National Institutes of Health

National Institute of Diabetes and Digestive and Kidney Diseases

The Science of Conditioning

Virtual Meeting
January 25, 2024

SUMMARY

Background and Overview

On January 25, 2024, the National Institutes of Health (NIH) sponsored a workshop on the Science of Conditioning, hosted by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Research into the treatment of nonmalignant hematological disease using hematopoietic stem cell (HSC) transplant has seen explosive growth during the past decade. Recent outcomes of clinical trials combining HSC transplantation (HSCT) and genome engineering to correct specific genetic defects have highlighted the critical need to understand the impact of bone marrow (BM) conditioning therapy on the microenvironment in these hematological disorders. NIDDK convened clinicians and researchers to share recent insights into the effects of current conditioning regimens, which can result in deleterious effects in patients with nonmalignant hematological disorders. The workshop highlighted emerging discoveries related to innovative conditioning regimens, as well as gaps in the science regarding how conditioning therapies affect both the BM microenvironment and the ability to graft HSCs. More than 110 participants attended the workshop.

Session I: Welcome Remarks and Introductions

Griffin P. Rodgers, M.D., M.A.C.P., Director, NIDDK, NIH
Shilpa Hattangadi, M.D., Program Director, Division of Kidney, Urologic, and Hematologic Diseases (KUH), NIDDK, NIH

NIDDK Director Griffin P. Rodgers, M.D., MACP, welcomed the participants. He noted the objective of the meeting, which is to provide a platform for establishing productive collaborations and stimulating new ideas to enable substantial progress in understanding the role of BM conditioning in outcomes of nonmalignant hematological diseases. The information shared in the meeting will have the potential to address ongoing challenges in the hematology field and improve the treatment of those living with hematological diseases. Dr. Rodgers expressed appreciation for the opportunity to convene leaders from different fields of research to consider new combinations of expertise, resources, and tools, and he thanked colleagues from the National Heart, Lung, and Blood Institute (NHLBI) and the National Institute on Aging (NIA) for their assistance in planning the meeting. He requested that the participants consider the possibility of collaborations that will lead to Multiple Principal Investigators applications and encouraged them to connect with NIDDK program staff to discuss funding opportunities, areas of mutual interest, and specific needs for this research area.

Dr. Shilpa Hattangadi, Program Director, KUH, NIDDK, thanked the planning committee—including the external organizers, Drs. Agnieszka Czechowicz and Matthew Porteus, Stanford University, and Dr. Andre Larochelle, NHLBI. She also thanked the team of NIH organizers who assisted her with the internal planning: Dr. Brian Bai, NHLBI; Dr. Cindy Roy, NIDDK; and Dr. John Williams, NIA. Dr. Hattangadi emphasized that those present—including NIH program staff, intramural investigators, NIH leadership, and members of the academic community and nonprofit and for-profit organizations—are listening to input from the meeting regarding areas of the field that require further development,

barriers to expanding the field, and possible needs and directions for the future state of the science. She listed three specific questions for participants to address:

1. What are the barriers confronted when examining the science of conditioning therapies used in HSCT for nonmalignant hematopoietic diseases?

2. What are the gaps in our knowledge of the effects of both novel and traditional conditioning regimens on the BM microenvironment and ability to engraft HSCs?

3. What tools, resources, or expertise might be needed for the hematology community to uncover new pathways of research to answer these open questions and close these gaps?

Dr. Hattangadi highlighted the Stimulating Hematology Investigation: New Endeavors (SHINE) programs developed by NIDDK, which were established in 2010 with support from NHLBI and NIA. The goals of SHINE are to recruit new investigators into nonmalignant hematology research, support emerging fields of related research, and build the field of classical hematology. Dr. Hattangadi noted that outcomes from the current meeting might result in a new SHINE notice of funding opportunity. She provided an overview of the day's agenda and encouraged all attendees to contribute to the discussion. She added that the success of the meeting will be measured by the number of novel hypotheses generated, the formation of new interdisciplinary teams, and an increase in hematology grant applications.

Session II: Setting the Stage—Selecting Conditioning Regimens for Specific Clinical Disorders

Suneet Agarwal, M.D., Ph.D., Co–Program Leader for the Stem Cell Transplant Center, Dana–Farber/ Boston Children's Cancer and Blood Disorders Center

Dr. Suneet Agarwal, Dana–Farber/Boston Children's Cancer and Blood Disorders Center, provided a historical and clinical overview of conditioning for hematopoietic cell therapies (HCTs) in nonmalignant diseases. He noted that the hematopoietic cell transplant paradigm was developed in the context of treating leukemia. Initial conditioning regimens—developed in an era of protection experiments against nuclear war and chemical agents—were designed to use radiation or chemotherapy to kill tumor cells and suppress the immune system for allogeneic transplantation. Conventional HCT-conditioning agents include DNA-alkylating agents (e.g., busulfan, cyclophosphamide, melphalan, thiotepa, treosulfan) and ionizing radiation (e.g., total-body irradiation [TBI], lymphoid irradiation). These cell cycle–independent, DNA-damaging agents are included in every allogeneic transplant regimen aiming to achieve myeloid engraftment. Such treatments are effective but result in collateral tissue damage, provoke graft-versus-host disease (GvHD), and are associated with short- and long-term sequelae (e.g., mucositis, infertility, endocrinopathy, secondary malignancy). Nontargeted genotoxic agents are a major contributor to short- and long-term morbidity and mortality in transplant patients.

