PROGRESS REPORT
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T cells and B cells called lymphocytes play essential roles in immune system. These lymphocytes are generated from hematopoietic stem cells (HSCs) which reside and are maintained in bone marrow (BM) throughout life. HSCs differentiate into T cells in the thymus and B cells in BM through successive series of lineage decision processes. Transcription factors (TFs) act in concert with epigenetic modifiers to regulate gene expression patterns that govern lymphocyte cell fates. Developmental disorders caused by the inactivation or acceleration of the key regulators often lead to the hematological malignancies, such as leukemia and lymphoma.

We have previously established a system that can be used to examine gene regulatory networks during lymphoid lineage specification from HSCs. We overexpressed Id3 protein fused with ERT2 (Estrogen receptor) protein, whose nuclear translocation is induced by 4-hydroxytamoxifen (4-OHT), in hematopoietic progenitors and cultured them in B cell differentiation conditions. B cell differentiation of Id3-transduced cells was blocked at an early developmental stage, but the cells grew enormously and maintained multipotency in the presence of 4-OHT (Ikawa et al. Stem Cell Reports, 2015). We named these multipotent progenitors induced leukocyte stem (iLS) cells. On the other hand, we also established T/NK progenitors which maintain the differentiation potential mainly for T and NK cells by culturing HSCs on OP9 feeder cells overexpressing Notch ligand, delta-like-1 (OP9/DLL1) in the presence of high concentration of cytokines (Ikawa et al. Science, 2010).

These novel systems enabled the analysis of a large set of regulatory molecules that control the generation of T and B lymphocytes. We have recently discovered the transcriptional network operative during B lineage commitment (Miyai et al. Genes Dev. 2018). The system can also be applied for *ex vivo* expansion of human hematopoietic stem/progenitors, which will be required for immune cell therapy or transplantation of HSCs. Thus, the aims of our study are 1) from a basic science perspective, to elucidate the mechanisms that orchestrate cell fate specification, commitment and differentiation during normal and neoplastic development of lymphocytes and 2) from a clinical medicine perspective, to establish a novel method to generate genetically-engineered lymphocytes such as chimeric antigen receptor (CAR)-T cells or CAR-NK cells using self-renewing hematopoietic progenitors such as iLS cells and T/NK progenitors for the development of immune cell therapy as a clinical strategy and to discover novel drugs for hematopoietic malignancies.

Roles of Polycomb Group Proteins in HSC development

Polycomb group (PcG) proteins are epigenetic chromatin modifiers that are important for maintaining cellular identities in stem cells mainly by regulating development- and differentiation-related genes that in general associate with CpG islands (CGIs) and are also linked to tumorigenesis. PcG proteins function by forming at least two catalytically different forms of multimeric protein complexes, namely Polycomb repressive complexes 1 (PRC1) and PRC2, which mediates mono-ubiquitination of

histone H2A at lysine 119 (H2AK119ub1) and trimethylation of the histone H3 at lysine 27 (H3K27me3), respectively. PRC1 has six alternative complexes depending on PCGF proteins (PRC1.1-1.6). It is previously shown that the PCGF4 is essential for the maintenance of HSCs, while physiological roles and functional relevance of each PRC1 complexes remain elusive. Thus, we are working on the roles of PCGF proteins during hematopoietic and immune cell development using several Cre-lox systems.

We have recently demonstrated that the PCGF1-PRC1 regulates DNA replication-coupled process to safeguard B cell fate specification (Takano et al. Nat Commun. 2022).

To examine whether PCGF1 has specific functions in B cell differentiation, we made B-cell specific PCGF1-deficient mice (mb1-cre PCGF1 fl/fl mice). Surprisingly, BM and spleen of the conditional PCGF1-deficient mice have normal numbers of B cells, indicating that the PCGF1 is dispensable for B cell differentiation after the commitment to the B cell lineage.

We next addressed whether PCGF1, 3, and 5 have redundant function during B cell development. Analysis of the B-cell specific PCGF/1/3/5-triple knock out (TKO) mice (mb1-cre PCGF1/3/5 fl/fl mice) at 2 months old revealed a slight reduction of $CD19^+$ B cells in BM. The B cell differentiation is mainly blocked at pro-B stage. In contrast, the number of B cells in spleen is severely reduced in the PCGF1/3/5 TKO mice. Of note, the pro-B cells were enormously expanded in BM and spleen of the TKO mice at 4 months old. The spleen was enlarged and pro-B ALL-like cells were occupied. The number of IgM^+IgD^+ mature B cells was reduced and the germinal center was completely diminished. The pro-B ALL-like cells had limited numbers of V_H-DJ_H rearrangement of IgH locus, indicating the oligoclonal expansion of the TKO pro-B cells. The RNA-seq analysis of the PCGF1/3/5 TKO pro-B cells demonstrated that the reduced expression of B-lineage associated genes, *Ebf1* and *Pax5*, whereas the expression of genes involved in inflammation and immune regulation was significantly upregulated. Moreover, tumor-associated genes, *Myc* and *Lmo2* were upregulated. These results indicated that PCGF1/3/5 are important for normal B cell differentiation and play key roles as a tumor-suppressor gene.

