Demystifying Medicine 2021
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Bone Marrow-Based Therapies Today and Tomorrow:

Gene Therapy for Inherited Immune Deficiencies.

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Unapproved/off label drugs statement:

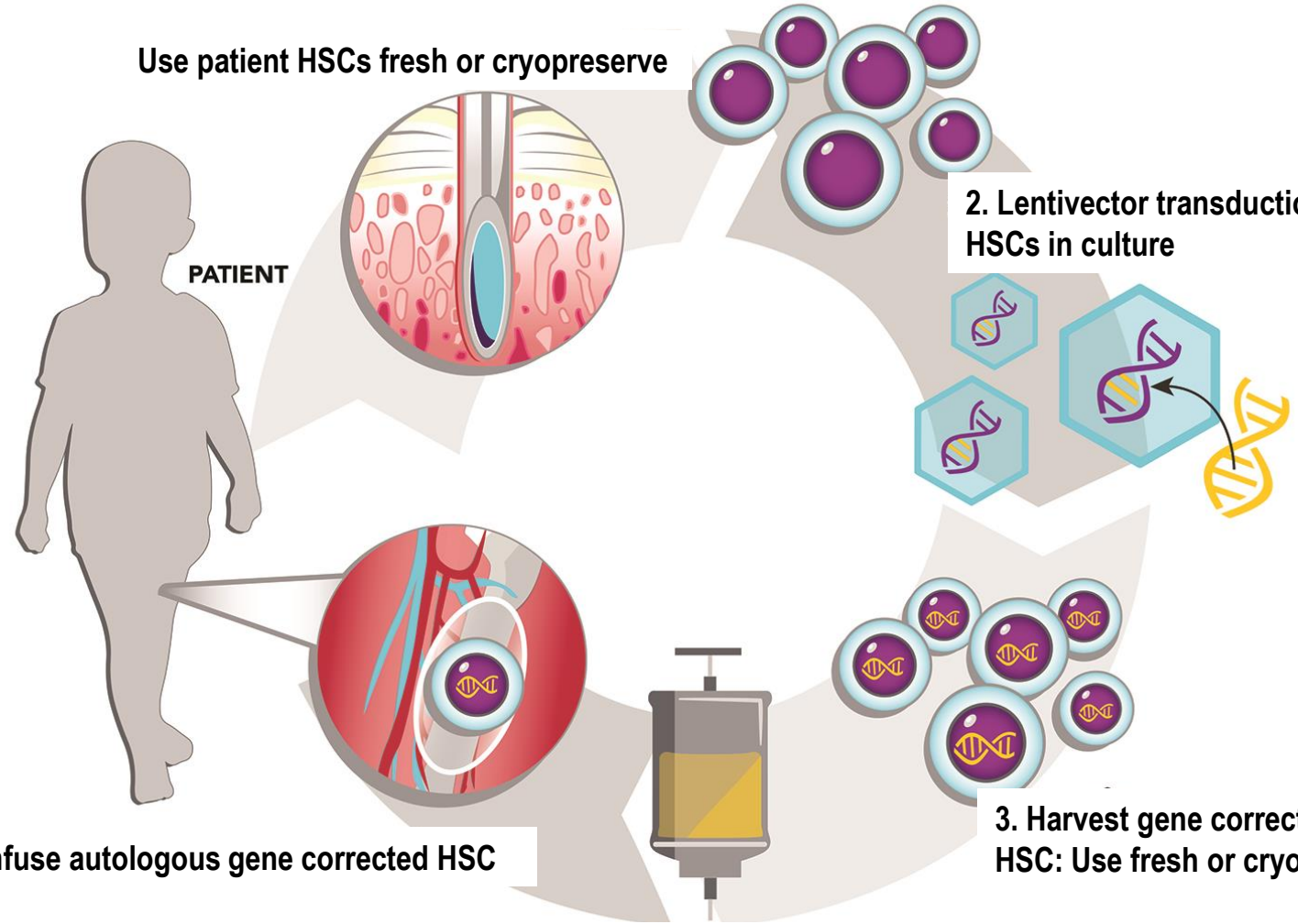
Genetically engineered autologous hematopoietic stem cells (HSC) used for gene therapy discussed today are experimental treatments, not standard of care, and are conducted under the auspices of Institutional Review Board (IRB) approved clinical protocols and US Food and Drug Administration (FDA) approved Institutional New Drug (IND) applications.

Learning Objectives

- **Describe collection and purification of autologous CD34+ hematopoietic stem cells (HSC) used for gene therapy.**
- **Define and describe *ex vivo* lentivector transduced HSC as used for gene therapy of primary immune deficiencies.**
- **Define the genetic cause, pathophysiology and clinical presentation of X-linked severe combined immune deficiency and X-linked chronic granulomatous disease.**
- **Discuss clinical outcome data from clinical trials of gene therapy for these disorders.**
- **As time permits, discuss gene editing of HSC for gene therapy.**

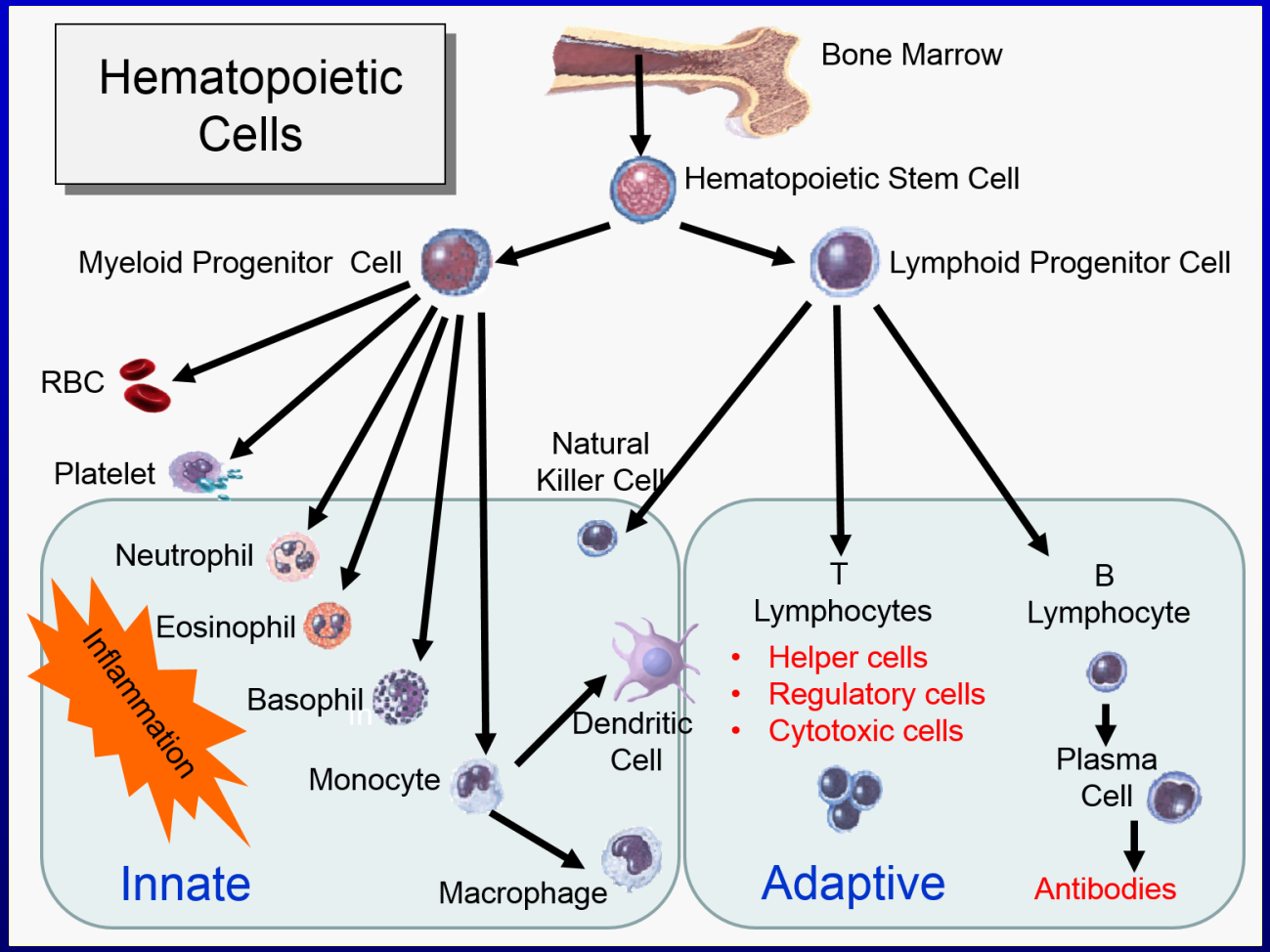
General Scheme for Lentivector Gene Therapy

1a. Mobilization / Apheresis / Selection of CD34+ hematopoietic stem cell (HSCs = Blood forming stem cells)
1b. Harvest bone marrow / Selection of CD34+ HSCs



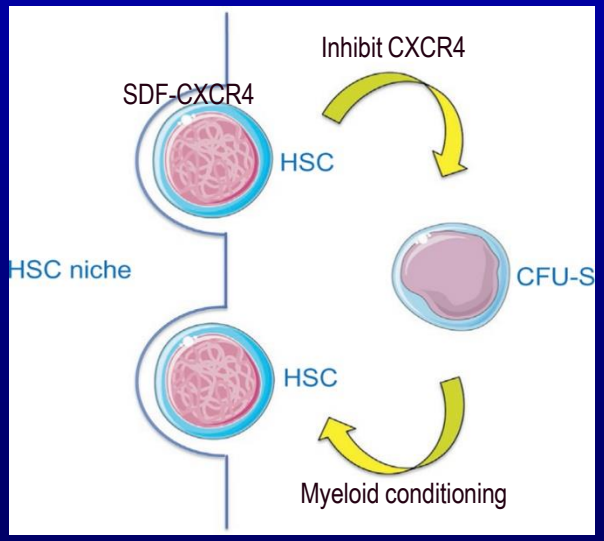
Bone Marrow – Blood and Immune Cell Generating Organ

Hematopoietic Stem Cells (HSC) in marrow produce mature blood and immune cells



HSC Occupy Specific Sites in the Bone Marrow (HSC Niche)

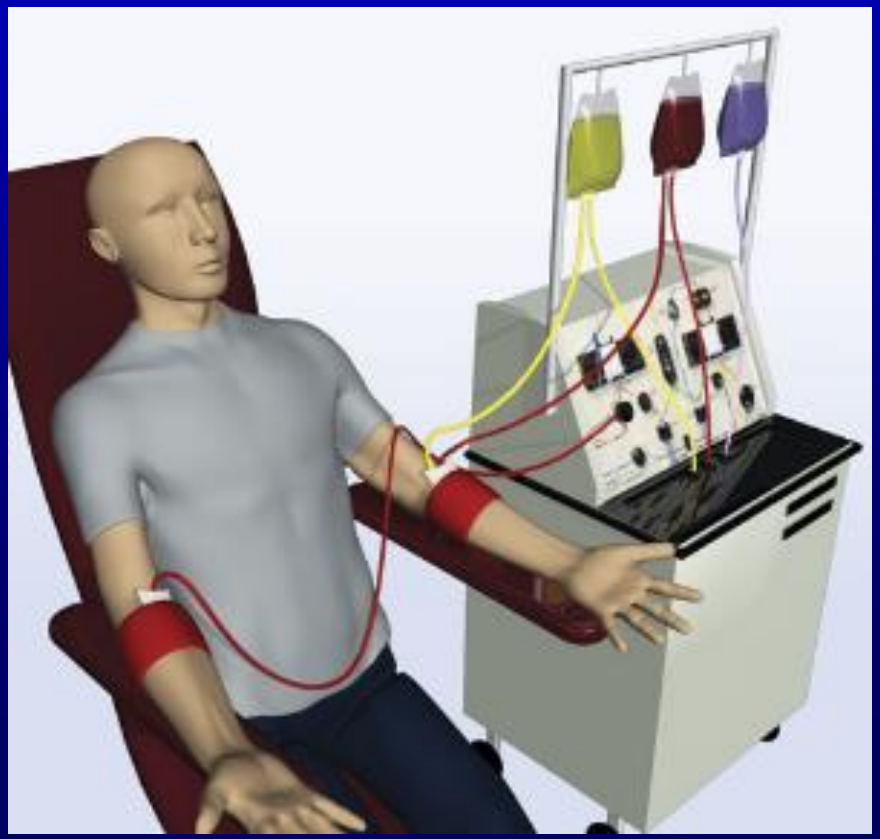
- HSC migrate in and out of the HSC niche.
- Stromal Derived Factor (SDF) binding to CXCR4 on HSC tethers HSC in the marrow.
- Treatment with G-CSF or plerixafor (CXCR4 inhibitor) disrupts the tether, enhancing release of HSC to the circulation. This is called HSC “mobilization”.
- Once in the circulation HSCs can be collected by apheresis.
- Successful engraftment of lentivector transduced HSC injected for gene therapy requires that HSC niches be “opened” by eliminating resident HSC using chemotherapy or radiation (Myeloid conditioning).

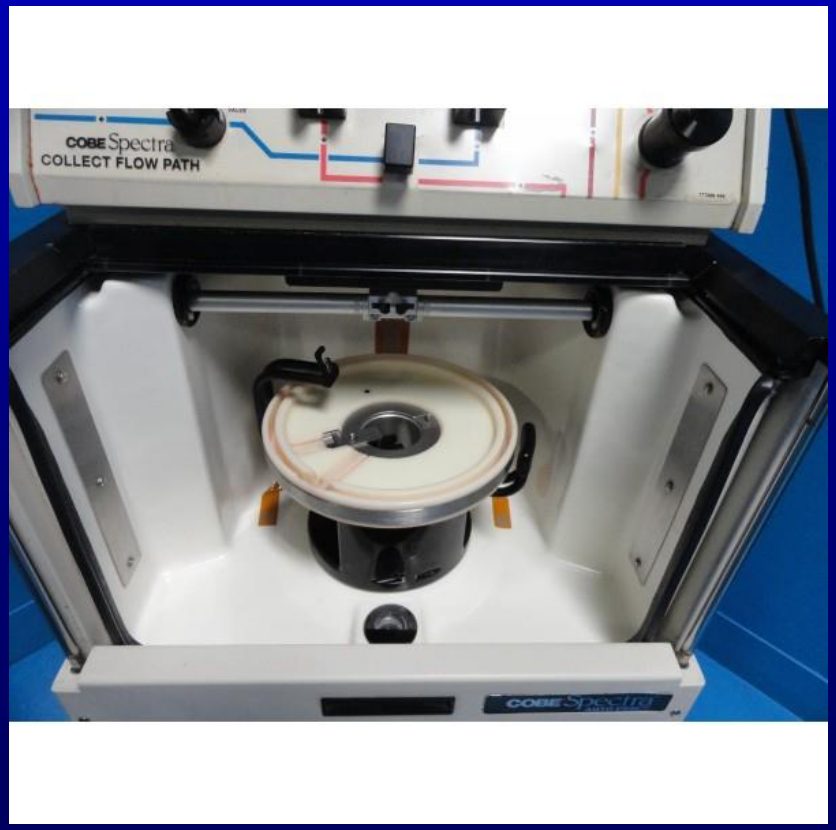


Modified from: Yu VWC and Scadden DT. Curr Top Dev Biol. 2016; 118: 21–44.

