Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Transplantation
幹細胞移植分野

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We are conducting clinical stem cell transplantation, especially using cord blood as a promising alternative donor for clinical use and investigating optimal strategies to obtain the best results in this area. We are also generating pre-clinical study to utilize virus-specific CTL for immune competent patients such as post-transplantation. Our goal is as allogeneic transplantation to be safer therapeutic option and to extend for older patients.

1. Cytokine profiles of pre-engraftment syndrome after single-unit cord blood transplantation for adult patients.

Konuma T[1], Kohara C[2], Watanabe E[3], Mizukami M[4], Nagai E[4], Oiwa-Monna M[2], Tanoue S[1], Isobe M[1], Kato S[1], Tojo A[1,2,5], Takahashi S[1,2,5]: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Division of Molecular Therapy, [3]IMSUT clinical flow cytometry laboratory, [4]Department of Laboratory Medicine, IMSUT Hospital, [5]Division of Stem Cell transplantation

Clinical manifestation of high-grade fever and skin rash before neutrophil engraftment, termed pre-engraftment syndrome (PES) or pre-engraftment immune reaction, has been frequently observed after cord blood transplantation (CBT). The pathophysiology of PES is poorly understood, but cytokine storm during the early phase of CBT is thought to be 1 of the main cause of PES. However, the cytokine profiles of PES after CBT are unclear. Therefore, we examined the relationship between serum cytokine profiles and PES in 44 adult patients who received CBT in our institution between February 2013 and June 2016. Serum levels of 21 cytokines, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-17F, IL-18, IL-21, IL-22, IL-23, IL-33, monocyte chemoattractant protein-1, IFN-α, IFN-γ, and TNF-α, were measured by multiplex bead assays using a flow cytometer. The median time until the absolute neutrophil count was $>.5\times10^9$/L was 21 days (range, 15 to 41 days). The cumulative incidence of PES was 79.6% (95% confidence interval, 63.3% to 88.5%) at 60 days after CBT. Serum levels of IL-5 (P=.009) and IL-6 (P =.01) at 2 weeks were significantly higher in patients who developed PES compared with those who did not develop PES. The conversion from naïve to effector or central memory phenotype of T cells was observed in PES. These data indicate that elevations of IL-5 and IL-6 around the time of clinical manifestation may be possible biomarkers for PES after CBT.

2. Cryopreserved CD34+ cell dose, but not total nucleated cell dose, influences hematopoietic recovery and extensive chronic GVHD after single-unit cord blood transplantation in adult patients.

Konuma T[1], Kato S[1], Oiwa-Monna M[2], Tanoue S[1], Ogawa M[2], Isobe M[1], Tojo A[1,2,3], Takahashi S[1,2,3]: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Division of Molecular Therapy, [3]Division of Stem Cell transplantation

Low cryopreserved total nucleated cell (TNC) dose in a cord blood (CB) unit has been shown to be associated with engraftment failure and mortality after single-unit cord blood transplantation (CBT) in adults. Although CB banks offer specific characteristics of cryopreserved cell dose, such as TNC, CD34+ cells, and colony-forming unit for granulocyte/macrophage (CFU-GM), the impact of each cell dose on engraftment and outcomes after single-unit CBT in adults remains unclear. We retrospectively analyzed the results of 306 CBTs for 261 adult patients in our institution between 1998 and 2016. The median age was 43 years (range, 16 to 68), the median actual body weight (ABW) was 56.2kg (range, 36.2 to 104.0), the median ideal body weight (IBW) was 62.3kg (range, 39.7 to 81.3), the median TNC dose was 2.46×107/ABW kg (range, 1.07 to 5.69), the median CD34+ cell dose was .91 ×105/ABW kg (range, .15 to 7.75), and the median CFU-GM dose was 24.46×103/ABW kg (range, .04 to 121.81). Among patients who achieved engraftment, the speed of neutrophil, platelet, and red blood cell engraftment significantly correlated with CD34+ cell dose, but not with TNC and CFU-GM dose, based on both ABW and IBW. In multivariate analysis, the incidence of extensive chronic graft-versus-host disease (GVHD) was significantly higher in patients receiving the highest CD34+ cell dose, based on both ABW and IBW. Nevertheless, no cell dose was associated with survival, transplantation-related mortality, and relapse. In conclusion, cryopreserved CD34+ cell dose was the best predictor for hematopoietic recovery and extensive chronic GVHD after CBT. The cryopreserved CD34 + cell dose should be used for unit selection criteria in single-unit CBT for adults.

3. Severe infusion-related toxicity after a second unrelated cord blood transplantation.

Tanoue S[1], Konuma T[1], Kato S[1], Oiwa-Monna M[2], Isobe M[1], Tojo A[1,2], Takahashi S[1,2]: [1]Department of Hematology/Oncology, IMSUT Hospital, [2]Division of Molecular Therapy

Several reports have demonstrated that severe and life-threating infusion-related toxicities, such as cardiac arrhythmias or cardiopulmonary events, rarely occur after cryopreserved CB infusion. In our patient, the main symptoms of infusion-related toxicity were hypoxemia and hypotension. Although urticaria was not observed, the rapid onset of symptoms after infusion and severe hypotension, which were probably not due to cardiogenic, hypovolemic and septic shock, suggested that the infusion-related toxicity in our patient was consistent with an anaphylactic reaction. Although the cause of infusion-related toxicities of cryopreserved cells was unclear, cryoprotective agents, such as DMSO and dextran, might be causative agents. Therefore, an initial sensitizing exposure to these agents after the first CB infusion might contribute to the development of anaphylactic reaction after a second CB infusion. t.

4. Alloimmune hemolysis due to major RhE incompatibility after unrelated cord blood transplantation.

Isobe M[1], Konuma T[1], Abe-Wada Y[2], Hirata K[2], Ogami K[2], Kato S1, Oiwa-Monna M[3], Tanoue S[1], Nagamura-Inoue T[2], Takahashi S[1,3], Tojo A[1,3].

