



FcγRII (CD32) modulates antibody clearance in NOD SCID mice leading to impaired antibody-mediated tumour cell deletion

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3 1 **FcγRII (CD32) modulates antibody clearance in NOD SCID mice leading to impaired antibody-**
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5 2 **mediated tumour cell deletion**
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11 4 Running title: CD32 and FcRn mediate rapid antibody clearance in NOD SCID mice
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3 **23 LIST OF ABBREVIATIONS**
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6 24 BMDM: bone marrow derived macrophage
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9 25 ELISA: enzyme-linked immunosorbent assay
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12 26 FcγR: Fc gamma receptor
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15 27 FcRn: neonatal Fc receptor
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18 28 HPLC: high performance liquid chromatography
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21 29 HRP: horseradish peroxidase
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24 30 IgG: immunoglobulin G
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27 31 LSEC: liver sinusoidal endothelial cells
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30 32 mAb: monoclonal antibody
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33 33 NK: natural killer
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36 34 NOD: non-obese diabetic
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39 35 NSG: non-obese diabetic severe combined immune deficient IL-2 γ^{-/-}
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42 36 PBS: phosphate buffered saline
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45 37 qPCR: quantitative polymerase chain reaction
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48 38 RBC: red blood cell
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51 39 SCID: severe combined Immune deficient
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54 40 SEC: size exclusion chromatography
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57 41 SPR: surface plasmon resonance
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3 **43 ABSTRACT**
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6 **44 Background**
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9 45 Immune compromised mice are increasingly used for the pre-clinical development of monoclonal
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11 46 antibodies (mAb). Most common are NOD SCID and their derivatives such as NOD SCID IL-2 $\gamma^{-/-}$
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13 47 (NSG), which are attractive hosts for patient derived xenografts. Despite their widespread use, the
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15 48 relative biological performance of mAb in these strains has not been extensively studied.
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18 **49 Methods**
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21 50 Clinically relevant mAb of various isotypes were administered to tumour and non-tumour bearing
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23 51 SCID and NOD SCID mice and the mAb clearance monitored by ELISA. Expression analysis of surface
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25 52 proteins in both strains was carried out by flow cytometry and immunofluorescence microscopy.
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27 53 Further analysis was performed *in vitro* by surface plasmon resonance to assess mAb affinity for Fc γ
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29 54 receptors (Fc γ R) at pH 6 and pH 7.4. NOD SCID mice genetically deficient in different Fc γ R were
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31 55 utilised to delineate their involvement.
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35 **56 Results**
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38 57 Here we show that strains on the NOD SCID background have significantly faster antibody clearance
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40 58 than other strains leading to reduced anti-tumour efficacy of clinically relevant mAb. This rapid
41
42 59 clearance is dependent on antibody isotype, the presence of Fc glycosylation (at N297) and
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44 60 expression of Fc γ RII. Comparable effects were not seen in the parental NOD or SCID strains,
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46 61 demonstrating the presence of a compound defect requiring both genotypes. The absence of
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48 62 endogenous IgG was the key parameter transferred from the SCID as reconstituting NOD SCID or
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50 63 NSG mice with exogenous IgG overcame the rapid clearance and recovered anti-tumour efficacy. In
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52 64 contrast, the NOD strain was associated with reduced expression of the neonatal Fc Receptor (FcRn).
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54 65 We propose a novel mechanism for the rapid clearance of certain mAb isotypes in NOD SCID mouse
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56 66 strains, based upon their interaction with Fc γ RII in the context of reduced FcRn.
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67 **Conclusions**

68 This study highlights the importance of understanding the limitation of the mouse strain being used
69 for pre-clinical evaluation, and demonstrates that NOD SCID strains of mice should be reconstituted
70 with IgG prior to studies of mAb efficacy.

72 **INTRODUCTION**

73 The growth in the numbers of monoclonal antibodies (mAb) being developed for the clinic,
74 particularly for use in cancer, has led to the concurrent development of *in vivo* models enabling their
75 pre-clinical evaluation.[1] These models have increasingly made use of immune-compromised mice
76 for growing patient-derived tumour xenografts and engrafting human immune or stem cells.[2, 3]

77 Commonly used models include non-obese diabetic (NOD) severe combined immunodeficient (SCID)
78 mice. The SCID mutation occurs in the *Prkdc* gene and impairs V(D)J recombination, leading to an
79 absence of functional B and T cells and resulting in mice lacking endogenous IgG.[4, 5] The NOD
80 phenotype results in reduced NK cell frequency and function and the absence of haemolytic
81 complement activity.[6] Whilst these immune deficient phenotypes make NOD SCID mice attractive
82 recipients for cell transfers (such as human PBMCs and tumour xenografts), they may be further
83 enhanced by additional genetic deletions such as the IL-2 γ -chain (NSG).[7, 8]

84 Whilst the effector function defects of NOD SCID mice and their related strains are often considered,
85 one aspect regularly overlooked is mAb clearance, despite the fact that genetic alterations, as well
86 as the lack of endogenous IgG in immune deficient strains, could readily impact on mAb
87 pharmacokinetics, resulting in altered efficacy.[9]

88 The primary receptors responsible for mediating IgG mAb activity are the Fc gamma receptor (Fc γ R)
89 family. It is comprised of 6 receptors in humans and 4 in mice, which vary in expression pattern and
90 affinity for IgG subclass.[10] Another receptor capable of interacting with IgG in both humans and

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3 91 mice is FcRn, which is widely expressed throughout the body. The pH dependent nature of FcRn-IgG
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5 92 interactions allows the receptor to scavenge IgG from lysosomes at an acidic pH, releasing it back
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7 93 into the circulation at neutral pH, providing the long *in vivo* half-life of antibodies.[11-13]
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10 94 In addition to the potential issue of altered efficacy arising from the lack of endogenous IgG (and
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12 95 reduced competition for FcγR with therapeutic mAb) in NOD SCID mice, previous reports indicate
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14 96 that immune-compromised mice, such as NOD SCID and NSG, have reduced mAb half-life compared
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16 97 to related strains.[14-16] More recently, it was reported that NOD SCID mice display an anomalous
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18 98 biodistribution of therapeutic antibodies, including reduced tumour targeting.[17] This suggests
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20 99 further work is required to understand the limitations of these models and develop strategies to
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22 100 overcome their shortcomings to make more translationally-relevant pre-clinical tumour models.
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27 101 During a recent project examining the efficacy of a tumour targeting antibody in NOD SCID mice, we
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29 102 noted rapid mAb clearance of human (h) IgG1 and mouse (m) IgG2a isotypes. Using a Eμ-Tcl1
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31 103 hCD20+ tumour model we found this rapid clearance resulted in reduced efficacy of clinically
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33 104 relevant mAb, such as rituximab. Employing genetically altered mice, we showed the rapid mAb
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35 105 clearance was dependent on the expression of the inhibitory FcγR, FcγRII. Additionally, we identified
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37 106 a reduced level of FcRn expression in NOD SCID mice, leading us to propose a novel hypothesis for
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39 107 how mAb half-life is regulated in these strains and means through which it can be overcome.
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3 110 **MATERIALS AND METHODS**
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6 111 **In vivo experiments.** Mice used in this study were bred and maintained in local facilities with
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8 112 experiments approved through local ethics committees and performed according to Home Office
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10 113 guidelines.
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16 115 **Generating bone marrow chimera.** Recipient mice were provided with acid water, pH 2.5 on day -7
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18 116 until 14 days after bone marrow receipt. Recipients received 1.1Gy radiation on days -1 and 0 using a
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20 117 MultiRad 350 X-ray Irradiator (Faxitron). Bone marrow was harvested from donor mice and $3-8 \times 10^6$
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22 118 cells injected I.V. into recipients. Systemic reconstitution was confirmed by flow cytometry 8-10
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24 119 weeks after engraftment.
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31 121 **E μ TCL-1 tumour model.** This model has been described previously.[18, 19] Briefly, 1×10^7
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33 122 cryopreserved E μ -TCL-1 transgenic (Tg) or hCD20⁺ E μ -TCL-1 Tg tumour splenocytes were injected I.P.
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35 123 into recipient mice. The presence of tumour was monitored in peripheral blood. Once tumour cells
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37 124 (CD19⁺CD5^{mid}) were detectable by flow cytometry, mice were treated. The white blood cell count
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39 125 was determined using a Coulter Z1 particle counter with red blood cells (RBC) lysed using ZAP-
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41 126 OGLOBIN II (both Beckman Coulter) or by flow cytometry using Precision Count beads (Biolegend).
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48 128 **In vivo antibodies.** All clinical antibodies were gifted from the Southampton General Hospital
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50 129 oncology pharmacy. Others were produced in-house. 18B12 and Rituximab isotype variants were
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52 130 cloned onto the appropriate IgG framework, produced in CHO cells and purified from culture
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54 131 supernatant with Protein A. Purity was assessed by electrophoresis (Beckman EP; Beckman) and lack
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56 132 of aggregation confirmed by SEC HPLC. Unless otherwise stated, all antibodies were administered
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58 133 I.P. in 200 μ l sterile PBS (Severn Biotech).
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56 135 **Flow cytometry.** Flow cytometry was performed using the antibodies listed in supplementary Table7
8 136 1. anti-mFcγR have been reported previously.[20, 21] Following staining, RBC lysis buffer was added9
10 137 (AbD Serotec) and cells washed before analysis on a FACS Canto or FACS Calibur flow cytometer (BD11
12 138 Biosciences). Alexafluor647 labelled MST-HN and H435A Abdegs for analysing FcRn expression were13
14 139 a kind gift from Prof Sally Ward (University of Southampton) and used at 5µg/ml with Fc block15
16 140 (2.4G2, 5µg/ml) prior to extracellular staining.
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2223 142 **Generating bone marrow derived macrophages (BMDM).** The tibia and femur of mice were flushed24
25 143 with sterile complete RPMI (RPMI 1640 (Life Technologies), 2mM L-glutamine, 1mM sodium26
27 144 pyruvate, 100U/ml penicillin, 100µg/ml streptomycin (all Life Technologies), 10% foetal calf serum28
29 145 (Sigma-Aldrich). Cells were plated in 6-well plates at 0.8×10^6 cells/ml in complete RPMI +20% L92930
31 146 conditioned media for 7-10 days.
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3435 147
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3738 148 **Determining plasma IgG concentration.** IgG concentration was determined by ELISA with reference39
40 149 to a standard curve of the same antibody as follows: for hIgG, maxisorp plates (Thermo Scientific)41
42 150 were coated with 5µg/ml goat anti-human Fc-specific polyclonal antibody (Sigma-Aldrich) and43
44 151 blocked with PBS +1% BSA before addition of serum for 1hour and washing. Detection was with45
46 152 Horseradish peroxidase (HRP) conjugated F(ab')₂ goat anti-hFc specific antibody (Jackson47
48 153 Immunoresearch). Plates were incubated with OPD substrate and OD₄₉₅ measured using an Epoch49
50 154 microplate spectrophotometer (Biotek). For quantification of mIgG, plates were coated with rabbit51
52 155 anti-mIgG and detected with HRP- rabbit anti-mIgG (both Jackson Immunoresearch).
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3 157 **Heat aggregation of IgG** Purified IgG was heated to 64°C for 30 minutes. The aggregated fraction
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5 158 was purified on a superdex S200 column (GE Healthcare). Aggregation was confirmed by HPLC using
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7 159 a Zorbax GF-250 column (Agilent).
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13 161 **Producing mFcγRII extracellular domain protein.** RNA was isolated and cDNA generated from SCID
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15 162 or NOD SCID BMDMs and the mFcγRII gene amplified using gene specific primers. Subsequently, the
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17 163 extracellular domain (residues 1-207) of mFcγRII were cloned with the addition of a 6xHis tag. The
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19 164 construct was transfected into MEXi -293E cells (IBA lifesciences) and FcγRII-His expressed according
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21 165 to the manufacturer's protocol and protein purified using a HisTrap HP column on an AKTA prime
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23 166 system (Both GE biosciences) and purity confirmed by SDS-PAGE.
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30 168 **Surface Plasmon resonance (SPR) analysis.** SPR was performed using a Biacore T100 system
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32 169 upgraded to a T200 (GE Life Sciences). For mFcγRII isoforms, anti-His capture antibody was
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34 170 immobilised on a CM5 chip (GE life Sciences). Purified FcγRII-His (10 µg/ml) was flowed over the chip
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36 171 at 30µl/min for capture. IgG was injected at 30µl/min. For all other analysis, IgG was immobilised via
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38 172 amine coupling with a target of 2000 RU. Recombinant mFcγRII or mFcRn (R&D systems) was flowed
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40 173 over the immobilised IgG in HBS-EP+ buffer (GE Life Sciences) at pH7.4 or pH6.0. Affinity constants
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42 174 were determined by analysis with Biacore Bioevaluation software assuming 1:1 binding.
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49 176 **Quantitative polymerase chain reaction (qPCR)** mRNA was extracted from SCID or NOD SCID
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51 177 splenocytes and hepatocytes using an RNeasy Mini Kit (Qiagen) and cDNA generated using a
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53 178 Superscript III reverse transcription kit (Life Technologies). qPCR was performed using GoTaq qPCR
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55 179 master mix (Promega) using gene specific primers, with HPRT1 as a control. Ct values were
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57 180 normalised using HPRT1 values and the $\Delta\Delta C_t$ method used to calculate fold change.
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182 **Western blotting.** Lysates were produced from 5×10^6 SCID or NOD SCID splenocytes and
183 hepatocytes using RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Deoxycholate, 0.1% SDS, 50 mM
184 Tris, pH 8) and run on a 12% Novex Nupage BIS-TRIS gel (Thermo Fisher) before transfer to a
185 methylcellulose membrane (GE Lifesciences). Primary antibodies were anti-mouse FcRn and Lamin B
186 with detection using an HRP-conjugated donkey anti-goat antibody (Supplementary Table 1).

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188 **Immunofluorescence.** Liver tissue from BALB/c or NSG mice was embedded in OCT (CellPath) and
189 frozen in isopentane on dry ice. $8 \mu\text{M}$ sections were cut and transferred to Superfrost plus slides
190 (Thermo Scientific), air dried overnight and fixed in 100% acetone. Following blocking, primary
191 antibodies against FcRn or Fc γ RII were added overnight before detection with Alexafluor488-
192 labelled secondary antibody (Supplementary Table 1) for 45 minutes. Subsequently, primary
193 antibodies against Clec4F or cytokeratin 8 were added for 2 hours before detection with AlexaFluor-
194 549 or AlexaFluor-568 conjugated secondary antibodies (Supplementary Table 1). Slides were
195 mounted using Vectashield hardset with DAPI (Vector Laboratories).

196 Images were collected using a CKX41 inverted microscope with a reflected fluorescence system
197 equipped with a DP22 camera running CellSens software, using Plan Achromat 10×0.25 and $40 \times$
198 0.65 objective lenses (all from Olympus). Images were transferred to ImageJ (Fiji) or Photoshop
199 (Adobe) where background autofluorescence was removed, contrast stretched and brightness
200 adjusted to maximise clarity, with all images treated equivalently.

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202 RESULTS

203 **Anti-tumour mAb therapy is less effective in NOD SCID mice due to rapid antibody clearance.**

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3 204 To explore potential differences of recipient mouse strains on immunotherapy efficacy, SCID and
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5 205 NOD SCID mice bearing established hCD20⁺ Eμ-TCL-1 tumours were treated with rituximab. Although
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7 206 initial tumour clearance was comparable between strains, 14 days after mAb treatment there were
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10 207 significantly more tumour cells in the peripheral blood of NOD SCID compared to SCID mice (Figure
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12 208 1A and B). To determine if this was associated with rituximab's type I nature,[22, 23] we repeated
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14 209 the experiment with the type II anti-hCD20 mAb, BHH2,[18] and observed the same reduced efficacy
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16 210 in NOD SCID compared to SCID mice (Figure 1B).

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19 211 To understand this difference in efficacy, the concentration of injected hIgG in the plasma of mice
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21 212 following treatment was determined (Figure 1C). This revealed that 7 days after mAb treatment
22
23 213 there was significantly less (~10 fold) hIgG in the plasma of NOD SCID compared to SCID mice (16.7 v
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25 214 1.6 μg/ml) suggesting that rapid hIgG1 clearance in NOD SCID mice was responsible for the less
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27 215 prolonged tumour deletion.

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31 216 To investigate whether the rapid clearance was related to the mAb, strain and/or tumour, an
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33 217 alternative hIgG1 mAb, cetuximab, was administered to non-tumour bearing SCID and NOD SCID
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35 218 mice. Cetuximab was also more rapidly cleared from NOD SCID compared to SCID mice with hIgG
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37 219 being undetectable in the plasma of NOD SCID mice by day 7 post-administration (Figure 1D). Similar
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39 220 results were also observed with other hIgG1 mAb including trastuzumab (Supplementary Figure 1),
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41 221 showing that the rapid clearance is directly related to the NOD SCID strain, independent of tumour
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43 222 and a common feature of therapeutically-relevant hIgG1 mAb. Importantly, the hIgG1 clearance in
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45 223 SCID and NOD mice was comparable to that of immune-competent BALB/c mice (Supplementary
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47 224 Figure 2 and previously shown [24]), confirming fast hIgG1 clearance in NOD SCID mice, rather than
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49 225 slow clearance in SCID or NOD mice. Furthermore, the lack of a difference in SCID mice
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51 226 demonstrates that rapid hIgG clearance does not result from the absence of endogenous IgG or
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55 227 immune deficiency *per se*.

