Advanced Clinical Research Center

Division of Molecular Therapy
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The main theme of our research is toward the development of novel therapeutic options against intractable malignant disorders including leukemia, lymphoma and various cancers. For this purpose, we are making every effort to master the mechanisms of normal and neoplastic stem cells on the basis of molecular and cellular biology as well as medical informatics. We also try to develop novel therapies in the field of regenerative medicine using bone marrow-derived mesenchymal stromal cells.

(1) Molecular and cellular analysis of hematological malignancies:
Tumor-specific genetic alterations often result in transcriptional dysregulation and activation of signal transduction pathways as well as defective tumor suppressors, which appear to be the primary cause of those tumors. We are studying the molecular and cellular aspects of hematological malignancies as a model system. Furthermore, we performed clinical sequencing in tight collaboration with Human Genome Center and Health Intelligence Center to establish a platform for precision medicine.

(2) Development of anti-cancer therapy using recombinant vaccinia virus:
Oncolytic virotherapy is an emerging type of cancer therapy in which a native or genetically modified virus selectively infects and replicates in the tumor and destroys tumor cells. The anti-tumor effects of oncolytic virus alone were generally insufficient in pre-clinical and clinical trials. Using genetic engineering, we loaded oncolytic viruses with foreign transgenes to increase the potency of the therapeutic effect.

(3) Development of a novel cell therapy using the genome editing with CRISPR/Cas9:
Cell therapy using mesenchymal stem cells and chimeric antigen receptor expressing-T cells (CAR-T cells) are promising therapeutic options for refractory diseases. While cell therapies are remarkably effective, very expensive cost hampers them to be applied for regular clinical use. We used CRISPR/Cas9 for the gene editing to generate a universal cell therapy.

(4) Clinical study of clonal evolution of HTLV-1-infected T cells into leukemia:
Adult T-cell leukemia is a T cell malignancy which develops in HTLV-1 infected individuals after long latency period. HTLV-1 infected cells are regarded to transform through multi-step oncogenesis process. We are analyzing HTLV-1 infected cells in different stages of transformation whose phenotypes such as CD7 and CADM1 expression vary in each stage by sorting them using flow cytometer. These analyses will provide useful information regarding molecular mechanism to develop ATL.

1. Therapeutic targeting of monokine production is a promising strategy to attenuate cytokine-release syndrome in CAR-T cell thrapy.

