Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Therapy
幹細胞治療分野

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Recent great progress in stem cell biology has brought about increase in the prospect for application of stem cell-based therapy. Especially the discovery of iPSCs, a great step forward in stem-cell research, holds out the promise of development of novel therapeutic strategies by generating iPSCs from patients. The goal of this laboratory is to provide new insights into stem cell biology as well as approaches to novel therapeutic intervention for various intractable diseases.

1. Investigation of bi-potent differentiation of hepatoblasts using inducible diphtheria toxin receptor-transgenic mice

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Hepatic progenitor cells, called hepatoblasts, are highly proliferative and exhibit bi-potential differentiation into hepatocytes and cholangiocytes in the fetal liver. Thus, they are the ideal source for transplantation therapy. Although several studies have been performed in vitro, the molecular mechanisms regulating hepatoblast differentiation in vivo following transplantation remain poorly understood. The aim of this study was to investigate an in vivo model to analyze hepatoblast bi-potency and proliferative ability.

Hepatic transplantation model using Cre-inducible diphtheria toxin receptor transgenic mice (iDTR), and albafpCre mice expressing Cre under the control of albumin and α-fetoprotein regulatory elements were established. Fresh hepatoblasts were transplanted into diphtheria toxin (DT)-injected iDTRalbafpCre mice and analyzed their differentiation and proliferation abilities by immunostaining and genes expression profiles.

Fresh hepatoblasts transplanted into DT-injected iDTRalbafpCre mice engrafted and differentiated into both hepatocytes and cholangiocytes. Additionally, the number of engrafted hepatoblast-derived hepatocytes increased following partial hepatectomy and serial DT injections. Expression levels of hepatic functional genes in transplanted hepatoblast-derived hepatocytes were similar to that of normal hepatocytes.

In our iDTRalbafpCre transplantation model, fresh hepatoblasts could differentiate into hepatocytes and cholangiocytes. In addition, these donor cells were induced to proliferate by the following liver injury stimulation. This result suggests that

this model is valuable for investigating hepatoblast differentiation pathways in vivo.

2. A Safeguard System for Induced Pluripotent Stem Cell-Derived Rejuvenated T Cell Therapy

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The discovery of induced pluripotent stem cells (iPSCs) has created promising new avenues for therapies in regenerative medicine. However, the tumorigenic potential of undifferentiated iPSCs is a major safety concern for clinical translation. To address this issue, we demonstrated the efficacy of suicide gene therapy by introducing inducible caspase-9 (iC9) into iPSCs. Activation of iC9 with a specific chemical inducer of dimerization (CID) initiates a caspase cascade that eliminates iPSCs and tumors originated from iPSCs. We introduced this iC9/CID safeguard system into a previously reported iPSC-derived, rejuvenated cytotoxic T lymphocyte (rejCTL) therapy model and confirmed that we can generate rejCTLs from iPSCs expressing high levels of iC9 without disturbing antigen-specific killing activity. iC9-expressing rejCTLs exert antitumor effects in vivo. The system efficiently and safely induces apoptosis in these rejCTLs. These results unite to suggest that the iC9/CID safeguard system is a promising tool for future iPSC-mediated approaches to clinical therapy.

3. Interspecific in vitro assay for the chimera-forming ability of human pluripotent stem cells. Development

Masaki H[1], Kato-Itoh M[1], Umino A[1], Sato H[1], Hamanaka S[1], Kobayashi T[1], Yamaguchi T[1], Nishimura K[2], Ohtaka M[2], Nakanishi M[2], Nakauchi H[3]: [1]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, [2]Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), [3]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine

Functional assay limitations are an emerging issue in characterizing human pluripotent stem cells (PSCs). With rodent PSCs, chimera formation using pre-implantation embryos is the gold-standard assay of pluripotency (competence of progeny to differentiate into all three germ layers). In human PSCs (hPSCs), however, this can only be monitored via teratoma formation or in vitro differentiation, as ethical concerns preclude generation of human-human or human-animal chimeras. To circumvent this issue, we developed a functional assay utilizing interspecific blastocyst injection and in vitro culture (interspecies in vitro chimera assay) that enables the development and observation of embryos up to headfold stage. The assay uses mouse pre-implantation embryos and rat, monkey and human PSCs to create interspecies chimeras cultured in vitro to the early egg-cylinder stage. Intra- and interspecific chimera assays with rodent PSC lines were performed to confirm the consistency of results in vitro and in vivo. The behavior of chimeras developed in vitro appeared to recapitulate that of chimeras developed in vivo; that is, PSC-derived cells survived and were integrated into the epiblast of egg-cylinder-stage embryos. This indicates that the interspecific in vitro chimera assay is useful in evaluating the chimera-forming ability of rodent PSCs. However, when human induced PSCs (both conventional and naïve-like types) were injected into mouse embryos and cultured, some human cells survived but were segregated; unlike epiblast-stage rodent PSCs, they never integrated into the epiblast of egg-cylinder-stage embryos. These data suggest that the mouse-

human interspecies in vitro chimera assay does not accurately reflect the early developmental potential/process of hPSCs. The use of evolutionarily more closely related species as host embryos might be necessary to evaluate the developmental potency of hPSCs.

4. Successful Reprogramming of Epiblast Stem Cells by Blocking Nuclear Localization of β-Catenin. Stem Cell Reports.

Murayama H[1], Masaki H[1], Sato H[1], Hayama T[1], Yamaguchi T[1], Nakauchi H[2]: [1]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, [2]Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine

Epiblast stem cells (EpiSCs) in mice and rats are primed pluripotent stem cells (PSCs). They barely contribute to chimeric embryos when injected into blastocysts. Reprogramming of EpiSCs to embryonic stem cell (ESC)-like cells (rESCs) may occur in response to LIF-STAT3 signaling; however, low reprogramming efficiency hampers potential use of rESCs in generating chimeras. Here, we describe dramatic improvement of conversion efficiency from primed to naive-like PSCs through upregulation of E-cadherin in the presence of the cytokine LIF. Analysis revealed that blocking nuclear localization of β-CATENIN with small-molecule inhibitors significantly enhances reprogramming efficiency of mouse EpiSCs. Although activation of Wnt/β-catenin signals has been thought desirable for maintenance of naive PSCs, this study provides the evidence that inhibition of nuclear translocation of β-CATENIN enhances conversion of mouse EpiSCs to naive-like PSCs (rESCs). This affords better understanding of gene regulatory circuits underlying pluripotency and reprogramming of PSCs.

5. Effective treatment against severe graft-versus-host disease with allele-specific anti-HLA monoclonal antibody in a humanized mouse model.

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Graft-versus-host disease (GVHD), mediated by donor-derived alloreactive T cells, is a major cause of nonrelapse mortality in allogeneic hematopoietic stem cell transplantation. Its therapy is not well-defined. We established allele-specific anti-human leukocyte antigen (HLA) monoclonal antibodies (ASHmAbs) that specifically target HLA molecules, with steady death of target-expressing cells. One such ASHmAb, against HLA-A*02:01 (A2-kASHmAb), was examined in a xenogeneic GVHD mouse model. To induce fatal GVHD, non-irradiated NOD/Shi-scid/IL-2Rγ(null) mice were injected with healthy donor human peripheral blood mononuclear cells, some expressing HLA-A*02:01, some not. Administration of A2-kASHmAb promoted the survival of mice injected with HLA-A*02:01-expressing peripheral blood mononuclear cells ($p < 0.0001$) and, in humanized NOD/Shi-scid/IL-2Rγ (null) mice, immediately cleared HLA-A*02:01-expressing human blood cells from mouse peripheral blood. Human peripheral blood mononuclear cells were again detectable in mouse blood 2 to 4 weeks after A2-kASHmAb administration, suggesting that kASHmAb may be safely administered to GVHD patients without permanently ablating the graft. This approach, different from those in existing GVHD pharmacotherapy, may open a new door for treatment of GVHD in HLA-mismatched allogeneic hematopoietic stem cell transplantation.

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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.

