clear.

We studied the role of IL-5 on mast cell and eosinophil activation and the role of activated mast cells in activation and survivability of eosinophils. First, we studied IgE-dependent production of histamine and cytokine (IL-5 and GM-CSF) by mast cells isolated from IL-5Rα KO and IL-5 KO mice to observe the function of IL-5 on releasability. We observed that mast cells, expressing detectable IL-5Rα, increased their steady state expression of IL-5 mRNA after cross -linking the IgE receptor. Although we observed IL-5, GM-CSF, and histamine release from these mast cells, there were no significant difference on each releasability, however, we found that IL -5 reduced 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 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 exclusion of expression by DNA parasites such as retoroviruses and transposons are now being recognized as an important host cell defense system in the cell nuclei. In Drosophila and plants, suppression of retrotransposon is known to involve RNA silencing (RNA interference), but it is not clear whether a similar mechanism is operating in human retroviral gene silencing. Our goal is to elucidate molecular mechanisms of human retroviral gene silencing in the infected human cells. The results would give us new ideas for latent infection observed in many human viruses and also for the design of unique retroviral vectors that would support long-term transgene expression providing strong tools for human gene therapy and regeneration medicine.

1. Epigenetical regulation and SWI/SNF chromatin remodeling complex

In multicellular organisms, epigenetic regulation of transcription supports distinct cell type-specific gene expression. Therefore, to understand viral strategies to proliferate and cause specific pathological effects in certain host cells, epigenetical analysis on both viral and host gene expression is essential in this post-genome era. While DNA methylation, histone acetylation and chromatin remodeling are expected to play major roles in these epigenetical regulations, their interaction with transcriptional factors as well as their mutual relationship remain largely unsolved.

We have been concentrated on the analysis of the major chromatin remodeling complex, SWI/SNF, which is composed of 10 protein subunits in human. The catalytic subunits, BRG1 and Brm, have ATPase activity with the helicase motif. Each SWI/SNF complex contain 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. Here 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. This year we showed that this chromatin remodeling complex is involved in the maintenance of retroviral gene expression and proposed that Brm-type SWI/SNF complex should be considered as “a trithorax-G complex

essential for cellular and viral memory", which is counteracting Polycomb-G complexes (a).

It is quite interesting that SW13, a human adrenal adenocarcinoma cell line, has been reported to be deficient in the expression of both *BRG1* and *Brm* genes. If SWI/SNF complex is indeed important for maintain "cellular memory", how can this cell line do this by lacking functional SWI/SNF complex? To answer this question, we intensively analyzed SW13 and found that this cell line actually encode functional *BRG1* and *Brm* and transcribes both genes constitutively. We further show that in a subtype of SW13 (SW13(vim−)), mRNA expression of *BRG1* and *Brm* genes is tightly suppressed at the post-transcription level. We have identifized another subtype of SW13, SW13(vim+), in which both *BRG1* and *Brm* mRNAs are expressed. Consistent with our previous observation that functional SWI/SNF complex is essential for transactivation through AP-1, the endogeneous *vimentin*, *CD44*, *c-met* and *collagenase* genes (know to be under the control of AP-1) were not expressed in SW13(vim−) but expressed in SW13(vim+). We will describe this unique epigenetical transition between two subtypes and discuss its biological meanings (b).

a. SWI/SNF chromatin remodeling complex and retroviral gene silencing.

Taketoshi Mizutani, Taiji Ito, Mitsue Yamamichi-Nishina, Nobutake Yamanichi, and Hideo Iba.

Silencing of transgenes, transposons and retroviruses is often considered as a host defense system in cellular nuclei. A DNA methylation-dependent mechanism is known to be involved in this process, but additional mechanisms remain largely unknown. Here we show that MuLV-based retrovirus vector transgene expression is rapidly silenced in human tumor cell lines lacking expression of Brm, a catalytic subunit of the SWI/SNF chromatin remodeling complex, even though these vectors can successfully enter, integrate, and initiate transcription. We detected this gene silencing as a reduction in the ratio of cells expressing the exogenous gene rather than a reduction in the average expression level, indicating that down-regulation occurs in an all-or-none manner. Retroviral gene expression was protected from silencing and maintained in Brm-deficient host cells by exogenous expression of Brm but not BRG1, an alternative ATPase subunit in the SWI/SNF complex. Introduction of exogenous Brm to these cells suppressed recruitment of protein complexes containing YY1 and histone deacetylase (HDAC) 1 and 2 to the 5′-LTR region of the integrated provirus, leading to the enhancement of acetylation of specific lysine residues in histone H4 located in this region. Consistent with these observations, treatment of Brm deficient cells with HDAC inhibitors but not DNA methylation inhibitors suppressed retroviral gene silencing. These results suggest that the Brm-containing SWI/SNF complex subfamily (trithorax-G) and a complex including YY1 and HDACs (Polycomb-G) counteract each other to maintain transcription of exogenously introduced genes.