To determine the role of canonical PRC1 in the hematopoietic system, ERT2-Cre-$PCGF2^{fl/fl}$ $PCGF4^{fl/fl}$ mice were generated. The BM cells of ERT2-Cre-$PCGF2^{fl/fl}$ $PCGF4^{fl/fl}$ mice (CD45.2) were transferred to lethally irradiated CD45.1 mice. After 4 weeks of transplantation, tamoxifen was administrated into the mice to delete PCGF2/4 alleles of the hematopoietic cells. The mice died around 5-6 weeks of tamoxifen injection. The absolute numbers of BM and spleen derived from PCGF2/4-deficient hematopoietic cells were two- to three-fold reduced compared to PCGF2/4-sufficient hematopoietic cells of the transplanted mice. We were not able to harvest T cells in the thymus due to thymus atrophy. Flow cytometric analysis of BM and spleen of the transplanted mice just before dead demonstrated the profound reduction of hematopoietic stem and progenitor cells (HSPCs) as well as mature hematopoietic cells. Especially, $Ter119^+$ erythroid lineage cells from PCGF2/4 deficient cells were dramatically reduced, indicating that the mice died due to anemia. The number of B cells was also severely reduced in BM and spleen. B cell differentiation was blocked at pro-B cells in BM. These results indicated that the PCGF2/4 are critical for the self-renewal activity and the differentiation of HSPCs.

These studies will dissect the functional relevance between canonical PRC1 and non-canonical PRC1 during hematopoietic and lymphocyte development.

Collaborators:

Sho Wakatsuki, Mizuki Sakihara, and Mayumi Hirakawa

Molecular mechanisms of B-precursor acute lymphoblastic leukemia (B-ALL) development

B-precursor acute lymphoblastic leukemia (B-ALL) is most common childhood tumors and the leading cause of cancer-related death in children and young adults. B-ALL is generated by a block in B cell differentiation, leading to accumulation of immature progenitor cells in BM, peripheral blood and occasionally the central nervous system. B-ALL represents about 85% of ALL cases, whereas the remaining 15% of the cases involve T-lineage ALL (T-ALL). A number of recurring chromosomal rearrangements are common in B-ALL and are critical events in leukemogenesis.

These rearrangements commonly perturb genes encoding regulators of hematopoiesis, tumor suppressors, oncogenes, or tyrosine kinases but commonly require additional genetic hits to establish the full leukemic phenotype. However, it is evident from experimental mouse models and through extensive genomic profiling of patient samples that these genetic driver lesions are usually insufficient to induce leukemia and require cooperating events. Thus, it remains to be determined how the chromosomal rearrangements trigger the induction of leukemia and generate pre-leukemic cells.

The translocation t(17;19)(q22;p13) results in the fusion of the transcriptional activation domain of the TF TCF3 (E2A) to the DNA-binding domain of HLF, (*TCF3::HLF*), defines a rare subtype of ALL (~0.5% of pediatric ALL) that is typically associated with relapse and death within two years from diagnosis. On the other hand, the translocation t(1;19)(q23;p13) results in a fusion of the gene, *TCF3::PBX1*, which occurs in about 5-10% of B-ALL patients. Previous studies indicated that possible direct targets of *TCF3::HLF* include the TF *Lmo2*, the transcriptional repressor *Snai1 (Slug)* and *Bcl2*. The *TCF3::HLF* fusion likely requires additional events to cause leukemia, because *TCF3::HLF* transgenic and knock-in mice did not recapitulate the human phenotype.

To understand the molecular mechanisms underlying the initiation of *TCF3::HLF* positive B-ALL, we have established a novel culture system. As described above, we have previously developed an inducible B cell differentiation system using iLS cells. The iLS cells are multipotent progenitors with lymphoid and myeloid lineage potential, which are maintained *in vitro* in the presence of 4-OHT, whereas they differentiate into B cells upon withdrawal of 4-OHT within 6 days. We retrovirally transduced the *TCF3::HLF* into iLS cells. The *TCF3::HLF*-transduced iLS cells proliferated well and maintained in the presence of 4-OHT just like normal iLS cells. However, the B cell differentiation potential was severely inhibited in the *TCF3::HLF*-transduced iLS cells.

To examine the impact of *TCF3::HLF*-transduced iLS cells or *TCF3::PBX1*-transduced iLS cells on leukemogenesis *in vivo*, we intravenously transferred the cells into sublethally-irradiated B6 mice. The mice transplanted with either *TCF3::HLF*-transduced iLS cells or *TCF3::PBX1*-transduced iLS cells succumbed to leukemia, displaying splenomegaly and leukemia infiltration into multiple organs at around 12 weeks of injection (*TCF3::HLF*) and 16 weeks of injection (*TCF3::PBX1*), respectively. BM, spleen and lymph nodes in the mice were infiltrated with immature blast cell population. Flow cytometric analysis revealed that most of the cells were pro-B or pre-B cells, indicating the generation of B lymphoid blasts.

