

1 **Human-like NSG mouse glycoproteins sialylation pattern changes the phenotype of human**  
2 **lymphocytes and sensitivity to HIV-1 infection**

3

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36

37 **Abstract**

38 **Background:**

39 The use of immunodeficient mice transplanted with human hematopoietic stem cells is an

40 accepted approach to study human-specific infectious diseases, like HIV-1, and to investigate

41 multiple aspects of human immune system development. However, mouse and human are

42 different in sialylation patterns of proteins due to evolutionary mutations of the CMP-N-

43 acetylneuraminic acid hydroxylase (*CMAH*) gene that prevent formation of N-

44 glycolylneuraminic acid from N-acetylneuraminic acid. How changes of mouse glycoproteins

45 chemistry will affect phenotype and function of transplanted human hematopoietic stem cells and

46 mature human immune cells in the course of HIV-1 infection is not known.

47

48 **Results:**

49 We mutated mouse *CMAH* on the most widely human cells transplantation strain NOD/scid-  
50  $IL2R\gamma_c^{-/-}$  (NSG) mouse background using the CRISPR/Cas9 system. The new strain provides a  
51 better environment for human immune cells. Transplantation of human hematopoietic stem cells  
52 leads to broad B cells repertoire, higher sensitivity to HIV-1 infection, and enhanced  
53 proliferation of transplanted peripheral blood lymphocytes. The mice showed low effects on the  
54 clearance of human immunoglobulins and enhanced transduction efficiency of recombinant  
55 adeno-associated viral vector rAAV2/DJ8.

56

57 **Conclusion:**

58 NSG-*cmah*<sup>-/-</sup> mice expand the mouse models suitable for human cells transplantation and this  
59 new model has advantages in generating a human B cell repertoire. This strain is suitable to  
60 study different aspects of the human immune system development, might provide advantages in  
61 patient-derived tissue and cell transplantation, and could allow studies of viral vectors and  
62 infectious agents that are sensitive to human-like sialylation of mouse glycoproteins.

63

64 **Keywords:** CMP-N-acetylneuraminic acid hydroxylase, NOD/scid- $IL2R\gamma_c^{-/-}$  mice,  
65 hematopoietic stem cells, HIV-1

66

67 **Background**

68 All vertebrate cell surfaces display a dense glycan layer often terminated with sialic acids that  
69 have multiple functions due to their location and diverse modifications [1]. The major sialic  
70 acids in most mammalian tissues are N-acetylneuraminic acid (Neu5Ac) and N-

71 glycolylneuraminic acid (Neu5Gc), the latter being derived from Neu5Ac via addition of one  
72 oxygen atom by CMP-Neu5Ac hydroxylase (Cmah). The pattern of proteins glycosylation  
73 affects the physiology of the cell, cell-to-cell communication, adhesion, migration, recognition  
74 by other cells and antibodies [2]. In infectious diseases, sialylation patterns influence how  
75 humans interact with some pathogens or viral vectors: including HIV-1, malaria, influenza, and  
76 streptococcus [3-13]. In xenotransplantation and stem cell biology, it is a key factor for graft  
77 acceptance and preservation of self-renewing properties [14]. Of the two most common sialic  
78 acids forms Neu5Gc is widely expressed on most mammalian tissues but has limited  
79 accumulation in human cells [15]. The human deficiency of Neu5Gc is explained by an  
80 inactivating mutation in the gene encoding CMP-N-acetylneuraminic acid hydroxylase (*CMAH*),  
81 the rate-limiting enzyme in generating Neu5Gc in cells of other mammals [16]. This deficiency  
82 also results in an excess of the precursor Neu5Ac in humans. This mutation appears universal to  
83 modern humans and happens to be one of the first known human-great ape genetic differences  
84 with an obvious biochemical readout. In particular, it is important for interaction with Sialic  
85 acid-binding Ig-like lectins, or Siglecs. Expression of such lectins vary in their specificity for  
86 sialic acid-containing ligands and are mainly expressed by cells of the immune system. For  
87 example, humans, compared to mice and rats, express a much larger set of CD33rSiglecs [17].  
88 CD33rSiglecs have immune receptor, tyrosine-based inhibitory motifs, and signal negatively  
89 [18]. Interaction with Siglec-7 has the potential to also affect monocyte migration and function  
90 [19] along with T-cell activation [8, 20, 21]. During B cells activation and germinal center  
91 formation (GC), Siglecs are important for appropriate activation of B cells and responses to T-  
92 cell-dependent and independent antigens [22]. In B-cell antigen receptor (BCR) engagement,  
93 interaction of CD22 and Siglec-G has been shown to inhibit the BCR signal [23]. Most

94 importantly, exposure to exogenous Neu5Gc is known to cause rapid phosphorylation of beta-  
95 catenin in both CMAH-overexpressing cells and bone marrow-derived mesenchymal stem cells,  
96 thereby inactivating Wnt/ $\beta$ -catenin signaling and, as a consequence, possibly forcing stem cells  
97 to lose pluri- or multipotency [24].

98 Immunodeficient mice transplanted with human hematopoietic stem/progenitor cells  
99 (HSPC) are an established model to study human-specific infections like HIV-1 [25]. However,  
100 if a particular sialic acid residue is missing in a donor species (Neu5Gc) and present in the  
101 recipient, biologic consequences can be difficult to delineate. Exclusion of mouse Neu5Gc has  
102 the potential to improve immune responses to pathogens with non-human patterns of  
103 glycosylation like HIV-1 and HCV (hepatitis C virus) and to study the pathogenicity of human-  
104 like sialylated pathogen surfaces [12, 17, 26, 27].

105 To distinguish the effect of the expression of CMP-N-acetylneuraminic acid hydroxylase  
106 in mice on human HSPC biology, improve the development of a human immune system in mice,  
107 and study responses to HIV-1 infection, we generated mutation in exon 6 of the gene on NSG  
108 strain using CRISP/Cas9 technology [28]. We compared original NSG and NSG-*cmah*<sup>-/-</sup> strains  
109 for multiple parameters and observed changes in the human lymphocyte phenotype and  
110 repertoire. Human lymphocytes generated from HSPC in a human-like sialylation environment  
111 exhibit persistence of naïve non-activated T-cell phenotypes and are more sensitive to HIV-1  
112 mediated depletion of CD4<sup>+</sup> T-cells. Alternatively, mature human lymphocytes derived from  
113 human peripheral blood expand more efficiently in the NSG-*cmah*<sup>-/-</sup> mice with higher levels of  
114 activation.

115 This new strain expands the utility of the NSG standard strain used to study human  
116 hematopoiesis and immunity and allows comparison of new viral vectors for gene therapy and  
117 sensitivity to a wider variety of pathogens.

118

## 119 **Results**

### 120 **Generation of NSG-*cmah*<sup>-/-</sup> mice.**

121 To generate a *Cmah* knockout mouse on NSG background, we designed two single guide RNAs  
122 (sgRNAs) targeting exon 6. Schematic of CRISPR targeting are shown in **Fig. 1**. Embryo  
123 isolation, microinjection, and generation of founder mice were performed as described in Harms  
124 et al [28]. Genotyping of pups showed that one founder contained a shorter sized amplicon.  
125 Sequencing of the shorter band revealed a deletion of 27 base-pair sequences in the exon.  
126 Genotyping of F1 offspring from this founder is shown in **Fig. 1b, 1c**.

127

### 128 **NSG-*cmah*<sup>-/-</sup> phenotype**

129 To confirm the inactivation of *CMAH* gene enzymatic activity and the absence of hydrolysis of  
130 Neu5Ac to Neu5Gc, we used the chicken anti-Neu5Gc antibody and anti-chicken  
131 immunoglobulin Y (IgY) antibody in different formats: horseradish peroxidase (HRP)-  
132 conjugated for Western blot (WB) and immunohistochemistry (IHC) of paraffin-embedded  
133 sections (**Fig. 2a and 2b**). FITC-conjugated antibodies were used for analysis of the surface  
134 expression Neu5Gc on immune cells in the peripheral blood (**Fig. 2c and 2d**). Neu5Gc  
135 expression was undetectable by WB and IHC in all tested tissues: spleen, liver, lung, kidney,  
136 heart, gut, and brain. The results were comparable with existing C57Bl/6-*Cmah*<sup>-/-</sup> animals.  
137 Moreover, flow cytometry showed better reduction of Neu5Gc expression on immune cells of

138 NSG-*cmah*<sup>-/-</sup> mice than on the existing strain of immune competent animals. By these three  
139 techniques, we confirmed the absence of CMAH gene activity and a human-like sialylation  
140 pattern of mouse biomolecules. Breeding for two years did not reveal any differences in fertility,  
141 body weight, or life span in comparison to the founder NSG mice.

142

### 143 **Comparison of human immune system development after CD34<sup>+</sup> hematopoietic** 144 **stem/progenitor cell transplantation**

145 Another wide application of NSG mice is transplantation of human CD34<sup>+</sup> HSPC for the  
146 development of human lymphopoiesis [29, 30]. The establishment of human B-cell  
147 lymphopoiesis in mouse bone marrow and T-cell lymphocytes in mouse thymus has been well  
148 demonstrated [29]. In this model, the expansion of human B cells occurs significantly earlier  
149 than CD3<sup>+</sup> T cells. We tested whether differences in cell surface glycoproteins sialylation would  
150 affect activation of newly generated human lymphocytes. NSG-*cmah*<sup>-/-</sup> and wild-type (wt) NSG  
151 mice were transplanted at birth with the same donor HSPC. At 3 and 6 months post-  
152 transplantation, the proportion of human T and B cells as well as activation status was  
153 determined (**Fig. 3**). The following markers were used: CD45RA for naïve CD3<sup>+</sup> T cells, CD22  
154 for CD19<sup>+</sup> B cells. CD22 is a BCR co-receptor that regulates B cell signaling, proliferation, and  
155 survival; it is required for T cell-independent antibody responses [31]. The frequency of human  
156 T cells in the peripheral blood of 3-month-old mice was higher in NSG wt mice, and the  
157 proportion of circulating B cells at the same time was higher in NSG-*cmah*<sup>-/-</sup> mice. By 6 months  
158 of age, there were similar proportions of T and B cells in the peripheral blood (**Fig. 3c**)  
159 regardless of sialylation status. In both strains the proportion of CD4<sup>+</sup> cells in peripheral blood  
160 increased. NSG and NSG-*cmah*<sup>-/-</sup> mice at this time had similar proportions of naïve

161 CD3<sup>+</sup>CD45RA<sup>+</sup> cells, which declined with time. Human B cells in NSG-*cmah*<sup>-/-</sup> mice showed a  
162 lower number of activated CD19<sup>+</sup>CD22<sup>+</sup> cells at 3 months of age. The differences in B cell  
163 activation (CD22 expression levels) were sustained at 6 months post-engraftment. The  
164 proportion of CD19<sup>+</sup>IgD<sup>+</sup> B cells was lower in NSG-*cmah*<sup>-/-</sup> mice at 3 months and did not decline  
165 by 6 months as was found in NSG wt humanized mice. The proportion of CD19<sup>+</sup>IgM<sup>+</sup>IgD<sup>+</sup> B  
166 cells in peripheral blood of both strains declined, and the proportion of CD19<sup>+</sup>IgM<sup>-</sup>IgD<sup>+</sup> cells  
167 [32, 33] increased. We did not observe significant differences in the numbers of CD19<sup>+</sup>IgM<sup>+</sup>IgD<sup>-</sup>  
168 mature B cells between strains at 6 months ( $5.9 \pm 0.9\%$  and  $7.7 \pm 1.3\%$  NSG-*cmah*<sup>-/-</sup> and NSG,  
169 respectively). The low levels of CD19<sup>+</sup>IgG<sup>+</sup> (0.7 – 3.8%, not shown) cells were found in both  
170 strains. The levels of IgM at 6 months of age varied from 1-350 µg/ml (**Fig. 3c**) and only low  
171 levels of IgG (1 – 50 µg/ml, not shown) were present in both strains at 6 months of age.