How is apheresis collection of HSC performed?

Apheresis collection of HSC: ***An apheresis catheter is placed in the arms or in a large vein in the neck with one line for blood to flow out of the patient into the apheresis machine and the other line for the return. Blood is anticoagulated with citrate coming out and then calcium/magnesium is added going back in to counteract the citrate anticoagulant. The apheresis machine spins the blood to cause it to separate into layers and a tiny "sipper" is situated to pull off the layer enriched in HSC.***





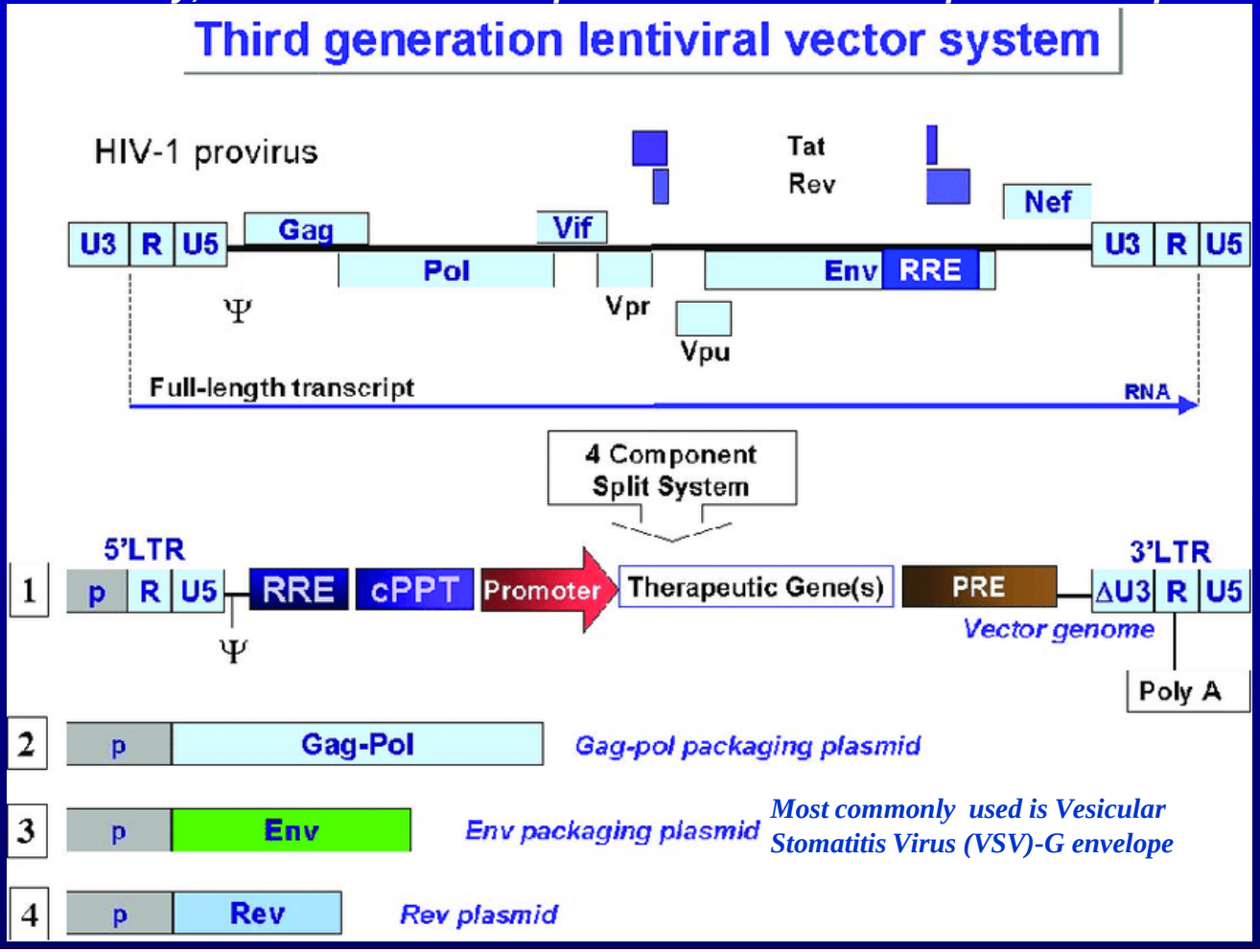
How are HSC purified from the apheresis collection?

Selection of HSC: ***Automated instruments are used to purify HSCs from the apheresis product using antibodies that bind to a cell surface molecule called CD34 that is found specifically on the surface of primitive blood HSCs.***

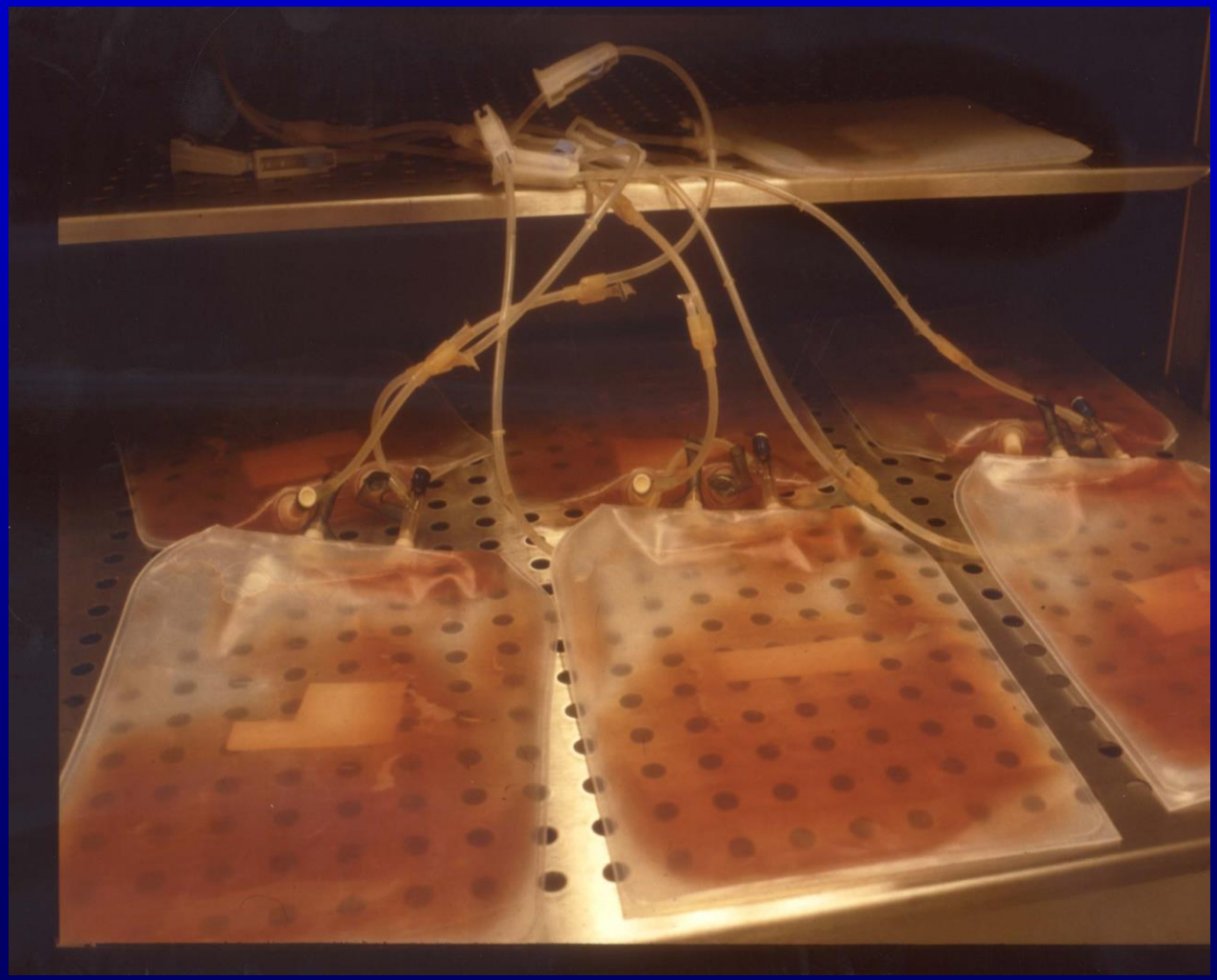
Purified patient CD34+ HSCs may be cryopreserved or used fresh grown in culture medium in flasks or thin gas permeable plastic culture bags where the cells are transduced with lentivector.

What is a Lentivector?

Self-inactivating (SIN) lentivector: ***Non-replicating virus extensively engineered from Human Immunodeficiency Virus (HIV) with many deletions to cripple the virus, but retaining the ability to enter the cell, reverse transcribe (make DNA from virus RNA), and insert itself (integrate) into the cell's genomic DNA. For safety, elements needed to produce the virus are split into 4 separate plasmids.***



Grow and Lentivector Transduce CD34+ HSC in Flasks or Gas Permeable Bags



How is the patient treated to enhance long term engraftment of gene corrected autologous HSC?

Myeloid conditioning: ***Refers to the use of chemotherapy or radiation to reduce resident bone marrow hematopoietic stem cells (HSC), thereby creating "empty" HSC niches, allowing transplanted genetically engineered HSC to engraft long term. Currently busulfan is the preferred alkylating chemotherapy agent used for myeloid conditioning for gene therapy as it does not target nor reduce T or B lymphocytes. However, gene therapy for some inherited immune disorders may also require use of agents that specifically target and remove autologous "misbehaving" T and/or B lymphocytes.***

Alternative approaches to myeloid conditioning are in pre-clinical development or early clinical studies that use antibodies targeting cKit (CD117) or other surface markers on HSC to replace the use of alkylating chemotherapy or radiation for myeloid conditioning.

Sustained significant clinical benefit has been demonstrated in completed or ongoing clinical trials of lentivector gene therapy to treat these inherited Primary Immune Deficiencies:

- **Adenosine Deaminase Deficiency (ADA) SCID;**
- **Wiskott-Aldrich Syndrome;**
- **X-linked Severe Combined Immune Deficiency; *Discuss today**
- **Artemis SCID;**
- **X-linked Chronic Granulomatous Disease; *Discuss today**
- **Leukocyte Adhesion Deficiency 1**

Many other trials of lentivector gene therapy for other PIDs are in planning or early clinical trials.

X-linked Severe Combined Immune Deficiency (XSCID)

- ***Most common genetic form of SCID comprising about 25% of SCID patients with frequency of about 1:200,000 births;***
- ***Mutations in IL2RG encoding the signaling common gamma chain of receptors for IL-2, -4, -7, -9, -15 and -21;***
- **Profound defect in T, B and NK cell immunity;**
- **Absent or few T and NK cells;**
- **B-cells immature; reduced numbers;**
- **Susceptible to severe life-threatening opportunistic infections;**

Infants with severe XSCID require urgent intervention: transplant or gene therapy. Those without a matched sibling donor or access to gene therapy receive a haploidentical lymphocyte-depleted bone marrow graft from a parent usually with little or no myeloid conditioning.

X-linked Severe Combined Immune Deficiency (XSCID)

- **Myeloid conditioning is not required for T cell reconstitution in XSCID infants.**

- **But without myeloid conditioning many infants as they age:**
  - **Have limited B cell function and require life-long IgG supplementation**
  - **Have little NK cell production**
  - **May experience decreasing donor T cell repertoire, function and number**
  - **May have chronic medical problems that include: growth and nutritional deficiencies, recurrent pneumonias and sinus infections, bronchiectasis, warts and molluscum, inflammatory bowel disease, protein-losing enteropathy, auto/allo-immune liver or renal disease, blood dyscrasias, chronic norovirus infection**

Lentivector Gene Therapy for XSCID: Two Clinical Trials

Older children and young adults with XSCID 2-40 years of age with incomplete immune reconstitution and chronic medical problems are the target population for our ongoing NIH clinical trial of lentivector gene therapy with non-myeloablative busulfan conditioning to be discussed today (ClinicalTrials.gov: NCT01306019).

Report of the first 5 patients:

De Ravin SS, Wu X, Moir S, Anaya-O'Brien S, Kwatemaa N, Littel P, Theobald N, Choi U, Su L, Marquesen M, Hilligoss D, Lee J, Buckner CM, Zarember KA, O'Connor G, McVicar D, Kuhns D, Throm RE, Zhou S, Notarangelo LD, Hanson IC, Cowan MJ, Kang E, Hadigan C, Meagher M, Gray JT, Sorrentino BP, Malech HL, Kardava L. Lentiviral hematopoietic stem cell gene therapy for X-linked severe combined immunodeficiency. Sci Transl Med. 2016 Apr 20;8(335):335ra57.

Informed by the NIH study of lentivector gene therapy for older children and young adults with XSCID that began in 2012, a clinical trial using this same lentivector plus busulfan myeloid conditioning to treat newly diagnosed infants was initiated in September 2016 by our collaborators at St. Jude Children's Research Hospital, Memphis, TN and Benioff Children's Hospital, Univ. of California, San Francisco, CA (ClinicalTrials.gov: NCT01512888)

Report of the first 8 patients:

Mamcarz E, Zhou S, Lockey T, Abdelsamed H, Cross SJ, Kang G, Ma Z, Condori J, Dowdy J, Triplett B, Li C, Maron G, Aldave Becerra JC, Church JA, Dokmeci E, Love JT, da Matta Ain AC, van der Watt H, Tang X, Janssen W, Ryu BY, De Ravin SS, Weiss MJ, Youngblood B, Long-Boyle JR, Gottschalk S, Meagher MM, Malech HL, Puck JM, Cowan MJ, Sorrentino BP. Lentiviral Gene Therapy Combined with Low-Dose Busulfan in Infants with SCID-X1. N Engl J Med. 2019 Apr 18;380(16):1525-1534. **The results from this trial will not be discussed today.**

NIH Trial of "Lentivector XSCID Gene Therapy for Older Children & Young Adults 2-40 years of Age"

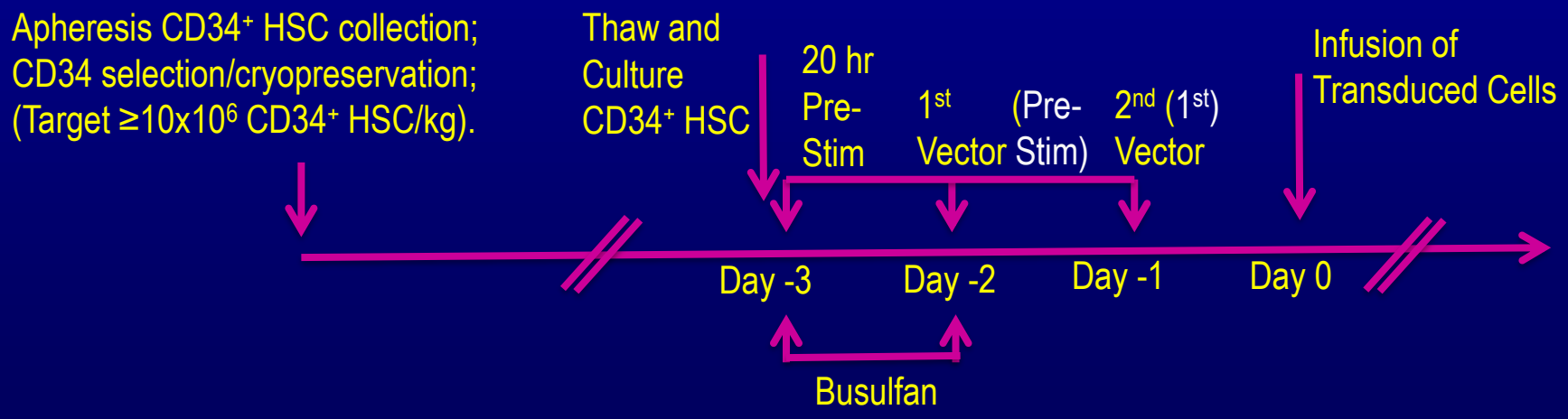
Target cell: Autologous peripheral blood mobilized CD34$^+$ HSC.