b. SW13 cells can transition between two distinct subtypes by switching expression of *BRG1* and *Brm* genes at the post-transcriptional level.

Mitsue Yamamichi-Nishina, Taiji Ito, Taketoshi Mizutani, Nobutake Yamamichi, Hirotaka Watanabe, and Hideo Iba

The human adrenal carcinoma cell line, SW13, has been reported to be deficient in both *BRG1* and *Brm* expression and therefore is considered to lack a functional SWI/SNF complex. We found that the original cell line of SW13 is composed of two subtypes: one that expresses neither BRG1 nor Brm (SW13(vim−)) and the another which does express both (SW13(vim+)). The presence of BRG1 and Brm in SW13 correlates completely with the cellular ability to express such genes as *vimentin*, *collagenase*, *c-met* and *CD44* that were under the control of a transcription factor, AP-1, which was previously shown to require a functional SWI/SNF complex for its transactivating activity. Transient treatment with inhibitors of histone deacetylase induced a stable transition of SW13(vim−) to a cell type indistinguishable from SW13(vim+), suggesting that these two subtypes are epigenetically different. Run-on analysis indicated that, unlike these four genes driven by AP-1, transcription of the *BRG1* and *Brm* genes are initiated in SW13(vim−) at a frequency comparable to SW13(vim+). No block in transcriptional elongation of either *BRG1* or *Brm* gene was detected in SW13(vim−) cells, indicating that their expression was completely suppressed at the post-transcriptional level in SW13(vim−) cells. We would like to propose that SW13 can spontaneously transition between two subtypes by switching expression of *BRG1* and *Brm* at the post-transcriptional level.

2. Function of oncogenes in epithelial cells. -Induction of epigenetical transition by v-*src*.

Yasuhito Shimizu, Nobutake Yamamichi, Kanako Saitoh, Akiko Watanabe, Taiji Ito, Mitsue Nishina, Toshihiko Suzuki[1], Chihiro Sasakawa[1], Sadao Yasugi[2], Masao Ichinose[3], Hideo Iba

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Rous sarcoma virus (RSV) is known to form exclusively sarcomas although it does not cause carcinomas (tumors originated from epithelium). But the molecular mechanisms supporting this as well as v-*src* function in the epithelium are largely unknown. We have recently developed recombination organ culture systems which enabled us to transfer genes specifically into primary epithelial cells of the developing chicken glandular stomach (proventriculus). This year, we concentrated how v-*src* expression affects the fate of epithelial cells both genetically and epigenetically. The following findings would explain at least in part why RSV does not apparently form exclusively carcinomas.

Recombination organ cultures in combination with the stable and transient gene transfer techniques by retrovirus and electroporation, respectively, enabled us to transfer oncogenes specifically into primary epithelial cells of the developing avian glandular stomach (proventriculus). In this system, the epithelium and mesenchyme are mutually dependent upon each other for their growth and differentiation. This system therefore offers an environment closed to *in vivo*. In this year, we found here that either stable or transient expression of v-*src* in the epithelium causes budding and migration of epithelial cells into mesenchyme. In response to the transient expression of v-Src or a constitutive active mutant of MEK, we observed immediate down-regulation of the *Sonic hedgehog* gene and subsequent elimination of *E-cadherine* expression in migrating cells, suggesting the involvement of MAP kinase signaling pathway in these processes. v-*src*-expressing cells that were retained in the epithelium underwent apoptosis (anoikis) and detached from the culture. Continuous expression of v-*src* by for example, Rous sarcoma virus was required for the epithelial cells to acquire the ability to express *type I collagen* and *fibronectin* genes (mesenchymal markers) and finally to establish the epithelial-mesenchymal transition.