Next-generation targeted antibodies for lymphodepletion without systemic toxicity were developed in the 1970s and 1980s. These include anti-thymocyte and anti-lymphocyte globulins, which were developed to enable renal allografts and enabled engraftment in allosensitized BM transplant (BMT) patients, and the humanized monoclonal, alemtuzumab, which was used in preparation for solid organ transplantation and allogeneic HCT. In the 1990s, DNA synthesis inhibitors (e.g., purine nucleoside analogs) were introduced into HCT regimens to maintain immune suppression while decreasing toxicity in infirm leukemia patients. Currently, conditioning regimens of varying intensities are chosen based on the desired antitumor or graft-versus-leukemia effect, as well as the tolerance of the patient based on their age and condition.

Dr. Agarwal discussed the goals of conditioning in nonmalignant disorders. Without the need to consider antitumor effects, the desired outcome shifts to generating a supportive niche for the transplanted cells. The level of immune suppression also should be considered. Several nonmalignant disorders are associated with an intact HSC niche (e.g., hemoglobinopathies, certain metabolic disorders, Diamond–Blackfan anemia, severe congenital neutropenia) or an intact but dysregulated lymphoid system (e.g., disorders of immune dysregulation, autoimmune disorders) and have increased requirements for ablation. Other conditions are associated with a depleted HSC niche (e.g., aplastic anemia, Fanconi anemia, dyskeratosis congenita [DC]) or an absent lymphoid system (e.g., severe combined immunodeficiency [SCID]) and might have a reduced requirement for ablation.

Dr. Agarwal discussed his group's work related to improving transplant outcomes in DC patients. DC is a telomere biology disorder associated with BM failure and pulmonary disease. Conditioning toxicity has proved to be a major HCT barrier in these patients, who experience poor survival due to the overlap between DC pathology and HCT toxicity. A multicenter trial initiated in 2012 determined that DC patients benefitted significantly from the elimination of radiation and DNA-alkylating agents from conditioning regimens, achieving durable engraftment (i.e., 97 percent of patients engrafted by day 42 post-HCT); sustained donor myeloid chimerism (i.e., more than 50 percent donor chimerism in all patients by day 100 post-HCT); and reconstitution of donor T lymphocytes (i.e., mixed or full cluster of differentiation 3 [CD3] protein chimerism in all but one patient). Low rates of GvHD were observed in the transplant patients; all cases were resolved with steroid treatment. In this first series of allogeneic BMT patients to achieve durable myeloid engraftment without exposure to alkylating agents or radiation, patient survival rates (i.e., 5-year post-BMT survival) improved from 60 percent observed with traditional conditioning methods to 89 percent. Such regimens, however, likely would be limited to patients with nonmalignant disorders with a failing hematopoietic system or a depleted HSC niche.

Dr. Agarwal reviewed several cases of niche establishment in gene-modified HCT for nonmalignant diseases. A novel treatment for cerebral adrenoleukodystrophy involves busulfan conditioning to open nonhematopoietic niches and enable engraftment of gene-corrected HSC-derived cells with super-physiological levels of the ATP-binding cassette subfamily D member 1 (ABCD1) protein. However, multiple patients who received this treatment developed myelodysplastic syndrome (MDS)—likely because of insertional oncogenesis. Busulfan conditioning also is being used in the context of sickle cell disease to enable engraftment of HSCs expressing wild-type globin, and cases of MDS also have been observed in these patients. Challenges regarding the leukemogenic potential of HCT conditioning in nonmalignant diseases have yet to be addressed.

In summary, Dr. Agarwal listed the ultimate goals of HCT conditioning for nonmalignant diseases: (1) eradicate host myeloid and/or lymphoid cells without injury to other cells and without causing cancer; (2) create a niche, if needed, in nonhematopoietic tissues; (3) enable engraftment of engineered autologous HSCs; and (4) achieve immune suppression sufficient to prevent graft rejection while maintaining infection control in allogeneic HCT. He noted that these goals will require novel agents and regimens, the development of which would be discussed in later sessions.

Questions and Answers

In response to a question about crosstalk between HSCs and mesenchymal stem and progenitor cells (MSPCs), Dr. Agarwal commented that in disorders like myelofibrosis, researchers theorize that patients are difficult to engraft because their niche is dysfunctional. Whether this is because the mesenchymal stem cell population is unclear. Overall, it has been rare for patients to experience engraftment challenges caused by a mesenchymal stem cell–depleted niche.