Conditioning: Busulfan 3mg/kg/d x 2 days with targeting on 2nd day to achieve Total AUC 9000 (μM/L)*(min) = ~32,200 (ng/ml)*(hr).

Vector: SIN insulated lentivector with codon optimized *IL2RG* cDNA.

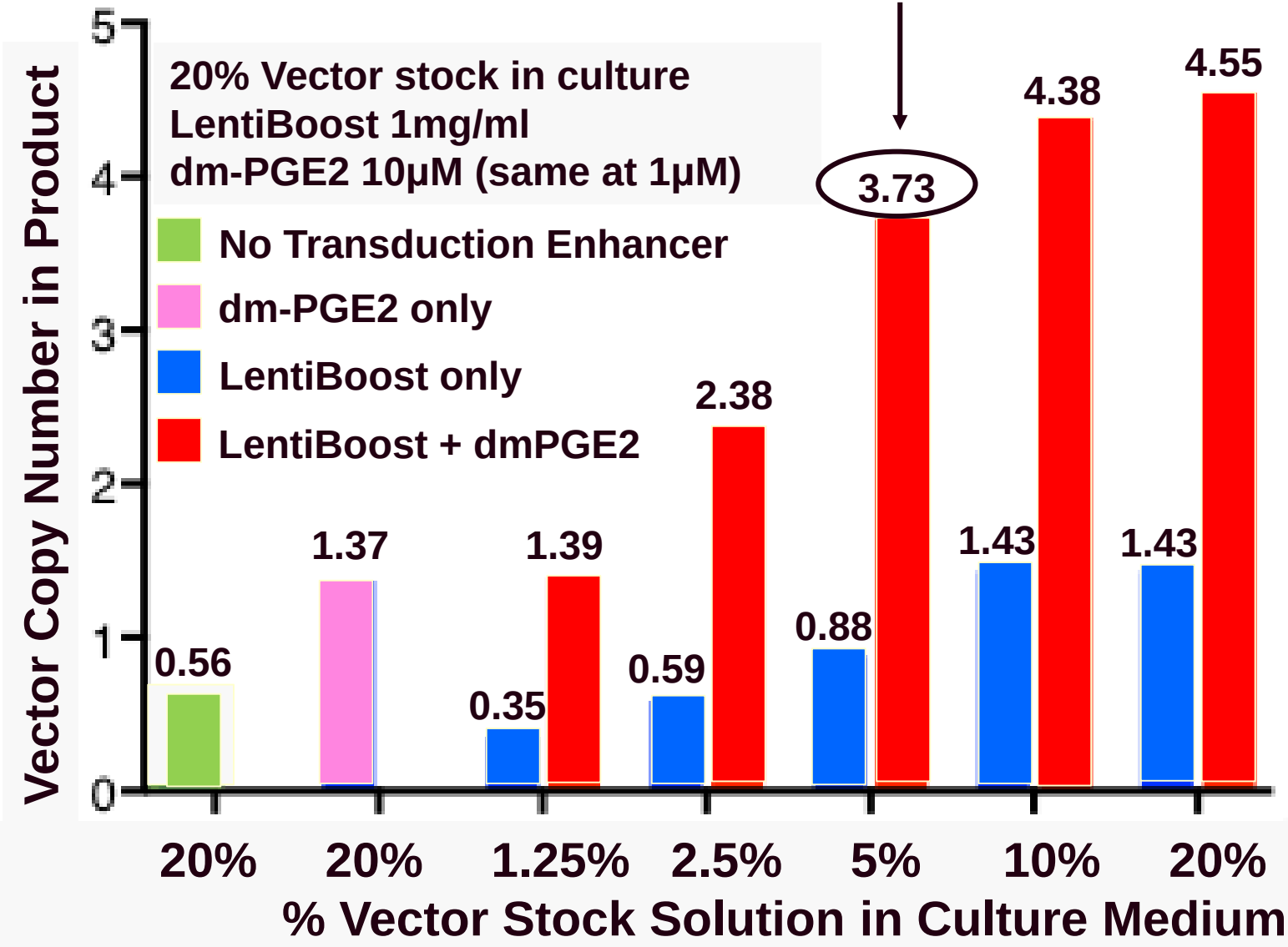
2012-2016 Original Transduction (OT) Regimen (8 Patients):
20hr pre-stim; 2 overnight transductions using 20-30% vector stock

2019-20 Enhancer Transduction (ET) Regimen (8 Patients):
44hr pre-stim; 1 overnight transduction using 5% vector stock
Enhancers: LentiBOOST™ 1mg/ml + 16,16-dmProstaglandin E2 1μM



LentiBoost (Kolliphor P338) and dimethyl-Prostaglandin E2 as Transduction Enhancers for XSCID Lentivector

Based on these studies the clinical SOP for GMP transduction uses 5% vector with LentiBoost 1mg/ml / dm-PGE2 1μM / Protamine Sulfate 4μg/ml for single overnight transduction at 2 x 10^6 $CD34^+$ cells/ml.



Treatment with OT Regimen (2012-2016): Patients 1-8

	Age Yrs	CD34+ 10^6/kg	VCN cells infused	Date Infused	PB Myeloid VCN at 3mo
P1	23	18	0.27	Oct 2012	0.07
P2	22	16	0.17	Jun 2013	0.06
P3	7	20	0.31	Mar 2015	0.07
P4	16	22	0.57	Jul 2015	0.23
P5	10	25	0.36	Jul 2015	0.29
P6	23	22	0.41	Feb 2016	0.33
P7	3	32	0.30	Sep 2016	0.04
P8	12	36	0.27	Oct 2016	0.05
n=8 median			0.31		0.07

*Treatment with ET Regimen (2019-20): Patients 9-15; *Retreat Patient 8*

	Age Yrs	CD34+ 10^6/kg	VCN cells infused	Date Infused	PB Myeloid VCN at 3mo
P9	19	5	3.19	Feb 2019	1.72
P10	13	9	4.70	Mar 2019	3.59
P11	25	25	14.28	Mar 2019	6.44
P12	34	28	3.07	Apr 2019	6.42
P13	24	17	4.22	Jul 2019	0.23**
P8*	15	10	4.78	Jun 2019	2.54
P14	9	21	2.57	Jan 2020	6.47
P15	4	14	3.73	Aug 2020	3.45
n=8 median			3.98		3.52

4-7 Year Clinical Outcome Followup Summary for the 8 Older Children/Young Adults Treated with the OT Regimen

Long term (4-8 yrs) positive achievements in the first group of patients treated with the OT Regimen:

- **6/7 cured chronic norovirus**
- **5/6 resolved protein losing enteropathy**
- **7/8 normal levels of IgM**
- **4/8 protective response to immunizations & ceased IgG supplements**
- **3/8 reduction in frequency and amount of IgG supplements**
- **6/8 increasing or normal $CD20^+$ $CD27^+$ IgG^+ memory B cells**
- **7/8 increased host T cells chimerism from 0-2% to 28-93%**
- **7/8 normal T cell proliferation response**
- **4/5 children made $CD4^+CD45RA^+CD31^+$ new thymic emigrants in normal range**
- **2/3 adults made detectable $CD4^+CD45RA^+CD31^+$ new thymic emigrants**

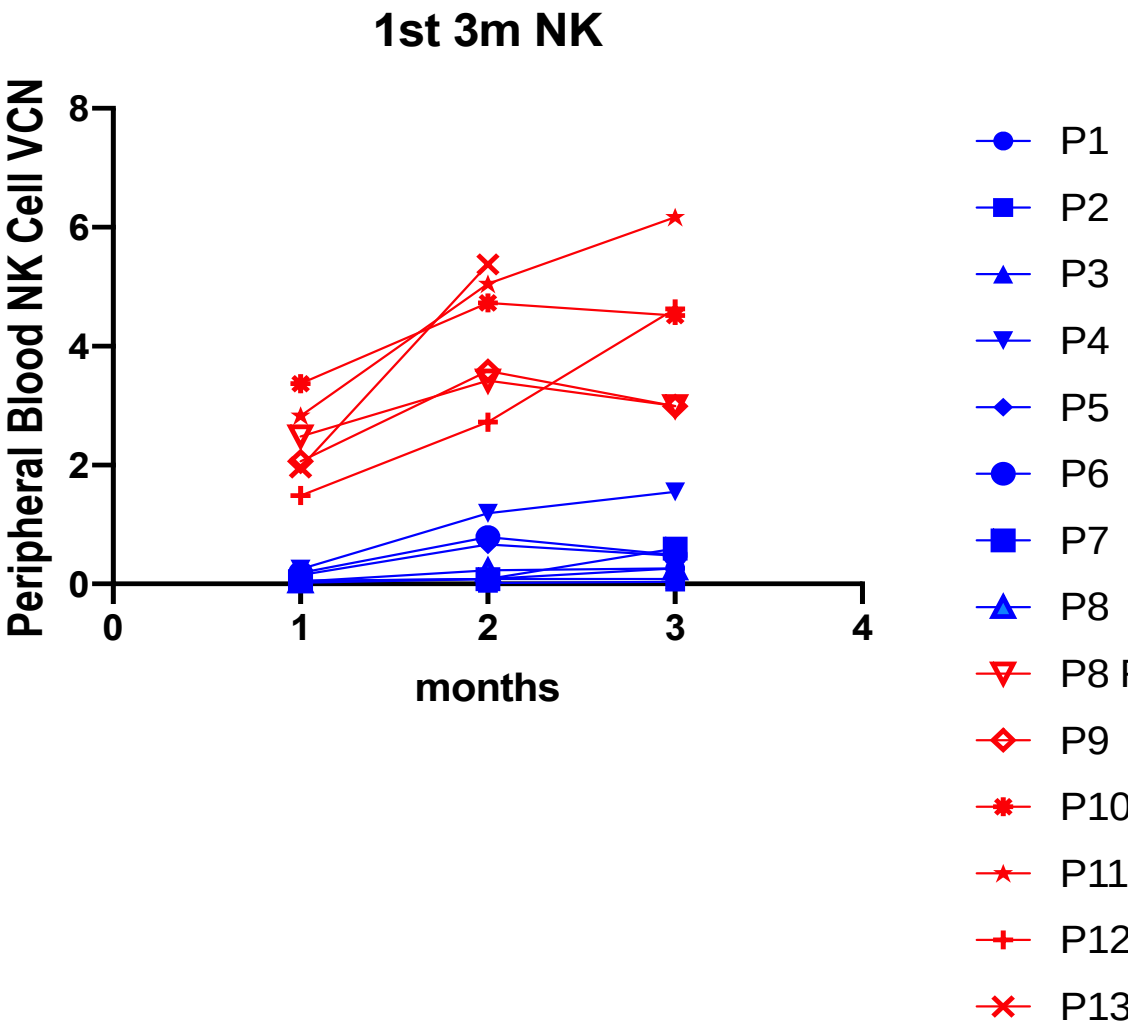
Original Transduction (OT) Regimen (no enhancers) 2012-2016

- **Transduced OT regimen product VCNs were relatively low at 0.17 to 0.57.**
- **P8 failed to achieve any of the laboratory or clinical landmarks.**
- **The pace to achieve laboratory & clinical landmarks was slow, in most patients was taking 2-3 years to occur.**
- **Slow increase in PB NK cells and only P1 at yr 7 achieved normal range of NK cells.**
- **Vector amount used per patient was an issue, particularly for adults.**

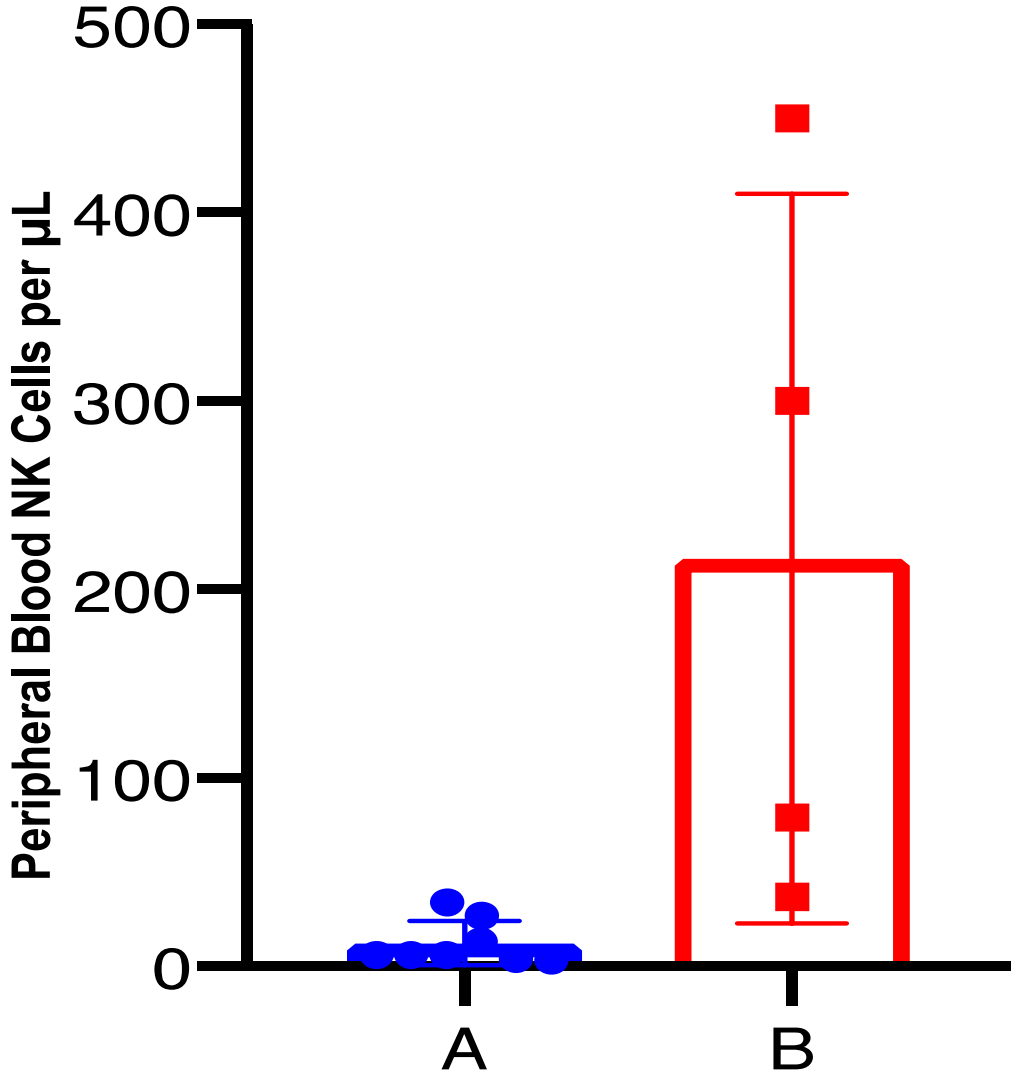
Enhancer Transduction (ET) Regimen 2019-Present Initiated to:

