Center for Experimental Medicine and Systems Biology

Laboratory of Innate Immunity
自然免疫研究分野

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Pathogen sensors, such as Toll-like receptor (TLR), play sentinel roles in detecting pathogenic ligands during infection and induce both innate and acquired immune responses. Meanwhile, excessive TLR responses are strongly associated with fatal diseases such as septic shock and autoimmune diseases. For this reason, immune system must strictly control TLR responses to avoid disruption of homeostasis. However, molecular mechanisms involved in TLR regulation are not fully elucidated. We have previously shown that TLRs are regulated by various TLR associating molecules including MD-2, PRAT4A and Unc93B1. Our goal is to uncover molecular mechanism which is indispensable for appropriate TLR responses using genetically engineered mice.

1. The meaning of TLR distribution.

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Toll-like receptor 5 (TLR5), a sensor for bacterial flagellin, mounts innate and adaptive immune responses, and has been implicated in infectious diseases, colitis and metabolic syndromes. Although TLR5 is believed to belong to cell surface TLRs, cell surface expression has never been verified. Moreover, it has remained unclear which types of immune cells express TLR5 and contribute to flagellin-dependent responses. For this reason, we established an anti-mouse/human TLR5 monoclonal antibody and studied cell surface expression of TLR5 on various immune cells. A macrophage cell line J774 expressed endogenous TLR5 on the cell surface and produced IL-6 and G-CSF in response to flagellin. In *in vivo*, cell surface TLR5 was mainly found on neutrophils and $CD11b^{hi}Ly6C^{hi}$ classical monocytes in the bone marrow, circulation, spleen and an inflammatory lesion. $Ly6C^{hi}$ classical monocytes, but not neutrophils, produced cytokines in response to flagellin. Splenic $CD8^{-}CD4^{+}$ conventional dendritic cells and $CD11c^{hi}CD11b^{hi}$ lamina propria DCs, also clearly expressed cell surface TLR5.

Unexpectedly, we also found that cell surface expression of TLR5 and flagellin-induced responses were completely abolished by silencing not only ER chaperon "PRotein Associated with TLR4 A (PRAT4 A)" but also "Unc93B1" which had been believed to regulate the trafficking of nucleic acid sensing intracellular TLRs. Collectively, Our study reveals that cell surface TLR5 is restricted to neutrophils, classical monocytes and specific DC subsets in mice, and the role of UNC93B1 is not limited to the TLRs signaling from the endolysosomes.

2. Characterization of cleaved forms of TLR7 and TLR9

Atsuo Kanno[1], Chikako Yamamoto[2], Yuji Motoi[2],

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Vertebrates have TLR3, 7, 8 and 9 as sensors of microbial nucleic acids, however it is suggested that TLR7/9 responses strongly associate with autoimmune diseases owing to inappropriate recognition of self nucleic acid. In endolysosome, TLR7 and TLR9 recognize a single-stranded RNA and an unmethylated CpG motif in microbial DNA, respectively. TLR7/9 ordinarily reside in Endoplasmic Reticulum, and ligand stimulation enhance the trafficking of TLR7/9 to endolysosome. Such strict regulation of TLR7/9 subcellular localization seems to have a role for blocking self nucleic acid recognition. Previous reports indicated novel posttranscriptional modification in TLR7/9 that ectodomains of TLR7/9 were cleaved in endolysosome. It seemed that ectodomain cleavage in TLR7/9 represents another strategy to restrict excessive TLR7/9 activation. Recently, we established new monoclonal anti-TLR7 and anti-TLR9 to detect endogenous or non-tagged TLRs and used these antibodies to clarify the mechanism of TLRs cleavage.