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Stem cells represent a valuable cell source in the field of regenerative medicine. Hematopoietic stem cells provide a good example of such usefulness of stem cell research, showing many successful cases in both hematopoietic cell transplantation and gene therapy. Pluripotent stem cells have become another possibility of cell sources in regenerative medicine that may be utilized either for the basic research or to cure the diseases. Our eventual goal is to establish safe and efficacious treatment for the patients suffering from various types of intractable diseases with no curative treatment available

1. Novel method for efficient production of multipotential hematopoietic progenitors from human pluripotent stem cells

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ESC are pluripotent cells derived from the inner cell mass of preimplantation embryos, and iPSC are induced from somatic cells by nuclear reprogramming. Since both have the ability to be maintained in culture indefinitely as undifferentiated cells, yet they are capable of forming more differentiated cell types, they are expected as a novel source of human transplantable cells for the regenerative medicine. We then planed to produce hematopoietic stem cells (HSC) for therapeutic HSC transplantation and functional blood cells for transfusion medicine from these human pluripotent stem cells. In result, we developed a novel method for the efficient production of hematopoietic progenitor cells (HPC) from hESC and hiPSC by co-culture with AGMS-3 stromal cells, which originate from murine aorta-gonad-mesonephros (AGM) region at 11 to 12 dpc. In the co-culture, various hematopoietic progenitors were generated, and this hematopoietic activity was concentrated in cobblestone-like (CS) cells within differentiated human ESC or iPSC colonies. A fraction of CS cells expressed CD34 and retained a potential for endothelial cells. They also contained HPC, especially erythroid and multipotential HPC at high frequency. The multipotential HPC abundant among the CS cells produced all types of mature blood cells, including adult type βglobin-expressing erythrocytes and tryptase and chymase-double positive mast cells (MC). They showed neither immature properties of PSC nor potentials to differentiate into endoderm and ectoderm at a clonal level. The developed co-culture system of hPSC can provide a novel source for hematopoietic and blood cells applicable to cellular therapies and drug screenings.

2. Derivation of blood cells from human pluripotent stem cells in culture without animal serum or cells

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It is inevitable to establish an *in vitro* culture method for the induction of hPSC, such as hESC or hiPSC, to differentiate into mature blood cells without animal serum and cells. To achieve this, we first induced hPSC to differentiate into mesenchymal stem cells (MSC). When human ES or iPS cells cultured on murine embryonic fibroblast (MEF) feeder cells were recultured on gelatin-coated culture dishes with platelet lysate (PL)-containing media in the absence of MEF feeder cells. Cells were passaged several times with PL containing media, and then MSC were induced after 6 to 8 weeks. The MSC were spindle-like shaped, revealed a phenotype of CD45−, CD34−, CD14−, CD105+, CD166+, CD31−, and SSEA-4−, and had the ability to differentiate into mesenchymal tissues such as bone, cartilage and fat in vitro. Murine MEF and undifferentiated hPSC were undetectable in the hPSC-derived MSC by reverse transcription polymerase chain reaction analysis. We then cocultured hPSC with MSC derived from hPSC themselves under serum-free condition. Two weeks later, a number of HPC appeared in the coculture. These HPC were cultured in hematopoietic colony assay using human serum. In result, hPSC-derived HPC produced various hematopoietic colonies, such as myeloid, erythroid and multilineage colonies, including all types of blood cells. The novel culture method must be useful for the clinical application of hPSC-derived blood cells.

3. Recapitulation of pathophysiological features of Wiskott Aldrich Syndrome using induced pluripotent stem cells (iPSCs)

Mozhgan Khalaj Amirhosseini[6], Haruna Takagi[6], Chieko Konishi[6], Huang-Ting Lin[6], Takashi Ishida[6], Chen-Yi Lai[6], Tomohiro Morio[7], Kohsuke Imai[7], Hiromitsu Nakauchi[4], Makoto Otsu: [6]Division of Stem Cell Bank, Center for Stem Cell Therapy and Regenerative Medicine, [7]Department of Pediatrics Perinatal and Maternal Medicine, Tokyo Medical and Dental University

Wiskott Aldrich syndrome (WAS) is an X-linked disease, which is caused by mutations in the gene encoding the WAS protein (WASp). As thrombocytopenia is the most typical feature of this disease, often exposing patients to a significant risk of life-threatening hemorrhage, it has been the main subject of study for many researchers. Because of limitations in disease modeling, precise mechanisms of the platelet abnormality remain to be elucidated. Here we established induced pluripotent stem cell (iPSC) lines from two XLT and one WAS patients as disease models to address the issues. We first confirmed that these disease-specific iPSCs retained gene mutations characteristic to each patient. Using our differentiation culture system, we demonstrated that numbers of both megakaryocyte (MK)s and platelets obtainable from both XLT- and WAS-iPSCs were significantly smaller than those from healthy iPSCs. Detailed analysis revealed that the observed defects were mainly due to insufficient production of proplatelet-bearing cells, but not to impaired platelet production per MK. Lentiviral-mediated gene transfer led to appearance of WASp expression in patient iPSC-derived MKs. The expression of WASp, however, did not reach the normal level that was seen in control-iPSC-MKs; yields of platelets showed some increase after gene transfer, but only marginally. Although further investigation is necessary, these results indicate the utility of iPSC-based disease modeling for WAS. We are now in the process of addressing how critically expression levels of WASp will affect the efficacy in platelet number recovery after gene transfer.

4. Demonstration of safe and efficacious gene therapy using X-CGD iPSCs.

Huang-Ting Lin[6], Haruna Takagi[6], Mozhgan Kharaj[6], Chieko Konishi[6], Takashi Ishida[6], Chen-Yi Lai[6], Makoto Otsu, Hiromitsu Nakauchi[4]

X-linked chronic granulomatous disease (XCGD) is caused by gp91phox deficiency. This compromises neutrophil (NEU) killing of phagocytosed pathogens due to impaired production of reactive oxygen species (ROS). In previous XCGD gene therapy clinical trials, the sustained persistence of gene marked cells could not be achieved without adversely triggering insertional mutagenesis. Additionally cellular recovery (gp91phox and ROS) had been incomplete on a per cell basis in comparison with healthy controls. This led to the hypothesis that the expression of ectopic gp91phox in developing NEUs could impede further differentiation into mature NEUs. To investigate this theory, a modeling system was established by generating patient autologous XCGD-iPSCs and its differentiation into NEUs. In this culture system, the hierarchical transition of NEU differentiation could be demonstrated from developing ($CD64^{dull}CD15^{dull}$) to mature ($CD64^{high}CD15^{high}$). Alpharetroviral vectors were used to transduce XCGD-iPSCs with the expression of codon optimized gp91phox cDNA driven by the ubiquitous EF1α short promoter. In healthy iPSC-derived NEUs, gp91phox expression and ROS production could only be detected in the mature fraction. In NEUs derived from transduced XCGD-iPSCs, functional recovery in the mature fraction was incomplete. Ectopic gp91phox expression could be detected in the developing fraction through in-

tracellular staining but not in healthy control cells. Most importantly, cell death was most prominent in developing NEUs ectopically expressing gp91phox. Mechanistic studies are under way to investigate the role of non-physiological ROS production in inducing endoplasmic reticulum (ER) stress resulting in cell apoptosis. Therefore, affording cellular protection from the detrimental effects of non-physiological ROS production may improve XCGD clinical outcomes.

5. A New Strategy To Overcome The Cell Dose Barrier To Umbilical Cord Blood Transplants

Takashi Ishida[6], Masaaki Higashihara[8], Hiromitsu Nakauchi[4], Makoto Otsu: [8]Department of Hematology, Kitasato University School of Medicine

Umbilical cord blood (UCB) serves as a suitable donor source in hematopoietic stem cell transplantation (HSCT). However, UCB has the major drawback that is delayed engraftment due to its low graft cell numbers, which often limits its use in HSCT. To overcome this cell dose barrier, double units UCB transplantation (UCBT) has been attempted, but the time to engraftment is still relatively delayed. Based on the report by Japan Red Cross Society, the majority of UCB units remain unused clinically because of their insufficient graft cell doses. Overall, these facts prompted us to seek for a new strategy to improve UCBT by using multiple (more than three) units. We here provide a proof of feasibility of such an approach using mouse transplantation models.

To mimic a clinical setting of UCBT, we first established an insufficient cell dose model by using mouse BM KSL (c-Kit$^+$, Sca-1$^+$, lineage-marker-negative) cells. In this model, C57BL/6 (B6-Ly5.2, H2^b) mice were the recipients, and congenic B6-Ly5.1 mice (H2^b) were the primary donors of cell grafts. The recipient mice were lethally irradiated, and thus could not survive with only the "insufficient cell dose" of B6-Ly5.1 graft. The outcomes in HSCT were tested by the addition of mixed allogeneic KSL cells (multi-allo HSPCs) in comparison with the addition of B6-Ly5.1 KSL cells (congenic HSPCs) with the equivalent doses. The effects of multi-allo HSPC transplants were evaluated by recipients' survival rate and complete blood counts over time. Detailed donor cell contribution in peripheral blood and BM was also determined by flow cytometry analysis.

Interestingly, addition of multi-allo HSPCs rescued otherwise lethal recipients as effectively as congenic-HSPCs with the equivalent acceleration of hematopoietic recovery. Chimerism analysis, however, revealed that this "KSL cells alone" transplantation led to long-term existence of multi-donor hematopoiesis, which was not ideal for a clinical setting. We then replaced B6-Ly5.1 KSL grafts with the whole BM (WBM) grafts. Titration experiments determined 50,000 WBM cells as a single unit, mimicking an "insufficient dose" of unmanipulated UCB. Addition of multi-allo HSPCs in this model also showed complete protection of recipients from lethality and enhanced early hematopoietic recovery. Remarkably, dominant B6-Ly5.1 chimerism was established and maintained in this modified model. Experiments using subfractionation of the B6-Ly5.1 grafts demonstrated that small numbers of T cells were responsible to single-donor chimerism formation possibly through graft versus graft reactions. Finally, to maximize transient early hematopoietic reconstitution by multi-allo HSPCs (we call this "bridging effect"), we tested whether cultured hematopoietic stem cells (HSC, defined as CD34$^{negatvie/low}$ KSL cells) worked better than uncultured KSL cells. Using our defined protocol compatible with stem cell amplification, we demonstrated that a mixture of cultured allo-HSCs exhibited the bridging effect even superior to that of an uncultured KSL cell mixture.