- **Assure all patients receive a product with high VCN (achieved as shown).**
- **Reduce amount of vector required (8-12 fold less vector used as shown).**
- **Enhance pace of NK cell generation where higher VCN may be required.**
- **Increase pace of immune reconstitution and achievement of clinical benefit.**

Accelerated Increase in NK cell VCN in the First 3 Months After Gene Therapy with ET Regimen compared to OC Regimen



Accelerated Increase in Number of Peripheral Blood NK Cells in the First 3 Months After Gene Therapy with ET Regimen compared to OC Regimen

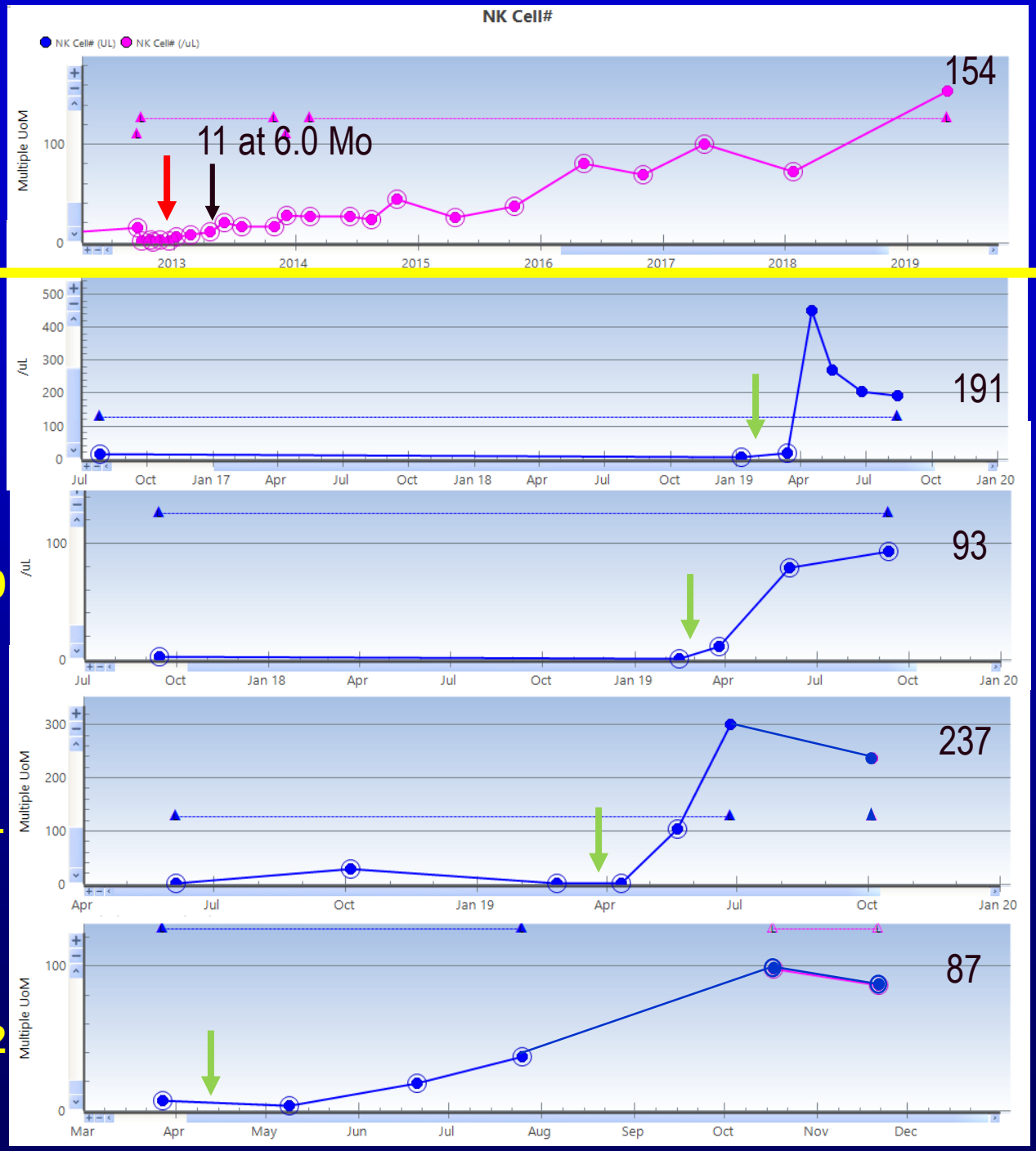


Transduction without enhancers with enhancers

Original Transduction (OT) Regimen Treatment Date

Enhanced Transduction (ET) Regimen Treatment Date

# of PB NK cells per µL



Follow up

7.0 yrs Post OT Regimen

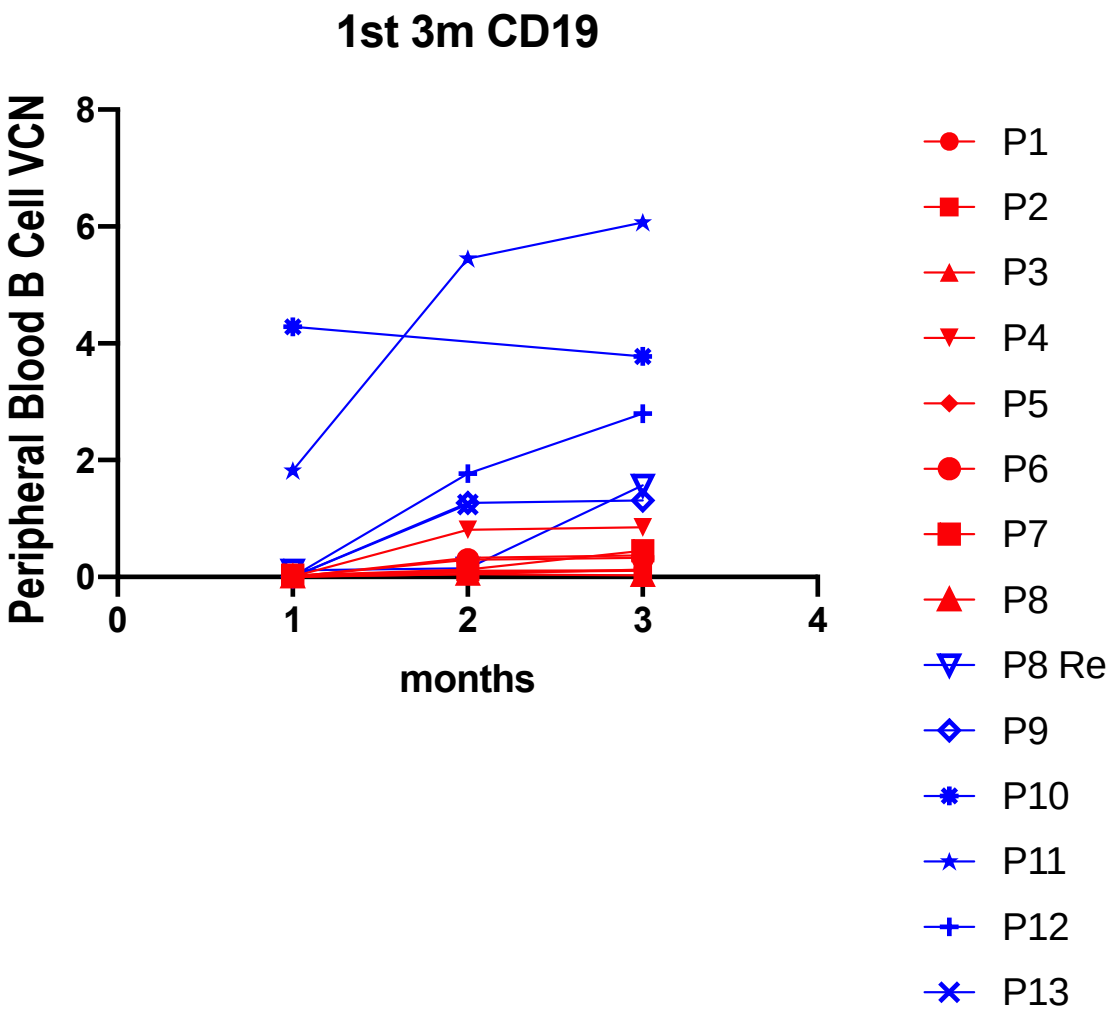
6.0 Months Post ET Regimen

6.0 Months Post ET Regimen

6.0 Months Post ET Regimen

6.0 Months Post ET Regimen

Accelerated Increase in B cell VCN in the First 3 Months After Gene Therapy with ET Regimen compared to OC Regimen



Accelerated IgM Increase with ET Regimen

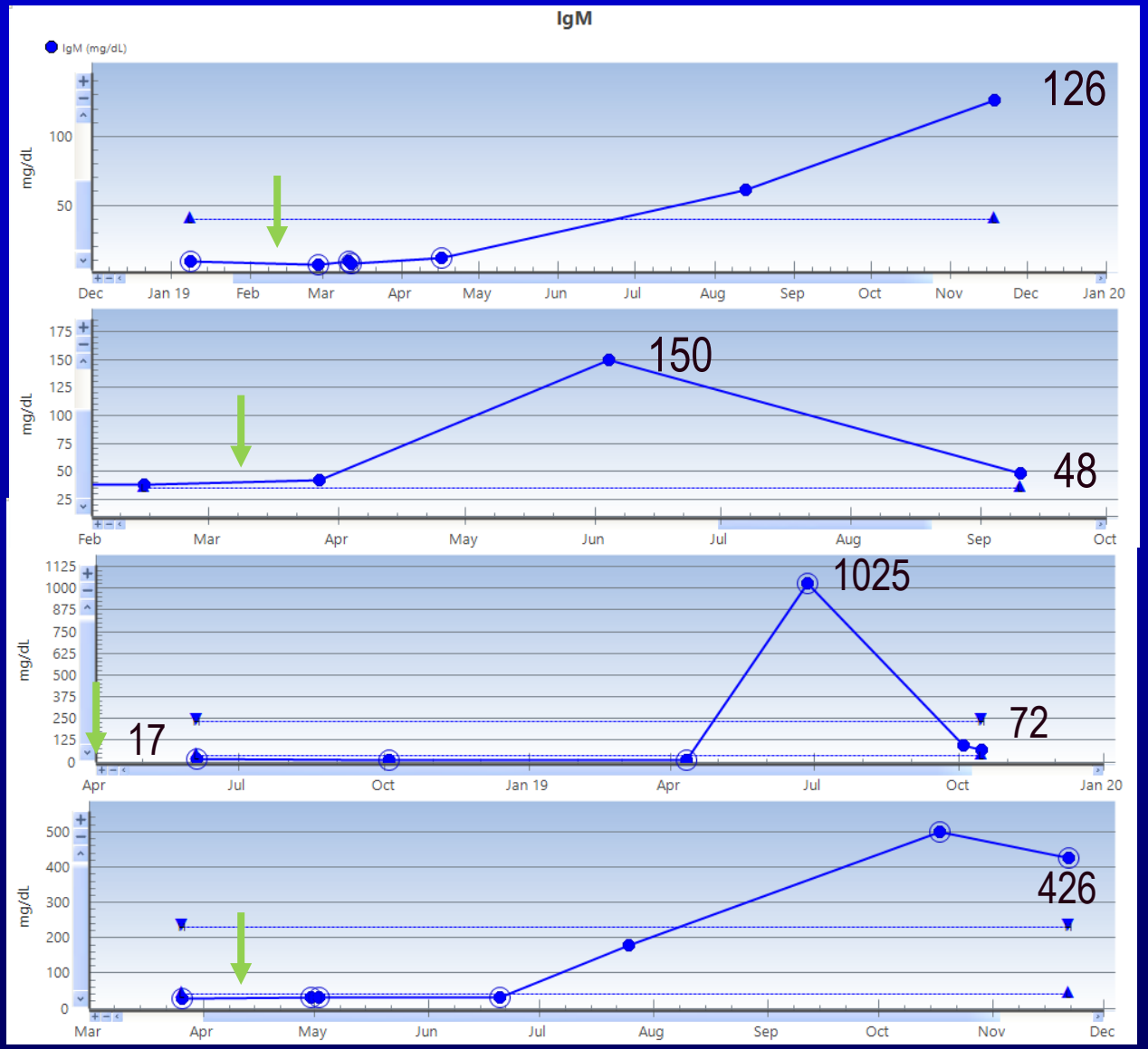
Enhanced Transduction (ET) Regimen Treatment Date