Our data showed that cleaved TLR7 N-terminal binds to C-terminal by its disulfide bond and the cysteines are important for response of TLR7 (Kanno et al., *Int. Immunol.* 2013). We focused on four cysteines of TLR7 (C98, C445, C475, and C722) and made serine mutants of these cysteines for analysis of cleavage pattern and the effect on response. As results, C98 and C475 were required for binding of TLR7 N-terminal to C-terminal, and proteolytic cleavage of TLR7. These cysteine mutants did not respond to TLR7 ligands, and also no response was observed in deletion mutants of TLR7 cleavage site or truncated form of TLR7 C-terminal alone. From these data, it is suggested that proteolytic cleavage of TLR7 and binding of N-terminal to C-terminal are essential for TLR7 response, and dependent on its cysteines.

In the case of TLR9, we found no effect of disulfide bond but the binding of N-terminal fragment to C-terminal fragment is important for TLR9 response. Now we are trying to confirm the relation between proteolytic cleavage of TLR9 and its response.

Furthermore, we generated the mice having mutation at cleavage sites of TLR7 or TLR9. To understand physiologic meanings of proteolytic cleavage in nucleic acid sensing-TLRs, these mice will be analyzed.

3. Roles for Unc93 homolog B1-dependent TLR 7/9 balance in vivo

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Nucleic acid sensing Toll-like receptor 7 (TLR7) and TLR9 recognize microbial RNA and DNA, respectively. These TLRs potentially recognize self-derived nucleic acid and have been shown to have a role in autoimmune diseases. For maintenance of homeostasis, it is important to keep responsiveness of the nucleic acid-sensing TLRs under tight control.

Unc93 homolog B1 (Unc93B1) has been reported to be indispensable for TLR7/9 responses in vitro. We have previously found that the alanine substitution for the 34th aspartic acid (D34A) of Unc93B1 enhanced TLR7 response but downregulated TLR9 response. These results suggest that Unc93B1 reciprocally regulate between TLR7 and TLR9 responses, and the TLR7/TLR9 balance is biased towards TLR9 in steady state.

To further clarify a role for Unc93B1-dependent TLR7/TLR9 balance *in vivo*, we started to generate knock-in mice harboring various types of mutations around D34 locus in the Unc93B1 gene which showed more accelerated phenotypes than D34A mutant mice. These knock-in mice were also designed that Unc93B1 knock-out mice were able to be generated by Cre-Lox recombination system. Therefore, we will be able to investigate the role of Unc93B1 in vivo for TLR7 and TLR9 responses. This mutant and knock-out mice were succeeded in germ line transmission, thus further analysis is now on going.

4. Identification of regulatory molecules for TLR responses and constructing genetically engineered mice.

Takuma Shibata[1,2], Yuji Motoi[1], Atsuo Kanno[1], Ryutaroh Fukui[1], Shin-ichiroh Saitoh[1], Nobuaki Yoshida[2] and Kensuke Miyake[1,2]: [1]Division of Infectious Genetics, Department of Microbiology and Immunology, [2]Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, TOKYO 1208-8639, Japan.

We are trying functional cloning and co-immunoprecipitation assay to comprehensively identify regulatory molecules associating with TLR responses. After checking the function of candidate genes in TLR response using knock-out cell lines by CRISPR/CAS9 *in vitro*, we construct conventional/conditional knock-out mice or knock-in transgenic mice using ROSA26 locus to reveal the physiological function of novel TLR associating molecules *in vivo*.

Publications

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"UNC93B1 is essential for the plasma membrane localization and signaling of Toll-like receptor 5" *PNAS*. in press, 2014

Center for Experimental Medicine and Systems Biology

Laboratory of Developmental Genetics
発生工学研究分野

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Gene targeting technology has revealed many aspects of gene functions in vivo. Knock out mice offer the opportunities of not only analyzing the complex gene functions in vivo, but also presenting various human disease models, where new therapeutic approaches can be explored. To allow more detailed dissection of gene function, we introduce a point mutation or disrupt genes in certain lineages (or stages) using Cre-loxP system, a method of conditional gene targeting. In the process of analyzing knock out mice, we have isolated spontaneous mutant mice which develop chylous ascites and edematous limbs. In order to understand the mechanism of lymphatic development and functions in more detail, we are also generating various knock-out/knock-in mouse lines including a conditional knock out mouse. In addition, we focus on analysis of neural development, aiming to understand the molecular mechanism of the maintenance of stemness and neural differentiation and to advance towards cell therapy of the damaged or degenerating nervous system. For this purpose, we are generating several conditional knock out mouse lines.