Extension to donors other than HLA-matched siblings following advanced immunosuppressive treatment has resulted in the emergence of viral infections as major contributors to morbidity and mortality after hematopoietic stem cell transplantation (HSCT). The degree of risk for infection is dictated by the degree of tissue mismatch between donor and recipient, and the resultant degree of immunosuppression. Reactivation of latent viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex and herpes zoster are common and often cause symptomatic disease. Respiratory viruses such as adenovirus, influenza and respiratory symptomatic virus also frequently cause infection. While pharmacological agents are standard therapy for some, they have substantial toxicities, generate resistant variants, and frequently ineffective and costly. Moreover, immune reconstitution is necessary for long-term protection especially after HSCT. As the delay in recovery of virus-specific cellular immune response is cleary associated with viral reactivation and disease, cellular immunotherapy to restore virus-specific immunity offers an attractive alternative to conventional drugs. Adoptive transfer of virus-specific lymphocytes (VSTs) from stem cell donors has been proved to be safe and effective to treat viral infection.

Manufacture of VSTs requires preparation of specialized antigen presenting cells (APCs), uses viruses or viral vectors to provide vial antigens to present on APCs. Recent report from a group of Baylor College of Medicine has introduced a new method to generate multiple VSTs by direct stimulation of peripheral blood mononuclear cells (PBMCs) with peptides to replace the complex and lengthy process above.

By this method, VSTs can be prepared by single stimulation with non-viral products and contain polyclonal mixture of T cells specific for a large number of epitopes in a multiple pathogenic viruses, which reduces the risk of immune escape by viral escape mutants and meet the requirement to treat viral infection after HSCT which occurs by broad pathogens. Moreover this method is as simple and fast as possible and takes only 10-14 days

for preparation which makes it clinically useful. With this method, polyclonal CTLs specific for multivirus antigens can be produced after single stimulation of PBMCs with a peptide mixture spanning the target antigens in the presence of IL4 and IL7.

We have introduced and verified this system to apply for clinical use in Japan.

The culture media for T cell expansion used in the studies reported by Baylor College of Medicine are supplemented with fetal bovine serum (FBS). While these serum products are traditionally used to expand T cells to promote cell growth and viability, there are some countries which the use of serum product is not allowed. Also, uncharacterized elements contained in the serum products may cause inconsistency in results from batch to batch. Cell expansion in serum-free media would therefore be preferable.

To meet the requirement for the viral infections after HSCT by broad viral antigens and in terms of regulation by the Japanese FDA, we established the method to generate multivirus-specific T cells targeting 7 viruses (CMV, EBV, AdV, HHV-6, BKV, JCV, and VZV) in serum-free medium.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Processing
幹細胞プロセシング分野

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Stem cells represent a valuable cell source in the field of regenerative medicine. Hematopoietic stem cells represent a valuable cell source for transplantation medicine, with which many diseases including primary immunodeficiency and hematologic malignancies can expect life-long cure by reconstitution of healthy hematopoiesis. Our eventual goal is to establish safe and efficacious transplantation strategies in a form of either allogeneic transplantation or gene therapy using autologous hematopoietic stem cells after gene correction.

1. Dissection of signaling events downstream of the c-Mpl receptor in murine hematopoietic stem cells via motif-engineered chimeric receptors

Koichiro Saka, Chen-Yi Lai, Masanori Nojima, Masahiro Kawahara, Makoto Otsu, Hiromitsu Nakauchi, Teruyuki Nagamune

Hematopoietic stem cells (HSCs) are a valuable resource in transplantation medicine. Cytokines are often used to culture HSCs aiming at better clinical outcomes through enhancement of HSC reconstitution capability. Roles for each signal molecule downstream of receptors in HSCs, however, remain puzzling due to complexity of the cytokine-signaling network. Engineered receptors that are non-responsive to endogenous cytokines represent an attractive tool for dissection of signaling events. We here tested a previously developed chimeric receptor (CR) system in primary murine HSCs, target cells that are indispensable for analysis of stem cell activity.

2. Evaluation of the Utility of Induced Pluripotent Stem Cells as a platform for modeling Wiskott Aldrich Syndrome

HONDA-OZAKI Fumiko, LIN Huan-Ting, OKUMURA Takashi, LAI Chen-Yi, OTSU Makoto

Wiskott Aldrich syndrome (WAS) is an X-linked disease, caused by mutations in the gene encoding the WAS protein (WASp). Thrombocytopenia is the main feature of this disease, often threatening patients to a significant risk of serious hemorrhage. It has been an issue of debate whether defective proplatelet release from megakaryocytes (MKs)(i.e., production) and/or destruction of platelets (PLTs) by the spleen macrophages (i.e., consumption) constitutes the major cause of thrombocytopenia. Due to the limitations inherent to classical experimental models, there have been conflicting results published; precise mechanisms causing thrombocytopenia in WAS therefore remain to be elucidated. To have an appropriate model in our hands and to elucidate mechanism(s) of thrombocytopenia in WAS to improve treatment of WAS patients in future, we here evaluated the utility of iPSCs as an emerging disease model for WAS. WASp-deficient iPSCs produced MKs and PLTs with similar yields as did control cells when differentiated from sorted MK progenitors. Interestingly, however, WASp-deficient iPSCs yielded only fewer MKs and PLTs than did healthy counterparts when differentiated from unsorted HPCs. In addition, we found that

WASp-deficiency variously affected phagocytosis function of iPSC-monocytes in a context specific manner, depending on what targets they would face. These data imply that not just MKs and PLTs but also other lineage cells such as phagocytic cells may play a role for WAS-thrombocytopenia. We are now trying to establish an in vitro co-culture system of iPSCs-derived monocytes and -platelets as a new experimental platform to clarify accurate roles of each cellular compartment.