Session III: Innovations in Conditioning

Preparing the Seed and Soil for Transplantation
Moderator: John Williams, Ph.D., Program Director, NIA, NIH

Illuminating the Functional Heterogeneity of the Bone Marrow Microenvironments
Anastasia Tikhonova, M.D., Ph.D., Assistant Professor, Department of Medical Biophysics, University of Toronto, Canada

Dr. Anastasia Tikhonova, University of Toronto, discussed recent advances and gaps in the understanding of the BM niche. Hematopoiesis, the dynamic process whereby HSCs give rise to immune and blood cells, occurs mainly in specialized BM niches. Based on imaging studies and functional evidence, two main elements—vascular endothelial cells and leptin receptor–positive ($Lepr^+$) MSPCs—are the main cellular contributors to the BM niche. Other cell types play a supportive role within the niche.

Dr. Tikhnova described her efforts to understand the heterogeneity of the BM niche, as well as its dynamic response to stress. Her team has used high-resolution, single-cell sequencing and protein studies to generate precise molecular maps of murine stem and progenitor niches. For example, the MSPC compartment can be divided into two major cell types: MSPCs expressing genes associated with adipogenesis (i.e., adipo-primed MSPCs) and MSPCs expressing genes associated with osteogenesis (i.e., osteo-primed MSPCs). Different types of MSPCs, as well as vascular endothelial cadherin–positive endothelial cells, were each shown to express a unique subset of pro-hematopoietic factors. Surprisingly, most of the niche cells were quiescent.

Dr. Tikhnova highlighted several other comprehensive studies of BM niche data sets but commented on the challenge of integrating results using inconsistent nomenclature from multiple experimental and analytical pipelines into a unified understanding of this microenvironment. To overcome this challenge, Dr. Tikhnova and a colleague performed a joint analysis of several murine data sets. An interactive browser of the integrated analysis is available at the Broad Institute Single Cell Portal. Current research efforts are focused on identifying niches for lymphoid and myeloid lineages.

Dr. Tikhnova described further studies of the murine BM niche following stress. Single-cell sequencing of the BM niche following treatment with 5-fluorouracil identified the perivascular cell population as particularly responsive to myoablation. Specifically, $LepR^+$ MSPCs underwent significant transcriptional reprogramming through the expansion of adipo-primed cluster, which was accompanied by a global reduction in osteolineage-associated gene expression. Stress-induced adipogenesis promotes stem cell regeneration and hematopoiesis via adipocyte secretion of stem cell factor (SCF), a critical growth factor necessary for HSCs. Following restoration of the BM niche, stress adipocytes dedifferentiate and return to their original MSPC state. Dr. Tikhnova emphasized that improving the efficiency of HSCT will require a deeper understanding of BM niche dynamics in the context of stress. Her group currently is investigating stress-mediated remodeling of the BM niche, which appears to respond differently to various chemotherapeutic agents.

The Other Side of the Coin: Engineering Hematopoietic Stem/Progenitor Cells (HSPCs) to Enhance Potency
Matthew Porteus, M.D., Sutardja Chuk Professor of Definitive and Curative Medicine, Stanford Medicine, Stanford University

Dr. Porteus emphasized that successful manipulation of HSCs can enable lifelong, durable re-engineering of the entire blood system. His research group focuses on the genetic engineering of HSCs using homology-directed repair (HDR), which can generate single-nucleotide changes and insert larger gene-

expression cassettes into precise locations in the genome. Recent studies by his group have demonstrated improvements in HDR (i.e., insertion/deletion-free gene correction) in the presence of AZD7648, a small molecule that inhibits the nonhomologous end joining pathway of DNA repair by blocking the activity of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Another group has shown that inhibition of p53-binding protein 1 (53BP1)—an important component of DNA double-strand break signaling—with a small peptide favors homology-dependent DNA repair and increases CRISPR-based genome editing efficiency.

One reason for favoring HDR-based genetic engineering is the ability to insert large portions of exogenous DNA. For example, members of Dr. Porteus's group performed whole-gene replacement (WGR) of *α-globin* with *β-globin* in β-thalassemia-derived human $CD34^+$ HSPCs, a twofold strategy that restored hemoglobin balance by increasing *β-globin* expression while simultaneously reducing *α-globin* overexpression and aggregate formation. The WGR experiments achieved a replacement efficiency of approximately 37 percent, even in the absence of small-molecule DNA repair inhibitors, but the cells failed to engraft successfully in immunodeficient mice.

Whereas future studies will aim to minimize challenges with engraftment, current studies aim to build a selective advantage into the targeted allele to enhance the potency of engrafted cells. For example, known mutations in the human *erythropoietin receptor* (*EpoR*) gene can be recreated in a murine model to confer an erythropoietin-dependent growth advantage to transgenic red blood cells (RBCs). When the *α-globin*/*β-globin* WGR was combined with the expression of mutant EpoR, modified cells exhibited a more than twofold selective advantage compared with cells that received only the *α-globin*/*β-globin* WGR. Conditions in which such a strategy might be used to reduce or eliminate chemotherapy include allogeneic HSCT for hemoglobinopathies (e.g., edit endogenous *EpoR* so that engrafted HSCs give rise to more RBCs than residual host HSCs), autologous settings for hemoglobinopathies (e.g., link *EpoR* to the therapeutic transgene), autologous settings for diseases in which RBCs could serve as a protein delivery vehicle, and other settings where HSCs can be conferred with selective advantages without increasing the probability of malignant transformation.