P9 IgM mg/dL

P10 IgM mg/dL

P11 IgM mg/dL

P12 IgM mg/dL



9.0 Months

6.0 Months

6.0 Months

6.0 Months

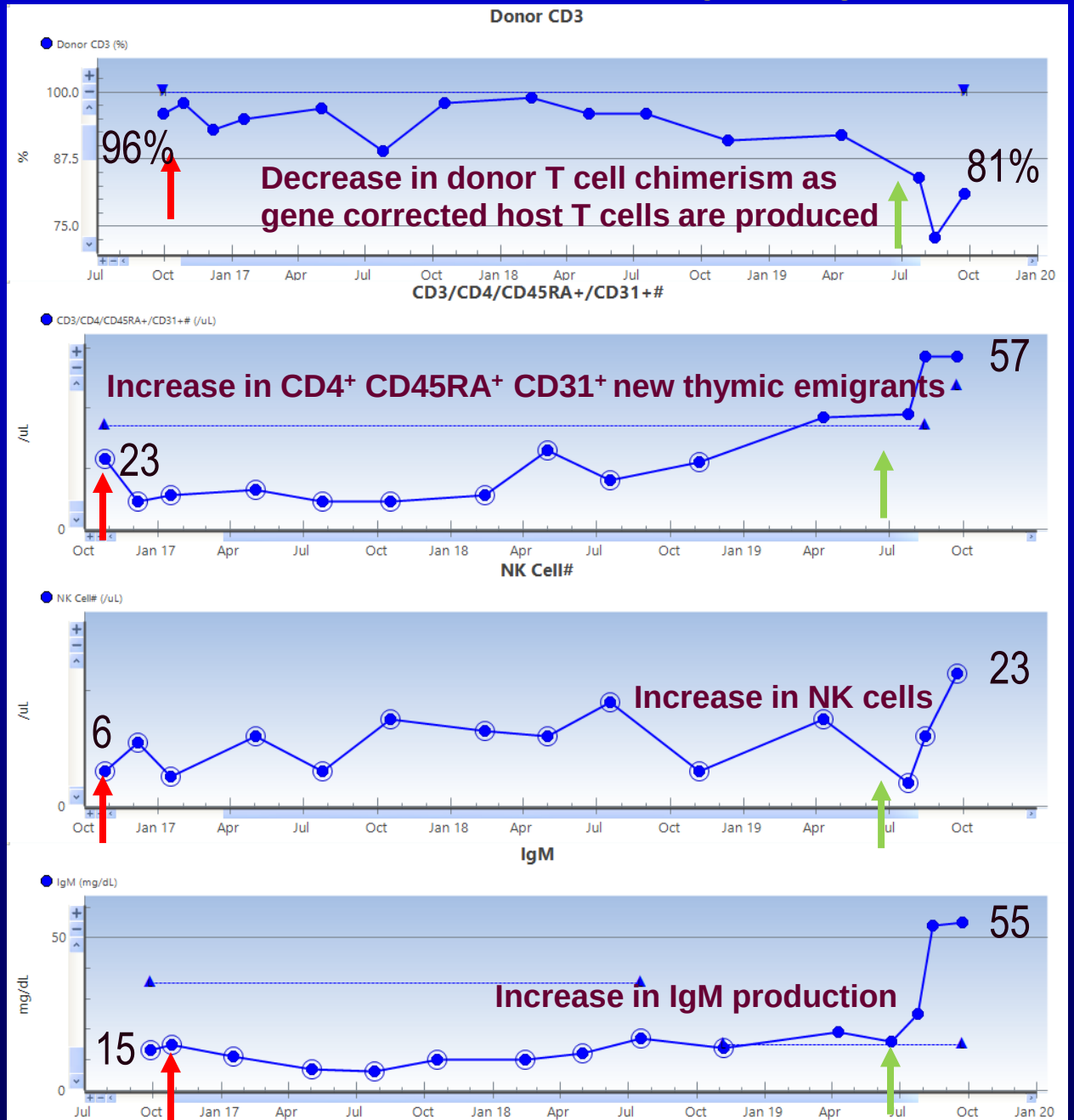
Retreatment of P8: Lineage VCN Following OT Regimen Treatment in Oct 2016 Compared to Lineage VCN Following ET Regimen Re-treatment in June 2019

#8 PT2725	Infused Product VCN 0.27											Infused Product VCN 4.8			
Summary	3w 10/31/16	2m 12/8/16	3m 1/23/17	6m 5/9/17	9m 7/28/17	12m 10/20/17	15m 2/15/18	18m 5/8/18	21m 7/20/18	24m 11/27/19	30m 4/15/19	RE_1m 7/29/19	RE-2m 8/15/19	RE-3m 9/30/19	RE-6m 1/22/20
CD3	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.04	0.10	0.09	0.19	0.34	0.21	0.39
CD14	0.04	0.05	0.05	0.06	0.06	0.06	0.07	0.06	0.06	0.07	0.07	3.52	2.71	2.54	4.13
PMN	0.03	0.04	0.05	0.05	0.05	0.05	0.06	0.06	0.06	0.06	0.07	3.58	2.77	2.18	3.23
CD19	0.00	0.05	0.03	0.03	0.04	0.07	0.12	0.11	0.14	0.13	0.15	0.12	0.15	1.57	2.16
NK	0.04	0.23	0.26	0.36	0.24	0.13	0.22	0.09	0.37	0.34	0.17	2.48	3.42	3.00	3.84
CD34	0.06	0.07	0.05	0.07	0.04	0.07	0.08	0.08	0.06	0.07	0.08	2.19	2.81	2.66	3.58

6 mo Post 1st Rx

6 mo Post 2nd Rx

Retreatment of P8: PB Immune Studies Following OT Regimen Treatment in Oct 2016 Compared to PB Immune Studies Following ET Regimen Re-treatment in June 2019



Original Transduction (OT) Regimen Treatment

Enhanced Transduction (ET) Regimen Treatment

2.75 years Between OT Regimen Treatment and ET Regimen Treatment

4.0 Months Post ET Regimen Treatment

Accelerated Clearance of Chronic Norovirus Infection with ET Regimen

- **Of the 7 patients with norovirus in the earlier OT regimen gene therapy group, the 6 who eventually cleared the infection required 1 to 3 years post gene therapy to achieve symptomatic improvement and cure their norovirus infection. P8 did not clear norovirus infection after the OT regimen treatment.**

- **P9 and P10 cured multiyear chronic norovirus or sapovirus infection, respectively, by 6 months post ET regimen gene therapy; and P11, P12 and re-treated P8 with norovirus were symptom free by 3-6 months. P14 improved GI symptoms by 2.5 months post gene therapy.**

Summary of Benefits of Enhanced Transduction (ET) Regimen

- **Requires 1/8 to 1/12 amount less vector.**
- **Achieves uniformly higher transduction product VCN.**
- **10-20 fold higher *in vivo* myeloid VCN with ET regimen; though P13 decreased myeloid VCN at mo 3 but stabilized at mo 4 same as 3 and NK VCN still 2.93.**
- **Accelerated production of NK cells with ET regimen.**
- **Accelerated increase in vector marking of B cells with ET regimen.**
- **Accelerated spike in IgM with ET regimen.**
- **Accelerated clearance of norovirus and GI symptoms with ET regimen.**

Longer follow up is needed to assess pace and extent of T cell correction, IgG production, response to immunization, overall clinical benefit and enhanced quality of life with ET regimen compared to OT regimen.

Longer follow up will be required to know if this increased vector marking using the ET regimen will prove to be stable and safe long term.

XSCID GENE THERAPY

Laboratory of Clinical Immunology and Microbiogy, NIAID

- Suk See De Ravin
- Elizabeth Kang
- Sandra Anaya-O'Brian
- Nana Kwatemaa
- Janet Lee
- Narda Theobald
- Joanna Peterson
- Taylor Liu
- Mary Garafalo
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- Dianne Hilligoss
- Martha Marquesen
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- Mort Cowan
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- Janel Long-Boyle

Texas Children's Hospital

- Celine Hanson

Hôpital Necker Enfants Malades

- Bénédicte Neven
- Alain Fischer

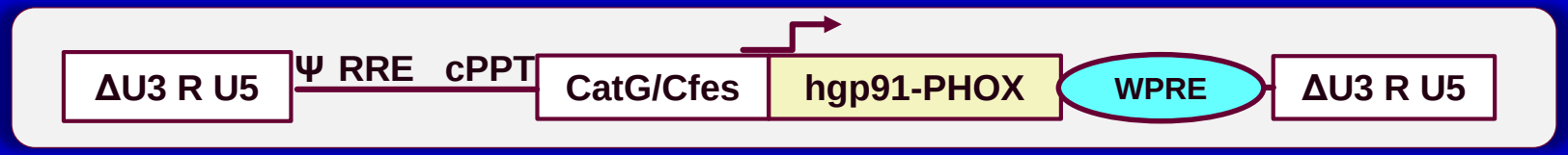
Univ Alabama Birmingham

- Fred Goldman

X-linked Chronic Granulomatous Disease (CGD)

- **Defective phagocyte NADPH-oxidase required for generation of superoxide derived microbicidal hydrogen peroxide by blood neutrophils and monocytes**
- **Recurrent life-threatening bacterial and fungal infections**
- **Granulomatous inflammation, especially inflammatory bowel disease.**
- **Incidence of ~1:200,000 births**
  - **X-linked CGD (X-CGD) affects ~70% of CGD patients; results from mutations in the *CYBB* gene encoding the gp91^{phox} subunit of phagocyte NADPH-oxidase.**

"G1XCGD" SIN Lentiviral Vector Developed for X-CGD



Self-inactivating (SIN) lentivector: Cathepsin G / cFes chimeric myeloid-specific promoter drives expression of gp91^{phox} from codon optimized *CYBB* cDNA.

Santilli G, Almarza E, Brendel C, Choi U, Beilin C, Blundell MP, Haria S, Parsley KL, Kinnon C, Malech HL, Bueren JA, Grez M, Thrasher AJ. Biochemical correction of X-CGD by a novel chimeric promoter regulating high levels of transgene expression in myeloid cells. Mol Ther 19:122, 2011.

International Multicenter Clinical Trial of Lentivector Gene Therapy for X-linked CGD

Treatment sites circled

Institute of Child Health and Great Ormond Street Hospital NHS Foundation Trust, London, UK
Adrian J Thrasher, Claire Booth, Giorgia Santilli, Karen F. Buckland, Diego Leon-Rico
Christine Rivat, Natalia Izotova, Kimberly Gilmour, Katie Snell, Jinhua Xu-Bayford Dip, Jinan Darwish

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Martha Marquesen, Elizabeth Quirk, Megan Crum,
Jean Ulrich, Jean Hammer, Christa Zerbe, Steven Holland,
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Georg-Speyer Haus, Frankfurt, Germany
Manuel Grez

Net4CGD Consortium

Lentivector Gene Therapy for X-CGD

- **$CD34^+$ collected by mobilization and apheresis;**
- ***Ex vivo* transduce purified $CD34^+$ HSC with G1XCGD;**
- **Busulfan conditioning pharmacokinetic-adjusted to total Area Under the Curve (AUC) 70-75,000 ng/ml*hr (sub-myeloablative);**
- **Transplantation with autologous gene modified $CD34^+$ HSC;**
- **Peripheral blood neutrophil oxidase activity assessed by dihydrorhodamine (DHR) flow cytometry assay that measures production of superoxide by blood neutrophils;**

Results of first 9 patients treated

Kohn DB, Booth C, Kang EM, Pai SY, Shaw KL, Santilli G, Armant M, Buckland KF, Choi U, De Ravin SS, Dorsey MJ, Kuo CY, Leon-Rico D, Rivat C, Izotova N, Gilmour K, Snell K, Dip JX, Darwish J, Morris EC, Terrazas D, Wang LD, Bauser CA, Paprotka T, Kuhns DB, Gregg J, Raymond HE, Everett JK, Honnet G, Biasco L, Newburger PE, Bushman FD, Grez M, Gaspar HB, Williams DA, Malech HL, Galy A, Thrasher AJ; Net4CGD consortium. Lentiviral gene therapy for X-linked chronic granulomatous disease. Nat Med. 2020 Feb;26(2):200-206.

- Distribution of the first 9 patients by center
  - 5 patients - US trial (ClinicalTrials.gov NCT02234934)
  - 4 patients - UK trial (ClinicalTrials.gov NCT01855685)
  - 2 patients passed away within 3 months post-gene therapy due to pre-existing conditions not related to GT
  - 7 patients were followed for ≥ 24 months and the results for these patients published in Feb 2020
  - One of these 7 patients died at 2.5 years post gene therapy from severe bullous/fibrotic pulmonary disease present pre-gene therapy
  - 4 additional patients were treated more recently in the US trial and will be discussed separately

First 9 Subjects Demographics Prior to Conditioning

Subject #	Age (yrs)	Prior Medical History	Treatment Date (mth/yr)	Status at Gene Therapy
1-UK	4	Pneumonectomy, EBV, Aspergillus, organomegaly, lymphadenopathy	7/13	Ongoing fever, organomegaly and lymphadenopathy
2-BOS	22	Klebsiella lymphadenitis, MRSA liver abscess, lung abscess, Nocardia abscess, Serratia abscess, GI and urethral granulomas	12/15	No active infections at GT
3-UK	18	Osteomyelitis, cerebral hemorrhage, colitis perianal abscess	7/16	Suspected fungal chest infection
4-NIH	18	Burkholderia cepacia, Nocardia and Aspergillus pneumonias	7/16	Stable
5-UK	2	Liver and splenic abscesses	4/17	Refractory spleen, liver abscesses, treatment with Linezolid
6-UCLA	27	Aspergillus, Nocardia and Serratia pneumonia, IBD, Crohn's disease	4/17	Bronchiectasis and pulmonary fibrosis with bullae
7-NIH	24	Multiple bacterial and fungal lung infections, lobectomy (lung), granulomatous liver, GI, recurrent lymphadenitis	6/17	Chronic persistent culture-positive Phellinus fungal pneumonia
8-NIH	3	BCGosis. >1yr hx of disseminated Aspergillus (bone, lungs, CNS)	8/17	Resistant Aspergillus at gene therapy. Status post: multiple granulocyte transfusions.
9-UK	22	Cerebral Aspergilloma, colitis, warts, granulomatous folliculitis; interstitial nephritis	9/17	Steroid- dependent colitis. Multi focal inflammatory changes on HRCT chest.