172

### 173 **Analysis of T and B cell repertoires in NSG-*cmah* and wild type NSG mice**

174 To characterize the global B and T cells receptor repertoires, we selected non-fractionated bone  
175 marrow cells suspension and spleen tissue samples. Human-specific primers were selected for  
176 analysis of human cells according to Adaptive Biotechnologies® (Seattle, WA, USA)  
177 technology [34]. We compared the repertoire profiles of bone marrow and spleen within one  
178 mouse and between NSG-*cmah*<sup>-/-</sup> and wt NSG mice. The generation of mature human  
179 lymphocytes requires a complex selection process in bone marrow (B cells) and thymus (T cells)  
180 and are highly dependent on the microenvironment. Glycosylation of stromal mouse counter-  
181 receptors is important for pre-B cells signaling and proliferation [35] and retention in bone  
182 marrow for B cells [36]. Maturation and activation of human B and T cells in mouse primary  
183 (bone marrow and thymus) and secondary lymphoid organs (spleen, lymph nodes) will likely



184 affect B and T cell receptors repertoire and development [37]. Using the same donor of HSPC,  
185 we compared the B and T cell receptors repertoires in the new and existing NSG strain at 8  
186 months of age. Spleen tissue and bone marrow pelleted cells were used for genomic DNA  
187 (gDNA) extraction and immunosequencing provided by Adaptive Biotechnologies®. Collected  
188 data were analyzed using immunoSEQ Analyzer (<https://www.adaptivebiotech.com/>). We used  
189 the common indexes to determine diversity of repertoires based on DNA sequences of the  
190 rearranged V-D-J gene segments encoding the third complementarity-determining region  
191 (CDR3) of IgH loci and T cell receptor beta chain (TCR $\beta$ ) in a given sample, the length of  
192 CDR3, and usage of IgH and TCR $\beta$  genes (**Fig. 4** and additional files 1-5 **Fig. S1 - S5**).

193 Gaussian-like distribution patterns of IgH CDR3 length in NSG-*cmah*<sup>-/-</sup> mice were  
194 similar to wt NSG (**Fig. 4a, 4b**) mice. In contrast, the frequency of clonal B-cell expansion was  
195 more common in the wt NSG mice, and the difference was close to statistical significance. A  
196 similar size distribution pattern of IgH CDR3 length was observed in bone marrow samples  
197 evaluated for five NSG-*cmah*<sup>-/-</sup> mice. The NSG animals analyzed also had significant variability  
198 (Additional file 1, **Fig. S1b**, two animals). The variability in NSG mice was also reflected by  
199 variable total number of templates (Additional file, **Fig. S1e**). We observed lower diversity of B  
200 cell repertoires in NSG mice compared to NSG-*cmah*<sup>-/-</sup> strain according to higher clonality of  
201 IgH genes in bone marrow and spleen (**Fig. 4c** and additional file 1, **Fig. S1c**). The average IgH  
202 CDR3 length naturally generated by the rearrangement machinery was found to be reduced  
203 during B cell development and we observed slight shift to the left of CDR3 length in 4 of 5  
204 analyzed NSG-*cmah*<sup>-/-</sup> mice by comparing bone marrow and spleen compartments (Additional  
205 file 2, **Fig. S2**). The use of IgH genes families were similar in spleen and bone marrow  
206 (Additional file 3, **Fig. S3C**) and correspond to the known human peripheral blood B cell data

207 [38, 39]. We observed similar changes in IgH D and J gene family usage between bone marrow  
208 (pre- and immature B cells) and spleen compartment with mature B cells (Additional file 3, **Fig.**  
209 **S3C**). The clonality of IgH gene loci were two folds higher in spleen compared to bone marrow,  
210 and in *cmah*<sup>-/-</sup> mice reached statistical significance (**Fig. 4d**).

211 We did not observe significant differences in TCRβ chain gene usage (Additional file 4,  
212 **Fig. S4**) nor repertoires in new strain compare to NSG mice (Additional file 5, **Fig. S5**). TCRβ  
213 clonality was lower in spleen compared to bone marrow in both strains, but these changes did not  
214 reach statistical significance (Additional file 5, **Fig. S5b**). The richness of repertoire was higher  
215 in spleen tissues compared to bone marrow samples in both strains (**Fig. 5d**). We did not observe  
216 statistically significant differences in TCRβ CDR3 length (Additional file 5, **Fig. S5a, 5b**) in  
217 spleen and bone marrow (not shown). Overall CDR3 length (nucleotides) was shorter in T cells  
218 compared to B cells. We did not find statistically significant differences in samples clonality and  
219 noted higher TCRβ richness in spleen compared to bone marrow as total number of TCRβ chain  
220 gene templates. Additionally, we observed better Pielou's evenness of TCRβ in NSG *cmah*<sup>-/-</sup>  
221 mice and overall increased evenness in spleen compared to bone marrow compartment  
222 (Additional file 5, **Fig. S5c, 5d, and 5e**).

223

## 224 **Comparison of human immune system responses to HIV-1 infection in NSG-*cmah*<sup>-/-</sup> and wt** 225 **NSG mice**

226 NSG mice, humanized by human hematopoietic stem cell transplantation, are a proven model to  
227 study the pathogenesis of HIV-1 infection [38]. We evaluated the effects of human-like  
228 sialylation of mouse tissues on the sensitivity of human cells to HIV-1. Two groups of mice with  
229 similar levels of reconstitution of circulating human CD45<sup>+</sup> cells in peripheral blood were

230 infected with the same dose of HIV-1<sub>ADA</sub> intraperitoneally at the age of 30-32 weeks when the  
231 majority of peripheral human cells were CD3<sup>+</sup>CD45RA<sup>+</sup>T cells. Four weeks post-infection,  
232 peripheral blood, spleen, and bone marrow samples were analyzed (**Fig. 5 and 6**, Additional files  
233 6 and 7, **Fig. S6, S7**). We observed a significant reduction in human T cells in all three  
234 compartments in NSG-*cmah*<sup>-/-</sup> mice including both CD3<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T-cells. The total  
235 number of CD4<sup>+</sup>CD45RO<sup>+</sup> cells and among this antigen-stimulated cells proportion of  
236 CD4<sup>+</sup>CD45RO<sup>+</sup>CD62L<sup>-</sup>CCR7<sup>-</sup> effector memory cells, the most sensitive to HIV-1 depletion,  
237 decline in *cmah*<sup>-/-</sup> mice compared to unaffected. The result was an increased frequency of central  
238 memory T cells CD4<sup>+</sup>CD45RO<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup> in the peripheral blood and spleen of HIV-1  
239 infected mice. This effect was not observed in NSG mice peripheral blood and spleen  
240 compartment. We found a higher HIV-1 viral load (VL) in the peripheral blood of NSG-*cmah*<sup>-/-</sup>  
241 mice at 4 weeks post-infection. In both strains, VL persisted at high levels for up to 9 weeks  
242 post-infection. As T cells decreased, we observed an increased frequency of B cells in all three  
243 compartments (blood, spleen, bone marrow). We also noted an increased proportion of human  
244 CD1c<sup>+</sup> cells in spleen of infected NSG-*cmah*<sup>-/-</sup> mice compared to wt NSG. Corresponding  
245 increases in myeloblasts (CD34<sup>+</sup>CD117<sup>+</sup>), promonocytes (CD4<sup>dim</sup>CD14<sup>neg or dim</sup>), and mature  
246 monocytes (CD4<sup>dim</sup>CD14<sup>bright</sup>) in bone marrow of HIV-1 infected NSG-*cmah*<sup>-/-</sup> mice were  
247 observed (**Fig. 6c**). We did not observe any differences in natural killer (NK) and NKT cells  
248 subpopulations (not shown) between the two strains. We also did not find differences in the  
249 levels of immune globulins and circulating HIV-1 specific binding antibodies. At nine weeks  
250 post-infection, in peripheral blood of HIV-1 infected NSG-*cmah*<sup>-/-</sup> mice, the decreased  
251 proportion of CD3<sup>+</sup> cells and specifically CD4<sup>+</sup>CD45RO<sup>+</sup> effector memory cells with increased  
252 number of monocytes CD14<sup>+</sup> were found (Additional file 7, **Fig. S7**).

253

254 **Effects of NSG-*cmah*<sup>-/-</sup> phenotype on transplanted human peripheral blood mononuclear**  
255 **cells behavior**

256 NSG mice are widely used for the transplantation of human peripheral blood immune cells  
257 (PBMC) [39]. We considered that the absence of Neu5Gc and human-like sialylation of  
258 glycoproteins could change the behavior of mature human immune cells derived from the adult  
259 donor. PBMC isolated from one donor were transplanted into the NSG and NSG-*cmah*<sup>-/-</sup> mice  
260 intraperitoneally. Starting from day 7 post-transplantation, mouse peripheral blood was collected  
261 and stained for the presence of human immune cells (**Fig. 7**). Human T lymphocytes were the  
262 major population with low absolute number of B cells and monocytes in peripheral blood. The  
263 expansion of human cells in NSG-*cmah*<sup>-/-</sup> mice significantly (~2 – 3.5 times) exceeded that seen  
264 in the NSG mice, and the absolute count of human CD45<sup>+</sup> cells (mean ± SEM) were 16 ± 2 vs 9  
265 ± 1 cells/μl at day 7, 513 ± 135 vs 146 ± 58 cells/μl at day 14, 474 ± 130 vs 168 ± 118 at day 21  
266 (P < 0.05) (**Fig. 7b**). The proportion of human cells in spleen were similar at the end-point of  
267 observation (**Fig. 7c**). Human T-cells in the mouse environment became activated and switched  
268 expression of CD45RA (naïve) to CD45RO (effector-memory). The activation of CD4<sup>+</sup> cells in  
269 *Cmah*<sup>-/-</sup> mice significantly exceeded the CD45RO to CD45RA switch in wt NSG mice. To a  
270 lower extent, the same was observed within CD8<sup>+</sup> T-cells (**Fig. 7d**). L-selectin (CD62L) is an  
271 adhesion molecule that recognizes sialylated carbohydrate groups, mediates the first steps of  
272 leukocyte homing to peripheral lymph nodes, and is programmed for recirculation through  
273 lymphoid organs, thus crucially controlling the initiation and maintenance of immune responses  
274 to pathogens [40]. CD62L<sup>+</sup> T-cells also were generated at higher frequency in *Cmah*<sup>-/-</sup> mice,  
275 including the splenic population of CD4<sup>+</sup>CD45RO<sup>+</sup>CD62L<sup>+</sup> (**Fig. 7e**). Overall, for this particular

276 donor, engraftment and expansion of mature human peripheral blood lymphocytes were more  
277 pronounced in the NSG-*cmah*<sup>-/-</sup> mouse strain.

278

279 **Host glycoproteins sialylation pattern affects the clearance of HIV-1 virus, half-life of**  
280 **infused human IgG and rAAV2/DJ8 biodistribution**

281 There is evidence that evolutionary loss via mutation of the CMAH genes changed the sensitivity  
282 of humans to viral and bacterial pathogens [41, 42]. We investigated the life-span of HIV-1 virus  
283 in the two mouse strains since the mouse is not a normal permissive host. We tested the  
284 concentration and time of HIV-1 VL in the peripheral blood of the non-humanized mouse as a  
285 parameter that potentially could influence viral pathogenicity. On the first and second day after  
286 intraperitoneally injection of 0.3 ml of HIV-1<sub>ADA</sub> viral stock, the detected copy numbers of HIV  
287 RNA were lower by ~0.3 log<sub>10</sub> in NSG-*cmah*<sup>-/-</sup> mice compared to NSG [4.98 ± 0.1 and 4.1. ±  
288 0.08 compared to 5.25. ± 0.06 and 4.4 ± 0.05 log<sub>10</sub>, respectively (P < 0.05, **Fig. 8a**)].

289 The clearance of sialylated glycoproteins going through the multiple types of receptors  
290 and changes of mouse sialylation could affect interaction with human immune globulins. We  
291 compared the time of circulation of human IgG in NSG-*cmah*<sup>-/-</sup> and wt mice (**Fig. 8b**). We were  
292 not able to determine the first minutes of injected IgG dose clearance, but overall clearance of  
293 human IgG was nearly identical in the two strains of mice. A slightly higher concentration  
294 remained in NSG-*cmah*<sup>-/-</sup> mice compared to NSG at the end of observation (1.1 ± 0.04 and 0.9 ±  
295 0.03 µg/ml for NSG-*cmah*<sup>-/-</sup> and wt mice, respectively) (P < 0.05).