Stem Cell Fitness and Longevity: The Role of Proteostasis
Robert Signer, Ph.D., Associate Professor, Medicine, University of California, San Diego

Dr. Robert Signer, University of California, San Diego, discussed challenges associated with preserving and enhancing stem cell fitness while maximizing the likelihood of rapid and robust long-term engraftment. The role of protein homeostasis—or proteostasis—in regulating stem cell fitness and longevity has been an underappreciated parameter for maximizing transplant success. Proteostasis describes the biological mechanisms that regulate the quality of the proteome by balancing protein synthesis, folding, trafficking, and degradation along with several proteotoxic stress response pathways. Genetic or environmental interventions that enhance the capacity to maintain proteostasis extend organismal longevity in a manner conserved by evolution.

Evidence that proteostasis confers benefits at the HSC level is accumulating. Compared to restricted progenitor cells, HSCs exhibit low levels of protein synthesis and high levels of aggrephagy (a specialized form of autophagy) that are necessary to minimize the presence of misfolded and unfolded proteins and maintain proteostasis and fitness. Even modest increases in protein synthesis result in catastrophic effects on HSC self-renewal and reconstituting potential. When cultured in basic media for only 18 hours, HSCs upregulate translational machinery and exhibit an approximately 20-fold increase in protein synthesis and a substantial increase in misfolded proteins compared with steady-state HSCs growing *in vivo*. Activation of the heat shock response—via expression of heat shock factor 1 (HSF1) or treatment with small molecules that inhibit heat shock protein 90 (HSP90) or T-complex protein Ring Complex (TriC)—promotes *ex vivo* maintenance of HSC fitness.

Session Panel Discussion

Dr. Williams asked about sex-based differences in hematopoietic lineage determination, which could be caused by differences in bone biology. Dr. Tikhnova answered that her laboratory has noted a difference in the number of MPSCs between male and female mice. She agreed that differences likely exist in humans.

Alternatives to Chemotherapy-based Conditioning: Approaches Based on Antibodies
Moderator: Cindy Roy, Ph.D., Program Director, KUH, NIDDK, NIH

Earliest Work on CD117 (c-Kit) Antibody Conditioning: Innovation in Conditioning Approaches for HSCT
Agnieszka Czechowicz, M.D., Ph.D., Assistant Professor of Pediatrics (Stem Cell Transplantation), Stanford Medicine, Stanford University

Dr. Czechowicz discussed her group's efforts to develop novel antibody conditioning regimens. One barrier to HSCT is that immune eradication is insufficient for high engraftment of purified HSCs; HSCs compete with other HSCs and not similar progenitors, with notable engraftment saturation in unconditioned settings. HSC-targeted conditioning aims to create space for HSCs using targeted, nonmyeloablative treatments developed using such compounds as antibody–drug conjugates (ADCs), bispecific antibodies, T-cell engagers, or chimeric antigen receptor (CAR) T cells.

One prime HSC target protein is CD117 (also known as c-Kit), a transmembrane receptor with tyrosine kinase activity known to be expressed primarily on HSCs and hematopoietic progenitors and function as a receptor for SCF. Early studies of a monoclonal antibody (termed ACK2)—which binds murine CD117 and antagonizes SCF binding—demonstrated profound depletion of host HSCs and substantially increased engraftment following conditioning with ACK2. A second-generation version of the antibody, which was conjugated to a ribosome-inactivating drug, resulted in an even more profound depletion of host HSCs and even higher levels of engraftment in both immunocompromised and immunocompetent murine models. Other groups have experimented with ACK2-based conditioning regimens in combination with radiation, nucleoside analogs, or other antibodies. Additionally, conditioning regimens involving anti-CD117 antibodies are extremely safe and well tolerated. Dr. Czechowicz did note, however, that CD117 is expressed on mast cells in the lung and that antihistamine treatments were given before most experiments.

Dr. Czechowicz's group advanced efforts to develop the first conditioning regimen using an antibody against human CD117 (anti-hCD117). A library of anti-hCD117 monoclonal antibodies was generated, and an antibody with antagonist qualities similar to ACK2 (termed SR-1) was identified. Murine xenograft studies were used to verify that SR-1 depletes wild-type and MDS human HSCs in xenografted mice. Through a partnership with Amgen, a humanized SR-1 monoclonal named AMG191 (now renamed JSP191/briquilimab) has been shown to exhibit anti-HSC activity and has undergone safety, efficacy, and kinetic studies in nonhuman primates (NHPs). Given the promising safety and efficacy profiles of this method, several companies are developing similar conditioning programs based on CD117 antibodies or ADCs. Data from a recent trial suggest that a single dose of 0.6 mg/kg JSP191 is extremely well tolerated, depletes host HSCs, has a predictable clearance after 10 days, enables engraftment of donor HSPCs, and improves donor myeloid chimerism.