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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. Selective incorporation of influenza virus RNA segments into virions

Fujii Y, Goto H, Watanabe T, Yoshida T, Kawaoka Y.

The genome of influenza A virus is comprised of eight viral RNA (vRNA) segments. Although the products of all eight vRNA segments must be present for viral replication, little is known about the mechanism(s) responsible for incorporation of these segments into virions. Two models have been proposed for the generation of infectious virions containing eight vRNA segments. The random-incorporation model assumes a common structural feature in all the vRNAs, enabling any combination of vRNAs to be incorporated randomly into virions. The selective-incorporation model predicts the presence of specific structures in each vRNA segment, leading to the incorporation of a set of eight vRNA segments into virions. Here we demonstrate that eight different vRNA segments must be present for efficient virion formation and that sequences within the coding region of (and thus unique to) the neuraminidase vRNA possess a signal that drives incorporation of this segment into virions. These findings indicate a unique contribution from individual vRNA segments and thus suggest a selective (rather than random) mechanism of vRNA recruitment into virions. The neuraminidase vRNA incorporation signal and others yet to be identified should provide attractive targets for the attenuation of influenza viruses in vaccine production and the design of new antiviral drugs.

2. Identification of protective epitopes on Ebola virus glycoprotein at the single amino acid level using recombinant vesicular stomatitis viruses

Takada A, Feldmann H, Stroeher U, Bray M, Watanabe S, Ito H, Mcgregor M, Kawaoka Y.

Ebola virus causes lethal hemorrhagic fever in humans, but currently there are no effective vaccines or antiviral compounds for this infectious disease. Passive transfer of monoclonal antibodies (MAbs) protects mice from lethal Ebola virus infection. However, the epitopes responsible for neutralization have been only partially charac-

terized because some of the MAbs do not recognize the short synthetic peptides used for epitope mapping. To identify the amino acids recognized by neutralizing and protective antibodies, we generated a recombinant vesicular stomatitis virus (VSV) containing the Ebola virus glycoprotein-encoding gene instead of the VSV G protein-encoding gene and used it to select escape variants by growing it in the presence of a MAb (133/3.16 or 226/8.1) that neutralizes the infectivity of the virus. All three variants selected by MAb 133/3.16 contained a single amino acid substitution at amino acid position 549 in the GP2 subunit. By contrast, MAb 226/8.1 selected three different variants containing substitutions at positions 134, 194, and 199 in the GP1 subunit, suggesting that this antibody recognized a conformational epitope. Passive transfer of each of these MAbs completely protected mice from a lethal Ebola virus infection. These data indicate that neutralizing antibody cocktails for passive prophylaxis and therapy of Ebola hemorrhagic fever can reduce the possibility of the emergence of antigenic variants in infected individuals.

3. High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza

Shiraishi K, Mitamura K, Sakai-Tagawa Y, Goto H, Sugaya N, Kawaoka Y.

Clinical samples from 15 amantadine-treated children were collected serially: before, during and/or after treatment, and studied to determine the actual prevalence, timing and clinical implications of M2 mutational events. Following viral RNA extraction and RT-PCR amplification of the viral RNA encoding the M2 protein, the products were cloned into plasmids and their sequences determined. Five mutations known to confer amantadine resistance in clinical samples were identified in 12 of 15 evaluable patients (80%), and 9 patients had more than a single (2 to 4) mutant viruses. The pattern of emergence of mutant strains was clarified from the study of six patients with at least four serial samples. Although viruses with M2 mutations tended to become the dominant populations, in two cases wild-type viruses became dominant after decreasing to low levels. These results suggest that resistant viruses emerge in a much higher proportion of amantadine-treated patients than suggested by previous studies.

4. The NB protein of influenza B virus is not necessary for virus replication in vitro

Hatta M, Kawaoka Y.

The NB protein of influenza B virus is thought to function as an ion channel and therefore would be expected to have an essential function in viral replication. Because direct evidence for its absolute requirement in the viral life cycle is lacking, we generated NB knockout viruses by reverse genetics and tested their growth properties both in vitro and in vivo. Mutants not expressing NB replicated as efficiently as the wild-type virus in cell culture, whereas in mice they showed restricted growth compared with findings for the wild-type virus. Thus, the NB protein is not essential for influenza B virus replication in cell culture but promotes efficient growth in mice.

