1 **A Restricted Role of FcγR in the Regulation of Adaptive Immunity**

2

3 Marieke F. Fransen[*], Hreinn Benonisson[†], Wendy W. van Maren[*1], Heng Sheng Sow[†], Cor

4 Breukel[†], Margot M. Linssen[†], Jill W. C. Claassens[†], Conny Brouwers[†], Jos van der Kaa[†], Marcel

5 Camps[*], Jan Willem Kleinovink[*] , Kelly K. Vonk[†], Sandra van Heiningen[†], Ngaisah Klar[¶], Lianne

6 van Beek[†], Vanessa van Harmelen[†], Lucia Daxinger[†], Kutty S. Nandakumar[‖,‡‡], Rikard

7 Holmdahl[‖], Chris Coward[#], Qingshun Lin[**], Sachiko Hirose [††], Daniela Salvatori[‡], Thorbald van

8 Hall[§], Cees van Kooten[¶], Piero Mastroeni[#], Ferry Ossendorp[*], J. Sjef Verbeek[†2]

9

10

11

12 * Department of Immunohematology & Blood Transfusion, †Department of Human Genetics

13 ‡ Department of Anatomy, §Department of Clinical Oncology, ¶Department of Nephrology,

14 Leiden University Medical Center, 2333 ZA, Leiden, the Netherlands

15 ‖ Department of Medical Biochemistry and Biophysics, Scheelesväg 2, B2, plan 4, Karolinska

16   Institute 17177, Stockholm, Sweden

17 #Department of Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES UK

18 **Department of Pathology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

19 ††Toin Human Science and Technology Center, Department of Biomedical Engineering, Toin

20   University of Yokohama, Yokohama 225-8502, Japan

21 ‡‡ School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China.

22

23 **Running title:** Phenotype of mouse lacking all FcγR

24

25 **Contact**

26 Dr. J. Sjef Verbeek

27 Department of Human Genetics Leiden University Medical Center

28 Albinusdreef 2; 2333 ZA, Leiden; the Netherlands Telephone: +31-71-5269410

29 Fax: +31-71-5268285

30 e-mail: j.s.verbeek@lumc.nl

31

32

33

Abstract

By their interaction with IgG immune complexes FcγR and complement link innate and adaptive immunity showing functional redundancy. In complement-deficient mice not only IgG downstream effector functions are often impaired but also adaptive immunity. Based on a variety of model systems utilising FcγR KO mice, it has been concluded that FcγR are also key regulators of both innate and adaptive immunity. However, several of the model-systems under-pinning these conclusions suffer from flawed experimental design. To address this issue in the absence of these caveats we generated a novel mouse model deficient for all FcγR (FcγRI/II/III/IV$^{-/-}$ mice).

These mice displayed normal development and lymphoid and myeloid ontogeny. Although IgG-effector pathways were impaired, adaptive immune responses to a variety of challenges, including bacterial infection and IgG-immune complexes, were not. Like FcγRIIb-deficient mice, FcγRI/II/III/IV$^{-/-}$ mice developed higher Ab titres, but no autoantibodies. These observations indicate a redundant role for activating FcγRs in the modulation of the adaptive immune response *in vivo*. We conclude that FcγR are downstream IgG-effector molecules with a restricted role in the ontogeny and maintenance of the immune system as well as regulation of adaptive immunity.

INTRODUCTION

Adequately defining the *in vivo* role of the receptors for IgG, FcγR, is severely hampered not only by the complexity of the FcγR gene family itself but also because of their functional redundancy with the complement system. FcγR and complement link innate and adaptive immunity on two levels. First of all, they mediate the activation of downstream effector pathways of innate immune cells by antigen (Ag) specific IgG. Secondly, they are involved in the IgG immune complex (IC) mediated regulation of adaptive immunity.

Four different FcγR, have been identified in the mouse. The IgG binding α-chains of the activating FcγRI, FcγRIII and FcγRIV, are associated with the FcR γ chain, a signal transduction subunit which is also required for cell surface expression (1). The activating FcγR are counterbalanced by the inhibiting receptor FcγRIIb. The four FcγR, are expressed in different combinations on a variety of immune cells, mainly myeloid effector cells.