6. Stage-specific roles for Cxcr4 signaling in murine hematopoietic stem/progenitor cells in the process of bone marrow repopulation

Chen-Yi Lai[6], Satoshi Yamazaki[4], Yasuaki Iimura[9], Masafumi Onodera[10], Shigeru Kakuta[11], Yoichiro Iwakura[11], Hiromitsu Nakauchi[4], Makoto Otsu: [9]Kushiro City General Hospital, [10]Department of Genetics, National Research Institute of Child Health and Development, [11]Laboratory of Molecular Pathogenesis, Center for Experimental Medicine and Systems,

Hematopoietic cell transplantation has proven beneficial for various diseases, but low-level engraftment would be problematic leading to patient mortality, requiring elucidation of the molecular determinant for successful engraftment. It remains unclear how hematopoietic stem/progenitor cells (HSPCs) home to the bone marrow (BM) microenvironment, initiate hematopoietic reconstitution, and maintain life-long hematopoiesis. By monitoring the *in vivo* kinetics of transplanted donor cells, we demonstrated that modification of Cxcr4 signaling in murine HSPCs did not affect homing/lodging events, but led to alteration in subsequent BM repopulation kinetics, with observations confirmed by both gain- and loss-of-function approaches. With the use of C-terminal truncated Cxcr4 as a gain-of-function effector, we showed that signal augmentation through Cxcr4 led to favorable *in vivo* repopulation of primitive cell populations in BM. These Cxcr4-augmented HSPCs exhibited *in vitro* enhanced seeding efficiencies in stromal cell co-cultures and altered ligand-mediated phosphorylation

kinetics of Extracellular signal-regulated kinases. Sustained signal enhancement, however, even with wild-type Cxcr4 overexpression resulted in poor peripheral blood reconstitution due to blunted release of donor hematopoietic cells from BM. We thus conclude that timely regulation of Cxcr4/CXCR4 signaling will provide donor HSPCs with enhanced repopulation potential, with preserving their ability to release progeny into peripheral blood for improved transplantation outcomes.

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Center for Stem Cell Biology and Regenerative Medicine

Laboratory of Stem Cell Regulation
幹細胞制御領域

Visiting Associate Professor Koichi Hattori, M.D, Ph.D. | 客員准教授 医学博士 服 部 浩 一

The goal of our laboratory is to identify novel therapeutic targets for diseases like cancer or inflammatory diseases by studying the role inflammatory and adult stem cells. Persistent inflammation is associated with diseases, including cancer, atherosclerosis, arthritis and autoimmune diseases. Recently, we identified plasmin as a novel therapeutic target for the treatment of inflammatory diseases like inflammatory bowel disease, sepsis and chronic graft-versus host disease after bone marrow transplantation, and during cancer.

1. Inhibition of plasmin attenuates murine acute graft-versus-host disease mortality by suppressing the matrix metalloproteinase-9-dependent inflammatory cytokine storm and effector cell trafficking

Aki Sato[1], Chiemi Nishida[2], Kaori Sato-Kusubata[2], Makoto Ishihara[1], Yoshihiko Tashiro[1], Ismael Gritli[2], Hiroshi Shimazu[1], Shinya Munakata[1], Hideo Yagita[3], Ko Okumura[3], Yuko Tsuda[4], Yoshio Okada[4], Arinobu Tojo[5], Hiromitsu Nakauchi[6], Satoshi Takahashi[5], Beate Heissig[2]#, Koichi Hattori[1,7]#: [1]Department of Stem Cell Regulation, The Institute of Medical Science, The University of Tokyo, [2]Department of Stem Cell Dynamics, The Institute of Medical Science, The University of Tokyo, [3]Atopy (Allergy) Research Center, Juntendo University School of Medicine, Tokyo, [4]Faculty of Pharmaceutical Sciences, Kobe Gakuin University, [5]Department of Hematology and Oncology, The Institute of Medical Science, The University of Tokyo, [6]Department of Stem Cell Therapy, The Institute of Medical Science, The University of Tokyo, [7]Center for Genome and Regenerative Medicine, Juntendo University School of Medicine, Tokyo.

The systemic inflammatory response observed during acute graft-versus-host disease (aGVHD) is driven by proinflammatory cytokines, a 'cytokine storm'. The function of plasmin in regulating the inflammatory response is not fully understood, and its role in the development of aGVHD remains unresolved. Here we show that plasmin is activated during the early phase of aGVHD in mice, and its activation correlated with aGVHD severity in humans. Pharmacological plasmin inhibition protected against aGVHD-associated lethality in mice. Mechanistically, plasmin inhibition impaired the infiltration of inflammatory cells, the release of membrane-associated proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and Fas-ligand directly, or indirectly via matrix metalloproteinases (MMPs) and alters monocyte chemoattractant protein-1 (MCP-1) signaling. We propose that plasmin and potentially MMP-9 inhibition offers a novel therapeutic strategy to control the deadly cytokine storm in patients with aGVHD, thereby preventing tissue destruction.

2. Inhibition of Plasmin Protects Against Colitis in Mice by Suppressing Matrix Metalloproteinase 9-mediated Cytokine Release From Myeloid Cells

Shinya Munakata[1], Yoshihiko Tashiro[1], Chiemi Nishida[2], Aki Sato[1], Hiromitsu Komiyama[3], Hiroshi Shimazu[1], Douaa Dhahri[2], Yousef Salama[2], Salita

Eiamboonsert[2], Kazuyoshi Takeda[4], Hideo Yagita[4], Yoko Tsuda[5], Yoshio Okada[5], Hiromitsu Nakauchi[6], Kazuhiro Sakamoto[3], Beate Heissig[2], Koichi Hattori[1,7]: [1]Division of Stem Cell Regulation, The Institute of Medical Science, The University of Tokyo, [2]Division of Stem Cell Dynamics, The Institute of Medical Science, The University of Tokyo, [3]Department of Coloproctological Surgery, Juntendo University Faculty of Medicine, Tokyo, [4]Department of Immunology, Juntendo University Graduate School of Medicine, Tokyo, [5]Faculty of Pharmaceutical Sciences, Kobe Gakuin University, [6]Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, [7]Center for Genome and Regenerative Medicine, Juntendo University School of Medicine, Tokyo

Activated proteases such as plasmin and matrix metalloproteinases (MMPs) are activated in intestinal tissues of patients with active inflammatory bowel diseases. We investigated the effect of plasmin on progression of acute colitis. Colitis was induced in $Mmp9^{-/-}$, $Plg^{-/-}$, and C57BL/6 (control) mice by administration of dextran sulfate sodium, trinitrobenzene sulfonic acid, or CD40 antibody. Plasmin was inhibited in control mice by intraperitoneal injection of YO-2, which blocks its active site. Mucosal and blood samples were collected and analyzed by reverse transcription polymerase chain reaction and immunohistochemical analyses, as well as for mucosal inflammation and levels of cytokines and chemokines. We showed that circulating levels of plasmin were increased in mice with colitis, compared with controls. Colitis did not develop in control mice injected with YO-2 or in $Plg^{-/-}$ mice. Colons from these mice had reduced infiltration of Gr1+ neutrophils and F4/80+ macrophages, and reduced levels of inflammatory cytokines and chemokines. Colonic inflammation and colitis induction required activation of endogenous MMP9. Following colitis induction, mice given YO-2, $Plg^{-/-}$ mice, and $Mmp9^{-/-}$ mice had reduced serum levels of tumor necrosis factor and CXCL5, compared to control mice. In summary, in mice, plasmin induces a feedback mechanism in which activation of the fibrinolytic system promotes development of colitis, via activation of MMP9 or proteolytic enzymes. The proteolytic environment stimulates influx of myeloid cells into the colonic epithelium and production of tumor necrosis factor and CXCL5. In turn, myeloid CD11b+ cells release the urokinase plasminogen activator, which accelerates plasmin production. Disruption of the plasmin-induced chronic inflammatory circuit might therefore be a strategy for treatment of colitis.

3. Role of mesenchymal stem cell-derived fibrinolytic factor in tissue regeneration and cancer progression

Beate Heissig[1], Douaa Dhahri[1], Salita Eiamboonsert[1], Yousef Salama[1], Hiroshi Shimazu[2], Shinya Munakata[2], Koichi Hattori[2,3]: [1]Division of Stem Cell Dynamics, The Institute of Medical Science, The University of Tokyo, Tokyo, [2]Laboratory of Stem Cell Regulation, The Institute of Medical Science, The University of Tokyo, [3]Center for Genome and Regenerative Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.