5. Antibody-dependent enhancement of Ebola virus infection

Takada A, Feldmann H, Ksiazek TG, Kawaoka Y.

Most strains of Ebola virus cause a rapidly fatal hemorrhagic disease in humans, yet there are still no biologic explanations that adequately account for the extreme virulence of these emerging pathogens. Here we show that Ebola Zaire virus infection in humans induces antibodies that enhance viral infectivity. Plasma or serum from convalescing patients enhanced the infection of primate kidney cells by the Zaire virus, and this enhancement was mediated by antibodies to the viral glycoprotein and by complement component C1q. Our results suggest a novel mechanism of antibody-dependent enhancement of Ebola virus infection, one that would account for the dire outcome of Ebola outbreaks in human populations.

6. Generation of influenza A viruses with chimeric (type A/B) hemagglutinins

Horimoto T, Takada A, Iwatsuki-Horimoto K, Hatta M, Goto H, Kawaoka Y.

To gain insight into the intertypic incompatibility between type A and B influenza viruses, we focused on the hemagglutinin (HA) gene, systematically studying the compatibility of chimeric (type A/B) HAs with a type A genetic background. An attempt to generate a reassortant containing an intact type B HA segment in a type A virus background by reverse genetics was unsuccessful despite transcription of the

type B HA segment by the type A polymerase complex. Although a type A virus with a chimeric HA segment comprising the entire coding sequence of the type B HA flanked by the noncoding sequence of the type A HA was viable, it replicated only marginally. Other chimeric viruses contained type A/B HAs possessing the type A noncoding region together with either the signal peptide or transmembrane/cytoplasmic region of type A virus or both, with the remaining regions derived from the type B HA. Each of these viruses grew to median tissue culture infectious doses of more than 105 per ml, but those with more type A HA regions replicated better, suggesting protein-protein interactions or increased HA segment incorporation into virions as contributing factors in the efficient growth of this series of viruses. All of these chimeric (A/B) HA viruses were attenuated in mice compared with wild-type A or B viruses. All animals intranasally immunized with a chimeric virus survived upon challenge with a lethal dose of wild-type type B virus. These results suggest a framework for the design of a novel live vaccine virus.

7. Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes.

Watanabe T, Watanabe S, Noda T, Fujii Y, Kawaoka Y.

At the final step in viral replication, the viral genome must be incorporated into progeny virions, yet the genomic regions required for this process are largely unknown in RNA viruses, including influenza virus. Recently, it was reported that both ends of the neuraminidase (NA) coding region are critically important for incorporation of this vRNA segment into influenza virions. To determine the signals in the hemagglutinin (HA) vRNA required for its virion incorporation, we made a series of deletion constructs of this segment. Subsequent analysis showed that 9 nucleotides at the 3' end of the coding region and 80 nucleotides at the 5' end are sufficient for efficient virion incorporation of the HA vRNA. The utility of this information for stable expression of foreign genes in influenza viruses was assessed by generating a virus whose HA and NA vRNA coding regions were replaced with those of vesicular stomatitis virus glycoprotein (VSVG) and green fluorescent protein (GFP), respectively, while retaining virion incorporation signals for these segments. Despite the lack of HA and NA proteins, the resultant virus, which possessed only VSVG on the virion surface, was viable and produced GFP-expressing plaques in cells even after repeated passages, demonstrating that two foreign genes can be incorporated and maintained stably in influenza A virus. These findings could serve as a model for the construction of influenza A viruses designed to express and/or deliver foreign genes.

Publications

Original papers

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Our research mainly focuses on a molecular mechanism underlying lipopolysaccharide (LPS) recognition. LPS is a membrane component of Gram-negative bacteria that potently activates the innate immune system. Endotoxin recognition molecules have been recently identified as Toll-like receptor 4 (TLR4) and MD-2. We have cloned MD-2 that is associated with the extracellular domain of TLR4. TLR4-MD-2, but not TLR4 alone, recognizes LPS. We recently showed that LPS directly binds to TLR4-MD-2 and trigger oligomerization of TLR4-MD-2.