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3 228 **Rapid mAb clearance in NOD SCID mice is isotype dependent and requires both SCID and NOD**
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5 229 **genotypes.**
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8 230 To determine if rapid mAb clearance in NOD SCID mice extended beyond hIgG1, isotype switch
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10 231 variants of rituximab were generated and administered to SCID or NOD SCID mice. Similar to hIgG1,
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12 232 mIgG2a also had a significantly faster mAb clearance in NOD SCID mice (Figure 1D), being no longer
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14 233 detectable in the plasma by day 14. In contrast, hIgG2 and mIgG1 had similar clearance rates in both
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16 234 strains. These results demonstrate that faster mAb clearance in NOD SCID mice is isotype
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18 235 dependent.
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22 236 We next assessed whether the rapid IgG clearance occurred in NOD and NSG strains. NOD mice had
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24 237 a normal hIgG1 clearance rate, akin to that seen in SCID and BALB/c (Figure 2A). However, NSG mice
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26 238 displayed rapid clearance, comparable to that in NOD SCID mice (Figure 2B). These data
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28 239 demonstrate that both NOD and SCID phenotypes are necessary to confer rapid IgG clearance.
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30 240 Moreover, the differences between isotypes in NOD SCID mice also occurred in the NSG strain, with
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32 241 hIgG1 and mIgG2a but not mIgG1 exhibiting rapid clearance (Figure 2B).
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39 243 **Rapid hIgG1 clearance is dependent on FcγR binding.**
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42 244 Given that mIgG2a and hIgG1 have similar FcγR binding profiles (binding to all mFcγR, with
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44 245 substantial affinity for several activatory FcγR), we hypothesised that the rapid mAb clearance of
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46 246 hIgG1 and mIgG2a isotypes in NOD SCID mice was mediated by FcγR.[25, 26] This was investigated
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48 247 using an N279Q (NQ)-mutant of rituximab which lacks glycosylation at N297 and does not robustly
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50 248 engage mFcγR (without compromising interaction with FcRn).[27] The NQ-mutant remained present
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52 249 in the plasma of NOD SCID mice at significantly higher concentrations at all time-points, supporting
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54 250 mFcγR involvement in the rapid hIgG1 clearance in NOD SCID mice (Figure 2C). Moreover, the
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3 251 concentration of rituximab-NQ was comparable between SCID and NOD SCID mice at all time-points
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5 252 suggesting that abrogation of mFcγR binding restored normal mAb clearance rate.
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11 254 **SCID and NOD SCID mice have comparable FcγR expression levels.**
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14 255 Having established that the rapid hIgG1 clearance rate in NOD SCID mice was likely dependent on
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16 256 mFcγR, the relative expressions levels of these receptors in SCID and NOD SCID mice was
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18 257 investigated (Figure 3A). Whilst there were no statistically significant differences in expression levels
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20 258 (2-way ANOVA $P > 0.05$) trends towards differential expression were observed. mFcγRII expression
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22 259 was lower on both Ly6C^{Hi} and Ly6C^{Lo} monocytes in NOD SCID compared to SCID mice (Figure 3B).
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24 260 Neutrophil and splenic macrophage FcγRIII expression was higher in SCID mice, with a similar
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26 261 expression profile for BMDMs (Supplementary Figure 3a). The expression of mFcγRI was not
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28 262 investigated as it is known to contain multiple polymorphisms in NOD SCID mice which prevent its
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30 263 detection using available antibodies.[28] The subtle differences in activatory mFcγR expression
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32 264 detailed above appear to be compensatory with a similar overall expression of activatory mFcγR in
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34 265 each strain. In summary, only monocyte FcγRII was found to differ between SCID and NOD SCID
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36 266 mice; the relevance of this to mAb clearance rate remains to be determined.
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44 268 **Rapid hIgG1 clearance in NOD SCID mice is dependent on FcγRII.**
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47 269 To understand the contribution of specific mFcγR to rapid antibody clearance in NOD SCID mice, we
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49 270 made use of animals lacking different classes of mFcγR. In NOD SCID FcR γ^{-/-} mice (which express no
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51 271 activatory FcγR at the cell surface[29]) there was no significant difference in the concentration of
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53 272 hIgG1 over time compared to NOD SCID mice (Figure 4A), demonstrating that a lack of activatory
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55 273 mFcγR does not influence hIgG1 clearance. However, in NOD SCID mice deficient in the inhibitory
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57 274 mFcγRII, the concentrations of hIgG1 were significantly increased compared to wild-type NOD SCID
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3 275 mice retaining mFcγRII (Figure 4B) and comparable with SCID mice. These results demonstrate that
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5 276 the rapid hlgG1 clearance in NOD SCID mice is dependent on mFcγRII. Moreover, this result suggests
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7 277 that the somewhat reduced FcγRII expression seen previously in NOD SCID mice is not responsible
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9 278 for the fast hlgG1 clearance rate.
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16 280 **The polymorphic variants of mFcγRII have comparable affinity for hlgG1.**
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18 281 A number of autoimmune strains, including NOD express the ly17.1 form of mFcγRII whilst most
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20 282 other in-bred strains, including BALB/c, express the ly17.2 variant.[30] These two polymorphic forms
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22 283 vary in four amino acids, three of which are located in the extracellular domain.[30] The extracellular
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24 284 domain of FcγRII from SCID and NOD SCID mice was cloned and expressed; their relative affinity for
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26 285 IgG was then determined by SPR. Neither heat aggregated, pooled hlgG or individual isotypes of IgG
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28 286 displayed substantially different binding affinities to the ly17.1 and ly17.2 variants (Table 1).
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	NOD SCID	SCID
	KD (M x10 ⁻⁶)	KD (M x10 ⁻⁶)
Aggregated hIgG	0.13	0.15
Cetux hIgG1	4.38	5.42
Ritux hIgG1	2.50	3.18
Ritux hIgG2	4.82	4.65
Ritux mIgG1	2.25	2.02
Ritux mIgG2a	2.43	2.63

Table 1. Affinity of mFcγRII variants for IgG subtypes. Recombinant mFcγRII extracellular domains from SCID or NOD SCID mice were captured on a Biacore CM5 chip using an immobilised anti-HIS antibody. IgG of specific isotypes or heat aggregated, pooled hIgG was flowed over the chip and the KD value calculated using Biacore evaluation software.

Absence of haematopoietic mFcγRII or phagocytes does not restore normal mAb clearance.

mFcγRII is expressed on both haematopoietic and non-haematopoietic cells.[30, 31] We therefore sought to determine which mFcγRII-expressing cells were responsible for the rapid clearance of hIgG1. Accordingly, NOD SCID mice were irradiated and reconstituted with bone marrow from NOD SCID FcγRII^{-/-} mice (Figure 4C and D). These mice, reconstituted with haematopoietic cells lacking mFcγRII displayed rapid clearance of hIgG1 and mIgG2a, indicating that mFcγRII on cells of the haematopoietic system was not responsible for the rapid mAb clearance (Figure 4E). We next considered whether phagocytes, particularly tissue resident macrophages, might be responsible and so deleted them with clodronate liposomes. This approach effectively removed macrophages

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3 303 (Supplementary Figure 3b) but only resulted in a small increase in circulating hlgG1 7 days after mAb
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5 304 administration, with no hlgG1 detectable by day 14 (Figure 4F). This suggests that phagocytes in
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7 305 NOD SCID mice are not primarily responsible for the rapid hlgG1 clearance and that a non-
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9 306 haematopoietic cell type is responsible. Given their high expression of mFcγII, the Liver Sinusoidal
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11 307 Endothelial cells (LSEC) seem the most likely candidate.[31] We confirmed high expression of
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13 308 mFcγRII on these cells by immunofluorescence of livers from both BALB/c and NOD SCID mice
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15 309 (Supplementary Figure 4 and b). Moreover, we found hlgG detectable at substantially higher levels
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17 310 within the liver of NSG than SCID mice following administration of hlgG1 mAb confirming a role for
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19 311 the liver as a site of hlgG1 accumulation (Supplementary Figure 4c).
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27 313 **NOD SCID mice have reduced FcRn expression.**

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29 314 As mFcγRII is not known to directly regulate mAb clearance, we next considered whether FcRn might
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31 315 be involved in the process of controlling clearance rate in the NOD SCID mouse. Importantly, FcRn in
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33 316 NOD SCID mice does not to contain any sequence variations compared to other strains and has
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35 317 normal binding to both human and mouse IgG.[14]. However, qPCR revealed significantly lower FcRn
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37 318 transcription in both spleen and liver of NOD SCID versus SCID mice (Figure 5A). This result was
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39 319 confirmed at the protein level by western blotting (Figure 5B) and flow cytometry using the MST-HN
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41 320 protein which maintains FcRn binding at both acidic and neutral pH (Figure 5C).[32] This latter
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43 321 approach demonstrated a lower expression of FcRn in Ly6C⁺ monocytes from NSG compared to SCID
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45 322 mice (MFI 354 v 2961, mean of N=4). Combined, these results demonstrate that there is a lower
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47 323 expression of FcRn in the tissues known to be important for IgG recycling (spleen and liver) of NOD
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49 324 SCID mice compared to SCID.[33]
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3 326 **mFcγRII has a pH dependent affinity for IgG isotypes and is expressed on the same cell types as**
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5 327 **FcRn.**
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8 328 Whilst reduced FcRn could explain rapid IgG clearance, it does not provide an explanation for the
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10 329 isotype dependent nature of the effects seen, as all isotypes should be affected equally. In contrast,
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12 330 mFcγRII is known to display differential affinity for IgG isotypes (high for mIgG1, low for mIgG2a and
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14 331 hIgG1) and so we considered if mFcγRII specificity might be involved in regulating the clearance of
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16 332 the different isotypes. Using SPR and two different mAb of each isotype, we confirmed that at pH7.4
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18 333 mFcγRII had ~10 fold higher affinity for mIgG1 ($2.74 \times 10^{-7} \text{M}$) versus mIgG2a ($1.18 \times 10^{-6} \text{M}$) and hIgG1
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20 334 (3.02×10^{-6}) whilst the affinity for hIgG2 was lower still ($7.65 \times 10^{-6} \text{M}$) (Table 2 and Figure 5D).
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24 335 We next considered that mFcγRII might internalise cell-surface bound IgG and by virtue of its higher
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26 336 affinity, preferentially protect mIgG1 from degradation following internalisation. To do this, it would
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28 337 need to remain bound to IgG in a low pH environment, akin to FcRn. We therefore repeated SPR
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30 338 analysis at pH6.0, and revealed that mFcγRII retained binding at low pH, with affinity for mIgG1,
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32 339 mIgG2a and hIgG1 ~100-fold higher than at pH7.4. Notably, the K_D for mIgG1 binding mFcγRII was
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34 340 $2.77 \times 10^{-9} \text{M}$, >10-fold higher than for hIgG1 and mIgG2a. This suggests that mFcγRII is capable of
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36 341 binding IgG at an acidic pH with the potential to protect IgG from degradation being greatest for
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38 342 mIgG1. Using previously published affinity data for IgG binding to mFcRn, we calculated the ratio of
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40 343 mFcγRII:FcRn binding for different isotypes at pH6.0 (Table 2 and Figure 5D).[34] hIgG2 exhibited a
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42 344 high mFcγRII:FcRn ratio, suggesting preferential binding for FcRn at an acidic pH. In comparison,
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44 345 hIgG1 had a ratio around 1 (indicating no overall preference) whereas mIgG1 had a low ratio,
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46 346 preferentially binding with a higher affinity to mFcγRII than to FcRn.
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52 347 Having hypothesised that the differential interaction with FcRn and FcγRII may play a role in the
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54 348 recycling of IgG, and with the knowledge that the liver expresses 75% of the FcγRII in the mouse, we
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56 349 sought to determine the distribution of these two receptors within the liver.[31] We found mFcγRII
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58 350 on a subset of Clec4F⁺ Kupffer cells but not on Cytokeratin 8⁺ hepatocytes (Supplementary Figure 4).
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3 351 However, the majority of mFcγRII was expressed by LSEC as determined by their morphology. This is
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5 352 consistent with a previous study reporting 90% of the liver mFcγRII as being expressed by LSEC when
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7 353 assessing immunofluorescence by pixel intensity.[31] We found FcRn to be widely expressed
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9 354 throughout the liver including on Kupffer cells and hepatocytes (Supplementary Figure 4). This is
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11 355 consistent with a previous report showing FcRn mRNA in various cell types, additionally identifying
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13 356 LSEC as having the highest expression level.[31] Together, these results suggest that LSEC may be the
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15 357 predominant cell type co-expressing FcγRII and FcRn.
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19 358 Overall, these data provided the possibility that antibody clearance in NOD SCID strains is controlled
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21 359 through differential engagement of the various isotypes by mFcγRII and FcRn. However, these
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23 360 effects have not been reported previously in standard in-bred strains, or the single NOD and SCID
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25 361 strains, indicating that the proposed pathway, which is at least in part mediated by isotype-
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27 362 dependent binding of mFcγRII, is only revealed in the absence of endogenous IgG.
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	FcγRII KD pH7.4 (M)	FcγRII KD pH6 (M)	Published FcRn KD pH6 (M)	KD ratio FcγRII/FcRn
hIgG1 #1	3.02 x10 ⁻⁶	4.92 x10 ⁻⁸	7.2x10 ⁻⁸	0.68
hIgG1 #2	6.61 x10 ⁻⁶	7.90 x10 ⁻⁸	7.2 x10 ⁻⁸	1.10
hIgG2 #1	7.65 x10 ⁻⁶	2.10 x10 ⁻⁷	6.3x10 ⁻⁸	3.33
hIgG2 #2	1.45 x10 ⁻⁵	2.18 x10 ⁻⁷	6.3x10 ⁻⁸	3.46
mIgG1 #1	2.74 x10 ⁻⁷	2.77 x10 ⁻⁹	1.57x10 ⁻⁶	0.0018
mIgG1 #2	4.98 x10 ⁻⁷	3.62 x10 ⁻⁹	1.57x10 ⁻⁶	0.0023
mIgG2a #1	1.18 x10 ⁻⁶	2.36 x10 ⁻⁸	4.9x10 ⁻⁷	0.054
mIgG2a #2	1.10 x10 ⁻⁶	2.41 x10 ⁻⁸	4.9x10 ⁻⁷	0.049

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3 363 **Table 2.** Affinity of mFcγRII and mFcRn for IgG subtypes. Antibodies were immobilised on a Biacore
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5 364 CM5 chip before flowing mFcγRII or mFcRn over the chip. FcRn was used at pH6 with FcγRII at pH6
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7 365 and pH7.4. KD was calculated using Biacore evaluation software.
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13 367 **Rapid hlgG1 clearance and reduced mAb efficacy can be overcome by IgG reconstitution.**

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16 368 We therefore hypothesised that the addition of exogenous mlgG would restore normal mAb
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18 369 clearance rate in NOD SCID mice. Accordingly, SCID and NOD SCID mice were reconstituted with
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20 370 mlgG1 and mlgG2a to a level equivalent to that seen in the plasma of wild type BALB/c mice
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22 371 (Supplementary Figure 5). Subsequently, the clearance of hlgG1 was investigated (Figure 5E).
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24 372 Reconstitution with mlgG overcame the rapid clearance of hlgG1 in NOD SCID mice such that it
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26 373 became comparable to that observed in SCID mice. In contrast, mlgG addition did not significantly
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28 374 alter the clearance of hlgG in SCID mice. Additionally, reconstitution with mlgG substantially reduced
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30 375 the accumulation of hlgG in the liver (Supplementary Figure 4c). Importantly, the expression of FcRn
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32 376 was not altered by reconstitution with mlgG (Figure 5c). Finally, we sought to determine if
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34 377 overcoming rapid clearance of hlgG1 by mlgG reconstitution could improve therapy, using an Eμ-
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36 378 TCL1 tumour and a hlgG1 antibody targeting mouse CD20 (18B12). Using this second tumour model
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38 379 we found the differences in duration of therapy between SCID and NSG mice was maintained, with
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40 380 tumour growth recurring in NSG before SCID mice (Figure 5F). We then compared the duration of
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42 381 tumour deletion in NSG mice versus NSG mice reconstituted with mlgG, using the same protocol as
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44 382 before. Reconstitution with mlgG was able to overcome the impaired therapy and restored
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46 383 comparable levels of tumour deletion and control to that observed in SCID mice both in terms of
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48 384 tumour (Figure 5F) and number of tumour cells (Supplementary Figure 6). This demonstrates that
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50 385 reconstitution with mlgG is able to restore mAb efficacy in NSG mice.
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DISCUSSION

Our observations with rituximab showed reduced tumour control in NOD SCID compared to SCID mice. Given that equivalent initial tumour clearance was observed, and deletion is known to be dose dependent, this impairment was considered to be a direct result of reduced serum persistence and insufficient mAb at later time points.[35, 36] Rituximab is a type I mAb, known to be internalised through cis-binding to FcγRII following target binding, reducing its efficacy.[18, 37] Importantly, reduced tumour control was also seen with a type II anti-CD20 reagent, BHH2, indicating mAb internalisation was not causal and that a separate phenomenon related to the mouse strain was responsible. In support of this, rapid clearance of wild-type and chimeric hlgG1 mAb was described recently in both NOD SCID and NSG mice.[14, 16] Furthermore, we saw the same rapid hlgG1 clearance for two additional hlgG1 mAb, lacking targets in the mouse, showing this phenomenon to be independent of the presence of tumour and unrelated to F(ab)-mediated antigen binding.