3. The functional analysis of dendritic cells developed from T-iPS cells from a single CD4 + T cell of Sjögren's syndrome

Mana Iizuka-Koga, Hiromitsu Asashima, Miki Ando, Chen-Yi Lai, Shinji Mochizuki, Mahito Nakanishi, Toshinobu Nishimura, Hiroto Tsuboi, Tomoya Hirota, Hiroyuki Takahashi, Isao Matsumoto, Makoto Otsu, Takayuki Sumida

Though it is important to clarify the pathogenic and/or regulatory functions of the T cells in human samples, their examination is frequently restricted in opportunity because the acquisition of sufficient quantity of dendritic cells (DCs), used as antigen presenting cells, might not be available, especially in autoimmune diseases. In this study, we developed the generation of mature DCs from induced pluripotent stem cells derived from T cells (T-iPSCs). We reprogramed only a single CD4 + T cell of a patient with Sjögren's syndrome (SS) into iPS cells, which were differentiated into DCs (TiPS-DCs) via TiPS-Sacs. Moreover, we examined their characterizations and functions. Under the microscope, the mature TiPS-DCs were dendritic cells-like morphology. The expression of CD11b, CD11c, HLA-ABC and CD40 was detected, and the CD80, CD86 and HLA-DR was also increased. The mature TiPS-DCs were able to produce TNF-α and IL-6 through stimulation. As compared with monocytes-derived DCs, TiPS-DCs was equal to the ability of antigen processing, and also the induction of robust proliferative response of allogeneic T cells in the dependent manner of cell numbers.

In conclusion, we succeeded to obtain an adequate amount of functional DCs from a single CD4 + T cell by way of iPS cells. This method should shed light on the characterization of pathogenic T cells for not only SS but also other diseases to elucidate the function of autoreactive T cells. The method shown in the study will enable us to identify autoreactive T cells in autoimmune diseases along with elucidate the function and pathogenicity of them.

4. Mutant iPS cells derived from patients with RALD show significance of KRAS for self-renewal and differentiation propensity

Kenji Kubara, Kazuto Yamazaki, Yasuharu Ishihara, Huan-Ting Lin, Masashi Ito, Kappei Tsukahara, Masatoshi Takagi, and Makoto Otsu

KRAS is widely known as a proto-oncogene and has been reported to play essential roles in stemness maintenance in some types of stem cells, including cancer stem cells. However, the roles of KRAS in pluripotent stem cells (PSCs) are largely unknown. Recently, somatic gain-of-function mutations of KRAS or NRAS in hematopoietic stem cells have been reported to cause RAS-associated autoimmune lymphoproliferative syndrome-like disease (RALD). Here, we investigated the roles of KRAS on stemness maintenance in the context of human PSCs using isogenic KRAS mutant (G13C/WT) and wild-type (WT/WT) induced PSCs (iPSCs), generated from the same RALD patients with the somatic KRAS mutation. Using the isogenic iPSC lines from two patients, we revealed that G13C/WT iPSCs displayed self-renewal and differentiation characteristics distinct from those of WT/WT iPSCs: expression of stemness markers including POU5F1 and NANOG, which is indicative of an undifferentiated state, was maintained at high levels in G13C/WT iPSCs under bFGF depletion; neuronal differentiation was clearly blunted from G13C/WT iPSCs. In addition, we generated wild-type (WTed/WT) and heterozygous knockout (Δed/WT) iPSCs from the same G13C/WT clone using gene-editing techniques. As expected, the G13C/WT-specific phenotypes were normalized in WTed/WT iPSCs. Interestingly, Δed/WT iPSCs showed lower potential to maintain undifferentiation status under bFGF depletion, with a higher tendency to differentiate into neuronal lineage than WTed/WT iPSCs. Biochemical analysis indicated hyper-activation of KRAS and subsequent increased phosphorylation levels of ERK in G13C/WT iPSCs. Pharmacological studies using specific kinase inhibitors demonstrated that the features compatible with enhanced stemness maintenance were canceled in mutant cells by pan-RAF and MEK inhibitors, but not by PI3K inhibitors. In addition, neuronal differentiation was improved in G13C/WT iPSCs with the MEK inhibitor treatment. These observations suggested that the KRAS-ERK pathway plays more critical roles than the KRAS-PI3K pathway in G13C/WT iPSCs. Collectively, the analyses on the isogenic and genome-edited iPSCs from the RALD patients revealed the crucial roles of the KRAS-ERK signaling on the stemness maintenance, having a strong impact on self-renewal and differentiation propensity in human iPSCs.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Signaling
幹細胞シグナル制御分野

Professor Toshio Kitamura, M.D., D.M.Sc.

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Our major interest is to elucidate the mechanisms of pluripotency, self-renewal and the control of cell division and differentiation of hematopoietic stem and progenitor cells. We have developed the retrovirus-mediated efficient gene transfer and several functional expression cloning systems, and utilized these system to our experiments. We are now conducting several projects related to stem cells to characterize stem cells, clarify underling mechanisms of maintenance of pluripotency, and differentiation.

1. Developing Analysis Tools for Cell Cycle and Cell Division of Hematopoietic Stem Cells: MgcRacGap - hmKusabiraOrange 2 (MRG - hmKuO2) fusion protein for midbody marker.

Yosuke Tanaka, Tsuyoshi Fukushima, Toshihiko Oki, Kotarou Nishimura, Asako Sakaue-Sawano[1], Atsushi Miyawaki[1], Toshio Kitamura: [1]Laboratory for Cell Function Dynamics, RIKEN, Wako, Saitama and ERATO Miyawaki Life Function Dynamics Project, JST.

Previously, we reported that MgcRacGap is a marker for midbody and that MgcRacGap-mVenus fusion protein visualized asymmetric inheritance and release of midbody during cytokinesis (Nishimura et al., 2013). We retrovirally introduced MRG-hmKuO2 into hematopoietic stem cells (HSCs), in order to examine whether midbody asymmetric inheritance and release is involved with asymmetric division of HSCs. HSCs showed high frequency of midbody release during cytokinesis in culture. Interestingly, one daughter cell releasing midbody differentiated earlier than the other daughter cell inheriting midbody. We generated Cre-inducible MRG-hmKuO2 mouse line. Briefly, the MRG-hmKuO2 fusion gene is inserted into Rosa 26 locus following a loxP-NEO-STOP-loxP cassette, in order to visualize asymmetric inheritance and release of midbody in vivo without retroviral infection. Crossing MRG-hmKuO2 mice with Vav-Cre mice, MRG-hmKuO2 nicely marked midbody asymmetric inheritance and release in HSCs in culture. We are planning to do paired-daughter assay using HSCs from MRG-hmKuO2 mice to examine whether inheritance and release of midbody link to asymmetric division of HSCs. Given that some problems were found in this new mouse line, we are planning to establish surrogate experimental models for this.

