Comprehensive evaluation of human immune system reconstitution in NSG™ and NSG™-SGM3 mouse models toward the development of a novel ONCO-HU™ xenograft model

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Abstract

The recent successes of immunotherapeutic approaches in the treatment of melanoma and the promise of similar treatments in a variety of other cancers underscore the importance of the immune system in cancer. Indeed, effective therapeutic design and evaluation require a comprehensive understanding of the interplay between the immune compartment and the proliferating tumor cells that comprise the tumor microenvironment. A humanized mouse strain engrafted with cancerous tissue from a patient derived xenograft (PDX) tumor provides researchers with a highly sophisticated tool, ideally suited to facilitate the design of treatment strategies that prevent tumor evasion of immune cells and improve cytotoxic responses.

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Severely combined immunodeficient mice such as NOD scid gamma (NSG™) and triple transgenic NSG™ mice expressing human cytokines KITLG, CSF2 and IL-3 (NSG™-SGM3) are proven hosts for the engraftment of human tumors and establishment of human immune system components following hematopoietic stem cell ($CD34^+$) transplantation. The endogenous expression of cytokines that support the development of myeloid lineages and regulatory T (Treg) cells potentially represents a substantial improvement over standard NSG™ mice.

Here, we employ three 14-color flow cytometry panels to perform a comprehensive and detailed analysis of the entire immune system. The three panels are designed to fully characterize specific branches of the immune system: 1) T cells 2) natural killer (NK) cells/dendritic cells (DCs)/B cells and 3) myeloid lineages. Blood, spleen and bone marrow tissue from both NSG™ and NSG™-SGM3 mice were evaluated at 10, 16, 21 and 31 weeks post-engraftment using each of the four phenotyping panels. Our results indicate that the triple transgenic NSG™-SGM3 mice exhibit a more completely humanized immune system as compared to NSG™ mice, with specific improvements in the distribution of T-cell subsets and overall representation of the myeloid lineage.

NSG™ mice engrafted with allogeneic human tumors represent a valuable preclinical testing platform for immuno-oncology.

Methods

Mice

NSG™ and NSG™ -SGM3 mice were humanized by transplantation of human cord-blood-derived $CD34^+$ hSC by The Jackson Laboratory (Bar Harbor, ME) and shipped to BD Technologies (Raleigh-Durham, NC) for processing.

Tissue processing

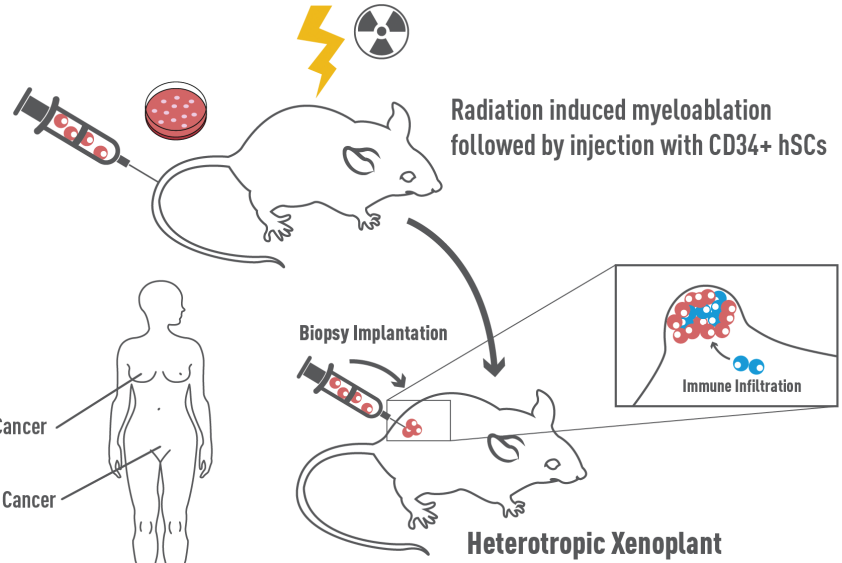
Mice were euthanized and spleen, bone marrow and peripheral blood were harvested. Spleen was placed between two glass slides and crushed. The slides were then rinsed into a 50 mL conical tube using 10-20 mL PBS, then filtered using a 70-µm sieve to collect a single cell suspension. Bone marrow was expelled from femurs and broken up using wide-bore pipette tips and trituration. It was then filtered through a 70-µm sieve to create a single cell suspension. 200-400 µL of peripheral blood was taken during terminal blood draw. Peripheral blood samples and single cell suspensions derived from spleen and bone marrow were treated with 4 mL ACK Buffer (Gibco A10492-01) for 7 minutes at room temperature in order to lyse all red blood cells. Samples were then washed once with 45 mL DPBS/2%FBS. Supernatant was aspirated, pellets were incubated in human FcR block, resuspended in PBS and then transferred to Falcon® 96-well plates for staining with antibody cocktails.