Tissue regeneration during wound healing or cancer growth and progression depends on the establishment of a cellular microenvironment. Mesenchymal stem cells (MSC) are part of this cellular microenvironment, where they functionally modulate cell homing, angiogenesis, and immune modulation. MSC recruitment involves detachment of these cells from their niche, and finally MSC migration into their preferred niches; the wounded area, the tumor bed, and the BM, just to name a few. During this recruitment phase, focal proteolysis disrupts the extracellular matrix (ECM) architecture, breaks cell-matrix interactions with receptors, and integrins, and causes the release of bioactive fragments from ECM molecules. MSC produce a broad array of proteases, promoting remodeling of the surrounding ECM through proteolytic mechanisms. The fibrinolytic system, with its main player plasmin, plays a crucial role in cell migration, growth factor bioavailability, and the regulation of other protease systems during inflammation, tissue regeneration, and cancer. Key components of the fibrinolytic cascade, including the urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1), are expressed in MSC. This review will introduce general functional properties of the fibrinolytic system, which go beyond its known function of fibrin clot dissolution (fibrinolysis). We will focus on the role of the fibrinolytic system for MSC biology, summarizing our current understanding of the role of the fibrinolytic system for MSC recruitment and the functional consequences for tissue regeneration and cancer. Aspects of MSC origin, maintenance, and the mechanisms by which these cells contribute to altered protease activity in the microenvironment under normal and pathological conditions will also be discussed.

4. Cancer therapy targeting the fibrinolytic system

Beate Heissig[1], Salita Eiamboonsert[1], Yousef Salama[1], Horoshi Shimazu[1], Douaa Dhahri[1], Shi-

nya Munakata[2], Yoshihiko Tashiro[2], Koichi Hattori[2]: [1]Division of Stem Cell Dynamics, The Institute of Medical Science, The University of Tokyo, [2]Laboratory of Stem Cell Regulation, The Institute of Medical Science, The University of Tokyo; Center for Genome and Regenerative Medicine, Juntendo University School of Medicine, Tokyo.

The tumor microenvironment is recognized as a key factor in the multiple stages of cancer progression, mediating local resistance, immune-escape and metastasis. Cancer growth and progression require remodeling of the tumor stromal microenvironment, such as the development of tumor-associated blood vessels, recruitment of bone marrow-derived cells and cytokine processing. Extracellular matrix breakdown achieved by proteases like the fibrinolytic factor plasmin and matrix metalloproteases is necessary for cell migration crucial for cancer invasion and metastasis. Key components of the fibrinolytic system are expressed in cells of the tumor microenvironment. Plasmin can control growth factor bioavailability, or the regulation of other proteases leading to angiogenesis, and inflammation. In this review, we will focus on the role of the fibrinolytic system in the tumor microenvironment summarizing our current understanding of the role of the fibrinolytic factors for the modulation of the local chemokine/cytokine milieu, resulting in myeloid cell recruitment, which can promote neoangiogenesis.

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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

Associate Professor Makoto Otsu
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Our major goal is to cure patients suffering from life-threatening diseases by the treatment with processing of various stem cells. Currently our efforts are directed toward the establishment of novel therapies using human pluripotent stem cells (hPSC), such as embryonic stem cells and induced pluripotent stem cells (ESC and iPSC, respectively), and the analysis of pathogenesis of a variety of disorders based on disease-specific iPS cells.

1. Novel method for efficient production of multipotential hematopoietic progenitors from human pluripotent stem cells

Shinji Mochizuki, Yasuhiro Ebihara[1], Feng Ma[2], Emiko Matsuzaka, Yuji Zaike[3], Hiromitsu Nakauchi[4], Kohichiro Tsuji[5], Makoto Otsu: [1]Department of Pediatric Hematology-Oncology, [2]Institute of Blood Transfusion Chinese Academy of Medical Sciences, Beijing Union Medical College, [3]Department of Laboratory Medicine, Research Hospital, [4]Division of Stem Cell Therapy, Center for Stem Cell Therapy and Regenerative Medicine, [5]Department of Pediatrics, National Hospital Organization Shinshu Ueda Medical Center

ESC are pluripotent cells derived from the inner cell mass of preimplantation embryos, and iPSC are induced from somatic cells by nuclear reprogramming. Since both have the ability to be maintained in culture indefinitely as undifferentiated cells, yet they are capable of forming more differentiated cell types, they are expected as a novel source of human transplantable cells for the regenerative medicine. We then planed to produce hematopoietic stem cells (HSC) for therapeutic HSC transplantation and functional blood cells for transfusion medicine from these human pluripotent stem cells. In result, we developed a novel method for the efficient production of hematopoietic progenitor cells (HPC) from hESC and hiPSC by co-culture with AGMS-3 stromal cells, which originate from murine aorta-gonad-mesonephros (AGM) region at 11 to 12 dpc. In the co-culture, various hematopoietic progenitors were generated, and this hematopoietic activity was concentrated in cobblestone-like (CS) cells within differentiated human ESC or iPSC colonies. A fraction of CS cells expressed CD34 and retained a potential for endothelial cells. They also contained HPC, especially erythroid and multipotential HPC at high frequency. The multipotential HPC abundant among the CS cells produced all types of mature blood cells, including adult type β globin-expressing erythrocytes and tryptase and chymase-double positive mast cells (MC). They showed neither immature properties of PSC nor potentials to differentiate into endoderm and ectoderm at a clonal level. The developed co-culture system of hPSC can provide a novel source for hematopoietic and blood cells applicable to cellular therapies and drug screenings.

2. Derivation of blood cells from human pluripotent stem cells in culture without animal serum or cells

Yasuhiro Ebihara[1], Shinji Mochizuki, Emiko Matsuzaka, Yuji Zaike[3], Hiromitsu Nakauchi[4], Kohichiro Tsuji[5], Makoto Otsu

It is inevitable to establish an *in vitro* culture method for the induction of hPSC, such as hESC or hiPSC, to differentiate into mature blood cells without animal serum and cells. To achieve this, we first induced hPSC to differentiate into mesenchymal stem cells (MSC). When human ES or iPS cells cultured on murine embryonic fibroblast (MEF) feeder cells were recultured on gelatin-coated culture dishes with platelet lysate (PL)-containing media in the absence of MEF feeder cells. Cells were passaged several times with PL containing media, and then MSC were induced after 6 to 8 weeks. The MSC were spindle-like shaped, revealed a phenotype of CD45−, CD34−, CD14−, CD105+, CD166+, CD31−, and SSEA-4−, and had the ability to differentiate into mesenchymal tissues such as bone, cartilage and fat in vitro. Murine MEF and undifferentiated hPSC were undetectable in the hPSC-derived MSC by reverse transcription polymerase chain reaction analysis. We then cocultured hPSC with MSC derived from hPSC themselves under serum-free condition. Two weeks later, a number of HPC appeared in the coculture. These HPC were cultured in hematopoietic colony assay using human serum. In result, hPSC-derived HPC produced various hematopoietic colonies, such as myeloid, erythroid and multilineage colonies, including all types of blood cells. The novel culture method must be useful for the clinical application of hPSC-derived blood cells.

3. Recapitulation of pathophysiological features of Wiskott Aldrich Syndrome using induced pluripotent stem cells (iPSCs)

Mozhgan Khalaj Amirhosseini[6], Haruna Takagi[6], Chieko Konishi[6], Huang-Ting Lin[6], Takashi Ishida[6], Chen-Yi Lai[6], Tomohiro Morio[7], Kohsuke Imai[7], Hiromitsu Nakauchi[4], Makoto Otsu: [6]Division of Stem Cell Bank, Center for Stem Cell Therapy and Regenerative Medicine, [7]Department of Pediatrics Perinatal and Maternal Medicine, Tokyo Medical and Dental University

Wiskott Aldrich syndrome (WAS) is an X-linked disease, which is caused by mutations in the gene encoding the WAS protein (WASp). As thrombocytopenia is the most typical feature of this disease, often exposing patients to a significant risk of life-threatening hemorrhage, it has been the main subject of study for many researchers. Because of limitations in disease modeling, precise mechanisms of the platelet abnormality remain to be elucidated. Here we established induced pluripotent stem cell (iPSC) lines from two XLT and one WAS patients as disease models to address the issues. We first confirmed that these disease-specific iPSCs retained gene mutations characteristic to each patient. Using our differentiation culture system, we demonstrated that numbers of both megakaryocyte (MK)s and platelets obtainable from both XLT- and WAS-iPSCs were significantly smaller than those from healthy iPSCs. Detailed analysis revealed that the observed defects were mainly due to insufficient production of proplatelet-bearing cells, but not to impaired platelet production per MK. Lentiviral-mediated gene transfer led to appearance of WASp expression in patient iPSC-derived MKs. The expression of WASp, however, did not reach the normal level that was seen in control-iPSC-MKs; yields of platelets showed some increase after gene transfer, but only marginally. Although further investigation is necessary, these results indicate the utility of iPSC-based disease modeling for WAS. We are now in the process of addressing how critically expression levels of WASp will affect the efficacy in platelet number recovery after gene transfer.