296 Biodistribution and expression levels of recombinant adeno-associated virus (rAAV)  
297 vector delivered genes could be affected by sialylation of host receptors [5, 10, 43]. We  
298 compared the luciferase expression delivered by rAAV2/DJ8. We were not able to detect

299 statistically significant differences in luminescence in liver and spleen between the two strains at  
300 7, 14, and 21 days post-inoculation (data not shown). However, at 32 days the expression of  
301 luciferase RNA was significantly higher in the spleen and brain of NSG-*cmah*<sup>-/-</sup> as determined by  
302 ddPCR (**Fig. 8c**). It was higher by 0.60 log<sub>10</sub> copies in spleen (4.72 ± 0.12 vs 4.12. ± 0.07 log<sub>10</sub>)  
303 and 0.54 log<sub>10</sub> in brain (1.40 ± 0.11 vs 0.85. ± 0.12 log<sub>10</sub>) of *cmah*<sup>-/-</sup> mice compared to NSG (P <  
304 0.05).

305

## 306 **Discussion**

307 We created NSG-*cmah*<sup>-/-</sup> mice with the intent to use this strain for different aspects of biomedical  
308 research. As initial phenotypic characterization, we compared several parameters involved in  
309 human immune system development in NSG-*cmah*<sup>-/-</sup> to the parent NSG strain. Glycoproteins-  
310 lectin interactions (for example, hematopoietic cells surface glycoproteins – bone marrow stromal  
311 cell lectins/selectins) are an important mechanism of human B-cell selection. The NSG mouse  
312 environment that contained Neu5Gc (non-human type of sialylation) exhibited a higher  
313 frequency of clonal B-cell outgrowth that may represent responses against the Neu5Gc moiety.  
314 In the absence of Neu5Gc, human B cells appear to remain less activated. This finding indicates  
315 that non-human sialylation has a negative effect on human B cell development, and the NSG-  
316 *cmah*<sup>-/-</sup> strain provides a more supportive environment with good repertoire development and less  
317 clonal outgrowth [44]. The observations of lower IgD<sup>+</sup> and CD22<sup>+</sup> human B cells and sustained  
318 naïve CD45RA<sup>+</sup>CD3<sup>+</sup> cells in the NSG-*cmah*<sup>-/-</sup> strain also support this conclusion.

319 We observed better bone marrow B-cell precursor engraftment in control NSG mice  
320 compared to NSG-*cmah*<sup>-/-</sup>, and better bone marrow T-cell engraftment in NSG-*cmah*<sup>-/-</sup>. The HIV-  
321 1 infected NSG-*cmah*<sup>-/-</sup> mice showed a significant reduction in bone marrow T-cells and a

322 subsequent increase in bone marrow B-cell precursor engraftment. These findings may indicate  
323 T-cell suppression of B-cell engraftment is occurring in this mouse strain but will require further  
324 study to confirm. In contrast, the NSG strain showed similar levels of bone marrow T-cell and B-  
325 cell engraftment regardless of infection with HIV-1.

326         The T cell TCR $\beta$  chain repertoires in NSG-*cmah*<sup>-/-</sup> and wt NSG mice were not different  
327 in clonality metrics and remain polyclonal in bone marrow and spleen. However, compared to wt  
328 NSG mice, NSG-*cmah*<sup>-/-</sup> exhibited better evenness of repertoire between spleen and bone  
329 marrow. We did not observe statistically significant differences in clonal T-cell expansion based  
330 on TCR $\beta$  CDR3 length frequency in bone marrow versus spleen. This was previously reported  
331 for NOD/scid mice transplanted with cord blood derived hematopoietic CD34<sup>+</sup> stem cells [45].

332         In the studies presented here, we are not showing effects of vaccination with the  
333 childhood vaccines DTaP, HiB, and MMRII, which were tested in comparison on both strains of  
334 humanized mice at 6 months of age. Only a few animals developed antigen-specific IgM. The  
335 inability of CD34-NSG humanized mice to efficiently respond to vaccination is related to  
336 multiple deficiencies recognized in the humanized animals. This includes the absence of  
337 supportive cytokines, human follicular dendritic cells responsible for accumulation of antigen  
338 and stimulation of germinal center B-cells, deficiency of follicular helper cells involved in T-cell  
339 dependent immune responses, deficiency of complement, and others (reviewed in [46]). Several  
340 approaches to improve human adaptive immune responses were explored by elimination of  
341 mouse tissue histocompatibility antigens and introducing human MHC class I and II, cytokines  
342 and co-transplantation of bone, spleen, liver, and thymus. These approaches improved adaptive  
343 responses to varying degrees. It is possible that the introduction of these additional factors on the

344 NSG-*cmah*<sup>-/-</sup> background will further optimize the function of the human immune system and  
345 development of adaptive immune responses.

346 In addition to the comparison of human immune system cell development and phenotype,  
347 we assessed the behavior of human mature peripheral blood mononuclear cells in their ability to  
348 colonize mouse spleen and peripheral blood. In contrast to lymphocytes generated from human  
349 stem cells transplanted into NSG-*cmah*<sup>-/-</sup> mice, which showed reduced levels of activation,  
350 transplanted mature lymphocytes very quickly expand and lose the naïve CD45RA<sup>+</sup> phenotype.  
351 This could be attributed to the selection of human T cells in mouse thymus and reduced  
352 responsiveness to the mouse MHC (major histocompatibility) antigens.

353 Sialic acid on cellular membrane molecules has been identified as an attachment receptor  
354 for several pathogens and toxins. The composition could influence the viral capture by different  
355 cell types and *trans*-infection [47]. We observed increased HIV-1 mediated depletion of human  
356 CD4<sup>+</sup> T-cells in the NSG-*cmah*<sup>-/-</sup> strain of mice compared to the parental NSG. We also found a  
357 reduction of circulating HIV-1 RNA in non-engrafted NSG-*cmah*<sup>-/-</sup> mice suggesting the viral  
358 particles may be more efficiently absorbed by cells with human-like sialylation patterns. We  
359 previously showed that mice transplanted with human hepatocytes also had enhanced clearance  
360 of virus from the circulation [48]. The increased pathogenicity of HIV-1 in NSG-*cmah*<sup>-/-</sup> mice  
361 may be related to both the properties of human cells raised in the more human-like modified  
362 mouse environment as well as interaction of virus with the modified mouse stroma.

363 As humanized mice serve as a model to test biologic activity of human therapeutic  
364 antibodies [49, 50], we compared the clearance of human IgG. Human IgG contain  
365 oligosaccharides with N-acetylneuraminic acid (Neu5Ac, NANA); whereas, rhesus, cow, sheep,  
366 goat, horse, and mouse IgGs contain oligosaccharides with N-glycolylneuraminic acid (Neu5Gc,



367 NGNA). The asialoglycoprotein receptor on hepatocytes [51] and FcRn on endothelial cells and  
368 placenta [52] could potentially be affected by human-like sialylation of receptors. Human FcRn  
369 binds to human, rabbit, and guinea pig IgG, but not significantly to rat, bovine, sheep, or mouse  
370 IgG (with the exception of weak binding to mouse IgG2b). In contrast, mouse FcRn binds to all  
371 IgG as previously analyzed [52]. Our observation of slower clearance of human IgG injected  
372 intravenously in NSG-*cmah*<sup>-/-</sup> mice suggests the model may be useful for evaluation of the  
373 therapeutic efficacy of human antibodies. Another miscellaneous application of NSG-*cmah*<sup>-/-</sup>  
374 mice could be testing of the transduction efficacy of viral vectors as well as viruses [53]. We  
375 used a common vector of rAAV serotype 2 with luciferase and expected to see the differences of  
376 transduction efficacy between wt NSG and NSG-*cmah*<sup>-/-</sup> mice. We found the expression of  
377 luciferase was not different in liver (the major affected organ), but we did observe differences in  
378 less commonly affected organs – such as spleen and brain. These findings suggest that  
379 endothelial and splenic hematopoietic cells with human-like sialylation profiles could be more  
380 sensitive to viral infection.

381

## 382 **Conclusions**

383 Humanized mice are widely used to study the human immune system responses to pathogens and  
384 therapeutics. However, mouse specific glycosylation affects the development of the human  
385 immune system and responses to various agents – such as viruses or biological, human-specific  
386 products like antibodies. We demonstrated that human-specific sialylation established by  
387 mutation of the *CMAH* gene supports naïve B and T cell generation with polyclonal receptors  
388 repertoires. In contrast to NSG wild type mouse sialylation background, we found the NSG-  
389 *cmah*<sup>-/-</sup> background increased sensitivity to HIV-1 infection and influenced rAAV vector

390 transduction patterns. As such, NSG-*Cmah*<sup>-/-</sup> mice may accelerate translational research that  
391 target human infections and therapeutics development.

392

### 393 **Material and Methods**

394

395 **Animals.** Strains obtained from the Jackson Laboratories C57Bl/6 (Stock No: 000664), CMAH  
396 knock-out (B6.129X1-*Cmah*<sup>tm1Avrk</sup>/J, Stock No: 017588), and NSG (Stock No:05557) were bred  
397 and housed in the pathogen-free animal facility at the University of Nebraska Medical Center  
398 (Omaha, NE, USA).

399 Generation of B6.129X1-*Cmah*<sup>tm1Avrk</sup>/J referred hereafter as C57Bl/6-*Cmah*<sup>-/-</sup> was  
400 described previously [54]. In this mouse, exon 6 was deleted by targeting a cassette containing  
401 LoxP flanked exon 6 with a neomycin cassette into 129/SvJ derived ES cells, followed by  
402 removal of the exon and the neomycin cassette through Cre enzyme and injection of deleted  
403 clone into blastocysts to generate chimera. The mice line was backcrossed to C57BL6/J strain for  
404 10 generations.

405

### 406 **CRISPR reagents, mice generation, and genotyping**

407 We deleted exon 6 using CRISPR approach in NSG strain background. Two guide RNAs were  
408 identified to target exon 6 (*Cmah* gRNA 1 CTTTGTGCATTTAACGGACCTGG and *Cmah*  
409 gRNA 2 TGAAATATATCAACCCTCCAGGG). The sgRNAs were transcribed from DNA  
410 templates generated by annealing two primers using the HiScribe™ T7 Quick High Yield RNA  
411 Synthesis Kit (New England Biolabs, Ipswich, MA) following manufacturer's instructions. Cas9  
412 mRNA was prepared using the pBGK plasmid as described in [28]. Injection mixture was

413 prepared by dilution of the components into injection buffer (5 mM Tris, 0.1 mM EDTA, pH 7.5)  
414 to obtain the following concentrations: 10 ng/ $\mu$ l Cas9 mRNA, 10 ng/ $\mu$ l Cmah Left Guide and  
415 Right Guide RNA. Female NSG mice 3–4 weeks of age (JAX Laboratories, Bar Harbor, ME,  
416 USA) were superovulated by intraperitoneal injection with 2.5IU pregnant mare serum  
417 gonadotropin (National Hormone & Peptide Program, NIDDK), followed 48 hours later by  
418 injection of 2.5 IU human chorionic gonadotropin (hCG, National Hormone & Peptide Program,  
419 NIDDK). Mouse zygotes were obtained by mating NSG stud males with superovulated NSG  
420 females. The animals were sacrificed 14 hours following hcG administration, and oviducts were  
421 collected to isolate fertilized embryos. Injection mixture was introduced into the pronuclei and  
422 cytoplasm of fertilized NSG embryos by microinjection using a continuous flow injection mode.  
423 Surviving embryos were surgically implanted into the oviducts of pseudopregnant CD-1  
424 recipient females. Genomic DNAs were extracted from tail biopsies and genotyping PCRs were  
425 performed as previously reported [28]. Cmah Forward TCCAGACCAGGAGGAGTTA and  
426 Cmah Reverse TCCACTCCGAGTTTCAGATCA primers were used for screening by PCR. The  
427 expected amplicon sizes were 455 bases and 428 bases respectively for wild type and mutant  
428 mice. The PCR products were column purified and were sequenced using the primers used for  
429 PCR amplification.