Separately, both SCID and NOD mice had normal clearance of hlgG1, comparable to that of immune competent BALB/c mice, as reported previously.[24] This indicated that the genetic background of NOD mice, coupled with a lack of endogenous IgG (as a result of the SCID mutation) combined to elicit rapid hlgG1 clearance in NOD SCID mice. Importantly, our observations were replicated in NSG mice, indicating that rapid hlgG1 clearance is a feature of all NOD SCID derived strains.

mlgG1 was found to have normal clearance in NOD SCID mice, whereas both hlgG1 and mlgG2a had short half-lives. Both humans and mice have multiple activatory FcγR, but a single inhibitory receptor, FcγRII (FcγRIIb in humans). These receptors interact differentially with the various mouse and human IgG isotypes: mlgG2a and hlgG1 bind preferentially to multiple activatory receptors and as such have high activatory:inhibitory (A:I) ratios.[26] In contrast, mlgG1 exhibits binding to only a single activatory FcγR (FcγRIII) whilst retaining binding to FcγRII, yielding a corresponding low A:I ratio.[38] These observations provided a potential clue towards the isotype-based effects observed.

A role for FcγR in this process was subsequently confirmed by using a hlgG1 N297Q mAb which

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3 412 abrogates binding to FcγRs.[27] Importantly, the N297Q mutation has been demonstrated not to
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5 413 alter hIgG1 clearance in immune compromised mice.[27] The normal clearance rate of N297Q mAb
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7 414 in NOD SCID mice suggested that the rapid clearance of hIgG1 (and mIgG2a) in NOD SCID mice was
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9 415 dependent on Fc:FcγR interaction.
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12 416 Despite establishing a likely role for mFcγR in rapid mAb clearance, no gross changes in activatory
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14 417 FcγR expression levels were observed in NOD SCID versus SCID mice. The subtle inter-strain
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16 418 differences in mFcγRIII and mFcγRIV expression are likely to be compensatory and do not result in a
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18 419 large difference in the total A:I ratio or amount of activatory FcγR on the cell surface. One caveat
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20 420 here was that the expression level of mFcγRI in NOD SCID mice could not be determined by flow
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22 421 cytometry as its sequence varies considerably from the canonical sequence seen in most other
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24 422 strains (by 17 residues), and so cannot be detected using our existing reagents.[39] mFcγRII
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26 423 expression was found to be lower on monocytes from NOD SCID compared to SCID mice, likely due
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28 424 to the previously reported alterations upstream of the gene in NOD SCID mice associated with lower
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30 425 expression.[40] However, subsequent results, particularly in mice lacking mFcγRII expression,
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32 426 demonstrate that the reduced expression of mFcγRII was unlikely responsible for the rapid mAb
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34 427 clearance.
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40 428 We additionally investigated the expression levels of FcRn, the primary receptor responsible for IgG
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42 429 recycling and long half-life.[41] Whilst it has a broad tissue distribution, expression is particularly
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44 430 prominent in the spleen and liver.[33, 41] Analysis of these tissues demonstrated that there was
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46 431 lower expression of FcRn in NOD SCID compared to SCID mice. SCID mice have previously been
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48 432 reported to have comparable FcRn expression and tissue distribution to immune-competent mice;
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50 433 indicating that the reduction is a result of the NOD phenotype rather than elevated expression in
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52 434 SCID mice.[33] However, given that the N279Q-mutated antibody which can interact with FcRn but
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54 435 not FcγR had a normal clearance rate in NOD SCID mice, the reduced FcRn expression in isolation
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56 436 cannot explain the differences in mAb clearance.
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3 437 To determine the receptor responsible, we used mice deficient in either the activatory or inhibitory
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5 438 mFcγR by employing mFcR γ-chain -/- or mFcγRII-/- mice, respectively. Only the absence of mFcγRII
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7 439 restored normal hlgG1 clearance in NOD SCID mice. This observation supports the implications of
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9 440 the N297Q-mutant data; i.e. failing to engage with mFcγRII and therefore phenocopying the effect in
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11 441 the mFcγRII-/- mice. Our observation that mFcγRII mediates this effect is in contrast to a previous
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13 442 study suggesting mFcγRIV is responsible.[16] The previous study used an Fc-engineered antibody
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15 443 reported to have reduced binding to FcγRIV; such mutations often result in broader changes to FcγR
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17 444 binding profiles with causal effects of specific FcγR difficult to define. In contrast, in the present
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19 445 study we were able to specifically define the role of FcγRII by using mice genetically deficient in
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21 446 FcγRII.

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26 447 Given the dependence on mFcγRII, we investigated this receptor in more detail. There are two
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28 448 polymorphic variants of mFcγRII; the ly17.1 haplotype expressed by NOD SCID mice, and the more
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30 449 common ly17.2 haplotype expressed by most other in-bred mouse strains, which differ by four
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32 450 amino acids, three of which are extracellular.[42, 43] We confirmed previous observations that these
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34 451 allotypes do not differ in their affinity for IgG. Whilst this SPR analysis assesses the likely effects of
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36 452 the extracellular polymorphisms, further investigation is needed into the role of the remaining I258S
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38 453 intracellular polymorphism to determine its influence on mAb internalisation. This could be of
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40 454 importance given the role of the intracellular I232T polymorphism in hFcγRIIB, which alters the
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42 455 ability of the receptor to cluster into lipid rafts and deliver inhibitory signals. [44]

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47 456 The data presented here indicate that no single factor can explain the isotype-dependent differences
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49 457 in mAb clearance in NOD SCID versus SCID mice. Instead, the data support a more complex model
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51 458 whereby multiple factors arising from the NOD and SCID backgrounds combine to deliver the
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53 459 observed defect. We propose a model whereby mFcγRII accelerates initial mAb internalisation. The
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55 460 ability of FcγRII to mediate mAb internalisation has been previously reported in DC mediated antigen
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57 461 presentation and the internalisation of rituximab.[37, 45] hlgG1 N297Q and hlgG2 do not bind
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3 462 appreciably to FcγRII, preventing receptor-mediated internalisation, maintaining equivalent
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5 463 clearance in both SCID and NOD SCID mice. Similarly, in SCID mice with a normal level of FcRn, the
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7 464 IgG internalised via FcγRII can be efficiently recycled by FcRn to maintain serum persistence.
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10 465 However, In NOD SCID mice, this efficient FcRn mediated recycling does not occur due to the
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12 466 reduced FcRn expression levels, resulting in more rapid serum loss.
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15 467 In trying to understand the isotype dependent nature of the rapid IgG clearance rate in NOD SCID
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17 468 mice, we unexpectedly observed an increased affinity of mFcγRII for all IgG at pH6.0. Crucially
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19 469 however, the affinity of mFcγRII for mIgG1 at pH6.0 was retained and ~10-fold higher than for the
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21 470 other isotypes investigated. This raises the possibility that mIgG1 is protected from degradation
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23 471 under acidic conditions due to continued association with mFcγRII following internalisation. In
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25 472 contrast, mIgG2a and hIgG1 would remain unprotected and become degraded following
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27 473 internalisation, due to mFcγRII having lower affinity for these isotypes at pH6.0. We propose that
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29 474 this reduced degradation occurs only in the presence of reduced FcRn expression in NOD SCID mice,
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31 475 further work is however required to confirm this hypothesis. These data therefore suggest a complex
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33 476 role for FcγRII in mAb clearance; it is required for the internalisation of IgG, preventing external
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35 477 catabolism, yet it also delivers IgG for internal lysosomal degradation unless it exhibits sufficient
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37 478 affinity for IgG binding at pH6.
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42 479 Prior experiments in conditional knock-out mice suggested that both endothelial and
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44 480 haematopoietic cells regulate IgG levels in mice.[46, 47] The transfer of mFcγRII^{-/-} NOD SCID bone
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46 481 marrow into NOD SCID mice resulted in mice deficient in haematopoietic mFcγRII but with mFcγRII
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48 482 expression on non-haematopoietic (predominantly endothelial) cells. hIgG1 clearance in these
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50 483 chimeras was unaltered compared to NOD SCID mice, demonstrating that haematopoietic cells were
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52 484 not responsible. The liver was previously found to be the main site of IgG clearance, accounting for
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54 485 30% of all antibody degradation.[48] The same organ has also been shown to contain 75% of the
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56 486 mFcγRII in the body, the receptor demonstrated here to be essential for rapid NOD SCID mAb
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3 487 clearance.[31] Additionally, we were able to demonstrate greater accumulation of hIgG in the liver
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5 488 of NSG compared to SCID mice, an effect that was overcome by the addition of exogenous mIgG.
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7 489 Our immunofluorescence studies, combined with previous reports, suggest that LSEC are the
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10 490 predominant cell type in the liver expressing both FcγRII and FcRn, the receptors regulating the fast
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12 491 mAb clearance.[31, 49] LSEC have been described as having the highest rates of endocytic uptake in
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14 492 the body. In addition, mFcγRII is required for the efficient clearance of small immune complexes. [31,
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16 493 50] This leads us to hypothesise that LSEC are the key cell type responsible for fast mAb clearance
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18 494 observed in the present study, with internalisation of IgG mediated by mFcγRII.
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21 495 Further investigation is required to determine if pH and isotype dependent affinity is restricted to
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23 496 mFcγRII or is common to the other FcγR. Moreover, it remains to be established if the same occurs
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25 497 with the inhibitory FcγRIIb in humans. This could have implications for mAb therapy as it is known
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27 498 that the A:I ratio of IgG binding to FcγR can determine the outcome of therapy, particularly where
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29 499 the expression of hFcγRIIb may increase, such as within the tumour microenvironment.[51]
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32 500 Moreover, in this context, the acidic pH of the tumour microenvironment may further modify this
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34 501 ratio of A:I binding by altering the relative binding to individual receptors.
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37 502 Given the importance of the rapid mAb clearance in NOD SCID mice on the therapeutic activity of
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39 503 direct targeting hIgG1 mAb, we sought a means of restoring normal pharmacokinetics. By
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41 504 reconstituting NOD SCID mice with physiological levels of mIgG, the rapid mAb clearance could be
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43 505 overcome, restoring persistence equivalent to that observed in SCID and BALB/c mice. Moreover,
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45 506 this increased persistence of therapeutic mAb was able to recover anti-tumour efficacy to the same
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47 507 level as seen in SCID mice. This result is in agreement with findings that the addition of human IVIg
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49 508 can restore the normal half-life of an antibody-drug conjugate.[16] Moreover, it has been previously
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51 509 reported that the addition of exogenous IgG is able to overcome anomalous antibody biodistribution
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53 510 in NOD SCID mice, adding to the potential benefits of IgG reconstitution in tumour models.[17] We
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55 511 suggest that in the presence of exogenous IgG, mFcγRII is occupied (most likely by the mIgG1
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3 512 component, due to its higher affinity) and less internalisation of hIgG1 can occur. Based on these
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5 513 observations, we propose that reconstitution with IgG should be a consideration when performing
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7 514 therapy experiments in NOD SCID mice in order to restore therapeutic antibody half-life.
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10 515 The implications for the findings presented here are wide-reaching. With an increasing use of
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12 516 immune-compromised mice in pre-clinical investigation of mAb therapeutics, it is essential to
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14 517 understand how the choice of host strain can influence the outcome. The clearance rate of the most
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16 518 clinically relevant deleting isotypes are significantly shorter in NOD SCID and NSG mice than other
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18 519 immune-compromised strains such as SCID. This is likely to underplay the therapeutic efficacy of
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20 520 mAb used in these models and complicate comparisons between strains. Additionally, there are
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22 521 significant efforts ongoing to understand the isotype requirements for mAb directed against
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24 522 different targets, and with different Fc requirements.[52] Our work suggests that NOD SCID mice
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26 523 may not be a suitable host strain for determining the optimal mAb isotype or therapeutic dose due
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28 524 to complications arising from different isotype-dependent clearance rates, unless exogenous mIgG
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30 525 reconstitution is also provided. Specifically, we suggest that caution should be exercised when
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32 526 interpreting results from immune compromised mice on the NOD SCID background with regard to
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34 527 differences in antibody activity that could be explained by mAb clearance rate.
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43 529 **DECLARATIONS**

44 45 46 530 **Ethics Approval**

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49 531 Animal experiments were cleared through local ethics committees and performed according to
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51 532 Home Office guidelines under project license PB24EEE31.
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55 56 57 534 **Consent for Publication**

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59 535 Not applicable
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56 537 **Availability of Data and Material**
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9 538 All datasets used and/or analysed during the current study are available from the corresponding
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11 539 author on reasonable request.
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14 540
1516
17 541 **Competing Interests**
1819
20 542 MSC is a retained consultant for Bioinvent and has performed educational and advisory roles for
21
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25
26 545 interest.
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4041
42 551
4344
45 552 **Author Contributions**
4647
48 553 RJO, CIM, SJ, KLC and VAP performed experiments. RJO analysed the data. RJO, MSC and MJG
49
50 554 interpreted the results. PD and HTC generated critical reagents. RJO and MSC wrote the manuscript.
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8
9
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39 681 **FIGURE LEGENDS**

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42 682 **Figure 1:** Antibody mediated therapy is reduced in NOD SCID mice due to faster mAb clearance. A -
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44 683 C) Eμ-Tcl1 x hCD20 Tg tumour cells were injected I.P. into SCID or NOD SCID mice. Once tumour was
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46 684 detectable in the peripheral blood animals were treated with 100μg rituximab or BHH2 I.P. A) 2 and
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48 685 14 days after treatment, the percentage of tumour cells in the blood was assessed. B) The number of
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50 686 tumour cells in the blood 14 days after treatment was determined (n=4-9). C) The concentration of
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52 687 hIgG in the plasma was measured by ELISA with a significantly lower concentration of hIgG on day 7
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54 688 in NOD SCID compared to SCID mice. hIgG was not detectable in the plasma of NOD SCID mice from
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56 689 day 14 onwards. D) In the absence of tumour, 100μg cetuximab or 100μg rituximab hIgG2, mIgG2a
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58 690 or mIgG1 was administered I.P. to SCID or NOD SCID mice. The concentration of human or mouse
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3 691 IgG in the plasma was determined by ELISA. (hlgG1 n=6-7; combined data from 2 independent
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5 692 experiments, hlgG2, mlgG1 and mlgG2a n=3, representative of 2 independent experiments). ND =
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7 693 not detectable. Statistics; 2 way ANOVA with multiple comparisons *P<0.05, **P<0.01, ***P<0.001.
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10 694 No significant differences were observed between SCID and NOD SCID mice receiving hlgG2 or
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12 695 mlgG1.

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18 697 **Figure 2:** Rapid antibody half-life requires both the NOD and SCID phenotypes as well as functional
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20 698 Fc. A) 100µg cetuximab injected I.P. into NOD or NOD SCID mice with the concentration of hlgG in
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22 699 the plasma determined by ELISA, over 14 days. (n=4-6). B) 100µg rituximab hlgG1, mlgG1 or mlgG2a
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24 700 was injected I.P. into NSG mice and the concentration of mouse or human IgG in the plasma
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26 701 determined by ELISA, (n=6-8), data combined from 2 independent experiments. (C) 100µg rituximab
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28 702 (hlgG1) or rituximab hlgG1 N297Q (NQ) was injected I.P. into SCID or NOD SCID mice and the
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30 703 concentration of hlgG in the plasma determined by ELISA. (SCID n=3-4, NOD SCID N=7 combined
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32 704 from 2 independent experiments). 2-way ANOVA with multiple comparisons, **P<0.01, ***P<0.001
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39 706 **Figure 3:** mFcyR expression profiling in SCID and NOD SCID mice. Splenocytes or peripheral blood
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41 707 from SCID and NOD SCID mice were stained with specific antibodies for Ly6c, Ly6G, CD11b or CD11c
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43 708 to identify monocytes, macrophages and neutrophils, concurrently with mAb to each mFcyR. N=3
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45 709 combined data from 2 independent experiments, mean +range. No statistically significant
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47 710 differences were observed between strains (2-way ANOVA with Tukeys multiple comparison test,
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49 711 p>0.05)
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56 713 **Figure 4:** Rapid mAb clearance in NOD SCID mice is dependent on expression of mFcyRII but not
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58 714 activatory mFcyR. A) NOD SCID and NOD SCID FcR γ -chain deficient mice (NS γ -/-) were injected with
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3 715 100µg trastuzumab hIgG1 I.V. Tail blood was collected and the concentration of hIgG in the plasma
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5 716 determined by ELISA. (n=3-4) mean +S.D. By day 14 hIgG was not detectable in the plasma of both
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7 717 strains. B) SCID, NOD SCID and NOD SCID mFcγRII^{-/-} mice were injected with 100µg cetuximab I.V.
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9 718 The concentration of hIgG in plasma was determined 2, 7 and 14 days later by ELISA. (n=3-4 per
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11 719 group), representative of 2 independent experiments. 2-way ANOVA with multiple comparisons
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13 720 **p<0.01, ***p<0.001. C) NOD SCID mice were irradiated and reconstituted with bone marrow cells
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15 721 harvested from NOD SCID FcγRII^{-/-} mice. D) Engraftment was confirmed by staining peripheral
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17 722 CD11b⁺ cells for mFcγRII expression. E) 100µg rituximab hIgG1 or mIgG2a was injected into these or
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19 723 control NOD SCID mice. The concentration of human or mouse IgG in the plasma was determined by
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21 724 ELISA. (n=3-4), mean +S.D. F) NOD SCID mice were injected I.V. with clodronate- or PBS-containing
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23 725 liposomes on days -3, -1, 6 and 13 to deplete macrophages. On day 0, 100µg rituximab was
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25 726 administered I.P. and the concentration of hIgG in the plasma determined by ELISA.
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34 728 **Figure 5:** NOD SCID mice have low expression of FcRn, associated with rapid mAb clearance, which
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36 729 can be overcome by IgG reconstitution. A) cDNA was produced from SCID and NOD SCID spleen and
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38 730 liver lysates with FcRn transcript expression analysed by qPCR using the ddCT method and expressed
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40 731 relative to that in SCID mice. (n=4-5), unpaired T-test **P<0.01, ***P<0.001. B) Splenocytes from
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42 732 SCID and NOD SCID spleens were lysed and Western blot performed on the lysates for FcRn and
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44 733 Lamin B as a loading control. C) Uptake of MST-HN Abdeg by splenocytes from SCID and NSG mice as
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46 734 well as mIgG reconstituted NSG mice, gated on CD11b⁺Ly6G⁻Ly6C⁺ cells. No protein (red), H435A
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48 735 control (blue) and MST-HN (orange) are shown. N=3-4 with a representative example for each group
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50 736 shown. D) Rituximab hIgG1, hIgG2, mIgG1 or mIgG2a was immobilised on Biacore CM5 chips and
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52 737 recombinant mFcγRII flowed over the chip at pH6 or pH7.4 with a highest receptor concentration of
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54 738 1000nM and 5-fold serial dilutions. E) SCID or NOD SCID mice were reconstituted with 400µg mIgG2a
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56 739 and 500µg mIgG1 on day 0. An additional 200µg mIgG2a was given on day 3, 6, 9, 12 and 15. 100µg
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3 740 rituximab was then given I.P. on day 0 and the concentration of hIgG in the plasma determined by
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5 741 ELISA. (n=4-8), data combined from 2 independent experiments, mean +S.D. 2-way ANOVA with
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7 742 multiple comparisons ***P<0.001, n.s. = not significant. F) E μ -Tcl1 tumour cells were injected I.P.
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9 743 into SCID or NSG mice. Once tumour was detectable in the peripheral blood a group of NSG mice
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11 744 were reconstituted with mIgG as described above, animals were then treated with 100 μ g hIgG1 anti-
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13 745 mCD20 (18B12) I.P. representative FACs plots are shown on the left. 14 days after treatment, the
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15 746 percentage of tumour cells in the blood was assessed and plotted. (n=5-6 per group), mean +S.D. 1-
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17 747 way ANOVA with multiple comparisons *P<0.05, **P<0.01.
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10 1 **FcγRII (CD32) modulates antibody clearance in NOD SCID mice leading to impaired antibody-**
11 **mediated tumour cell deletion**
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16 4 Running title: CD32 and FcRn mediate rapid antibody clearance in NOD SCID mice
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20 6 Robert J. Oldham¹, C. Ian Mockridge¹, Sonya James¹, Patrick J. Duriez², HT Claude Chan¹, Kerry L.
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22 7 Cox¹, [Vicentiu A Pitic¹](#), Martin J Glennie¹, Mark S. Cragg¹
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48 20 Keywords: FcγRII, CD32, NSG, NOD, SCID, antibodies, FcRn, antibody clearance, isotype
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10 23 **LIST OF ABBREVIATIONS**