4. Demonstration of safe and efficacious gene therapy using X-CGD iPSCs.

Huang-Ting Lin[6], Haruna Takagi[6], Mozhgan Kharaj[6], Chieko Konishi[6], Takashi Ishida[6], Chen-Yi Lai[6], Makoto Otsu, Hiromitsu Nakauchi[4]

X-linked chronic granulomatous disease (XCGD) is caused by gp91phox deficiency. This compromises neutrophil (NEU) killing of phagocytosed pathogens due to impaired production of reactive oxygen species (ROS). In previous XCGD gene therapy clinical trials, the sustained persistence of gene marked cells could not be achieved without adversely triggering insertional mutagenesis. Additionally cellular recovery (gp91phox and ROS) had been incomplete on a per cell basis in comparison with healthy controls. This led to the hypothesis that the expression of ectopic gp91phox in developing NEUs could impede further differentiation into mature NEUs. To investigate this theory, a modeling system was established by generating patient autologous XCGD-iPSCs and its differentiation into NEUs. In this culture system, the hierarchical transition of NEU differentiation could be demonstrated from developing ($CD64^{dull}CD15^{dull}$) to mature ($CD64^{high}CD15^{high}$). Alpharetroviral vectors were used to transduce XCGD-iPSCs with the expression of codon optimized gp91phox cDNA driven by the ubiquitous EF1α short promoter. In healthy iPSC-derived NEUs, gp91phox expression and ROS production could only be detected in the mature fraction. In NEUs derived from transduced XCGD-iPSCs, functional recovery in the mature fraction was incomplete. Ectopic gp91phox expression could be detected in the developing fraction through

intracellular staining but not in healthy control cells. Most importantly, cell death was most prominent in developing NEUs ectopically expressing gp91phox. Mechanistic studies are under way to investigate the role of non-physiological ROS production in inducing endoplasmic reticulum (ER) stress resulting in cell apoptosis. Therefore, affording cellular protection from the detrimental effects of non-physiological ROS production may improve XCGD clinical outcomes.

5. A New Strategy To Overcome The Cell Dose Barrier To Umbilical Cord Blood Transplants

Takashi Ishida[6], Masaaki Higashihara[8], Hiromitsu Nakauchi[4], Makoto Otsu: [8]Department of Hematology, Kitasato University School of Medicine

Umbilical cord blood (UCB) serves as a suitable donor source in hematopoietic stem cell transplantation (HSCT). However, UCB has the major drawback that is delayed engraftment due to its low graft cell numbers, which often limits its use in HSCT. To overcome this cell dose barrier, double units UCB transplantation (UCBT) has been attempted, but the time to engraftment is still relatively delayed. Based on the report by Japan Red Cross Society, the majority of UCB units remain unused clinically because of their insufficient graft cell doses. Overall, these facts prompted us to seek for a new strategy to improve UCBT by using multiple (more than three) units. We here provide a proof of feasibility of such an approach using mouse transplantation models.

To mimic a clinical setting of UCBT, we first established an insufficient cell dose model by using mouse BM KSL (c-Kit$^+$, Sca-1$^+$, lineage-marker-negative) cells. In this model, C57BL/6 (B6-Ly5.2, H2^b) mice were the recipients, and congenic B6-Ly5.1 mice (H2^b) were the primary donors of cell grafts. The recipient mice were lethally irradiated, and thus could not survive with only the "insufficient cell dose" of B6-Ly5.1 graft. The outcomes in HSCT were tested by the addition of mixed allogeneic KSL cells (multi-allo HSPCs) in comparison with the addition of B6-Ly5.1 KSL cells (congenic HSPCs) with the equivalent doses. The effects of multi-allo HSPC transplants were evaluated by recipients' survival rate and complete blood counts over time. Detailed donor cell contribution in peripheral blood and BM was also determined by flow cytometry analysis.

Interestingly, addition of multi-allo HSPCs rescued otherwise lethal recipients as effectively as congenic-HSPCs with the equivalent acceleration of hematopoietic recovery. Chimerism analysis, however, revealed that this "KSL cells alone" transplantation led to long-term existence of multi-donor hematopoiesis, which was not ideal for a clinical setting. We then replaced B6-Ly5.1 KSL grafts with the whole BM (WBM) grafts. Titration experiments determined 50,000 WBM cells as a single unit, mimicking an "insufficient dose" of unmanipulated UCB. Addition of multi-allo HSPCs in this model also showed complete protection of recipients from lethality and enhanced early hematopoietic recovery. Remarkably, dominant B6-Ly5.1 chimerism was established and maintained in this modified model. Experiments using subfractionation of the B6-Ly5.1 grafts demonstrated that small numbers of T cells were responsible to single-donor chimerism formation possibly through graft versus graft reactions. Finally, to maximize transient early hematopoietic reconstitution by multi-allo HSPCs (we call this "bridging effect"), we tested whether cultured hematopoietic stem cells (HSC, defined as CD34$^{negatvie/low}$ KSL cells) worked better than uncultured KSL cells. Using our defined protocol compatible with stem cell amplification, we demonstrated that a mixture of cultured allo-HSCs exhibited the bridging effect even superior to that of an uncultured KSL cell mixture.

6. Stage-specific roles for Cxcr4 signaling in murine hematopoietic stem/progenitor cells in the process of bone marrow repopulation

Chen-Yi Lai[6], Satoshi Yamazaki[4], Yasuaki Iimura[9], Masafumi Onodera[10], Shigeru Kakuta[11], Yoichiro Iwakura[11], Hiromitsu Nakauchi[4], Makoto Otsu: [9]Kushiro City General Hospital, [10]Department of Genetics, National Research Institute of Child Health and Development, [11]Laboratory of Molecular Pathogenesis, Center for Experimental Medicine and Systems

Hematopoietic cell transplantation has proven beneficial for various diseases, but low-level engraftment would be problematic leading to patient mortality, requiring elucidation of the molecular determinant for successful engraftment. It remains unclear how hematopoietic stem/progenitor cells (HSPCs) home to the bone marrow (BM) microenvironment, initiate hematopoietic reconstitution, and maintain life-long hematopoiesis. By monitoring the *in vivo* kinetics of transplanted donor cells, we demonstrated that modification of Cxcr4 signaling in murine HSPCs did not affect homing/lodging events, but led to alteration in subsequent BM repopulation kinetics, with observations confirmed by both gain- and loss-of-function approaches. With the use of C-terminal truncated Cxcr4 as a gain-of-function effector, we showed that signal augmentation through Cxcr4 led to favorable *in vivo* repopulation of primitive cell populations in BM. These Cxcr4-augmented HSPCs exhibited *in vitro* enhanced seeding efficiencies in stromal cell co-cultures and altered ligand-mediated phosphorylation

kinetics of Extracellular signal-regulated kinases. Sustained signal enhancement, however, even with wild-type Cxcr4 overexpression resulted in poor peripheral blood reconstitution due to blunted release of donor hematopoietic cells from BM. We thus conclude that timely regulation of Cxcr4/CXCR4 signaling will provide donor HSPCs with enhanced repopulation potential, with preserving their ability to release progeny into peripheral blood for improved transplantation outcomes.

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Center for Stem Cell Biology and Regenerative Medicine

Division of Stem Cell Transplantation
幹細胞移植分野

Professor	Arinobu Tojo, M.D., D.M.Sc.	教　授　医学博士	東　條　有　伸
Associate Professor	Satoshi Takahashi, M.D., D.M.Sc.	准教授　医学博士	高　橋　　聡

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. Effective treatment against severe graft-versus-host disease with allele-specific anti-HLA monoclonal antibody in a humanized mouse model.

Nakauchi Y, Yamazaki S, Napier SC, Usui JI, Ota Y, Takahashi S, Watanabe N, Nakauchi H.

Graft-versus-host disease (GVHD), mediated by donor-derived alloreactive T cells, is a major cause of nonrelapse mortality in allogeneic hematopoietic stem cell transplantation. Its therapy is not well-defined. We established allele-specific anti-human leukocyte antigen (HLA) monoclonal antibodies (ASHmAbs) that specifically target HLA molecules, with steady death of target-expressing cells. One such ASHmAb, against HLA-A*02:01 (A2-kASHmAb), was examined in a xenogeneic GVHD mouse model. To induce fatal GVHD, non-irradiated NOD/Shi-scid/IL-2Rγ(null) mice were injected with healthy donor human peripheral blood mononuclear cells, some expressing HLA-A*02:01, some not. Administration of A2-kASHmAb promoted the survival of mice injected with HLA-A*02:01-expressing peripheral blood mononuclear cells ($p < 0.0001$) and, in humanized NOD/Shi-scid/IL-2Rγ (null) mice, immediately cleared HLA-A*02:01-expressing human blood cells from mouse peripheral blood. Human peripheral blood mononuclear cells were again detectable in mouse blood 2 to 4 weeks after A2-kASHmAb administration, suggesting that kASHmAb may be safely administered to GVHD patients without permanently ablating the graft. This approach, different from those in existing GVHD pharmacotherapy, may open a new door for treatment of GVHD in HLA-mismatched allogeneic hematopoietic stem cell transplantation.