Flow cytometry

Cell suspensions from each of the three sample types (blood, spleen and bone marrow) were stained for 30 minutes with each of the 14 color panels described in the Results section. Fluorochrome selection for each of the panels was performed using the BD Horizon™ Guided Panel Solution; you can learn more about the tool at **bdbiosciences.com/us/tools/s/gps**. Antibody cocktails were diluted in 50 µL of BD Horizon™ Brilliant Stain Buffer (BD Biosciences Cat. No. 659611). After staining, cells were washed two times with PBS and recovered via centrifugation (7 min at 300*g*). Samples were acquired on a Special Order BD LSRFortessa™ X-20 flow cytometer (BD Biosciences Cat. No. 658226R1) and data analysis was performed using BD FACSDiva™ software.

NSG™ Mouse

The IL-2rg null allele was crossed from B6.129S4-Il2rgtm1Wjl/J to NOD.CB17-Prkdcscid/J for > 8 generations.



PDX Mouse Model

Patient Derived Xenoplant

NSG™-SGM3 Mouse

1) Transgenes injected into C57BL/6xC3H/HeN oocytes

2) Founders backcrossed to BALB/c-scid/scid, then to NOD.CB17-Prkdcscid mice for at least 11 generations

3) The NOD.CB17-Prkdcscid –SGM3 mice were bred to NSG mice to create NSG-SGM3

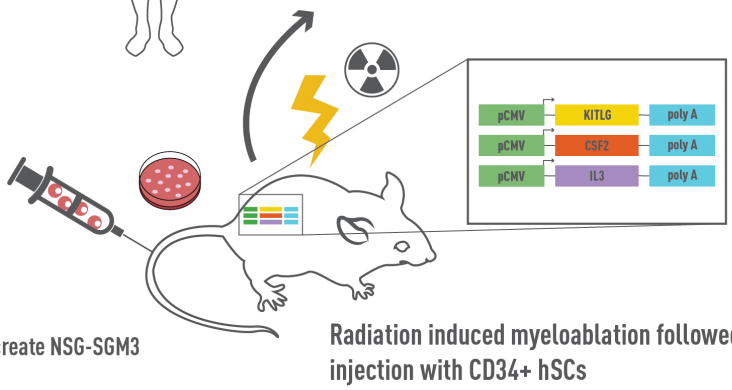


Figure 1. NSG™ vs NSG™-SGM3 mice

NSG™ mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, 005557) are devoid of mature B, T and NK cells. NSG™-SGM3 (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ, 013062) are NSG™ mice that contain transgenes expressing human Stem Cell Factor (KITLG), GM-CSF (CSF2) and IL-3. Recipient mice were irradiated at 3-4 weeks of age and engrafted with human cord-blood-derived CD34$^+$ hematopoietic stem cells. Prior to co-engraftment of human tumors, mice are validated for human multilineage engraftment and establishment of human immunity. *Figure courtesy of The Jackson Laboratory.*

Results

T-cell panel

Sample tissues (bone marrow, spleen and peripheral blood) were stained with a 14-color panel (see table on next page for reagent list). The panel was designed to enumerate T-cell subsets. Representative flow plots (spleen, 23-week old NSG™ mouse), demonstrate the gating strategy used for analysis. Enumerated populations are plotted as boxplots (next page, bottom). The green bands across each plot represent the range of each subpopulation in peripheral blood as measured in normal healthy adult donors (n=6) using the same panel. Not all enumerated population plots are shown. Those that showed little change or were in clear agreement with reference values (green band) were not shown. The intermediate 16-17 week and 23-week timepoints are not shown.