The *in vivo* role of FcγR has been extensively studied by analyzing the phenotype of mice deficient either for one or combinations of two or three FcγR or the FcR γ chain. By establishing a variety of disease models such as arthritis, hemolytic anemia, anaphylaxis and lupus like disease in these KO mice, we and others have shown that FcγR play an important role in the downstream antibody (Ab) effector pathways which drive the pathogenesis in these diseases (2). However, by using antibodies with a mutation in their Fc domain, destroying FcγR binding without affecting interactions with complement, it has recently been shown that several IgG downstream effector functions can be mediated also by complement (3). Mice deficient in the early pathway components C1q, C3 and C4 and the complement receptors Cr1/Cr2 have impaired humoral responses to T cell dependent and T cell independent Ag (4-6) indicating that the complement system plays an important role in priming and regulation of the adaptive immune response (7). Moreover, C1q deficient mice develop spontaneously lupus like

4

80   disease. A series of observations suggest that FcγR also play a role in priming and

81   regulation of adaptive immunity and the maintenance of immune tolerance. Ag-specific IgG1,

82   IgG2a, and IgG2b enhance Ab and CD4$^+$ T cell responses to soluble protein Ag via activating

83   FcγRs, probably by increasing Ag presentation by dendritic cells to Th cells (8). With Ag-

84   specific IgG3, an IgG subclass not interacting with FcγR, this process is complement dependent

85   (9). In FcR γ chain KO mice, immunized with the model Ag KLH, the delayed-type

86   hypersensitivity (DTH) response after challenge is significantly decreased compared to the DTH

87   in WT mice. Moreover, the secondary responses of CD4$^+$ T cells to Ag and Ab formation were

88   also reduced in these mice (10). These data suggest that activating FcγRs on antigen presenting

89   cells (APCs) facilitate Ag presentation resulting in efficient priming of Th cell responses *in vivo*

90   in an IC-dependent manner which is required for a full-blown Ab response. We and others have

91   shown that soluble IgG-IC enhance cross presentation by DCs resulting in a strong induction of

92   the proliferation of antigen specific CTLs (11-14). It is generally believed that FcγR play an

93   important role in this process (15).

94   Combined, these observations suggest an important role of activating FcRs in modulating the

95   adaptive immune response. In addition, cross-linking the B cell receptor with FcγRIIb by IgG-

96   IC results in down regulations of Ab production. FcγRIIb deficient mice develop higher Ab

97   titers compared to WT mice (16). Moreover it has been shown that FcγRIIb deficient mice when

98   backcrossed into C57BL/6 background develop spontaneously lupus like disease (17).

99   In conclusion, many *in vivo* observations in WT and FcγR KO mice suggest a pleiotropic role of

100   FcγR in the immune system. However, many of these studies have some flaws. Several studies

101   were performed in FcR γ chain deficient mice. The FcR γ chain is a promiscuous signal

102   transduction subunit, associated with at least nine other receptor complexes (18). Most FcγR

103   KO mice have been generated by gene targeting in 129-derived ES cells, and subsequently

5

104   backcrossed into C57BL/6 background. We have shown that after backcrossing the remaining

105   129-derived sequences flanking the FcγRIIb KO allele, including the hypomorphic autoimmune

106   susceptibility SLAM locus (19) cause the autoimmune phenotype of the FcγRIIb KO mouse on

107   mixed 129/C57BL/6 background whereas the FcγRIIb deficiency only enhances the lupus like

108   disease (20). In many in vivo cross-presentation studies bone marrow derived DCs ex vivo

109   loaded with IgG-IC of the model antigen chicken Ovalbumine (OVA) induced strong

110   proliferation of adoptively transferred OVA antigen specific T cells (11,12,14). However, the

111   endogenous anti-OVA cytotoxic T cell response was very low (21). Moreover, *in vivo* cross

112   presentation of IgG-IC derived Ag was impaired in C1q deficient mice (22).

113   To address these issues in the absence of these caveats we generated a novel C57BL/6 mouse

114   model deficient for all four FcγR but expressing the FcR γ chain and analyzed its phenotype.

115   Although, as expected, we could confirm that a variety of IgG downstream effector pathways

116   were impaired, the overall characteristics of the immune system of these mice and WT control

117   mice were very similar. Their B and T cell responses were not impaired. Like FcγRIIb-

118   deficient mice, FcγRI/II/III/IV^{-/-} mice developed higher Ab titres, but no autoantibodies with

119   age. We conclude that, in contrast to complement, FcγR have little or no role in the ontogeny

120   and the maintenance of the immune system. Their role in priming and regulation of the

121   adaptive immune response appears redundant.