1. Lnk prevents inflammatory CD8+ T-cell proliferation and contributes to intestinal homeostasis.

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The intracellular adaptor Lnk (also known as SH2B3) regulates cytokine signals that control lymphohematopoiesis, and Lnk−/− mice have expanded B-cell, megakaryocytes and hematopoietic stem-cell populations. Moreover, mutations in the LNK gene are found in patients with myeloproliferative disease, whereas LNK polymorphisms have recently been associated with inflammatory and autoimmune diseases, including celiac disease. Here, we describe a previously-unrecognized function of Lnk in the control of inflammatory CD8+ T-cell proliferation and in intestinal homeostasis. Mature T cells from newly-generated Lnk-Venus reporter mice had low but substantial expression of Lnk, whereas Lnk expression was down-regulated during homeostatic T-cell proliferation under lymphopenic conditions. The numbers of CD44hi IFN-γ+ CD8+ effector or memory T cells were found to be increased in Lnk−/− mice, which also exhibited shortening of villi in the small intestine.

Lnk−/− CD8+ T cells survived longer in response to stimulation with interleukin-15 (IL-15) and proliferated even in non-lymphopenic hosts. Transfer of Lnk−/− CD8+ T cells together with wild-type CD4+ T cells into Rag2-deficient mice recapitulated a sign of villous abnormality. Our results reveal a link between Lnk and immune cell-mediated intestinal tissue destruction.

2. Conditional ablation of HMGB1 in mice reveals its protective function against endotoxemia and bacterial infection.

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High-mobility group box 1 (HMGB1) is a DNA-binding protein abundantly expressed in the nucleus that has gained much attention for its regulation of immunity and inflammation. Despite this, whether and how HMGB1 contributes to protective and/or pathological responses in vivo is unclear. In this study, we constructed *Hmgb1*-floxed (*Hmgb1*f/f) mice to achieve the conditional inactivation of the gene in a cell- and tissue-specific manner by crossing these mice with an appropriate Cre recombinase transgenic strain. Interestingly, although mice with HMGB1 ablation in myeloid cells apparently develop normally, they are more sensitive to endotoxin shock compared with control mice, which is accompanied by massive macrophage cell death. Furthermore, these mice also show an increased sensitivity to *Listeria monocytogenes* infection. We also provide evidence that the loss of HMGB1 in macrophages results in the suppression of autophagy, which is commonly induced by lipopolysaccharide stimulation or *L. monocytogenes* infection. Thus, intracellular HMGB1 contributes to the protection of mice from endotoxemia and bacterial infection by mediating autophagy in macrophages. These newly generated HMGB1 conditional knockout mice will serve a useful tool with which to study further the in vivo role of this protein in various pathological conditions.

3. Smad2 Is Essential for Maintenance of the Human and Mouse Primed Pluripotent Stem Cell State

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Human embryonic stem cells and mouse epiblast stem cells represent a primed pluripotent stem cell state that requires TGF-β/activin signaling. TGF-β and/or activin are commonly thought to regulate transcription through both Smad2 and Smad3. However, the different contributions of these two Smads to primed pluripotency and the downstream events that they may regulate remain poorly understood. We addressed the individual roles of Smad2 and Smad3 in the maintenance of primed pluripotency. We found that Smad2, but not Smad3, is required to maintain the undifferentiated pluripotent state. We defined a Smad2 regulatory circuit in human embryonic stem cells and mouse epiblast stem cells, in which Smad2 acts through binding to regulatory promoter sequences to activate Nanog expression while in parallel repressing autocrine bone morphogenetic protein signaling. Increased autocrine bone morphogenetic protein signaling caused by Smad2 down-regulation leads to cell differentiation toward the trophectoderm, mesoderm, and germ cell lineages. Additionally, induction of Cdx2 expression, as a result of decreased Smad2 expression, leads to repression of Oct4 expression, which, together with the decreased Nanog expression, accelerates the loss of pluripotency. These findings reveal that Smad2 is a unique integrator of transcription and signaling events and is essential for the maintenance of the mouse and human primed pluripotent stem cell state.