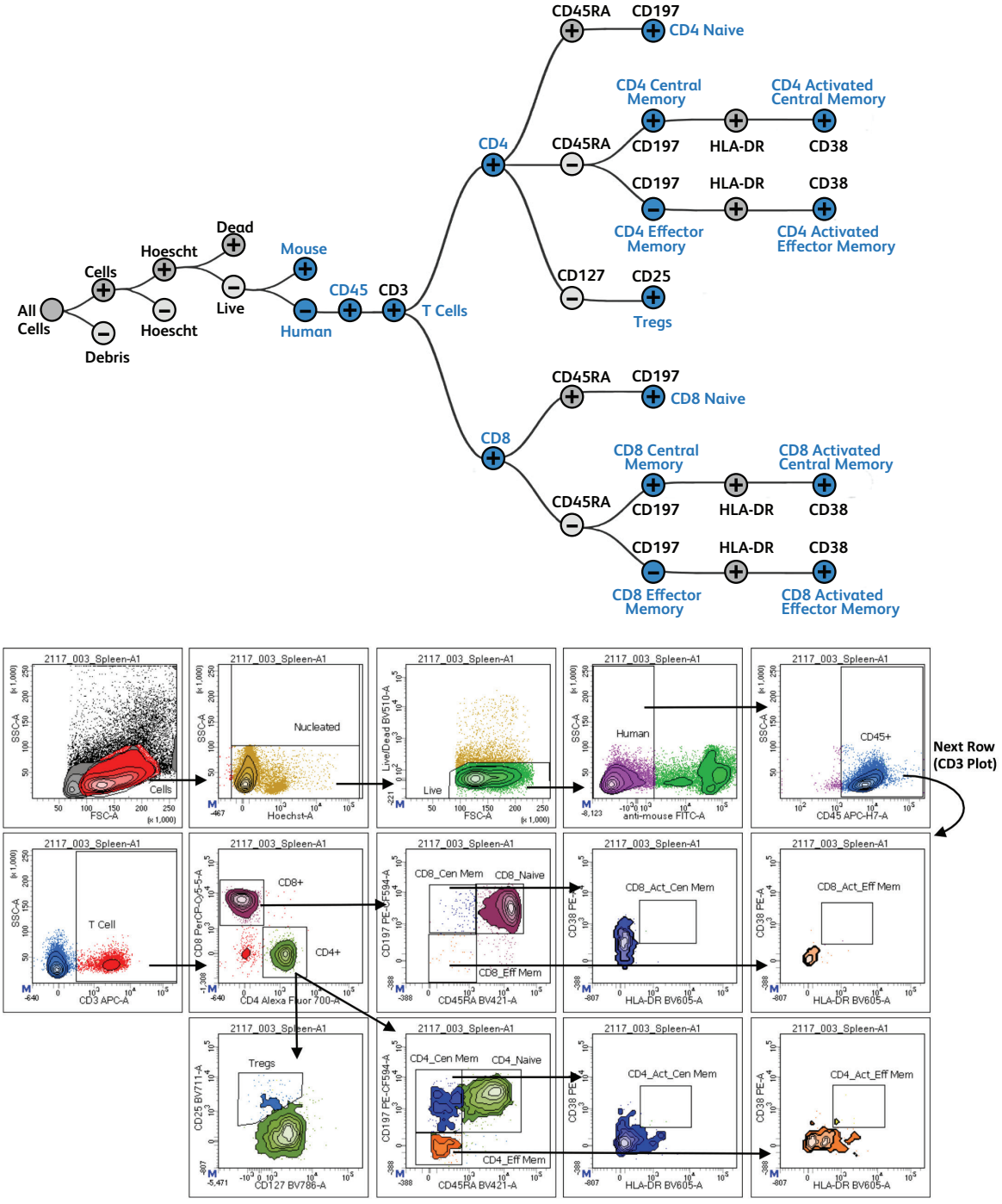


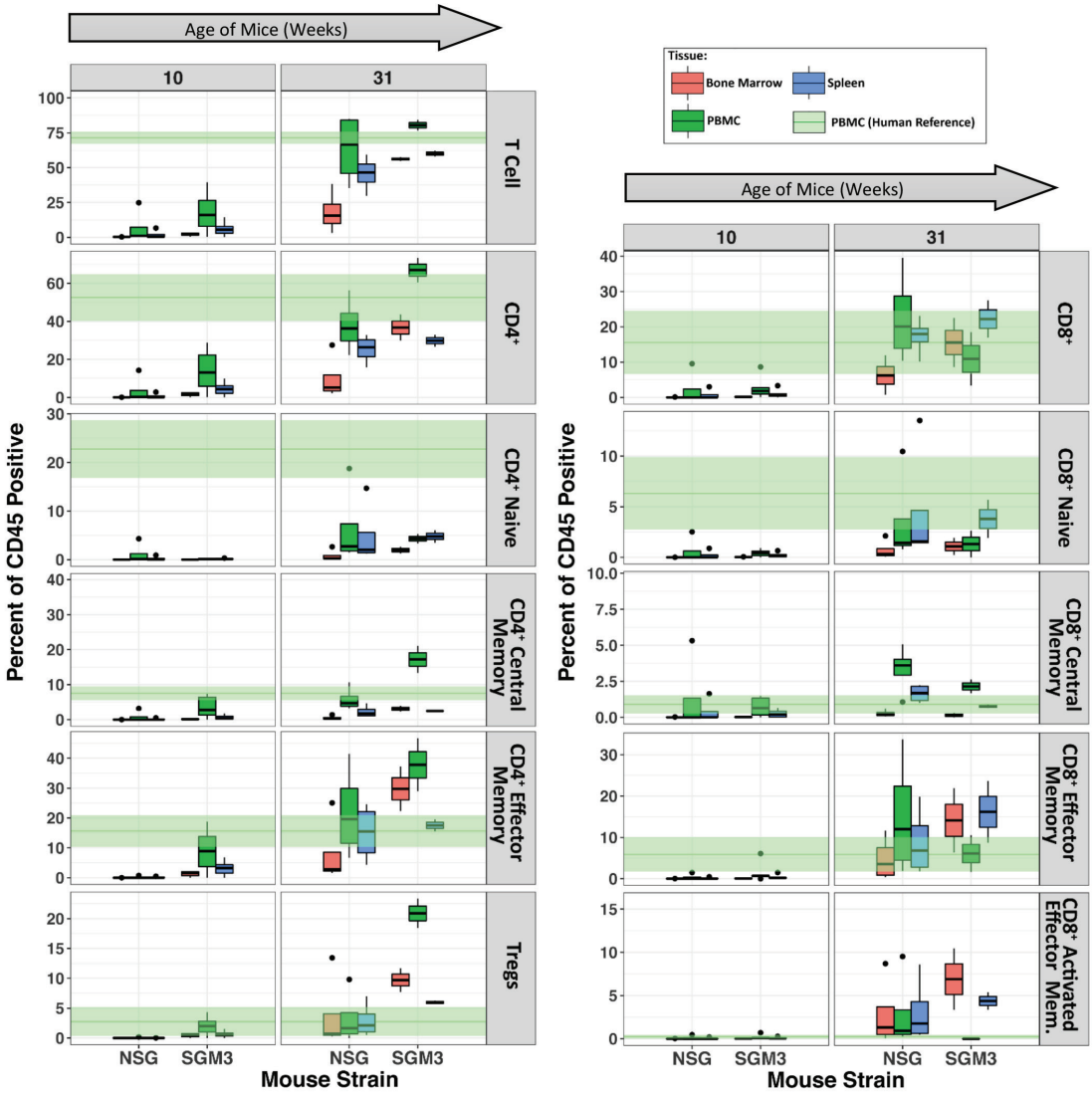
Figure 2. T-cell panel population hierarchy and gating strategy