To determine the molecular mechanisms of B-ALL development, RNA-seq analysis was performed. The data demonstrated that the expressions of several inflammatory cytokines, including *Il1b, Il6* and *Ifng*, were significantly upregulated in *TCF3::HLF* B-ALL cells. Public transcriptomic data using pediatric clinical samples also showed a higher expression of IL1B in *TCF3::HLF* B-ALL compared to other B-ALL.

Deletion of IL1B by using CRISPR-Cas9

system or a blocking antibody against IL1B strongly restrained the growth of YCUB2 cells (a *TCF3::HLF* B-ALL cell line), suggesting that the IL1B contributed to the aggressive growth of *TCF3::HLF* B-ALL.

These results indicate that inflammatory cytokines may promote the development and proliferation of *TCF3::HLF* B-ALL and could be a novel therapeutic target of B-ALL

Collaborators:

Aisa Suzuki, Hitomi Otomo, Tsukasa Shigehiro, Masatoshi Takagi (Tokyo medical and dental Univ.), and Takeshi Inukai (Yamanashi Univ.)

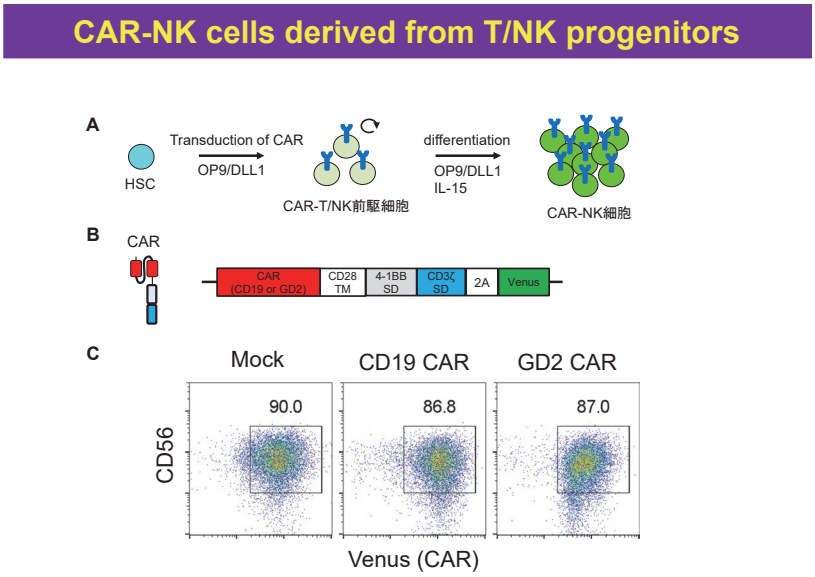
Development of a novel immunotherapy using self-renewing hematopoietic progenitors

CAR-T cells and CAR-NK cells are promising immune cell therapies for cancer treatment. Compared to CAR-T cells, CAR-NK cells could offer some significant advantages, including: (1) safer with minimal cytokine syndrome and less possibilities of graft-versus-host disease, (2) natural cytotoxic activities, and (3) high feasibility for "off-the-shelf" manufacturing. However, there are several barriers to clinical application of CAR-NK cells, such as mass production of NK cells and the long-term cytotoxicities of CAR-NK cells *in vivo*.

We have developed a method to produce a large number of NK cells by expanding T/NK cell progenitors derived from HSPCs in mouse BM or human cord blood (hCB) (Ikawa et al. Science, 2010; Unpublished). The T/NK progenitors were efficiently generated by culturing HSPCs of hCB on OP9/DLL1 cells in the presence of various cytokines. T/NK progenitors were expanded and maintained *in vitro* for at least several months and the cells easily differentiated into NK cells within 7 days. Of note, T/NK progenitors retained a potential to generate at least 200 times more NK cells than hCB HSPCs. The human NK cells generated from T/NK progenitors were activated by the NK cell target, K562 cells. We succeeded in generating CAR-NK cells against CD19 from T/NK progenitors derived from hCB HSPCs. The CD19 CAR-NK cells had normal cytotoxic activities against B-ALL cell line, YCUB2 or NALM6. Thus, these CAR-NK cells derived from T/NK progenitors will provide novel "off-the-shelf" lymphocytes for cancer immunotherapy.

Collaborators:

Yuki Narita, Shogo Tanimori, Hiroyuki Kadota and Tsukasa Shigehiro



Publications
Original articles

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3. Ogawa T, Ochiai K, Iwata T, **Ikawa T**, Tsuzuki T, Shiroguchi K and Takahashi K. Different cell imaging methods did not significantly improve immune cell image classification performance. ***PLOS ONE.*** 17(1):e0262397, 2022