4. PTB Deficiency Causes the Loss of Adherens Junctions in the Dorsal Telencephalon and Leads to Lethal Hydrocephalus

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Polypyrimidine tract-binding protein (PTB) is a well-characterized RNA-binding protein and known to be preferentially expressed in neural stem cells (NSCs) in the central nervous system; however, its role in NSCs in the developing brain re-

mains unclear. To explore the role of PTB in embryonic NSCs in vivo, Nestin-Cre-mediated conditional Ptb knockout mice were generated for this study. In the mutant forebrain, despite the depletion of PTB protein, neither abnormal neurogenesis nor flagrant morphological abnormalities were observed at embryonic day 14.5 (E14.5). Nevertheless, by 10 weeks, nearly all mutant mice succumbed to hydrocephalus (HC), which was caused by a lack of the ependymal cell layer in the dorsal cortex. Upon further analysis, a gradual loss of adherens junctions (AJs) was observed in the ventricular zone (VZ) of the dorsal telencephalon in the mutant brains, beginning at E14.5. In the AJs-deficient VZ, impaired interkinetic nuclear migration and precocious differentiation of NSCs were observed after E14.5. These findings demonstrated that PTB depletion in the dorsal telencephalon is causally involved in the development of HC and that PTB is important for the maintenance of AJs in the NSCs of the dorsal telencephalon.

5. FGF2-induced Ras-MAPK signalling maintains lymphatic endothelial cell identity by upregulating endothelial-cell-specific gene expression and suppressing TGFβ signalling through Smad2.

Taeko Ichise, Nobuaki Yoshida, Hirotake Ichise

The lymphatic endothelial cell (LEC) fate decision program during development has been described. However, the mechanism underlying the maintenance of differentiated LEC identity remains largely unknown. Here, we show that fibroblast growth factor 2 (FGF2) plays a fundamental role in maintaining a differentiated LEC trait. In addition to demonstrating the appearance of LECs expressing α-smooth muscle actin in mouse lymphedematous skin in vivo, we found that mouse immortalised LECs lose their characteristics and undergo endothelial-to-mesenchymal transition (EndMT) when cultured in FGF2-depleted medium. FGF2 depletion acted synergistically with transforming growth factor (TGF) β to induce EndMT. We also found that H-Ras-overexpressing LECs were resistant to EndMT. Activation of H-Ras not only upregulated FGF2-induced activation of the Erk mitogen activated protein kinases (MAPK3 and MAPK1), but also suppressed TGFβ-induced activation of Smad2 by modulating Smad2 phosphorylation by MAPKs. These results suggest that FGF2 regulates LEC-specific gene expression and suppresses TGFβ signalling in LECs through Smad2 in a Ras-MAPK-dependent manner. Taken together, our findings provide a new insight into the FGF2-Ras-MAPK-dependent mechanism that maintains and modulates the LEC trait.