1. Lipopolysaccharide Interaction with Cell Surface Toll-like Receptor 4-MD-2: Higher Affinity than that with MD-2 or CD14

Sachiko Akashi,[1] Shin-ichiroh Saitoh,[1] Yasutaka Wakabayashi,[1] Takane Kikuchi,[1] Noriaki Takamura,[1] Yoshinori Nagai,[1] Yutaka Kusumoto,[1] Koichi Fukase,[2] Shoichi Kusumoto,[2] Yoshiyuki Adachi,[3] Atsushi Kosugi,[4,5] and Kensuke Miyake[1,5]: [1]Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [2]Department of Chemistry, Graduate School of Science, Osaka University; [3]Laboratory of Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science; [4]Medical Technology and Science, Graduate School of Medicine, Osaka University; [5]CREST, Japan Science and Technology Corporation, Tokyo, Japan

Toll-like receptors (TLRs) are innate recognition molecules for microbial products, but their direct interactions with corresponding ligands remain unclarified. Lipopolysaccharide (LPS), a membrane constituent of Gram-negative bacteria, is the best-studied TLR ligand and recognized by TLR4 and MD-2, a molecule associated with the extracellular domain of TLR4. Although TLR4-MD-2 recognizes LPS, little is known for physical interaction between LPS and TLR4-MD-2. We here demonstrate cell surface LPS/TLR4-MD-2 complexes. CD14 greatly enhances the formation of LPS/TLR4-MD-2 complexes, but is not coprecipitated with LPS/TLR4 -MD-2 complexes, suggesting a role for CD14 in LPS loading onto TLR4-MD-2 but not in the interaction itself between LPS and TLR4-MD-2. A tentative dissociation constant (K_D) for LPS/TLR 4-MD-2 complexes was about 3 nM, which is about 10-20 times lower than the reported K_d for LPS/MD-2 or LPS/CD14. The presence of detergent disrupts LPS interaction with CD14 but not with TLR4-MD-2. E5531, a lipid A antagonist developed for therapeutic intervention of endotoxin shock, blocks LPS interaction with TLR4-MD-2 at a concentration 100 times lower than that required for blocking LPS interaction with CD14. These results reveal direct LPS interaction with cell surface TLR4-MD-2 that is distinct

from that with MD-2 or CD14.

2. Protective role of mast cells in mice infected with *Plasmodium berghei* ANKA

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Malaria is the most serious parasitic disease of humans in the world, and about 3 million people died from the disease every year, most of them are children under 5 years old. It has been reported that approximately 85% of children and adults living in areas of Gambia, Liberia, Madagascar, or Thailand in which *Plasmodium falciparum* malaria is endemic have elevated IgE levels in the blood, comprising both total and IgE antimalaria antibodies. Although experimental malaria infections of mice also give rise to IgE elevation, no direct evidence regarding to the possible role of IgE for protection or pathogenesis is available. The importance of IgE production in relation to other protective mechanisms in malaria remains to be clarified. In the present study, we have investigated the association of IgE level and parasite growth, and the possible roles of mast cells in malaria using animal models. To assess the protective roles of IgE in malaria, the congenitally selective IgE-deficient SJA/9 and the background SJL/J mice were infected with murine malaria parasite, *P. berghei* ANKA. After the *P. berghei* infection, since appearance of *P. berghei* parasites in SJA/9 mice was earlier than SJL/J mice, and the mortality of SJA/9 mice were significantly higher than that of SJL/J mice, these results suggested the protective role of IgE in *P. berghei* infection. IgE has many biological functions in infectious and allergic diseases via mast cells bearing Fc ε RI on their cell membranes. To directly evaluate the in vivo role of mast cells in malaria, mast cell-deficient WBB6/F1-W/Wv and their congenic wild-type WBB6/F1-W/W+ mice were infected with *P. berghei* ANKA. W/Wv mice developed significantly severe disease than wild-type mice, as indicated by higher daily parasitemia in the blood. However, the production of anti-IgE antibody was same levels in the W/Wv and W/W+ mice after the infection, but significant increased cytokine productions such as TNF-α and IFN-γwere observed in W/W+ mice. If mast cell deficiency accounts for the differences of parasite growth in W/Wv and W/W+ mice, reconstitution of the mast cell population in these animals should restore disease severity to the level of wild-type animals. To examine the this possibility, we performed mast cell reconstitution in W/Wv recipients by intravenous injection of cultured bone marrow mast cells, and then the parasite growth was examined. Since the mast cell-reconstituted W/Wv mice showed a similar growth of parasites as compared with W/W+ mice, these data suggested that mast cells have protective roles in malaria. These data provide a new host defense mechanism for the malaria.