Future studies will explore expansion into other indications (e.g., combination with immune suppression for allogeneic HSCT, combination in genetically modified HSCs for autologous HSCT). Initial results from a study of antibody conditioning in patients with Fanconi anemia show that JSP191 eliminates the need for TBI or busulfan conditioning regimens, and the combined approach of JSP191 conditioning with

immunosuppression and T-cell receptor $\alpha\beta^+$ T cell/$CD19^+$ B cell–depleted hematopoietic grafts appears to be feasible, safe, and effective.

ADC Conditioning for Allogeneic Transplantation
John Dipersio, M.D., Ph.D., Director, Gene and Cellular Therapy, Washington University School of Medicine in St. Louis

Dr. John Dipersio, Washington University School of Medicine in St. Louis, discussed his group's efforts to develop chemotherapy- and radiation-free conditioning for allogeneic transplantation. Initial studies showed that c-Kit–saporin and CD45–saporin ADCs are effective at depleting murine HSCs and conditioning animals for syngeneic HSCT. However, ADCs alone are insufficient to immunosuppress for allogeneic HSCT. Previous studies indicated that two signaling pathways—interferon gamma receptor (IFNγR) and interleukin-6 receptor (IL6R)—could be targeted via inhibitors of the downstream Janus kinases 1 and 2 (JAK1/JAK2) to improve outcomes in major histocompatibility complex (MHC)–mismatched allogeneic HSCT models by inhibiting host immune responses. Indeed, the JAK1/JAK2 inhibitor baricitinib permitted engraftment in mice conditioned with CD45–SAP by impairing IL15-mediated survival of natural killer cells in the host.

To further improve the efficacy of ADC conditioning for allogeneic HSCT, more toxic ADC conjugates were sought. Pyrrolobenzodiazepines (PBDs) are covalent DNA crosslinkers that are active in both dividing and quiescent cells. Because PBDs are too toxic for stand-alone use, they were tested in the context of targeted therapy. When combined with the JAK1/JAK2 inhibitor ruxolitinib, both CD45– and c-Kit–PBD enabled fully MHC-mismatched allogeneic HSCT. A single dose of CD45–PBD provided durable protection from acute myeloid lymphoma (AML) *in vivo*.

Even low-level systemic exposure to ADC toxins can cause deleterious side effects via killing of healthy cells expressing the target, nonspecific ADC uptake, and premature ADC payload release. For the treatment of nonmalignant diseases, less severe conditioning might enable successful HSCT while minimizing toxicity. For this reason, CD47 and c-Kit antibodies combined with ruxolitinib were evaluated and shown to enable fully MHC-mismatched transplantation with high levels of multilineage engraftment in preclinical murine models.

Anti-cMPL Immunotoxin Effectively and Safely Depletes Human and Rhesus HSCs: Implications for Pre-transplant Conditioning
Daisuke Araki, M.D., Staff Clinician, NHLBI, NIH
Andre Larochelle, M.D., Ph.D., Investigator, Regenerative Therapies for Inherited Blood Disorders, NHLBI, NIH

Dr. Larochelle reminded participants that beyond the established function of the myeloproliferative leukemia protein (also known as the thrombopoietin [TPO] receptor or cMPL) in platelet production, its activation plays a key role in HSPC survival and proliferation. Eltrombopag, a cMPL agonist, has shown efficacy in improving myeloid hematopoiesis in patients with BM failure. Building on this observation, his group hypothesized that inhibition of the TPO–cMPL signaling axis could reduce HSPC survival and serve as a conditioning regimen.

Dr. Daisuke Araki, NHLBI, explained that the cMPL protein is present in HSPCs, platelets, and megakaryocytes. Flow cytometry analysis showed that high expression of cMPL marks adult human long-term HSCs, a unique population of HSCs with remarkable proliferative potential. To test the hypothesis that targeting cMPL can enhance the specificity of HSPC depletion *in vivo*, a bivalent form of the antigen-binding portion of an antibody against human/NHP cMPL was linked to a diphtheria toxin fragment (DT390-biscFV[cMPL]). Treatment with DT390-biscFV(cMPL) resulted in cMPL-dependent

toxicity in *in vitro* studies, as well as efficient and persistent depletion of certain HSPCs (and not the majority of progenitor cells and mature blood cells) in an NHP model. Pharmacokinetic studies indicated that DT390-biscFV(cMPL) has a short serum half-life and a favorable safety profile. Platelet counts in experimental animals decreased immediately after treatment but rebounded within 10 days of treatment. Proof-of-concept transplants in NHPs using a DT390-biscFV(cMPL) conditioning regimen demonstrated that long-term engraftment of lentiviral (LV)–transduced HSPCs can be achieved. Additional transplant studies are ongoing to further assess safety and efficacy.

Session Panel Discussion

Dr. Saar Gill, Perelman School of Medicine, asked Dr. Czechowicz about cytopenias observed after a single dose of JSP191. Dr. Czechowicz answered that minimal evidence of cytopenia was detected in the SCID study patients (in which the effects of JSP191 as a single treatment could be observed). Cytopenias observed in the Fanconi anemia trial likely were caused by other agents (e.g., TBI, cyclophosphamide) or due to existing BM failure.