122

123

124

125

126

127

128 **Material and Methods**

129

130 **Mice**

131 Mice were housed and all experiments were performed at the SPF animal facilities of the

132 laboratory animal facility (PDC) of the Leiden University Medical Center (LUMC) or

133 University of Cambridge (Salmonella infection). The health status of the animals was monitored

134 over time. Animals tested negative for all agents listed in the FELASA (Federation of European

135 Laboratory Animal Science Associations) guidelines for SPF mouse colonies (23).

136 All mouse studies were approved by the animal ethics committee of the LUMC. Experiments

137 were performed in accordance with the Dutch Act on Animal Experimentation and EU

138 Directive 2010/63/EU ('On the protection of animals used for scientific purposes'). C57BL/6J

139 mice were purchased from Charles River the Netherlands. All FcγR KO mice were generated in

140 the transgenic mouse facility of the LUMC (**Figures 1 and S1**). The EIIaCre deleter strain

141 (n=20 on C57BL/6J background), was a kind gift of Dr. Heiner Westphal. The Flp deleter strain

142 C57BL/6-Tg(CAG-flpe)36Ito/ItoRbrc, was purchased from Jackson Laboratories Bar Harbor,

143 Me. Mice were routinely checked for their genotype by PCR.

144

145 **Cells and cell lines**

146 B3Z is an ovalbumin (OVA) 257-264 (SIINFEKL) –specific H-2Kb restricted co-stimulatory

147 independent T cell hybridoma cell line. Intraperitoneal thioglycolate elicited macrophages of

148 WT C57BL/6 and FcγRI/II/III/IV$^{-/-}$ mice were isolated by abdominal lavage with 5 ml PBS 48h

149 after intraperitoneal injection of 1.5 ml 4% thioglycollate medium (Becton Dickinson, Mountain

150 View CA).

151

Collagen Ab Induced Arthritis (CAIA)

Mice were injected intravenously (i.v.) with 4 mg of a cocktail of four different mouse anti-mouse collagen type II IgG monoclonal Abs (Equimolar mix of M2139 (IgG2b) + CIIC1 (IgG2a) + CIIC2 (IgG2b) + UL1 (IgG2b) on day 0 and in addition on day 3 with 100μg of LPS from E.coli 055:B5 (Sigma-Aldrich L2880) in 100μl of PBS intraperitoneally. At day 10 an additional amount of 4 mg of a cocktail of four different mouse anti-mouse collagen type II IgG monoclonal Abs was injected IP to boost the response. From day 7 onwards development of arthritis was monitored daily in a blind manner using a caliper to measure footpad swelling (24).

Anaphylaxis

Mice were sensitized by injecting iv 400 μg of pyrogen-free mouse anti-TNP IgG2a in saline and challenged 4 hrs. later by i.v. injection of 1 mg pyrogene-free DNP-HSA (2,4-dinitrophenylated human serum albumin) (A6661 Sigma-Aldrich) in saline per mouse (25). For the monitoring of blood pressure, the mice were anesthetized by i.p. injection of ketamine (75mg/kg), dexdomitor (0.2mg/kg) and atropine (0.5mg/kg) in saline. After induction of anesthesia, the femoral artery and femoral vein were catheterized. The artery catheter was connected to the blood pressure monitor and blood pressure was allowed to stabilize for ≥5 min. Subsequently, the mice were injected i.v. via the femoral vein catheter with DNP-HSA. Blood pressure was monitored for ≥30 min after OVA injection, using a physiological pressure transducer (AD Instruments, Colorado Springs, CO). The signal was acquired and digitized in PowerLab (AD Instruments), sampled at 200 Hz, and analyzed offline using LabChart (AD Instruments).

Ab dependent Cellular phagocytosis (ADCP)

WT C57Bl/6 and FcγRI/II/III/IV$^{-/-}$ mice were injected with 25 µg of the rat IgG2b 2.43 Ab (in-house production) intraperitoneally to deplete CD8$^+$ T cells. One day before, and three days after the depleting Ab injection CD8$^+$ T cell numbers were analyzed in blood using flowcytometry and quantified as a percentage of total CD3$^+$ cells. As determined with Surface Plasmon Resonance, the rat IgG2b antibody has a binding preference for activating mouse FcγR (activating-to-inhibitory FcγR binding [A/I] = 40) (26).

