291. A Novel Safety Assay for Retroviral Vectors That Reproduces Lmo2 Proto-Oncogene Insertional Activation Events

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Gene therapies for X-linked severe combined immunodeficiency (SCID-X1) and Wiskott-Aldrich syndrome (WAS) with gammaretroviral vectors have caused a number of cases of acute lymphoid leukemias due to insertional activation of oncogenes, mostly LMO2. Transduction of murine bone marrow hematopoietic cells and subsequent culture for myeloid immortalization or transplant into recipient mice detected the oncogenic activity of the spleen focus-forming virus (SFFV) vector, which was used in the WAS trial, but failed to detect the oncogenic activity of the SCID-X1 gene therapy vector MFG-gc. The lack of the most relevant Lmo2 insertion by gammaretroviral vectors in these assays demands more relevant and sensitive vector safety assays. It has been shown that murine early thymic progenitor cells (ETP), when cultured on OP9-DL1 stroma cells, progress through the DN1, DN2, DN3, DN4, DP and SP differentiation stages, and that overexpression of LMO2 in ETP cells caused a differentiation block at the DN2 stage (CD4-CD8-CD25+CD44+). We tested whether transduction of ETP cells with gammaretroviral or lentiviral vectors could reproduce Lmo2 integrations and induce DN2 differentiation block. We tested three different gamma-retroviral vectors MSCV-GFP, SFFV-GFP and MFG-gc and two lentiviral vectors Cl20-SFFV-mCherry and Cl20i4r-SFFV-mCherry. The two self-inactivating lentiviral vectors contain a single internal SFFV long terminal repeat but differ in that one contains the cHS400 chromatin insulators. We transduced ETP cells with these vectors and cultured them on OP9-DL1 cells for up to 40 days. The range of vector copy number was 1.07-10.78 for the MSCV-GFP vector, 0.24-1.22 for the SFFV-GFP vector, 0.59-7.98 for the MFG-gc vector, 3.97-13.04 for the Cl20-SFFV-mCherry vector and 4.57-18.21 for the Cl20i4r-SFFV-mCherry vector at days 10-11. Between days 20-35, a distinct DN2-blocked cell subpopulation was clearly detected by flow cytometry in all the 20 gamma-retroviral groups and also in 3/9 lentiviral groups. None of the 6 mock groups had any evidence of DN2 blockade. Insertion site mapping of sorted DN2 cells showed that in the 10 MSCV-GFP groups, 3 had Lmo2 insertions, 3 had Mef2c insertions, and 3 had both Lmo2 and Mef2c insertions; in the 6 SFFV-GFP groups, 3 had Lmo2 insertions; in the 4 MFG-gc groups, 3 had Lmo2 insertions; both Cl20-SFFV-mCherry groups had Mef2c insertions and the one Cl20i4r-SFFV-mCherry group had Lmo2 insertion. These insertions occurred either in the introns or within +/- 50kb window of the gene. Transplant of the DN2-blocked cells from selected MSCV-GFP groups into recipient mice led to acute lymphoid leukemias. These results show that the transduction of ETP cells and the resultant DN2 blockade represent a more relevant and sensitive assay for vector safety assessment. Our result also suggests that an ETP-like subpopulation may be present in the bone marrow of SCID-X1 and WAS patients and explain the enhanced propensity of these disorders to oncogenic transformations.