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

Shin-ichiroh Saitoh,[1] Sachiko Akashi,[1] Takenao Yamada,[2] Natsuko Tanimura,[2] Makiko Kobayashi,[1] Kazunori Konno,[1] Fumi Matsumoto,[2] Koichi Fukase,[3] Shoichi Kusumoto,[3] Yoshinori Nagai,[1] Yutaka Kusumoto,[1] Atsushi Kosugi,[2,4] and Kensuke Miyake[1,4]: [1]Division of Infectious Genetics, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [2]Medical Technology and Science, Graduate School of Medicine, Osaka University; [3]Department of Chemistry, Graduate School of Science, Osaka University; [4]CREST, Japan Science and Technology Corporation, Tokyo, Japan

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 to-

gether, 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. Analysis of MD-2 functions utilizing newly established monoclonal antibodies

Yutaka Kusumoto[1,2], Kazunori Konno[1], Sachiko Akashi[1,2], Yoshinori Nagai[1,2], Shin-Ichiroh Saitoh[1,2] and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, The Institute of Medical Science, The university of Tokyo, [2]CREST, Japan Science & Technology corporation

MD-2 makes the complex with TLR4 and is important to recognize and signaling gram negative bacterial LPS. Not only the mechanisms of recognition of LPS, but other functions of MD-2 do not clarified. In order to study the functions of MD-2, we have tried to establish the rat monoclonal antibody (mAb) against mouse MD-2 (mMD-2). Until now, we could establish some mAbs against mMD-2. One (HR 452) of these is useful to immunoprecipitation, westernblotting and ELISA. This mAb revealed that, in addition to TLR4/MD-2 complex, mMD-2 exist on the membrane of cell surface or cytoplasmic organs as homo dimmer. On the other hand, it was also proved that MD-2 in the TLR4 /MD-2 complex is monomer. Immunoprecipitation by HR452 and the treatment of some kinds of glycosidase indicated that the patterns of glycosylation were different between in the membrane-associated dimerized MD-2 and in MD-2 in the complex with TLR4. In addition, another unknown modification may need to the maturation of MD-2 in TLR4/MD-2 complex. Immunoprecipitation also indicated the candidates of associated molecules with membrane-associated MD-2 or MD-2 in TLR4/MD-2 complex. In addition to proteins, it was suggested that some of fatty acid were also associate to MD-2. Now we are studying to confirm that these candidates physically and functionally associate with the membrane-associated MD-2 or TLR4/MD-2 complex. Newly established mAb against mMD-2 has given us some additional information about MD-2. We do research the functions of MD-2 from various viewpoints utilizing these mAb, MD-2KO mouse or purified mMD-2.

5. The Toll-like receptor protein RP105 regulates responses to bacterial lipopolysaccharide and lipopeptides in B cells

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Recent studies have revealed that the Toll-like receptors (TLR), homologues of *Drosophila* Toll protein, play an important role in recognizing microorganisms. For example, TLR4 recognizes lipopolsaccharide (LPS) in a variety of cell types, and TLR2 and TLR9 recognize lipopeptides and bacterial DNA, respectively. We previously reported that the responses of B cells to LPS were also regulated by another TLR protein, RP105 because RP105-deficient B cells showed hyporesponsiveness to LPS. Here, we report that RP105 -deficient B cells are impaired not only in LPS-induced responses, but also in bacterial lipopeptide-induced responses. We first studied an in vitro B cell response to lipopeptides or bacterial DNA. RP105-deficient B cells revealed partial responses in proliferation and CD86 up-regulation induced by lipopeptides, Pam_3CSK_4 or MALP-2. In contrast to lipopeptides, RP105-deficient B cells revealed normal responses to bacterial DNA. Next we also studied an in vivo B cell response to Pam_3CSK_4 or MALP-2. Mice were immunized with FITC-Pam_3CSK_4 or FITC-MALP-2, and the production of FITC-specific antibodies was measured with ELISA. In contrast to in vitro responses to Pam_3CSK_4 or MALP -2, RP105-deficient mice were severely impaired in antibody production to FITC-Pam_3CSK_4 or FITC-MALP-2 as well as to TNP-LPS. In this study, we clearly demonstrate that RP105 functionally interacts with TLR4 and TLR2 but not TLR9, and has important roles in responses to LPS and lipopeptides, especially in antibody production to them.