Cell populations shown in blue (top) were measured across all tissue types and across all strain groups (10 weeks, 16-17 weeks, 23 weeks and 31 weeks). The population hierarchy was generated using the BD Horizon GPS tool (see Methods). Each population was defined according to the gating strategy shown above. The data shown is a representative set of plots from the spleen of a 23-week old NSG™ mouse. This gating strategy does not include all markers in the panel. Some markers were included to identify specific activated phenotypes that will be relevant in future comparative studies using tumor bearing mice.

Reagents used in T-cell panel

Laser (nm)	Fluorchrome	T-Cell Panel	Cat. No.
488	FITC	*Mouse Dump	(at right)
	PerCP-Cy™5.5	CD8	560662
561	PE	CD38	555480
	PE-CF594	CD197	562381
	PE-Cy™7	CD28	560684
652	APC	CD3	555342
	APC-H7	CD45	560274
	AF700	CD4	557922
405	BV421	CD45RA	562885
	BV510	FVS Live/Dead	564406
	BV605	HLA-DR	562846
	BV711	CD25	563159
	BV786	CD127	563324
355	BUV395	Hoescht 33342	561908

*Mouse Dump	
Antibody	Cat. No.
mCD45	553079
mH2Kd	553592
mTer119	561032
mCD31	558738
mCD41	561849
mCD71	553266



NK/DC/B cell panel

Sample tissues (bone marrow, spleen and peripheral blood) were stained with a 14-color panel (see table on next page for reagent list). The panel was designed to enumerate DC, NK-cell and B-cell subsets. Representative flow plots (spleen, 23-week-old NSG™ mouse) demonstrate the gating strategy used for analysis. Enumerated populations are plotted as boxplots (next page, bottom). The green bands across each plot represent the range of each subpopulation in peripheral blood as measured in normal healthy adult donors (n=6) using the same panel. Not all enumerated population plots are shown. Those that showed little change or were in clear agreement with reference values (green band) were not shown. The intermediate 16-17 week and 23 week timepoints are not shown.