292. Lentivirus Vector Mediated Gene Correction in Artemis-Deficient Severe Combined Immunodeficiency

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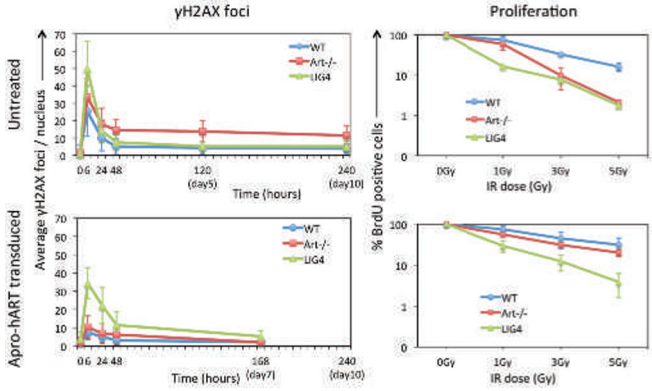
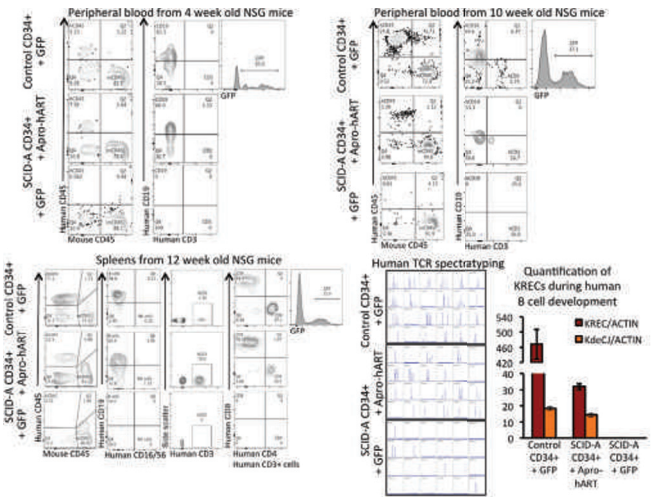
Mutations in DCLRE1C/Artemis, a DNA repair gene, cause T-B-NK+ SCID by preventing V(D)J recombination in T and B cell progenitors and also confer heightened sensitivity to irradiation and alkylator chemotherapy. SCID newborn screening identifies Artemis-deficient SCID (ART-SCID) early in life and while allogeneic hematopoietic cell transplantation can cure ART-SCID, preparative regimens for conditioning are poorly tolerated. Without alkylating chemotherapy patients may have graft failure while toxic effects of chemotherapy include increased mortality, short stature and abnormal dental development. Thus, building on experience with X-linked and ADA deficient SCID, we found addition of a normal Artemis gene to hematopoietic stem cells (HSC) an attractive strategy to treat ART-SCID.

Since overexpression of Artemis protein causes cellular cytotoxicity, a lentivirus vector with human Artemis cDNA and its endogenous promoter (Apro-hART) was produced and used to transduce fibroblasts from ART-SCID patients and controls. Apro-hART transduced ART-SCID fibroblasts showed correction of radiosensitivity by enumeration of foci of DNA damage and proliferation assays. Radiosensitivity of cells lacking the DNA repair enzyme Ligase-4 was not corrected.



Mobilized peripheral blood CD34+ cells from an ART-SCID patient, incapable of differentiation into T and B cells, were transduced with Apro-hART or GFP lentivirus and cultured on OP9 cells or injected into irradiated newborn NSG mice. OP9 cocultures and blood and spleen cells from NSG mice showed that Apro-hART-corrected, but not GFP-transduced, ART-SCID CD34+ cells differentiated into B cells and T and B cells, respectively, as did GFP-transduced control CD34+ cells. Lymphocyte maturation was proven by lineage specific surface markers and measures of V(D)J diversity and recombination, T cell receptor Vbeta spectratyping and Kappa chain receptor excision circles (KRECs), respectively.



Colony forming assays with transduced cells revealed transduction efficiency of 28.6%, a mean vector copy number of 3/cell and a diverse profile of lentivirus integration sites.

This successful gene correction and restoration of Artemis function in fibroblasts and HSCs from ART-SCID patients supports institution of a clinical trial of gene addition therapy for ART-SCID.