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Sachiko Akashi, Shin-ichiroh Saitoh, Yasutaka Wakabayashi, Takane Kikuchi, Noriaki Taka-

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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. Uniqueness of gut-associated lymphoreticular tissues

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The mucosal immune system is consisted with two functionally distinctive anatomical/histological sites, namely inductive and effector compartments. Gut-associated lymphoreticular tissues, such as Peyer's patches and cecal patches, are important inductive sites for the initiation of antigen-specific mucosal immune responses. The gut-associated lymphoreticular tissues may have an epithelial barrier different from that of villous epithelium. Unique profiles of claudin-2, -3, and -4 and occludin expression were noted in the tight junctions of follicle-associated epithelium (FAE) of Peyer's patches: claudin-4 was preferentially expressed in the apex region; claudin-2 was only weakly expressed on the crypt side of the FAE compared with stronger expression on the crypt side of villous epithelial cells; and claudin-3 and occludin were found throughout the dome region of FAE. These unique expression patterns were present also in cecal patch FAE. In addition, claudin-4 expression in the FAE of Peyer's patches and cecal patches correlated with the presence of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)-positive apoptotic cells, and Peyer's patch-deficient mice exhibited expression patterns of claudin and occludin in villous epithelia similar to those in wild-type mice. These results show that claudin-4 expression was preferentially associated with the dome region of FAE, the mucosal inductive site of the murine intestine. In that location it might correlate with the cell life cycle, help maintain the apex configuration of the dome, or be a factor favoring the uptake of antigens by the FAE. These new results provide new aspect of the

tight junction associated molecules as a part of the innate immunity in the mucosal immune system.

2. Mucosal Vaccine

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Invasion of infectious agents including HIV, Influenza and *E. Coli* O157 through mucosal surfaces can be prevented by use of the common mucosal immune system (CMIS), which interconnects inductive tissues, including Peyer's patches (PPs) and nasopharyngeal-associated lymphoreticular tissue (NALT), and effector tissues of the intestinal and respiratory tracts. In order for the CMIS to induce maximal protective mucosal immunity, co-administration of mucosal adjuvant has been shown to be essential. Our recent efforts at mucosal vaccine development have focused on nasal administration of vaccine antigen together with nontoxic mutant-based adjuvants for the induction of the protective immunity. To this end, a chimeric form of a nontoxic adjuvant combining the merits of mutant cholera toxin A subunit (mCT-A) and heat labile toxin B subunit (LT-B) was created as the second generation of detoxified toxin-based mucosal adjuvant. Furthermore, our results have demonstrated that fusogenic liposome containing with HIV gp-160 induced virus-specific mucosal IgA antibody responses in the reproductive tissues of nasally immunized mice. In addition, mice nasally immunized with fusogenic liposome containing HIV gp-160 exhibited virus antigen-specific CTL responses. These results suggest fusogenic liposome is an effective and attractive vaccine delivery vehicle for the development of mucosal vaccines. Finally, our recent effort is aimed toward the generation of MHC class II tetramer with toxin associated peptide for the elucidation of vaccine and pathogen antigen-specific Th cell responses in both mucosal and systemic compartments.

