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A Case Study on Variance and its Implications on Study Design in the huNOG-EXL

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Objective: to understand the factors and the degree of variance indicative in the use of humanized immune system (HIS) mice and the implications of these on study design

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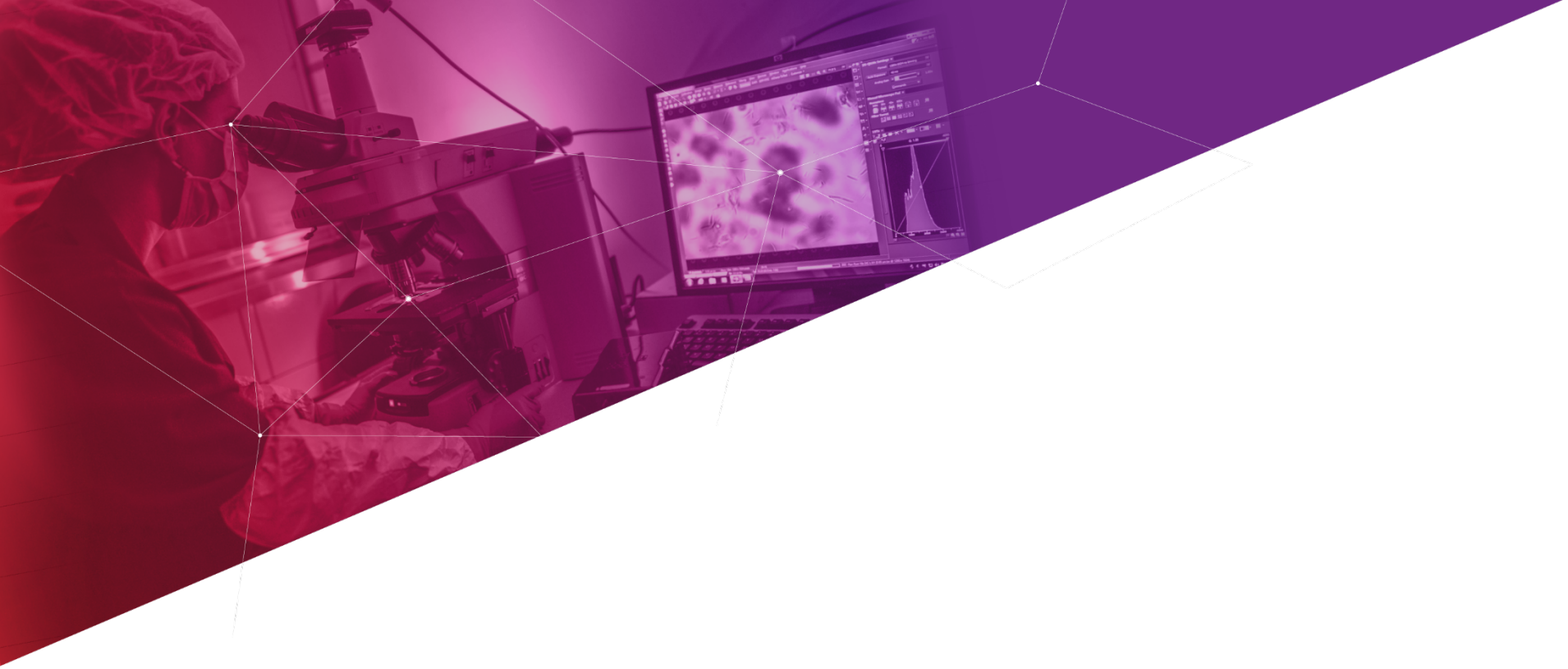

What are HIS mice?

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HIS mouse overview

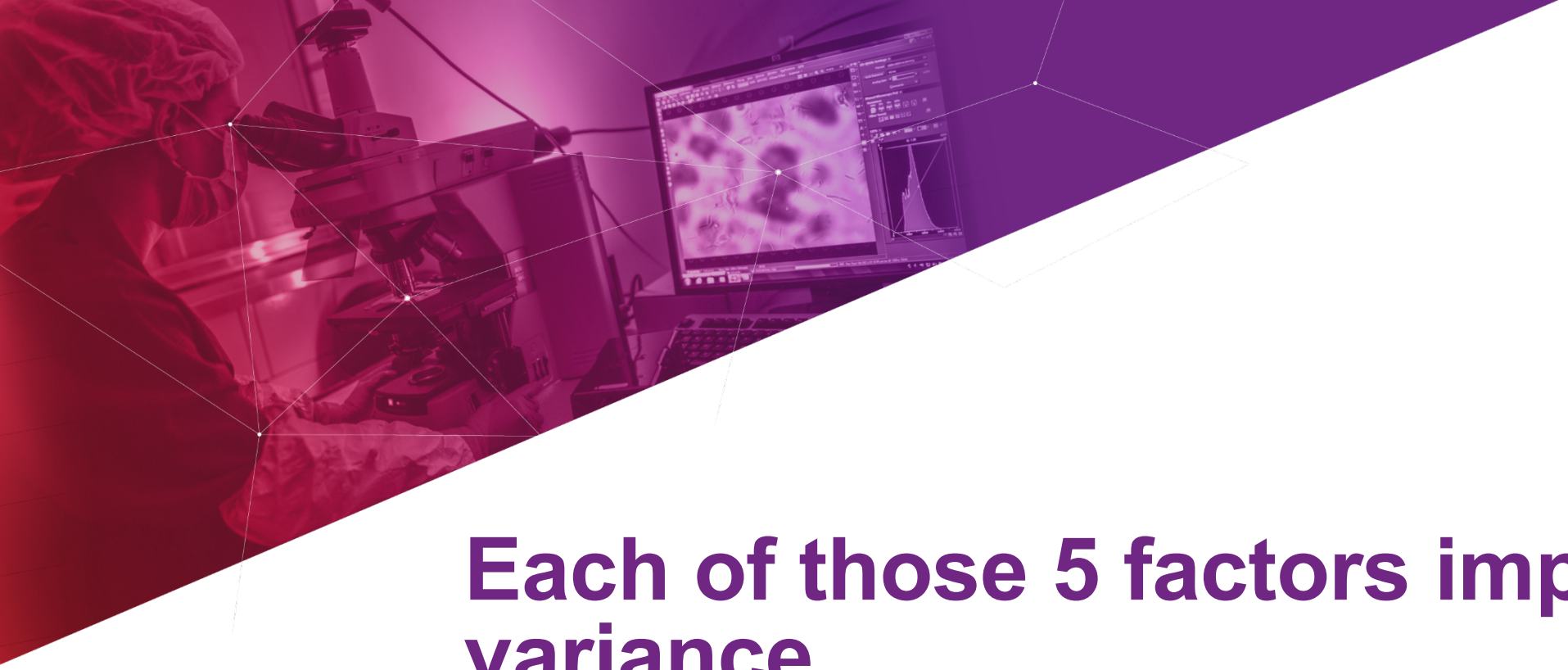
- Humanized immune system derived from engraftment with human immune cells
- Requires the use of a superimmunodeficient strain (IL-2rγ^{null}; NK cell deficient; murine innate impairment) with preservation of human CD47 binding
- **The utility and phenotypic characteristics of these models are dependent on the underlying methodology in their creation**
- To compare the results of one independent HIS study to another (even with the same strain) ask "**how were they made**" and evaluate the differences/similarities within the methods section
- Established utility within the immuno-oncology space (e.g. bispecifics, checkpoint inhibitors)
- Currently, heavy interest in utility for immunology (auto-immune) directed therapeutics. Early evaluation as IND-enabling model, first in human dosing and immuno-toxicity.

What question are you trying to ask with the HIS mouse?

The answer will point to:

- Strain
- Myeloablation requirements
- Type/dose of human cells for engraftment
- Reconstitution/kinetics desired
- Desire for other (PDX, CDX, thymus, etc.)

What human cells do you need, how long do you need them to be present, and what is the functionality that you need from them?



Each of those 5 factors imparts a variance

The level of variance across an experiment can impact the power required

Reference on Determining Sample Size: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3826013/

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The engraftment process

It all starts with how you make the mice

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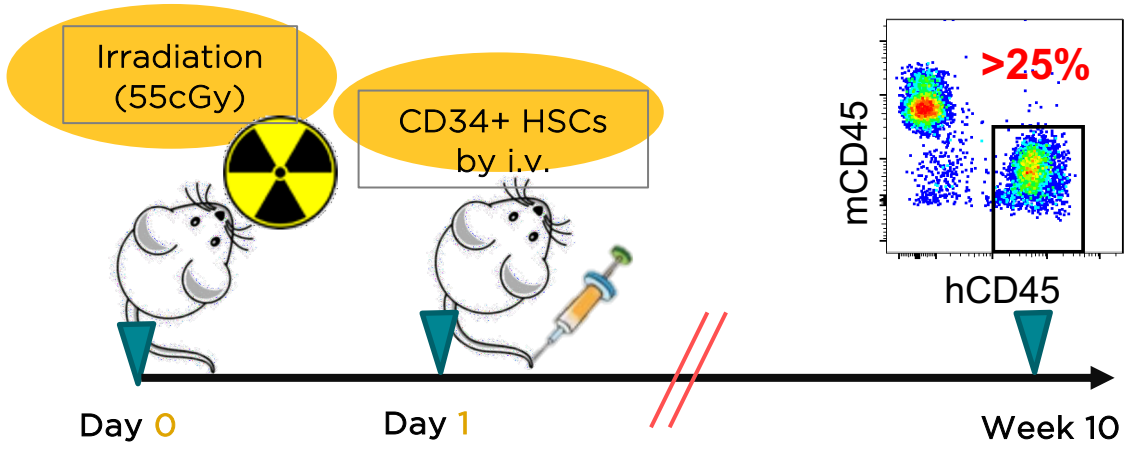
Overview of Taconic's engraftment process

This is specific to the huNOG-EXL - our process differs based on strain

huNOG-EXL

HUMAN CD34+ HSC-ENGRAFTED NOG-EXL



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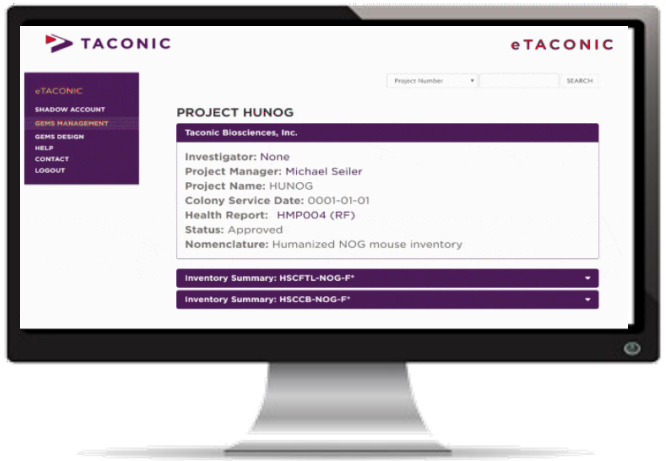

- Select your study cohorts from live online inventory
- Chimerism data provided for model selection
- HLA-A2 status (+ or -) provided for model selection
- Receive QC data for every huNOG mouse ordered

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Factors to consider for HIS mouse creation

Each of these can impact the overall utility and characteristics of the model

- Human **immune** cells to be engrafted
  - PBMCs, CD34+ from cord blood, CD34+ from fetal liver, CD34+ from bone marrow, G-CSF mobilized CD34+, NK cells, etc.
- Characteristics of human donor
  - HLA status, age, ethnicity, latent viral infections, disease or healthy
- Characteristics of human **immune** cells
  - Single or pooled source, purification method, fresh or cryopreserved, dose, *ex vivo* expansion/activation, genetic modifications
- Strain of mouse to be used
  - NOG/NSG or human transgene expressing, age, sex, microbiota
- Actual engraftment procedure
  - Depth/type of anesthesia, experience of technicians, route of injection/introduction, type/duration/dose of preconditioning, cytokine administration, use of antibiotics, etc.

Factors to consider for HIS mouse creation

Each of these can impact the overall utility and characteristics of the model

- Human **immune** cells to be engrafted

EXAMPLES:
- NOG with 150,000 CD34+ CB cells, 110 cGy irradiation, tail vein i.v. at 6 wks F
- NOG with 1,000,000 PBMC cells, no irradiation, tail vein i.v. at 6 wks F
- NSG with 200,000 CD34+ fetal liver cells, 325 cGy irradiation, tail vein i.v. at 6 wks F + fetal thymus kidney capsule
- NOG-EXL with 100,000 CD34+ CB cells, 20 mg/kg busulfan i.p., tail vein i.v. at 6 wks F

obilized CD34+,

/activation,

  - NOG/NSG or human transgene expressing, age, sex, microbiota

- Actual engraftment procedure
  - depth/type of anesthesia, experience of technicians, route of injection/introduction, type/duration/dose of preconditioning, cytokine administration, use of antibiotics, etc.

Factors to consider for HIS mouse creation

Each of these can impact the overall utility and characteristics of the model

- Human **immune** cells to be engrafted

EXAMPLES:
- NOG with 150,000 CD34+ CB
- NOG with 1,000,000 PBMC
- NSG with 200,000 CD34+ fet
  + fetal thymus kidney capsule
- NOG-EXL with 50,000 CD34+ CB cell

EACH OF THESE MODELS IS VERY DIFFERENT

Level of chimerism
Type of human cells present
Duration of study window
Expected variance
Overall utility

Even if we could unify on the same donor: the results produced with one model may NOT carry over to another.

  - NOG/NSG or human transgene exp

- Actual engraftment procedure
  - depth/type of anesthesia, experience of technicians, route of injection/introduction, type/duration/dose of preconditioning, cytokine administration, use of antibiotics, etc.

Publication: HIS mouse reporting standards (MISHUM)

Stripecke et al. EMBO Mol Med (2020) 12: e8662; DOI 10.15252/emmm.201708662

Review

Innovations, challenges, and minimal information for standardization of humanized mice

Renata Stripecke[1,2,†,*], Christian Münz[3,†], Jan Jacob Schuringa[4,†], Karl-Dimiter Bissig[5,†], Brian Soper[6,†], Terrence Meeham[7,†], Li-Chin Yao[8], James P Di Santo[9], Michael Brehm[10], Estefania Rodriguez[11], Anja Kathrin Wege[12], Dominique Bonnet[13], Silvia Guionaud[14], Kristina E Howard[15], Scott Kitchen[16], Florian Klein[17], Kourosh Saeb-Parsy[18], Johannes Sam[19], Amar Deep Sharma[1], Andreas Trumpp[20,21], Livio Trusolino[22,23], Carol Bult[6] & Leonard Shultz[6,†]

Minimal Information for Standardization of Humanized Mice (MISHUM)

- Article reviews:
  - Types of humanization (cells/tissues/tumors/viruses)
  - Common data endpoints based on use
- Goal: to align the scientific community on the methodological and analysis outputs so that information could be interpreted and assessed compared to other independent humanized mouse studies.
- Challenge:

"we looked at cytokine release syndrome in humanized mice but it didn't work…

"we tried anti-PD1 in humanized mice but it didn't work…"

Remember this publication was needed because of the **lack** of uniform/minimal reporting in publications

- These conclusive statements towards humanized mice are unfortunately common. Without knowing how they asked the question, how could one tell either way? I highlighted broad limitation statements, but it is also common to see the opposite: "*it can do X, we saw it in our experiment and published it*."

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Our answer to our question needs to be rejected or accepted.

Our design needs to be capable of doing that.

HIS mice have factors that will impact the effect.

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A case study:

How did we assess our own experimental hypothesis with the huNOG-EXL in an IO application?

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Question: How does factor X impact the reconstitution and potential IO utility of the model?

Experimental Hypothesis: Factor X significantly effects the reconstitution (chimerism/phenotype/TILs), kinetics, and/or tumor (CDX or PDX) growth profile.

Null Hypothesis: There is no significant difference between animals with factor X and those without.

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The limiting factor to our study design is the maximum n-value produced from a given HSC donor

The only way to do a direct comparison of two or more variables is to retain the impact of the variables within a single donor. Sample size cannot exceed what a donor can produce!