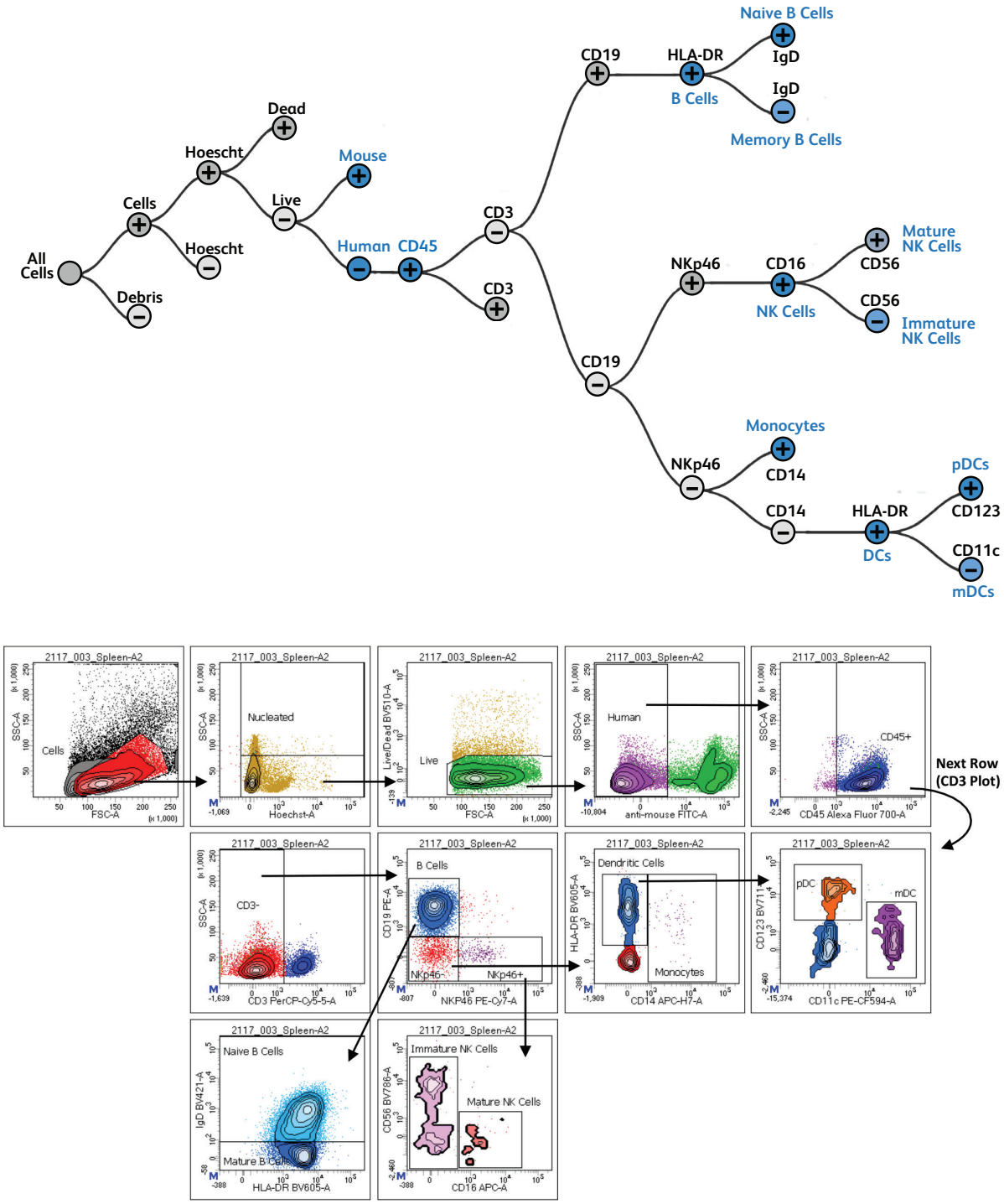


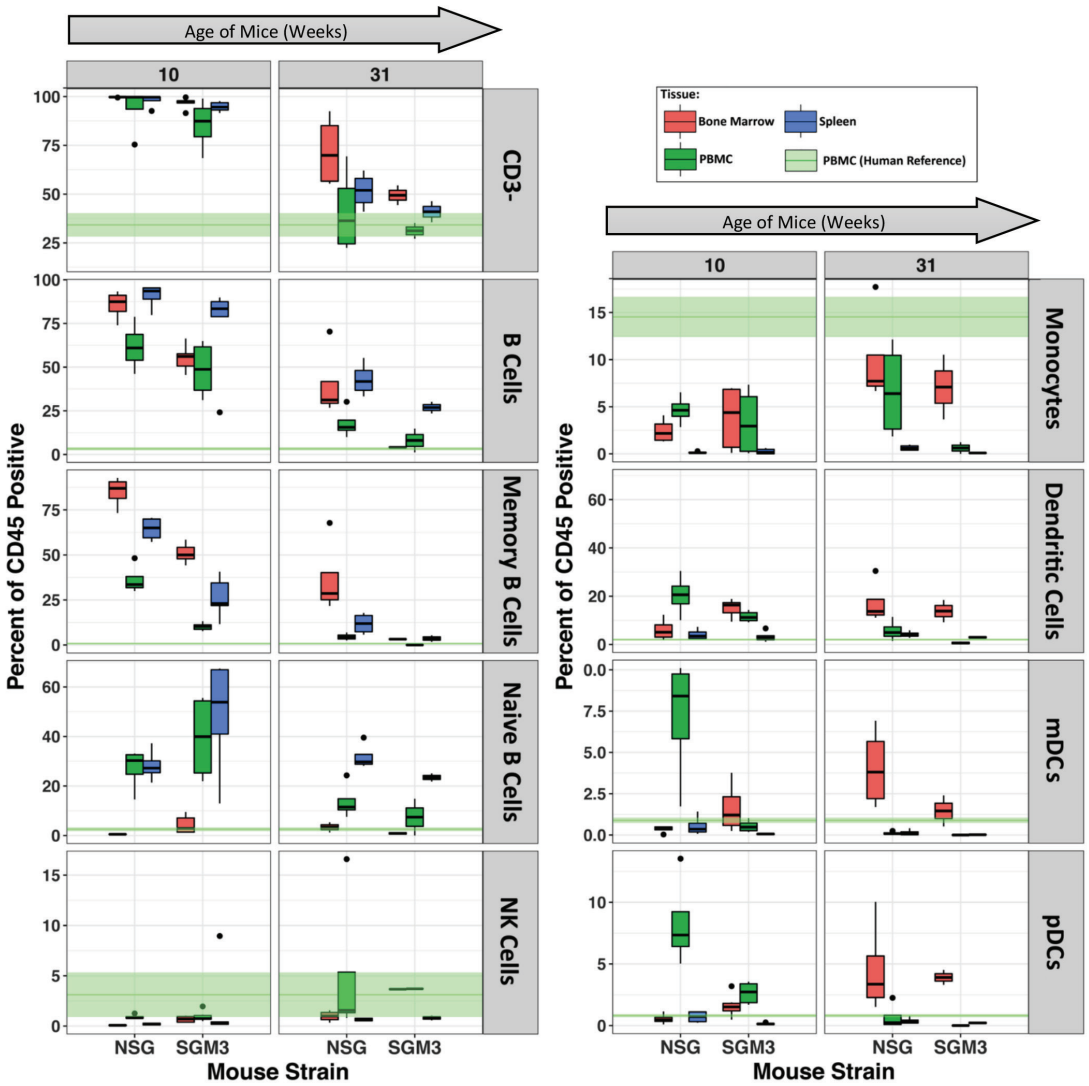
Figure 3. NK/DC/B cell population hierarchy and gating strategy