2. Developing Analysis Tools for Cell Cycle and Cell Division of Hematopoietic Stem Cells: A novel G0 marker, mVenus-p27K- and its transgenic mouse

Tsuyoshi Fukushima, Yosuke Tanaka, Toshihiko Oki, Kotarou Nishimura, Asako Sakaue-Sawano[1], Atsushi Miyawaki[1], Toshio Kitamura: [1]Laboratory for Cell Function Dynamics, RIKEN, Wako, Saitama and ERATO Miyawaki Life Function Dynamics Project, JST.

One of the common features of the stem cells is that they are in quiescent (G0) phase of cell cycle. Several reports indicate that tissue specific stem cells like hematopoietic stem cells and cancer stem cells with tumor initiating potentials are in G0

phase.

We have developed a novel G0 marker, mVenus-p27K- (Oki et al, 2013). The mVenus-p27K- clearly marked G0 and very early G1 in NIH3T3 cells. To examine G0 status in HSCs, we generated a Cre-inducible mVenus-p27K- mouse line that carried mVenus-p27K- fusion gene in Rosa26 locus following a loxP-NEO-STOP-loxP cassette. After crossing with Vav-Cre mice, we analyzed mVenus-p27K- expression in HSCs. We expected most of the HSCs are mVenus-p27K- positive because most of the HSCs are dormant. However, surprisingly, three different populations (mVenus-p27K-high (70%), mVenus-p27K-low (20%), mVenus-p27K-negative (10%)) were given from HSC fraction (CD150 + CD 48-cKit + Sca-1 + Lineage-). These three populations were in G0 stage judged by Pyronin Y/Hoechst double-staining. To examine difference among these three populations, we performed bone marrow reconstitution assay. To our surprise, only mVenus-p 27K-high population showed high bone marrow reconstitution ability. Moreover, mVenus-p27K-high population was only detected in HSC fraction in the bone marrow, not in other hematopoietic fractions. This indicates that our mVenus-p27K- marker could be a good marker for isolation of transplantable HSCs. To characterize differences among the three fractions in HSCs, we performed single-cell RNASeq (scRNASeq) analysis. The tSNE analysis from scRNASeq data showed that mVenus-p27K-high single-cells and mVenus-p27K-low single-cells were plotted into similar cluster, whereas mVenus-p27K-negative single-cells were plotted into a different cluster from other two. However, HSC-related genes, such as Lyl1, Hlf, Hhex, were highly expressed in mVenus-p27K-high single-cells than mVenus-p27K-low single-cells. Other new unknown genes were also plotted in the same position, suggesting that those genes are strong candidate to define mVenus-p27K-high HSCs. Small-cell Mass Spec analysis and metabolome analysis for these cell fractions are ongoing. We also plan to examine where mVenus-p27K-high HSCs locate in bone marrow using 4D imaging.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Dynamics
幹細胞ダイナミクス解析分野

Associate Professor　Beate Heissig, M.D.

准教授　医学博士　　ハイジッヒ，ベアーテ

Proteases perform highly selective and limited cleavage of specific substrates including growth factors and their receptors, cell adhesion molecules, cytokines, apoptotic ligand and angiogenic factors. The goal of our laboratory is to identify novel therapeutic targets for diseases like cancer or inflammatory diseases by studying the role of proteases and growth factors during inflammation, tissue regeneration, and in stem cell biology.

1. The angiogenic factor Egfl7 alters thymogenesis by activating Flt3 signaling

Yousef Salama, Koichi Hattori, Beate Heissig

Thymic regeneration is a crucial function that allows for the generation of mature T cells after myelosuppression like irradiation. However molecular drivers involved in this process remain undefined. Here, we report that the angiogenic factor, epidermal growth factor-like domain 7 (Egfl7), is expressed on steady state thymic endothelial cells (ECs) and further upregulated under stress like post-irradiation. Egfl7 overexpression increased intrathymic early thymic precursors (ETPs) and expanded thymic ECs. Mechanistically, we show that Egfl7 overexpression caused Flt3 upregulation in ETPs and thymic ECs, and increased Flt3 ligand plasma elevation in vivo. Selective Flt3 blockade prevented Egfl7-driven ETP expansion, and Egfl7-mediated thymic EC expansion in vivo. We propose that the angiogenic factor Egfl7 activates the Flt3/Flt3 ligand pathway and is a key molecular driver enforcing thymus progenitor generation and thereby directly linking endothelial cell biology to the production of T cell-based adaptive immunity.

2. The role of plasmin in the pathogenesis of murine multiple myeloma

Salita Eiamboonsert, Yousef Salama, Hiroshi Watarai, Douaa Dhahri, Yuko Tsuda, Yoshio Okada, Koichi Hattori, Beate Heissig

Aside from a role in clot dissolution, the fibrinolytic factor, plasmin is implicated in tumorigenesis. Although abnormalities of coagulation and fibrinolysis have been reported in multiple myeloma patients, the biological roles of fibrinolytic factors in multiple myeloma (MM) using in vivo models have not been elucidated. In this study, we established a murine model of fulminant MM with bone marrow and extramedullar engraftment after intravenous injection of B53 cells. We found that the fibrinolytic factor expression pattern in murine B53 MM cells is similar to the expression pattern reported in primary human MM cells. Pharmacological targeting of plasmin using the plasmin inhibitors YO-2 did not change disease progression in MM cell bearing mice although systemic plasmin levels was suppressed. Our findings suggest that although plasmin has been suggested to be a driver for disease progression using clinical patient samples in MM using mostly in vitro studies, here we demonstrate that suppression of plasmin generation or inhibition of plasmin cannot alter MM progression in vivo.