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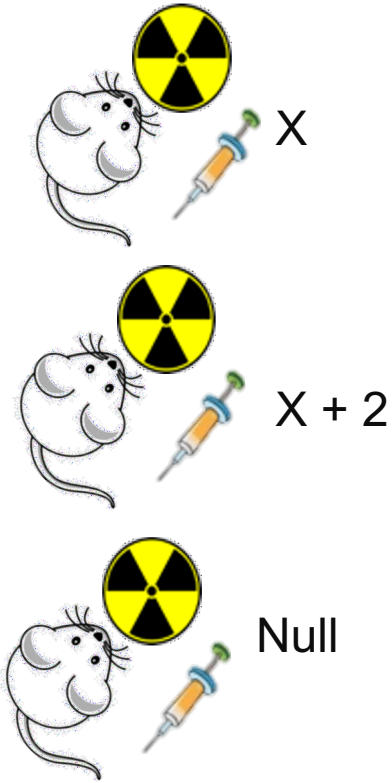
Our study design

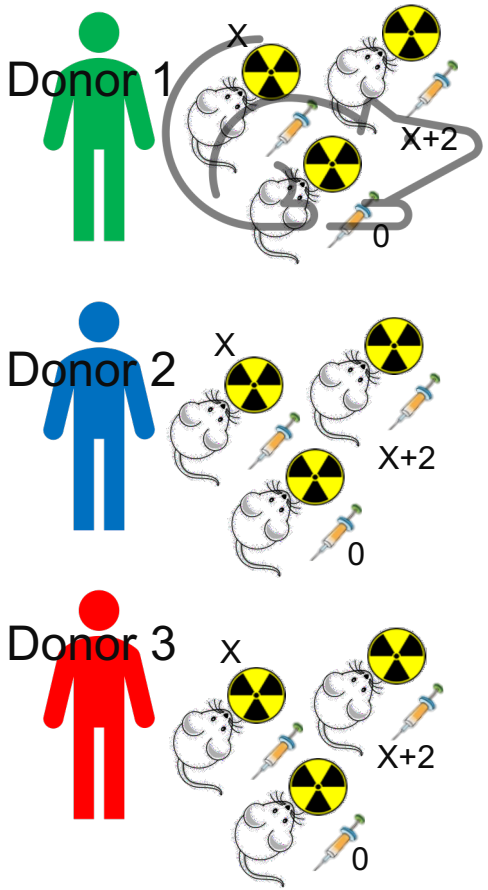
Balance between n-value/donor, critical ask, # variables, degree of variance

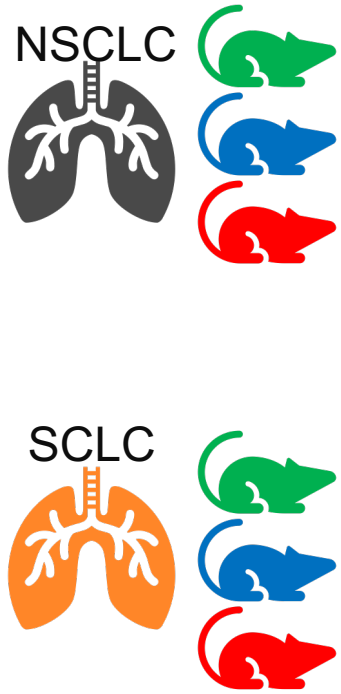
- Essential to question = 3 different arms (Factor X, Factor X+2, and Vehicle)
- Tumor growth = 2 arms (NSCLC CDX and SCLC PDX)
- Treatment = 2 arms (anti-PD1 and isotype control)
- Complete study is a 3 x 2 x 2
- Use of a power analysis software (G-power: two-way repeated measures ANOVA 2x3, effect moderate, alpha 0.01)
- ***Rate limiting step of a study design sample size = the titer of CD34+ cells of a given donor**
- Higher titer donors (>2M) do exist but they are rare; we made use of these
- Case study: total n=180, n= 60/donor x **3 donors**; sample size **n=20** (calculated n=13 + 54% attrition factor)
- Results: adequate power for essential question (Factor X), less desirable for 2' variable (*tumor)
- Sample size was not adequate for treatment (3' variable)
- *Overage for study constrained to 50%, recommend (especially for PDXs) 100%; SD chimerism 20, failure <10%

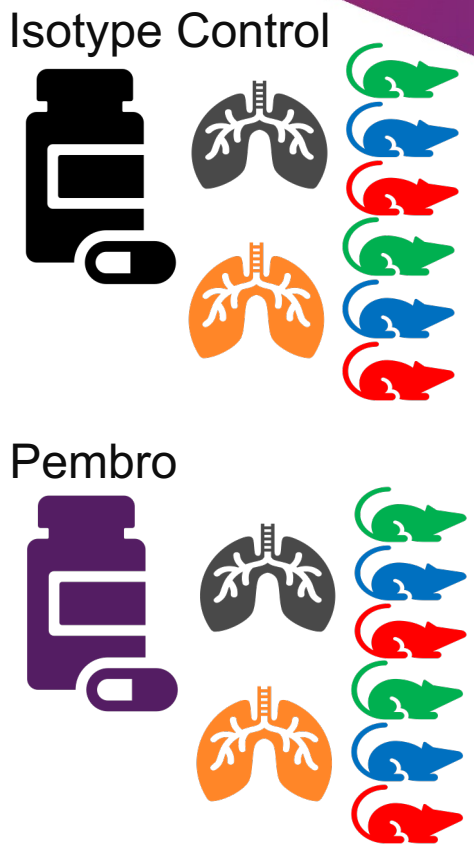
Recap of our study design

Graphical representation of variables









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huNOG-EXL Study

Production Plan		Group ID	Model	Factor X+2	Factor X	Mice per Donor (Study)
		A	13395-F	X	N/A	20
Donors on Study	3	B	13395-F	N/A	N/A	20
Total Mice Engrafted	180	C	13395-F	N/A	X	20
						60

STUDY DESIGN			Donor 1	Donor 2	Donor 3		Total Flow Samples		
			n=60	n=60	n=60		210		
Study Groups & Interventions			Mouse Quantites				Flow Samples Quantities		
Group ID	CDX	Condition	Donor 1	Donor 2	Donor 3	N	Blood	Spleen	Tumor
A	A549	Veh	4	4	4	12	4	4	3
A	A549	Pembro	4	4	4	12	0	0	3
A	SCLC PDX	Veh	4	4	4	12	4	4	3
A	SCLC PDX	Pembro	4	4	4	12	0	0	3
A	OVERAGE	OVERAGE	2	2	2	6	0	0	0
	50% OVERAGE		6	6	6	18			
B	A549	Veh	4	4	4	12	4	4	3
B	A549	Pembro	4	4	4	12	0	0	3
B	SCLC PDX	Veh	4	4	4	12	4	4	3
B	SCLC PDX	Pembro	4	4	4	12	0	0	3
B	OVERAGE	OVERAGE	2	2	2	6	0	0	0
	50% OVERAGE		6	6	6	18			
C	Select CDX	Veh	4	4	4	12	4	4	3
C	Select CDX	Pembro	4	4	4	12	0	0	3
C	OVERAGE	OVERAGE	2	2	2	6	0	0	0
	50% OVERAGE		2	2	2	6			
						Flow samples/group ID:	20	20	30
						Flow samples/group ID/donor:	60	60	90

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huNOG-EXL Study

Production Plan		Group ID	Model	1' Factor X+2	Factor X	Mice per Donor (Study)
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2'		3'	n=60	n=60	n=60		210		
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B	A549	Pembro	4	4	4	12	0	0	3
B	SCLC PDX	Veh	4	4	4	12	4	4	3
B	SCLC PDX	Pembro	4	4	4	12	0	0	3
B	OVERAGE	OVERAGE	2	2	2	6	0	0	0
	50% OVERAGE		6	6	6	18			
C	Select CDX	Veh	4	4	4	12	4	4	3
C	Select CDX	Pembro	4	4	4	12	0	0	3
C	OVERAGE	OVERAGE	2	2	2	6	0	0	0
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						Flow samples/group ID:	20	20	30
						Flow samples/group ID/donor:	60	60	90

Across 3 variables (1' + 2' + 3')
n=4/donor
started with 60 -> 4

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huNOG-EXL Study

Production Plan		Group ID	Model	Factor X+2	Factor X	Mice per Donor (Study)
		A	13395-F	X		
Donors on Study	3	B	13395-F	N/		
Total Mice Engrafted	180	C	13395-F	N/		

STUDY DESIGN			Donor 1	Donor 2	Don			
			n=60	n=60	n=			
Study Groups & Interventions			Mouse Quantites					
Group ID	CDX	Condition	Donor 1	Donor 2	Donor 3	N		
A	A549	Veh	4	4	4	1		
A	A549	Pembro	4	4	4	1		
A	SCLC PDX	Veh	4	4	4	1		
A	SCLC PDX	Pembro	4	4	4	1		
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	50% OVERAGE		2	2	2	6			
						Flow samples/group ID:	20	20	30
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Donors have both INTRA- and INTER- variance

Unless the question is specific to a single donor, **MUST** use multiple donors to assess.

For example: donor has unique characteristics which impact results e.g. resistant to tumor growth or high responsiveness/resistance to treatment.

Treat a donor like an independent n-value. An n=1 is not a wise decision.

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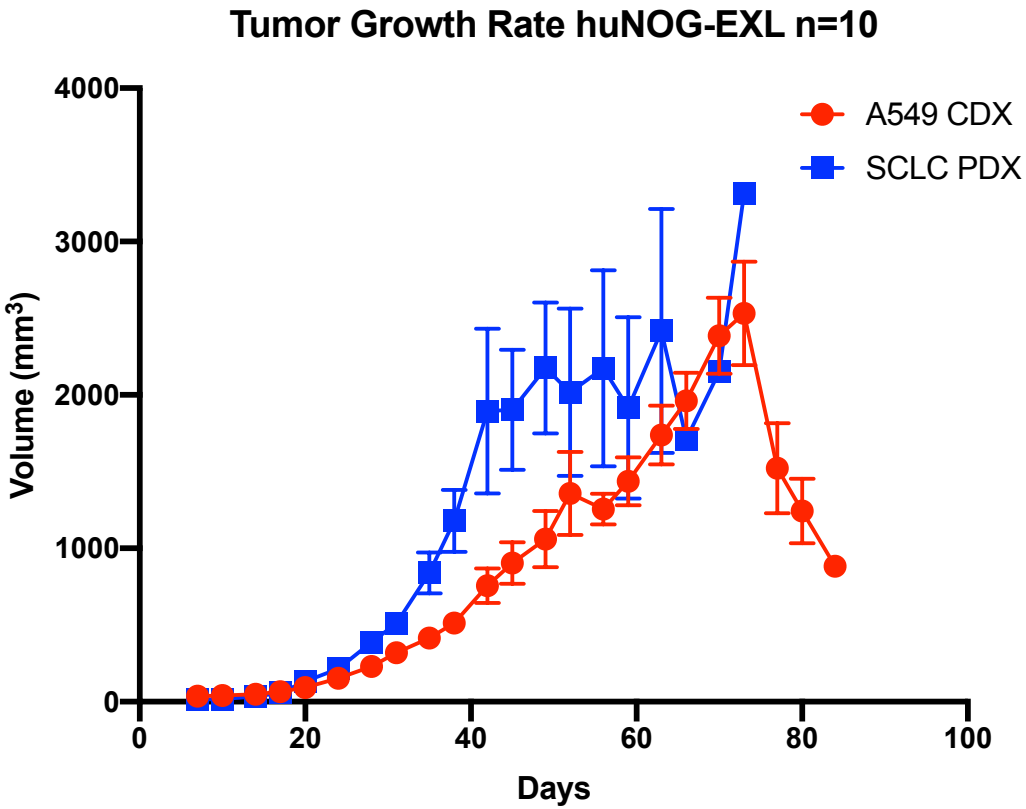
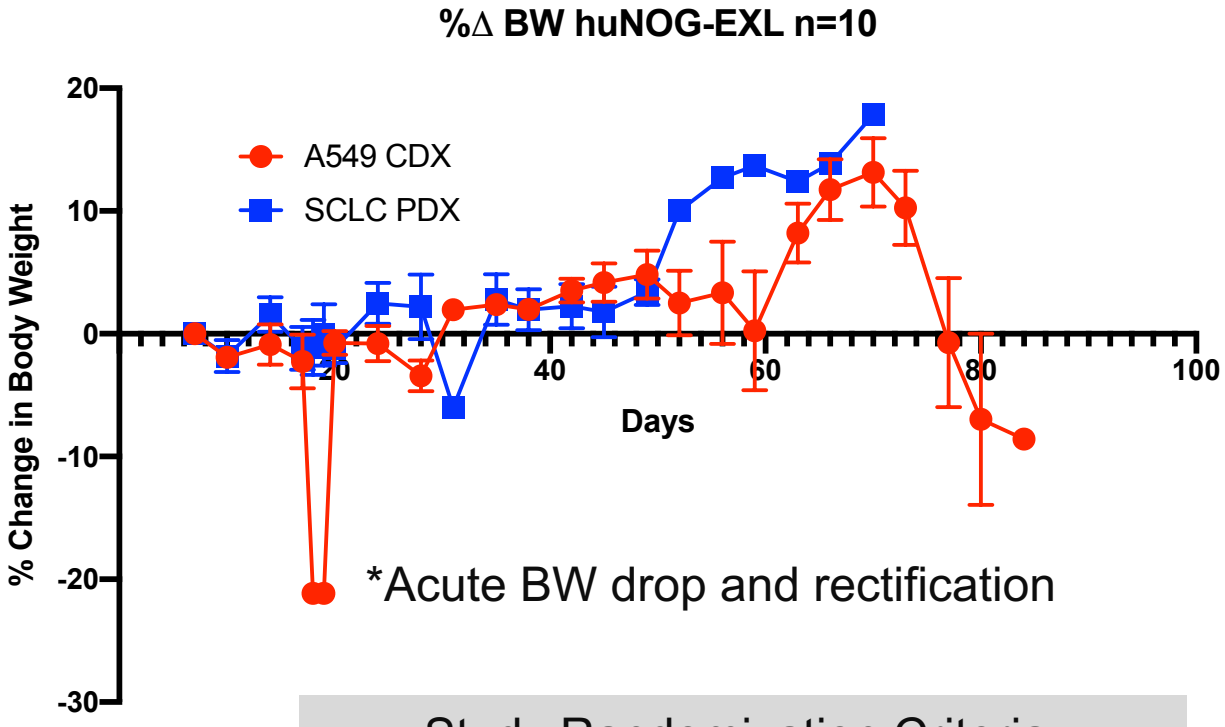
Tumor growth kinetics

- Don’t assume tumor growth kinetics in one mouse strain will equal another or be the same in HIS version of mouse
- Ideal to run a pilot tumor growth curve study to determine timeline and volume for treatment with HIS mice
- The donors used in the pilot tumor growth curve were independent of those for our study
- Fast-growing tumors probably not as relevant vs. slow-growing and/or complex TME support
- PDX: origins, patient/tx info, is it primary or passaged? What is the passage #?
- Overage: 50% CDX and 100% PDX
- SCLC PDX used (not owned by Taconic) Caucasian, male, 68 yo, right upper lobe, naïve, P5. Tumor cryopreserved cells were SQ inoculated into NSG to generate warm tumors, vol. 600-1000 mm^3 were harvested and cut into $3mm^3$ chunks. These were then SQ inoculated into huNOG-EXL for the pilot tumor growth study. The mice within our growth study then provided the $3mm^3$ tumor pieces that were SQ inoculated into the study huNOG-EXL animals.
- NSCLC CDX (A549; ATCC CCL-185) Caucasian, male, 58 yo, P11, *in vitro* expansion of cells. Inoculum of $5x10^6$ cells were SQ injected into pilot and study huNOG-EXL animals.

NSCLC CDX and SCLC PDX growth

huNOG-EXL female, 2 donors, 12 WPE, growth kinetics & body weights





*Acute BW drop and rectification

Study Randomization Criteria:
SCLC PDX ~150-250 mm^3
NSCLC CDX ~80-120 mm^3

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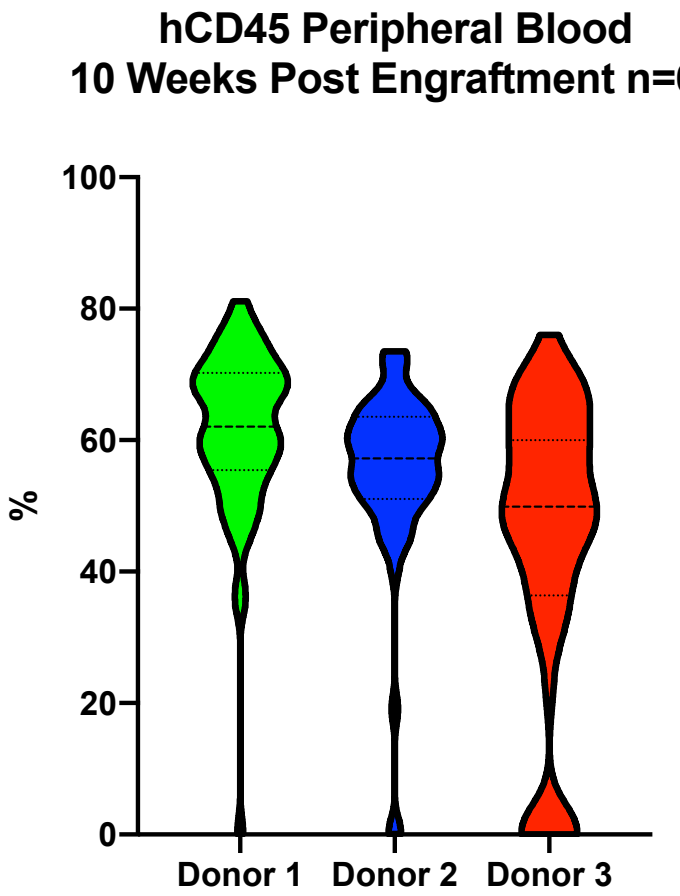
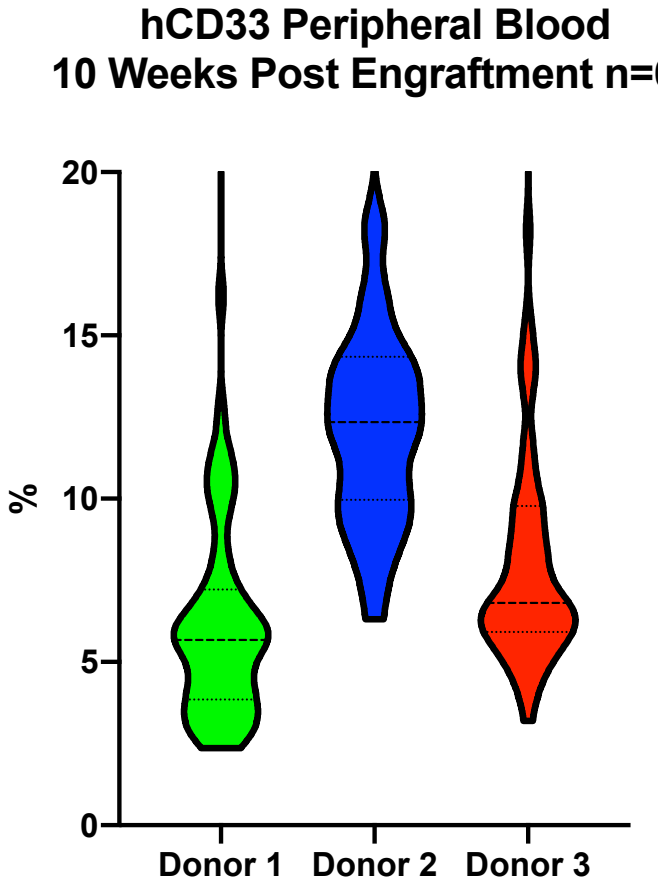
Human chimerism and myeloid lineage