In vitro uptake and cross presentation of immune complex derived Ag

In vitro uptake and cross-presentation of OVA-IC derived Ag by DCs were studied by pre-forming complexes of OVA and rabbit polyclonal anti-OVA ab (Cappel) in a ratio of 1 to 30, and incubating 5 µg of these complexes with 25.000 BM-DC cells in normal DC conditioned medium, as described (27). Rabbit IgG binds to all mouse FcγR (27,28). For uptake, Alexa488-labeled OVA was used, and measured using flow cytometry, with or without quenching of extracellularly bound fluorescent OVA by addition of trypan blue (Sigma-Aldrich). For Cross presentation, 25.000 BMDC were incubated with 50.000 B3Z cells. B3Z cells recognize the minimal SIINFEKL OVA-CTL epitope in MHC class I. Recognition leads to up-regulation of the transcription factor NFAT which activates a LacZ-reporter gene by binding to its IL-2 promoter (29). After overnight incubation with BM-DC the B3Z cells were incubated with a lysis buffer containing the CPRG substrate for β-gal (PBS +1% 9 mg/mL CPRG + 0.9% 1m MgCl2 + 0.125% NP40 + 0.71% 14.3m β-mercaptoethanol) at 37 °C until the color reaction had progressed sufficiently for readout in a plate reader measuring the optical density at 590 nm. A peptide with the minimal OVA epitope SIINFEKL (100 ng/mL in PBS) that directly binds to MHC class I was used as a positive control and unstimulated D1 cells (a dendritic cell line, as

200   described (27) as negative controls.

201

In vivo cross-presentation of immune complex derived Ag

203   CD8$^+$ T cells were isolated from spleen and lymph nodes from OT-I/CD45.1 mice with the BD

204   CD8$^+$ lymphocyte enrichment kit and labeled with CFSE. Three million OT-1 T cells were i.v.

205   injected in recipient mice. One day later either 200 µg rabbit polyclonal anti-OVA Ab (Cappel)

206   or non-specific rabbit sera (negative control) was injected i.v. followed 30 minutes later by 5 µg

207   OVA (Worthington Biochemicals) or in house synthesized peptide with the minimal SIINFEKL

208   OVA epitope (positive control). Three days later mice were sacrificed, spleens were isolated and

209   proliferation of CFSE-labelled OT-1 T lymphocytes was analysed in single cell suspensions by

210   flow cytometry gated on CD8$^+$ and CD45.1$^+$ cells.

211

Quantitation of Immune complex clearance

213   Age- and weight-matched naive mice received an i.v. injection of 100 μg rabbit

214   IgG anti-ovalbumin (Cappel) followed by an i.v. injection of 5 μg Alexa Fluor 488/647 labeled

215   ovalbumin (Life Technologies) 15 minutes later. At indicated time points blood was drawn and

216   serum was collected. 5 μl serum was mixed with sample buffer, heated at 95°C for 5 minutes

217   and loaded on SDS/PAGE. Fluorescent ovalbumin was quantified directly from the SDS/PAGE

218   gels by a Typhoon 9410 Variable mode imager (GE Healthcare Bio-Sciences) and ImageQuant

219   TL v8.1 software (GE Healthcare Life Sciences).

220   At different time-points after injection, mice were sacrificed and a single lobe of liver was

221   isolated and imaged by IVIS Spectrum (PerkinElmer) using excitation at 605 nm and measuring

222   emission at 680 nm with an exposure time of 2 seconds.

223

Flow cytometry

Single cell suspensions were made from spleen, thymus, lymph nodes, bone marrow and from

lavage of peritoneal cavity 24hr after injection of 1.5ml thioglycolate. For analysis of myeloid

cells from spleen, organs were incubated for 30 minutes with Liberase (Sigma-Aldrich)

according to manufacturer's protocol. Cells were blocked with 10% normal mouse serum. 7-

AAD (Life Technologies) was used to exclude dead cells.