3. Pharmacological targeting of plasmin prevents lethality and tissue damage in a murine model of macrophage activation syndrome

Hiroshi Shimazu, Shinya Munakata, Yoshihiko Tashiro, Yousef Salama, Salita Eiamboonsert, Yasunori Ota, Haruo Onoda, Yuko Tsuda, Yoshio Okada, Hiromitsu Nakauchi, Beate Heissig, Koichi Hattori

Macrophage activation syndrome (MAS) is a life-threatening disorder characterized by a cytokine storm and multiorgan dysfunction due to excessive immune activation. Although abnormalities of coagulation and fibrinolysis are major components of MAS, the role of the fibrinolytic system and its key player, plasmin, in the development of MAS remains to be solved. We established a murine model of fulminant MAS by repeated injections of Toll-like receptor-9 (TLR-9) agonist and d-galactosamine (DG) in immunocompetent mice. We found plasmin was excessively activated during the progression of fulminant MAS in mice. Genetic and pharmacological inhibition of plasmin counteracted MAS-associated lethality and other related symptoms. We show that plasmin regulates the influx of inflammatory cells and the production of inflammatory cytokines/chemokines. Collectively, our findings identify plasmin as a decisive checkpoint in the inflammatory response during MAS and a potential novel therapeutic target for MAS.

4. Plasminogen activator inhibitor-1 regulates macrophage-dependent postoperative adhesion by enhancing EGF-HER1 signaling in mice

Kumpei Honjo, Shinya Munakata, Yoshihiko Tashiro, Yousef Salama, Hiroshi Shimazu, Salita Eiamboonsert, Douaa Dhahri, Ichimura, A., Takashi Dan, Toshio Miyata, Kazuyoshi Takeda, Kazuhiro Sakamoto, Koichi Hattori, Beate Heissig

Adhesive small bowel obstruction remains a common problem for surgeons. After surgery, platelet aggregation contributes to coagulation cascade and fibrin clot formation. With clotting, fibrin degradation is simultaneously enhanced, driven by tissue plasminogen activator-mediated cleavage of plasminogen to form plasmin. The aim of this study was to investigate the cellular events and proteolytic responses that surround plasminogen activator inhibitor (PAI-1; *Serpine1*) inhibition of postoperative adhesion. Peritoneal adhesion was induced by gauze deposition in the abdominal cavity in C57BL/6 mice and those that were deficient in fibrinolytic factors, such as $Plat^{-/-}$ and $Serpine1^{-/-}$ In addition, C57BL/6 mice were treated with the novel PAI-1 inhibitor, TM5275. Some animals were treated with clodronate to deplete macrophages. Epidermal growth factor (EGF) experiments were performed to understand the role of macrophages and how EGF contributes to adhesion. In the early phase of adhesive small bowel obstruction, increased PAI-1 activity was observed in the peritoneal cavity. Genetic and pharmacologic PAI-1 inhibition prevented progression of adhesion and increased circulating plasmin. Whereas $Serpine1^{-/-}$ mice showed intra-abdominal bleeding, mice that were treated with TM5275 did not. Mechanistically, PAI-1, in combination with tissue plasminogen activator, served as a chemoattractant for macrophages that, in turn, secreted EGF and up-regulated the receptor, HER1, on peritoneal mesothelial cells, which led to PAI-1 secretion, further fueling the vicious cycle of impaired fibrinolysis at the adhesive site. Controlled inhibition of PAI-1 not only enhanced activation of the fibrinolytic system, but also prevented recruitment of EGF-secreting macrophages. Pharmacologic PAI-1 inhibition ameliorated adhesion formation in a macrophage-dependent manner.

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<Beate Heissig Group>

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Cellomics
幹細胞セロミクス分野

Project Associate Professor Hiroshi Watarai, Ph.D.　特任准教授　博士(医学)　渡　会　浩　志

Single cell analysis has become increasingly important for cellular biologists doing basic, translational, and clinical research. It was once believed that cell populations were homogeneous, but the latest evidence shows that heterogeneity does in fact exist even within small cell populations. Gene expression measurements based on the homogenized cell population are misleading averages and don't account for the small but critical changes occurring in individual cells. Individual cells can differ dramatically in size, protein levels, and expressed RNA transcripts, and these variations are key to answering previously irresolvable questions in cancer research, stem cell biology, immunology, and developmental biology. We are also trying to develop new advanced techniques by the integration of photonics, chemistry, electrical engineering, mechanical engineering, bioinformatics, and others.

1. The role of plasmin in the pathogenesis of murine multiple myeloma.

Salita Eiamboonsert[1], Yousef Salama[1], Hiroshi Watarai[2], Douaa Dhahri[1], Yuko Tsuda[3], Yoshio Okada[3], Koichi Hattori[4], Beate Heissig[1]: [1]Division of Stem Cell Dynamics, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, [2]Division of Stem Cell Cellomics, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, [3]Faculty of Pharmaceutical Sciences, Kobe Gakuin University, [4]Center for Genome and Regenerative Medicine, Juntendo University School of Medicine.

Aside from a role in clot dissolution, the fibrinolytic factor, plasmin is implicated in tumorigenesis. Although abnormalities of coagulation and fibrinolysis have been reported in multiple myeloma patients, the biological roles of fibrinolytic factors in multiple myeloma (MM) using in vivo models have not been elucidated. In this study, we established a murine model of fulminant MM with bone marrow and extramedullar engraftment after intravenous injection of B53 cells. We found that the fibrinolytic factor expression pattern in murine B53 MM cells is similar to the expression pattern reported in primary human MM cells. Pharmacological targeting of plasmin using the plasmin inhibitors YO-2 did not change disease progression in MM cell bearing mice although systemic plasmin levels was suppressed.