430

#### 431 **Western blot and flow cytometry analyses of NeuGc expression**

432 Tissue samples from NSG and NSG-*cmah*<sup>-/-</sup> mice were collected and homogenized in ice cold  
433 RIPA buffer with HALT protease inhibitor (cat#78430, ThermoFisher Scientific, Waltham, MA,  
434 USA). 20  $\mu$ g of protein/sample was denatured in Laemmli sample buffer, then loaded and ran on  
435 a 12% SDS-polyacrylamide gel. The separated proteins were transferred to polyvinylidene

436 difluoride (PVDF) membrane, blocked in 0.5% cold fish gelatin in TRIS buffered saline with  
437 0.05% Tween-20 (TBS-T) for 2 hours at room temperature and then incubated in the primary  
438 antibody, Neu5GC (1:4000 in 0.5% cold fish gelatin TBS-T; chicken polyclonal IgY antibody,  
439 Biologend Cat. No 146901) for overnight at 4 °C. After washes, the membrane was incubated in  
440 HRP-conjugated goat-anti-chicken IgY (GeneTex Cat. GTX26877, Lucerna-Chem AG,  
441 Switzerland) secondary antibody (1:4000) for 1 hour at room temperature. The immunoblot was  
442 developed with chemiluminescence and imaged using FluorChem M imager (Proteinsimple, San  
443 Jose, CA, USA). GAPDH was used as housekeeping control (clone GA1R, cat #MA5-15738,  
444 ThermoFisher, Waltham, MA USA). For flow cytometry, whole blood was obtained by  
445 bleeding the mice from the facial vein. After spinning it down at 1800 rpm for 8 minutes and  
446 removing the excess serum, 50 µl of the 1:1 mixture of the whole blood and 0.5% gelatin from  
447 cold water fish was incubated in the Fc block for 15 minutes on ice. Next, the primary anti-  
448 Neu5Gc was added and incubated for 30 minutes on ice. Washing was done by re-suspending the  
449 samples in 1ml of the 0.5% gelatin from cold water fish and spinning it at 1500 rpm for 5  
450 minutes for 3 times. The secondary FITC-labeled anti-chicken reagent (**ab46969**) staining  
451 followed by red blood cells lysis using red blood cell lysis (Cat. #349202 from BD Bioscience,  
452 San Jose, CA) were performed. Further, acquisition was done on BD LSR II flow cytometer cells  
453 and analyzed on gated leukocytes using FLOWJO, analysis software (Tree Star, Ashland, OR,  
454 USA).

455

#### 456 **Immunohistology**

457 Organs collected from NSG-*cmah*<sup>-/-</sup>, NSG wt and C57Bl/6 mice were fixed and paraffin  
458 embedded; 5 µ thick sections were stained with antibodies for Neu5Gc (anti-Neu5Gc antibody,

459 Biolegend, San Diego, CA, USA, Cat. No: 146903, dilution 1:100) at 4 °C overnight. Secondary  
460 anti-chicken antibodies (Biolegend Cat. No. 146901) were used at dilution 1:100 and DAB as  
461 chromogen. Tissues counterstained with hematoxylin.

462

### 463 **Human PBMC transplantation and engraftment analyses**

464 NSG-*cmah*<sup>-/-</sup> and wild type NSG mice at 8 weeks of age were transplanted with single donor  
465 human PBMC intraperitoneally ( $20 \times 10^6$  cells/mouse). Blood samples were collected by facial  
466 vein bleeding for immunophenotyping at variable intervals (7, 14, 24, 31 days). Five-week post-  
467 injection, mice were sacrificed; blood, liver and spleen tissues were collected for  
468 immunophenotyping and immunohistochemistry. Briefly, 50  $\mu$ L of whole blood was stained in  
469 EDTA-coated tubes with two different monoclonal antibody panels (Table 1) in a final volume  
470 of approximately 100 $\mu$ l. Cells not stained with any of the antibody were initially used to define  
471 the gating strategy. Compensation beads (BD Biosciences, cat. #552843) were stained with each  
472 antibody separately and run at each acquisition to calculate the compensation matrix.  
473 Immunophenotyping was performed to determine the absolute count and frequency of blood  
474 cells: leukocytes (CD45, BD Biosciences, Cat. #555482); CD3 (#557943); CD4 (#560650); CD8  
475 (#562428); CD19 (#562653) and CD14 (eBioscience, San Diego, CA, Cat. #17-0149-42) for  
476 blood and spleen, respectively. The gating strategy for identification of cell subsets is presented  
477 in Figure 1A. Presence of activation markers CD45RA/CD45RO (BD Biosciences, #560674;  
478 #563215) and CD62L (#555544) were also checked for CD4<sup>+</sup> and CD8<sup>+</sup> T cells subpopulations,  
479 CD22 on B cells (#563940) in blood and spleen as absolute count and frequency of parent,  
480 respectively. After staining of cells, red blood cells were lysed with FACS Lysing solution (BD  
481 Biosciences), and cells were washed with PBS and resuspended in 1% paraformaldehyde. For

482 absolute counting of the blood samples, CountBright™ absolute counting beads (Invitrogen,  
483 Carlsbad, CA, USA; catalog C36950) were added to each sample before acquisition. Acquisition  
484 of stained cells was performed on BD LSR II (BD Biosciences) using acquisition software FACS  
485 Diva (BD Biosciences); and data were analyzed using FLOWJO, analysis software (Tree Star).  
486 Event counts of each cell population were exported, and absolute count/ $\mu$ l of blood were  
487 calculated using the following formula: [(Number of cell events / number of bead events)  $\times$   
488 (assigned bead count of the lot (beads/50  $\mu$ l) / Volume of sample)].

489

#### 490 **Human CD34<sup>+</sup> cell transplantation and engraftment analyses**

491 NSG-*cmah*<sup>-/-</sup> and wild type NSG mice reconstituted with the same cord blood sample derived  
492 CD34<sup>+</sup> cells ( $5 \times 10^4$ /mouse intrahepatically) were bled at three and again at six months of age.  
493 For both time points  $\sim$ 100  $\mu$ l of blood was collected into MiniCollect 0.5 mL EDTA tubes  
494 (Greiner Vacuette North America Inc., Monroe, NC, USA; Cat. #450475). Plasma was collected  
495 and stored at  $-80^\circ$  for future use. Remaining cell samples were diluted at a 1:1 ratio with 50  $\mu$ l  
496 FACS buffer (2% fetal bovine serum in PBS) and mixed thoroughly. The samples were divided  
497 into two panels, B cell and T cell. The B cell panel consisted of mouse anti-human CD45-FITC  
498 (BD Pharmingen; Cat. #555482), CD19-BV605 (BD Biosciences; Cat. #562653), CD22-  
499 BV421(#563940), IgG-PerCP/Cy5.5 (Biolegends, # 409312), IgD-PE (eBiosciences, #12-9868-  
500 42), IgM-APC (eBiosciences, #17-9998-42), and Brilliant Stain Buffer (BD Horizon, #563794)  
501 cocktails. The T cell panel consisted of: anti-human CD45-FITC (BD Pharmigen, #555482),  
502 CD3-Alexa Fluor 700 (BD Biosciences #557943), CD4-APC (BD Pharmingen #555349), CD8-  
503 BV421 (BD Horizons #562428), CD45RA-APC-H7 (BD Biosciences #560674), CD14-PE (BD  
504 Pharmingen #555398), and Brilliant Stain Buffer. After 30 minutes incubation at 4  $^\circ$ C, red blood

505 cell lysis (BD Bioscience #349202) and two washes, samples were fixed with 2%  
506 paraformaldehyde and acquisition was performed on BD LSR II flow cytometer.

507

### 508 **HIV-1 infection**

509 Animals with comparable peripheral blood repopulation with human leucocytes were infected  
510 with HIV-1<sub>ADA</sub> at 10<sup>4</sup> 50% tissue culture infectious (TCID<sub>50</sub>) dose intraperitoneally. At four and  
511 nine weeks post infection, animals were euthanized for the VL analysis (COBAS®  
512 AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0, Roche Molecular Systems Inc., Pleasanton,  
513 CA, USA) and human cells phenotypes by FACS. Peripheral blood, spleen, and bone marrow  
514 cells were analyzed as described above.

515 Additional bone marrow human population analysis was performed by flow cytometry at  
516 the end of observation at 5-6 weeks post infection. Approximately 10<sup>6</sup> isolated bone marrow cells  
517 were aliquoted into three tubes for each mouse evaluated. The cells were washed once with 2 mL  
518 phosphate buffered saline (PBS) and were then incubated with the 8 antibody cocktails for 30  
519 minutes at room temperature in 200 uL of PBS with 10% fetal calf serum (PBS-FCS). The  
520 following antibodies were used in combination: T-NK cocktail consisting of APC-H7 conjugated  
521 anti-CD3 (clone SK7), PE-Cy7 conjugated anti-CD4 (clone SK3), PE conjugated anti-CD7  
522 (clone 8G12), PerCP-CY5-5 conjugated anti-CD8 (clone SK1), FITC conjugated anti-CD14  
523 (clone M□P9), V450 conjugated anti-CD16 (clone 3G8) APC conjugated anti-CD56 (clone  
524 NCAM16.2) and V500c–conjugated CD45 (clone 2D1); MYELO cocktail consisting of APC-  
525 H7 conjugated anti-HLA-DR (clone L243), PE conjugated anti-CD10 (clone H10a), PE-Cy7  
526 conjugated anti-CD13 (clone L138), APC conjugated anti-CD24 (clone 2G5), V450 conjugated  
527 anti-CD33 (clone WM53), FITC conjugated anti-CD34 (clone 8G12 ), PerCP-CY5-5 conjugated

528 anti-CD117 (clone 95C3) and V500c–conjugated CD45 (clone 2D1) and the BCL tube  
529 consisting of FITC conjugated anti-kappa (clone TB28-2), PE conjugated anti-lambda (clone 1-  
530 155-2), PE-Cy7 conjugated anti-CD10, PerCP-Cy5.5 conjugated anti-CD19 (clone SJ25C1),  
531 APC-H7 conjugated anti-CD20 (clone L27), APC conjugated anti-CD24 (clone 2G5), V450  
532 conjugated anti-CD38 (clone HB7) and V500c–conjugated CD45 (clone 2D1). All antibodies  
533 were obtained from BD Biosciences (Franklin Lakes, NJ, USA) except CD24 and CD117, which  
534 were obtained from Beckman Coulter (Brea, CA, USA).

535 After incubation, the cells were washed twice with 1 mL PBS and were resuspended in  
536 500 uL PBS containing stabilizer (BD Biosciences) to fix the cells. Fifty thousand to 100,000  
537 cell events were collected for each sample on a Becton Dickinson FACS Canto II (Franklin  
538 Lakes, NJ, USA). Analysis of the FCS files was performed using Kaluza 1.3 analysis software  
539 (Beckman Coulter).

540 Gating schemes for the bone marrow analysis are shown in the additional file 6 **Fig. S6**.

541 For all bone marrow cell populations characterized, total cell events were derived based  
542 on gating and logical antigen (Boolean) profiles. Population frequencies were then derived by  
543 dividing the specific cell population events by the total human cell events after CD45 and singlet  
544 gating.

545

#### 546 **HIV-1 viral clearance**

547 Viral clearance naïve non-humanized animals were injected with HIV-1 stock  $3 \times 10^3$   
548 TCID<sub>50</sub>/mouse), and blood was collected on days 1, 2, 5, and 7 post inoculation. Number of viral  
549 RNA copies were determined as described above.

550



551 **Recombinant adeno-associated virus biodistribution**

552 NSG-*cmah*<sup>-/-</sup> and wild type NSG mice were injected intravenously with rAAV2/DJ EF1a-  
553 luciferase 1.825E+11 GE/mouse (Viral Vector Core, University of Iowa, Iowa City, IA; Cat No.  
554 Uiowa-6161: Lot AAV3240; <http://www.medicine.uiowa.edu/vectorcore/>). D-Luciferin  
555 Bioluminescent Substrate (Cat 770504, PerkinElmer, Waltham, MA) was used for in vivo  
556 detection. The biodistribution of luminescence were analyzed by IVIS® Spectrum an in vivo  
557 imaging system at 1, 2, and 4 weeks after rAAV administration to validate and compare  
558 transduction efficiency. Liver, spleen, and brain tissues were collected at day 32 for detection of  
559 luciferase gene on droplet digital PCR (ddPCR) (QX200™ Droplet Digital™ PCR System, Bio-  
560 Rad, Hercules, CA, USA) with forward primer 5'CTTCGAGGAGGAGCTATTCTT-3', reverse  
561 primer 5'-GTCGTA CTTGATGAGAGTG-3', and luciferase probe 5'-/56  
562 FAM/TGCTGGTGC/ZEN/CCACACTATTTAGCT/3IABKFQ/-3' (Integrated DNA  
563 Technologies, Inc., Coralville, IAUSA). Briefly, isolation of RNA was performed using a  
564 RNeasy Plus Universal Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.  
565 The final PCR reaction was comprised of ddPCR supermix (Bio-Rad), 20U/μl reverse  
566 transcriptase, 15mM Dithiothreitol (DTT), 900nM primers, 250nM probe and 100ng of RNA  
567 template in a final volume of 20 μl and loaded into an eight-channel disposable droplet generator  
568 cartridge (Bio-Rad). Generated droplets were then transferred into a 96-well PCR plate, heat-  
569 sealed with foil and then amplified to endpoint using a BioRad C1000 Touch PCR cycler at 95  
570 °C for 10 minutes then 40 cycles of 94 °C for 15 seconds and 60 °C for 1 minute (2 °C/s ramp  
571 rate) with a final step at 98 °C for 10 minutes and 4 °C hold. Plates containing amplified droplets  
572 were loaded into a QX200 droplet reader (Bio-Rad). Discrimination between negative droplets  
573 (no luciferase copies) and positives (with luciferase copies) was used to estimate concentration

574 of targets (luciferase copies/ul) using QuantaSoft analysis software (Bio-Rad). The resulted  
575 copies were normalized to input RNA and represented as luciferase copies in log scale.