2. Myeloablative unrelated cord blood transplantation for Philadelphia chromosome-positive acute lymphoblastic leukemia: comparison with other graft sources from related and unrelated donors.

Konuma T, Kato S, Ooi J, Oiwa-Monna M, Tojo A, Takahashi S.

Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia (ALL) is a distinct clinical entity among ALL and is associated with adverse outcomes and higher rates of relapse when conventional chemotherapy is used alone. Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for patients with Ph + ALL, the impact of graft sources, particularly cord blood transplantation (CBT), on allo-

HSCT for patients with Ph + ALL has yet to be clarified. We retrospectively compared clinical outcomes after unrelated CBT (n = 20), unrelated bone marrow transplantation (n = 7), and related bone marrow and peripheral blood stem cell transplantations (n = 13) following myeloablative conditioning in 40 patients with Ph + ALL. Although graft source had no significant impact on survival or relapse, disease status at transplantation did significantly affect outcomes. These data suggest that unrelated CBT is feasible and should be considered early in the course of patients with Ph + ALL when HLA-compatible related and unrelated donors are not available.

3. The Adult Myelodysplastic Syndrome and Adult Acute Myeloid Leukemia Working Group of the Japan Society for Hematopoietic Cell Transplantation. Effect of granulocyte colony-stimulating factor combined conditioning in cord blood transplantation for myelodysplastic syndrome and secondary acute myeloid leukemia: a retrospective study in Japan. Biol Blood Marrow Transplant. 2015 Sep; 21(9): 1632-40. doi: 10.1016/j.bbmt.2015.05.009. Epub 2015 May 16.

Konuma T, Kato S, Ooi J, Oiwa-Monna M, Ebihara Y, Mochizuki S, Yuji K, Ohno N, Kawamata T, Jo N, Yokoyama K, Uchimaru K, Tojo A, Takahashi S.

Granulocyte colony-stimulating factor (G-CSF) increases the susceptibility of dormant malignant or nonmalignant hematopoietic cells to cytarabine arabinoside (Ara-C) through the induction of cell cycle entry. Therefore, G-CSF-combined conditioning before allogeneic stem cell transplantation might positively contribute to decreased incidences of relapse and graft failure without having to increase the dose of cytotoxic drugs. We conducted a retrospective nationwide study of 336 adult patients with myelodysplastic syndrome (MDS) and secondary acute myeloid leukemia (sAML) after single-unit cord blood transplantation (CBT) who underwent 4 different kinds of conditioning regimens: total body irradiation (TBI) ≥8 Gy + Ara-C/G-CSF + cyclophosphamide (CY) (n = 65), TBI≥8 Gy + Ara-C + CY (n = 119), TBI≥8 Gy + other (n = 104), or TBI < 8 Gy or non-TBI (n = 48). The TBI≥8 Gy + Ara-C/G-CSF + CY regimen showed significantly higher incidence of neutrophil engraftment (hazard ratio, 1.52; 95% confidence interval [CI], 1.10 to 2.08; P = .009) and lower overall mortality (hazard ratio,. 46; 95% CI, .26 to .82; P = .008) rates compared with those without a G-CSF regimen. This retrospective study shows that the G-CSF-combined conditioning regimen provides better engraftment and survival results in CBT for adults with MDS and sAML.

4. Generation of multivirus-specificT Cells by a single stimulation of PBMCs with a peptide mixture utilizing serum-free medium.

Fujita Y, Tanaka Y, Takahashi S.

Restoration of virus-specific immunity offers an attractive alternative to conventional drugs. Recently, the system of rapid generation of multivirus-specific T cells has been reported (Gerdemann, U, 2012). With this system, polyclonal CTLs specific for multivirus antigens can be produced after a 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. To meet the requirement for the viral infections after HSCT by broad viral antigens and in terms of regulation by the Japanese FDA, we generated mutivirus-specific T-cells targeting 7 viruses (CMV, EBV, AdV, HHV-6, BKV, JCV, and VZV) in serum-free medium. PBMCs were stimulated with peptide mixture spanning the target antigens of 3 (CMV, EBV, AdV) or 7 viruses as above and cultured in serum-free medium with IL4 and IL7 for 9-12 days. After single-stimulation and culture, the cells were analyzed with cell number, phenotypes, TCR repertoires, and virus-specific responses by FACS and ELISpot. Starting from 20 × 10^6 of PBMCs, 3 or 7 viruses' antigens stimulated cells increased to averages of 92.0 × 10^6 and 112.7 × 10^6, respectively after 9-12 days. These cells were mostly CD3T, which contained both CD4T and CD8T with a dominant phenotype of central memory (CD62L + CD45RO +). Actually, the cells demonstrated specificity toward all the 7 virus antigens by IFNγ production or CD107a degranulation assay. Furthermore, we confirmed the CD107a + IFNγ + effector-cells against specific virus antigens displayed a wide range of TCR diversities including several predominant TCR repertoire populations depending on the kinds of specific-antigens. We could rapidly and easily generate polyclonal 7 viruses-specific T cells with a single stimulation of PBMCs without using any serum products. This system is ready to go to the clinical trials after allogeneic transplantation setting.

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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.

教 授 医学博士 北 村 俊 雄

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. Development of new retroviral vectors.

Toshikhiko Oki, Jiro Kitaura, Yutaka Enomoto, Tomoyuki Uchida, Fumi Shibata-Minoshima, and Toshio Kitamura:

We previously developed an effective retroviral transduction system consisted of vectors named as pMXs, pMYs, pMZs and pMCs and packaging cells named as PLAT-E, PLAT-A, and PLAT-F. PLAT-E and A produce ecotropic and amphotropic retroviruses, respectively, upon transient transfection of retrovirus vectors. PLAT-F can produce retroviruses whose envelopes are derived from feline leukemia virus RD114 which somehow has a high-affinity binding to human CD34. Therefore, the viruses produced from CD34 can efficiently infect CD34+ cells. In fact, multiple infections of PLAT-F-produced retroviruses can infect CD34+ human bone marrow cells with nearly 100% infection efficiencies. Based on this system, we developed new vectors including vectors with luciferase maker (pMX-IL), vectors for GFP or RFP fusion proteins, vectors with lox sequences for deletion of inserted genes with Cre-loxP, Tet-On and Tet-Off systems, vectors for expression, inhibition, and monitoring the expression of miroRNA (pMXe series). We utilized these vectors in studying stem cell biology and also in developing the innovative tools for regenerative medicine

2. Co-ordinate control of cell division and cell fate of by the Rho family small GTPases.

Toshihiko Oki, Kohtaro Nishimura, Toshiyuki Kawashima, and Toshio Kitamura:

We previously identified MgcRacGAP through functional cloning as a protein that enhances or induces macrophage differentiation of leukemic cell lines M1 and HL60. Interestingly, MgcRacGAP plays distinct roles depending on the cell cycle. In the interphase, it plays critical roles in activation and nuclear translocation of STAT3 and STAT5 as a Rac-GAP. In the mitotic phase, MgcRacGAP plays essential roles in completion of cytokinesis as a Rho-GAP. Interestingly, Aurora B-mediated phosphorylation of S387 converts MgcRacGAP from Rac-GAP to Rho-GAP.

We have recently shown that expression of MgcRacGAP is regulated by a cell cycle-dependent manner: MgcRacGAP expression increases in S/G2/M phase and decreases in early G1 phase, suggesting that MgcRacGAP may play some roles in G1 check point. In addition to the transcriptional control, MgcRacGAP protein levels are controlled by ubiquitin-dependent degradation, leading to its decrease in G1 phase. Using the proteome analysis

and retroviral transduction, we identified APC/CDH1 as an E3 ligase involved in regulation of MgcRacGAP and the degron in MgcRacGAP. Now we are investigating the physiological roles of this regulation. In summary, our results implicate MgcRacGAP in coordination of cell cycle progression and cell fate determination.