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Cancer immunotherapy using chimeric antigen receptor-armed T cells (CAR-T cells) have shown excellent outocomes in hematological malignancies. However, cytokine release syndrome (CRS), characterized by excessive activation of CAR-T cells and macrophages remains to be overcome. Steroid administration usually resolves signs and symptoms of CRS but abrogates CAR-T cell expansion and persistence. Tocilizumab, a humanized monoclonal antibody against interleukin-6 receptor (IL-6R), attenuates CRS without significant loss of CAR-T cell activities, while perfect rescue of CRS symptoms cannot be achieved by IL-6/IL-6R blockade. There is actual need for novel strategies to prevent or cure CRS. TO-207, an N-benzoyl-L-phenylalanine derivative compound, significantly inhibits inflammatory cytokine production in a human monocyte/ macrophage-specific manner. Here we tested TO-207 for its ability to inhibit cytokine production without impaired CAR-T cell function in a CRS-simulating co-culture system consisting of CAR-T cells, target leukemic cells and monocytes. To observe a precise pattern of cytokine release from CAR-T cells and monocytes, we first established a co-culture system that mimics CRS using K562/CD19 cells, 19-28z CAR-T cells, and peripheral blood $CD14^+$ cells. IFN-γ was produced exclusively from CAR-T cells, and TNF-α, MIP-1α, M-CSF, and IL-6 were produced from both CAR-T cells and monocytes, but monocytes were the major source of these cytokine production. MCP-1, IL-1β, IL-8, and IL-10 were released exclusively from monocytes. To observe the effect of drugs on cytokine production, prednisolone (PSL), TO-207, tocilizumab, and anakinra (an IL-1R antagonist) were added to the co-culture. PSL exhibited suppressive effects on TNF-α, IFN-γ, and MCP-1 production. Tocilizumab did not suppress these cytokines. Anakinra up-regulated IL-6 and IL-1β production, probably due to activation of negative feedback loops. Interestingly, TO-207 widely suppressed all of these monocyte-derived cytokines including TNF-α, IFN-γ, IL-6, IL-1β, MCP-1, IL-8, and GM-CSF. Next, we observed whether the cytokine inhibition by TO-207 attenuates killing effect of CAR-T cells. PSL attenuated killing effect of $CD4^+$ CAR-T cells and $CD8^+$ CAR-T cells toward K562/CD19 cells. In contrast, TO-207 did not exhibit any change in cytotoxicity of $CD4^+$ CAR-T cells and $CD8^+$ CAR-T cells. To determine whether the effect of PSL and TO-207 on cytotoxicity changes in the presence of $CD14^+$ monocytes, $CD14^+$ cells were added to the co-culture. In the absence of CAR-T cells, PSL induced a modest attenuation of cytotoxicity, whereas to the CAR-T cells, PSL exhibited a significant attenuation of cytotoxicity. TO-207 exhibited a minimal effect on cytotoxicity in the absence or presence of CAR-T cells. These results suggested that CAR-T cells play a major role in the cytotoxicity toward leukemia cells, and drugs that do not affect CAR-T cell functions, such as TO-207, maintain their cytotoxic effects on leukemia cells. In conclusion, our present co-culture model with K562/CD19 cells, 19-28z CAR-T cells, and $CD14^+$ monocytes accurately recapitulated killing effect and cytokine release profiles. IFN-γ was produced exclusively by CAR-T cells, but most of cytokines such as TNF-α, MIP-1α, M-CSF, IL-6, MCP-1, IL-1β, IL-8, and IL-10 were from $CD14^+$ monocytes/macrophages. Because killing effect was largely dependent on CAR-T cells while cytokine production was dependent on monocytes/macrophages, selective inhibition of pro-inflammatory cytokines from monocytes by TO-207 would be ideal for treatment of CAR-T-related CRS. These results encourage us to consider a clinical trial for the use of CRS.

2. Development of a novel cell therapy using the genome editing with CRISPR/Cas9

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Cell therapies using mesenchymal stem cells (MSCs) are effective for the treatment of graft versus host disease (GvHD) following allogeneic stem cell transplantation. However, human leukocyte antigen (HLA) alleles usually mismatch between patients and donors, and transplanted MSCs are eventually rejected by the host immunity. The knockout (KO) of HLA gene would unlock the HLA restriction and facilitate the development of universal cell therapy. For a longer retention of transplanted MSCs in the recipient, a genome editing to knockout HLA molecule was performed. As HLA class I molecules are expressed on the cell surface together with β-2 microglobulin (B2M), knockout (KO) of B2M leads to loss of expression of HLA. Using the electroporation, MSCs were transfected with Cas9 protein and a short guide RNA (sgRNA) targeting B2M. Successful KO of B2M and HLA class I was confirmed on day 7. We confirmed that B2M-/- MSCs retains the immunosuppressive effect as strong as parental MSCs using the mixed lymphocyte reaction (MLR) in the presence of MSCs. Although loss of HLA would protect MSCs from cytotoxic T lymphocytes (CTLs), loss of HLA deprives a protective effect of HLA through the binding to inhibitory receptors on the natural killer (NK) cells. To avoid from both CTLs and NK cells, HLA-G, an almost invariant non-classical HLA, was fused with B2M, and the B2M/HLA-G fusion was successfully transduced into MSCs using a lentiviral vector. We are also attempting to insert the B2M/HLA-G fusion into MSCs with a genome editing method using CRIS-