11
12 24 BMDM: bone marrow derived macrophage

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14 25 ELISA: enzyme-linked immunosorbent assay

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16 26 FcγR: Fc gamma receptor

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18 27 FcRn: neonatal Fc receptor

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20 28 HPLC: high performance liquid chromatography

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22 29 HRP: horseradish peroxidase

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24 30 IgG: immunoglobulin G

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26 31 LSEC: liver sinusoidal endothelial cells

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28 32 mAb: monoclonal antibody

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30 33 NK: natural killer

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32 34 NOD: non-obese diabetic

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34 35 NSG: non-obese diabetic severe combined immune deficient IL-2 γ ^{-/-}

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36 36 PBS: phosphate buffered saline

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38 37 qPCR: quantitative polymerase chain reaction

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40 38 RBC: red blood cell

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42 39 SCID: severe combined Immune deficient

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44 40 SEC: size exclusion chromatography

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46 41 SPR: surface plasmon resonance

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60**43 ABSTRACT****44 Background**

45 Immune compromised mice are increasingly used for the pre-clinical development of monoclonal
46 antibodies (mAb). Most common are NOD SCID and their derivatives such as NOD SCID IL-2 $\gamma^{-/-}$
47 (NSG), which are attractive hosts for patient derived xenografts. Despite their widespread use, the
48 relative biological performance of mAb in these strains has not been extensively studied.

49 Methods

50 Clinically relevant mAb of various isotypes were administered to tumour and non-tumour bearing
51 SCID and NOD SCID mice and the mAb clearance monitored by ELISA. Expression analysis of surface
52 proteins in both strains was carried out by flow cytometry and immunofluorescence microscopy.
53 Further analysis was performed *in vitro* by surface plasmon resonance to assess mAb affinity for Fc
54 receptors (Fc γ R) at pH 6 and pH 7.4. NOD SCID mice genetically deficient in different Fc γ R were
55 utilised to delineate their involvement.

56 Results

57 Here we show that strains on the NOD SCID background have significantly faster antibody clearance
58 than other strains leading to reduced anti-tumour efficacy of clinically relevant mAb. This rapid
59 clearance is dependent on antibody isotype, the presence of Fc glycosylation (at N297) and
60 expression of Fc γ RII. Comparable effects were not seen in the parental NOD or SCID strains,
61 demonstrating the presence of a compound defect requiring both genotypes. The absence of
62 endogenous IgG was the key parameter transferred from the SCID as reconstituting NOD SCID or
63 NSG mice with exogenous IgG overcame the rapid clearance and recovered anti-tumour efficacy. In
64 contrast, the NOD strain was associated with reduced expression of the neonatal Fc Receptor (FcRn).
65 We propose a novel mechanism for the rapid clearance of certain mAb isotypes in NOD SCID mouse
66 strains, based upon their interaction with Fc γ RII in the context of reduced FcRn.

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10 67 **Conclusions**

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12 68 This study highlights the importance of understanding the limitation of the mouse strain being used
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14 69 for pre-clinical evaluation, and demonstrates that NOD SCID strains of mice should be reconstituted
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16 70 with IgG prior to studies of mAb efficacy.
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20 72 **INTRODUCTION**

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22 73 The growth in the numbers of monoclonal antibodies (mAb) being developed for the clinic,
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24 74 particularly for use in cancer, has led to the concurrent development of *in vivo* models enabling their
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26 75 pre-clinical evaluation.[1] These models have increasingly made use of immune-compromised mice
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28 76 for growing patient-patient-derived tumour xenografts and engrafting human immune or stem
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30 77 cells.[2, 3]

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32 78 Commonly used models include non-obese diabetic (NOD) severe combined immunodeficient (SCID)
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34 79 mice. The SCID mutation occurs in the Prkdc gene and impairs V(D)J recombination, leading to an
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36 80 absence of functional B and T cells, and resulting in mice lacking endogenous IgG.[4, 5] The NOD
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38 81 phenotype results in reduced NK cell frequency and function and the absence of haemolytic
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40 82 complement activity.[6] Whilst these immune deficient phenotypes make NOD SCID mice attractive
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42 83 recipients for cell transfers (such as human PBMCs and tumour xenografts), they may be further
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44 84 enhanced by additional genetic deletions such as the IL-2 γ -chain (NSG).[7, 8]

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46 85 Whilst the effector function defects of NOD SCID mice and their related strains are often considered,
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48 86 one aspect regularly/often overlooked is mAb clearance, despite the fact that genetic alterations, as
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50 87 well as the lack of endogenous IgG in immune deficient strains, could readily impact on mAb
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52 88 pharmacokinetics, resulting in altered efficacy.[9]

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54 89 The primary receptors responsible for mediating IgG mAb activity are the Fc gamma receptor (Fc γ R)
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56 90 family. It is comprised of 6 receptors in humans and 4 in mice, which vary in expression pattern and

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91 affinity for IgG subclass.[10] Another receptor capable of interacting with IgG in both humans and
92 mice is FcRn, which is widely expressed throughout the body. The pH dependent nature of FcRn-IgG
93 interactions allows the receptor to scavenge IgG from lysosomes at an acidic pH, releasing it back
94 into the circulation at neutral pH, providing the long *in vivo* half-life of antibodies.[11-13]
95 In addition to the potential issue of altered efficacy arising from the lack of endogenous IgG (and
96 reduced competition for FcγR with therapeutic mAb) in NOD SCID mice, previous reports indicate
97 that immune-compromised mice, such as NOD SCID and NSG, have reduced mAb half-life compared
98 to related strains.[14-16] More recently, it was reported that NOD SCID mice display an anomalous
99 biodistribution of therapeutic antibodies, including reduced tumour targeting.[17] This suggests
100 further work is required to understand the limitations of these models and develop strategies to
101 overcome their shortcomings to make more translationally-relevant pre-clinical tumour models.
102 During a recent project examining the efficacy of a tumour targeting antibody in NOD SCID mice, we
103 noted rapid mAb clearance of human (h) IgG1 and mouse (m) IgG2a isotypes. Using a Eμ-Tcl1
104 hCD20+ tumour model we found this rapid clearance resulted in reduced efficacy of clinically
105 relevant mAb, such as rituximab. Employing genetically altered mice, we showed the rapid mAb
106 clearance was dependent on the expression of the inhibitory FcγR, FcγRII. Additionally, we identified
107 a reduced level of FcRn expression in NOD SCID mice, leading us to propose a novel hypothesis for
108 how mAb half-life is regulated in these strains and means through which it can be overcome.

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10 111 **MATERIALS AND METHODS**

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12 112 **In vivo experiments.** Mice used in this study were bred and maintained in local facilities with
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14 113 experiments approved through local ethics committees and performed according to Home Office
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16 114 guidelines.

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20 116 **Generating bone marrow chimera.** Recipient mice were provided with acid water, pH 2.5 on day -7
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22 117 until 14 days after bone marrow receipt. Recipients received 1.1Gy radiation on days -1 and 0 using a
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24 118 MultiRad 350 X-ray Irradiator (Faxitron). Bone marrow was harvested from donor mice and $3-8 \times 10^6$
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26 119 cells injected I.V. into recipients. Systemic reconstitution was confirmed by flow cytometry 8-10
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28 120 weeks after engraftment.

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31 122 **hCD20⁺ E μ TCL ϵ 1-1 tumour model.** This model has been described previously.[18, 19] Briefly, 1×10^7
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33 123 cryopreserved E μ -TCL-1 transgenic (Tg) or hCD20⁺ E μ -TCL ϵ 1-1 Tg tumour splenocytes were injected
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35 124 I.P. into recipient mice. The presence of tumour was monitored in peripheral blood. Once tumour
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37 125 cells (CD19⁺CD5^{mid}) were detectable by flow cytometry, mice were treated. The white blood cell
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39 126 count was determined using a Coulter Z1 particle counter with red blood cells (RBC) lysed using ZAP-
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41 127 OGLOBIN II (both Beckman Coulter) or by flow cytometry using Precision Count beads (Biolegend).

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44 129 **In vivo antibodies.** All clinical antibodies were gifted from the Southampton General Hospital
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46 130 oncology pharmacy. Others were produced in-house. 18B12 and Rituximab isotype variants were
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48 131 cloned onto the appropriate IgG framework, produced in CHO cells and purified from culture
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50 132 supernatant with Protein A. Purity was assessed by electrophoresis (Beckman EP; Beckman) and lack
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52 133 of aggregation confirmed by SEC HPLC. Unless otherwise stated, all antibodies were administered
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54 134 I.P. in 200 μ l sterile PBS (Severn Biotech).

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136 **Flow cytometry.** Flow cytometry was performed using the antibodies listed in supplementary Table

137 1. anti-mFcγR have been reported previously.[20, 21] Following staining, RBC lysis buffer was added

138 (AbD Serotec) and cells washed before analysis on a FACS Canto or FACS Calibur flow cytometer (BD

139 Biosciences). Alexafluor647 labelled MST-HN and H435A Abdegs for analysing FcRn expression were

140 a kind gift from Prof Sally Ward (University of Southampton) and used at 5µg/ml with Fc block

141 (2.4G2, 5µg/ml) prior to extracellular staining.

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143 **Generating bone marrow derived macrophages (BMDM).** The tibia and fibia-femur of mice were

144 flushed with sterile complete RPMI (RPMI 1640 (Life Technologies), 2mM L-glutamine, 1mM sodium

145 pyruvate, 100U/ml penicillin, 100µg/ml streptomycin (all Life Technologies), 10% foetal calf serum

146 (Sigma-Aldrich). Cells were plated in 6-well plates at 0.8x10⁶ cells/ml in complete RPMI +20% L929

147 conditioned media for 7-10 days.

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149 **Determining plasma IgG concentration.** IgG concentration was determined by ELISA with reference

150 to a standard curve of the same antibody as follows: Fer-for hIgG, maxisorp plates (Thermo

151 Scientific) were coated with 5µg/ml goat anti-human Fc-specific polyclonal antibody (Sigma-Aldrich)

152 and blocked with PBS +1% BSA before addition of serum for 1hour and washing. Detection was with

153 Horseradish peroxidase (HRP) conjugated F(ab')₂ goat anti-hFc specific antibody (Jackson

154 Immunoresearch). Plates were incubated with OPD substrate andwith OD₄₉₅ measured using an

155 Epoch microplate spectrophotometer (Biotek). For quantification of mIgG, plates were coated with

156 rabbit anti-mIgG and detected with HRP- rabbit anti-mIgG (both Jackson Immunoresearch).

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10 158 **Heat aggregation of IgG** Purified IgG was heated to 64°C for 30 minutes. The aggregated fraction
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12 159 was purified on a superdex S200 column (GE Healthcare). Aggregation was confirmed by HPLC using
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14 160 a Zorbax GF-250 column (Agilent).

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18 162 **Producing mFcγRII extracellular domain protein.** RNA was isolated and cDNA generated from SCID
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20 163 or NOD SCID BMDMs and the mFcγRII gene amplified using gene specific primers. Subsequently, the
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22 164 extracellular domain (residues 1-207) of mFcγRII were cloned with the addition of a 6xHis tag. The
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24 165 construct was transfected into MEXi -293E cells (IBA lifesciences) and FcγRII-His expressed according
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26 166 to the manufacturer's protocol and protein purified using a HisTrap HP column on an AKTA prime
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28 167 system (Both GE biosciences) and purity confirmed by SDS-PAGE.

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31 169 **Surface Plasmon resonance (SPR) analysis.** SPR was performed using a Biacore T100 system
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33 170 upgraded to a T200 (GE Life Sciences). For mFcγRII isoforms, anti-His capture antibody was
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35 171 immobilised on a CM5 chip (GE life Sciences). Purified FcγRII-His (10 µg/ml) was flowed over the chip
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37 172 at 30µl/min for capture. IgG was injected at 30µl/min. For all other analysis, IgG was immobilised via
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39 173 amine coupling with a target of 2000 RU. Recombinant mFcγRII or mFcRn (R&D systems) was flowed
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41 174 over the immobilised IgG in HBS-EP+ buffer (GE Life Sciences) at pH7.4 or pH6.0. Affinity constants
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43 175 were determined by analysis with Biacore Bioevaluation software assuming 1:1 binding.

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45 177 **Quantitative polymerase chain reaction (qPCR)** mRNA was extracted from SCID or NOD SCID
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47 178 splenocytes and hepatocytes using an RNeasy Mini Kit (Qiagen) and cDNA generated using a
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49 179 Superscript III reverse transcription kit (Life Technologies). qPCR was performed using GoTaq qPCR
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51 180 master mix (Promega) using gene specific primers, with HPRT1 as a control. Ct values were
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53 181 normalised using HPRT1 values and the $\Delta\Delta C_t$ method used to calculate fold change.