Cell populations shown in blue (top) were measured across all tissue types and across all strain groups (10 weeks, 16-17 weeks, 23 weeks and 31 weeks). The population hierarchy was generated using the BD Horizon GPS tool (see Methods). Each population was defined according to the gating strategy shown above. Data shown is a representative set of plots from the spleen of a 23-week-old NSG™ mouse. This gating strategy does not include all markers in the panel. Some markers were included to identify specific activated phenotypes that will be relevant in future comparative studies using tumor bearing mice.

Reagents used in NK/DC/B cell panel

Laser (nm)	Fluorchrome	NK/DC/B-Cell Panel	Cat. No.
488	FITC	*Mouse Dump	(at right)
	PerCP-Cy™5.5	CD3	560835
561	PE	CD19	555413
	PE-CF594	CD11c	562393
	PE-Cy™7	NKp46	562101
652	APC	CD16	561304
	APC-H7	CD14	560180
	AF700	CD45	560566
405	BV421	IgD	562518
	BV510	FVS Live/Dead	564406
	BV605	HLA-DR	562845
	BV711	CD123	563161
	BV786	CD56	564058
355	BUV395	Hoescht 33342	561908

*Mouse Dump	
Antibody	Cat. No.
mCD45	553079
mH2Kd	553592
mTer119	561032
mCD31	558738
mCD41	561849
mCD71	553266



Myeloid panel

Sample tissues (bone marrow, spleen and peripheral blood) were stained with a 14-color panel (see table on next page for reagent list). The panel was designed to enumerate myeloid subsets. Representative flow plots (bone marrow, 10-week-old NSG™ mouse), demonstrate the gating strategy used for analysis. Enumerated populations are plotted as boxplots (next page, bottom). The green bands across each plot represent the range of each subpopulation in peripheral blood as measured in normal healthy adult donors (n=6) using the same panel. Not all enumerated population plots are shown. Those that showed little change or were in clear agreement with reference values (green band) were not shown. The 16-17-week and 23-week timepoints are not shown.