6. Role of PTBs, an alternative splicing factor, on spermatogenesis

Takashi Takijiri, Yoshiki Nagata, Manabu Ozawa, Reiko Sakamoto and Nobuaki Yoshida

Germ cells are the only cells which can transfer genomic information to the next generation. In males, sustainable sperm production is maintained by exquisite balances of self-renewal or differentiation of spermatogonial stem cells in the testis. Polypyrimidine tract-binding proteins (PTB(s)) are RNA-binding proteins that bind specifically to pyrimidine-rich sequences of RNAs and play multiple important roles such as RNA processing including alternative splicing regulation. In Drosophila, PTB is necessary for spermatid individualization, and PTB-mutant males are infertile. Since PTB is expressed higher in testis in mammals, we hypothesized that PTB(s) act for homeostasis of spermatogenesis/spermiogenesis. Interestingly, expression of PTBP1 in testicular germ cells is restricted in spermatonogia, and phenotype of the gene knockout male mouse showed drastic reduction of gonocyte or spermatogonia soon after birth and showing subfertile phenotype. These results suggest that PTBP1 has important roles on primordial germ cell development in fetus. In addition, all fertile male of PTBP1 null mice become infertile until 7-month of age, suggestion that PTBP1 also has important role for maintaining sustainable spermatogonial stem cells self renew. On the other hand, PTBP2, a homolog protein of PTBP1 possessing the same set of functional domains, expression in spermatogonia is less evident (or absent) but shows a drastic increase according to progression of meiosis. PTBP2-null male mice are totally infertile, and having very few sperms in epididymis. Immunohistological and immunocytological analysis revealed that a large part of spermatocytes in the PTBP2 null male is arrested their meiosis at pachytene stage, and having large number of abnormal multinucleated giant cells in seminiferous tubules. Further and more detail determination how the abnormality in spermatogenesis occurs by the lacking of PTBP1 or PTBP2 is now under estimated.

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Center for Experimental Medicine and Systems Biology

Laboratory of Systems Biology
システムズバイオロジー研究分野

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Gene-modified mice are considered to be powerful tools for understanding of pathophysiological function of the targeted gene(s) in vivo. Our research focus is the understanding of pathogenesis of rejection and immune disorders such as allergy and autoimmunity using gene-modified mice.

IL-33, but not IL-25, is crucial for the development of house dust mite antigen-induced allergic rhinitis

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Both interleukin (IL)-33 and IL-25 induce Th2 cytokine production by various cell types, suggesting that they contribute to development of allergic disorders. However, the precise roles of IL-33 and IL-25 in house dust mite (HDM)-induced allergic rhinitis (AR) remain unclear. Both IL-33 and IL-25 were produced mainly by nasal epithelial cells during HDM-induced AR. Eosinophil and goblet cell counts in the nose and IL-5 levels in lymph node cell culture supernatants were significantly decreased in IL-33-deficient, but not IL-25-deficient, mice compared with wild-type mice during HDM-induced AR, but the serum IgE and IgG1 levels did not differ. On the other hand, HDM-induced AR developed similarly in wild-type mice transferred with either IL-33-deficient BM cells or wild-type BM cells. IL-33, but not IL-25, produced by nasal epithelial cells was crucial for the development of murine HDM-induced AR. These observations suggest that IL-33 neutralization may be a potential approach for treatment of HDM-induced AR in humans.

Galectin-9 enhances cytokine secretion, but suppresses survival and degranulation, in human mast cell line

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Galectin-9 (Gal-9), a lectin having a β-galactoside-binding domain, can induce apoptosis of Th1 cells by binding to TIM-3. In addition, Gal-9 inhibits IgE/Ag-mediated degranulation of mast cell/basophilic cell lines by binding to IgE, thus blocking IgE/Ag complex formation. However, the role of Gal-9 in mast cell function in the absence of IgE is not fully understood. Here, we found that recombinant Gal-9 directly induced phosphorylation of Erk 1/2 but not p38 MAPK in a human mast cell line, HMC-1, which does not express FcεRI. Gal-9 induced apoptosis and inhibited PMA/ionomycin-mediated degranulation of HMC-1 cells. On the other hand, Gal-9 induced cytokine and/or chemokine production by HMC-1 cells, dependent on activation of ERK1/2 but not p38 MAPK. In addition, the lectin activity of Gal-9 was required for Gal-9-mediated cytokine secretion by HMC-1 cells. These observations suggest that Gal-9 has dual properties as both a regulator and an activator of mast cells.

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