PROGRESS REPORT
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Lymphocytes including T cells, B cells and NK cells 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 and NK 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, B and NK cells. We 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, CAR-NK cells, or TCR-T cells using self-renewing hematopoietic progenitors, which will lead to groundbreaking advancements in cancer treatment in the future.

Roles of Polycomb Group Proteins in the hematopoietic system

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 (one of non-canonical PRC1) regulates DNA replication-coupled process to safeguard B cell fate specification (Takano et al. Nat Commun. 2022). We also found that PCGF1/3/5 are important for normal B cell differentiation and play key roles as a tumor-suppressor gene using the B-cell specific PCGF/1/3/5-triple knock out (TKO) mice (mb1-cre PCGF1/3/5 fl/fl mice), suggesting that non-canonical PRC1 works together to promote B cell commitment and differentiation (manuscript in preparation).

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 conditional PCGF2/4-double knockout (cDKO) mice died around 5-6 weeks of tamoxifen injection. The absolute numbers of BM and spleen derived from cDKO hematopoietic cells were two- to three-fold reduced compared to PCGF2/4-sufficient hematopoietic cells of the transplanted mice. Of note, we were not able to harvest cDKO T cells in the thymus due to thymus atrophy. Flow cytometric analysis of BM and spleen of the cDKO 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 as well as Megakaryocyte-Erythroid progenitor (MEP) cells from cDKO BM were dramatically reduced, indicating that the mice died due to anemia. The number of cDKO B cells was also severely reduced in BM and spleen. B cell differentiation was blocked at pro-B cells in BM. To determine the involvement of the cell cycle inhibitor *Cdkn2a*, which is one of the target genes of PCGF4, ERT2-Cre PCGF2$^{fl/fl}$PCGF4$^{fl/fl}$CDKN2A$^{-/-}$ conditional triple knockout (cTKO) mice were generated. Intriguingly, they represented a substantial recovery in the number of lymphocytes but not in long term HSCs and MEPs, suggesting that there must have other targets in the maintenance of HSCs and MEP differentiation. RNA-seq analysis demonstrated that red blood cell-related genes were downregulated in erythroid progenitors of the cTKO mice. These results indicated that the PCGF2/4 act in together in the maintenance of HSCs and differentiation of HSPCs toward erythroid cells.

Our studies will dissect the functional relevance between canonical PRC1 and non-canonical PRC1 during hematopoietic and immune cell development.

Collaborators:

Sho Wakatsuki, Yutaro Ohashi, 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. Chromatin immunoprecipitation (ChIP)-seq analysis revealed that *TCF3::HLF* bound to a previously-unrecognized regulatory region of the IL1B gene locus, and deletion of this B-ALL specific enhancer region dramatically reduced IL1B expression, leading to cell death of *TCF3::HLF* ALL.

Taken together, our findings highlight the functional importance of inflammatory cytokines,

particularly IL-1β, in the progression and exacerbation of *TCF3::HLF* ALL, offering new therapeutic strategies for this aggressive and fatal disease.

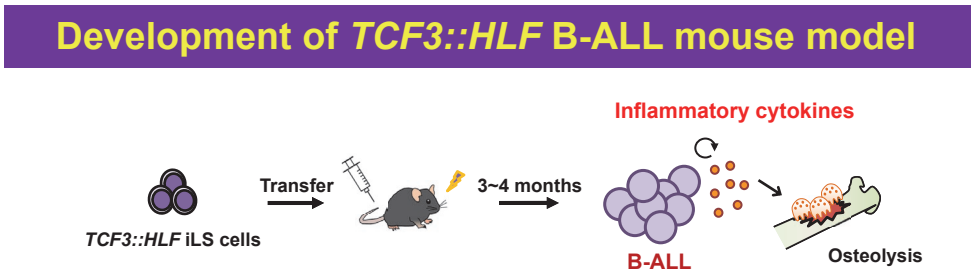
Collaborators:

Aisa Suzuki, Daisuke Sato, 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 have demonstrated significant success in treatment of certain hematologic malignancies. Compared to CAR-T cells, CAR-NK cells offer several distinct advantages, including: (1) safer with minimal cytokine release syndrome and neurotoxicity and less possibilities of graft-versus-host disease, (2) natural antitumor activities, and (3) high feasibility for "off-the-shelf" manufacturing. However, they still face several limitations for clinical application, such as mass production of NK cells and the persistency of CAR-NK cells *in vivo*.

We previously developed a method to produce a large number of NK cells by expanding T/NK progenitor (pT/NK) cells derived from HSPCs in mouse BM or human cord blood (hCB) (Ikawa et al. Science, 2010; Unpublished). The pT/NK cells were efficiently generated by culturing HSPCs of hCB on OP9/DLL1 cells in the presence of various cytokines. pT/NK cells were expanded and maintained *in vitro* for at least several months and the cells easily differentiated into NK cells within 7 days. Of note, pT/NK cells retained a potential to generate at least 200 times more NK cells than hCB HSPCs. The human NK cells generated from pT/NK cells exhibited high cytotoxic activity against the NK cell target, K562 cells at a comparable level to NK cels from peripheral blood or CB. Furthermore, we succeeded in generating CAR-NK cells against CD19 from pT/NK cells derived from hCB HSPCs. The CD19 CAR-NK cells had normal cytotoxic activities against B-ALL cell line, NALM6. To determine the antitumor activity of pT/NK-derived CAR-NK cells against solid tumors, we generated HER2 (human epidermal growth factor receptor type2) CAR-NK cells from hCB-derived pT/NK cells. The HER2 CAR-NK cells showed a significant antitumor activity



against HER2-positive MDA-MB-453 cells both in vitro and in vivo. Thus, the pT/NK cells will provide a fundamental source for "off-the-shelf" CAR-NK cell therapy.

Collaborators:

Shogo Tanimori, Hiroyuki Kadota, Jia Han, Karin Noma and Tsukasa Shigehiro

Publications

Original articles

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2. Nakajima-Takagi Y, Oshima M, Takano J, Koide S, Itokawa N, Uemura S, Yamashita M, Andoh S, Aoyama K, Isshiki Y, Shinoda D, Saraya A, Arai F, Yamaguchi K, Furukawa Y, Koseki H, **Ikawa T**, and Iwama A. Polycomb repressive complex 1.1 coodinates homeostatic and emergency myelopoiesis. ***eLife***. 12:e83004, 2023

3. Jin J, Ogawa T, Hojo N, Kryukov K, Shimiz K, **Ikawa T**, Imanishi T, Okazaki T, and Shiroguchi K. Robotic data acquisition with deep learning enables cell image-based prediction of transcriptomic phenotypes. ***PNAS***. 120(1):e2210283120. doi: 10.1073/pnas.2210283120, 2023