An early perspective of the degree of chimerism and variance





hCD45	Donor 1	Donor 2	Donor 3
Mean	61.4	55.14	44.96
St. Dev.	12.74	13.80	22.08
Median	62.00	57.25	49.90

hCD33	Donor 1	Donor 2	Donor 3
Mean	6.34	13.94	10.42
St. Dev.	3.51	8.32	12.85
Median	5.68	12.35	6.81

Donors Significantly DIFFERENT
One-way ANOVA $p<0.0001$

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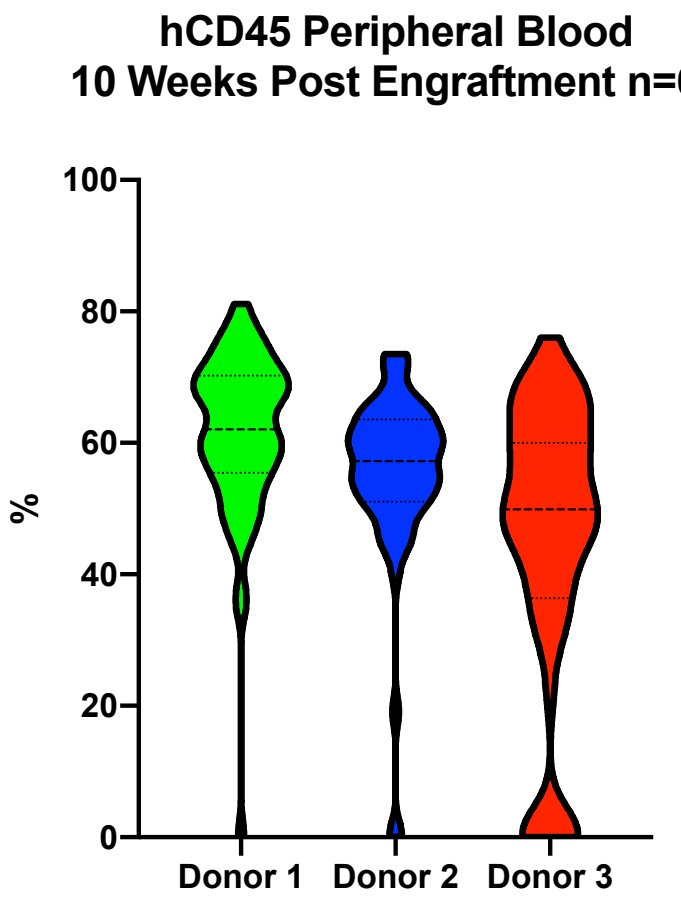
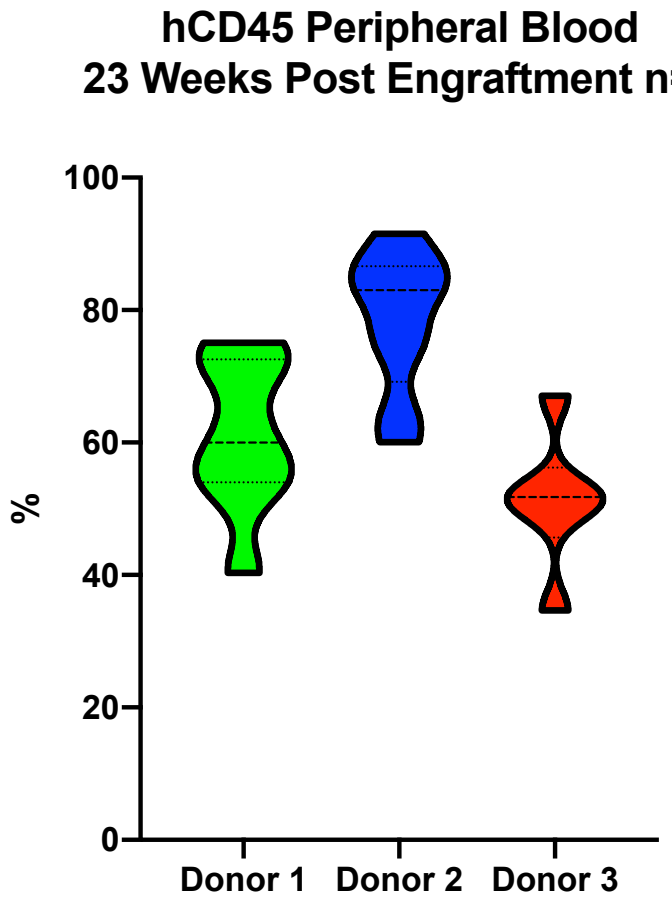
Human chimerism is stable in huNOG-EXL

10 WPE vs. 23 WPE whole blood %hCD45





hCD45 @ 10	Donor 1	Donor 2	Donor 3
Mean	61.4	55.14	44.96
St. Dev.	12.74	13.80	22.08
Median	62.00	57.25	49.90

hCD45 @ 23	Donor 1	Donor 2	Donor 3
Mean	60.97	78.48	51.20
St. Dev.	11.84	10.69	10.31
Median	60.02	83.00	51.77

Donors Significantly DIFFERENT
One-way ANOVA $p<0.0001$

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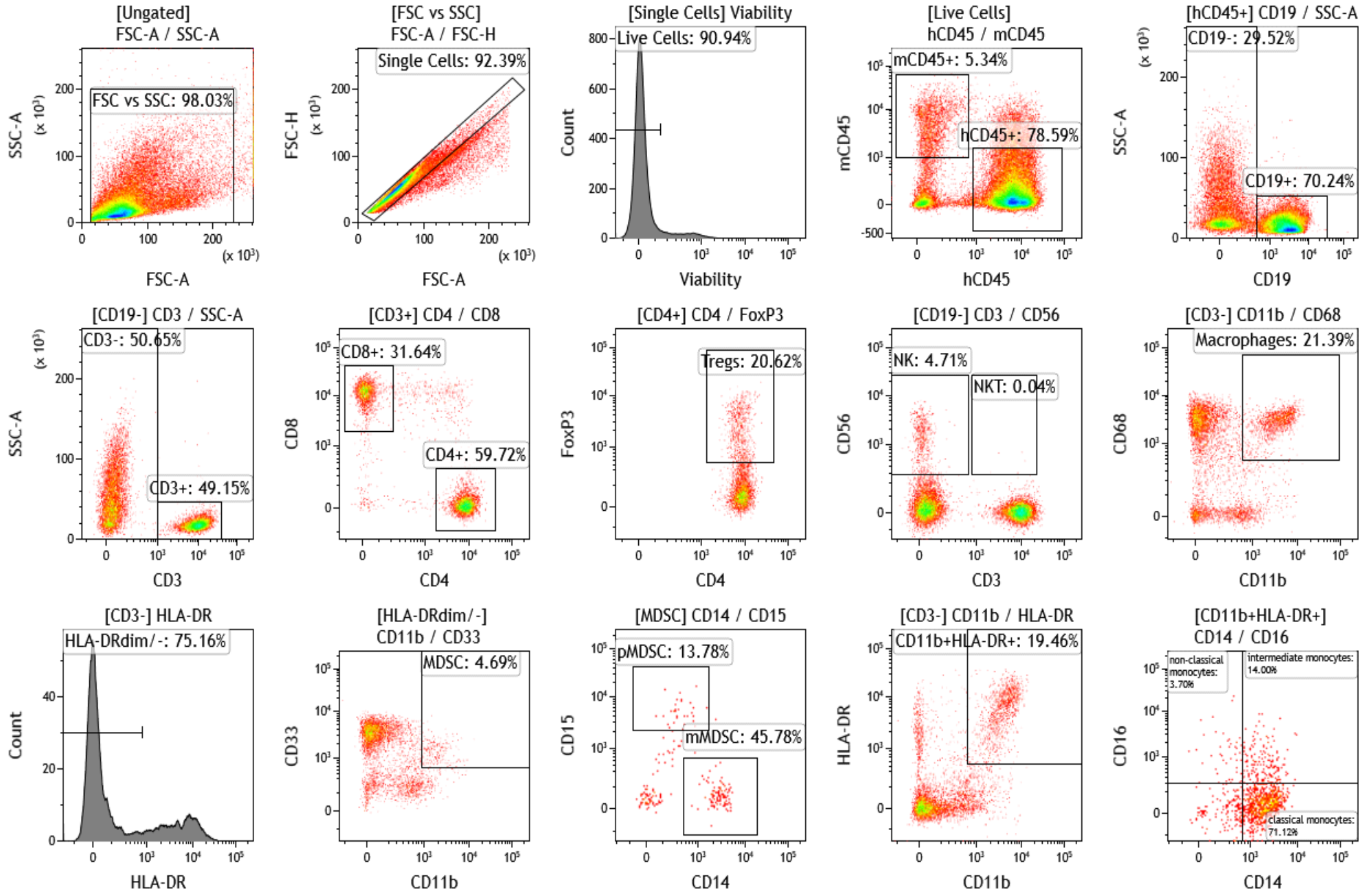
Markers:
mCD45
hCD45
CD3
CD4
CD8
FoxP3
CD56
CD19
CD33
CD11b
CD68
HLA-DR
CD14
CD15
CD16
L/D



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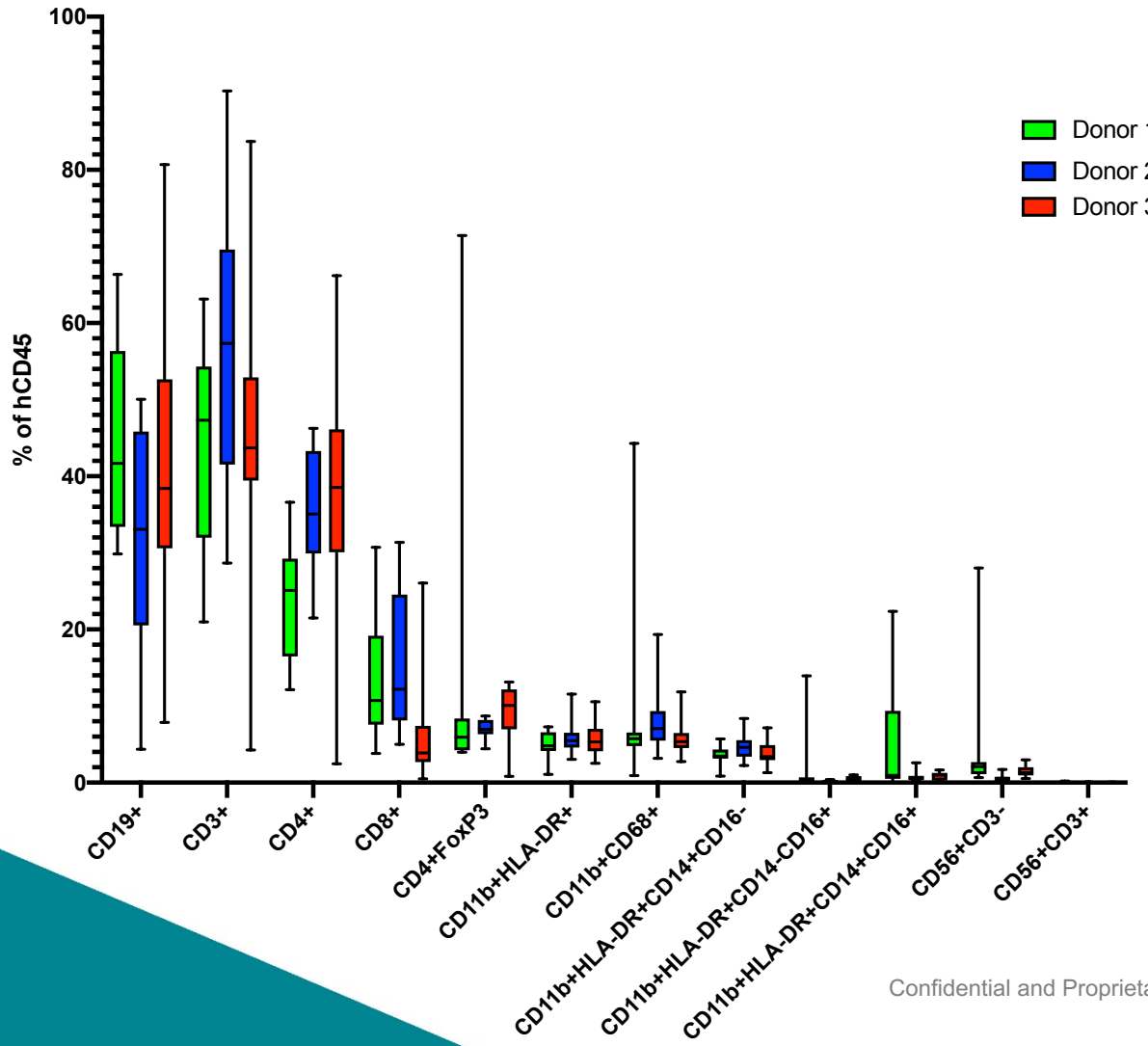
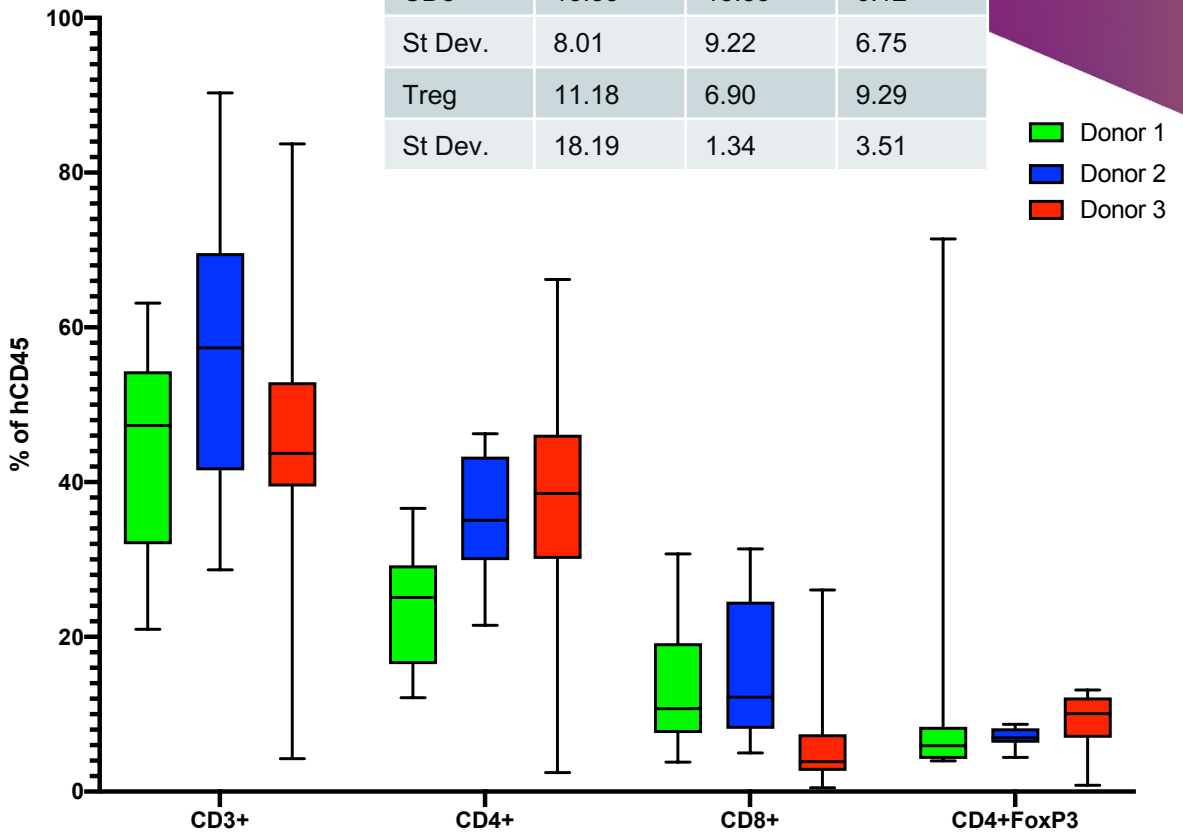
Detailed snapshot: 23 WPE blood