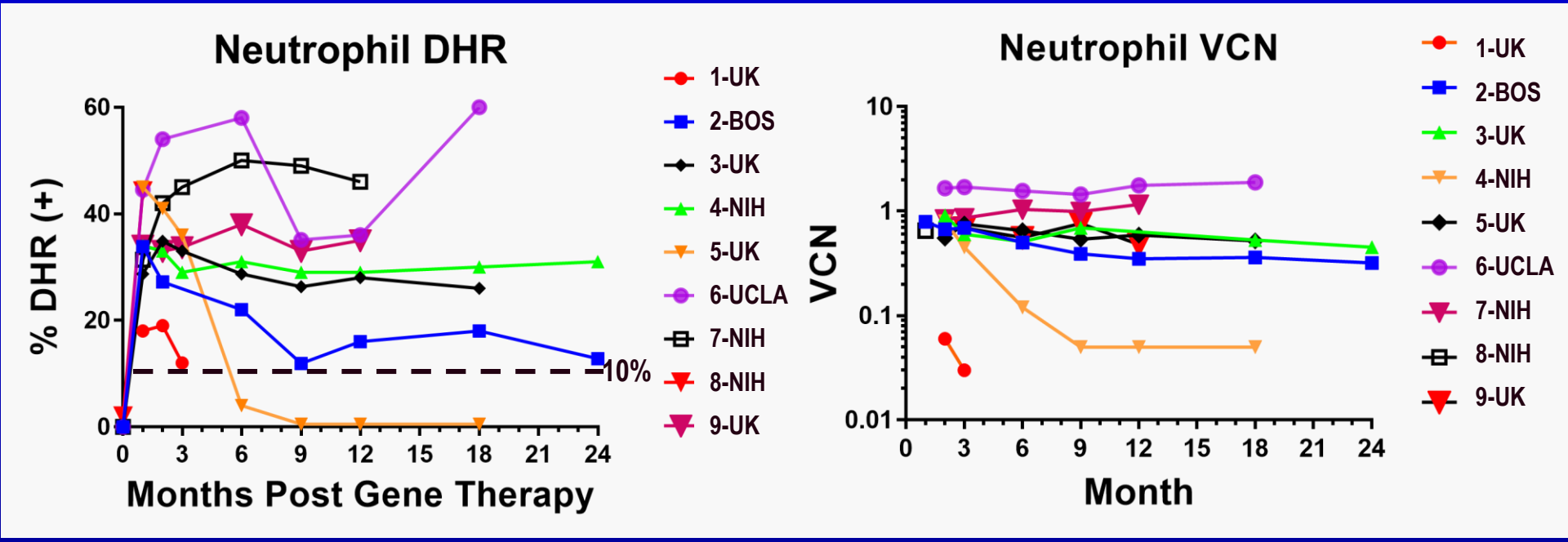
UK (Patients treated at UCL-GOSH); **BOS** (Patients treated at Boston Children's Hospital)
UCLA (Patients treated at UCLA Children's Hospital); **NIH** (Patients treated at NIH CC)

Cell Product Characteristics / Conditioning Dosing

Subject #	Cell Product Formulation	Cell Product Dose (x 10^6/kg)	Product Vector Copy Number	%PCR+ Colony Forming Units	Busulfan AUC* (ng/ml*hr)
1-UK	Fresh	15.6	1.5	69	75,000
2-BOS	Fresh	19.5	1.3	74	65,117
3-UK	Cryo	12.5	1.7	Not done	75,000
4-NIH	Fresh	32.6	1.0	49	66,191
5-UK	Cryo	6.5	5.5	Not done	75,000
6-UCLA	Fresh	10.1	3.1	79	67,666
7-NIH	Fresh	11.0	0.7	61	77,378
8-NIH	Fresh	4.62 (fresh source) 24.5 (thawed source)	2.0 1.4	71	62,349
9-UK	Cryo	7.75	2.9	Not done	75,000

*AUC = area under the curve (cumulative exposure) – Targeted 65-75,000

Patient Blood Neutrophils: % DHR$^+$ and Vector Copy Number



1-UK achieved 12% DHR$^+$ neutrophils at month 3, but died soon thereafter from progression of severe lung inflammation.

8-NIH achieved 38% DHR$^+$ neutrophils at month 1 but died soon thereafter from intracerebral bleed at site of brain fungal infection secondary to autoimmune thrombocytopenia.

5-UK achieved 35% DHR$^+$ neutrophils at month 3, but had significant loss of marking through month 12 stabilizing at ~0.5% DHR$^+$ neutrophil and is alive and well.

6-UCLA maintained high level 60% DHR$^+$, but died at 2.5 years post treatment from progressive bullous, fibrotic pulmonary disease.

The 5 other patients are alive and well maintaining a steady level of %DHR$^+$ neutrophils and VCN from last data point shown to present.

Summary

- **7 of the first 9 patients were alive & well ≥24 mo post gene therapy (GT) though one died from chronic pulmonary disease at 2.5 years.**
  - **No new CGD-related infections to the present**
  - **No vector-related complications**
  - **No molecular evidence of clonal dysregulation**

- **Corrected neutrophil function observed in all patients within 1 month in X-CGD patients treated with autologous GT**
  - **At 24 months, 6/7 surviving patients demonstrated persistence of 16–46% oxidase positive neutrophils though one of these died at 2.5 years after therapy from progression of pulmonary disease.**
  - **At last follow up all of the 5 of living patients with curative levels of oxidase activity no longer require CGD-related prophylactic antibiotics, and none have had any CGD related infections.**

Example of Clinical Benefit from Gene Therapy:

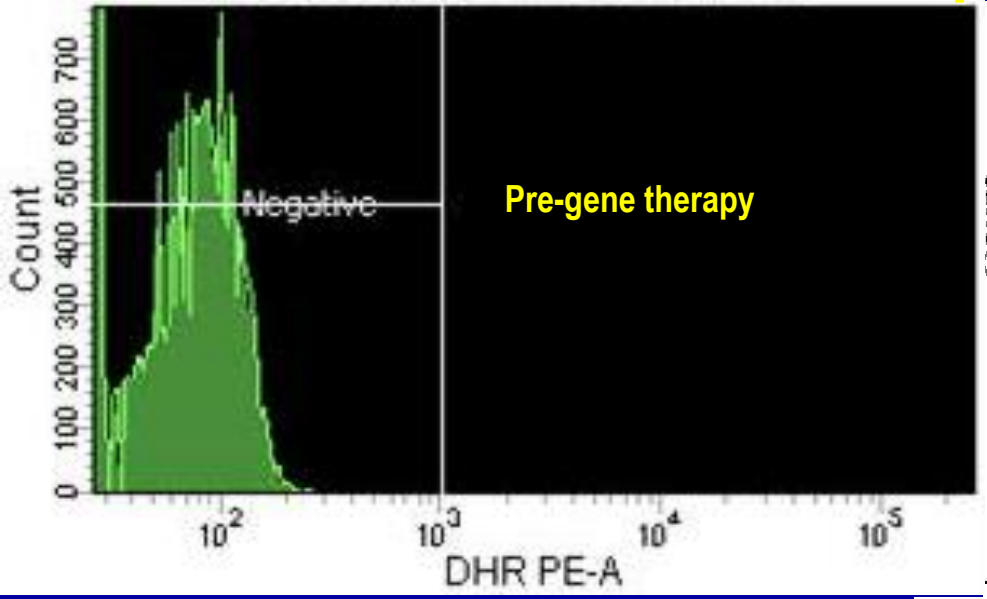
Subject 7-NIH: 24 yo man with X-CGD followed at NIH.

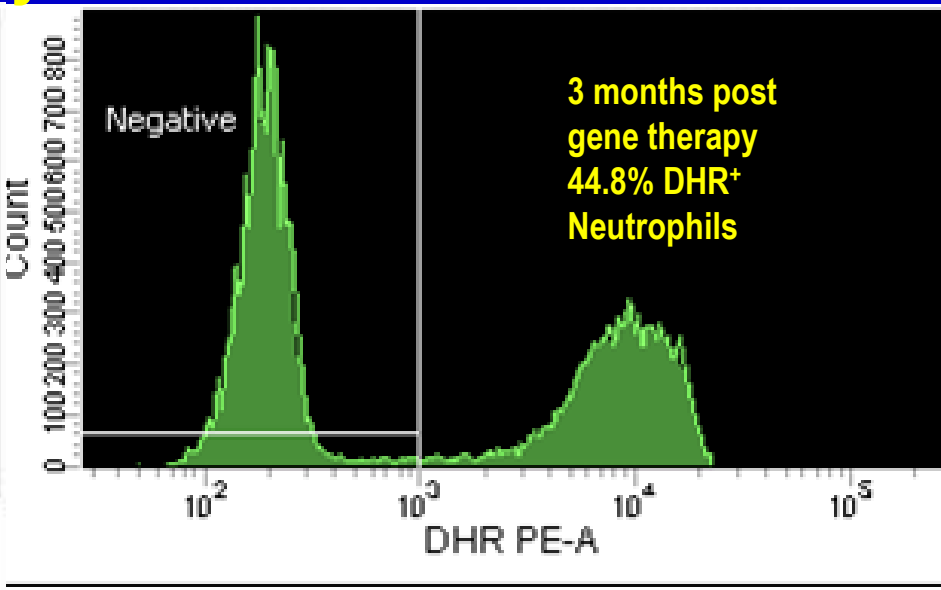
Clinical history: multiple bacterial and fungal lung infections; past lung lobe resection; granulomatous inflammation of liver, small and large intestines; chronic hidradenitis.

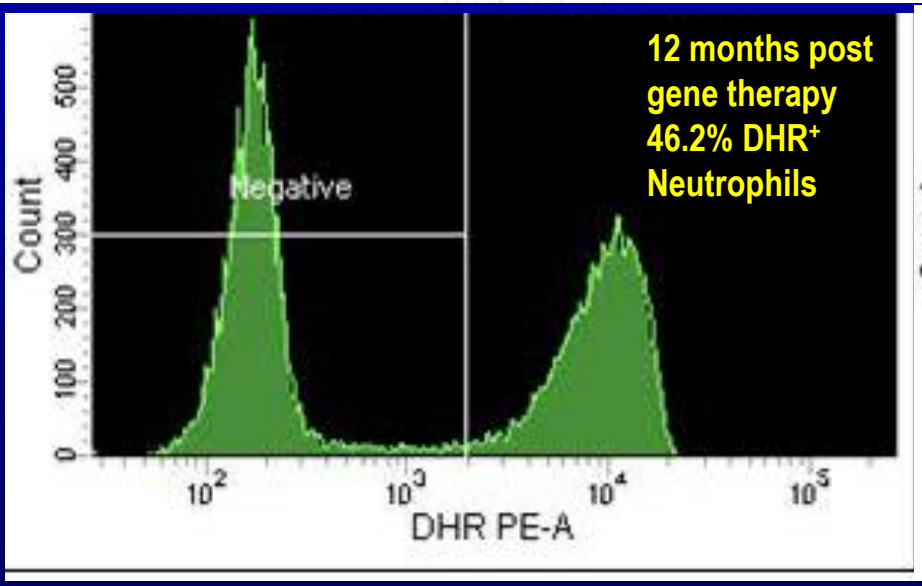
Ongoing infection not responsive to antibiotic therapy: October 2016 developed Inocutis (Phellinus group) fungus right side pneumonia with persistence and worsening of pneumonia from Dec 2016 to May 2017 when lung biopsy was still positive for fungus growth in culture.

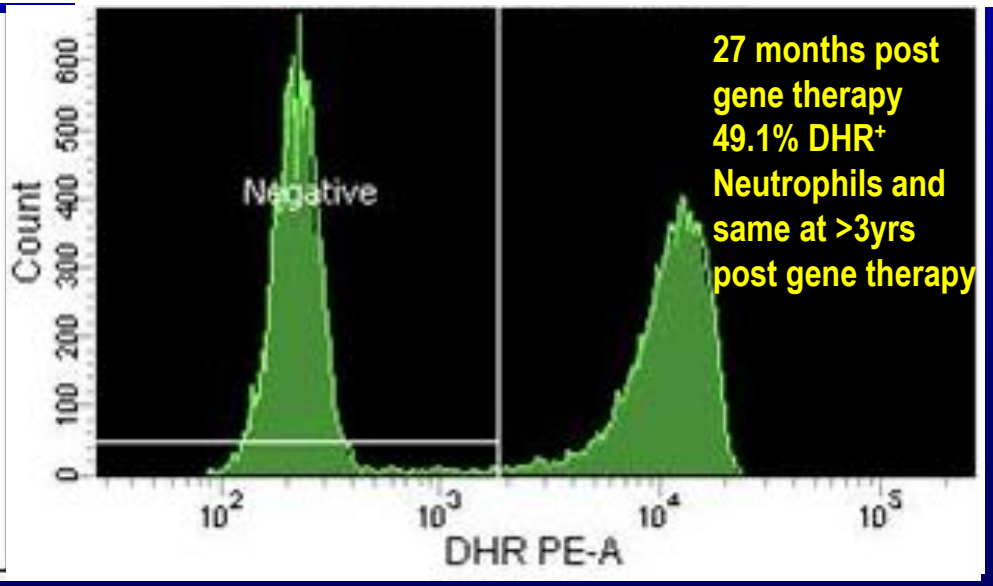
Gene therapy: Mid-June 2017.

XCGD 7-NIH: DHR Assay of Peripheral Blood Neutrophils Before and After Gene Therapy of 06/17/2017