PR/Cas9. To establish a sophisticated method by which efficient and safe gene KO and/or knock-in (KI) are carried out, we examined several methods using CRISPR/Cas9. While a high efficiency of KO could be achieved by the transfection of either a CRISPR/Cas9-expressing plasmid (pX330) or a mixture of sgRNA and Cas9 protein, the efficiency of KI was very low using a conventional electroporation of sgRNA, Cas9 protein, and donor DNA (in the form of plasmid). The limiting factor in KI seemed to be the cytotoxicity due to the large DNA size that was transfected as a DNA donor. We compared several different types of DNA donors, including plasmids with homology arms (HA) on both sides of the inserted gene, plasmids without HA on both sides, the linear double strand DNAs, and the single strand DNAs. We found that transfection of sgRNA/Cas9 with a plasmid that have sgRNA recognition sites on both sides of transgene (with no HAs) showed a relatively low cytotoxicity and a good KI efficiency (homology-independent transgene insertion). Using the method that we have confirmed, we will put forward our genome editing experiments for the development of a new cell therapy.

3. CD4$^+$CADM1$^+$ cell percentage predicts disease progression in asymptomatic HTLV-1 carriers and indolent adult T-cell leukemia/lymphoma.

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We recently took advantage of the universal expression of cell adhesion molecule 1 (CADM1) by CD4$^+$ cells infected with HTLV-1 and the downregulation of CD7 expression that corresponds with the oncogenic stage of HTLV-1-infected cells to develop a flow cytometric system using CADM1 versus CD7 plotting of CD4$^+$ cells. We risk-stratified HTLV-1 asymptomatic carriers (AC) and indolent adult T-cell leukemia/lymphoma (ATL) cases based on the CADM1$^+$ percentage, in which HTLV-1-infected clones are efficiently enriched. AC and indolent ATL cases were initially classified according to their CADM1$^+$ cell percentage. Follow-up clinical and flow cytometric data were obtained for 71 cases. In G1 (CADM1$^+$ ≤ 10%) and G2 (10% < CADM1$^+$ ≤ 25%) cases, no apparent clinical disease progression was observed. In G3 (25% < CADM1$^+$ ≤ 50%) cases, five out of nine (55.5%) cases progressed from AC to smoldering-type ATL. In G4 (50% < CADM1$^+$) cases, the cumulative incidence of receiving systemic chemotherapy at 3 years was 28.4%. Our results indicate that the percentage of the CD4$^+$ CADM1$^+$ population predicts clinical disease progression: G1 and G2 cases, including AC cases, are stable and considered to be at low risk; G3 cases, including advanced AC cases and smoldering-type ATL cases based on the Shimoyama criteria, are considered to have intermediate risk; and G4 cases, which are mainly indolent ATL cases, are unstable and at high risk of acute transformation.

4. Prognostic impact of circulating tumor DNA status post allogeneic hematopoietic stem cell transplantation in acute myeloid leukemia and myelodysplastic syndrome.

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This study was performed to assess the utility of tumor-derived fragmentary DNA, or circulating tumor DNA (ctDNA), for identifying high-risk patients for relapse of acute myeloid leukemia and myelodysplastic syndrome (AML/MDS) after undergoing myeloablative allogeneic hematopoietic stem cell transplantation (alloSCT). We retrospectively collected tumor and available matched serum samples at diagnosis and 1 and 3 months post-alloSCT from 53 patients with AML/MDS. After identifying driver mutations in 51 patients using next-generation sequencing, we designed at least 1 personalized digital polymerase chain reaction assay per case. Diagnostic ctDNA and matched tumor DNA exhibited excellent correlations with variant allele frequencies. Sixteen patients relapsed after a median of 7 months post-alloSCT. Both mutation persistence (MP) in bone marrow (BM) at 1 and 3 months post-alloSCT and corresponding ctDNA persistence (CP) in the matched serum (MP1 and MP3; CP1 and CP3, respectively) were comparably associated with higher 3-year cumulative incidence of relapse (CIR) rates (MP1 vs non-MP1, 72.9% vs 13.8% [P = .0012]; CP1 vs non-CP1, 65.6% vs 9.0% [P = .0002]; MP3 vs non-MP3, 80% vs 11.6% [P = .0002];

CP3 vs non-CP3, 71.4% vs 8.4% [$P < .0001$]). We subsequently evaluated whether subset analysis of patients with 3 genes associated with clonal hematopoiesis, *DNMT3A*, *TET2*, and *ASXL1* (DTA), could also be helpful in relapse prediction. As a result, CP based on DTA gene mutations also had the prognostic effect on CIR. These results, for the first time, support the utility of ctDNA as a noninvasive prognostic biomarker in patients with AML/MDS undergoing alloSCT.