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12 183 **Western blotting.** Lysates were produced from 5×10^6 SCID or NOD SCID splenocytes and
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14 184 hepatocytes using RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Deoxycholate, 0.1% SDS, 50 mM
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16 185 Tris, pH 8) and run on a 12% Novex Nupage BIS-TRIS gel (Thermo Fisher) before transfer to a
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18 186 methylcellulose membrane (GE Lifesciences). Primary antibodies were anti-mouse FcRn and Lamin B
19 187 with detection using an HRP-conjugated donkey anti-goat antibody (Supplementary Table 1).
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23 189 **Immunofluorescence.** Liver tissue from BALB/c or NSG mice was embedded in OCT (CellPath) and
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25 190 frozen in isopentane on dry ice. $8 \mu\text{M}$ sections were cut and transferred to Superfrost plus slides
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27 191 (Thermo Scientific), air dried overnight and fixed in 100% acetone. Following blocking, primary
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29 192 antibodies against FcRn or Fc γ RII were added overnight before detection with Alexafluor488-
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31 193 labelled secondary antibody (Supplementary Table 1) for 45 minutes. Subsequently, primary
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33 194 antibodies against Clec4F or cytokeratin 8 were added for 2 hours before detection with AlexaFluor-
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35 195 549 or AlexaFluor-568 conjugated secondary antibodies (Supplementary Table 1). Slides were
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37 196 mounted using Vectashield hardset with DAPI (Vector Laboratories).
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39 197 Images were collected using a CKX41 inverted microscope with a reflected fluorescence system
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41 199 0.65 objective lenses (all from Olympus). Images were transferred to ImageJ (Fiji) [or Photoshop](#)
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43 200 [\(Adobe\)](#) where background autofluorescence was removed, contrast stretched and brightness
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45 201 adjusted to maximise clarity, with all images treated equivalently.
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48 203 **RESULTS**

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51 204 **Anti-tumour mAb therapy is less effective in NOD SCID mice due to rapid antibody clearance.**
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10 205 To explore potential differences of recipient mouse strains on immunotherapy efficacy, SCID and
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12 206 NOD SCID mice bearing established hCD20⁺ Eμ-TcCL1-1 tumours were treated with rituximab.
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14 207 Although initial tumour clearance was comparable between strains, 14 days after mAb treatment
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16 208 there were significantly more tumour cells in the peripheral blood of NOD SCID compared to SCID
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18 209 mice (Figure 1A and B). To determine if this was associated with rituximab's type I nature,[22, 23] we
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20 210 repeated the experiment with the type II anti-hCD20 mAb, BHH2,[18] and observed the same
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22 211 reduced efficacy in NOD SCID compared to SCID mice (Figure 1B).
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24 212 To understand this difference in efficacy, the concentration of injected hIgG in the plasma of mice
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26 213 following treatment was determined (Figure 1C). This revealed that 7 days after mAb treatment
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28 214 there was significantly less (~10 fold) hIgG in the plasma of NOD SCID compared to SCID mice (16.7 v
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30 215 1.6 µg/ml) suggesting that rapid hIgG1 clearance in NOD SCID mice was responsible for the less
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32 216 prolonged tumour deletion.
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34 217 To investigate ~~if-whether~~ the rapid clearance was related to the mAb, strain and/or tumour, an
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36 218 alternative hIgG1 mAb, cetuximab, was administered to non-tumour bearing SCID and NOD SCID
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38 219 mice. Cetuximab was also more rapidly cleared from NOD SCID compared to SCID mice with hIgG
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40 220 being undetectable in the plasma of NOD SCID mice by day 7 post-administration (Figure 1D). Similar
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42 221 results were also observed with other hIgG1 mAb including trastuzumab (Supplementary Figure 1),
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44 222 showing that the rapid clearance is directly related to the NOD SCID strain, independent of tumour
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46 223 and a common feature of ~~therapeutically-therapeutically~~-relevant hIgG1 mAb. Importantly, the
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48 224 hIgG1 clearance in SCID and NOD mice was comparable to that of immune-competent BALB/c mice
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50 225 (Supplementary Figure 2 and previously shown [24]), confirming fast hIgG1 clearance in NOD SCID
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52 226 mice, rather than slow clearance in SCID or NOD mice. Furthermore, the lack of a difference in SCID
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54 227 mice demonstrates that rapid hIgG clearance does not result from the absence of endogenous IgG or
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56 228 immune deficiency *per se*.
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229 **Rapid mAb clearance in NOD SCID mice is isotype dependent and requires both SCID and NOD**
230 **genotypes.**

231 To determine if rapid mAb clearance in NOD SCID mice extended beyond hlgG1, isotype switch
232 variants of rituximab were generated and administered to SCID or NOD SCID mice. Similar to hlgG1,
233 mlgG2a also had a significantly faster mAb clearance in NOD SCID mice (Figure 1D), being no longer
234 detectable in the plasma by day 14. In contrast, hlgG2 and mlgG1 had similar clearance rates in both
235 strains. These results demonstrate that faster mAb clearance in NOD SCID mice is isotype
236 dependent.

237 We next assessed if-whether the rapid IgG clearance occurred in NOD and NSG strains. NOD mice
238 had a normal hlgG1 clearance rate, akin to that seen in SCID and BALB/c (Figure 2A). However, NSG
239 mice displayed rapid clearance, comparable to that in NOD SCID mice (Figure 2B). These data
240 demonstrate that both NOD and SCID phenotypes are necessary to confer rapid IgG clearance.
241 Moreover, the differences between isotypes in NOD SCID mice also occurred in the NSG strain, with
242 hlgG1 and mlgG2a but not mlgG1 exhibiting rapid clearance (Figure 2B).

244 **Rapid hlgG1 clearance is dependent on FcγR binding.**

245 Given that mlgG2a and hlgG1 have similar FcγR binding profiles (binding to all mFcγR, with
246 substantial affinity for several activatory FcγR), we hypothesised that the rapid mAb clearance of
247 hlgG1 and mlgG2a isotypes in NOD SCID mice was mediated by FcγR.[25, 26] This was investigated
248 using an N279Q (NQ)-mutant of rituximab which lacks glycosylation at N297 and does not robustly
249 engage mFcγR (without compromising interaction with FcRn).[27] The NQ-mutant remained present
250 in the plasma of NOD SCID mice at significantly higher concentrations at all time-points, supporting
251 mFcγR involvement in the rapid hlgG1 clearance in NOD SCID mice (Figure 2C). Moreover, the

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10 252 concentration of rituximab-NQ was comparable between SCID and NOD SCID mice at all time-points
11 suggesting that abrogation of mFcγR binding restored normal mAb clearance rate.

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16 255 **SCID and NOD SCID mice have comparable FcγR expression levels.**

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18 256 Having established that the rapid hlgG1 clearance rate in NOD SCID mice was likely dependent on
19 mFcγR, the relative expressions levels of these receptors in SCID and NOD SCID mice was

20 257 investigated (Figure 3A). **Whilst there were no statistically significant differences in expression levels**

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22 258 **(2-way ANOVA P>0.05) trends towards differential expression were observed.** mFcγRII expression

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24 259 was lower on both Ly6C^{hi} and Ly6C^{lo} monocytes in NOD SCID compared to SCID mice (Figure 3B).

25 260 Neutrophil and splenic macrophage FcγRIII expression was higher in SCID mice, with a similar

26 261 expression profile for BMDMs (Supplementary Figure 3a). The expression of mFcγRI was not

27 262 investigated as it is known to contain multiple polymorphisms in NOD SCID mice which prevents its

28 263 detection using available antibodies.[28] The subtle differences in activatory mFcγR expression

29 264 detailed above appear to be compensatory with a similar overall expression of activatory mFcγR in

30 265 each strain. In summary, only monocyte FcγRII was found to **substantially** differ between SCID and

31 266 NOD SCID mice; **the relevance of this to mAb clearance rate remains to be determined.**

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35 270 **Rapid hlgG1 clearance in NOD SCID mice is dependent on FcγRII.**

36 271 To understand the contribution of specific mFcγR to rapid antibody clearance in NOD SCID mice, we

37 272 made use of animals lacking different classes of mFcγR. In NOD SCID FcγR γ^{-/-} mice (which express no

38 273 activatory FcγR at the cell surface[29]) there was no significant difference in the concentration of

39 274 hlgG1 over time compared to NOD SCID mice (Figure 4A); **], demonstrating that a lack of activatory**

40 275 mFcγR does not influence hlgG1 clearance. However, in NOD SCID mice deficient in the inhibitory

41 276 mFcγRII, the concentrations of hlgG1 were significantly increased compared to wild-type NOD SCID

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Commented [OR4]: Reviewer 3 point 4: Here we make it clear that at this point in the manuscript the relevance of expression level to half- life has not yet been determined

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10 276 mice retaining mFcγRII (Figure 4B) and comparable with SCID mice. These results demonstrate that
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12 277 the rapid hlgG1 clearance in NOD SCID mice is dependent on mFcγRII. Moreover, this result suggests
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14 278 that the somewhat reduced FcγRII expression seen previously in NOD SCID mice is not responsible
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16 279 for the fast hlgG1 clearance rate.
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Commented [OR5]: Reviewer 3 point 4. At this point it is apparent that the reduced FcγRII expression is not responsible for the effects observed, therefore we have clarified this point here.

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20 281 **The polymorphic variants of mFcγRII have comparable affinity for hlgG1.**

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22 282 A number of autoimmune strains, including NOD express the ly17.1 form of mFcγRII whilst most
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24 283 other in-bred strains, including BALB/c, express the ly17.2 variant.[30] These two polymorphic forms
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26 284 vary in four amino acids, three of which are located in the extracellular domain.[30] The extracellular
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28 285 domain of FcγRII from SCID and NOD SCID mice was cloned and expressed; their relative affinity for
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30 286 IgG was then determined by SPR. Neither heat aggregated, pooled hlgG or individual isotypes of IgG
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32 287 displayed substantially different binding affinities to the ly17.1 and ly17.2 variants (Table 1).
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	NOD SCID KD (M x10 ⁻⁶)	SCID KD (M x10 ⁻⁶)
Aggregated hlgG	0.13	0.15
Cetux hlgG1	4.38	5.42
Ritux hlgG1	2.50	3.18
Ritux hlgG2	4.82	4.65
Ritux mlgG1	2.25	2.02
Ritux mlgG2a	2.43	2.63

Table 1. Affinity of mFcγRII variants for IgG subtypes. Recombinant mFcγRII extracellular domains from SCID or NOD SCID mice were captured on a Biacore CM5 chip using an immobilised anti-HIS antibody. IgG of specific isotypes or heat aggregated, pooled hlgG was flowed over the chip and the KD value calculated using Biacore evaluation software.

Absence of haematopoietic mFcγRII or phagocytes does not restore normal mAb clearance.

mFcγRII is expressed on both haematopoietic and non-haematopoietic cells.[30, 31] We therefore sought to determine which mFcγRII-expressing cells were responsible for the rapid clearance of hlgG1. Accordingly, NOD SCID mice were irradiated and reconstituted with bone marrow from NOD SCID FcγRII^{-/-} mice (Figure 4C and D). These mice, reconstituted with haematopoietic cells lacking mFcγRII displayed rapid clearance of hlgG1 and mlgG2a, indicating that mFcγRII on cells of the haematopoietic system was not responsible for the rapid mAb clearance (Figure 4E). We next considered whether phagocytes, particularly tissue resident macrophages, might be responsible and so deleted them with clodronate liposomes. This approach effectively removed macrophages

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(Supplementary Figure 3b) but only resulted in a small increase in circulating hlgG1 7 days after mAb administration, with no hlgG1 detectable by day 14 (Figure 4F). This suggests that phagocytes in NOD SCID mice are not primarily responsible for the rapid hlgG1 clearance and that a non-haematopoietic cell type is responsible. Given their high expression of mFcγII, the Liver Sinusoidal Endothelial cells (LSEC) seem the most likely candidate.[31] We confirmed high expression of mFcγRII on these cells by immunofluorescence of livers from both BALB/c and NOD SCID mice (Supplementary Figure 4 and b). **Moreover, we found hlgG detectable at substantially higher levels within the liver of NSG than SCID mice following administration of hlgG1 mAb confirming a role for the liver as a site of hlgG1 accumulation (Supplementary Figure 4c).**

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NOD SCID mice have reduced FcRn expression.

As mFcγRII is not known to directly regulate mAb clearance, we next considered whether FcRn might be involved in the process of controlling clearance rate in the NOD SCID mouse. Importantly, FcRn in NOD SCID mice does not contain any sequence variations compared to other strains and has normal binding to both human and mouse IgG.[14]. However, qPCR revealed significantly lower FcRn transcription in both spleen and liver of NOD SCID versus SCID mice (Figure 5A). This result was confirmed at the protein level by western blotting (Figure 5B) and flow cytometry using the MST-HN protein which maintains FcRn binding at both acidic and neutral pH (Figure 5C).[32] This latter approach demonstrated a lower expression of FcRn in Ly6C⁺ monocytes from NSG compared to SCID mice (MFI [3545706](#) v [29611957](#), mean of N=3-4). Combined, these results demonstrate that there is a lower expression of FcRn in the tissues known to be important for IgG recycling (spleen and liver) of NOD SCID mice compared to SCID.[33]

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Commented [OR6]: Reviewer 3 point 5 – we have now referenced the new part of supplementary figure 4 showing staining for hlgG in the liver.

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10 327 **mFcγRII has a pH dependent affinity for IgG isotypes and is expressed on the same cell types as**
11 **FcRn.**

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14 329 Whilst reduced FcRn could explain rapid IgG clearance, it does not provide an explanation for the
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16 330 isotype dependent nature of the effects seen, as all isotypes should be affected equally. In contrast,
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18 331 mFcγRII is known to display differential affinity for IgG isotypes (high for mIgG1, low for mIgG2a and
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20 332 hIgG1) and so we considered if mFcγRII specificity might be involved in regulating the clearance of
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22 333 the different isotypes. Using SPR and two different mAb of each isotype, we confirmed that at pH7.4
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24 334 mFcγRII had ~10 fold higher affinity for mIgG1 ($2.74 \times 10^{-7} \text{M}$) versus mIgG2a ($1.18 \times 10^{-6} \text{M}$) and hIgG1
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26 335 (3.02×10^{-6}) whilst the affinity for hIgG2 was lower still ($7.65 \times 10^{-6} \text{M}$) (Table 2 and Figure 5D).

27 336 We next considered that mFcγRII might internalise cell-surface bound IgG and by virtue of its higher
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29 337 affinity, preferentially protect mIgG1 from degradation following internalisation. To do this, it would
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31 338 need to remain bound to IgG in a low pH environment, akin to FcRn. We therefore repeated SPR
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33 339 analysis at pH6.0, and revealed that mFcγRII retained binding at low pH, with affinity for mIgG1,
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35 340 mIgG2a and hIgG1 ~100-fold higher than at pH7.4. Notably, the KD for mIgG1 binding mFcγRII was
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37 341 $2.77 \times 10^{-9} \text{M}$, >10-fold higher than for hIgG1 and mIgG2a. This suggests that mFcγRII is capable of
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39 342 binding IgG at an acidic pH with the potential to protect IgG from degradation being greatest for
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41 343 mIgG1. Using previously published affinity data for IgG binding to mFcRn, we calculated the ratio of
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43 344 mFcγRII:FcRn binding for different isotypes at pH6.0 (Table 2 and Figure 5D). [34] hIgG2 exhibited a
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45 345 high mFcγRII:FcRn ratio, suggesting preferential binding for FcRn at an acidic pH. In comparison,
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47 346 hIgG1 had a ratio around 1 (indicating no overall preference) whereas mIgG1 had a low ratio,
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49 347 preferentially binding with a higher affinity to mFcγRII than to FcRn.

50 348 Having hypothesised that the differential interaction with FcRn and FcγRII may play a role in the
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52 349 recycling of IgG, and with the knowledge that the liver expresses 75% of the FcγRII in the mouse, we
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54 350 sought to determine the distribution of these two receptors within the liver. [31] We found mFcγRII
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56 351 on a subset of Clec4F⁺ Kupffer cells but not on Cytokeratin 8⁺ hepatocytes (Supplementary Figure 4).

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10 352 However, the majority of mFcγRII was expressed by LSEC as determined by their morphology. This is
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12 353 consistent with a previous study reporting 90% of the liver mFcγRII as being expressed by LSEC when
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14 354 assessing immunofluorescence by pixel intensity.[31] We found FcRn to be widely expressed
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16 355 throughout the liver including on Kupffer cells and hepatocytes (Supplementary Figure 4). This is
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18 356 consistent with a previous report showing FcRn mRNA in various cell types, additionally identifying
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20 357 LSEC as having the highest expression level.[31] Together, these results suggest that LSEC may be the
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22 358 predominant cell type co-expressing FcγRII and FcRn.
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24 359 Overall, these data provided the possibility that antibody clearance in NOD SCID strains is controlled
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26 360 through differential engagement of the various isotypes by mFcγRII and FcRn. However, these
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28 361 effects have not been reported previously in standard in-bred strains, or the single NOD and SCID
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30 362 strains, indicating that the proposed pathway, which is at least in part mediated by isotype-
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32 363 dependent binding of mFcγRII, is only revealed in the absence of endogenous IgG.

	FcγRII KD pH7.4 (M)	FcγRII KD pH6 (M)	Published FcRn KD pH6 (M)	KD ratio FcγRII/FcRn
hlgG1 #1	3.02 x10 ⁻⁶	4.92 x10 ⁻⁸	7.2x10 ⁻⁸	0.68
hlgG1 #2	6.61 x10 ⁻⁶	7.90 x10 ⁻⁸	7.2 x10 ⁻⁸	1.10
hlgG2 #1	7.65 x10 ⁻⁶	2.10 x10 ⁻⁷	6.3x10 ⁻⁸	3.33
hlgG2 #2	1.45 x10 ⁻⁵	2.18 x10 ⁻⁷	6.3x10 ⁻⁸	3.46
mIgG1 #1	2.74 x10 ⁻⁷	2.77 x10 ⁻⁹	1.57x10 ⁻⁶	0.0018
mIgG1 #2	4.98 x10 ⁻⁷	3.62 x10 ⁻⁹	1.57x10 ⁻⁶	0.0023
mIgG2a #1	1.18 x10 ⁻⁶	2.36 x10 ⁻⁸	4.9x10 ⁻⁷	0.054
mIgG2a #2	1.10 x10 ⁻⁶	2.41 x10 ⁻⁸	4.9x10 ⁻⁷	0.049

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10 364 **Table 2.** Affinity of mFcγRII and mFcRn for IgG subtypes. Antibodies were immobilised on a Biacore
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12 365 CM5 chip before flowing mFcγRII or mFcRn over the chip. FcRn was used at pH6 with FcγRII at pH6
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14 366 and pH7.4. KD was calculated using Biacore evaluation software.
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18 368 **Rapid hlgG1 clearance and reduced mAb efficacy can be overcome by IgG reconstitution.**

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20 369 We therefore hypothesised that the addition of exogenous mlgG would restore normal mAb
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22 370 clearance rate in NOD SCID mice. Accordingly, SCID and NOD SCID mice were reconstituted with
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24 371 mlgG1 and mlgG2a to a level equivalent to that seen in the plasma of wild type BALB/c mice
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26 372 (Supplementary Figure 5). Subsequently, the clearance of hlgG1 was investigated (Figure 5E).
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28 373 Reconstitution with mlgG overcame the rapid clearance of hlgG1 in NOD SCID mice such that it
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30 374 became comparable to that observed in SCID mice. In contrast, mlgG addition did not significantly
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32 375 alter the clearance of hlgG in SCID mice. Additionally, reconstitution with mlgG substantially reduced
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34 376 the accumulation of hlgG in the liver (Supplementary Figure 4c). Importantly, the expression of FcRn
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36 377 was not altered by reconstitution with mlgG (Figure 5c). Finally, we sought to determine if
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38 378 overcoming rapid clearance of hlgG1 by mlgG reconstitution could improve therapy, using an Eμ-
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40 379 TCL1 tumour and a hlgG1 antibody targeting mouse CD20 (18B12). Using this second tumour model
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42 380 we found the differences in duration of therapy between SCID and NSG mice was maintained, with
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44 381 tumour growth recurring in NSG before SCID mice (Figure 5F). We then compared the duration of
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46 382 tumour deletion in NSG mice versus NSG mice reconstituted with mlgG, using the same protocol as
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48 383 before. Reconstitution with mlgG was able to overcome the impaired therapy and restored
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50 384 comparable levels of tumour deletion and control to that observed in SCID mice both in terms of
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52 385 tumour (Figure 5F) and number of tumour cells (Supplementary Figure 6). This demonstrates that
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54 386 reconstitution with mlgG is able to restore mAb efficacy in NSG mice.
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60**DISCUSSION**

Our observations with rituximab showed reduced tumour control in NOD SCID compared to SCID mice. Given that equivalent initial tumour clearance was observed, and deletion is known to be dose dependent, this impairment was considered to be a direct result of reduced serum persistence and insufficient mAb at later time points.[35, 36] Rituximab is a type I mAb, known to be internalised through cis-binding to FcγRII following target binding, reducing its efficacy.[18, 37] Importantly, reduced tumour control was also seen with a type II anti-CD20 reagent, BHH2, indicating mAb internalisation was not causal and that a separate phenomenon related to the mouse strain was responsible. In support of this, rapid clearance of wild-type and chimeric hlgG1 mAb was described recently in ~~NOD-SCID mice although the mechanism was not discussed~~ both NOD SCID and NSG mice.[14, 16] Furthermore, we saw the same rapid hlgG1 clearance for two additional hlgG1 mAb, lacking targets in the mouse, showing this phenomenon to be independent of the presence of tumour and unrelated to F(ab)-mediated antigen binding.