% of hCD45: vast majority of human cells are T and B cells

%hCD45	Donor 1	Donor 2	Donor 3
CD3 mean	43.18	55.82	46.18
St Dev.	13.79	18.82	20.14
CD4 mean	24.26	36.22	36.87
St Dev.	7.71	7.45	15.77
CD8	13.35	15.85	6.12
St Dev.	8.01	9.22	6.75
Treg	11.18	6.90	9.29
St Dev.	18.19	1.34	3.51



n=12/donor



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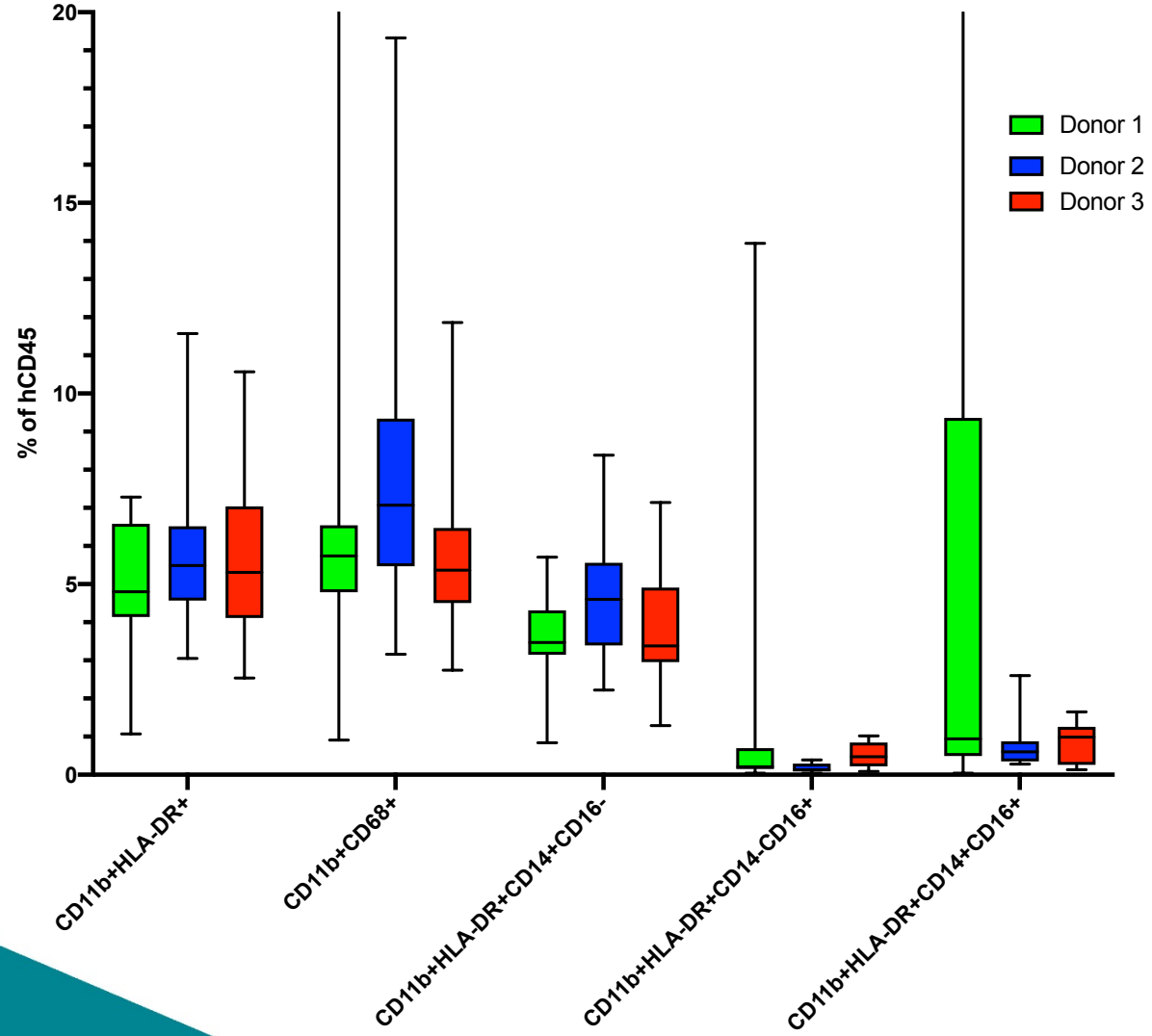
However, human myeloid is present at 23 WPE in blood

Monocytes (classical, **intermediate,** non-classical) **and macrophages**



%hCD45	Donor 1	Donor 2	Donor 3
Mono mean	4.99	6.08	5.63
St Dev.	1.66	2.42	2.21
Macro mean	8.40	8.00	5.85
St Dev.	10.94	4.09	2.30
Classical	3.63	4.64	3.80
St Dev.	1.19	1.58	1.58
Non-Classical	1.42	0.18	0.52
St Dev.	3.78	0.11	0.31
Intermediate	4.63	0.73	0.86
St Dev.	7.04	0.58	0.53

n=12/donor

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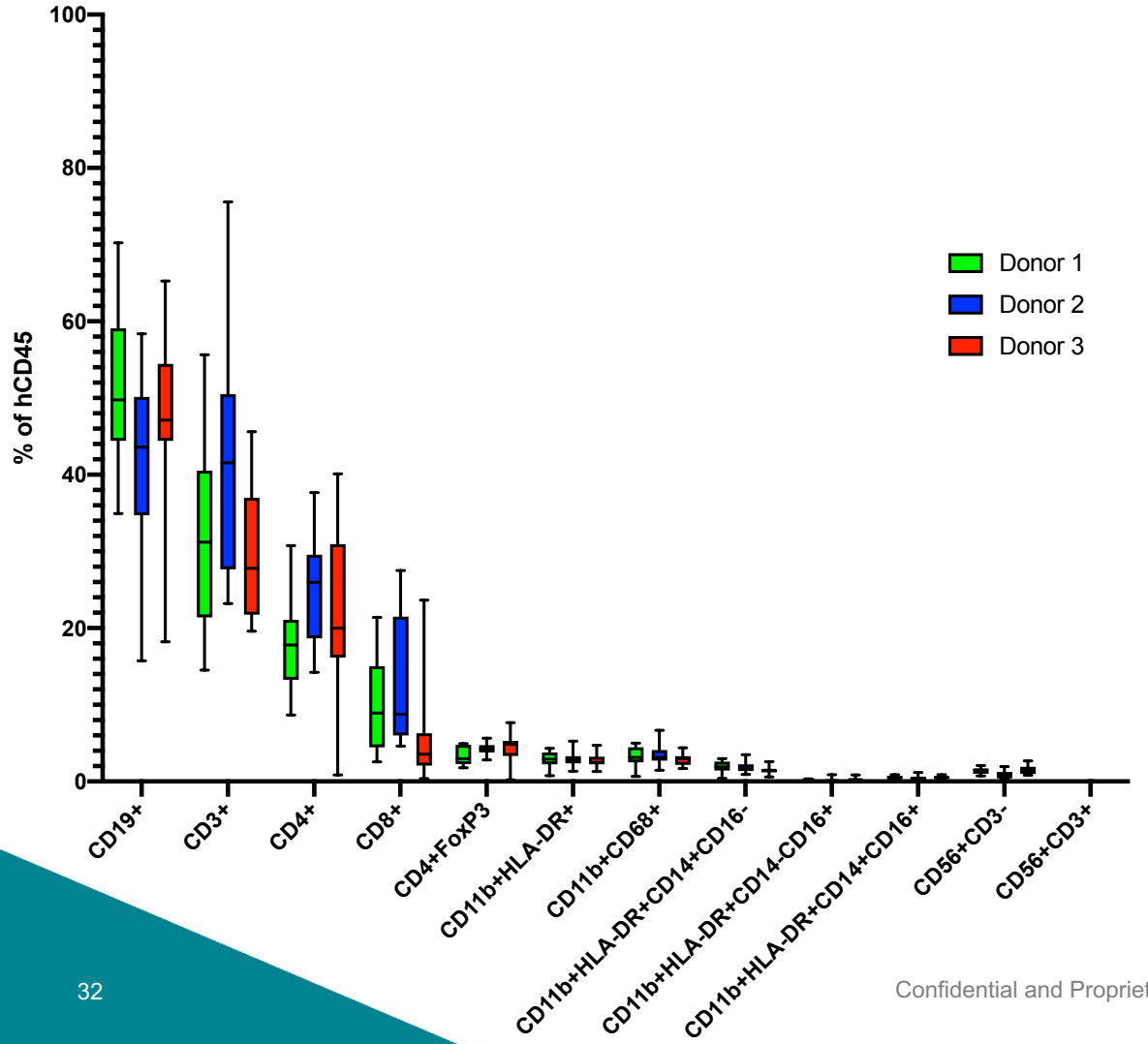
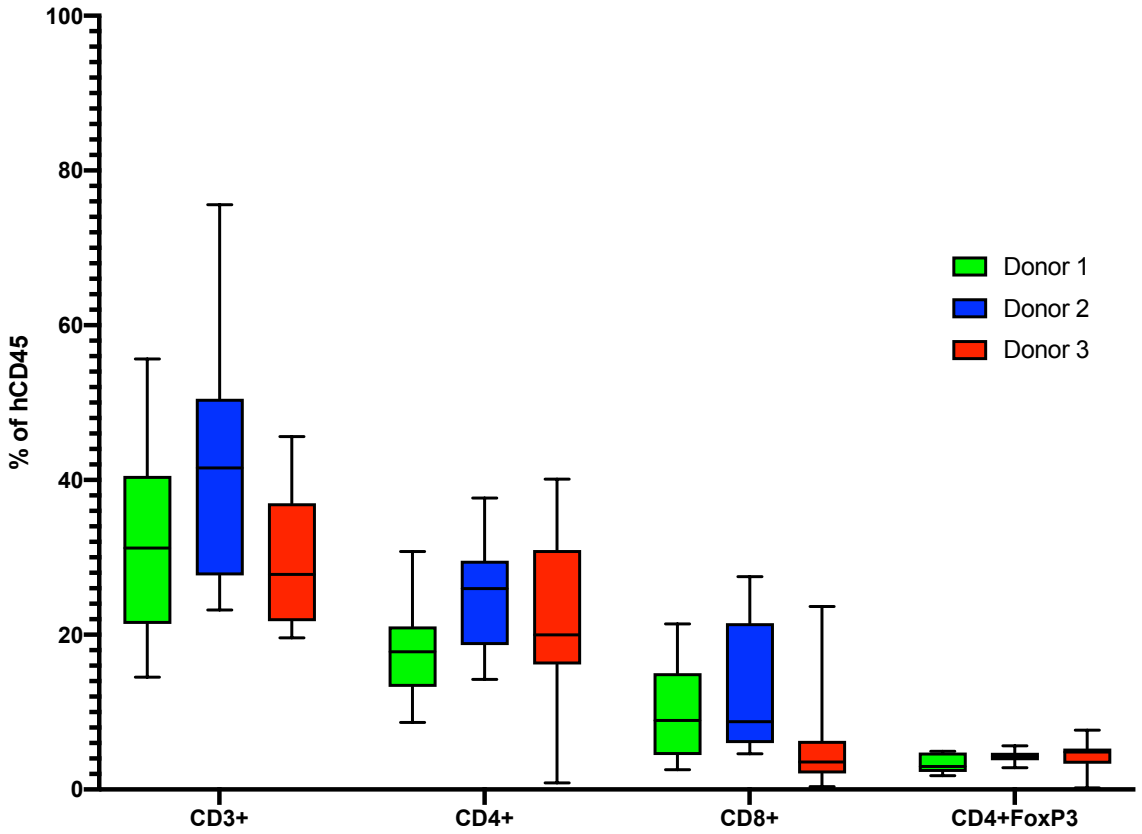
Detailed snapshot: 23 WPE spleen % hCD45

Note the consistency of phenotype between blood (previous slides) and spleen





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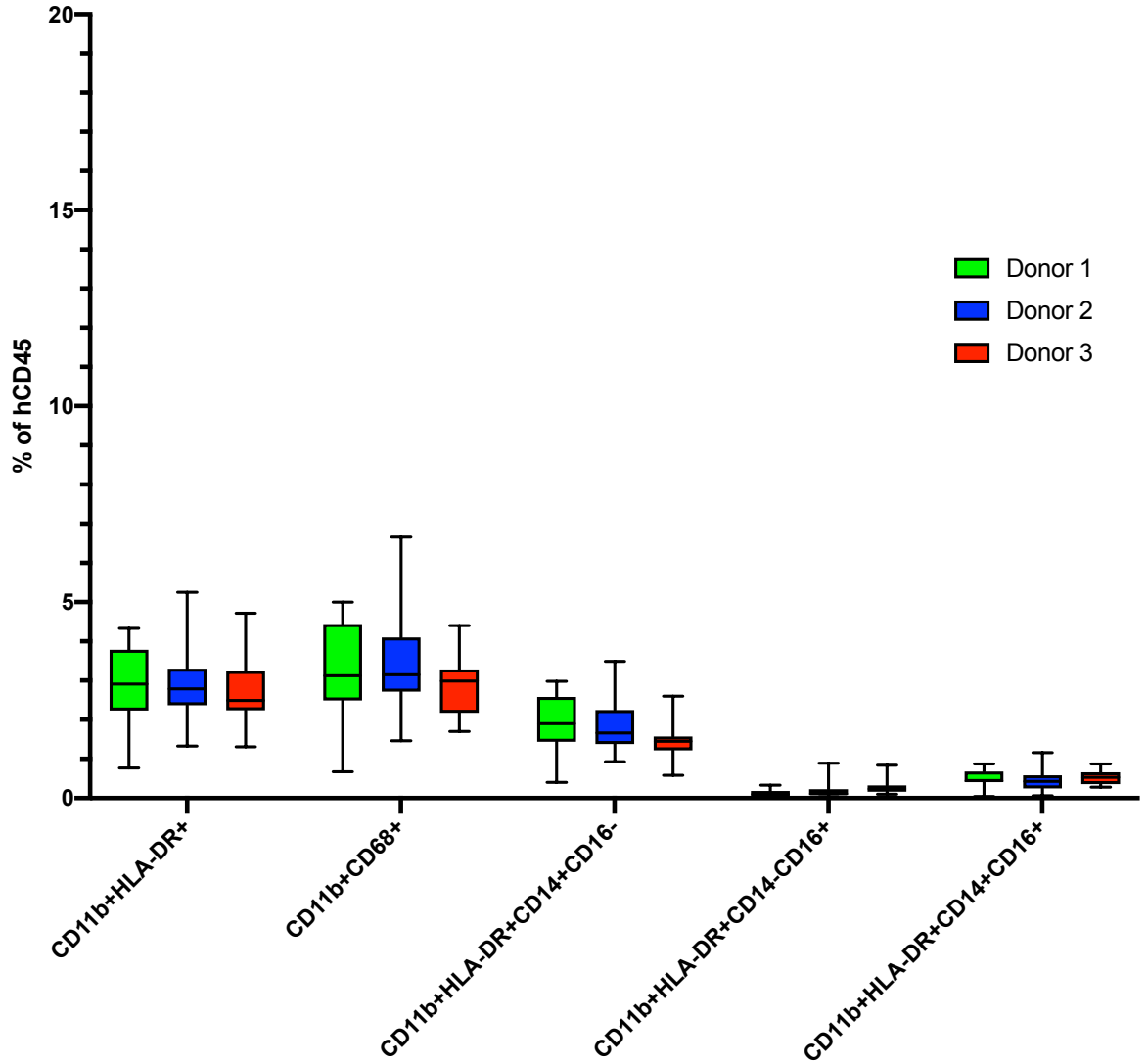
Myeloid cells also present 23 WPE in spleen

Monocytes (classical, **intermediate**, non-classical) **and macrophages**



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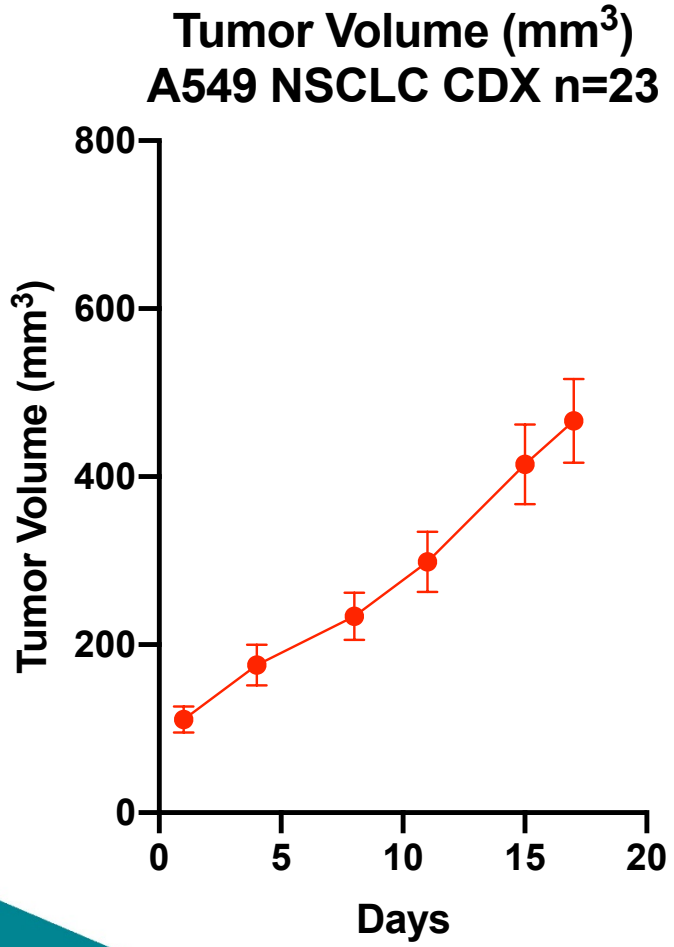
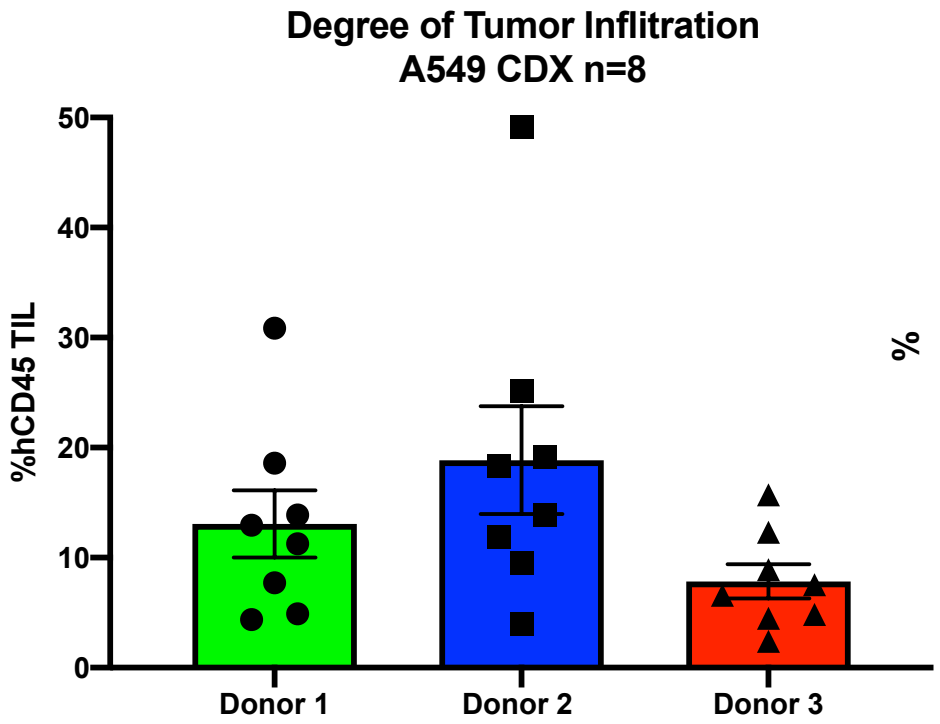
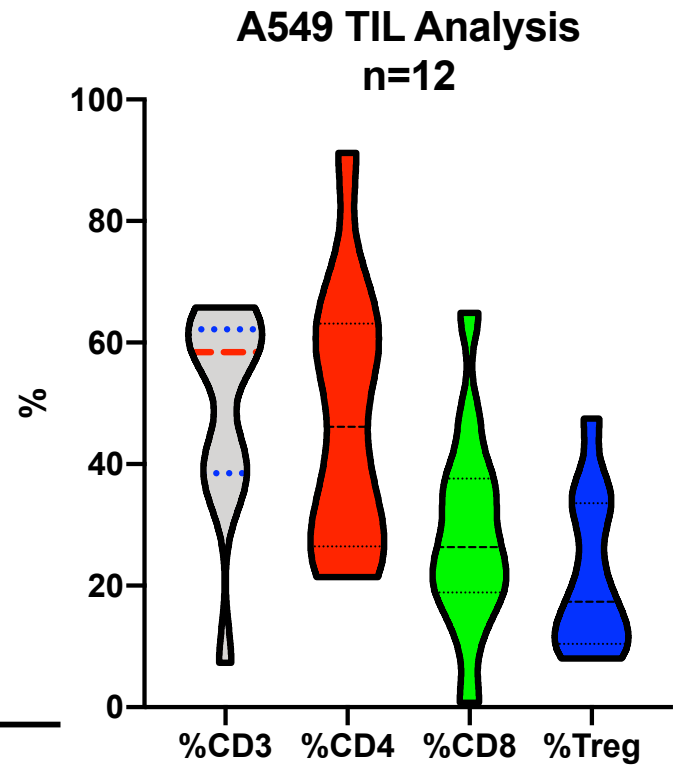
Analysis of tumor infiltrates: A549 NSCLC CDX