Our findings suggest that although plasmin has been suggested to be a driver for disease progression using clinical patient samples in MM using mostly in vitro studies, here we demonstrate that suppression of plasmin generation or inhibition of plasmin cannot alter MM progression in vivo.

2. IL-22BP dictates characteristics of Peyer's patch follicle-associated epithelium for antigen uptake.

Toshi Jinnohara[1,2], Takashi Kanaya[1,2], Koji Hase[3,4], Sayuri Sakakibara[1], Tamotsu Kato[1], Naoko Tachibana[1], Takaharu Sasaki[1], Yusuke Hashimoto[1,2], Toshiro Sato[7], Hiroshi Watarai[5], Jun Kunisawa[6,8], Naoko Shibata[6], Ifor R. Williams[9], Hiroshi Kiyono[6,10], Hiroshi Ohno[1,2]: [1]Laboratory for Intestinal

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Interleukin-22 (IL-22) acts protectively and harmfully on intestinal tissue depending on the situation; therefore, IL-22 signaling needs to be tightly regulated. IL-22 binding protein (IL-22BP) binds IL-22 to inhibit IL-22 signaling. It is expressed in intestinal and lymphoid tissues, although its precise distribution and roles have remained unclear. In this study, we show that IL-22BP is highly expressed by $CD11b^{+}CD8\alpha^{-}$ dendritic cells in the subepithelial dome region of Peyer's patches (PPs). We found that IL-22BP blocks IL-22 signaling in the follicle-associated epithelium (FAE) covering PPs, indicating that IL-22BP plays a role in regulating the characteristics of the FAE. As expected, FAE of IL-22BP-deficient (*Il22ra2*$^{-/-}$) mice exhibited altered properties such as the enhanced expression of mucus and antimicrobial proteins as well as prominent fucosylation, which are normally suppressed in FAE. Additionally, *Il22ra2*$^{-/-}$ mice exhibited the decreased uptake of bacterial antigens into PPs without affecting M cell function. Our present study thus demonstrates that IL-22BP promotes bacterial uptake into PPs by influencing FAE gene expression and function.

3. Analyses of a mutant Foxp3 allele reveal BATF as a critical transcription factor in the differentiation and accumulation of tissue regulatory T cells.

Norihito Hayatsu[1], Takahisa Miyao[1], Masashi Tachibana[1], Ryuichi Murakami[1,6], Akihiko Kimura[1], Takako Kato[1], Eiryo Kawakami[2,5], Takaho A. Endo[3], Ruka Setoguchi[4], Hiroshi Watarai[7], Takeshi Nishikawa[1], Takuwa Yasuda[4], Hisahiro Yoshida[4], Shohei Hori[1,6]: [1]Laboratory for Immune Homeostasis, RIKEN Center for Integrative Medical Sciences, [2]Laboratory for Disease Systems Modeling, RIKEN Center for Integrative Medical Sciences, [3]Laboratory for Integrative Genomics, RIKEN Center for Integrative Medical Sciences, [4]Laboratory for Immunogenetics, RIKEN Center for Integrative Medical Sciences, [5]Disease Biology Group, RIKEN Medical Sciences Innovation Hub Program, [6]Laboratory of Immunology and Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, [7]Division of Stem Cell Cellomics, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Sciences, The University of Tokyo.

Foxp3 controls the development and function of regulatory T (Treg) cells, but it remains elusive how Foxp3 functions in vivo. Here, we established mouse models harboring three unique missense Foxp3 mutations that were identified in patients with the autoimmune disease IPEX. The I363V and R397W mutations were loss-of-function mutations, causing multi-organ inflammation by globally compromising Treg cell physiology. By contrast, the A384T mutation induced a distinctive tissue-restricted inflammation by specifically impairing the ability of Treg cells to compete with pathogenic T cells in certain nonlymphoid tissues. Mechanistically, repressed BATF expression contributed to these A384T effects. At the molecular level, the A384T mutation altered Foxp3 interactions with its specific target genes including Batf by broadening its DNA-binding specificity. Our findings identify BATF as a critical regulator of tissue Treg cells and suggest that sequence-specific perturbations of Foxp3-DNA interactions can influence specific facets of Treg cell physiology and the immunopathologies they regulate.

4. A novel mouse model of iNKT cell deficiency generated by CRISPR/Cas9 reveals a pathogenic role of iNKT cells in metabolic disease.

Yue Ren[1,2], Etsuko Sekine-Kondo[1], Risa Shibata[1,3], Megumi Kato-Itoh[4], Ayumi Umino[4], Ayaka Yanagida[4,9], Masashi Satoh[5], Komaki Inoue[6], Tomoyuki Yamaguchi[4], Keiichi Mochida[6], Susumu Nakae[3], Luc Van Kaer[7], Kazuya Iwabuchi[5], Hiromitsu Nakauchi[4,8], Hiroshi Watarai[1]: [1]Division of Stem Cell Cellomics, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, [2]The Neurological Institute of Jiangxi Province, Department of Neurology, Jiangxi Provincial People's Hospital, [3]Laboratory of Systems Biology, Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo, [4]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, [5]Department of Immunology, Kitasato University School of Medicine, [6]Cellulose Production Research Team, RIKEN Center for Sustainable Resource Science, [7]Depart-

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iNKT cells play important roles in immune regulation by bridging the innate and acquired immune systems. The functions of iNKT cells have been investigated in mice lacking the Traj18 gene segment that were generated by traditional embryonic stem cell technology, but these animals contain a biased T cell receptor (TCR) repertoire that might affect immune responses. To circumvent this confounding factor, we have generated a new strain of iNKT cell-deficient mice by deleting the *Traj18* locus using CRISPR/Cas9 technology, and these animals contain an unbiased TCR repertoire. We employed these mice to investigate the contribution of iNKT cells to metabolic disease and found a pathogenic role of these cells in obesity-associated insulin-resistance. The new *Traj18*-deficient mouse strain will assist in studies of iNKT cell biology.