5. Combined inhibition of HDAC and AKT as a strategy to overcome multi-drug resistance in patients with multiple myeloma

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Patients with multiple myeloma (MM) have multiple choices of therapy including monoclonal antibodies, proteasome inhibitors, and immunomodulatory drugs (IMiDs), whereas some patients still develop resistance to these drugs and require novel therapeutic modalities. Here, we focused on inhibition of HDAC and AKT to overcome drug resistance. Lenalidomide (Len) selectively binds to cereblon (CRBN), which mediates recruitment of specific substrates like IKZF1 to E3 ubiquitin ligase and subsequent degradation, resulting in downregulation of IRF-4 and c-Myc. Then, we developed Len-resistant myeloma cells by RNAi-mediated downregulation of CRBN. Treatment of these cells with HDAC inhibitors reduced IKZF1 mRNA, suggesting potential efficacy of HDAC inhibitors against CRBN-low expressing or mutated MM. According to the integrated database for expression profile and disease prognosis (GenomicScape, http://www.genomicscape.com), higher expression of MICA was significantly associated with better overall survival in MM. MICA is an NK cell-activating ligand and plays an important role in ADCC. We observed that ADCC activity of both daratumumab and elotuzumab against MM cells was enhanced in the presence of HDAC inhibitors, which was compatible with our previous data that HDAC inhibitors upregulated MICA mRNA expression via inhibition of IKZF1 (ASH2018 abstract #4435). We also observed that HDAC inhibitors upregulated MICA mRNA in CRBN-deficient cells, suggesting promise of the combination of HDAC inhibitors and monoclonal antibodies against Len-resistant MM. Len-resistance is also affected by phosphorylation status of GSK-3. PI3K/AKT pathway is frequently activated in MM cells, and AKT inactivates GSK-3 by direct phosphorylation, resulting in c-Myc stabilization. Enhanced phosphorylation of GSK-3 was observed in CRBN-deficient H929 (MM) cells after long-term culture with Len, and such a phosphorylation status of GSK-3 was correlated with less CRBN amount and higher Len concentration (Figure 1). Afuresertib, an AKT inhibitor, suppressed GSK-3 phosphorylation (p-GSK-3) with or without ACY-1215, an HDAC inhibitor, leading to a substantial decrease of c-Myc (Figure 2). On the other hand, CHIR 99021, a GSK-3 inhibitor, partially counteracted to cytotoxic effect of afuresertib on H929 cells (Figure 3). These results suggest that increased p-GSK-3 is involved in acquired Len-resistance, and that combined inhibition of HDAC and AKT can overcome Len-resistance through decreased p-GSK-3. Furthermore, we examined the efficacy of CUDC-907, a dual HDAC and PI3K inhibitor. CUDC-907 had a cytotoxic effect on the MM cell lines including those had low CRBN expression. Bortezomib, doxorubicin, and dexamethasone resistant MM cell lines were also sensitive to CUDC-907. CUDC-907 upregulated MICA mRNA expression, but downregulated IKZF1 mRNA expression. Treatment of RPMI-8226 cells with CUDC-907 enhanced the ADCC activity of daratumumab (Figure 4). Furthermore, CUDC-907 was effective on primary MM cells which were resistant to bortezomib and Len (Figure 5). Thus, dual inhibition of HDAC and AKT with or without monoclonal antibodies is a promising therapeutic approach to multi-drug resistant MM.