3. Role of innate immunity in the mucosal immune system: biological contribution of non-classical MHC molecule in the intestinal immunity

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Mucosal epithelium provides a fist line of defense which is considered to be the site for the initiation of both innate and acquired immunity. Thus, epithelial cells are considered to be one of key immunological cells for the mucosal immune system. MICA is a nonclassical MHC class I molecule expressed in various tumors and intestinal epithelium. It is reported that MICA is a ligand for NKG2D expressed on NK or T cells in humans and its expression is increased by stresses such as heat shock or viral infection. However, it is not yet proven that what is the biological contribution of MICA, especially in gut environments. Therefore, we generated a transgenic model expressing human MICA in mouse intestine under the control of $T3^b$ promoter ($T3^b$: a nonclassical MHC class Ib molecule expressed in mouse intestine) in order to understand the precise role of MICA *in vivo*. The $T3^b$-MICA transgenic mice expressed the MICA molecules selectively in epithelial cells of small and large intestines. Moreover, the MICA expressed in small intestine induced a clonal expansion of CD4CD8αα (DP) IELs. We are currently examining the regulatory functions of MICA-induced IELs for sustaining host homeostasis against the immunological and infectious diseases occurred in intestine. This unique *in vivo* model will enable investigation of possible influences of stress-inducible MICA on the gut

immune surveillance.

4. Mucosally-induced allergic diarrhea

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Systemically primed mice develop severe diarrhea following repeated oral challenge of ovalbumin (OVA). This murine diarrhea model has been used to clarify the underlying mechanism of intestinal hypersensitivity. Histological analysis demonstrated that dramatic infiltration of eosinophils selectively occurred in the large intestine (see Figure). Large intestinal $CD4^+\alpha\beta$T lymphocytes elicited a brisk synthesis of IL-4, and IL-13 but little or no IFN-γ synthesis, whereas small intestinal $CD4^+\alpha\beta$T lymphocytes produced no detectable levels of antigen-induced cytokines. As would be expected from the high levels of Th2-type cytokines, brisk levels of IgE were detected in sera and IgE antibody-producing cells were detected in the large but not small intestine of mice with diarrhea. Strikingly, identically treated signal transducers and activators of transcription 6 (STAT6) gene-disrupted mice failed to develop OVA-induced diarrhea. To understand molecular mechanisms for the predominant Th2 environment in the large intestinal tract, we hypothesized that intestinal IL-12p40, antagonist of IL-12 p70 may create a dominant Th2 environment. To this end, over-expression of IL-12p40 was appeared in the large intestine of OVA-induced diarrhea mice. It was interesting to note that large but not small intestinal epithelial cells produce IL-12p40 in the allergic diarrhea mice. Our finding provided new evidence that over-expression of IL-12p40 is an important contributing factor for the generation of the dominant Th2-type environment in the large intestine of mice with allergic diarrhea. In addition to the molecular and cellular elucidation of immunological mechanisms for the development of mucosally induced allergic diseases in the intestinal tract, our goal is to combine the information generated through the basic and clinical investigation of the mucosal immune system for the development of new generation of mucosal vaccine and mucosal immune-therapy for the prevention and control for mucosally-induced allergic disease.

5. Antigen processing for MHC class I-restricted antigen presentation

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An important arm of the mucosal immune system is of course provided by cell-mediated immunity including those of $CD8^+$ cytotoxic T lymphocytes (CTL). However, very minimum information is currently available molecular aspect of MHC class I (MHC I) mediated antigen presentation at the mucosal epithelium. Our recent efforts have been aimed to elucidate the fundamental aspect of MHC I mediated antigen presentation at mucosal epithelium. MHC I molecules display antigenic epitopes on the cell surface as ligands for $CD8^+$ CTL. Antigen processing in cytoplasm and endoplasmic reticulum (ER) is required for the generation of epitope/MHC I complexes, but the underlying mechanism is still obscure. To address this, we have recently developed a novel enzymatic method to detect antigenic intermediates generated in cytoplasm and ER. This novel method revealed that intracellular proteolysis yielded a mixture of antigenic intermediates containing N-terminal franking residues, which was mediated by multicatalytic proteasome. We also showed that these intermediates were associated with cytosolic chaperones like TRiC (TCP-1 ring complex) and heat shock protein 90 (hsp90). Destabilization of TRiC using a specific small interfering RNA resulted in a degradation of the associated intermediates, subsequently leading to the inhibition of surface antigen presentation. Thus, TRiC plays a pivotal role for the protecting proteolytic intermediates in the MHC I antigen processing pathway. Our current effort is focused on the role of TRiC in the induction of CTL in the mucosal epithelium following virus infection.

Publications

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