5. Ultrafast confocal fluorescence microscopy beyond the fluorescence lifetime limit.

Hideharu Mikami[1], Jeffrey Harmon[1], Hirofumi Kobayashi[1], Syed Hamad[1], Yisen Wang[1,2], Osamu Iwata[3], Kengo Suzuki[3], Takuro Ito[4], Yuri Aisaka[5], Natsumaro Kutsuna[5], Kazumichi Nagasawa[6], Hiroshi Watarai[6], Yasuyuki Ozeki[7], Keisuke Goda[1,4,8]: [1]Department of Chemistry, The University of Tokyo, [2]College of Precision Instrument and Optoelectronic Engineering, Tianjin University, [3]Euglena Co., Ltd., [4]Japan Science and Technology Agency, [5]LPixel Inc., [6]Division of Stem Cell Cellomics, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, The University of Tokyo, [7]Department of Electrical Engineering and Information Systems, The University of Tokyo, [8]Department of Electrical Engineering, University of California.

Laser-scanning confocal fluorescence microscopy is an indispensable tool for biomedical research by virtue of its high spatial resolution. Its temporal resolution is equally important, but is still inadequate for many applications. Here we present a confocal fluorescence microscope that, for the first time to our knowledge, surpasses the highest possible frame rate constrained only by the fluorescence lifetime of fluorophores (typically a few to several nanoseconds). This microscope is enabled by integrating a broadband, spatially distributed, dual-frequency comb or spatial dual-comb and quadrature amplitude modulation for optimizing spectral efficiency into frequency-division multiplexing with single-pixel photodetection for signal integration. Specifically, we demonstrate confocal fluorescence microscopy at a record high frame rate of 16,000 frames/s. To show its broad biomedical utility, we use the microscope to demonstrate 3D volumetric confocal fluorescence microscopy of cellular dynamics at 104 volumes/s and confocal fluorescence imaging flow cytometry of hematological and microalgal cells at 2 m/s.

6. Generation and validation of novel anti-bovine CD163 monoclonal antibodies ABM-1A9 and ABM-2D6.

Yoshinori Shimamoto[1], Junko Nio-Kobayashi[4], Hiroshi Watarai[5], Masashi Nagano[6], Natsuko Saito[2], Eiki Takahashi[7], Hidetoshi Higuchi[3], Atsushi Kobayashi[6], Takashi Kimura[6], Hiroshi Kitamura[2]: [1]Laboratory of Animal Therapeutics, Department of Veterinary Science, Rakuno Gakuen University, [2]Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Rakuno Gakuen University, [3]Laboratory of Animal Health, Department of Veterinary Medicine, Rakuno Gakuen University, [4]Laboratory of Histology and Cytology, Department of Functional Morphology, Graduate School of Medical Sciences, Hokkaido University, [5]Division of Stem Cell Cellomics, The Institute of Medical Science, The University of Tokyo, [6]Laboratory of Theriogenology and Laboratory of Pathology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, [7]Research Resources Center, RIKEN Brain Science Institute.

The scavenger receptor CD163 is widely used as a cell signature of alternatively active "M2" macrophages in mammals. In this study, we generated two monoclonal antibodies, namely ABM-1A9 and ABM-2D6, against the extracellular region of bovine CD163. Conventional Western blotting using the antibodies gave immune reactive bands corresponding to the transmembrane (～150 kDa) and soluble (～120 kDa) forms of bovine CD163 when tested using spleen protein lysate extracted from cattle with mastitis. The minimum limit of detectable concentration of both antibodies was relatively lower (5.0 ng/mL) than that of the anti-human CD163 monoclonal antibody AM-3K (> 1.0 μg/mL), which has been used previously for the detection of bovine CD163. An immunohistochemical study using formalin-fixed paraffin-embedded sections indicated that ABM-1A9 and ABM-2D6 clearly stained some Iba1＋ macrophages in the lymph nodes of cattle with mastitis. Moreover, the CD163-stained macrophages were frequently observed engulfing leukocytes. ELISA using ABM-2D6 distinguished levels of circulating soluble CD163 between healthy cattle (< 16.9 pmol/mL) and cattle with mastitis (> 33.7 pmol/mL). These new monoclonal antibodies

can be used for the diagnosis and evaluation of prognosis of inflammatory diseases in cattle, using immunohistological analysis and blood test applications.

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Center for Stem Cell Biology and Regenerative Medicine

Stem Cell Bank
ステムセルバンク

Associate Professor	Makoto Otsu, M.D., Ph.D.		准教授	博士(医学)	大 津 真
Project Assistant Professor	Chen-Yi Lai, Ph.D.		特任助教	博士(生命科学)	賴 貞 儀

Stem cells represent a valuable cell source in the field of regenerative medicine. Pluripotent stem cells are newly emerging types of stem cells that may be utilized either for the basic research or to cure the diseases. In this laboratory, we have been focusing especially on the utilization of induced pluripotent stem cells as a research platform to elucidate pathophysiology of intractable diseases based on their proper modeling. Our eventual goal is to establish safe and efficacious treatment for the patients suffering from various types of diseases currently with no curative treatment available

1. Dissection of signaling events downstream of the c-Mpl receptor in murine hematopoietic stem cells via motif-engineered chimeric receptors

Koichiro Saka, Chen-Yi Lai, Masanori Nojima, Masahiro Kawahara, Makoto Otsu, Hiromitsu Nakauchi, Teruyuki Nagamune

Hematopoietic stem cells (HSCs) are a valuable resource in transplantation medicine. Cytokines are often used to culture HSCs aiming at better clinical outcomes through enhancement of HSC reconstitution capability. Roles for each signal molecule downstream of receptors in HSCs, however, remain puzzling due to complexity of the cytokine-signaling network. Engineered receptors that are non-responsive to endogenous cytokines represent an attractive tool for dissection of signaling events. We here tested a previously developed chimeric receptor (CR) system in primary murine HSCs, target cells that are indispensable for analysis of stem cell activity.