3. Molecular therapy targeting signal transduction pathways using small molecule compounds

Toshiyuki Kawashima, Susumu Goyama, Akiho Tsuchiya, Toshihiko Oki, Jiro Kitaura, and Toshio Kitamura:

STAT3 is frequently activated in many cancers and leukemias, and is required for transformation of NIH3T3 cells. Therefore, we have started searching for STAT3 inhibitors. We already established an efficient screening protocol for identification of STAT3 inhibitors, and identified several compounds that inhibit STAT3 activation. Through the screening of a library of small molecule compounds, we found the compounds RJSI-1 and RJSI-2 that inhibited STAT3 activation. RJSI-2 also inhibited activation of STAT1, STAT5, JAK1 and JAK2, however RJSI-2 is not a kinase inhibitor. On the other hand, RJSI-1 inhibited nuclear transport of phosphorylated STAT proteins, implicating a novel mechanism in inhibiting STAT proteins. We have also shown that these compounds are effective in a tumor-burden mouse model. In addition, we collaborate with a US biotech venture company in modification of RSJI-1 for optimization to develop anticancer drugs, and have developed JP1156 which kill the tumor cells with much lower IC50. In addition to STAT3 inhibitors, we have started a new project to develop STAT5 inhibitors in collaboration with a pharmaceutical company. To this end, we are now in the process to establish a screening method to search for STAT5 inhibitors.

In addition to STAT3 inhibitors, we have recently started a new project to develop STAT5 inhibitors in collaboration with a pharmaceutical company. To this end, we have developed a screening method to search for STAT5 inhibitors. In addition to STAT3/5 inhibitors, we have started several collaborations with several domestic and global pharmaceutical companies to evaluate the efficacies of a variety of molecular targeted therapies in our established mouse MDS/AML/MPN models.

4. Development of G0 indicator

Toshihiko Oki, Kotarou Nishimura, Takeshi Fukushima, Yosuke Tanaka, 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 hematopietic stem cells and cancer stem cells with tumor initiating potentials are in G0 phase.

Recently we have developed the system to indicate cells in G0 phase. It is a system to monitor the amount of p27, which is destructed during G0 to G1 phase and is not expressed in S/G2/M phase, using the cells retrovirally trasduced with the fusion protein between a fluorescent protein like mVenus and p27K- (a p27 mutant lacking CDK inhibitory activities) as a similar cell cycle indicator system, fluorescent, ubiquitination-based cell cycle indicator, (Fucci). mVenus-p27K- positive cells are Ki67 negative quiescent cells and mVenus-p27K- signals are enhanced when the cycling cell enter G0 phase in response to serum starvation or contactinhibition.

Using this system, we identified genetic signatures of G0 cells. Several genes specifically expressed in G0 cells are now being investigated in terms of their functions and biological significance in G0 phase. The mVenus-p27K- trasgenic mice were generated to track several kinds of tissue specific stem cells in vivo. We identified muscle satellite and colon mVenus-p27K- positive Now we are generating a Rosa26-knock in mouse.

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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

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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. We demonstrated that the matrix metalloproteinase-9 is activated during leukemic cell progression. In addition, the serine proteinase plasmin plays a role in the myeloid cell recruitment in inflamed tissues during inflammatory bowel disease, sepsis and graft versus host disease after bone marrow transplantation and during cancer progression.

1. Role of mesenchymal stem cell-derived fibrinolytic factor in tissue regeneration and cancer progression

Beate Heissig[1], Douaa Dhahri[1], Salita Eiamboonsert[1], Yousef Salama[1], Hiroshi Shimazu[2], Shinya Munakata[2], Koichi Hattori[2,3]: [1]Division of Stem Cell Dynamics, The Institute of Medical Science, The University of Tokyo, Tokyo, [2]Division of Stem Cell Regulation, The Institute of Medical Science, The University of Tokyo, [3]Center for Genome and Regenerative Medicine, Juntendo University School of Medicine.

Tissue regeneration during wound healing or cancer growth and progression depends on the establishment of a cellular microenvironment. Mesenchymal stem cells (MSC) are part of this cellular microenvironment, where they functionally modulate cell homing, angiogenesis, and immune modulation. MSC recruitment involves detachment of these cells from their niche, and finally MSC migration into their preferred niches; the wounded area, the tumor bed, and the BM, just to name a few. During this recruitment phase, focal proteolysis disrupts the extracellular matrix (ECM) architecture, breaks cell-matrix interactions with receptors, and integrins, and causes the release of bioactive fragments from ECM molecules. MSC produce a broad array of proteases, promoting remodeling of the surrounding ECM through proteolytic mechanisms. The fibrinolytic system, with its main player plasmin, plays a crucial role in cell migration, growth factor bioavailability, and the regulation of other protease systems during inflammation, tissue regeneration, and cancer. Key components of the fibrinolytic cascade, including the urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1), are expressed in MSC. This review will introduce general functional properties of the fibrinolytic system, which go beyond its known function of fibrin clot dissolution (fibrinolysis). We will focus on the role of the fibrinolytic system for MSC biology, summarizing our current understanding of the role of the fibrinolytic system for MSC recruitment and the functional consequences for tissue regeneration and cancer. Aspects of MSC origin, maintenance, and the mechanisms by which these cells contribute to altered protease activity in the microenvironment under normal and pathological conditions will also be discussed.

2. Cancer therapy targeting the fibrinolytic system

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The tumor microenvironment is recognized as a key factor in the multiple stages of cancer progression, mediating local resistance, immune-escape and metastasis. Cancer growth and progression require remodeling of the tumor stromal microenvironment, such as the development of tumor-associated blood vessels, recruitment of bone marrow-derived cells and cytokine processing. Extracellular matrix breakdown achieved by proteases like the fibrinolytic factor plasmin and matrix metalloproteases is necessary for cell migration crucial for cancer invasion and metastasis. Key components of the fibrinolytic system are expressed in cells of the tumor microenvironment. Plasmin can control growth factor bioavailability, or the regulation of other proteases leading to angiogenesis, and inflammation. In this review, we will focus on the role of the fibrinolytic system in the tumor microenvironment summarizing our current understanding of the role of the fibrinolytic factors for the modulation of the local chemokine/cytokine milieu, resulting in myeloid cell recruitment, which can promote neoangiogenesis.

3. Inhibition of plasmin attenuates murine acute graft-versus-host disease mortality by suppressing the matrix metalloproteinase-9-dependent inflammatory cytokine storm and effector cell trafficking

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The systemic inflammatory response observed during acute graft-versus-host disease (aGVHD) is driven by proinflammatory cytokines, a 'cytokine storm'. The function of plasmin in regulating the inflammatory response is not fully understood, and its role in the development of aGVHD remains unresolved. Here we show that plasmin is activated during the early phase of aGVHD in mice, and its activation correlated with aGVHD severity in humans. Pharmacological plasmin inhibition protected against aGVHD-associated lethality in mice. Mechanistically, plasmin inhibition impaired the infiltration of inflammatory cells, the release of membrane-associated proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and Fas-ligand directly, or indirectly via matrix metalloproteinases (MMPs) and alters monocyte chemoattractant protein-1 (MCP-1) signaling. We propose that plasmin and potentially MMP-9 inhibition offers a novel therapeutic strategy to control the deadly cytokine storm in patients with aGVHD, thereby preventing tissue destruction.

4. Inhibition of Plasmin Protects Against Colitis in Mice by Suppressing Matrix Metalloproteinase 9-mediated Cytokine Release From Myeloid Cells

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Activated proteases such as plasmin and matrix metalloproteinases (MMPs) are activated in intestinal tissues of patients with active inflammatory bowel diseases. We investigated the effect of plasmin on progression of acute colitis. Colitis was induced in Mmp9-/-, Plg-/-, and C57BL/6 (control) mice by administration of dextran sulfate sodium, trinitrobenzene sulfonic acid, or CD40 antibody. Plasmin was inhibited in control mice by intraperitoneal injection of YO-2, which blocks its active site.

Mucosal and blood samples were collected and analyzed by reverse transcription polymerase chain reaction and immunohistochemical analyses, as well as for mucosal inflammation and levels of cytokines and chemokines. We showed that circulating levels of plasmin were increased in mice with colitis, compared with controls. Colitis did not develop in control mice injected with YO-2 or in Plg-/- mice. Colons from these mice had reduced infiltration of Gr1+ neutrophils and F4/80+ macrophages, and reduced levels of inflammatory cytokines and chemokines. Colonic inflammation and colitis induction required activation of endogenous MMP9. Following colitis induction, mice given YO-2, Plg-/- mice, and Mmp9-/- mice had reduced serum levels of tumor necrosis factor and CXCL5, compared to control mice. In summary, in mice, plasmin induces a feedback mechanism in which activation of the fibrinolytic system promotes development of colitis, via activation of MMP9 or proteolytic enzymes. The proteolytic environment stimulates influx of myeloid cells into the colonic epithelium and production of tumor necrosis factor and CXCL5. In turn, myeloid CD11b+ cells release the urokinase plasminogen activator, which accelerates plasmin production. Disruption of the plasmin-induced chronic inflammatory circuit might therefore be a strategy for treatment of colitis.

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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. Interleukin-17B antagonizes interleukin-25-mediated mucosal inflammation.

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The pro-inflammatory functions of interleukin-17 (IL-17) have been well documented. Here, we have demonstrated further functional complexity among the IL-17 family of cytokines. Adding to a well-established role in type 2 immunity, we found that IL-25 was pathogenic in acute colitis, whereas IL-17 B was conversely protective. These opposing effects were similar in *Citrobacter rodentium* infection and allergic asthma. Notably, we have found a unique role for IL-17B in inhibiting IL-25-mediated IL-6 production by CECs, making IL-17B an antagonistic and anti-inflammatory cytokine in this family.