Separately, both SCID and NOD mice had normal clearance of hlgG1, comparable to that of immune competent BALB/c mice, as reported previously.[24] This indicated that the genetic background of NOD mice, coupled with a lack of endogenous IgG (as a result of the SCID mutation) combined to elicit rapid hlgG1 clearance in NOD SCID mice. Importantly, our observations were replicated in NSG mice, indicating that rapid hlgG1 clearance is a feature of all NOD SCID derived strains.

mlgG1 was found to have normal clearance in NOD SCID mice, whereas both hlgG1 and mlgG2a had short half-lives. Both humans and mice have multiple activatory FcγR, but a single inhibitory receptor, FcγRII (FcγRIIb in humans). These receptors interact differentially with the various mouse and human IgG isotypes: mlgG2a and hlgG1 bind preferentially to multiple activatory receptors and as such have high activatory:inhibitory (A:I) ratios.[26] In contrast, mlgG1 exhibits binding to only a single activatory FcγR (FcγRIII) whilst retaining binding to FcγRII, yielding a corresponding low A:I ratio.[38] These observations provided a potential clue towards the isotype-based effects observed.

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10 413 A role for FcγR in this process was subsequently confirmed by using a hlgG1 N297Q mAb which
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12 414 abrogates binding to FcγRs.[27] Importantly, the N297Q mutation has been demonstrated not to
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14 415 alter hlgG1 clearance in immune compromised mice.[27] The normal clearance rate of N297Q mAb
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16 416 in NOD SCID mice suggested that the rapid clearance of hlgG1 (and mlgG2a) in NOD SCID mice was
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18 417 dependent on Fc:FcγR interaction.

19 418 Despite establishing a likely role for mFcγR in rapid mAb clearance, no gross changes in activatory
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21 419 FcγR expression levels were observed in NOD SCID versus SCID mice. The subtle inter-strain
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23 420 differences in mFcγRIII and mFcγRIV expression are likely to be compensatory and do not result in a
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25 421 large difference in the total A:I ratio or amount of activatory FcγR on the cell surface. One caveat
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27 422 here was that the expression level of mFcγRI in NOD SCID mice could not be determined by flow
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29 423 cytometry as its sequence varies considerably from the canonical sequence seen in most other
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31 424 strains (by 17 residues), and so cannot be detected using our existing reagents.[39] mFcγRII
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33 425 expression was found to be lower on monocytes from NOD SCID compared to SCID mice, likely due
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35 426 to the previously reported alterations upstream of the gene in NOD SCID mice associated with lower
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37 427 expression.[40] However, subsequent results, particularly in mice lacking mFcγRII expression,
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39 428 ~~demonstratesuggest~~ that the reduced expression of mFcγRII was unlikely ~~to be~~ responsible for the
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41 429 ~~rapidfast~~ mAb clearance.

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40 430 We additionally investigated the expression levels of FcRn, the primary receptor responsible for IgG
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42 431 recycling and long half-life.[41] Whilst it has a broad tissue distribution, expression is particularly
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44 432 prominent in the spleen and liver.[33, 41] Analysis of these tissues demonstrated that there was
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46 433 lower expression of FcRn in NOD SCID compared to SCID mice. SCID mice have previously been
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48 434 reported to have comparable FcRn expression and tissue distribution to immune-competent mice;
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50 435 indicating that the reduction is a result of the NOD phenotype rather than elevated expression in
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52 436 SCID mice.[33] However, given that the N279Q-mutated antibody which can interact with FcRn but

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10 437 not FcγR had a normal clearance rate in NOD SCID mice, the reduced FcRn expression [in](#)
11 [isolation alone](#) cannot explain the differences in mAb clearance.
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14 439 [To confirm a role for mFcγR in rapid mAb clearance in NOD SCID mice, To determine the receptor](#)
15 [responsible, we](#) used mice deficient either the activatory or inhibitory mFcγR by employing mFcR
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17 441 γ-chain -/- or mFcγRII-/- mice, respectively. Only the absence of mFcγRII restored normal hIgG1
18 442 clearance in NOD SCID mice. This observation supports the implications of the N297Q-mutant data;
19 443 i.e. failing to engage with mFcγRII and therefore phenocopying the effect in the mFcγRII-/- mice. [Our](#)
20 444 [observation that mFcγRII mediates this effect is in contrast to a previous study suggesting mFcγRIV is](#)
21 445 [responsible.](#)[16] [The previous study used an Fc-engineered antibody reported to have reduced](#)
22 446 [binding to FcγRIV; such mutations often result in broader changes to FcγR binding profiles with](#)
23 447 [causal effects of specific FcγR difficult to define. In contrast, in the present study we were able to](#)
24 448 [specifically define the role of FcγRII by using mice genetically deficient in FcγRII. However, this](#)
25 449 [mechanism does not explain the isotype differences in IgG clearance between SCID and NOD SCID](#)
26 450 [mice.](#)[16]

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35 451 Given the dependence on mFcγRII, we investigated this receptor in more detail. There are two
36 452 polymorphic variants of mFcγRII; the Iy17.1 haplotype expressed by NOD SCID mice, and the more
37 453 common Iy17.2 haplotype expressed by most other in-bred mouse strains, which differ by four
38 454 amino acids, three of which are extracellular.[42, 43] We confirmed previous observations that these
39 455 allotypes do not differ in their affinity for IgG. Whilst this SPR analysis assesses the likely effects of
40 456 the extracellular polymorphisms, further investigation is needed into the role of the remaining I258S
41 457 intracellular polymorphism to determine its influence on mAb internalisation. This could be of
42 458 importance given the role of the intracellular I232T polymorphism in hFcγRIIB, which alters the
43 459 ability of the receptor to cluster into lipid rafts and deliver inhibitory signals. [44]

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50 460 The data presented here indicate that no single factor can explain the isotype-dependent differences
51 461 in mAb clearance in NOD SCID versus SCID mice. Instead, the data support a more complex model

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10 462 whereby multiple factors arising from the NOD and SCID backgrounds combine to deliver the
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12 463 observed defect. We propose a model whereby mFcγRII accelerates initial mAb internalisation. The
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14 464 ability of FcγRII to mediate mAb internalisation has been previously reported in DC mediated antigen
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16 465 presentation and the internalisation of rituximab.[37, 45] hIgG1 N297Q and hIgG2 do not bind
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18 466 appreciably to FcγRII, preventing receptor-mediated internalisation, maintaining equivalent
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20 467 clearance in both SCID and NOD SCID mice. Similarly, in SCID mice with a normal level of FcRn, the
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22 468 IgG internalised via FcγRII can be efficiently recycled by FcRn to maintain serum persistence.
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24 469 However, in NOD SCID mice, this efficient FcRn mediated recycling does not occur due to the
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26 470 reduced FcRn expression levels, resulting in more rapid serum loss.

26 471 In trying to understand the isotype dependent nature of the rapid IgG clearance rate in NOD SCID
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28 472 mice, we unexpectedly observed an increased affinity of mFcγRII for all IgG at pH6.0. Crucially
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30 473 however, the affinity of mFcγRII for mIgG1 at pH6.0 was retained and ~10-fold higher than for the
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32 474 other isotypes investigated. This raises the possibility that mIgG1 is protected from degradation
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34 475 under acidic conditions due to continued association with mFcγRII following internalisation. In
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36 476 contrast, mIgG2a and hIgG1 would remain unprotected and become degraded following
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38 477 internalisation, due to mFcγRII having lower affinity for these isotypes at pH6.0. We propose that
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40 478 this reduced degradation occurs only in the presence of reduced FcRn expression in NOD SCID mice,
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42 479 further work is however required to confirm this hypothesis. These data therefore suggest a complex
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44 480 role for FcγRII in mAb clearance; it is required for the internalisation of IgG, preventing external
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46 481 catabolism, yet it also delivers IgG for internal lysosomal degradation unless it exhibits sufficient
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48 482 affinity for IgG binding at pH6.

46 483 Prior experiments in conditional knock-out mice suggested that both endothelial and
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48 484 haematopoietic cells regulate IgG levels in mice.[46, 47] The transfer of mFcγRII -/- NOD SCID bone
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50 485 marrow into NOD SCID mice resulted in mice deficient in haematopoietic mFcγRII but with mFcγRII
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52 486 expression on non-haematopoietic (predominantly endothelial) cells. hIgG1 clearance in these

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10 487 chimeras was unaltered compared to NOD SCID mice, demonstrating that haematopoietic cells were
11 not responsible. The liver was previously found to be the main site of IgG clearance, accounting for
12 488 not responsible. The liver was previously found to be the main site of IgG clearance, accounting for
13 489 30% of all antibody degradation.[48] The same organ has also been shown to contain 75% of the
14 mFcγRII in the body, the receptor demonstrated here to be essential for rapid NOD SCID mAb
15 490 mFcγRII in the body, the receptor demonstrated here to be essential for rapid NOD SCID mAb
16 clearance.[31] **Additionally, we were able to demonstrate greater accumulation of hlgG in the liver**
17 491 **of NSG compared to SCID mice, an effect that was overcome by the addition of exogenous mlgG.**
18 492 **Our immunofluorescence studies, combined with previous reports, suggest that LSEC are the**
19 493 **predominant cell type in the liver expressing both FcγRII and FcRn, the receptors regulating the fast**
20 494 **mAb clearance.[31, 49] LSEC have been described as having the highest rates of endocytic uptake in**
21 495 **the body. In addition, mFcγRII is required for the efficient clearance of small immune complexes. [31,**
22 496 **50] This leads us to hypothesise that LSEC are the key cell type responsible for fast mAb clearance**
23 497 **observed in the present study, with internalisation of IgG mediated by mFcγRII.**
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25 499 Further investigation is required to determine if pH and isotype dependent affinity is restricted to
26 500 mFcγRII or is common to the other FcγR. Moreover, it remains to be established if the same occurs
27 501 with the inhibitory FcγRIIb in humans. This could have implications for mAb therapy as it is known
28 502 that the A:I ratio of IgG binding to FcγR can determine the outcome of therapy, particularly where
29 503 the expression of hFcγRIIb may increase, such as within the tumour microenvironment.[51]
30 504 Moreover, in this context, the acidic pH of the tumour microenvironment may further modify this
31 505 ratio of A:I binding by altering the relative binding to individual receptors.

32 506 Given the importance of the rapid mAb clearance in NOD SCID mice on the therapeutic activity of
33 507 direct targeting hlgG1 mAb, we sought a means of restoring normal pharmacokinetics. By
34 508 reconstituting NOD SCID mice with physiological levels of mlgG, the rapid mAb clearance could be
35 509 overcome, restoring persistence equivalent to that observed in SCID and BALB/c mice. **Moreover,**
36 510 **this increased persistence of therapeutic mAb was able to recover anti-tumour efficacy to the same**
37 511 **level as seen in SCID mice. This result is in agreement with findings that the addition of human IVIg**

Commented [OR11]: Reviewer 3 point 5. Added reference in the discussion to our additional immunofluorescence experiments.

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10 512 can restore the normal half-life of an antibody-drug conjugate.[16] Moreover, it has been previously
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12 513 reported that the addition of exogenous IgG is able to overcome anomalous antibody biodistribution
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14 514 in NOD SCID mice, adding to the potential benefits of IgG reconstitution in tumour models.[17] We
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16 515 suggest that in the presence of exogenous IgG, mFcγRII is occupied (most likely by the mIgG1
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18 516 component, due to its higher affinity) and less internalisation of hIgG1 can occur. Based on these
19 517 observations, we propose that reconstitution with IgG should be a consideration when performing
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21 518 therapy experiments in NOD SCID mice in order to restore therapeutic antibody half-life.

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23 519 The implications for the findings presented here are wide-wide-reaching. With an increasing use of
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25 520 immune-compromised mice in pre-clinical investigation of mAb therapeutics, it is essential to
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27 521 understand how the choice of host strain can influence the outcome. The clearance rate of the most
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29 522 clinically relevant deleting isotypes are significantly shorter in NOD SCID and NSG mice than other
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31 523 immune-compromised strains such as SCID. This is likely to underplay the therapeutic efficacy of
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33 524 mAb used in these models and complicate comparisons between strains. Additionally, there are
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35 525 significant efforts ongoing to understand the isotype requirements for mAb directed against
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37 526 different targets, and with different Fc requirements.[52] Our work suggests that NOD SCID mice
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39 527 may not be a suitable host strain for determining the optimal mAb isotype or therapeutic dose due
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41 528 to complications arising from different isotype-dependent clearance rates, unless exogenous mIgG
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43 529 reconstitution is also provided. Specifically, we suggest that caution should be exercised when
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45 530 interpreting results from immune compromised mice on the NOD SCID background with regard to
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47 531 differences in antibody activity that could be explained by mAb clearance rate.

532 533 **DECLARATIONS**

534 **Ethics Approval**

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535 Animal experiments were cleared through local ethics committees and performed according to
536 Home Office guidelines under project license PB24EEE31.

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538 **Consent for Publication**

539 Not applicable

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541 **Availability of Data and Material**

542 All datasets used and/or analysed during the current study are available from the corresponding
543 author on reasonable request.

544

545 **Competing Interests**

546 MSC is a retained consultant for Bioinvent and has performed educational and advisory roles for
547 Boehringer Ingelheim, Merck KGaA, Baxalta and GLG. He has received research funding from
548 Bioinvent, Roche, Gilead, Iteos, UCB and GSK. The other authors have no financial conflicts of
549 interest.

550

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554 (A24721) as well as CRUK centre support C328/A25139.

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556 **Author Contributions**

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10 557 RJO, CIM, SJ, and KLC and VAP performed experiments. RJO analysed the data. RJO, MSC and MJG
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12 558 interpreted the results. PD and HTC generated critical reagents. RJO and MSC wrote the manuscript.

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17
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19
20 562 Sciences, Faculty of Medicine, University of Southampton, UK), Prof Falk Nimmerjahn and Prof Sally
21
22 563 Ward for helpful discussions relating to the experiments reported herein. SW additionally provided
23
24 564 the Abdeg reagents for quantifying FcRn expression.

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26 565 **REFERENCES**

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60**FIGURE LEGENDS**

Figure 1: Antibody mediated therapy is reduced in NOD SCID mice due to faster mAb clearance. A - C) E μ -Tcl1 x hCD20 Tg tumour cells were injected I.P. into SCID or NOD SCID mice. Once tumour was detectable in the peripheral blood animals were treated with 100 μ g rituximab or BHH2 I.P. A) 2 and 14 days after treatment, the percentage of tumour cells in the blood was assessed. B) The number of tumour cells in the blood 14 days after treatment was determined (n=4-9). C) The concentration of hlgG in the plasma was measured by ELISA with a significantly lower concentration of hlgG on day 7 in NOD SCID compared to SCID mice. hlgG was not detectable in the plasma of NOD SCID mice from day 14 onwards. D) In the absence of tumour, 100 μ g cetuximab or 100 μ g rituximab hlgG2, mlgG2a or mlgG1 was administered I.P. to SCID or NOD SCID mice. The concentration of human or mouse IgG in the plasma was determined by ELISA. (hlgG1 n=6-7; combined data from 2 independent experiments, hlgG2, mlgG1 and mlgG2a n=3, representative of 2 independent experiments). ND = not detectable. Statistics; 2 way ANOVA with multiple comparisons *P<0.05, **P<0.01, ***P<0.001. No significant differences were observed between SCID and NOD SCID mice receiving hlgG2 or mlgG1.