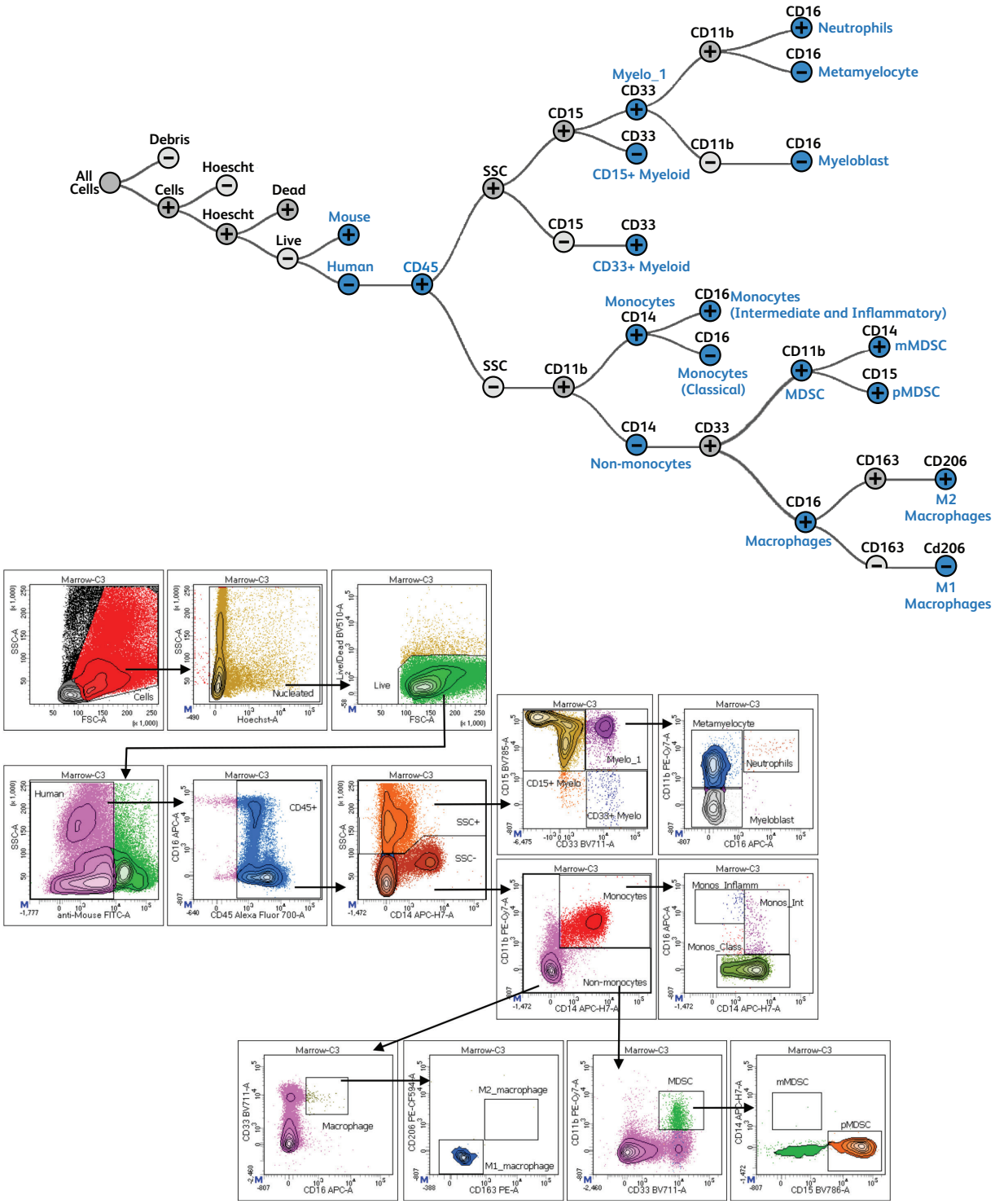


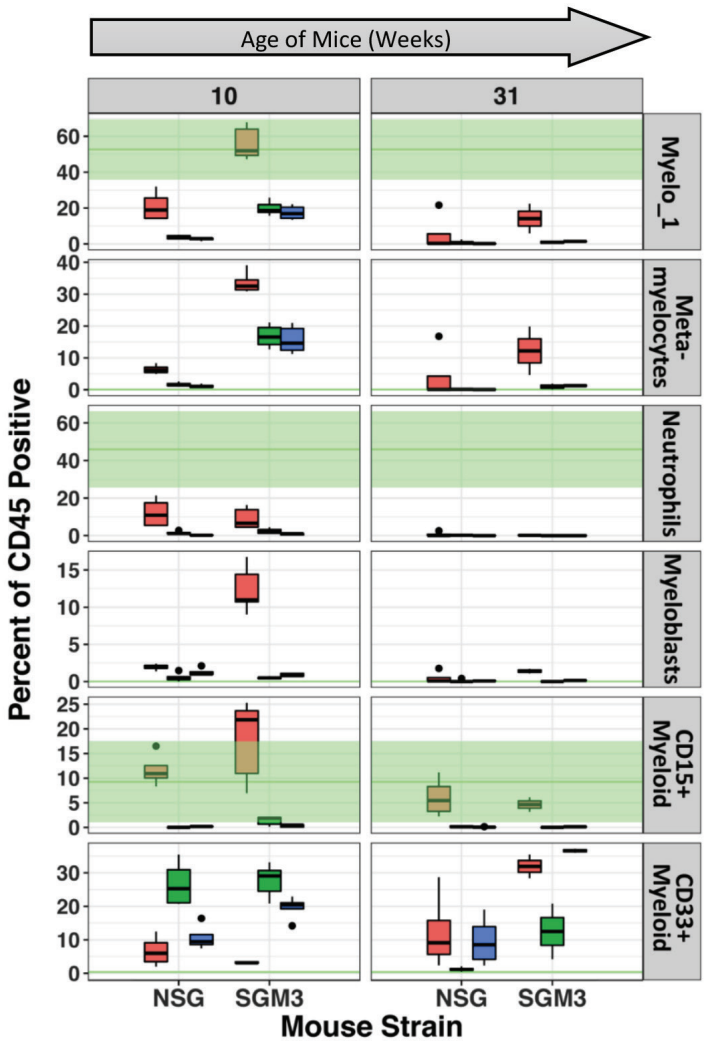
Figure 4. Myeloid population hierarchy and gating strategy