Dr. Gill asked Dr. Araki why neutropenia was not observed after treatment with DT390-biscFV(cMPL). He also wondered whether the NHPs were susceptible to the diphtheria toxin. Dr. Araki noted that neutrophils do not express surface cMPL and are not likely to be targeted by the compound. He and Dr. Larochelle agreed that higher doses of DT390-biscFV(cMPL) might result in side effects like neutropenia.

Dr. Roy asked whether preexisting antibodies against the diphtheria toxin might neutralize the DT390-biscFV(cMPL) compound. Dr. Araki noted that the diphtheria toxin fragment was chosen partly because it already had undergone clinical safety trials in humans. The possibility of neutralization by preexisting antibodies has not yet been examined.

Several participants asked about positive and negative aspects associated with HSC versus HSPC depletion. Dr. Czechowicz answered that the differences between these two conditioning modalities still are not well understood. She noted that in her group's experiments with drug-conjugated ACK2, only CD117-expressing HSCs (rather than all stem and myeloid progenitor cells expressing CD117) were depleted—leading to the conclusion that depletion of HSCs alone can be sufficient for engraftment. For this reason, Dr. Czechowicz recommended that the field discontinue the use of the phrase "myeloablative transplant." Dr. Larochelle added that he agreed with Dr. Czechowicz based on results from his group's anti-cMPL immunotoxin experiments (i.e., targeted depletion of certain HSPCs and not the majority of progenitor cells and mature blood cells enabled persistent engraftment of engineered HSPCs). He suggested that a deeper understanding of competition among HSPCs might enable the transplantation of a more defined population of cells. As a final comment, Dr. Larochelle pointed out that patients with Fanconi anemia present a unique scenario in which specific antibodies could be used to eliminate all residual HSCs that are at high risk for malignant transformation.

Alternatives to Chemotherapy-based Conditioning: Other Approaches
Moderator: Brian Bai, Ph.D., Program Director, NHLBI, NIH

Long-term Follow-up of Fanconi Anemia Patients Treated with LV-mediated Gene Therapy
Paula Rio, Ph.D., Head, Bone Marrow Aplasia Unit at the Biomedical Innovation Unit, Centre for Energy, Environmental and Technological Research (CIEMAT), Madrid, Spain

Dr. Paula Rio, CIEMAT, provided an overview of Fanconi anemia, a rare hereditary disease that involves genomic instability due to an impaired DNA damage response. Clinical outcomes of the disease include congenital abnormalities, a predisposition to cancer, and hematological defects that lead to early-onset BM failure. Treatment of BM failure with allogeneic HSCT is associated with several challenges

(e.g., low probability of finding a suitable donor, increased cancer risk), and gene therapy with autologous cells is being explored as a more suitable alternative. Dr. Rio noted that conditioning is not required for gene therapy treatment of Fanconi anemia. Somatic mosaicism in patients often provides a natural source of BM and blood cells with a functional DNA repair capacity; conditioning often is associated with an increased risk of head and neck squamous cell carcinoma; the proliferative advantage of Fanconi anemia HSCs is corrected by LV-mediated gene therapy; and patients with Fanconi anemia harbor a limited number of HSPCs.

Results from the FANCOSTEM and FANCOLEN-I clinical trials showed that mobilization of patients' HSPCs with granulocyte colony-stimulating factor (G-CSF) and plerixafor resulted in the collection of sufficient cells for gene therapy purposes. $CD34^+$ HSPCs were transduced with a wild-type copy of the *Fanconi anemia, complementation group A* (*FANCA*) gene before infusion into patients, who exhibited progressive engraftment that was observed in peripheral blood cells. Higher doses of corrected cells were associated with higher levels of corrected cells detected in the gene therapy patients. Phenotypic analysis was used to confirm the phenotypic correction of BM progenitors and peripheral blood T cells. Whereas patients in the early stages of the disease experienced reversion of BM failure, patients with advanced BM failure who received delayed engraftment (due to lower levels of corrected CD34+ HSPCs) experienced continued progression of the disease. Single-cell analysis showed that gene therapy normalizes the transcriptional signature of HSCs, restoring regulatory pathways that typically are altered in Fanconi anemia. Current data from the FANCOLEN-II trial suggest that the infusion of higher corrected cell doses in the earlier stages of the disease will prevent BM failure.

Targeted Radiation-based Conditioning to Enhance Donor Mobilization and Engraftment and Preserve Organ Function
Susanta Hui, Ph.D., DABR, Professor, City of Hope

Dr. Susanta Hui, City of Hope, shared the results of his studies on organ-specific radiation to overcome challenges associated with myeloablative conditioning regimens in HSCT. His group developed a targeted radiation system to deliver TBI and total-marrow irradiation (TMI) in a murine model and evaluate the dosimetric differences between TBI and TMI. Compared to TBI, TMI resulted in similar dose exposure to bone and a 50 percent reduction in dose compared with other organs. TBI and TMI were compared in an HCT model with or without cyclophosphamide conditioning. In both the presence and absence of cyclophosphamide, a significant increase in donor BM cell engraftment was observed in the TMI-treated mice compared with the TBI-treated mice. Significantly higher levels of stromal-derived factor-1 (SDF-1), a chemokine involved in the recruitment of HSCs to the BM after HSCT, also were observed in the TMI-treated mice. Notably, TMI-treated mice expressed higher levels of SDF-1 non-BM organs than TBI-treated mice. Dr. Hui's group hypothesized that increased expression of SDF-1 in nontargeted organs following TBI attracts migratory donor cells away from the BM; after TMI, the reverse is true and donor cells are attracted to the BM niche. Further studies indicated that TMI modulates immune cell trafficking, shifting the mobilization of cytotoxic T cells from the gastrointestinal system toward the BM. As a result, GvHD is reduced, and survival is improved in the TMI model.