6. Genomic Analysis of therapy-related myeloid neoplasms and tracking of he founder clone by circulating tumor DNA (ctDNA)

Kondo K[1], Yokoyama K[2], Yusa N[3], Nakamura S[1], Ogawa M[1], Takei T[1], Kobayashi A[1], Ito M[1], Shimizu E[4], Kasajima R[5], Wada Y[6], Yamaguchi R[4], Imoto S[5], Nagamura-Inoue T[6], Uchimaru K[2], Miyano S[4], Tojo A[1, 2].

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We retrospectively collected tumor samples, including bone marrow (BM), tumor-rich peripheral blood (PB), or alternatively, serum samples, at diagnosis and before diagnosis from 15 tMNs patients in our hospital. We subjected tumor DNA and control buccal swab DNA to comparative whole-exome sequencing (WES) and/or whole-genome sequencing (WGS). After identifying somatic driver mutations, we designed droplet digital PCR (ddPCR) assays for each mutation identified. All 15 patients had a history

of primary hematological malignancies (malignant lymphoma, n=9; acute leukemia, n=4; multiple myeloma, n=2) and had received prior chemotherapy and/or radiotherapy with or without autologous stem cell transplantation (ASCT). The median age at presentation of tMNs was 53 years (range, 6-74), and the median latent period between prior malignancy and tMNs was 45 months (range, 10-161). Conventional cytogenetic analysis revealed high incidence of complex karyotype (38.4%) and *MLL* rearrangement (30.7%). WES and/or WGS revealed that 93.3% (n=14/15) of the cases contained at least one putative driver mutation in 17 genes (median of 1 mutation per patient [range 1-4]). The most frequent mutations found in *TP53* and epigenetic modifier gene (*KMT2D*/*KDM6A*/*ASXL1*/*ASXL2*), mutated in 33% of the samples, followed by signal transduction proteins (*MPL*/*BRAF*/*FLT3-TKD*/*KRAS*, 26.7%). On the other hand, none of the patients with tMNs had mutations characteristic for *de novo*-AML or -MDS (e.g., *FLT3-ITD*, *NPM1*, or spliceosome factors). Together, the spectrum of driver mutations in our cohort was consistent with previous reports in tMNs. We tried to trace back mutant clone using BM and/or serum before diagnosis of tMNs in 2 patients. In UPN-5 who developed MDS-EB1 after ASCT for lymphoma, *ETV6* p.E153fs, a putative founder mutation of tMNs, was applied to liquid biopsy to trace back. *ETV6* ctDNA could be detected as early as 7 months prior to the development of MDS with variant allele frequency (VAF) of 0.06% (blue arrowhead in figure 1A). Most intriguingly, the proportion of *ETV6* ctDNA varied with or without G-CSF administration during the clinical course; VAF increase from 0 to 47.0% on G-CSF and decrease from 47.0 to 1.2% off G-CSF. In UPN-10 who had been clinically diagnosed as tMNs (MDS-EB2) after intensive chemotherapy for prior AML, not otherwise specified with normal karyotype, WGS combined with WES identified four driver mutations in BM at diagnosis of tMNs. Then, four driver mutations, *WT1* p.A365fs, *MLL* rearrangement, inv(3), and del(20q) were all applied to combined analysis of ctDNA and BM as well. Unexpectedly, we could find the presence of the founder clone, inv(3), with high allele burden in BM at initial diagnosis of AML-NOS with normal karyotype. On the contrary, we could not detect other three genes alterations until 4 months before diagnosis of tMNs. These findings would contribute to outline the genetic landscape of tMNs, and especially suggest the role of cytokine-related selective pressures after chemotherapy and of the potential pre-tMNs conditions in the pathogenesis of tMNs.