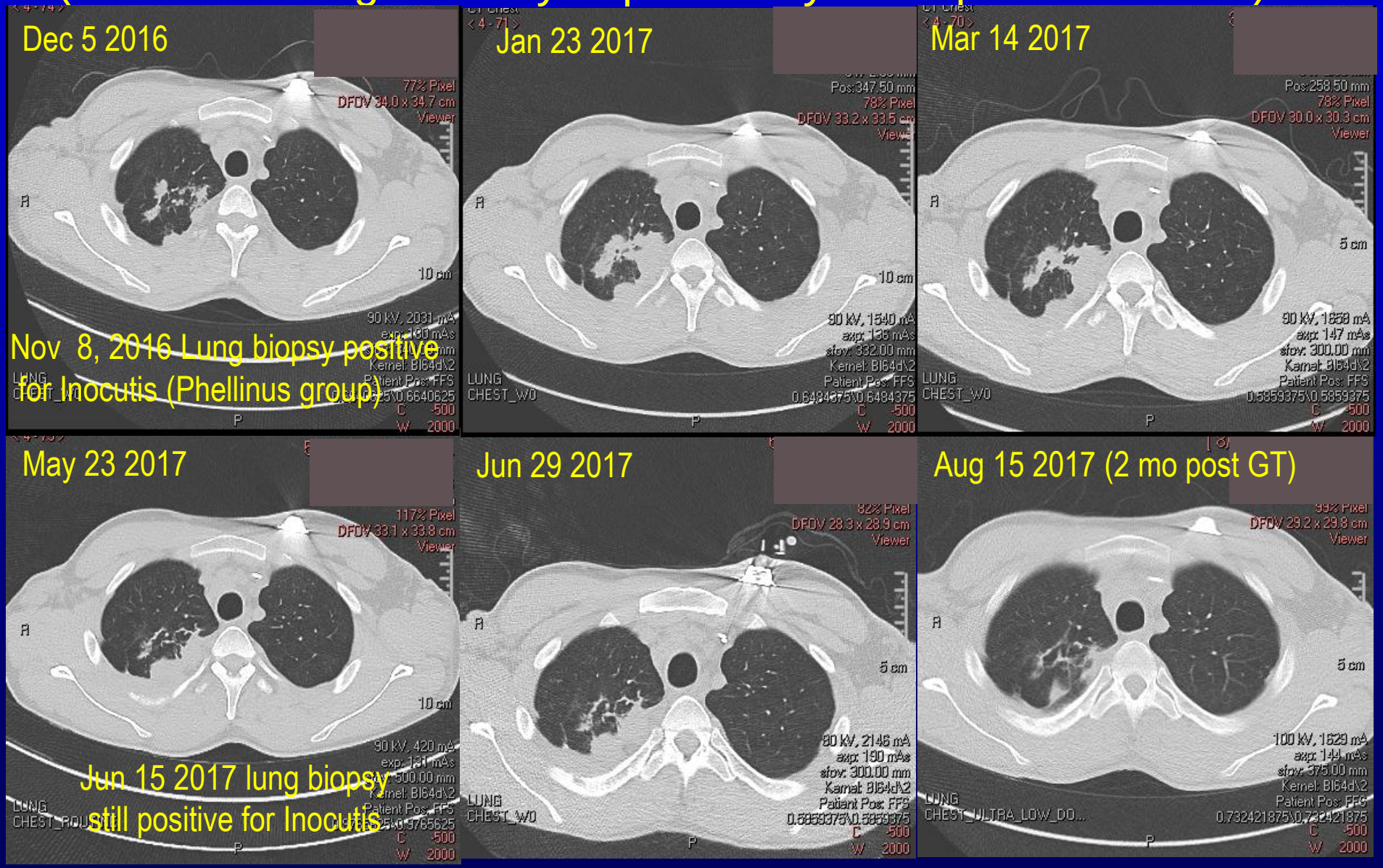




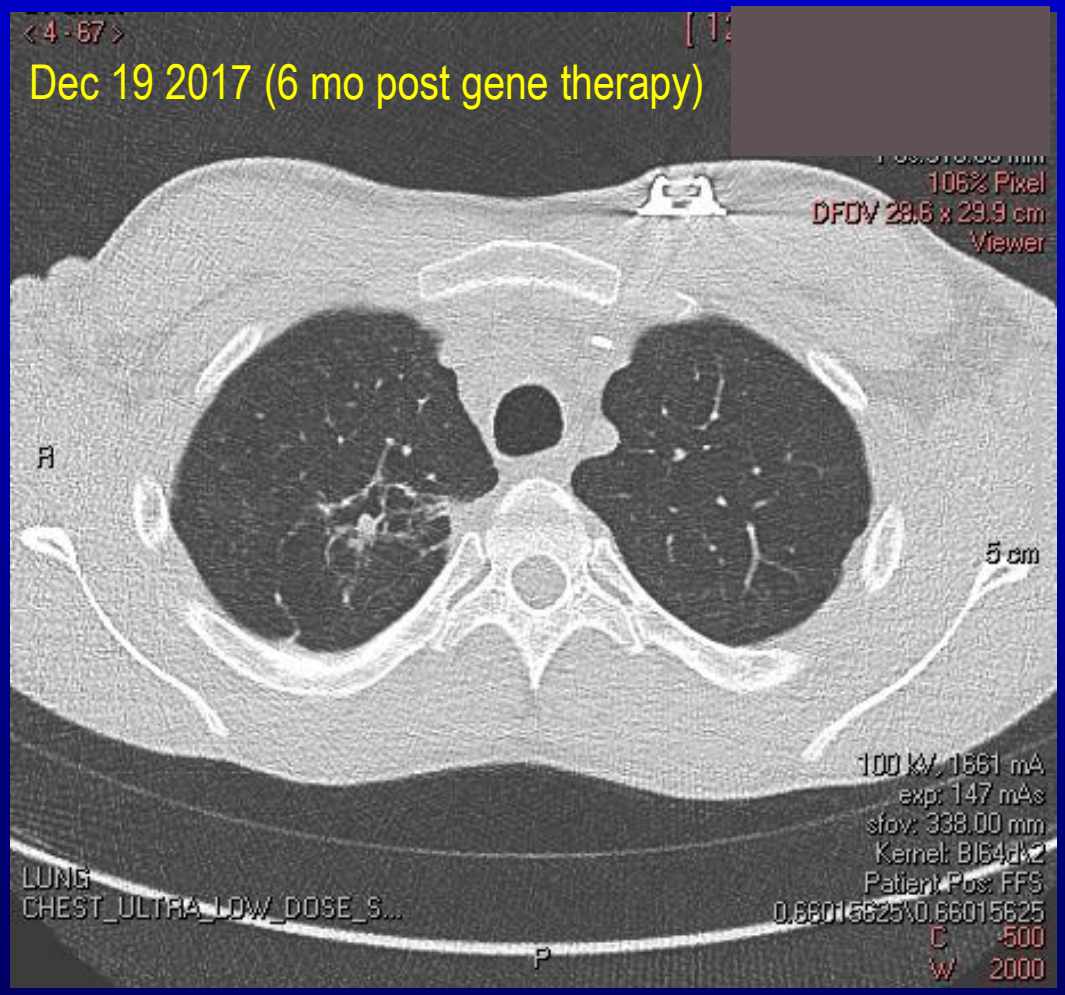




XCGD-N104: Time course of Inocutis (Phellinus group fungus) pneumonia Dec 2016 to Aug 2017 (Gene therapy 6/17/2017) (Pneumonia significantly improved by 2 mo post treatment)

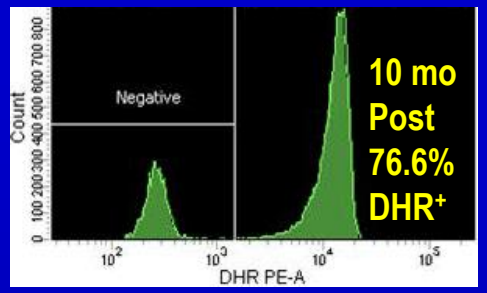


XCGD-N104: Inocutis (Phellinus group fungus) pneumonia fully resolved by 6 mo post gene therapy; chronic hidradenitis and IBD resolved by 12 months post gene therapy

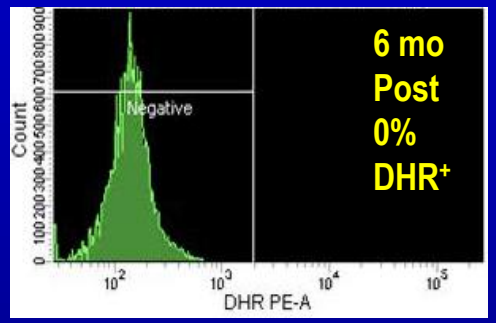


More Recent Additional 4 Patients

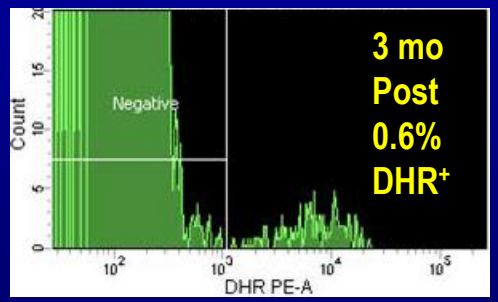
- **A-NIH: 31yo; Bone/lung apergillus/Inocutis (Phellinus) & B. cepacia pneumonia**
  Maintained >75% DHR$^+$



- **B-UCLA: 8 yo; recurrent infections**
  Early >30% DHR$^+$ progressed to 0%



- **C-UCLA: 3 yo; recurrent infections**
  Early >30% DHR$^+$ progressed to 0.3%



- **D-BOS: 11yo; recurrent infections** **>6 mo Post 0% DHR$^+$**
  Early >30% DHR$^+$ progressed to 0%

Updated Summary

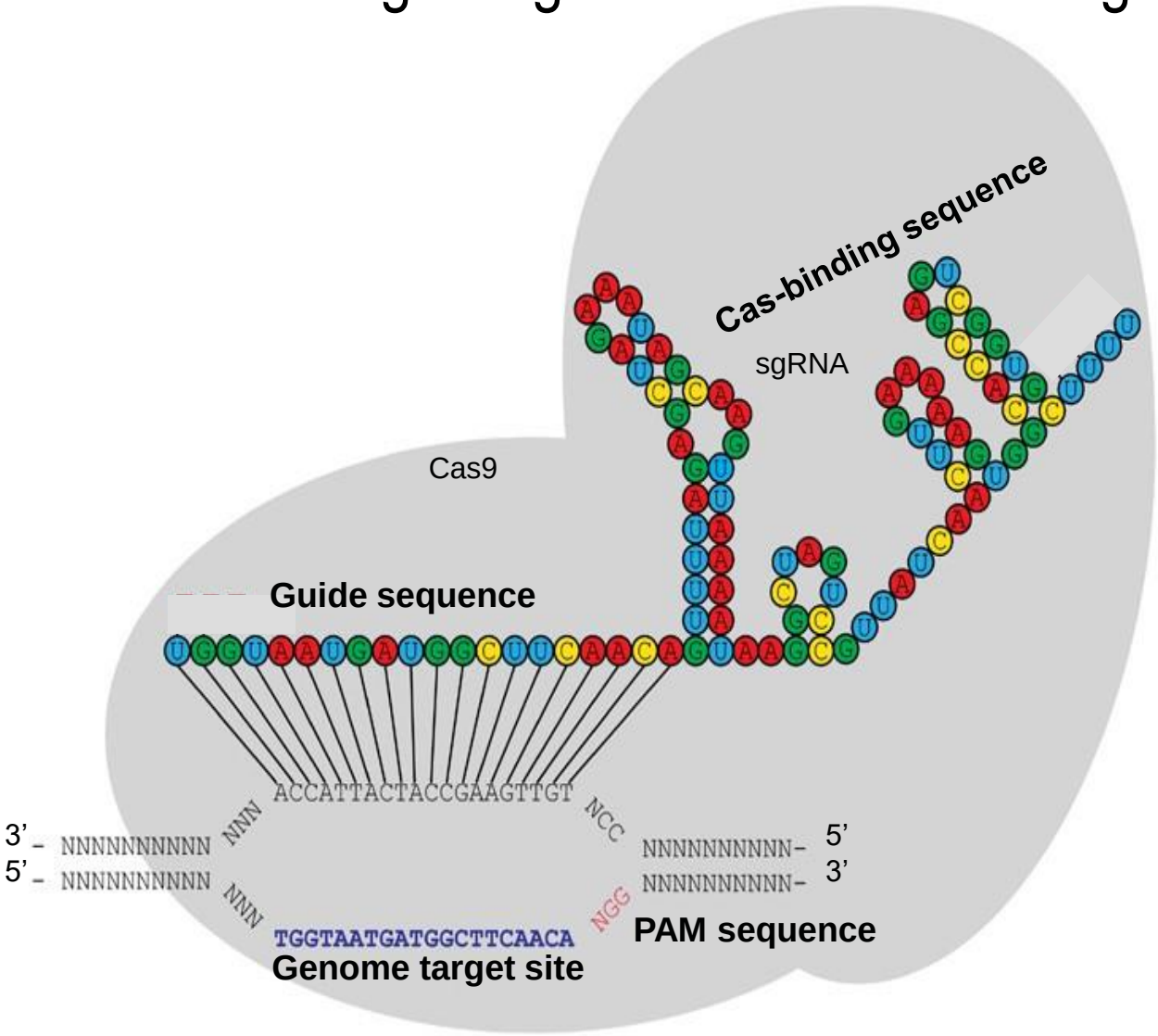
- **Of the 4 additional patients (1 adult / 3 children) treated in the last 9-18 months the adult patient has maintained high levels of correction/marking (>76%) at >1.5yrs, but 2 children have no detectable DHR^+ neutrophils by 2 to 6 months, and 1 child has 0.3% DHR^+ neutrophils at >12 months.**

- **Thus, of the entire group of 13 patients treated, 4 (all children) have experienced early loss of gene corrected neutrophils and vector marking (all at ≤6 months post GT)**

- **The protocol has been on hold with respect to treatment of additional patients but will re-open soon with plan to add anti-inflammation treatment in the form of Stelara (ustekinumab) pre-treatment and sirolimus (± steroids) post treatment to reduce CGD related inflammation and to prevent immune rejection of the corrected cells.**

CRISPR Gene Editing

Gene editing using CRISPR-Cas9 and sgRNA



Modified from: Hendel et al., Nat Biotechnol. 2015 Sep;33(9):985-9. (Porteus lab)

Gene Editing Nuclease Targets a Site for Double Strand DNA Break

Mostly high fidelity repair; but nuclease keeps cutting till an insert or deletion changes sequence sufficiently to stop recognition.

Non-Homologous End Joining (NHEJ = indels)

Provide <u>DNA donor</u> template with homology arms flanking desired changes near nuclease recognition site cutting.

Homologous Recombination (HR)

Electroporation is used to deliver CRISPR/Cas9 (either as mRNA or as recombinant protein), sgRNA (as the "guide") and either a short single strand oligodeoxynucleotide (ssODN) or AAV6 as the "donor" into $CD34^+$ hematopoietic stem/progenitor cells. In our lab we use the MaxCyte instrument, but other electroporation devices are available.

MaxCyte, Inc: MaxCyte® GT™ high efficiency electroporation, GMP clinical scale-up to $>10^9$ cells.

Repair a Specific Mutation Causing X-CGD

HDR of a "hot-spot" mutation in Exon 7 of the *CYBB* gene causing X-CGD using CRISP/Cas9 with ssODN donor

De Ravin SS, Li L, Wu X, Choi U, Allen C, Koontz S, Lee J, Theobald-Whiting N, Chu J, Garofalo M, Sweeney C, Kardava L, Moir S, Viley A, Natarajan P, Su L, Kuhns D, Zarember KA, Peshwa MV, Malech HL. CRISPR-Cas9 gene repair of hematopoietic stem cells from patients with X-linked chronic granulomatous disease. Sci Transl Med. 2017 Jan 11;9(372):eaah3480.

We have identified 20 patients (14 kindreds) followed at NIH with X-CGD caused by a single bp mutation in Exon 7 *CYBB* (a relative "hot-spot" causing ~7% of X-CGD cases).

1. **Mutation:** GAA ***CGA*** A **to** GAA ***TGA*** A C>T 676 leading to Arg(226)stop at start of Exon 7 of *CYBB* gene.

2. **Oxidase activity:** All patients are gp91phox protein null and have no residual oxidase activity.

3. **Clinical significance:** 3 have died; 3 have received successful allogeneic bone marrow transplant at NIH; 1 was successfully corrected by lentivector gene therapy at NIH (~48% of circulating neutrophils oxidase positive); the remaining 13 living patients including both children and adults have had recurrent life-threatening infections.