### 576 **Human IgG clearance**

577 We compared the circulation time of human IgG (IVIG, PRIVIGEN, CSL Behring LLC). NSG-  
578 *cmah*<sup>-/-</sup> and control mice were injected with 100 µl of 10% IVIG in saline. Blood samples were  
579 collected at 30 minutes after IVIG injection as point day 0. The actual concentration of human  
580 IgG in peripheral blood was measured at days 1, 2, 3, 6, 7, and 14. Plasma human IgG  
581 concentration was determined by ELISA kit (Bethyl Laboratories, Inc. Montgomery, TX, USA,  
582 cat# E80-104).

583

### 584 **List of abbreviations**

585 **CMAH:** CMP-N-acetylneuraminic acid hydroxylase

586 **HRP:** Horseradish peroxidase

587 **HSPC:** Hematopoietic stem/progenitor cells

588 **HIV-1:** Human immunodeficiency virus type 1

589 **IHC:** Immunohistochemistry

590 **Neu5Ac:** N-acetylneuraminic acid

591 **Neu5Gc:** N-glycolylneuraminic acid

592 **NSG:** NOD/scid-IL2R $\gamma_c$ <sup>-/-</sup> mice

593 **PBMC:** Peripheral blood immune cells

594 **rAAV:** Recombinant adeno-associated viral vector

595 **Siglecs:** Sialic acid-binding Ig-like lectins

596 **WB:** Western blot

597 **WT mice:** Wild-Type mice

598

599 **Declarations**

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610

611 **Availability of data and materials**

612 All data generated or analyzed during this study are included in this published article and its  
613 additional files.

614 **Authors' contributions**

615 SG and LYP conceived and designed the study. RSD, ABW, and YC performed in vivo studies  
616 on mice, flow cytometry. RSD, SM, and PSJ performed immunohistochemistry, qPCR, and  
617 Western blot procedures. CBG, RMQ, and DWH performed embryo isolation, microinjection,  
618 and generation of founder mice. ABW, SM, YC, and SMM performed cord blood collection,  
619 CD34<sup>+</sup> cells isolation, FACS analysis of peripheral blood. SJP performed flow cytometry of

620 bone marrow. RSD, SJP, CBG, SG, and LYP interpreted the results and wrote the manuscript.

621 All authors have read and given approval of the final version of the manuscript.

### 622 **Ethics approval and consent to participate**

623 All animal experiments were conducted following *NIH guidelines for housing and care of*  
624 *laboratory animals* and in accordance with the University of Nebraska Medical Center protocols  
625 approved by the institution's Institutional Animal Care and Use Committee (protocol numbers  
626 13-009, 14-100 and 06-071) in animal facilities accredited by the Association for Assessment  
627 and Accreditation of Laboratory Animal Care International.

### 628 **Consent for publication**

629 Not applicable.

### 630 **Competing interests**

631 The authors declare that they have no competing interests.

632

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801  
802 **Additional files**

803 Additional file 1. **Fig. S1.** Human immune cells IGH genes CDR3 length frequency distribution  
804 in bone marrow. **a** Histograms of IgH CDR3 length in bone marrow of NSG-*cmah*<sup>-/-</sup> and **b** NSG  
805 mice. **c** The NSG-*cmah*<sup>-/-</sup> background was associated with a lower clonal B-cell outgrowth, and  
806 subsequently a lower maximal frequency of templates. **d** The frequency of CDR3 lengths can be  
807 seen to show a more gaussian distribution in the NSG-*cmah*<sup>-/-</sup> mice although ~~and~~ the number of  
808 total IGH templates was not statistically different **e** Statistical analysis was performed  
809 immunoSEQ Analyzer (<https://www.adaptivebiotech.com/>). Five animals per strain were used.

810  
811 Additional file 2. **Fig. S2.** IgH CDR3 length in bone marrow and spleen in NSG-*cmah*<sup>-/-</sup> (m158,  
812 103, 96, 159, 154) and NSG wild type mice (M376, 3578, 3571, 3577, 3573). Arrows indicate  
813 shortening of CDR3 length in spleen (orange bars) compared to the bone marrow (blue bars)  
814 samples in the same animal.

815

816 Additional file 3. **Fig. S3.** IGH genes family usage.



817 **a** Individual IgH genes families usage in NSG-*cmah*<sup>-/-</sup> and **b** NSG wt mice represent the  
818 summary of productive rearrangement frequency in spleen tissue samples. Similar profiles were  
819 found in bone marrow (not shown). **c** The frequency of V, D and J genes family usage in bone  
820 marrow and spleen tissue are highly similar in both strains as expected. As shown, there are no  
821 significant differences between NSG and NSG-*cmah*<sup>-/-</sup> mice in overall IgH gene family usage. In  
822 both strains, a significant reduction in the frequency of IGHD02 usage was apparent in splenic  
823 B-cells compared to bone marrow. Mild increased frequencies in IGHD01, IGD06, and IGD07  
824 usage was also noted in NSG-*cmah*<sup>-/-</sup> splenic tissue. In NSG wt mice, only IGD03 usage was  
825 increased in splenic B-cells compared to bone marrow. For J family usage, an increase in  
826 IGHJ03 frequency was found in NSG-*cmah*<sup>-/-</sup> splenic B-cells with a reduction of IGHJ05 and  
827 IGHJ06 usage. Statistical analysis was done by two-way ANOVA with Fisher's LSD test.  $P <$   
828 0.05 (\*) considered significant.

829

830 Additional file 4. **Fig. S4.** Spleen TCR $\beta$  chain gene usage.

831 **a** Individual TCR $\beta$  chain gene families usage in NSG-*cmah*<sup>-/-</sup> and **b** NSG wt mice represent the  
832 frequency of productive rearrangements TCR $\beta$  in spleen tissue samples. Similar profiles were  
833 found in bone marrow (not shown).

834

835 Additional file 5. **Fig. S5.** Human TCR $\beta$  chain genes CDR3 size frequency distributions in  
836 spleen and repertoire characteristic in bone marrow and spleen. **a** Histograms of TCR $\beta$  chain  
837 gene CDR3 length in bone marrow of NSG-*cmah*<sup>-/-</sup> and **b** NSG mice. **c** NSG-*cmah*<sup>-/-</sup> background  
838 was associated with lower clonal frequencies in spleen (Spl) compared to bone marrow (BM),  
839 which was statistically significant by one-way paired t-test ( $P = 0.0381$ ) for NSG-*cmah*<sup>-/-</sup> mice.

840 Five animals per strain were used. **d** The richness of TCR $\beta$  chain gene repertoire in both strains  
841 was higher in spleen compared to bone marrow (\* $P < 0.05$ ), while the number of total  
842 TCR $\beta$  chain gene templates was not statistically different between strains (not shown). **e** The  
843 Pielou' evenness of repertoires in the bone marrow and splenic compartments was not  
844 significantly different in both strains; however, the evenness was higher in the spleens of NSG-  
845 *cmah*<sup>-/-</sup> mice (one tail paired t-test,  $P = 0.0312$ ). Red dashed line highlights higher evenness of  
846 TCR $\beta$  in NSG-*cmah*<sup>-/-</sup> strain compared to wild type NSG by one-tail Mann Whitney test ( $P =$   
847 0.0446).

848

849 Additional file 6. **Fig. S6.** Humanized bone marrow gating strategies.

850 For all tubes, the human cells were isolated based on human CD45 expression. The CD45-  
851 positive cells were secondarily isolated using a singlet gate (forward angle peak height vs.  
852 forward angle area) to eliminate cell doublets and triplets. The percentage of lymphocytes was  
853 enumerated based on two CD45 by light scatter displays so that cells had to be present in both  
854 gates to be considered lymphocytes. T-cells, NK-cells, and monocytes were enumerated using a  
855 T-cell/NK-cell cocktail containing CD3, CD4, CD7, CD8, CD14, CD16, and CD56. T-cells were  
856 identified as CD3-positive, low side light scatter events. The CD3-positive T-cells were further  
857 characterized for CD4 and CD8 expression to enumerate the helper and cytotoxic T-cell subsets.  
858 NK-cells were isolated using a low side light scatter (SS) gate on the CD45 by side light scatter  
859 histogram. The low SS cells were characterized for CD56 and CD16 expression to enumerate the  
860 two NK-cell subsets (not shown here). Monocytes were isolated using a Boolean logic gate as  
861 CD4<sup>dim</sup> and not CD3-positive cells. Promonocytes and mature monocytes were further identified  
862 based on CD14 expression density. CD19-positive, CD10-positive and CD20 positive B-cells

863 were enumerated using a B-cell specific cocktail containing kappa, lambda, CD10, CD19, CD20,  
864 CD24 and CD38. CD19-positive, low SS cells were gated to enumerate total B-cells and  
865 precursors. The CD19-positive B-cells were further characterized for expression of CD10 to  
866 identify the B-cell precursors and CD20 to identify transitional to mature B-cells. Myeloblasts,  
867 proB-cells, preB-cells, mast cells, and granulocytes were enumerated using a myeloid cell  
868 cocktail containing HLA-DR, CD10, CD13, CD24, CD33, CD34 and CD117. CD34-positive  
869 events were gated on a CD34 by SS histogram and were further characterized as myeloblasts or  
870 pro B-cells based on the CD45 by SS profile. Total B-cell precursors were isolated based on  
871 HLA-DR, CD10, and CD24 co-expression. PreB-cells were calculated using Boolean logic as  
872 total B-cell precursors and not proB-cells. Mast cells were enumerated as CD117<sup>bright</sup> events on a  
873 CD34 by CD117 display. Finally, the granulocytes were estimated based on a CD45 by high SS  
874 gate that excluded the CD117<sup>bright</sup> mast cells. Samples were run on a Becton Dickenson Canto II  
875 flow cytometer and analyzed offline using Kaluza software from Beckman Coulter.

876

877 Additional file 7. **Fig. S7.** Profile of human cells in NSG-*cmah*<sup>-/-</sup> at 9 weeks post HIV-1  
878 infection. NSG-*cmah*<sup>-/-</sup> mice were infected with HIV-1<sub>ADA</sub> intraperitoneally at 5 months of age.  
879 At 9 weeks post-infection, samples were collected for FACS analyses of the peripheral blood,  
880 spleen, and bone marrow. **A**, Human cell profile in peripheral blood. FACS gating strategies  
881 used: human CD45/CD3/CD14; CD3/CD4/CD8; CD4/CD45RO. **B**, For spleen additional  
882 analysis included: human CD45/CD14/CD123/CD1c; and CD45/CD19. **C**, Bone marrow  
883 analyses was done for CD45/CD3/CD19 and lineage negative CD3<sup>-</sup>/CD19<sup>-</sup> human CD34<sup>+</sup> and  
884 CD33-positive cells. Individual mouse and means with SEM are shown. P values were  
885 determined with Mann-Whitney test.  $P \leq 0.05$  were considered significant. Reconstituted at

886 variable levels, NSG-*cmah*<sup>-/-</sup> mice showed high sensitivity to HIV-1 infection with significantly  
887 decreased numbers of human T-cells (predominantly helper T-cells) and CD4<sup>+</sup>CD45RO<sup>+</sup>  
888 memory T-cells in peripheral blood and spleen. In contrast, CD19-positive mature B-cells  
889 (spleen) and B-cell precursors (bone marrow) were significantly increased in NSG-*cmah*<sup>-/-</sup> mice  
890 following HIV exposure.