Tumor growth curve and TIL as %hCD45

hCD45	Donor 1	Donor 2	Donor 3
Mean	13.07	18.86	7.846
St. Dev.	8.641	13.83	4.385
Median	12.11	16.08	7.065







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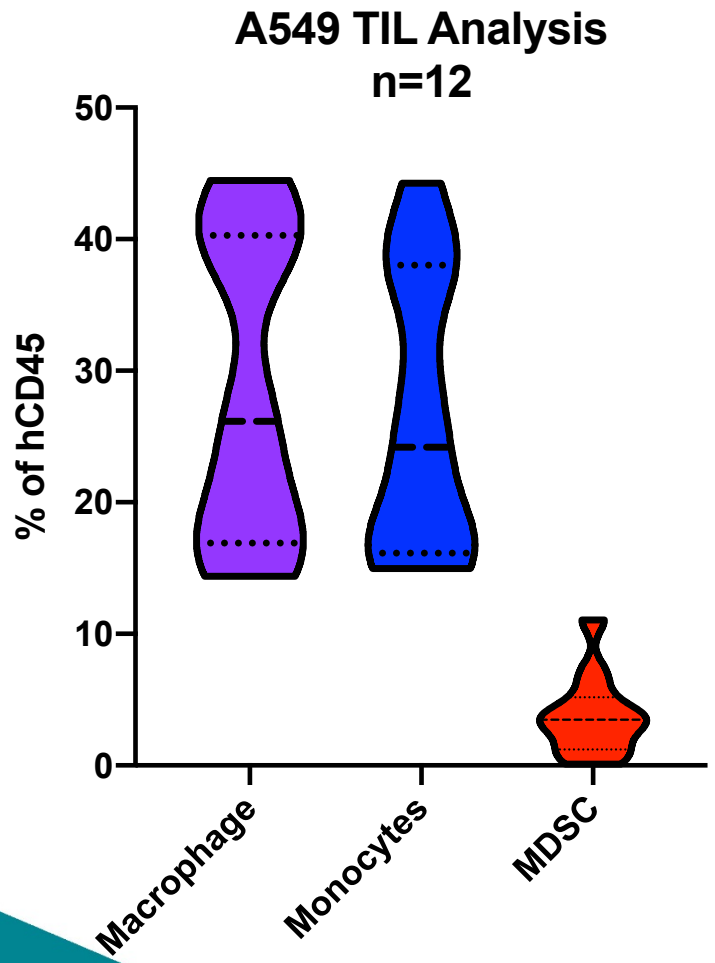
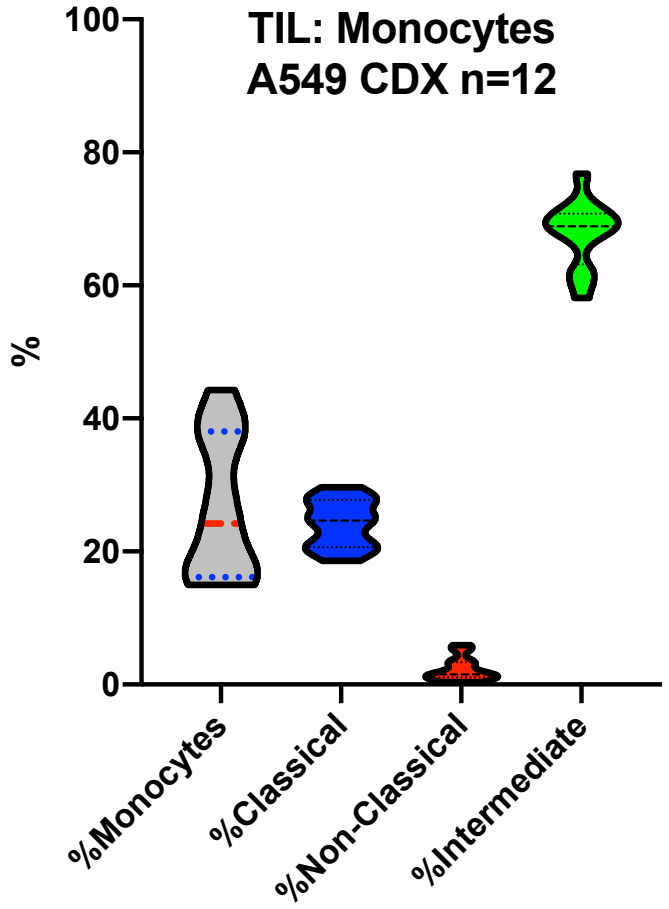
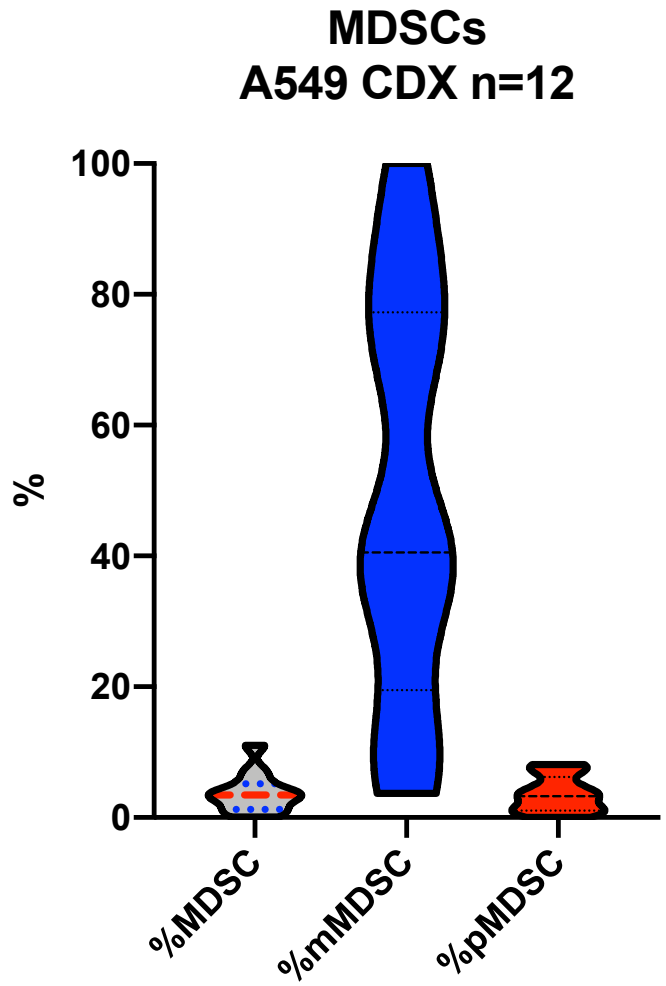
Human myeloid cells infiltrate A549 tumor

Myeloid lineage







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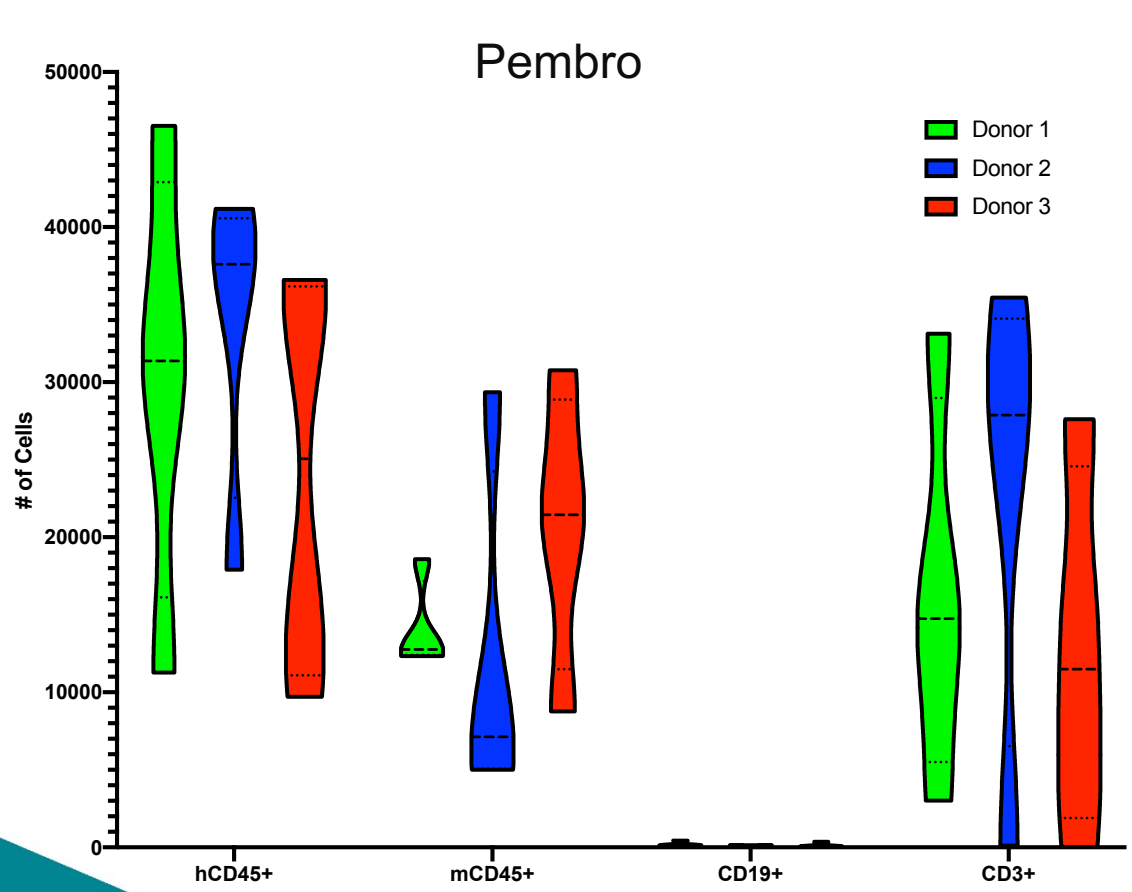
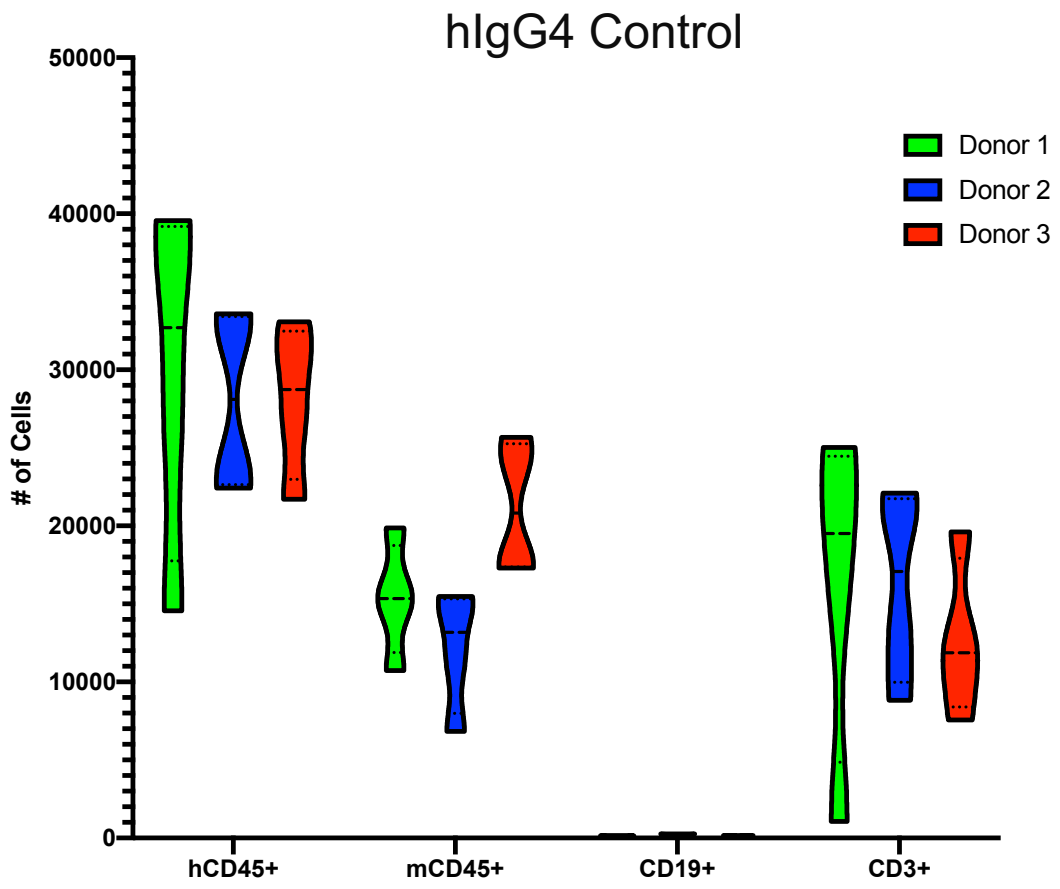
A549 NSCLC CDX TIL analysis

Pembrolizumab 10mg/kg IP vs. hIgG4 treated

Dose Schedule: BIW x 7
Actual: 17 days (5 dose admin)





TACONIC
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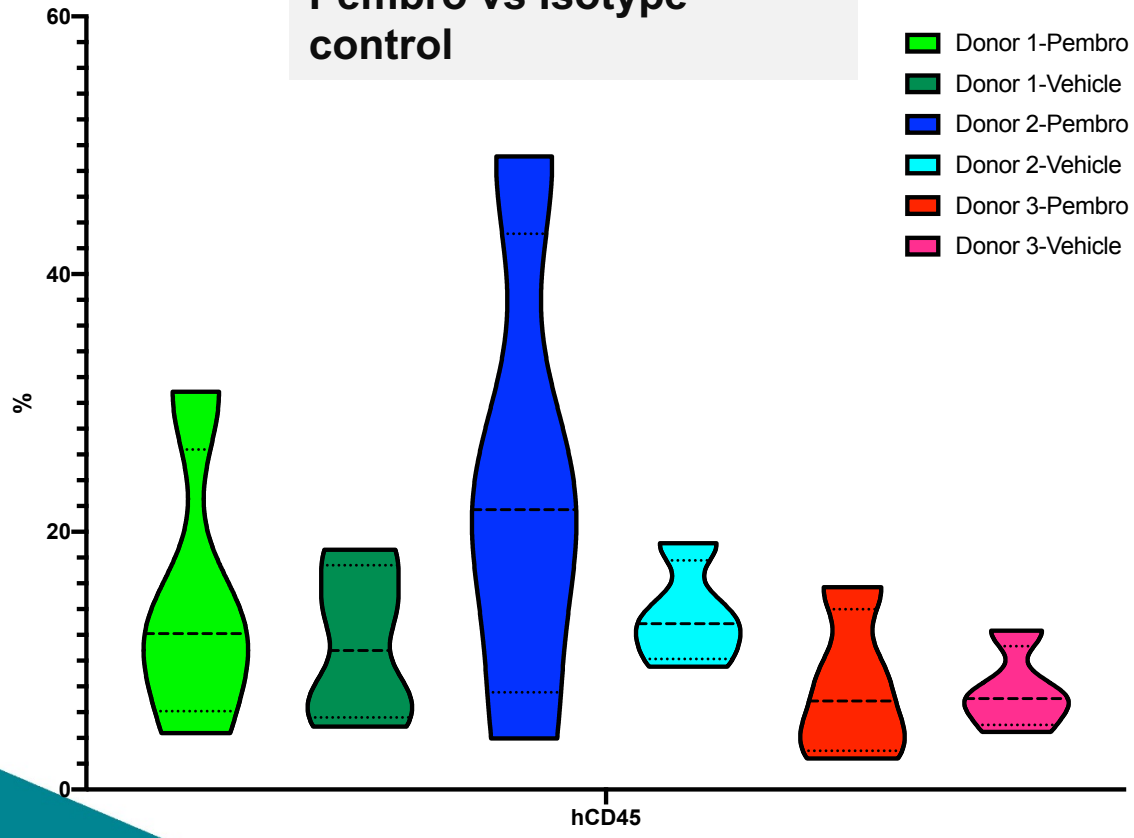
A549 NSCLC CDX TIL analysis