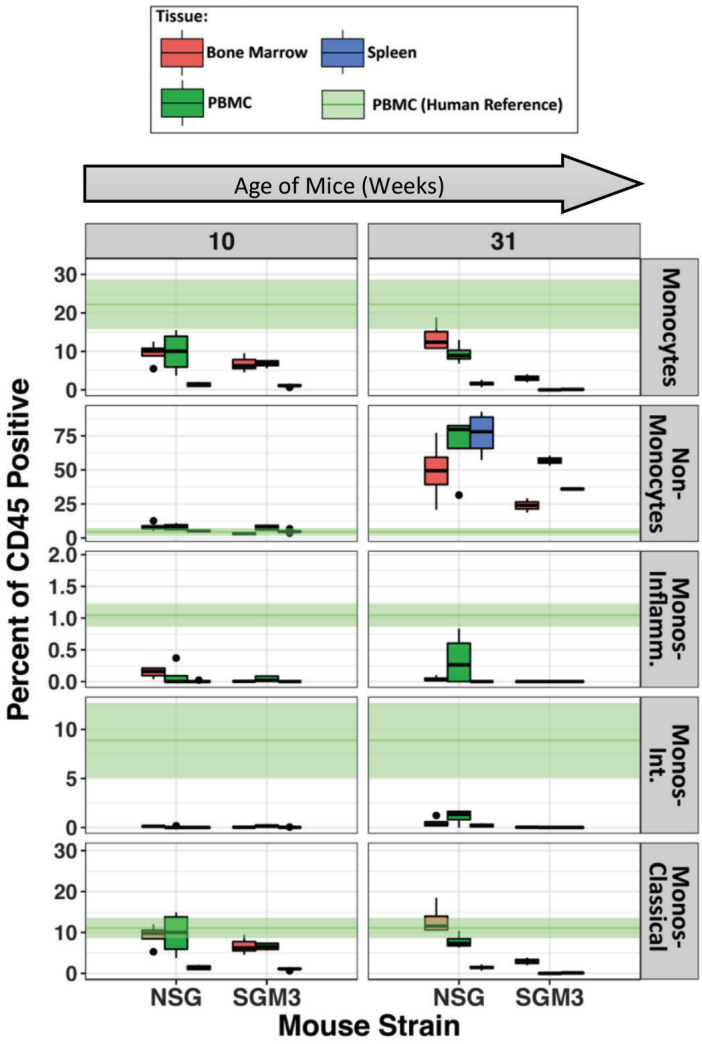
Cell populations shown in blue (top) were measured across all tissue types and across all strain groups (10 weeks, 16-17 weeks, 23 weeks and 31 weeks). The population hierarchy was generated using the BD Horizon GPS tool (see Methods). Each population was defined according to the gating strategy shown above. Data shown is a representative set of plots from the bone marrow of a 10-week-old NSG™ mouse. This gating strategy does not include all markers in the panel. Some markers were included to identify specific activated phenotypes that will be relevant in future comparative studies using tumor bearing mice.

Reagents used in myeloid cell panel

Laser (nm)	Fluorochrome	Myeloid Panel	Cat. No.
488	FITC	*Mouse Dump	(at right)
	PerCP-Cy™5.5	CD195	560635
561	PE	CD163	560933
	PE-CF594	CD206	564063
	PE-Cy™7	CD11b	557743
652	APC	CD16	561306
	APC-H7	CD14	560180
	AF700	CD45	560566
405	BV421	CD192	564067
	BV510	FVS Live/Dead	564406
	BV605	HLA-DR	562845
	BV711	CD33	563171
	BV786	CD15	563383
355	BUV395	Hoescht 33342	561908

*Mouse Dump	
Antibody	Cat. No.
mCD45	553079
mH2Kd	553592
mTer119	561032
mCD31	558738
mCD41	561849
mCD71	553266





Conclusions

The expression of three human growth factors (SCF, GM-CSF and IL-3) in the NSG™ mouse model represents a significant improvement in the development of a suitable xenograft model. BD's sophisticated and flexible flow cytometry tools facilitated a deep and comprehensive analysis of the immune system in these mice. NSG™-SGM3 mice exhibit improved reconstitution compared to NSG™ mice as measured by the frequency of most major immune subsets including T cells, B cells, NK cells and DCs. In terms of percentage, significant deficiencies of the myeloid compartment (most evident in the reduced frequencies of monocytes and neutrophils) persist in both NSG™ and NSG™-SGM3 strains of mice. NSG™-SGM3 mice exhibit increased frequencies of Tregs (not evident in NSG™ mice), and an expanded memory T-cell pool with a concomitant reduction in naïve T cells ($CD4^+$ and $CD8^+$). While absolute cell numbers were not measured, there were qualitative increases in the total numbers of $CD45^+$ cells as a whole in the NSG™-SGM3 derived samples. These observations clearly merit a more quantitative investigation into the absolute cell numbers of each of the target cell populations measured. The overall improvement in immune reconstitution seen in the NSG™-SGM3 mice compared to NSG™ mice demonstrates the utility of this strain as a potential host for PDX tumors and may improve the ability of researchers to study the complex interplay between immune and cancer cells within the tumor microenvironment, which could help in the design and development of immunotherapeutic approaches to cancer treatment.

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