Figure 2: Rapid antibody half-life requires both the NOD and SCID phenotypes as well as functional Fc. A) 100 μ g cetuximab injected I.P. into NOD or NOD SCID mice with the concentration of hlgG in the plasma determined by ELISA, over 14 days. (n=4-6). B) 100 μ g rituximab hlgG1, mlgG1 or mlgG2a was injected I.P. into NSG mice and the concentration of mouse or human IgG in the plasma determined by ELISA, (n=63-84), representative of data combined from 2 independent experiments. (C) 100 μ g rituximab (hlgG1) or rituximab hlgG1 N297Q (NQ) was injected I.P. into SCID or NOD SCID mice and the concentration of hlgG in the plasma determined by ELISA. (SCID n=3-4, NOD SCID N=7 combined from 2 independent experiments) -results representative of 2 independent experiments. 2-way ANOVA with multiple comparisons, **P<0.01, ***P<0.001

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12 713 **Figure 3:** mFcyR expression profiling in SCID and NOD SCID mice. Splenocytes or peripheral blood
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14 714 from SCID and NOD SCID mice were stained with specific antibodies for Ly6c, Ly6G, CD11b or CD11c
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16 715 to identify monocytes, macrophages and neutrophils, concurrently with mAb to each mFcyR. N=32
17 716 representative of 3 combined data from 2 independent experiments, mean +range. No statistically
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19 717 significant differences were observed between strains (2-way ANOVA with Tukeys multiple
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21 718 comparison test, p>0.05)

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25 720 **Figure 4:** Rapid mAb clearance in NOD SCID mice is dependent on expression of mFcyRII but not
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27 721 activatory mFcyR. A) NOD SCID and NOD SCID FcR γ -chain deficient mice (NS γ -/-) were injected with
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29 722 100 μ g trastuzumab hlgG1 I.V. Tail blood was collected and the concentration of hlgG in the plasma
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31 723 determined by ELISA. (n=3-4) mean +S.D. By day 14 hlgG was not detectable in the plasma of both
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33 724 strains. B) SCID, NOD SCID and NOD SCID mFcyRII-/- mice were injected with 100 μ g cetuximab I.V.
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35 725 The concentration of hlgG in plasma was determined 2, 7 and 14 days later by ELISA. (n=3-4 per
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37 726 group), representative of 2 independent experiments. 2-way ANOVA with multiple comparisons
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39 727 **p<0.01, ***p<0.001. C) NOD SCID mice were irradiated and reconstituted with bone marrow cells
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41 728 harvested from NOD SCID FcyRII-/- mice. D) Engraftment was confirmed by staining peripheral
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43 729 CD11b+ cells for mFcyRII expression. E) 100 μ g rituximab hlgG1 or mlgG2a was injected into these or
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45 730 control NOD SCID mice. The concentration of human or mouse IgG in the plasma was determined by
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47 731 ELISA. (n=3-4), mean +S.D. F) NOD SCID mice were injected I.V. with clodronate- or PBS-containing
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49 732 liposomes on days -3, -1, 6 and 13 to deplete macrophages. On day 0, 100 μ g rituximab was
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51 733 administered I.P. and the concentration of hlgG in the plasma determined by ELISA.

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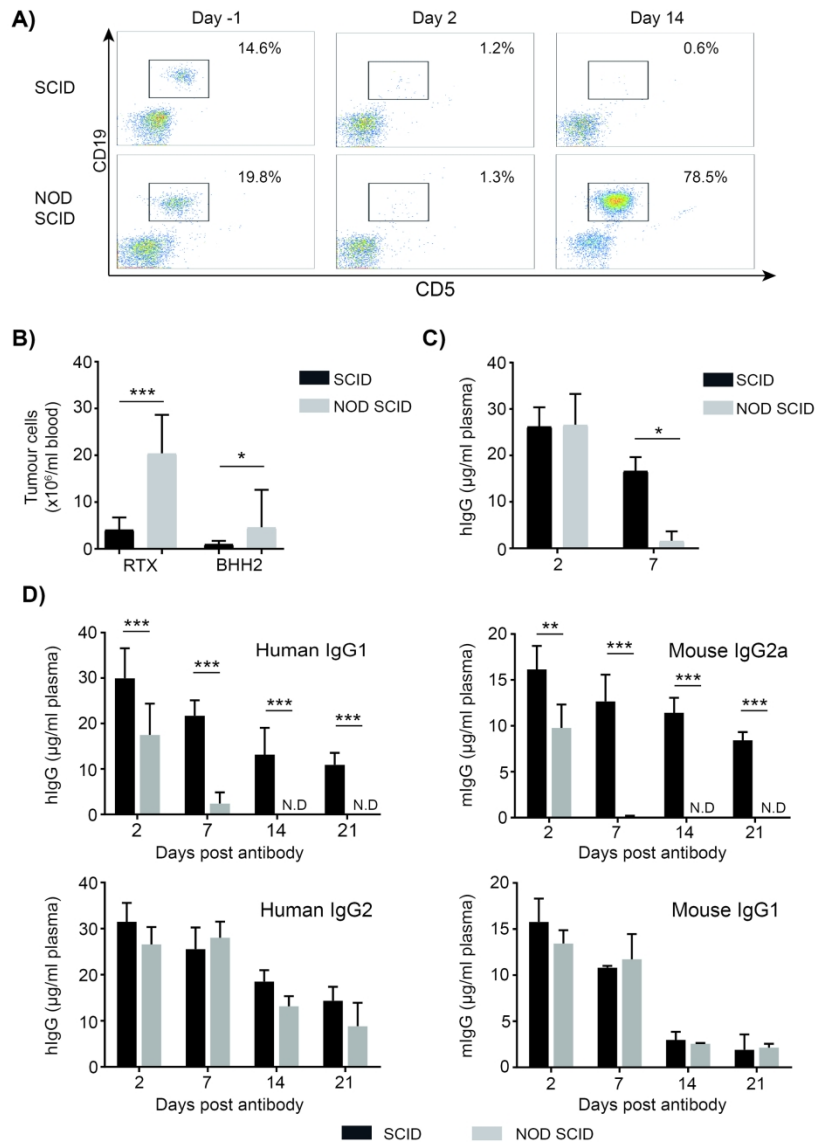
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Figure 5: NOD SCID mice have low expression of FcRn, associated with rapid mAb clearance, which can be overcome by IgG reconstitution. A) cDNA was produced from SCID and NOD SCID spleen and liver lysates with FcRn transcript expression analysed by qPCR using the ddCT method and expressed relative to that in SCID mice. (n=4-5), unpaired T-test **P<0.01, ***P<0.001. B) Splenocytes from SCID and NOD SCID spleens were lysed and Western blot performed on the lysates for FcRn and Lamin B as a loading control. C) Uptake of MST-HN Abdeg by splenocytes from SCID and NSG mice as well as mIgG reconstituted NSG mice, gated on CD11b+Ly6G-Ly6C+ cells. No protein (greyred), H435A control (redblue) and MST-HN (Blueorange) are shown. N=3-4 with a representative example for each group shown Representative example of N=4. D) Rituximab hlgG1, hlgG2, mlgG1 or mlgG2a was immobilised on Biacore CM5 chips and recombinant mFcγRII flowed over the chip at pH6 or pH7.4 with a highest receptor concentration of 1000nM and 5-fold serial dilutions. E) SCID or NOD SCID mice were reconstituted with 400µg mlgG2a and 500µg mlgG1 on day 0. An additional 200µg mlgG2a was given on day 3, 6, 9, 12 and 15. 100µg rituximab was then given I.P. on day 0 and the concentration of hlgG in the plasma determined by ELISA. (n=4-8), data combined from 2 independent experiments, mean +S.D. 2-way ANOVA with multiple comparisons ***P<0.001, n.s. = not significant. F) Eµ-Tcl1 tumour cells were injected I.P. into SCID or NSG mice. Once tumour was detectable in the peripheral blood a group of NSG mice were reconstituted with mlgG as described above, animals were then treated with 100µg hlgG1 anti-mCD20 (18B12) I.P. representative FACS plots are shown on the left. 14 days after treatment, the percentage of tumour cells in the blood was assessed and plotted. (n=5-6 per group), mean +S.D. 1-way ANOVA with multiple comparisons *P<0.05, **P<0.01.

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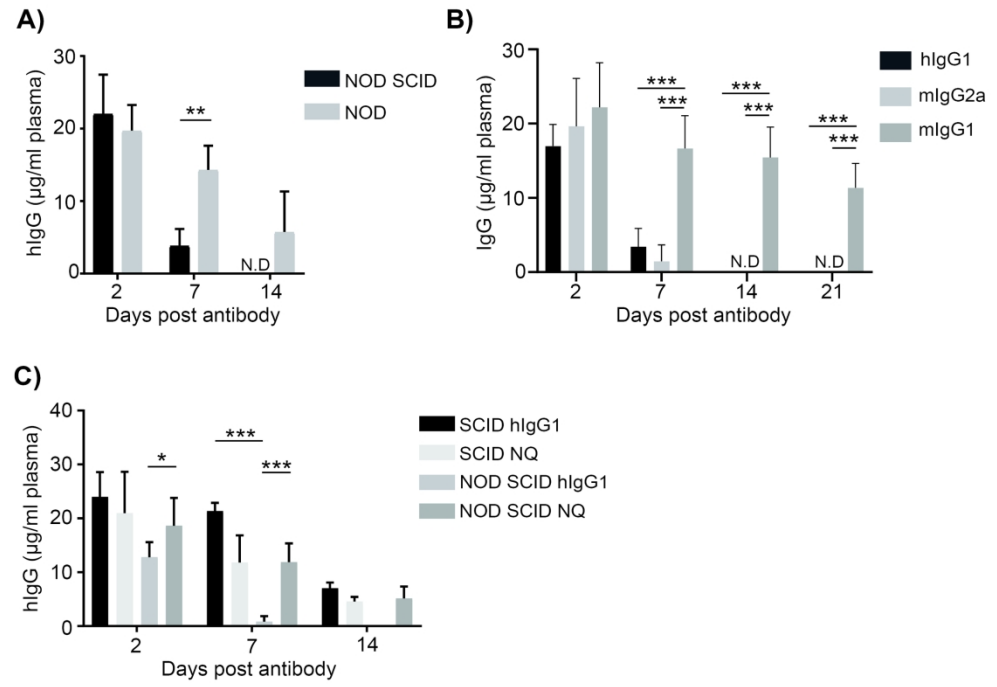
Figure 1



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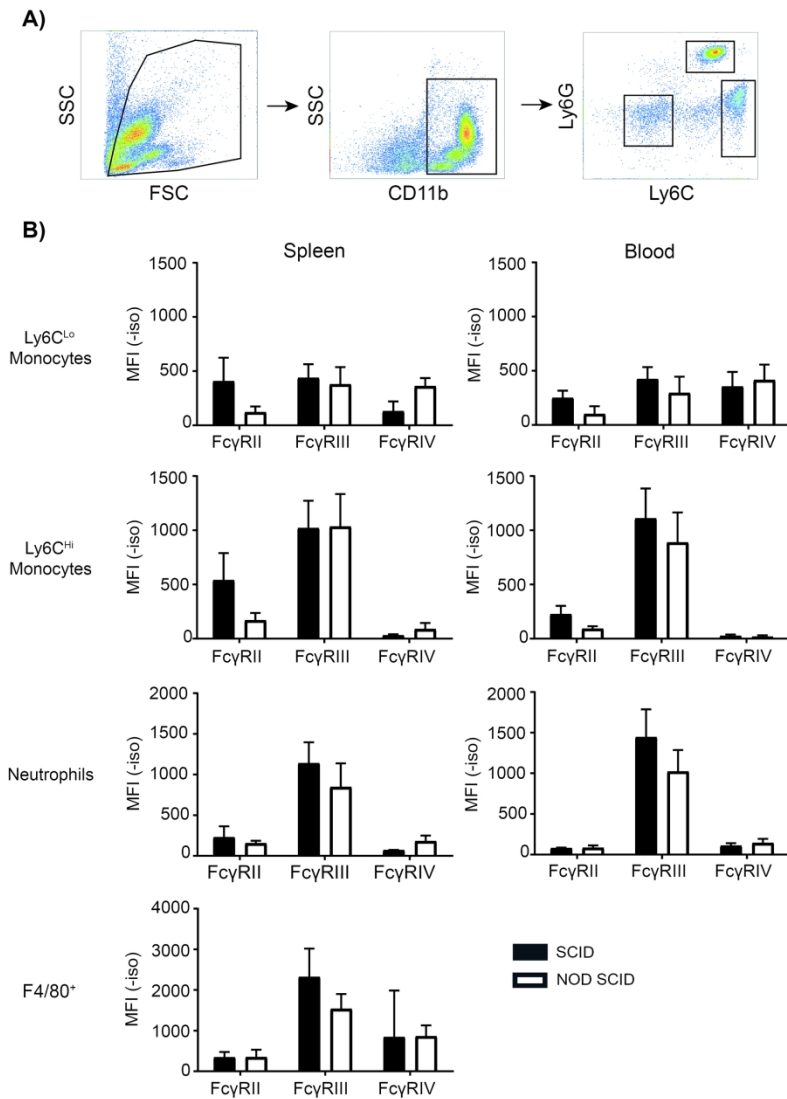
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Figure 2



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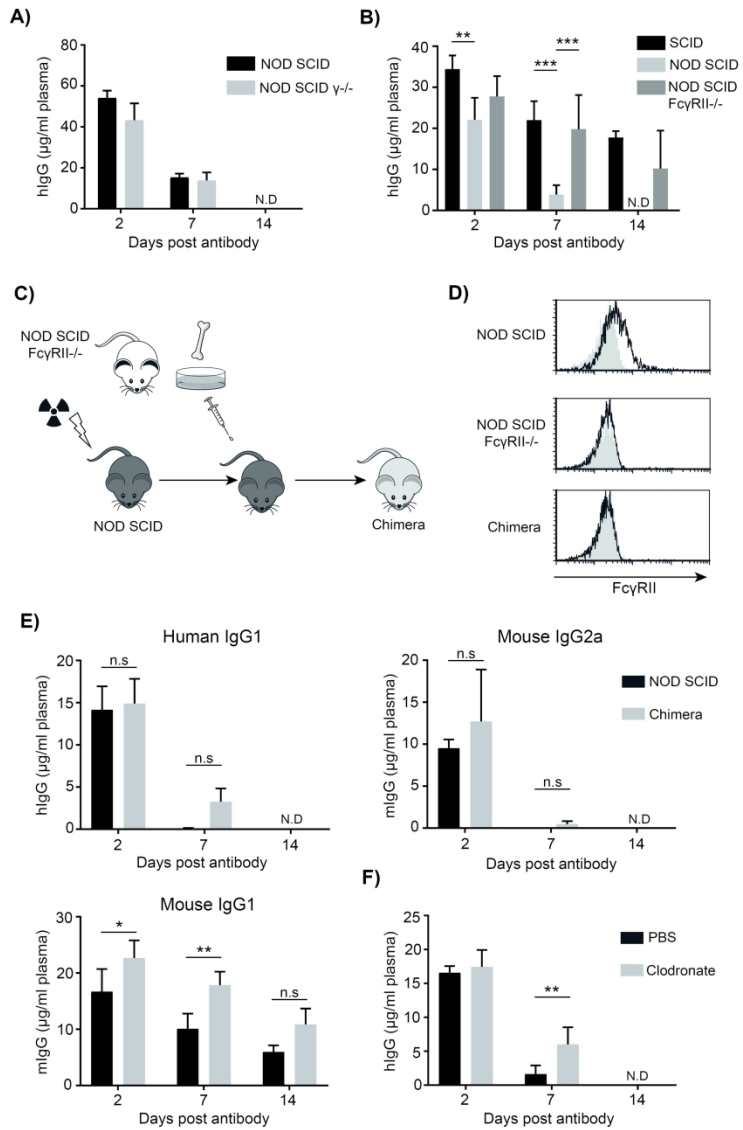
Figure 3



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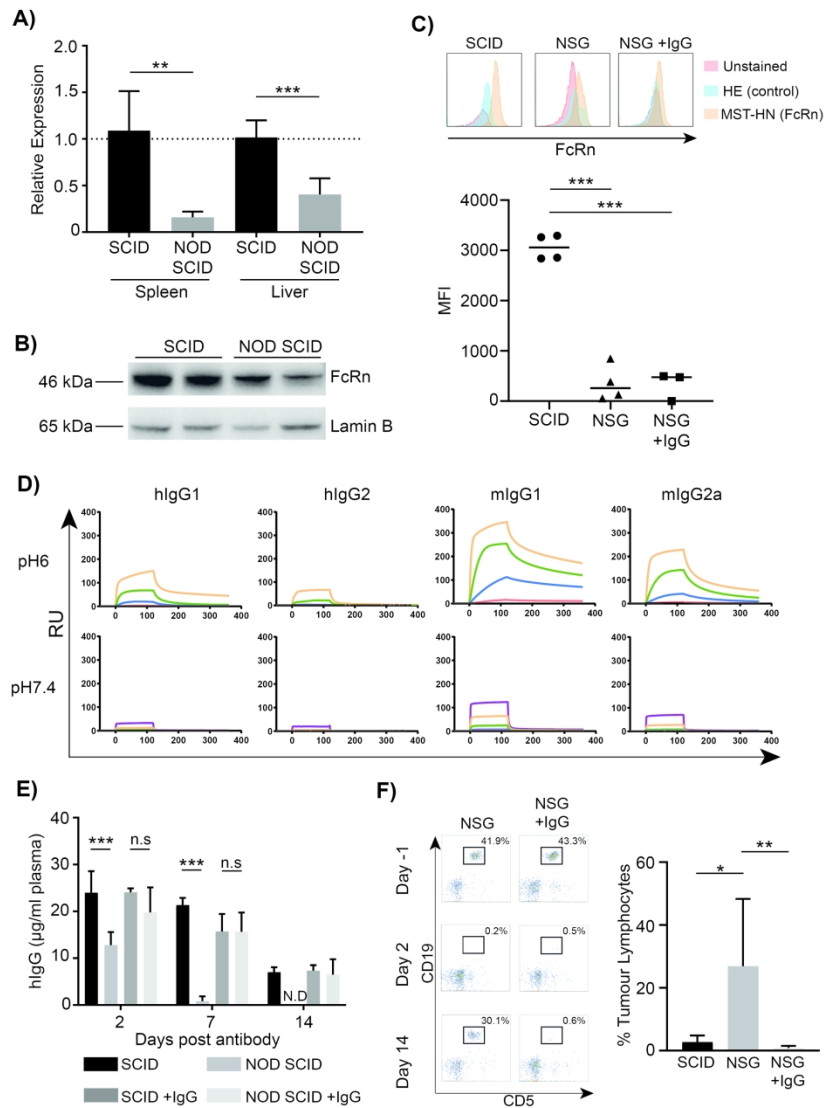
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Figure 4