Dr. Hui's group assessed the use of TMI in the humanized homozygous Berkeley sickle cell disease murine model, which exhibits homozygosity for *hemoglobin S* (*HbSS*) and mimics sickle cell anemia in human patients. *HbSS* mice were treated with varying TBI and TMI doses and transplanted with BM from donor mice expressing wild-type *hemoglobin A* (*HbAA*). Although increased doses of TBI were lethal, mice tolerated escalated doses of TMI. Only mice treated with high doses of TMI exhibited sustained donor chimerism and expressed HbA protein in peripheral blood cells. Compared with untreated *HbSS* mice, high-dose TMI mice (post-BMT) had higher levels of differentiated RBCs and reduced immature reticulocytes in the peripheral blood.

Dr. Hui added that methods for total marrow irradiation and total marrow and lymphoid irradiation (TMLI) are under clinical development. A phase 1 clinical trial to evaluate the safety and efficacy of TMLI and alemtuzumab as a conditioning regimen for sickle cell disease is currently underway. Future studies will investigate whether this approach can be used as conditioning for standard HCT and gene therapy to reduce toxicity and enhance donor cell homing. Additionally, further characterization of spatial and temporal heterogeneity in the skeletal system might enable even more targeted marrow irradiation therapies.

Mobilization-based Conditioning Coupled with Transient Engraftment Enhancers
Luigi Naldini, M.D., Director, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy

Dr. Luigi Naldini, San Raffaele Telethon Institute for Gene Therapy, explained that his group is addressing HSPC mobilization in a humanized nonobese diabetic/SCID/gamma (or NSG) murine model. Mobilization leverages biological and chemical agents to interfere with the homing of HSPCs. For example, administration of G-CSF is thought to trigger proteolysis within the extracellular matrix to promote HSPC egress into the bloodstream. The goal is to transplant HSPCs at the peak of mobilization, which might enable competition with mobilized cells to repopulate BM niches, establishing some level of donor chimerism.

Dr. Naldini noted that *ex vivo* culture endows HSPCs with homing advantages against freshly mobilized cells. When engrafted HSPCs that have been mobilized out of their niche are compared with the LV-transduced cells from the original donor (which were mobilized themselves to establish the original transplant but recovered in cell culture while being transduced), the mobilized HSPCs express lower levels of C-X-C chemokine receptor type 4 (CXCR4) than the transduced cells because the mobilization protocol involves partial proteolysis of CXCR4 (a major component of the BM retention axis) and other homing receptors. Further studies indicated that HSCT following mobilization of host HSPCs (i.e., mobilization-based HSCT) results in cultured donor HSPCs' outcompeting mobilized cells for engraftment in depleted BM niches and the establishment of stable partial chimerism at potentially therapeutic levels. Furthermore, transient overexpression of CXCR4 (especially of mobilization-resistant CXCR4 variants) enhances donor HSPC migration and can be coupled with gene editing to increase the migration potential of HSPCs.

Preliminary studies of mobilization-based HSCT in a clinically relevant NHP model have shown similar results to those seen in the NSG murine model. Potential first clinical applications include HSCT settings that require a low chimerism threshold for disease correction and do not require lymphodepletion or immune suppression (e.g., SCID, DNA repair diseases), and the protocol can be further developed to address such diseases as hemoglobinopathies. Future studies will be required to address lower rates of chimerism associated with mobilization-based HSCT and patients who are resistant to mobilizing agents.

Targeting CD45 as an Immunotherapeutic Conditioning Regimen
Saar Gill, M.D., Ph.D., Associate Professor of Medicine (Hematology–Oncology) at the Hospital of the University of Pennsylvania, Perelman School of Medicine

Dr. Gill listed two main challenges in CAR T-cell therapy: (1) no cell surface antigens are truly cancer specific, and (2) the development of new CAR T cells for each hematological malignancy or disease is inefficient. To overcome these issues, Dr. Gill's goal was to identify a single cell–surface antigen target that would be specific to virtually any blood malignancy in any patient. CD45 is a promising target expressed on the surface of most hematopoietic cells, including cancer cells, but CAR T cells' targeting CD45 would eliminate HSCs and cause severe pancytopenia. An epitope-editing strategy was used to render CD45 invisible to CAR T cells to generate a functional CAR T cell–resistant hematopoietic system. Sequential truncation followed by alanine scanning of the CD45 extracellular domain was used to

map the target epitope of the antibody of interest (BC8) to CD45's protein tyrosine phosphatase domain 1. Nonsynonymous mutations were introduced into the epitope using CRISPR-based gene editing, and disruption of BC8 binding to base-edited CD45 ($CD45^{BE}$) was confirmed. $CD45^{BE}$ is fully functional and capable of rescuing CAR T-cell dysfunction associated with CD45 knockout. Base-edited CD45 CAR T cells (BE CART45) demonstrated *in vitro* cytotoxicity against AML, B-cell lymphoma, and T-cell lymphoma cells.