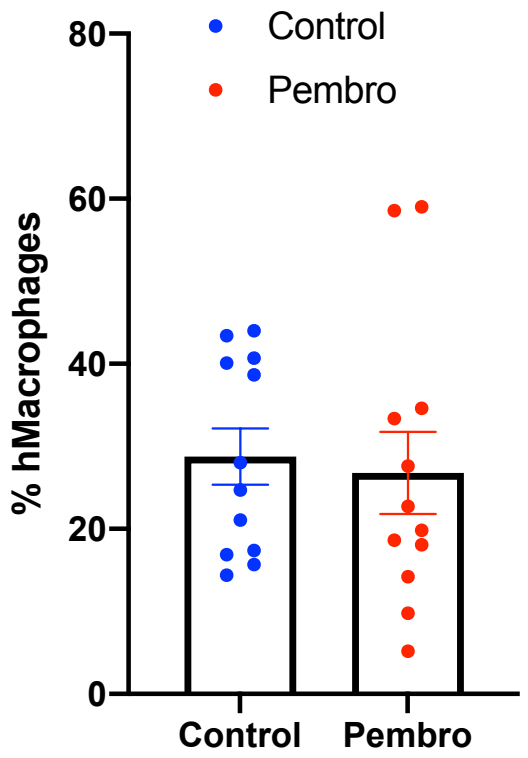
Donor: Pembrolizumab 10mg/kg IP vs. hIgG4 treated

Dose Schedule: BIW x 7
Actual: 17 days (5 dose admin)

A549 TIL Analysis n=4
Pembro vs Isotype control



Tumor Associated Macrophages
A549 huNOG-EXL 3 donors, n=12



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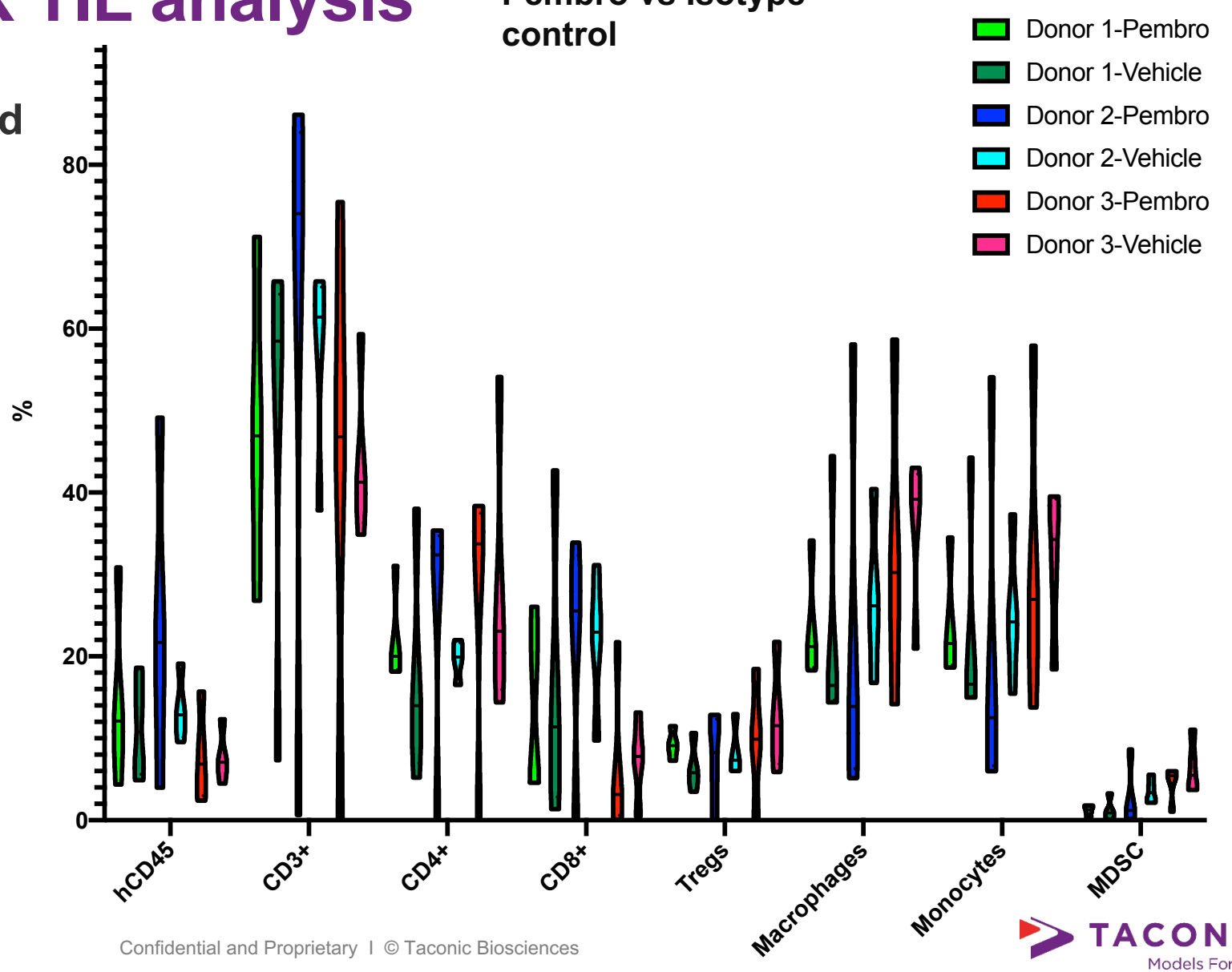
A549 NSCLC CDX TIL analysis

Donor: Pembrolizumab 10mg/kg IP vs. hIgG4 treated



Dose Schedule: BIW x 7
Actual: 17 days (5 dose admin)

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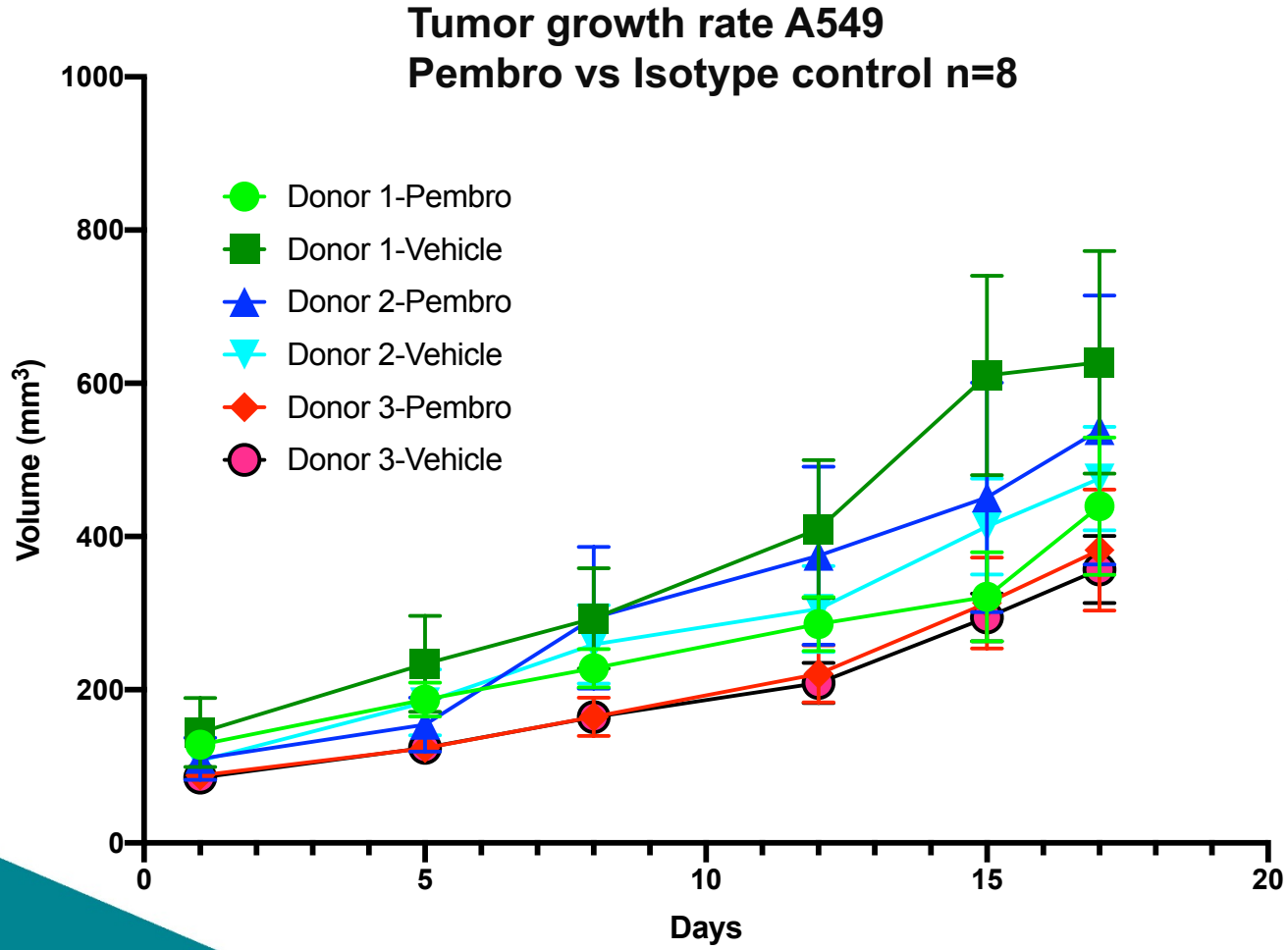
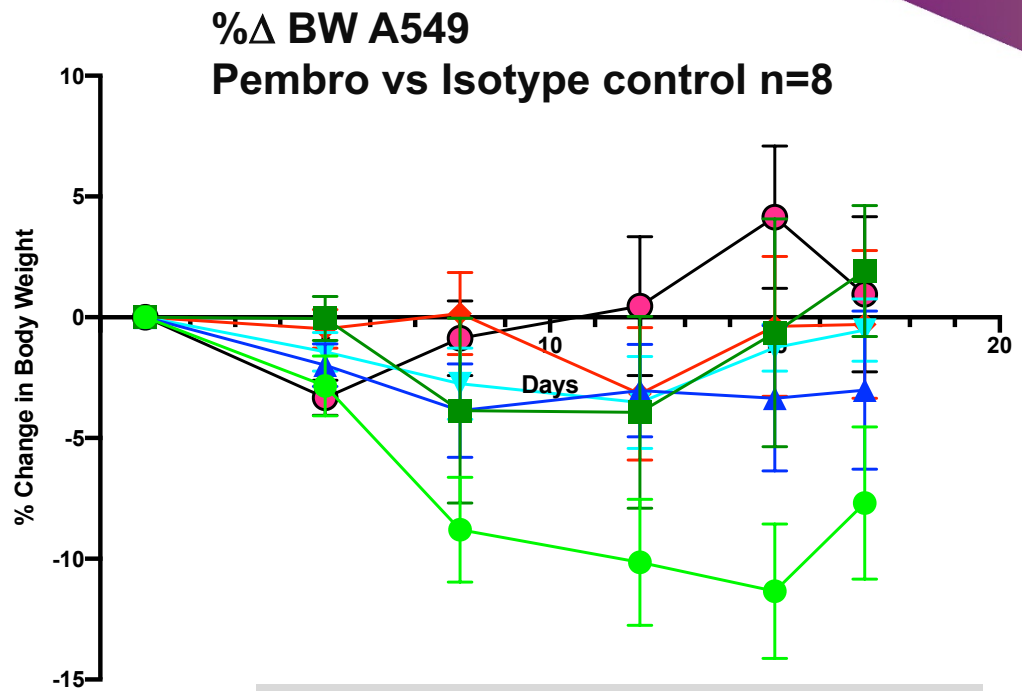
A549 CDX tumor kinetics

Donor: Pembrolizumab 10mg/kg IP vs. hIgG4 treated





Dose Schedule: BIW x 7
Actual: 17 days (5 dose admin)

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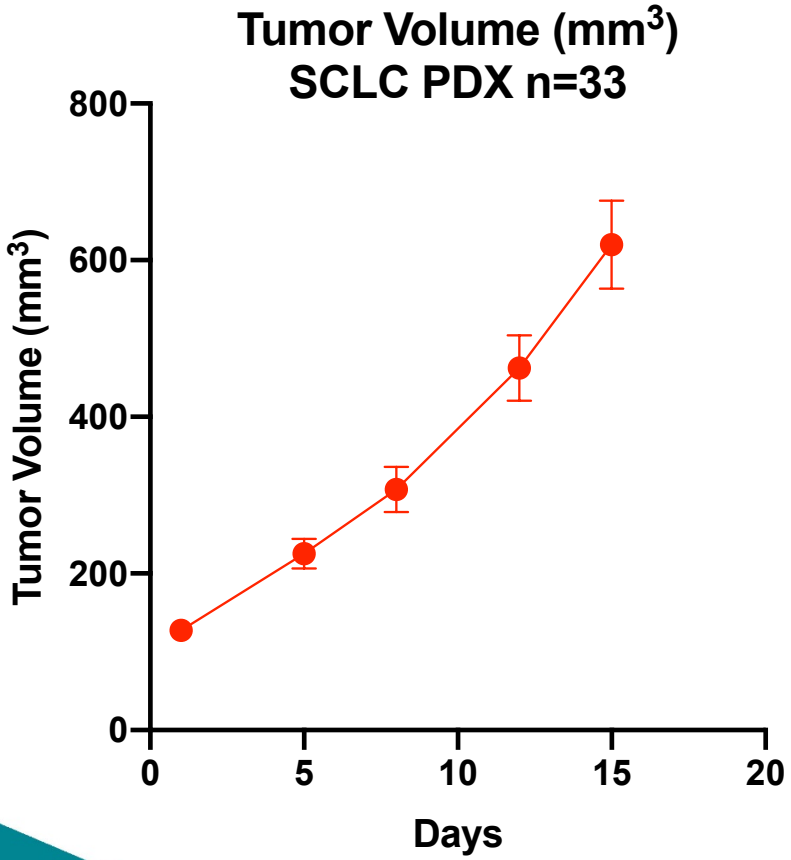
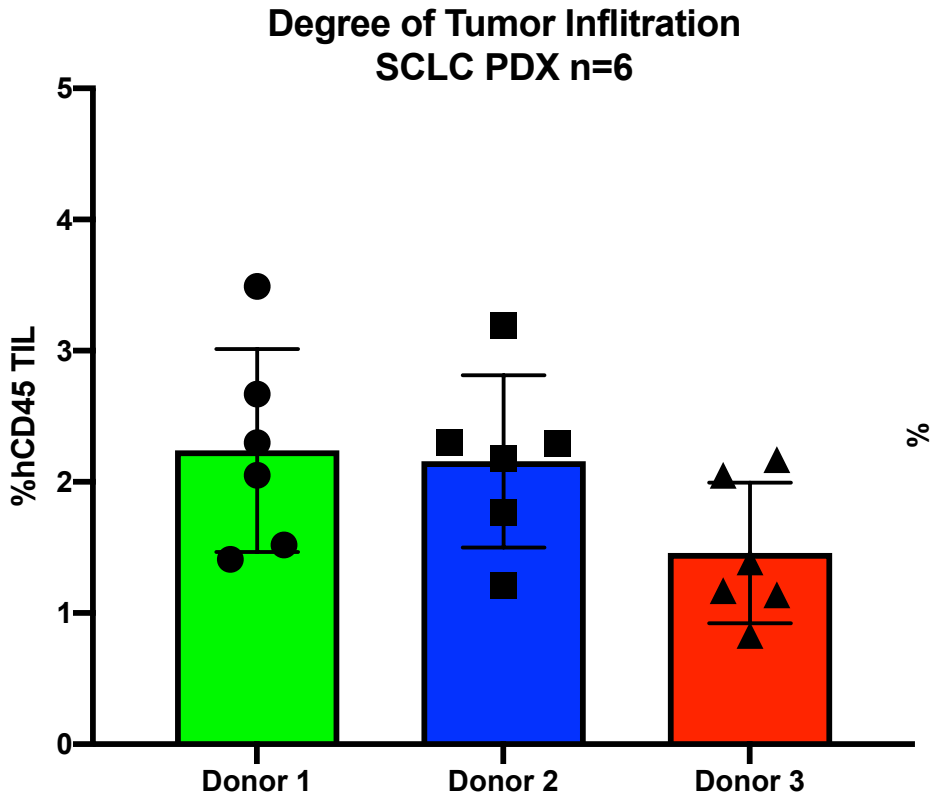
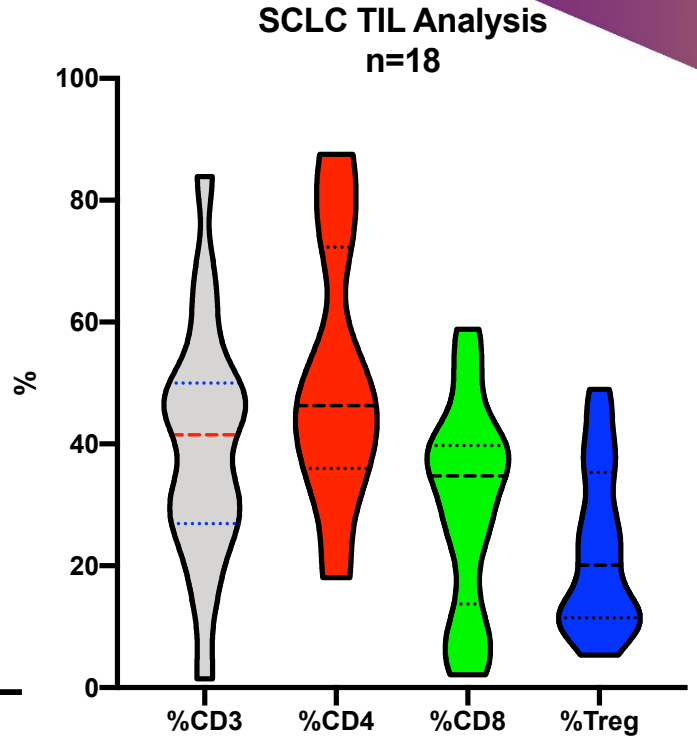
SCLC PDX TIL analysis