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Figure 5



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Supplementary Table

Table S1. List of antibodies for flow cytometry and Immunofluorescence

Target	Isotype	Conjugate	Clone	Company
CD19	Rat IgG2a	PE	1D3	In-house
CD19	Rat IgG2a	APC	1D3	Biolegend
CD5	Rat IgG2a	FITC	53-7.3	Biolegend
CD11b	Rat IgG2b	Pacific Blue	M1/70	Biolegend
Ly6C	Rat IgG2c	PerCP/Cy5.5	HK1.4	Biolegend
Ly6G	Rat IgG2a	APC/Cy7	1A8	Biolegend
FcγRII	Mouse F(ab') ₂	FITC	AT130-2	In-house
FcγRII	Human IgG2	Unconjugated	AT130-2	In-house
FcγRII	Rat IgG2a	Unconjugated	AT130-2	In-house
FcRn	Goat	Unconjugated	AF6775	R&D systems
Lamin B	Goat	Unconjugated	Not-applicable	Santa Cruz Biotechnology
Goat IgG	Donkey	HRP	Not-applicable	Santa Cruz Biotechnology
Clec4F	Human IgG1	Unconjugated	4M23	In-house
Cytokeratin 8	Rabbit	Unconjugated	EP1628Y	Abcam
Rat IgG	Goat	Alexafluor 488	polyclonal	Invitrogen
Human IgG	Goat	Alexafluor 488	polyclonal	Abcam
Goat IgG	Chicken	Alexafluor 488	polyclonal	Invitrogen
Human IgG	Goat	Alexafluor 549	polyclonal	Abcam
Rabbit IgG	Goat	Alexafluor 568	polyclonal	Invitrogen

SUPPLEMENTARY FIGURES

Supplementary Figure 1 100µg trastuzumab was administered I.P. to SCID or NOD SCID mice. The concentration of human or mouse IgG in the plasma was then determined by ELISA 2-21 days later. (n=4; 2 way ANOVA with multiple comparisons ***P<0.001

Supplementary Figure 2 SCID, NOD or BALB/c mice were injected I.P. with 100µg cetuximab. The concentration of hIgG in the plasma was determined by ELISA 7 days later. N=4, representative of 2 independent experiments. One-way ANOVA P>0.05

Supplementary Figure 3 A) Bone marrow derived macrophages from SCID and NOD SCID mice were stained with fluorescent antibodies specific for individual mouse FcγR. MFI with isotype control subtracted N=2, representative of 2 independent experiments. B) NOD SCID mice were injected I.V.

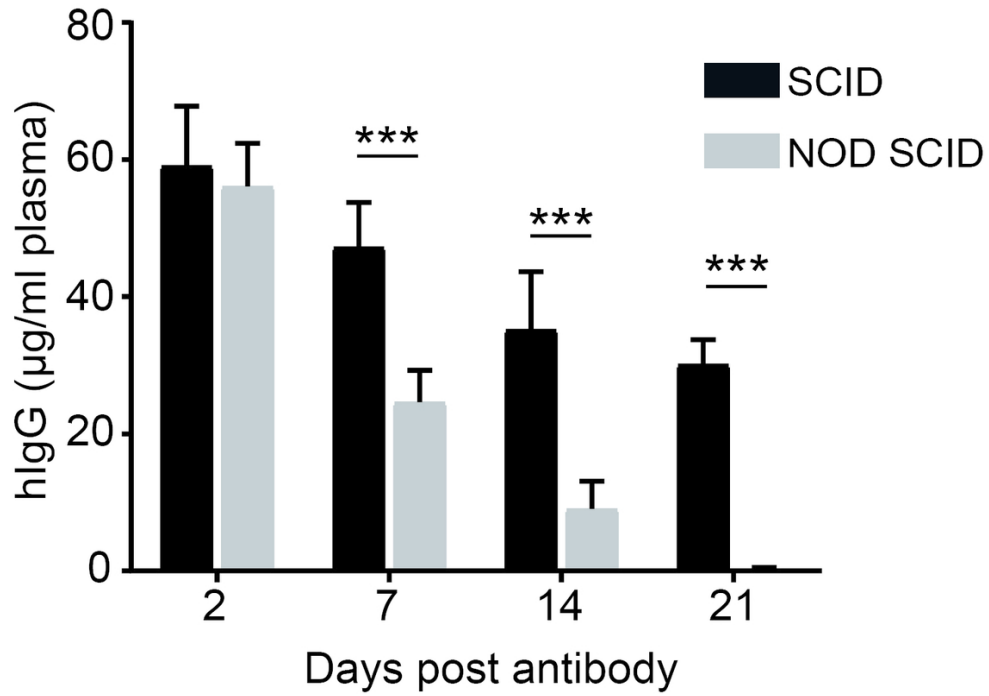
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3 with clodronate- or PBS-containing liposomes on days -3, -1, 6 and 13. Splenic macrophages
4 (CD11b+, F4/80+) were quantified on day 14, representative of 2 animals.
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8 **Supplementary Figure 4.** A and B) Sections were cut from frozen, OCT embedded liver from BALB/c
9 (A) and NSG (B) mice. Sections were stained using primary antibodies against FcRn, FcγRII,
10 Cytokeratin 8 and Clec4F which were detected using fluorescently conjugated secondary antibodies.
11 Sections were counterstained with DAPI. Mounted slides were imaged at 10x or 40x magnification
12 with the highlighted area expanded in the right hand image. C) SCID or NSG mice were reconstituted
13 with 400µg mIgG2a and 500µg mIgG1 prior to administration of 100µg rituximab hIgG1. After 48
14 hours livers were harvested and embedded in OCT. Sections were stained using fluorescently
15 conjugated goat anti-hIgG before being counterstained with DAPI, mounted and imaged as
16 described above. Images from 3 mice from each treatment group are shown at 10x magnification
17 with a representative single mouse from each group shown below at 40x magnification.
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23 **Supplementary Figure 5.** SCID or NOD SCID mice were reconstituted with 400µg mIgG2a and 500µg
24 mIgG1 on day 0. An additional 200µg mIgG2a was given on day 3, 6, 9, 12 and 15. The concentration
25 of mIgG in the plasma was determined by ELISA and compared to that of a BALB/c mouse. N=3.
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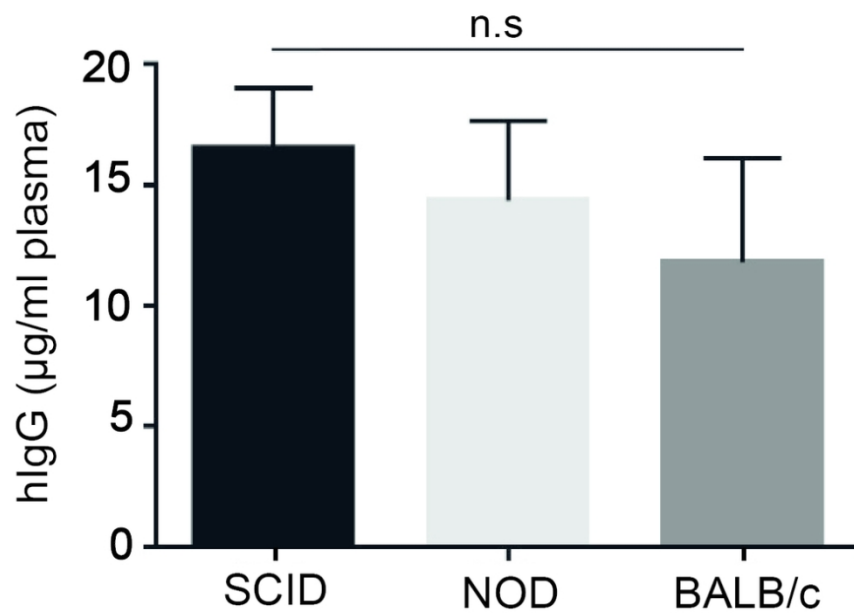
30 **Supplementary Figure 6.** Eµ-Tcl1 tumour cells were injected I.P. into SCID or NSG mice. Once tumour
31 was detectable in the peripheral blood a group of NSG mice were reconstituted with mIgG as
32 follows: 400µg mIgG2a and 500µg mIgG1 was administered on day 0, an additional 200µg mIgG2a
33 was given on day 3, 6, 9, 12. Animals were treated on day 0 with 100µg hIgG1 anti-mCD20 (18B12)
34 14 days after treatment, the number of tumour cells in the blood was assessed. (n=5-6 per group),
35 mean +S.D. 1-way ANOVA with multiple comparisons *P<0.05.
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Supplementary Figure 1



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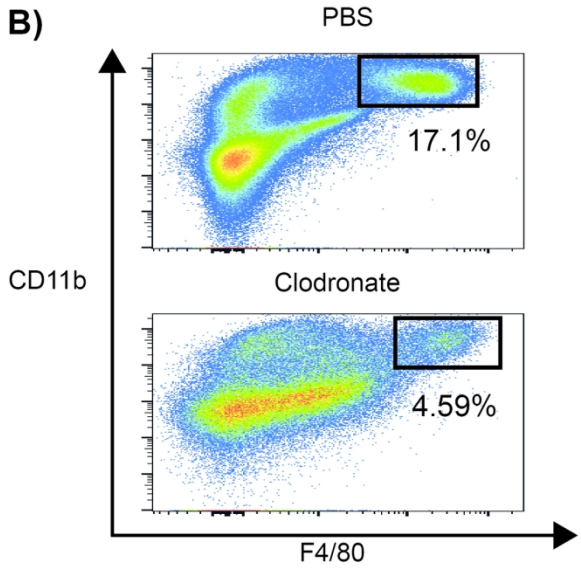
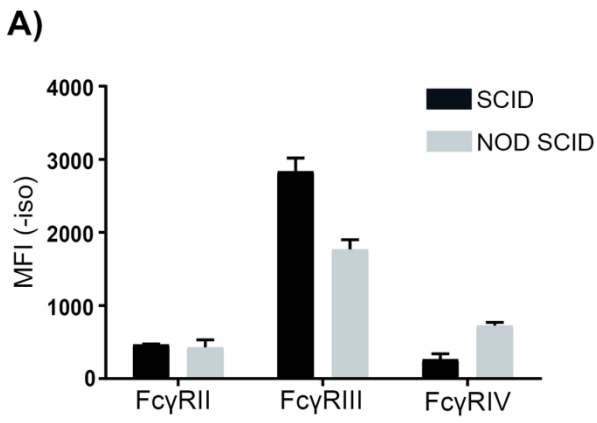
Supplementary Figure 2



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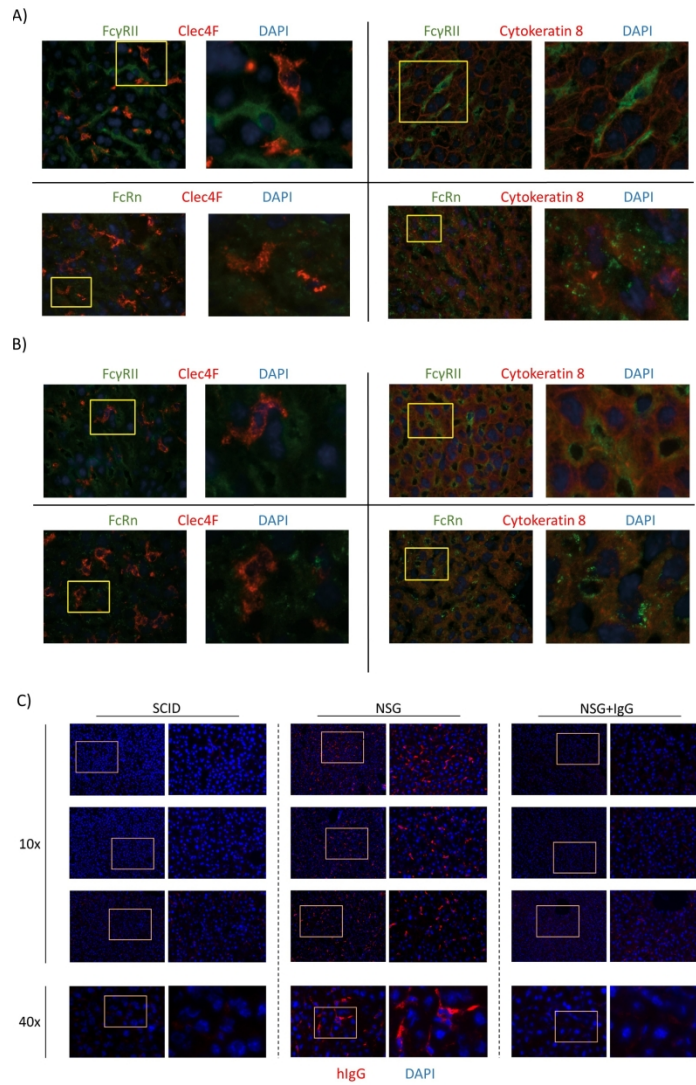
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Supplementary Figure 3



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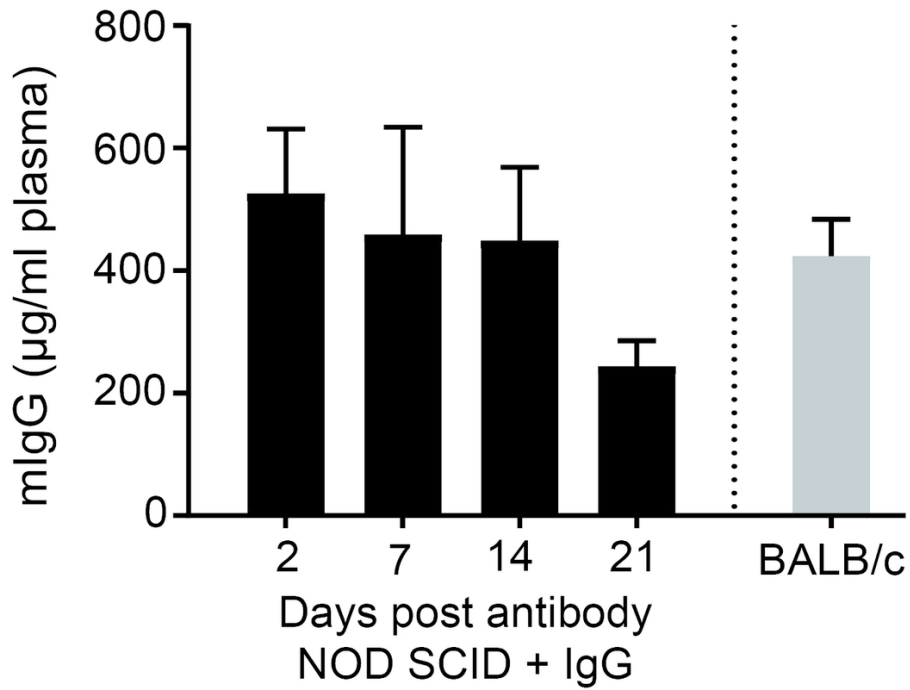
Supplementary Figure 4



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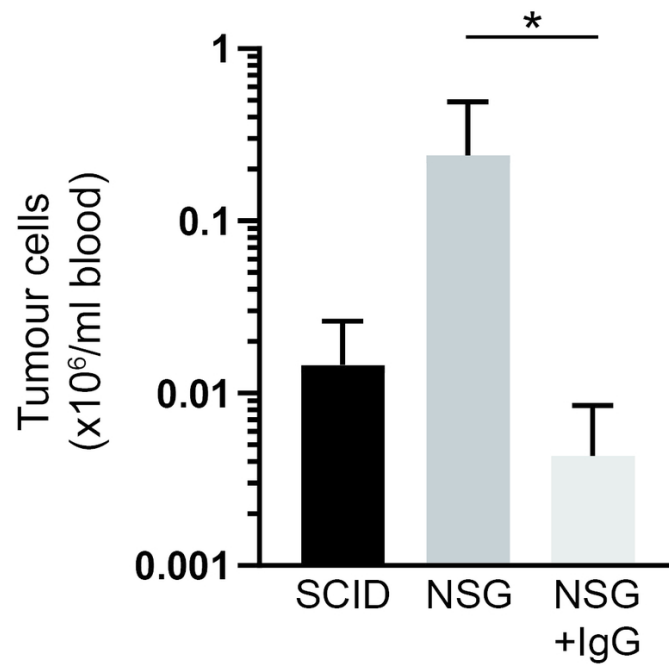
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Supplementary Figure 5



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Supplementary Figure 6



98x89mm (300 x 300 DPI)