To determine whether $CD45^{BE}$-expressing HSPCs could generate a functional CAR T cell–resistant hematopoietic system, human $CD34^{+}$ HSPCs were base edited before being grafted into mice. $CD45^{BE}$ HSPCs maintain colony-forming potential after engraftment and differentiate into functional myeloid cells, B cells, and T cells *in vivo*. Following treatment with BE CART45, engrafted $CD45^{BE}$ HSPCs and human hematopoietic cells in the peripheral blood and BM were protected; unedited HSCs and progeny cells were eliminated. In the same model, BE CART45 eliminated engrafted human leukemia cells while simultaneously sparing healthy hematopoietic cells. The $CD45^{BE}$ HSPC strategy has also been shown to be effective with bispecific T cell–engaging antibody technology, which is more tractable in a clinical setting.

Molecular Cell Shielding for Conditioning
Lukas Jeker, M.D., Ph.D., Assistant Professor, University of Basel, Switzerland

Dr. Lukas Jeker, University of Basel, re-emphasized the primary challenge associated with antigen-specific depletion modalities: They cannot discriminate between diseased and healthy cells. Molecular epitope engineering can be used as a strategy to shield cell surface receptors while retaining their expression and function in healthy, therapeutic cells. This technique has several applications in HSCT, including pretransplant conditioning and posttransplant elimination of residual disease or targeting of previously inaccessible antigens. For example, with antibody-based conditioning, patients require an antibody washout period and receiving only a single dose is possible. Transplantation of shielded HSPCs can occur earlier in the washout period and is compatible with repetitive dosing.

As described earlier, CD117 is an attractive target for antibody conditioning. Dr. Jeker's group successfully screened for CD117 variants that inhibit SR-1 antibody binding while preserving SCF binding and signaling (and, therefore, SCF-dependent cell proliferation). The variants were introduced to $CD34^{+}$ human HSPCs via CRISPR and detected by cell sorting as a population of cells that expressed CD117 but did not bind SR-1. The edited HSPCs engraft during primary and secondary transplantation. A novel SCF-blocking humanized monoclonal antibody (CIM058) was engineered. Treatment with CIM058 depletes engrafted wild-type HSCs *in vivo* but causes enrichment of CD117-edited cells when compared with treatment with an isotype control. Similarly, HSPCs engineered to be shielded from a CD45-targeted ADC enabled selective wild-type tumor cell ablation with preserved hematopoiesis in engrafted cells.

Session Panel Discussion

Dr. Brian Bai, NHLBI, asked Dr. Naldini whether donor HSPCs overexpressing CXCR4 are capable of differentiation into peripheral cells. Dr. Naldini answered that overexpression of CXCR4 in this system is transient and does not appear to alter the behavior of the HSPCs.

Session IV: Brainstorming Gaps in the Field and Needed Future Resources

Workshop participants gathered in three breakout rooms to discuss the following questions:

1. **Transplant Outcomes:** How can the field improve current technologies to measure the success of transplant outcomes?

2. **Model Systems:** Which questions are best answered using which *in vivo* or *in vitro* models? What biological or biophysical barriers must be included for the question being addressed?

3. **Other Diseases:** What other nonmalignant hematological diseases could be new applications for novel conditioning regimens?

The full audience reviewed the group discussions to discuss resources required for future studies and ensure that the comments and suggestions proffered in the breakout rooms had been accurately recorded.

Summary and Conclusion

The goals of conditioning in nonmalignant hematopoietic disorders are to generate a supportive niche for transplanted cells and address the level of immunosuppression required for the treatment. Current studies are evaluating every aspect of the field: whether harsh or leukemogenic conditioning regimens are necessary for certain treatment applications, how established signaling pathways contribute to hematopoiesis in novel ways, and how such elements as functional heterogeneity in the BM niche and stem cell proteostasis and mobilization can affect transplantation outcomes. Other studies are addressing technical aspects of HSCT, including adapting diverse genetic engineering techniques; assessing new methods, like targeted irradiation; and exploring novel antigen-based targets, antibodies, and small molecules that can be incorporated into targeted conditioning treatments for improved HSCT efficacy and reduced toxicity.

New ideas will be needed to address gaps in the field. Workshop participants agreed that future efforts must address such top priorities as the limitations of common *in vivo* and *in vitro* models and inconsistent protocols that differ between humans and animals. To move the field forward, fundamental questions—such as which animal model is the most applicable to human diseases, how different conditioning agents act and interact within the niche, and how different states of health and disease alter the BM microenvironment—must be asked and answered. The workshop set the stage for the generation of novel hypotheses and the formation of new collaborations to address critical gaps in the science of conditioning for nonmalignant hematological diseases.