293. Optimal In Vivo Treg Induction and Suppression of Immune Responses By Synergistic Use of Rapamycin and FLT3 Ligand

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Immune-mediated rejection of therapeutic proteins is a major hurdle for gene and protein replacement therapies for genetic disease. However, optimal induction and expansion of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) counteract effector responses. We have previously shown that co-administration of antigen with rapamycin simultaneously promotes deletion of conventional $CD4^+$ T cells and induction of Treg, so that we were able to induce tolerance to factors VIII and IX in treatment of the X-linked bleeding disorders hemophilia A and B. Here, we found in DO11.10 $Rag^{-/-}$ mice (homozygous for ovalbumin-peptide specific TCR, deficient in endogenous mature B, T or Treg cells) that the cytokine FMS-like receptor tyrosine kinase ligand (Flt3L) enhances the in vivo effect of rapamycin via selective expansion of plasmacytoid dendritic cells (pDC), resulting in substantially improved induction of OVA-specific Treg. This regimen, consisting of co-administration of rapamycin/Flt3L/OVA 3x/week for 1 month, also prevented OVA-specific $CD8^+$ T cell responses in immune competent mice after intrasmuscular OVA gene transfer with an AAV1 vector. Flt3L signaling is critical to the generation and steady state expansion of both the conventional ($CD11c^+$, $CD8^+CD11c^+$) and plasmacytoid ($CD11c^{mid\text{-}lo}PDCA\text{-}1^+$) subsets of DC, which indirectly expands naturally occurring Treg. Interestingly, Flt3L-induced signaling in DC and DC precursors occurs through the mTOR pathway and can therefore be blocked by rapamycin, posing the question of why we are able to use these drugs synergistically for Treg induction. We find that limited doses of rapamycin selectively block Flt3L-induced expansion of conventional DC (cDC) but not pDC. Induced mTOR signaling is effectively blocked in cDC, while pDC have increased mTOR activity and are thus more resistant to inhibition by rapamycin. Consequently, Flt3L and rapamycin synergistically promote induction of antigen-specific Treg via selective expansion of pDC. During Flt3L/rapamycin/antigen co-administration, Treg induction is abrogated upon pDC depletion, thus confirming that Treg induction is pDC dependent. Similarly, Flt3L/antigen by itself fails to induce Treg, indicating that both pDC and rapamycin are required for Treg induction in this regimen.

Finally, we are able to demonstrate in a therapeutic model for FVIII protein replacement therapy that combined Flt3L/rapamycin/FVIII treatment is superior in prevention of inhibitory antibody responses in hemophilia A mice (BALB/c $F8e16^{-/-}$) when compared to rapamycin/FVIII only. In contrast, Flt3L/antigen administration in the absence of rapamycin, while enhancing activation induced cell death (AICD) of conventional $CD4^+$ T cells, fails to induce Treg and also fails to induce tolerance to OVA or to FVIII. Thus, the differential effect of rapamycin on DC subsets can be exploited to improve tolerance induction via enhanced Treg induction, creating new opportunities for development of immune tolerance therapies with wide applicability.

294. Myeloablative Conditioning Is Required for Efficient Engraftment of Gene-Modified Cells and Prevention of Antibody Production Against Transgene Products in a Rhesus Stem Cell Gene Therapy Model

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Reduced intensity conditioning (RIC) regimens are desirable for hematopoietic stem cell (HSC)-targeted gene therapy. However, in previous gene therapy trials, low gene marking was reported in peripheral blood cells, raising the question that RIC might be insufficient for (1) opening niches for efficient engraftment and (2) inducing immunological tolerance to transgenes. Therefore, we sought to evaluate both engraftment and tolerance for genetically modified cells using our rhesus HSC gene therapy model following RIC.

We performed a dose de-escalation of total body irradiation (TBI) (10, 8, 6, and 4Gy) as RIC transplantation (total 19 animals), in which mobilized rhesus $CD34^+$ cells were transduced with a VSV-G-pseudotyped chimeric HIV-1 vector encoding GFP (or YFP) and these cells were transplanted into autologous animals following TBI. We evaluated GFP-positive rates (%GFP), average vector copy number per cell (VCN), and antibody titers against GFP and VSV-G in peripheral blood 6 months after transplantation.

When we evaluated *in vitro* %GFP in transduced rhesus $CD34^+$ cells, efficient transduction (22-71%) was observed among all TBI groups (without correlation). After transplantation of the transduced $CD34^+$ cells, GFP-positive cells were detected in peripheral blood cells in all animals. Increasing doses of TBI resulted in higher %GFP with logarithmic regression in both granulocytes (R^2=0.64, $p<0.01$) and lymphocytes (R^2=0.67, $p<0.01$)(Figure). Similar results were observed between TBI dose and VCNs in both granulocytes (R^2=0.54, $p<0.01$) and lymphocytes (R^2=0.52, $p<0.01$). These data suggest that higher doses of TBI improve engraftment of gene-modified hematopoietic repopulating cells.

To evaluate immunological tolerance for gene-modified cells, we measured both anti-GFP and anti-VSV-G antibody titers using serum samples from transplanted animals. Lower dose TBI allowed anti-GFP antibody production with logarithmic regression (R^2=0.84, $p<0.01$)(Figure), while no significant effect of anti-VSV-G antibody was observed among all TBI groups (R^2=0.003, p=0.87). These data demonstrate that higher dose TBI is important to induce immunological tolerance for gene-modified cells in a rhesus transplantation model.

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