Tumor growth curve and infiltration %hCD45







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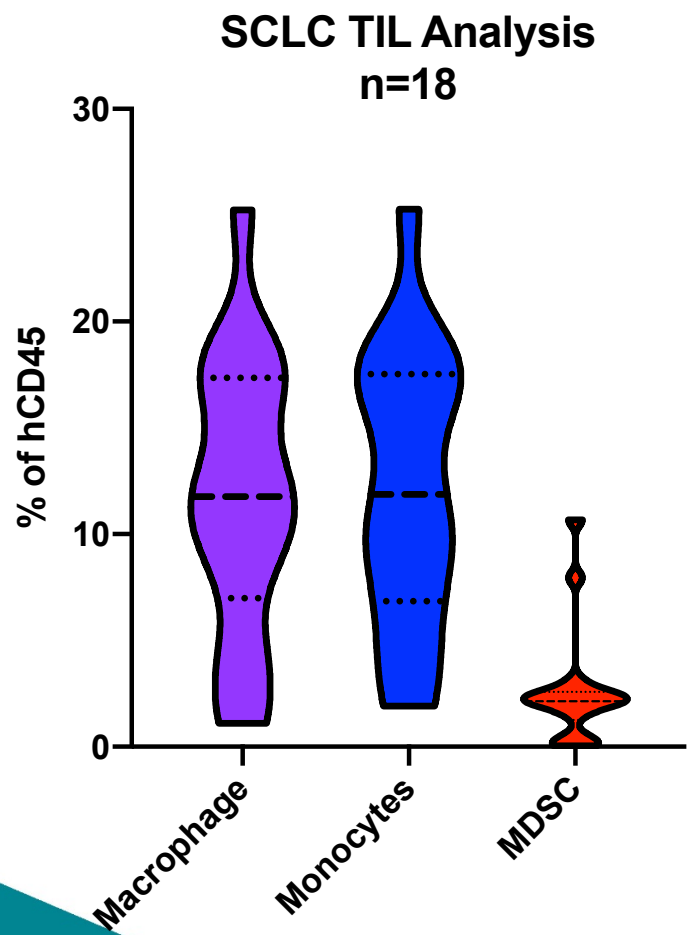
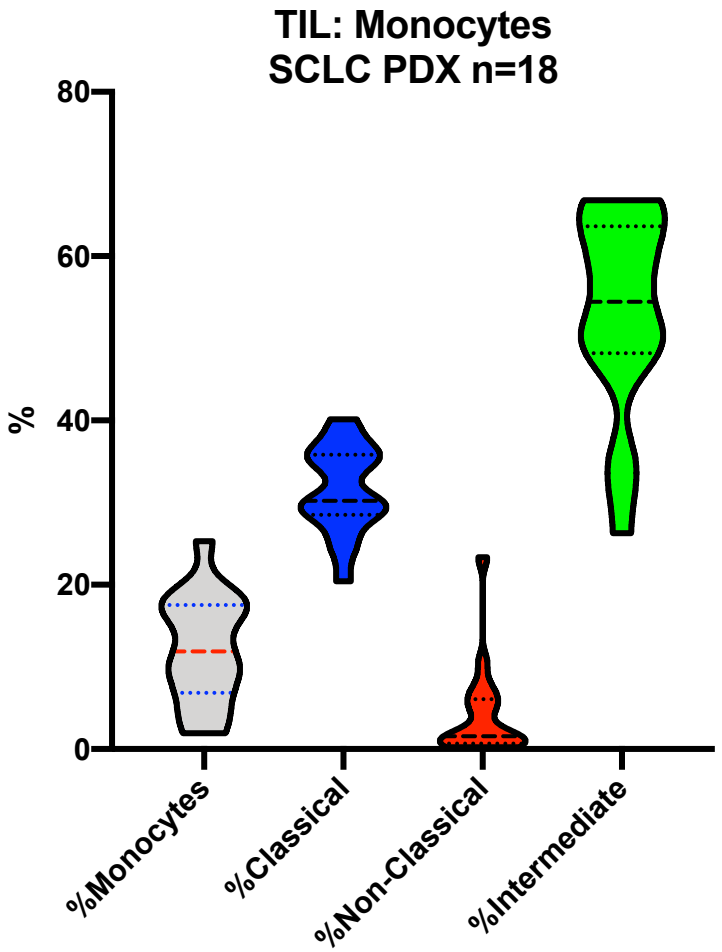
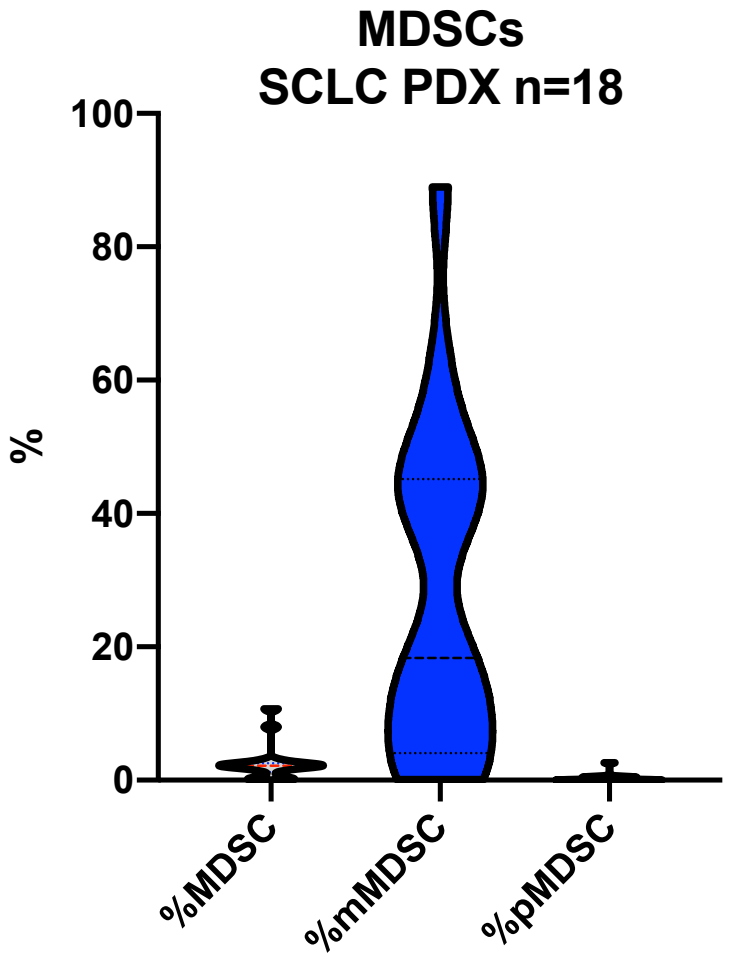
SCLC PDX TIL analysis

Myeloid lineage







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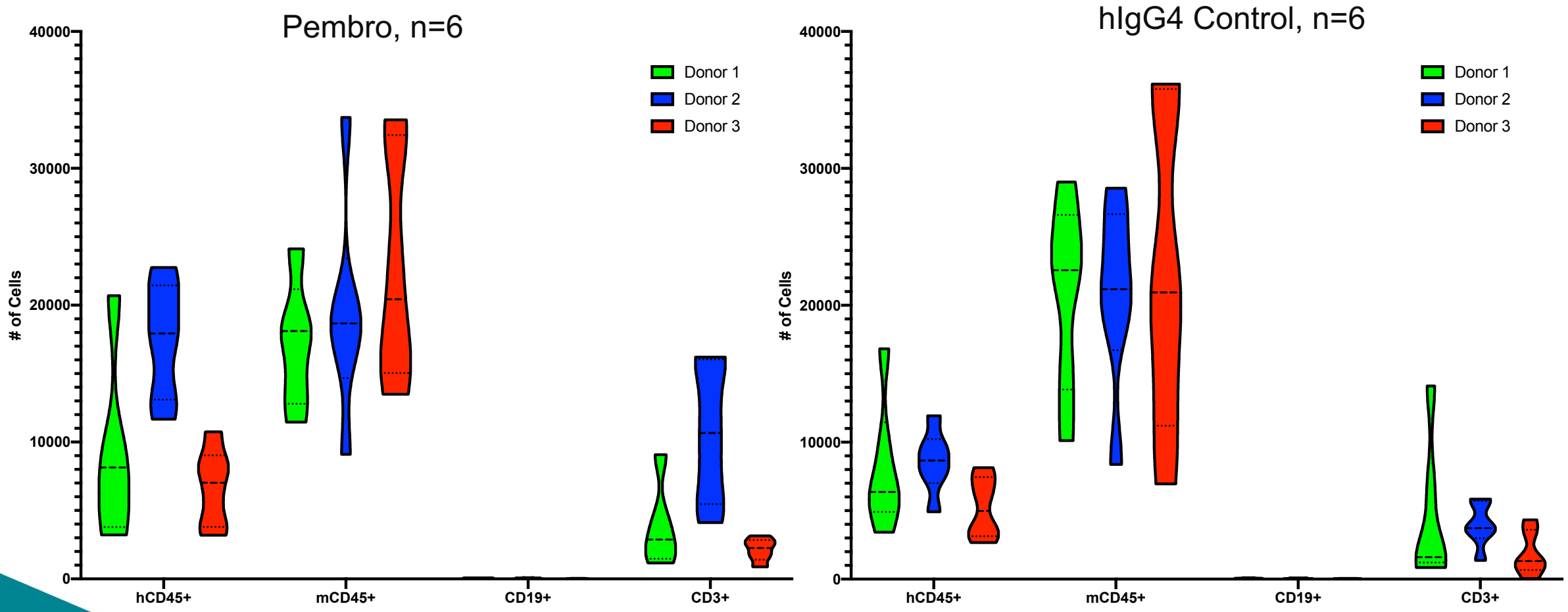
SCLC PDX TIL analysis

Pembrolizumab 10mg/kg IP vs. hIgG4 treated

Dose Schedule: BIW x 7
Actual: 15 days (4 dose admin)



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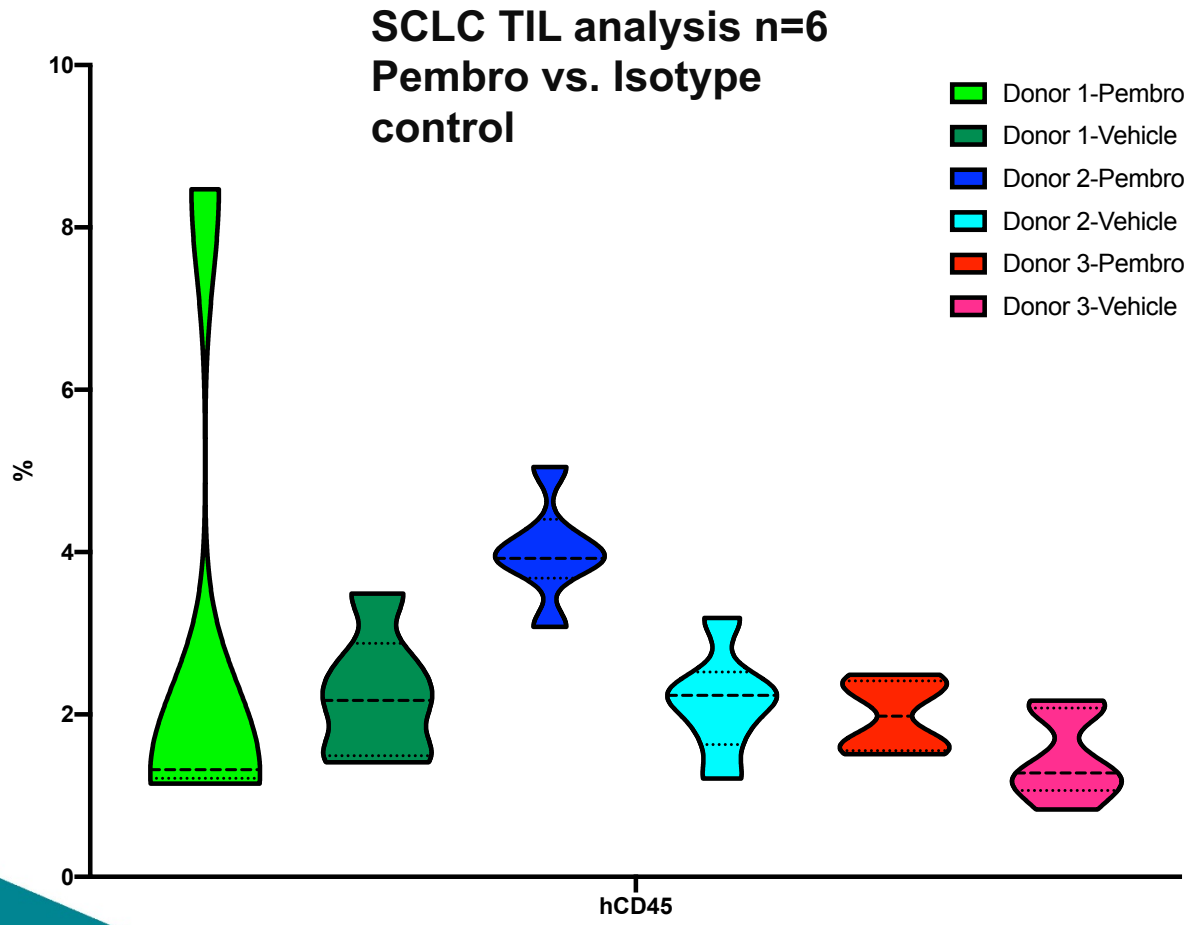
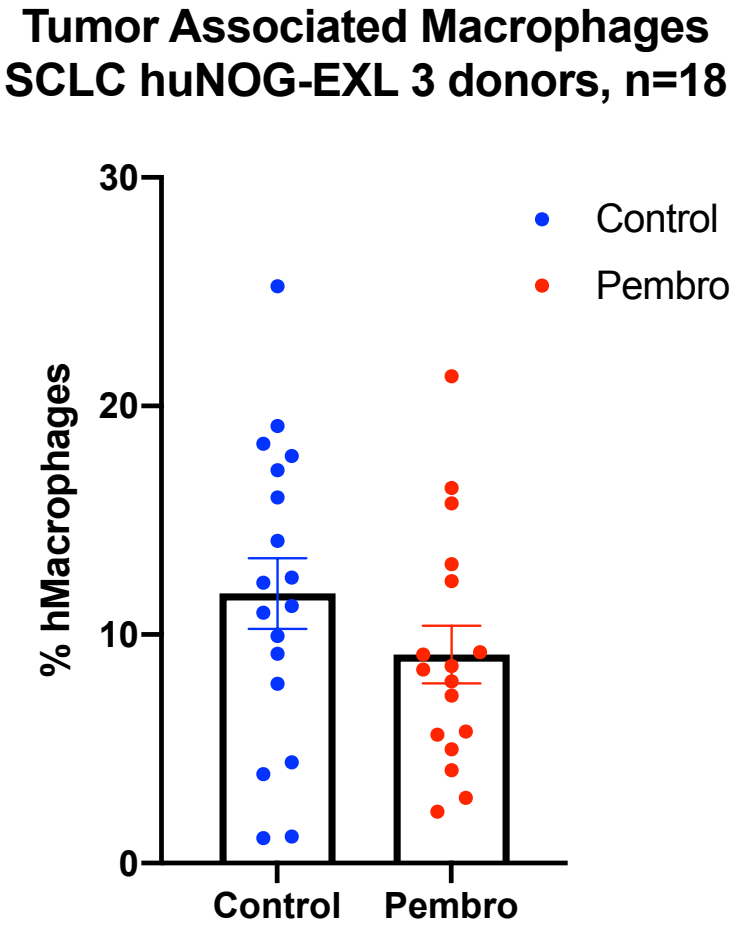
SCLC PDX TIL analysis

Donor: Pembrolizumab 10mg/kg IP vs. hIgG4 treated

Dose Schedule: BIW x 7
Actual: 15 days (4 dose admin)





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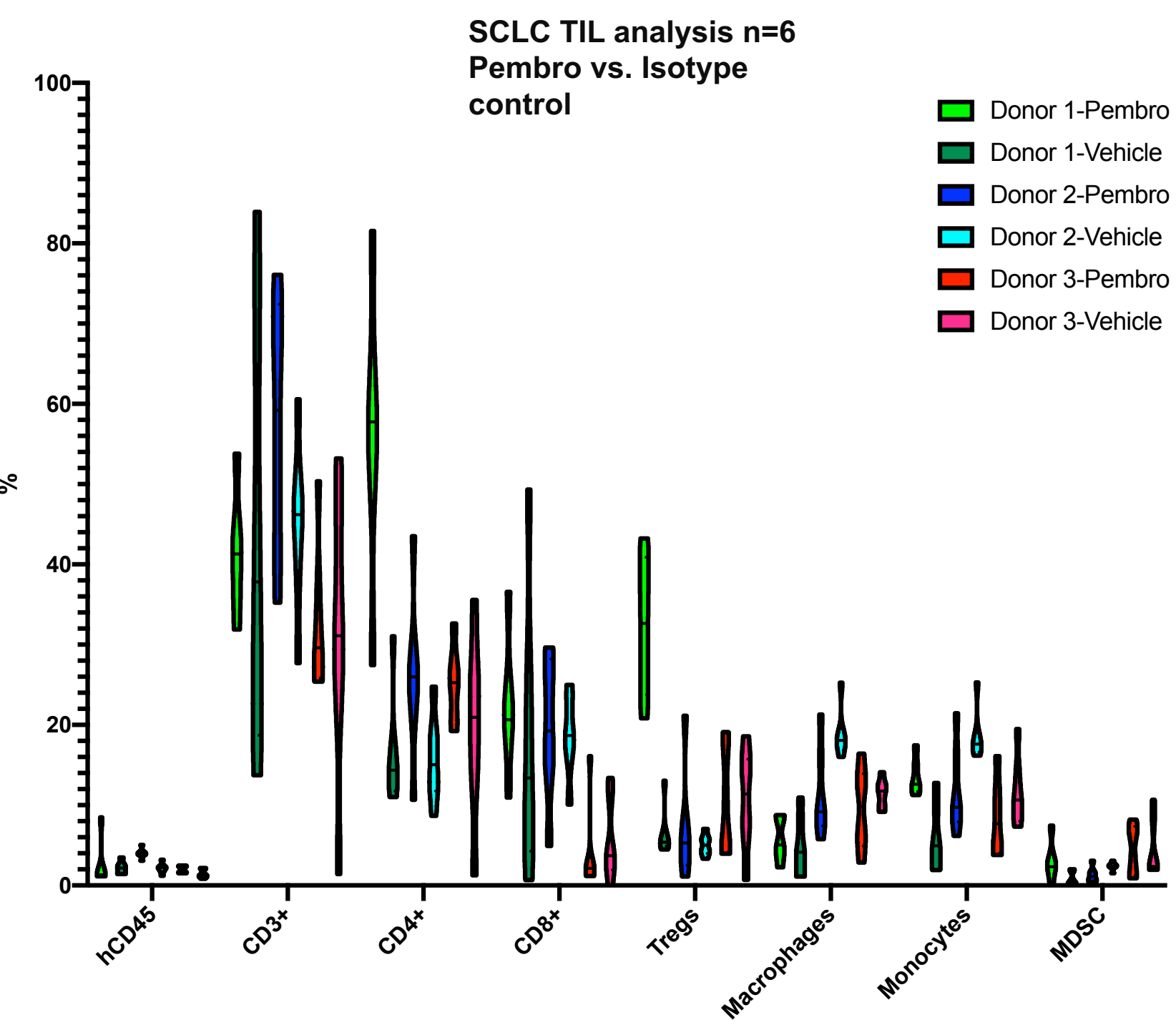
SCLC PDX TIL analysis