2. Evaluation of the Utility of Induced Pluripotent Stem Cells as a platform for modeling Wiskott Aldrich Syndrome

HONDA-OZAKI Fumiko, LIN Huan-Ting, OKUMURA Takashi, LAI Chen-Yi, OTSU Makoto

Wiskott Aldrich syndrome (WAS) is an X-linked disease, caused by mutations in the gene encoding the WAS protein (WASp). Thrombocytopenia is the main feature of this disease, often threatening patients to a significant risk of serious hemorrhage. It has been an issue of debate whether defective proplatelet release from megakaryocytes (MKs)(i.e., production) and/or destruction of platelets (PLTs) by the spleen macrophages (i.e., consumption) constitutes the major cause of thrombocytopenia. Due to the limitations inherent to classical experimental models, there have been conflicting results published; precise mechanisms causing thrombocytopenia in WAS therefore remain to be elucidated. To have an appropriate model in our hands and to elucidate mechanism(s) of thrombocytopenia in WAS to improve treatment of WAS patients in future, we here evaluated the utility of iPSCs as an emerging disease model for WAS. WASp-deficient iPSCs produced MKs and PLTs with similar yields as did control cells when differentiated from sorted MK progenitors. Interestingly, however, WASp-deficient iPSCs yielded only fewer MKs and PLTs than did healthy counterparts when differentiated

from unsorted HPCs. In addition, we found that WASp-deficiency variously affected phagocytosis function of iPSC-monocytes in a context specific manner, depending on what targets they would face. These data imply that not just MKs and PLTs but also other lineage cells such as phagocytic cells may play a role for WAS-thrombocytopenia. We are now trying to establish an in vitro co-culture system of iPSCs-derived monocytes and -platelets as a new experimental platform to clarify accurate roles of each cellular compartment.

3. The functional analysis of dendritic cells developed from T-iPS cells from a single CD4+ T cell of Sjögren's syndrome

Mana Iizuka-Koga, Hiromitsu Asashima, Miki Ando, Chen-Yi Lai, Shinji Mochizuki, Mahito Nakanishi, Toshinobu Nishimura, Hiroto Tsuboi, Tomoya Hirota, Hiroyuki Takahashi, Isao Matsumoto, Makoto Otsu, Takayuki Sumida

Though it is important to clarify the pathogenic and/or regulatory functions of the T cells in human samples, their examination is frequently restricted in opportunity because the acquisition of sufficient quantity of dendritic cells (DCs), used as antigen presenting cells, might not be available, especially in autoimmune diseases. In this study, we developed the generation of mature DCs from induced pluripotent stem cells derived from T cells (T-iPSCs). We reprogramed only a single CD4+ T cell of a patient with Sjögren's syndrome (SS) into iPS cells, which were differentiated into DCs (TiPS-DCs) via TiPS-Sacs. Moreover, we examined their characterizations and functions. Under the microscope, the mature TiPS-DCs were dendritic cells-like morphology. The expression of CD11b, CD11c, HLA-ABC and CD40 was detected, and the CD80, CD86 and HLA-DR was also increased. The mature TiPS-DCs were able to produce TNF-α and IL-6 through stimulation. As compared with monocytes-derived DCs, TiPS-DCs was equal to the ability of antigen processing, and also the induction of robust proliferative response of allogeneic T cells in the dependent manner of cell numbers.

In conclusion, we succeeded to obtain an adequate amount of functional DCs from a single CD4+ T cell by way of iPS cells. This method should shed light on the characterization of pathogenic T cells for not only SS but also other diseases to elucidate the function of autoreactive T cells. The method shown in the study will enable us to identify autoreactive T cells in autoimmune diseases along with elucidate the function and pathogenicity of them.

4. Mutant iPS cells derived from patients with RALD show significance of KRAS for self-renewal and differentiation propensity

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KRAS is widely known as a proto-oncogene and has been reported to play essential roles in stemness maintenance in some types of stem cells, including cancer stem cells. However, the roles of KRAS in pluripotent stem cells (PSCs) are largely unknown. Recently, somatic gain-of-function mutations of KRAS or NRAS in hematopoietic stem cells have been reported to cause RAS-associated autoimmune lymphoproliferative syndrome-like disease (RALD). Here, we investigated the roles of KRAS on stemness maintenance in the context of human PSCs using isogenic KRAS mutant (G13C/WT) and wild-type (WT/WT) induced PSCs (iPSCs), generated from the same RALD patients with the somatic KRAS mutation. Using the isogenic iPSC lines from two patients, we revealed that G13C/WT iPSCs displayed self-renewal and differentiation characteristics distinct from those of WT/WT iPSCs: expression of stemness markers including POU5F1 and NANOG, which is indicative of an undifferentiated state, was maintained at high levels in G13C/WT iPSCs under bFGF depletion; neuronal differentiation was clearly blunted from G13C/WT iPSCs. In addition, we generated wild-type (WTed/WT) and heterozygous knockout (Δed/WT) iPSCs from the same G13C/WT clone using gene-editing techniques. As expected, the G13C/WT-specific phenotypes were normalized in WTed/WT iPSCs. Interestingly, Δed/WT iPSCs showed lower potential to maintain undifferentiation status under bFGF depletion, with a higher tendency to differentiate into neuronal lineage than WTed/WT iPSCs. Biochemical analysis indicated hyper-activation of KRAS and subsequent increased phosphorylation levels of ERK in G13C/WT iPSCs. Pharmacological studies using specific kinase inhibitors demonstrated that the features compatible with enhanced stemness maintenance were canceled in mutant cells by pan-RAF and MEK inhibitors, but not by PI3K inhibitors. In addition, neuronal differentiation was improved in G13C/WT iPSCs with the MEK inhibitor treatment. These observations suggested that the KRAS-ERK pathway plays more critical roles than the KRAS-PI3K pathway in G13C/WT iPSCs. Collectively, the analyses on the isogenic and genome-edited iPSCs from the RALD patients revealed the crucial roles of the KRAS-ERK signaling on the stemness maintenance, having a strong impact on self-renewal and differentiation propensity in human iPSCs.

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