Gene editing using MaxCyte ® GT electroporation instrument for C>T 676 Exon 7 mutation repair

1. **Used CRISPR-Cas9 mRNA or CRISPR-Cas9 RNP.**

2. **Mutation-specific 20 bp gRNA which overlaps and directs efficient cutting near the mutation site and which does not mediate cutting of normal sequence.**

3. **100 bp single-stranded oligodeoxynucleotide donor DNA with normal *CYBB* sequence centered on the mutation site that was 2p phosphorothioate end-modified to direct HDR.**

gRNA homology relationship to mutation site

Splice acceptor

TTCACCCAGACGAATTGTACGTGGGCAGACC **Normal**

TTCACCCAGA*T*GAATTGTACGTGGGCAGACC **Mutant**

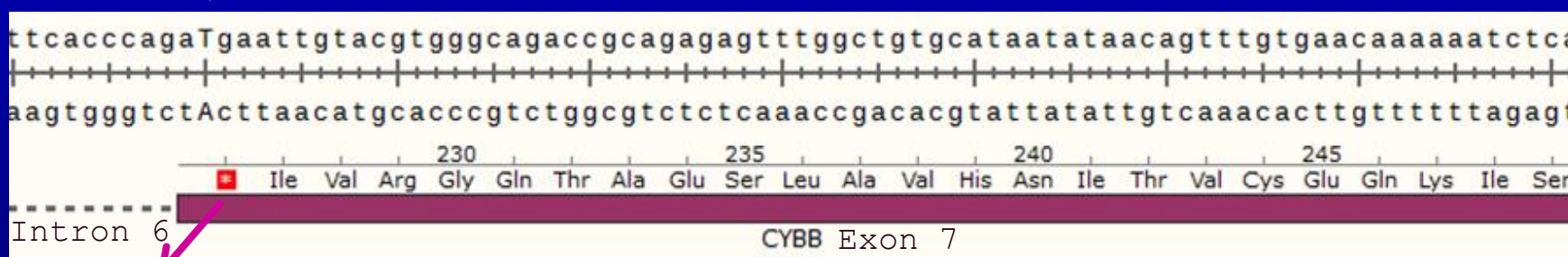
CACCCAGA*T*GAATTGTACGT **gRNA**

Mutation

Mutation C>T 676

Cut site

PAM



TGA Stop Codon

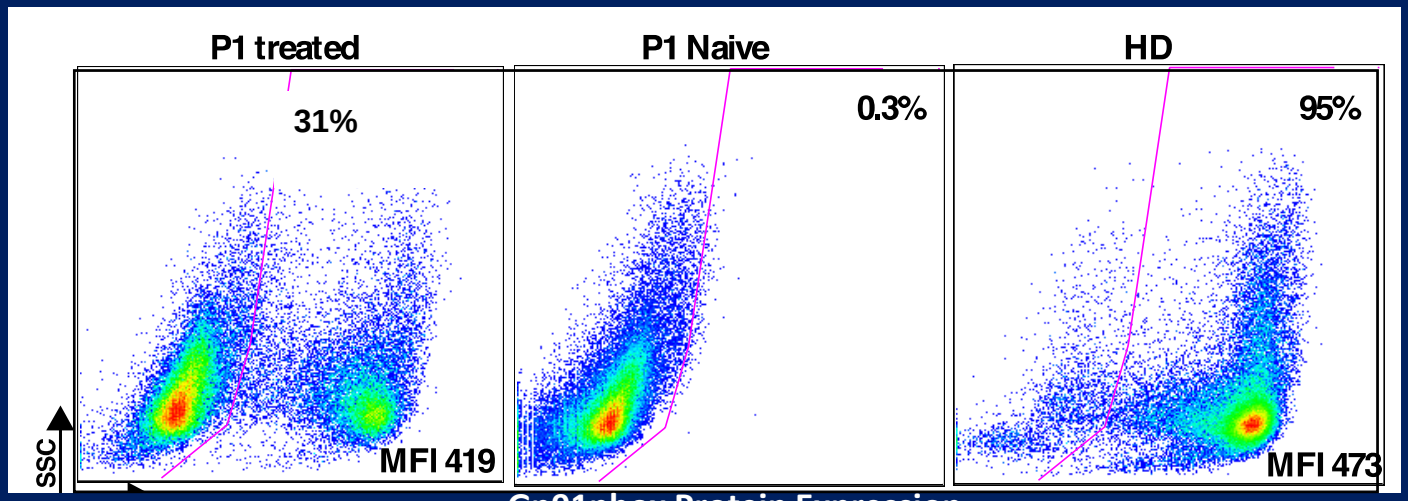
CGA Arg

g91 expression

Extensive studies performed first in patient derived EBV-B cells and then in patient CD34+ HSC to optimize correction:
Electroporate on culture day 2 with CRISPR/Cas9 mRNA100 µg/ml, sgRNA 200 µg/ml, phosphorothioate-modified ssODN donor 200 µg/ml.

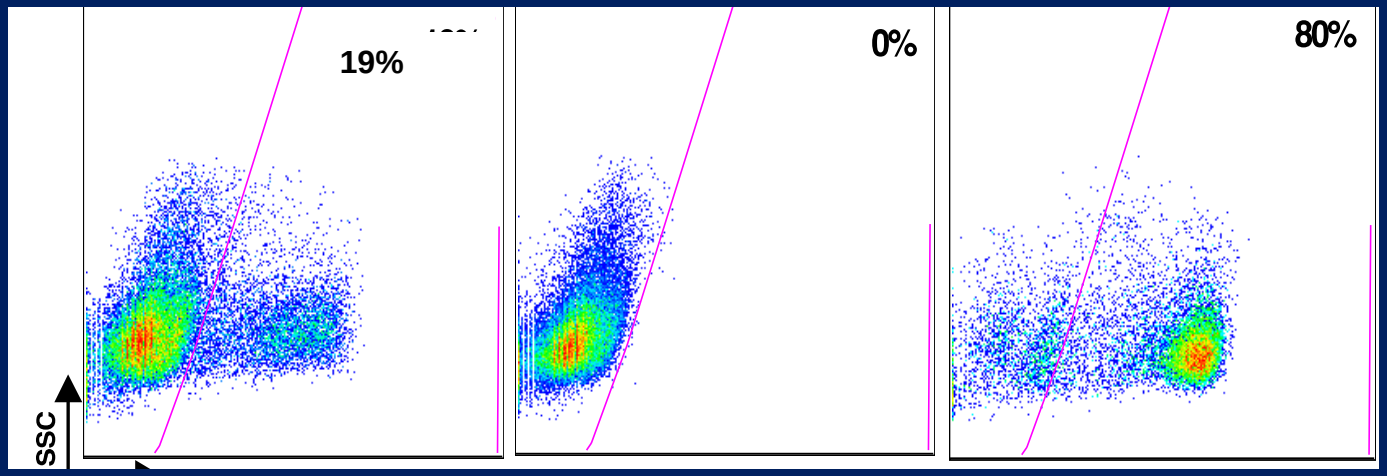
Mutation repair in X-CGD CD34^{+} cells: *In vitro*

Patient Repaired **Patient Naïve** **Healthy Donor**



Gp91phox Protein Expression

Restored gp91^{phox} protein expression in PMNs differentiated *in vitro*



DHR Assay of Oxidase Activity

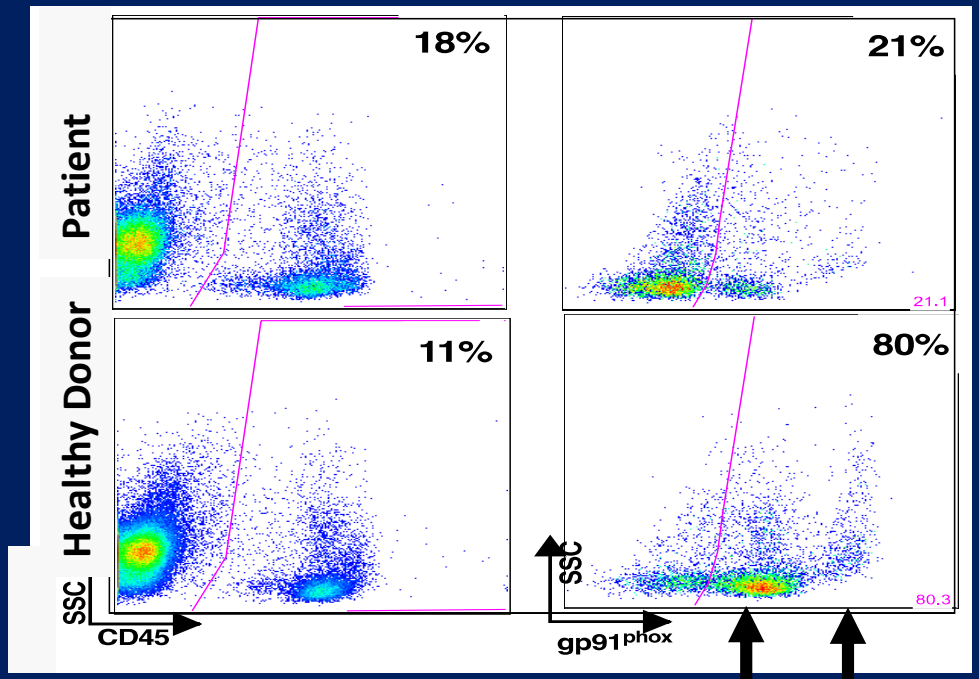
Restored NADPH oxidase activity (DHR+) in PMNs differentiated *in vitro*

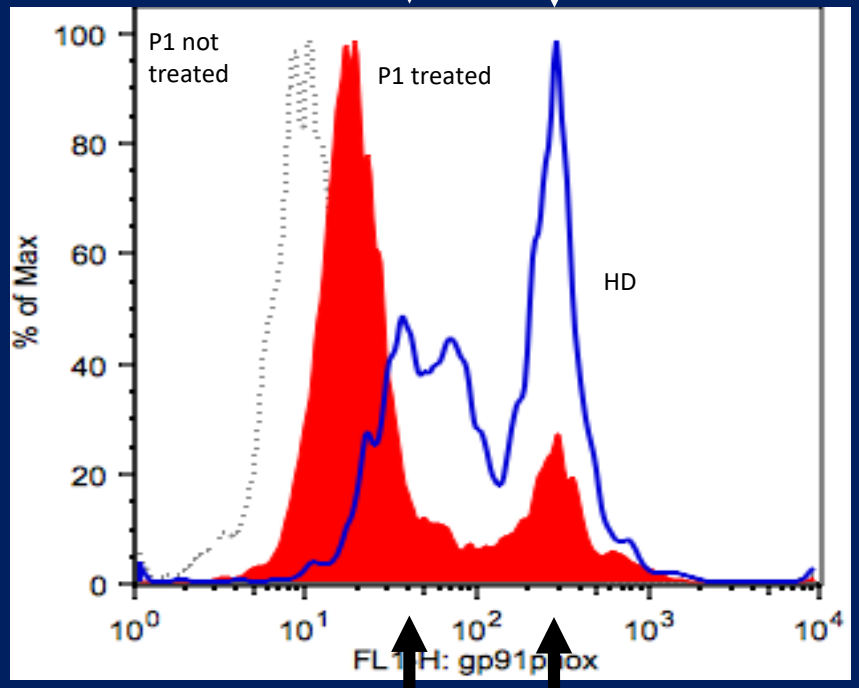
Repair of C>T 676 Arg(226)stop mutation in X-CGD patient CD34+ HSC transplanted 20 wks in NSG mice

Human CD45+ cell engraftment (no effect of editing on engraftment)

Human CD45+ cells expressing gp91phox (high levels of correction in patient myeloid cells)

Corrected X-CGD patient neutrophils and monocytes in NSG mouse peripheral blood have normal per cell expression of gp91phox





Monocytes Neutrophils

Monocytes Neutrophils

5 month *In Vivo* corrected X-CGD is 21%/80% = 26% of healthy donor.
Compared to *Ex Vivo* of about 32% of healthy donor transplanted into the NSG mice.

Current Optimization of CD34+ HSC CRISPR/Cas9 Editing

Thaw cryopreserved X-CGD CD34$^+$ cells

2 days expansion

Electroporate cells with CYBB sgRNA plus CRISPR/Cas9 mRNA or Ribonuclear Protein plus ssODN (if ssODN is used as donor)

Immediately transduce with AAV2/6 containing cDNA (if cDNA is used as the donor) and retain in the culture for 12-24 hours

Change medium to remove AAV

1-2 days culture

Collect DNA

Indel assay

2 weeks differentiation with G-CSF

Analyze correction in mature neutrophils

<u>CD34$^+$ cell culture</u>:
Low density culture (0.2 to 0.5 x 10^6 cells/mL)

StemSpan II serum-free medium containing 0.75 µM of StemRegenin-1, 35 nM of UM171, 100 ng/mL each of SCF, Flt3-L, TPO, and IL-6

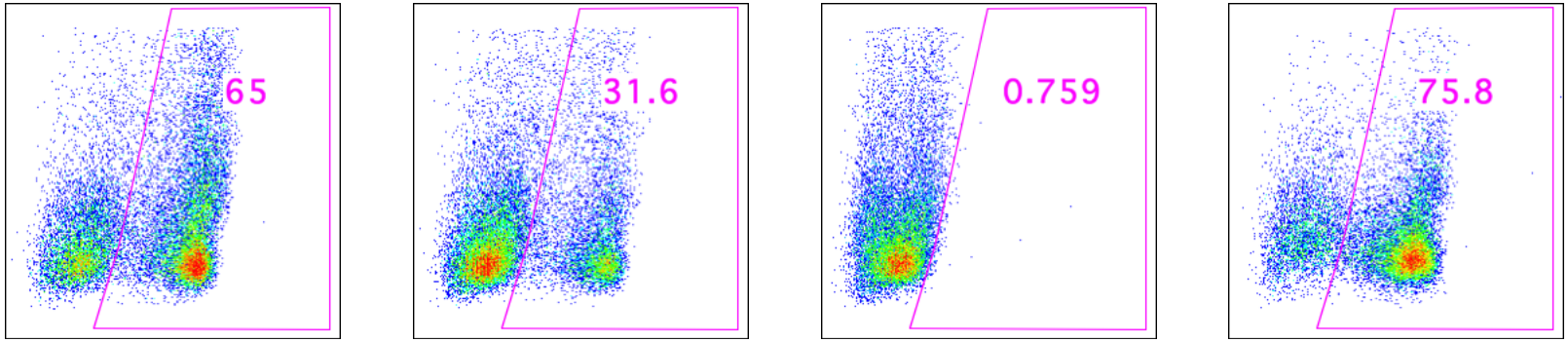
<u>Electroporation (MaxCyte GT system)</u>:
3-10 million cells in 100 µL MaxCyte d buffer with
100 pmol (=16.4 µg) Cas9 protein (or mRNA)
400 pmol modified sgRNA (complexed for 10-15 minutes at room temperature)
i53 mRNA 10 µg (enhances HR: see Canny MD, et al. Nat Biotechnol 36:95, 2018)

<u>AAV transduction</u>:
10^5 AAV particles per cell (AAV6 capsid with Y705+761F mutations)

Use of Most Current Conditions Including i53 (Canny et al) for enhancement of HDR by ssODN Donor HR Correction of CYBB Exon 7 C>T 676 Mutation in Gene Edited X-CGD CD34^{+} HSCs: *In vitro* myeloid differentiated to neutrophils

X-CGD CD34+ cells (shown is % of cells in + gate)

Healthy donor CD34+ cells



+ i53 (inhibits 53PB1)

No i53

Naïve CGD (no edit)

Healthy Donor

NADPH oxidase p91phox subunit expression →

***Ex Vivo* corrected X-CGD is 65%/75% = 87% of healthy donor.**

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