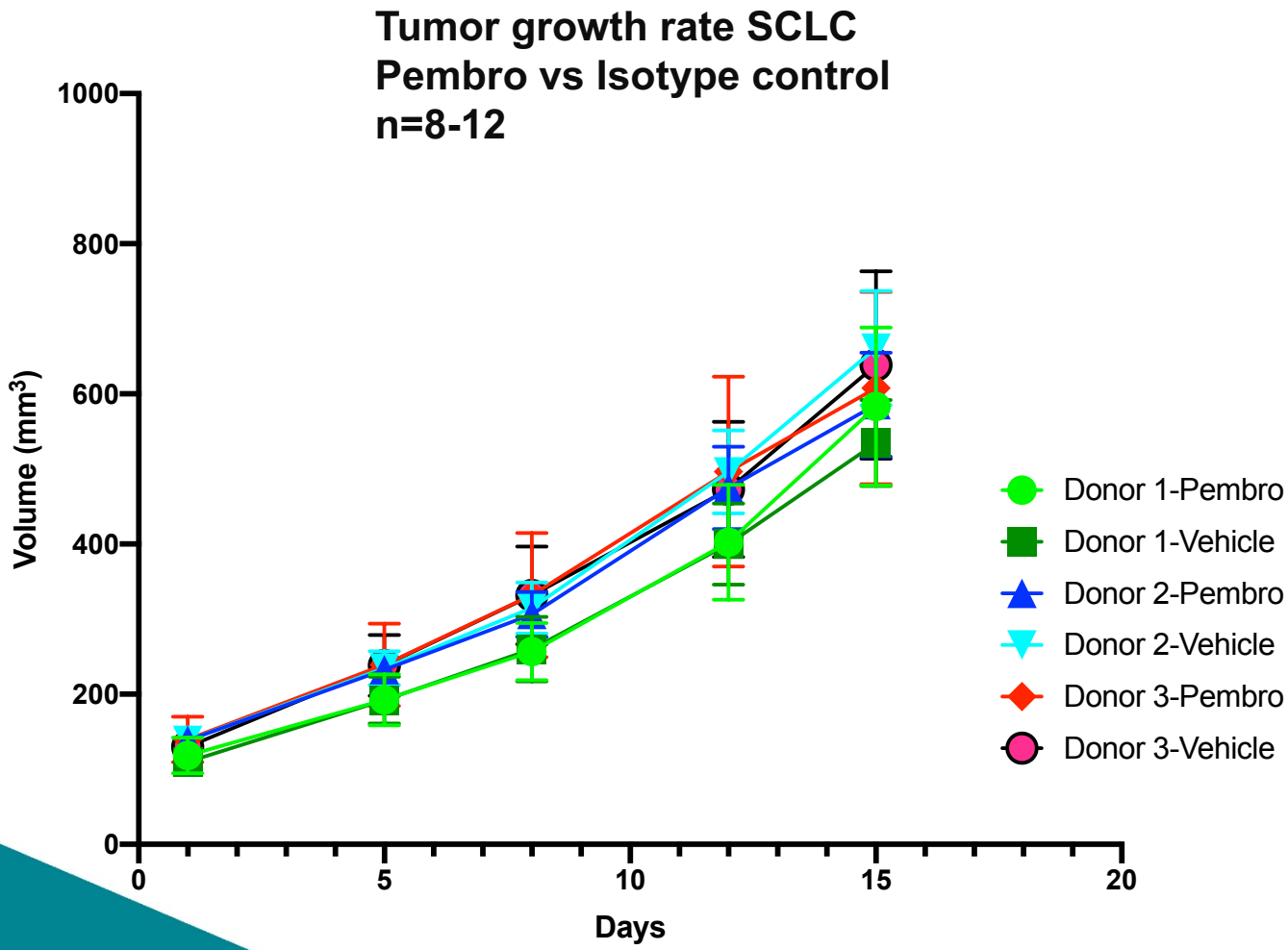


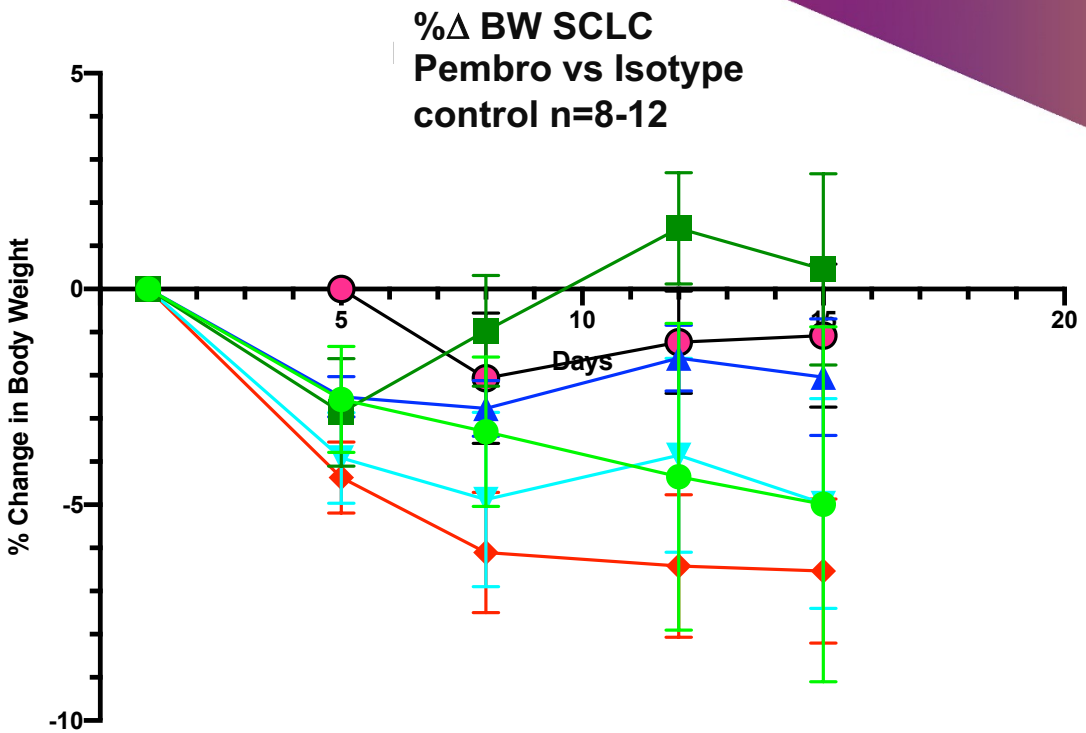
- Donor: Pembrolizumab 10mg/kg IP vs. hIgG4 Treated

Dose Schedule: BIW x 7
Actual: 15 days (4 dose admin)

SCLC PDX tumor kinetics

Donor: Pembrolizumab 10 mg/kg IP vs. hIgG4 treated





Dose Schedule: BIW x 7
Actual: 15 days (4 dose admin)

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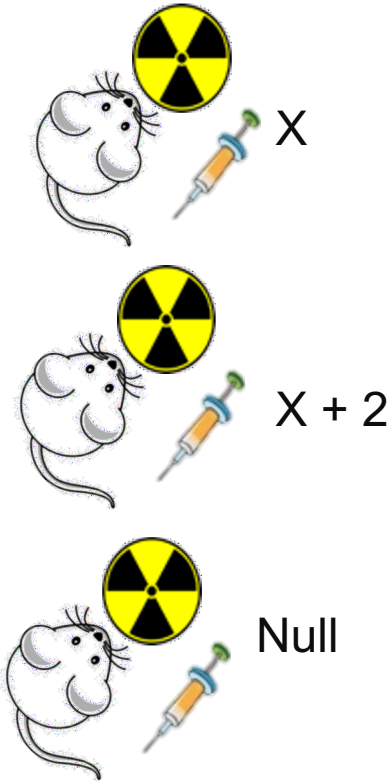
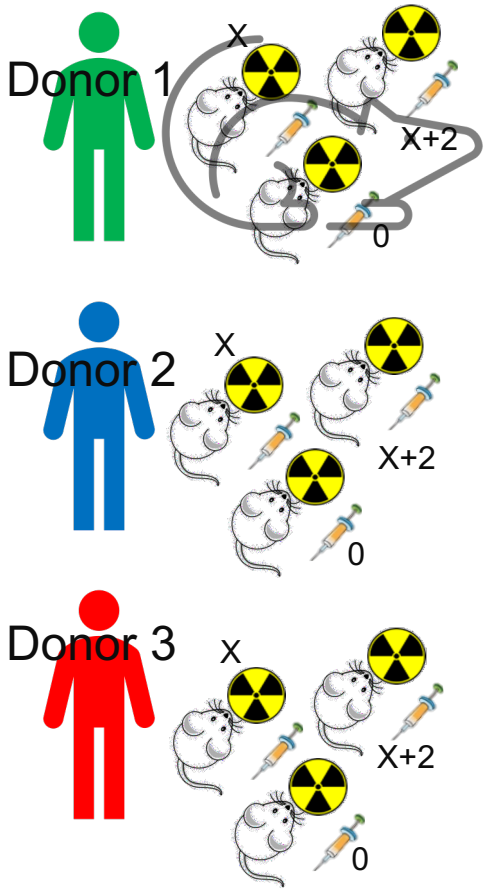
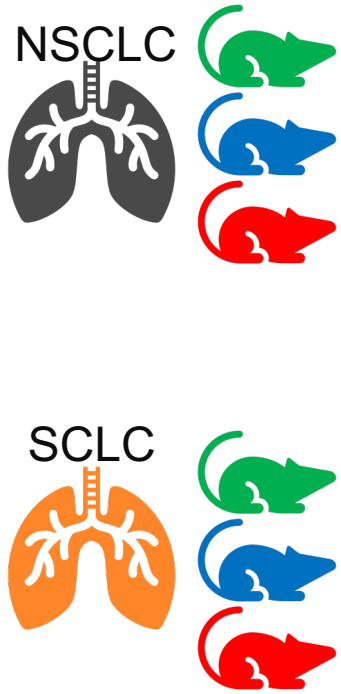
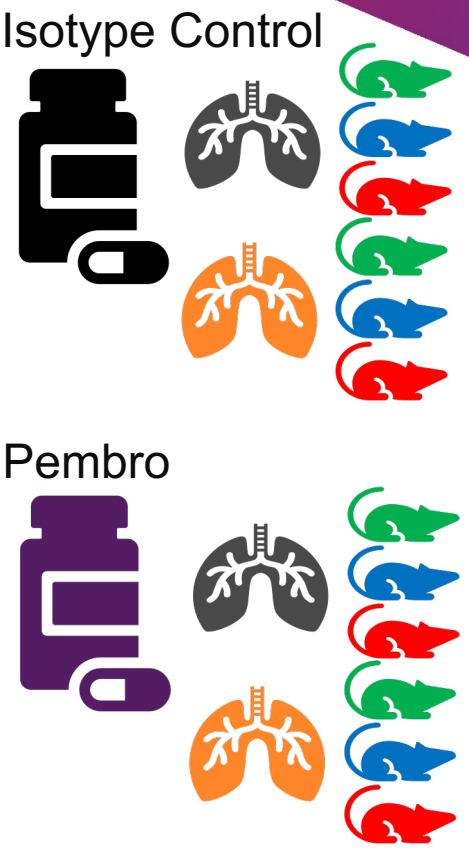
Recap of our study design

Graphical representation of variables









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Results

- Accepted the null hypothesis and rejected the experimental (1' question, Factor X)
- A power can easily be diluted based on the # of questions n=60 to n=20 to n=4
- We asked too many questions (3 of them to be exact)
- More than likely we wouldn't have been able to adequately power Factor X and pembro efficacy
- Thus, for drug efficacy remember n-values must be sufficiently powered to account for animal health, donor variance, and variance of effect
- Tumors were not engrafted into our study animals until ~16 WPE, logistics are important
- A549 has been engrafted in the huNOG-EXL previously http://mct.aacrjournals.org/content/early/2018/12/22/1535-7163.MCT-18-0836 and showed TGI of ~26% with anti-PD1 (not same formulation)
- The SCLC PDX had been previously shown in CD34+ engrafted NSG to show a response to anti-PD1
- Recapitulating checkpoint inhibitor efficacy studies published in humanized immune system mice requires maintenance of methodology, vision of variables, and adequate powering of an experiment accounting for both

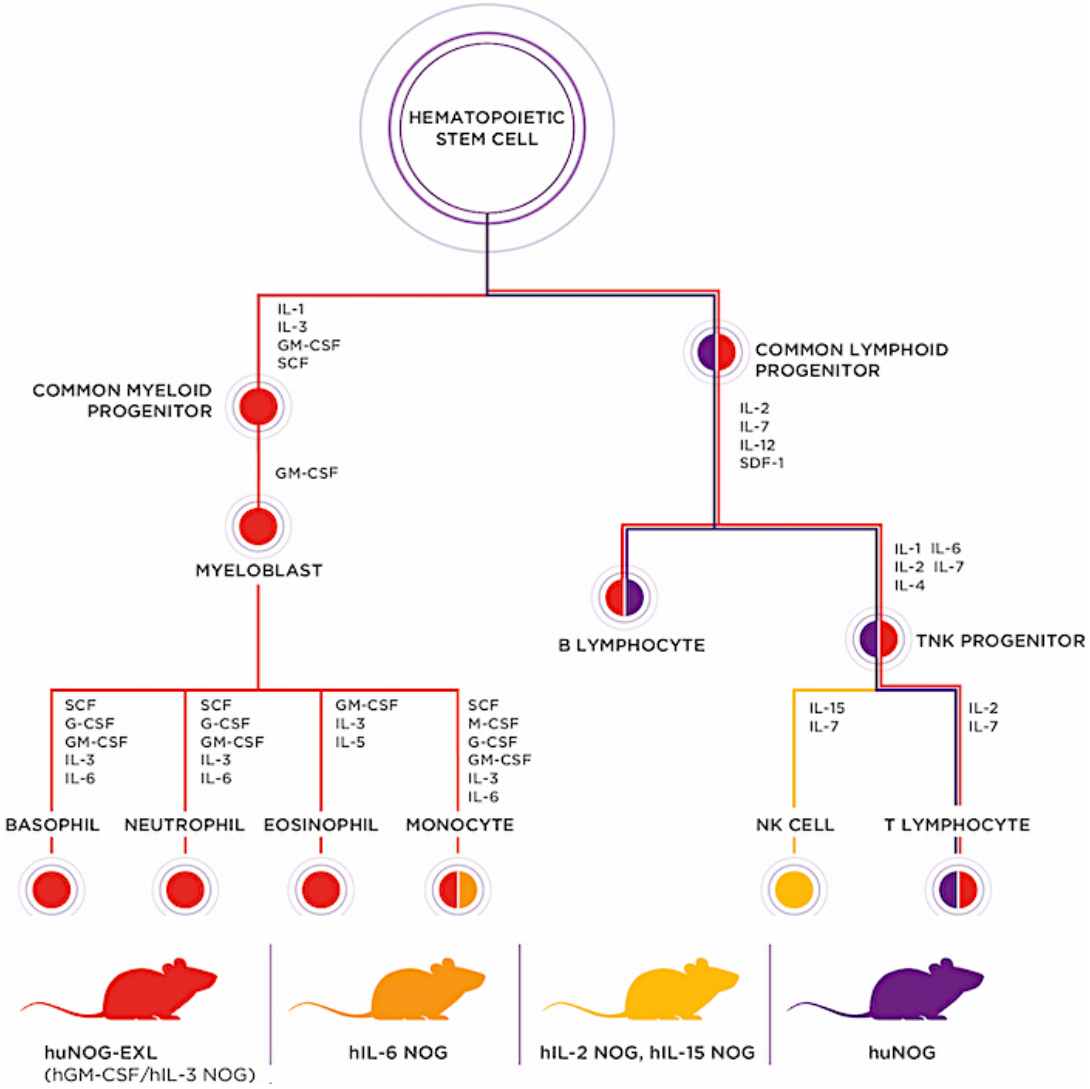
Summary

- The METHODS MATTER!!
- Unless the effect is predicted to be large, a sufficient n-value must account for both variance of the introduced variables **and** that imparted by the donor
- The rate limiting step to the power (n-value) of a HIS study design is the # of CD34+ cells of a donor
- Tumor growth and infiltration can vary based on tumor type, HIS donor, tumor donor, passage #, strain, orthotopic vs. SQ, etc.
- Study duration vs. model kinetics/health outcomes must align
- What we haven't talked about yet is very relevant to your ability to ask a question: environmental stress, microbiota impact, serial bleeding frequency, housing/bedding

- Taconic offers a wide range of host strains and study-ready HIS models
- HIS models offer tremendous value, but must be applied appropriately
- Taconic's field application scientists are experts in applying HIS models to both drug discovery and basic research applications

Complex experiments require complex models.

Let us help you pick the right one.



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Thank you

Janell.Richardson@Taconic.com

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