891

892

### 893 **Figure legends**

894 **Fig. 1.** Disruption of *Cmah* gene in NSG strain mouse using the CRISPR/Cas9 system. **a** Design  
895 of 27bp deletion in *CMAH* gene in exon 6 by sgRNAs. **b** Genotyping PCR of F1 offspring  
896 showing wild type and deletion (lines 1, 3-5). **c** Sequence alignment of the wild type and the  
897 deletion allele. The guide sequences are shown in red and the protospacer adjacent motif (PAM)  
898 sequences are in green.

899

900 **Fig. 2.** NSG-*cmah*<sup>-/-</sup> mice phenotype.

901 **a** Western blot analysis of Neu5Gc presence in NSG wild type mice (*cmah*<sup>+</sup>) and NSG-*cmah*<sup>-/-</sup>  
902 (*cmah*<sup>-</sup>) tissue samples. All tested mouse tissues with chicken anti-Neu5Gc antibody (Biolegend,  
903 CA, USA, 1:4000) were negative. **b** Confirmation of *CMAH* knockout on NSG background by  
904 immunohistology. Spleen, kidney, and lung tissues were fixed, paraffin embedded and 5 μ thick  
905 sections of NSG-*Cmah*<sup>-/-</sup> generated strain (left), wt NSG (right) mouse and *Cmah*<sup>-/-</sup>C57/Bl6  
906 original immune competent strain (middle column) were stained with antibodies for Neu5Gc  
907 (anti-Neu5Gc antibody, Biolegend, CA, USA, 1:100) at 4 °C overnight. Images captured by  
908 Nikon E800 microscope at objective magnification 20×. New generated strain-derived tissues

909 deficient for Neu5Gc as existing *cmah*<sup>-/-</sup>C57/Bl6 strain. Wt NSG mice express Neu5Gc epitopes  
910 and tissue sections have brown color. **c** Confirmation of *CMAH* gene knockout on NSG  
911 background by FACS. We compared expression of Neu5Gc on white blood cells by staining  
912 with anti-Neu5Gc antibody and secondary FITC-labeled anti-chicken reagent. Panel shows  
913 Neu5Gc staining for C57Bl/6 *cmah*<sup>-/-</sup> original strain obtained from the Jackson Laboratories  
914 (Stock No: 017588) (red) compare to C57Bl/6 wild type (orange). **d** Panel shows the similar  
915 pattern of the absence of Neu5Gc expression on NSG-*cmah*<sup>-/-</sup> mice (cyan) and the presence of  
916 Neu5Gc on cells derived from heterozygous mice that retain enzyme activity and have Neu5Gc  
917 on the surface of leucocytes (green and red).

918

919 **Fig. 3.** Effects of the *cmah*<sup>-/-</sup> background on human immune cells expansion and activation after  
920 HSPC transplantation.

921 At 3 and 6 months post CD34<sup>+</sup> cell transplantation, the frequency of circulating human  
922 lymphocyte subsets were analyzed. **a** and **b** Representative plots and gating strategies for human  
923 B-cell and T-cell enumeration. **c** Individual mouse NSG-*cmah*<sup>-/-</sup> (open symbol) and NSG wild  
924 type mice (closed symbol) and means with SEM are shown for human B and T cells in mouse  
925 peripheral blood. Naïve phenotype of CD3<sup>+</sup>CD45RA<sup>+</sup> T-cells exhibited better persistence at 6  
926 months of age in the NSG-*cmah*<sup>-/-</sup> strain. Lower proportions of mature CD19<sup>+</sup>IgD<sup>+</sup> B cells in  
927 peripheral blood of *cmah*<sup>-/-</sup> mice at 6 months of age, as well as a lower frequency of CD22 B-cell  
928 expression at 3 and 6 months of age, were also observed. P values were determined with  
929 Kruskal-Wallis test and Dunn's multiple comparisons tests (\*) P values determined by Mann-  
930 Whitney test (#) and paired t-test (@) are shown.

931

932 **Fig. 4.** Effects of the NSG-*cmah*<sup>-/-</sup> background on human immune cells IGH genes repertoires.

933 **a** Histograms of IgH CDR3 length (nucleotides) in spleen of NSG-*cmah*<sup>-/-</sup> and **b** NSG mice. **c**  
934 and NSG-*cmah*<sup>-/-</sup> background was associated with a lower frequency of clonal B-cell expansion  
935 in both bone marrow and spleen. **d** After transition from bone marrow to spleen clonal B-cell  
936 expansion increased in NSG- *cmah*<sup>-/-</sup> mice. Statistical analysis of CDR3 length was performed  
937 immunoSEQ Analyzer (<https://www.adaptivebiotech.com/>). P < 0.05 considered a statistically  
938 significant. Five animals per strain were used. Individual CDR3 length in spleen (mature  
939 compartment) and bone marrow (developmental compartment) are shown on **Fig. S2**.

940

941 **Fig. 5.** Effects of the *cmah*<sup>-/-</sup> background on human cell responses to HIV-1 infection.

942 NSG-*cmah*<sup>-/-</sup> and NSG wt mice were infected with HIV-1<sub>ADA</sub> intraperitoneally at 6 months of  
943 age. **a** At 4 weeks post infection, blood samples were collected for FACS analyses of the  
944 peripheral blood. **b** Four to seven animals per group were euthanized for the spleen profile  
945 analysis. Bone marrow data shown on **Fig. 6**. FACS gating strategies were used: human  
946 CD45/CD3/CD19; CD3/CD4/CD8. For available blood samples, additional analyses for human  
947 cells subpopulations CD4/CD45RA/CD45RO/CD62L/CCR7 were done. For spleen, additional  
948 analysrs included: human CD45/CD14/CD123/CD1c. Available plasma samples were analyzed  
949 for the HIV-1 RNA copies number, human IgM, IgG and HIV-1 specific antibodies at 1:10 times  
950 dilution (last panel **a**). Individual mouse and means with SEM are shown. P values were  
951 determined with Kruskal-Wallis test and Dunn's multiple comparisons tests (\*) and Mann-  
952 Whitney test (#). P ≤ 0.05 were considered significant. Viral load at 4 weeks post infection was  
953 compared with unpaired t test with Welch's correction. In comparison to NSG mice, NSG-*cmah*<sup>-/-</sup>  
954 mice showed a higher sensitivity to HIV-1 infection with increased viral load at 4 weeks post

955 infection and a significant decrease in numbers of CD4<sup>+</sup> T cells including effector  
956 CD4<sup>+</sup>CD45RO<sup>+</sup>CD62L<sup>-</sup>CCR7<sup>-</sup> cells post-infection. Profiling results of animals euthanized at 9  
957 weeks post-infection are shown in Supplemental Material (**Fig. S7**).

958

959 **Fig. 6.** Effects of the *cmah*<sup>-/-</sup> background on human cell responses to HIV-1 infection in bone  
960 marrow.

961 **a** Human differentiated T cells, **b** precursors and mature B cells, **c** myeloid lineage cells FACS  
962 analyses. Mice bone marrow were reconstituted with comparable proportions of human CD45<sup>+</sup>  
963 cells; HIV-1 mediated depletion of T cells were more pronounced in NSG-*cmah*<sup>-/-</sup> animals  
964 compared to NSG wt. HIV-1 infection increased the relative proportion of B cell precursors and  
965 mature B-cells (CD20<sup>+</sup>) and the proportion of CD14<sup>+</sup> macrophages compared to NSG mice.  
966 Individual mouse and means with SEM are shown. P values were determined with Kruskal-  
967 Wallis test and Dunn's multiple comparisons tests (\*) and Mann-Whitney test (#). P ≤ 0.05 were  
968 considered significant. The gating strategies are shown in Supplemental Material (**Fig. S6**).

969

970 **Fig. 7.** Human immune cells expansion and phenotype changes in NSG-*cmah*<sup>-/-</sup> mice.

971 Males NSG-*cmah*<sup>-/-</sup> and wt NSG 5 weeks of age were transplanted intraperitoneally with single-  
972 donor human peripheral blood mononuclear cells. The number and phenotype of human cells in  
973 peripheral blood were analyzed up to 31 days post transplantation and percentage of human cells  
974 in the spleen at the end point of observation. **a** Gating strategy to identify human CD45<sup>+</sup> cells,  
975 CD19<sup>+</sup>, CD14<sup>+</sup>, and CD3<sup>+</sup> T cells and their subpopulations. **b** Absolute counts of human cells in  
976 peripheral blood with accelerated expansion of human T cells and CD4<sup>+</sup> cells in the NSG-*cmah*-  
977 *-/-* compared to NSG. **c** Proportion of human cells in the spleens at the end-point were similar in

978 both strains. **d** Proportion of memory CD45RO<sup>+</sup> and central memory CD45RO<sup>+</sup>CD62L<sup>+</sup> were  
979 increased in NSG-*cmah*<sup>-/-</sup> mice among CD4<sup>+</sup> T-cells in blood and spleen. **e** Proportion of CD8<sup>+</sup>  
980 T-cells were also increased but to a lesser degree. Six animals per group were used and **b** and **d**  
981 represent means with SEM, **c** and **e** individual mouse data. Statistical analysis done by 2-way  
982 ANOVA with Sidak's multiple comparison test, \* - P < 0.05.

983

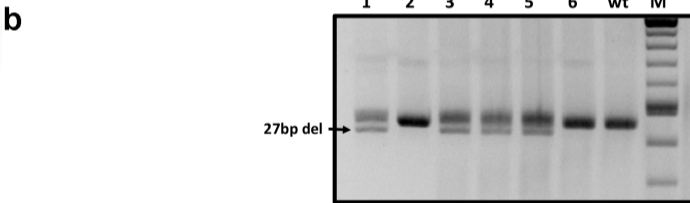
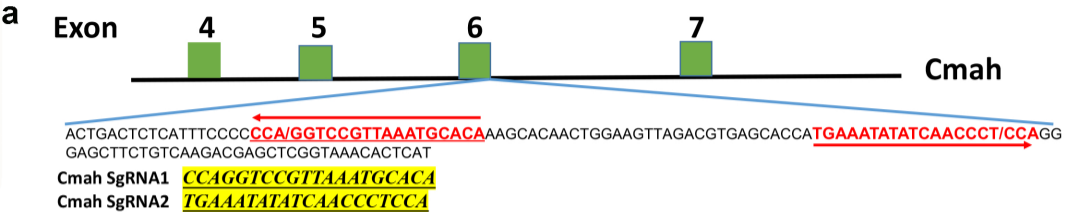
984 **Fig. 8.** Effects of *cmah*<sup>-/-</sup> background on HIV-1 and IgG clearance from blood circulation and  
985 tissue luciferase expression delivered by rAAV2/DJ8.

986 **a** Clearance of HIV-1 from mouse peripheral blood. **b** Clearance of human IgG from the  
987 peripheral blood (n = 5). Percentage of human IgG determined at 30 minutes after injection of  
988 100 µl of a 10% human IgG (10 mg/mouse) were 10.6 ± 1.2 and 11.0 ± 0.9 µg/ml for NSG-  
989 *cmah*<sup>-/-</sup> and wild type mice, respectively. Five to three blood samples were collected for days 1 –  
990 14 time points, mean and SEM are shown. End-point IgG concentrations in peripheral blood  
991 were 1.1 ± 0.04 and 0.9 ± 0.03 µg/ml for NSG-*cmah*<sup>-/-</sup> and wild type mice, respectively. **c**  
992 Luciferase RNA expression at 32 days post intravenous injection in liver, spleen, and brain. (#) P  
993 ≤ 0.05 were considered significant by one-tail Mann-Whitney test.