7. Utility of whole exome sequencing of ctDNA in drug-resistant and/or advanced phase chronic myeloid leukemia

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Despite the success of tyrosine kinase inhibitors (TKIs), some patients with chronic myeloid leukemia (CML) reveal drug resistance and/or progress to advanced phase, which is partly attributed to pre-existing or acquired genomic alterations, separate from the *BCR-ABL1* gene. Circulating tumor DNA (ctDNA) is now applied to a less-invasive and unbiased evaluation of cancer-associated mutations in various malignancies. Herein, we performed comparative whole exome sequencing of bone marrow (BM) and matched ctDNA from 10 patients with CML, most of which were TKI-resistant: chronic phase (CP, n=5); accelerated phase (AP, n=1); blast phase (BC, n=4). We identified seven mutations in two BC, one AP, and one CP patients. These include *ABL1 (2)*, *ASXL1* (3), *SETD2* (1), and *TP53* (1), among which six mutations were shared between BM and ~~matched~~ ctDNA, but the *TP53* mutation was solely detected in ctDNA. Intriguingly, all the patients with these mutations exhibited poor prognosis following TKI therapy (dead, n=3; relapse, n=1). Our results suggest that ctDNA may be comparable to BM in evaluating genomic alterations associated with TKI-resistance and/or disease progression in CML.

8. Granulocyte colony-stimulating factor-asociated aortitis in the Japanes Adverse Drug Event Report database.

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Granulocyte colony-stimulating factor (G-CSF) is the standard-of-care therapy for chemotherapy-associated neutropenia in patients with malignancies. Recent case reports have implied that G-CSF treatment may be associated with the development of aortitis, but the precise nature of the relationship is unclear. We investigated the association between G-CSF and risk for aortitis in patients with various malignancies. We performed an observational study of 102,014 subjects with malignant neoplasms documented in the Japanese Adverse Drug Event Report (JADER) database between April 2004 and February 2018. The ad-

justed odds ratio (OR) and 95% confidence interval (CI) for aortitis in patients treated and not treated with G-CSF were estimated using multivariate logistic regression with R software. Among the 102,014 subjects, 25 developed aortitis. Of the 3409 and 98,630 subjects treated and not treated with G-CSF, 16 (0.47% [95% CI; 0.27, 0.76]) and 9 (0.01% [0.00, 0.02]) developed aortitis, respectively. Multivariate logistic regression indicated an association between G-CSF and aortitis (adjusted OR 45.87 [19.16, 109.8], $p < 0.001$). The values for filgrastim, pegfilgrastim, and lenograstim were 0.25% (0.07, 0.63), 1.58% (0.79, 2.81), and 0.24% (0.05, 0.69), respectively. G-CSF treatment was associated with a signal of increased risk for aortitis among patients with malignant neoplasms. Three different G-CSF agents were associated with such risk, implying that it is a class effect. However, we do not recommend changing G-CSF prescriptions, because our results may have been influenced by the limitations of the JADER database and because the benefit of G-CSF treatment outweighs the potential risk.

9. T memory stem cells after allogeneic haematopoietic cell transplantation: unique long-term kinetics and influence of chronic graft-versus-host disease.

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T memory stem cells (TSCMs) are a subset of primitive T cells capable of both self-renewal and differentiation into all subsets of memory and effector T cells. Therefore, TSCMs may play a role in immune reconstitution and graft-versus-host disease (GVHD) in patients receiving allogeneic haematopoietic cell transplantation (HCT). We conducted a cross-sectional study to evaluate the proportions, absolute counts, phenotypes and functions of TSCMs in 152 adult patients without disease recurrence at least 12 months after undergoing HCT. $CD4^+$ TSCMs were negatively correlated with number of months after transplantation in HCT patients that received cord blood transplantation, but not in patients that received bone marrow transplantation or peripheral blood stem cell transplantation. The proportions and absolute counts of $CD4^+$ TSCMs and expression levels of inducible co-stimulator (ICOS) in $CD8^+$ TSCMs were significantly higher in patients with mild and moderate/severe cGVHD compared to patients without cGVHD. These data suggested that, more than 12 months after allogeneic HCT, the kinetics of $CD4^+$ TSCMs were dependent on the type of donor source, and further that $CD4^+$ TSCMs and ICOS levels in $CD8^+$ TSCMs were associated with cGVHD.

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