IL-25 is generally thought of as a Th2-cell-derived factor, promoting allergic asthma responses but also protecting against parasitic infection. Here, we have shown that IL-25 in acute colitis promoted IL-6 production from CECs, which is consistent with a report of IL-25 promoting the release of IL-6 from eosinophils. Throughout the course of this study, IL-25-dependent IL-6 production was the most consistently inhibited by IL-17B treatment. However, at this time we cannot rule out the influence of other mediators in promoting the protective and pathogenic phenotypes observed in IL-25- and IL-17B-deficient mice, respectively. Most likely, IL-17B antagonism of IL-25 is influencing multiple facets of the mucosal immune response, which is the subject of our current investigations.

Our finding that IL-25 deletion protects against

dextran sodium sulfate (DSS) differs from another showing that injecting IL-25 protects against the development of DSS colitis. Though we cannot fully explain the discrepancy between reports, IL-25 stimulation of primary CECs in vitro promoted the production of IL-6, consistent with decreased inflammation observed in *Il25*$^{-/-}$ mice. Indeed, when we injected WT mice directly with IL-25 or IL-17B, we observed phenotypes opposite to those found in cytokine-deficient mice. Thus, different housing conditions or microbiota might influence results obtained from recombinant protein injection.

IL-17B has long been a mysterious member of the IL-17 cytokine family. Previous work has demonstrated that IL-17RB might be oncogenic whereas IL-17B might contribute to tumor cell growth. The original report characterizing IL-17B demonstrates that this cytokine stimulates the release of TNF-α and IL-1β from THP-1 cells. However, we did not observe production of pro-inflammatory mediators after IL-17B stimulation, consistent with IL-17B being unable to induce IL-6 from fibroblasts. Instead, the primary effect we observed for IL-17B treatment in CECs was direct inhibition of IL-25-mediated IL-6 production. This of course begs the question of how exactly IL-17B can inhibit IL-25-mediated IL-6 production in CECs. One possibility is that IL-17B competes with IL-25 for binding IL-17RB. Indeed, we found that IL-17B inhibited IL-25 binding to IL-17RB or IL-17RA and IL-17RB complexes. Because previous work has demonstrated that IL-17A and IL-17F form heterodimers, we investigated this possibility as well. However, exhaustive attempts to identify IL-17B and IL-25 heterodimers were unsuccessful, suggesting that competition for IL-17RB binding causes functional antagonism. IL-17B had higher activity in blocking IL-25 binding to the IL-17RA and IL-17RB heterodimer compared to IL-17 RB alone. Conversely, IL-25 was capable of binding IL-17RB in the absence of IL-17RA, suggesting that IL-17RA was more important for signaling. These binding assays have limitations considering that we cannot rule out the contribution of endogenous IL-17 receptors that might be expressed in the human HEK cells, because we do not know whether the murine IL-17RB could form heterodimers with endogenous human IL-17RA. Another caveat is that only mouse receptors were co-expressed in these cells and more direct studies need to be performed in the future on both mouse and human IL-17RA and IL-17RB similar to studies on IL-17 and IL-17F. Finally, we need to determine whether IL-17RB is internalized after binding of IL-17B, IL-25, or both. Differential IL-17RB internalization could represent another mechanism governing IL-17B regulation of IL-25.

Our current model is that colon inflammation enhances IL-25 pro-inflammatory signaling while IL-17B is concurrently induced to restrain IL-25. IL-25 has a higher affinity for IL-17RB alone compared to IL-17B. However, treatment with IL-17B throughout the course of DSS colitis led to protection in WT animals, indicating that a shift in the IL-25 to IL-17 B ratio can influence the course of inflammation. Previous work has demonstrated that IL-17F in some situations antagonizes IL-17 signaling, including forming heterodimeric complexes with IL-17 that have weaker activity compared to IL-17 alone. We believe that the antagonism exerted on IL-25 signaling by IL-17B, however, is unique to this family because IL-17F by itself can activate signaling pathways important for inflammation but we have yet to find a standalone function for IL-17B. Moreover, IL-17B could not antagonize IL-6 production induced by IL-17 in CECs, demonstrating the specificity of IL-17B action. Therefore, further study is needed to determine whether IL-17B alone can transmit a functional signal to any cell type and to determine the precise mechanism for how IL-17B is inhibiting IL-25 binding to IL-17RB.

The prospect of modulating IL-17RB signaling for the treatment of colitis at this time seems promising. Our findings that recombinant IL-17B injections throughout the course of DSS administration can improve disease outcome in WT animals further supports this theory. Whether IL-17B is as important in human inflammatory bowel disease (IBD) is currently under investigation. The production of IL-25 has been linked to human IBD even though polymorphism is not believed to be a risk factor. Two recent reports have shown that IBD patients have decreases in IL-25. These results might suggest that IL-25 is a protective factor in human IBD; however, IL-17B expression in these patients has yet to be determined. Decreased IL-25 expression in human IBD might also be representative of a failure of IL-17B to control inflammation. Thus, further investigation is necessary to determine the role of IL-17B in human IBD as well as determining whether IL-17RB represents a viable therapeutic target.

2. In vivo and in vitro analyses of α-galactosylceramide uptake by conventional dendritic cell subsets using its fluorescence-labeled derivative.

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Dendritic cells (DCs) are antigen-presenting cells

(APCs) that exist widely in the body and survey pathogen invasions. Upon recognition of pathogens, DCs present peptide antigens via major histocompatibility complex (MHC) molecules to the T cells. DCs also present lipid antigen via nonclassical MHC CD1 to natural killer T cells (NKT cells), inducing robust cytokine production.

DCs are primarily divided into two populations: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs are further subdivided into two groups, $CD8\alpha^+$ and $CD8\alpha^-$ DCs, by the expression of CD8α. NKT cells are divided into two subsets by the pattern of T cell antigen receptor (TCR) expression. Type I NKT cells express invariant TCR, so they are designated as invariant NKT (iNKT) cells. iNKT cells recognize marine sponge α-galactosylceramide (α-GalCer) on the CD1d molecule of cDCs, inducing antitumor activity via the robust production of IFN-γ. During interaction with cDCs, iNKT cells express CD40 ligand (CD40L), which stimulates cDCs via their CD40, subsequently inducing IL-12 production from $CD8\alpha^+$ DCs. In turn, IL-12 stimulates iNKT cells, resulting in the strong production of IFN-γ. Furthermore, using Langerin-eGFP-diphtheria toxin receptor (DTR) mice, depletion of $Langerin^+$ cDCs decreased IL-12 and IFN-γ production after stimulation with injection of α-GalCer, suggesting that $Langerin^+$ $CD8\alpha^+$ DCs are involved in IFN-γ production of iNKT cells in vivo. However, it is unclear why $CD8\alpha^+$ DCs induce higher IFN-γ production of iNKT cells than $CD8\alpha^-$ DCs.

After i.v. injection, antigens in the blood are captured by the splenic marginal zone (MZ) cells, causing a rapid immune response. MZ consists of $SIGNR1/ER\text{-}TR9^+$ macrophages, MZB cells, and cDCs, forming special equipment to sense/capture antigens in the blood flowing into the sinus between the MZ and marginal $CD169/SER4^+$ metallophil zone. Interestingly, $Langerin^+$ $CD8\alpha^+$ DCs were observed in the MZ, and iNKT cells migrated to the MZ after injection of α-GalCer.

Here, we compared α-GalCer uptake and degradation between $CD8\alpha^-$ and $CD8\alpha^+$ DC subsets using Cy5-conjugated α-GalCer (Cy5-α-GalCer). We also performed histological analyses to observe the uptake of Cy5-α-GalCer in the spleen. Our results suggest that both cellular properties and localization of $CD8\alpha^+$ DCs enable efficient stimulation of iNKT cells in response to α-GalCer. In cDC subsets, $CD8\alpha^+$ DCs uptake α-GalCer effectively compared with $CD8\alpha^-$ DCs in vivo and in vitro. In addition, α-GalCer is more stable in $CD8\alpha^+$ DCs than in $CD8\alpha^-$ DCs. It is possible that these two properties of $CD8\alpha^+$ DCs result in their efficient accumulation and presentation of α-GalCer. Furthermore, $CD8\alpha^+$ DCs strategically localize in the MZ, which is specialized in capturing blood-borne antigens, and preferentially uptake α-GalCer injected i.v.. Furthermore, $CD8\alpha^+$ DCs in the spleen express higher levels of membrane-bound CXCL16 than $CD8\alpha^-$ DCs, possibly enhancing activation of iNKT cells. These cellular properties and localization of $CD8\alpha^+$ DCs seem to enable the robust induction of IFN-γ from iNKT cells in vivo.

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