994



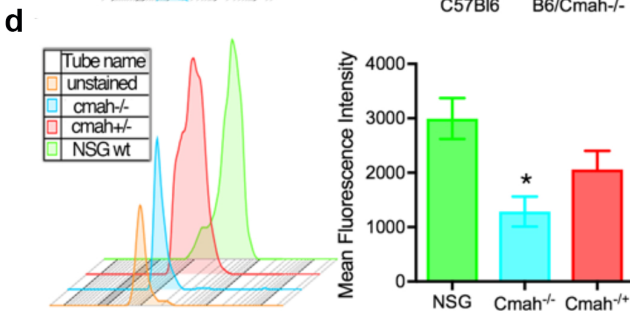
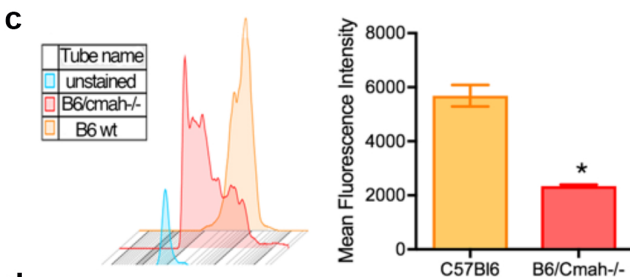
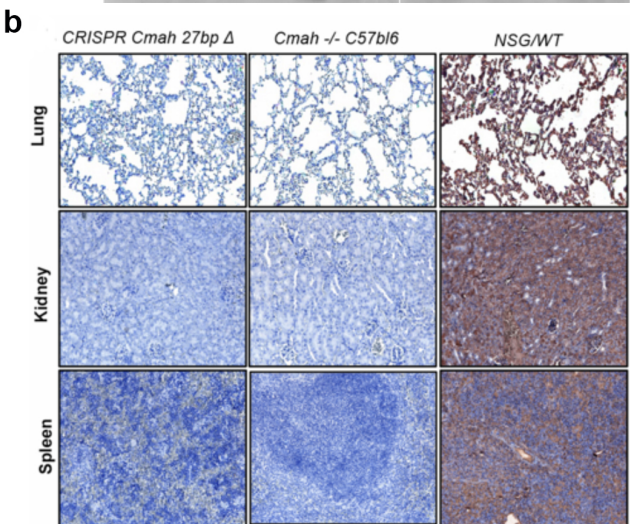
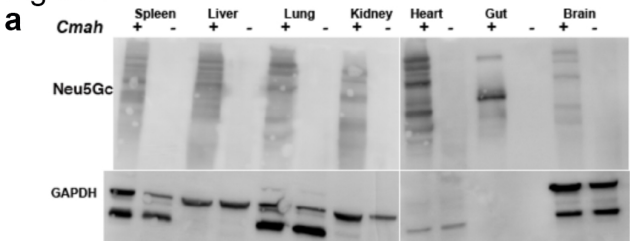
Figure 1



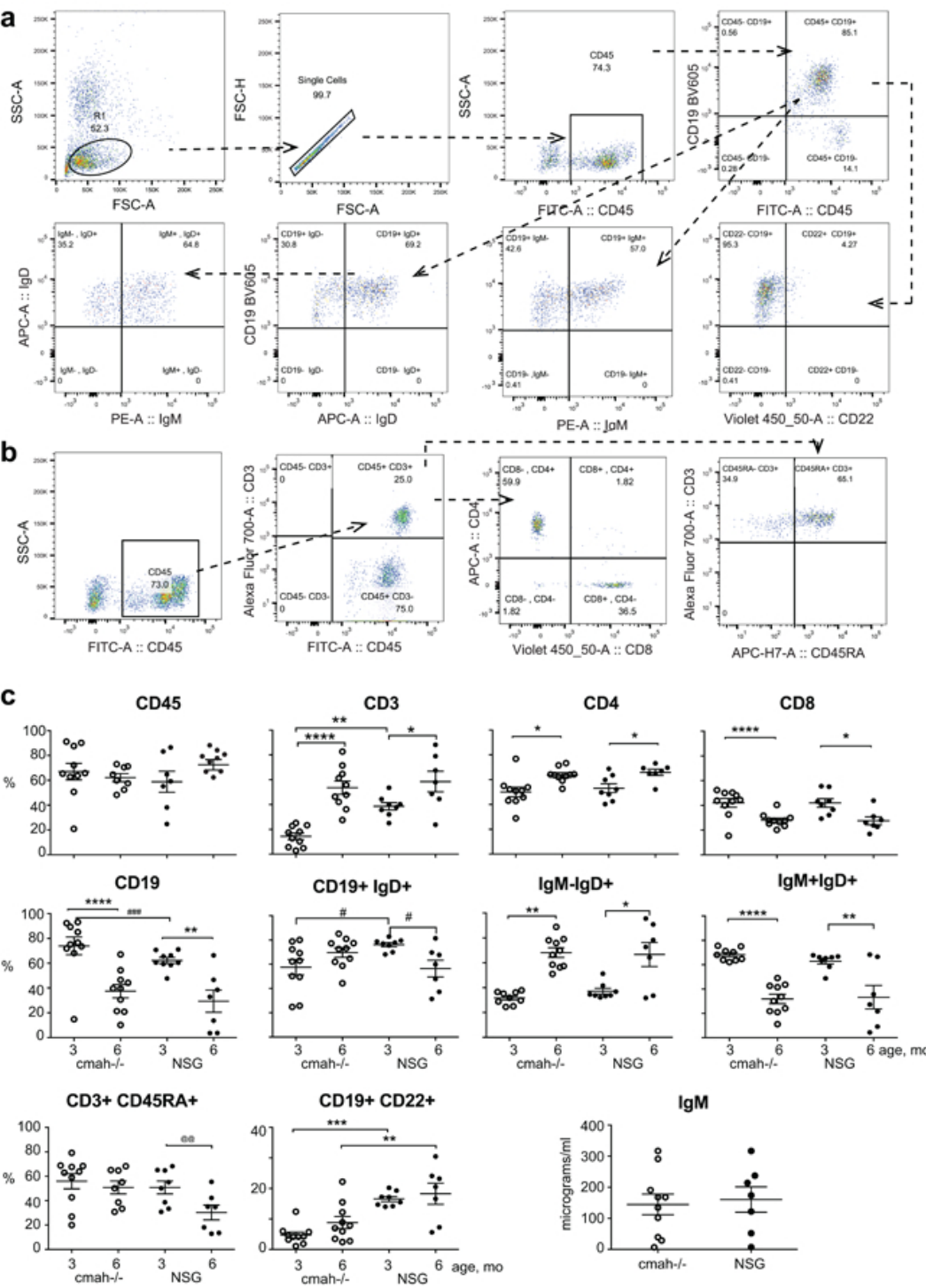
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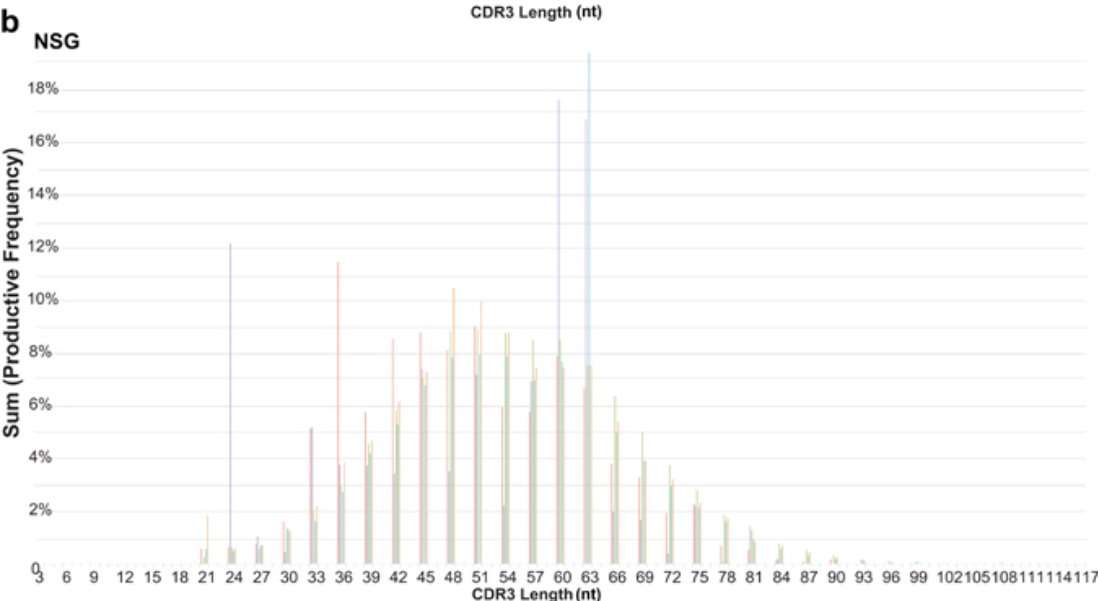
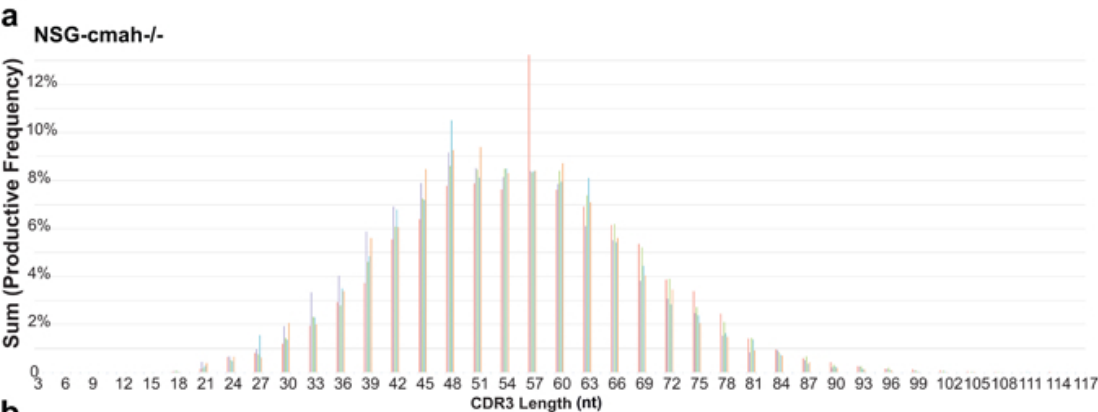
**Wild Type** ACTGACTCTCATTTCCCC **CGA** **GGTCCGTTAAATGCACA** AAGCACA ACTGGAAGTTAGACGTGAGCACCA **ATGAAATATATCAACCCTCCA** **GGG** AGCTTCTG  
**27bp Del** ACTGACTCTCATTTCCCCCCA-----GGAAGTTAGACGTGAGCACCATGAAATATATCAACCCTCCAGGGAGCTTCTG

**Figure 2**

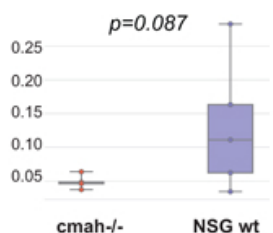


**Figure 3**



**Figure 4**

**c** IGH clonality in spleen



**d** IGH clonality changes from bone marrow to spleen

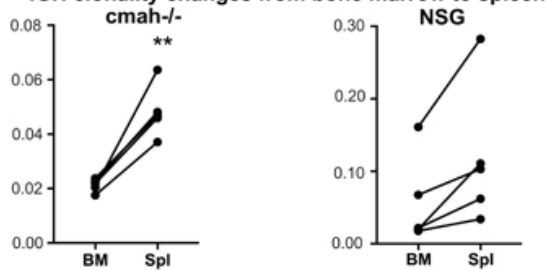
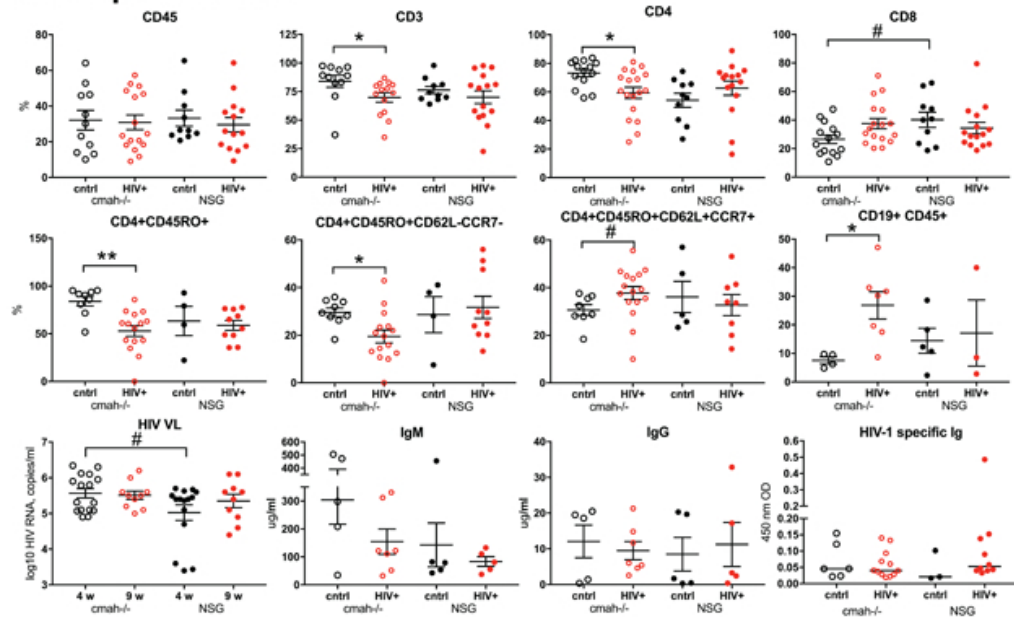


Figure 5

## a. Peripheral blood



## b. Spleen

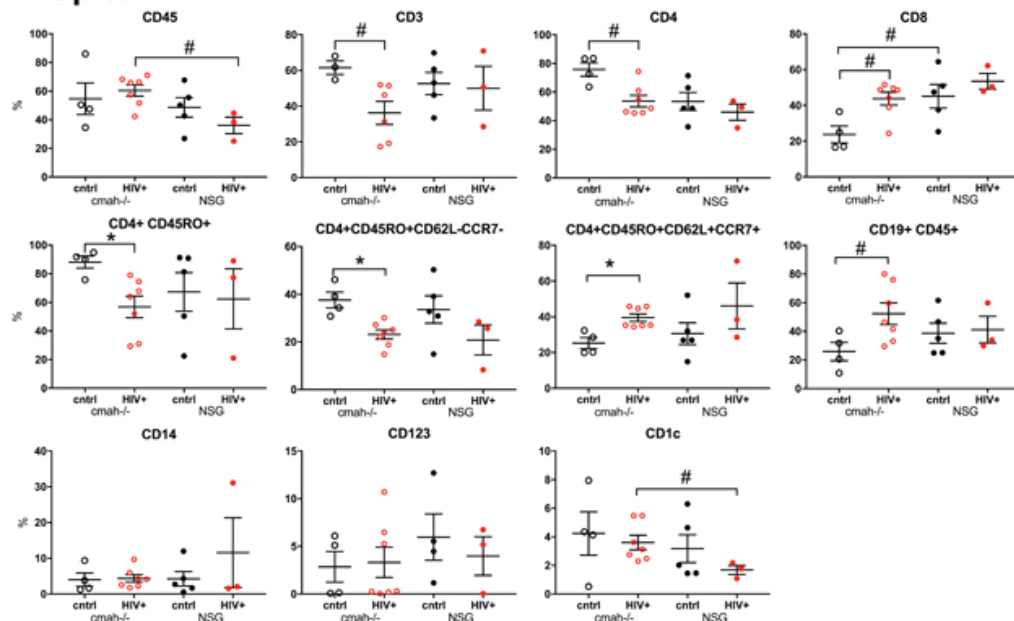


Figure 6

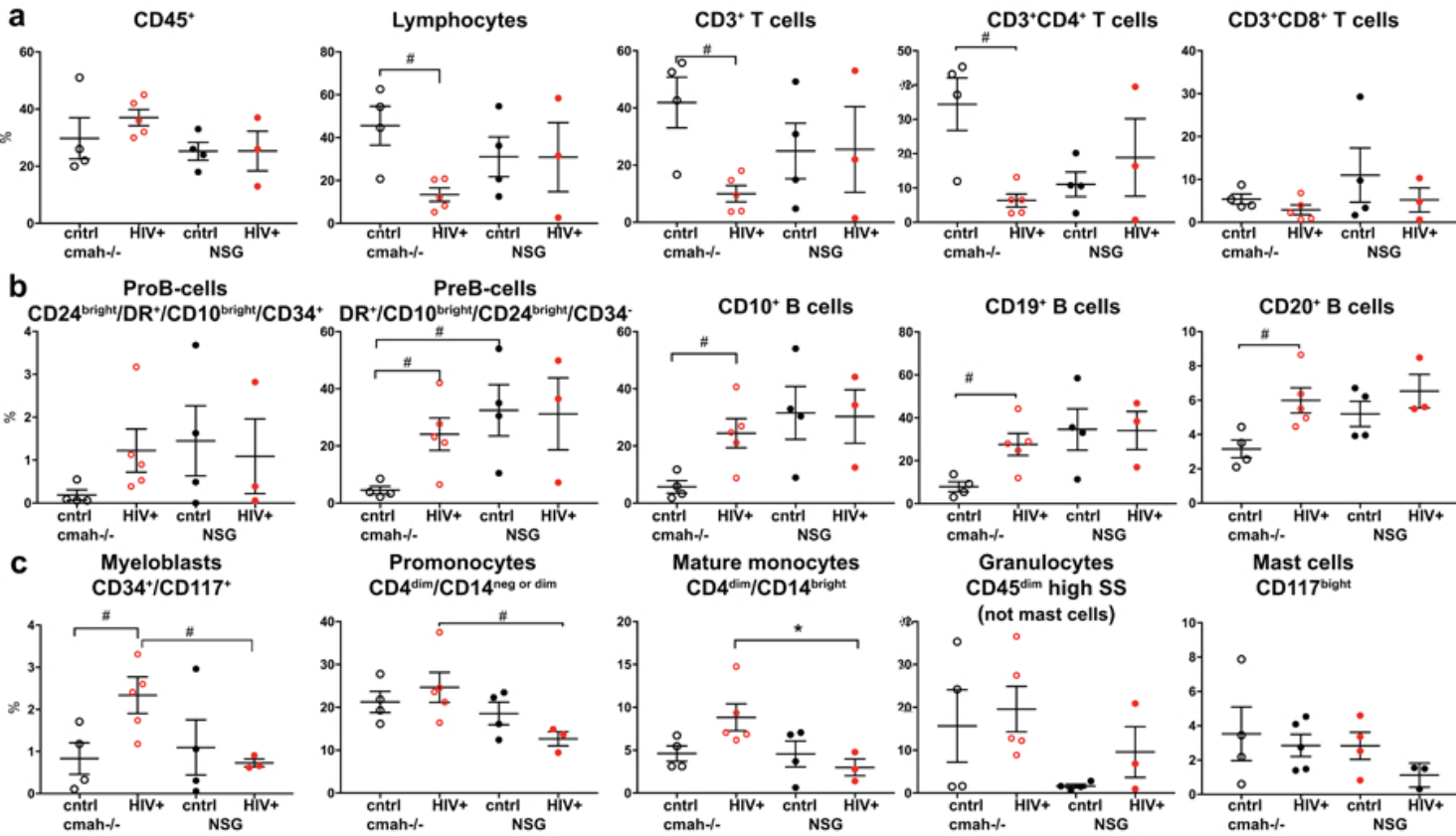
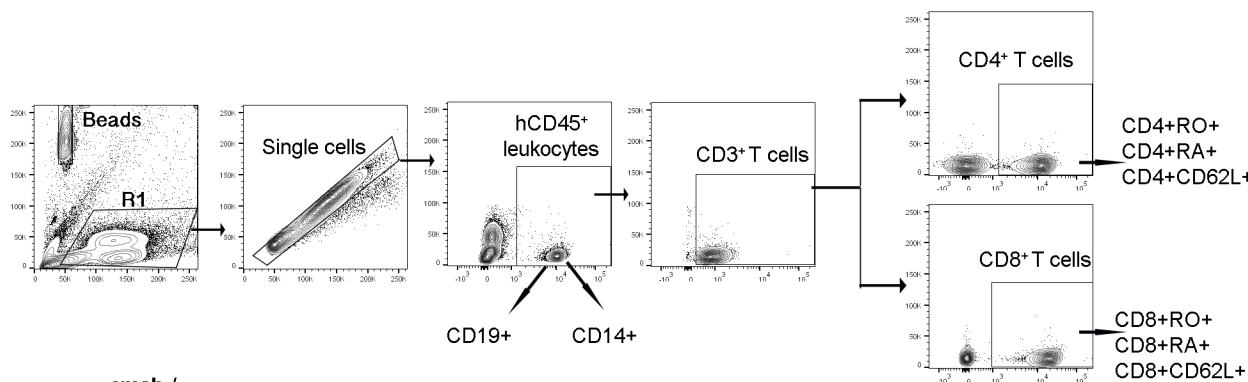
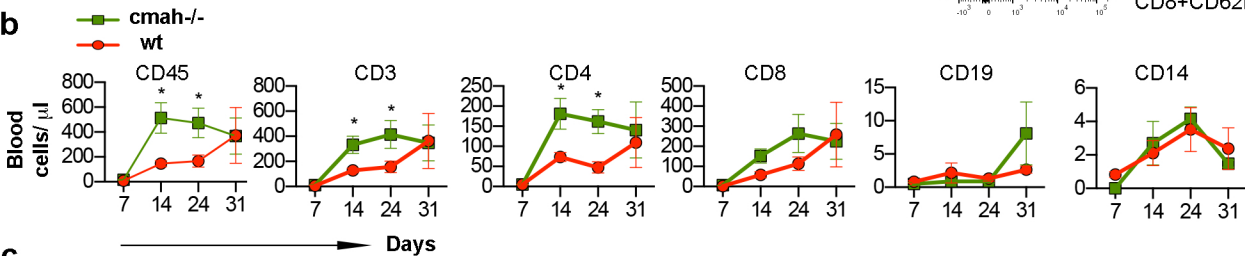


Figure 7

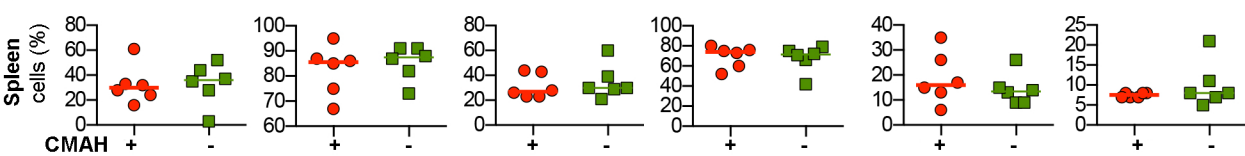
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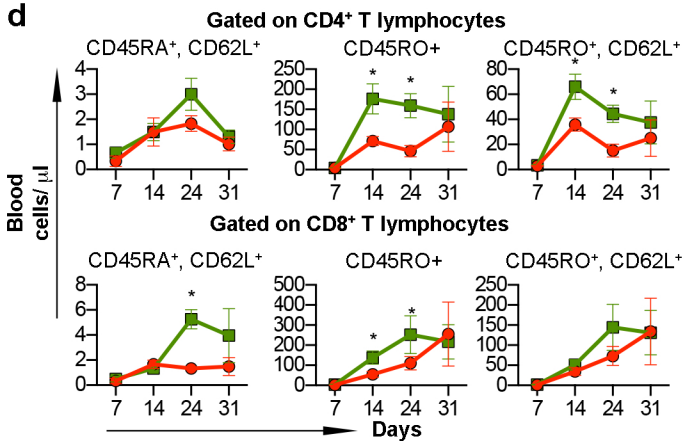
**b**



**c**



**d**



**e**

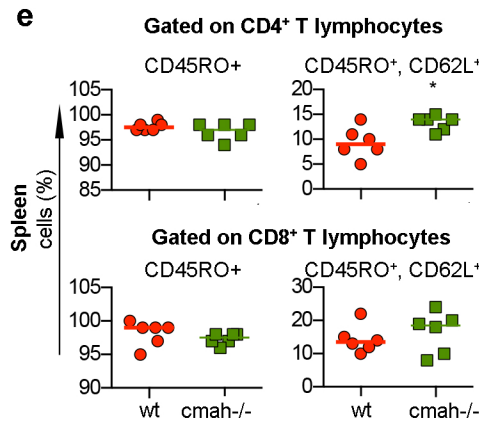
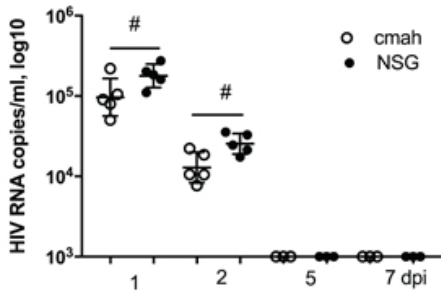
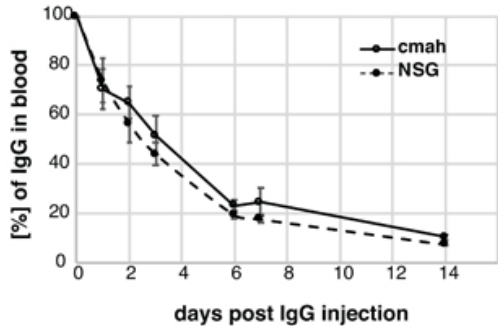


Figure 8

**a**



**b**



**c**