Abs for the following surface markers were used in this study: CD11c (clone HL3), CD8b

(clone 53-5.8), CD19 (clone 1D3), CD90.1 (clone H1S51), and FcγRI clone X54-5/7.1 (all

from Becton Dickinson). CD3ε (145-2c11), B220 (RA3-6B2), and CD45.2 (clone 104) (all from

eBioscience). CD4 (clone RM4-4), F4-80 (clone BM8), Ly6C (HK1.4), and Ly6G (clone 1A8)

(all from Biolegend), FcgRIIb (clone Ly17.2 produced in house); FcγRIII (clone 275003, from

R&D) and FcγRIV (clone 012, from Sino Biological).

Serum levels of IgG subclasses

Serum was collected from 10 months old naive FcγRI/II/III/IV$^{-/-}$ and WT C57Bl/6 mice.

Elisa was performed with goat anti mouse IgG (Becton Dickinson), and goat anti mouse IgG,

IgG1, IgG2a or IgG2b HRP (Southern Biotech) and TMB substrate (Dako). Reaction was

stopped with 1M H$_2$SO$_4$ and absorption was measured at 450 nm.

For antigen-specific antibody titres in serum; Mice were immunized with 50 μg TNP-BSA in

100 μl CFA (1:1 emulsion with PBS) injected s.c. at day 0 and boosted at day 14 and 28 with

25 μg TNP-BSA in 100 μl IFA (1:1 emulsion with PBS). Ab titres in sera collected at day 36

were assessed with ELISA. Streptavidin-coated 96-wells plates were incubated with 1nmol/ml

biotin-BSA and blocked with 5% non-fat milk. Secondary Ab was goat-anti-mouse horseradish

peroxidase (HRP). Substrate ABTS (Code no. S1599, Dako) was added, and absorption was

248 measured at 415nmSerum levels of IgG class autoantibodies were determined using ELISA

249 plates coated with 5μg/ml of dsDNA (Sigma-Aldrich) or 5μg/ml of histone (Sigma-Aldrich) or

250 4μg/ml of chromatin, respectively, as previously described (30). Serum levels of binding

251 activities against dsDNA, histone, and chromatin were expressed in units by reference to a

252 standard curve obtained by serial dilution of a standard serum pool from (NZB × NZW)F1 mice

253 ages >8 months, containing 1,000 unit activities/ml. Serum levels of IgG Abs were measured

254 using HRP conjugated anti-mouse IgG secondary Abs (Southern Biotech, Birmingham AL,

255 USA) and detected at OD^{450} nm by using TMB Substrate Reagent(BD).

256

257 **Infection with *Salmonella* live vaccine**

258 Mice were infected with the *Salmonella* Typhimurium (STm) SL3261, and attenuated *aroA*

259 strain (31). Live bacteria for parenteral immunization were prepared from a 16 hr. static culture

260 of STm SL3261 in LB Broth, diluted 1/100 in PBS and administered by *i.v.* injection into the

261 tail vein (~10^6 CFU/mouse). Actual inoculum dose was determined by plating dilutions on LB

262 Agar.

263 Salmonella induces Th1 T-cell responses. IFNγ and IL2 production correlate well with T-h1

264 responses to *Salmonella*. T-cell stimulation assays and cytokine measurement after infection

265 with Salmonella live vaccine were performed as follows: CD4$^+$ T cells were positively enriched

266 from spleens using magnetic bead-conjugated Abs (Miltenyi Biotec), according to

267 manufacturer's instructions. Purity was assessed by flow cytometry on a FACSCalibur machine

268 (Becton Dickinson). CD4$^+$ T cells were stimulated with *Salmonella* Ag or anti-CD3e (clone

269 145-2C11, eBioscience) and anti-CD28 (clone 37.51, eBioscience) as positive control in the

270 presence of mitomycin C-treated (25 mg/ml; 37°C for 30 min) splenic Ag presenting cells.

271 *Salmonella* Ag was alkali-treated *Salmonella* Typhimurium SL1344. Levels of IFN-γ and IL-2

272   produced at 72 hr and 24 hr were determined using Duoset ELISA kits (R & D Systems)

273   according to manufacturer's instructions.

274   Anti-LPS Abs after infection with Salmonella live vaccine were detected by ELISA as follows:

275   *Salmonella* Typhimurium LPS (Sigma-Aldrich) was dissolved in water containing sodium

276   deoxycholate (0.5% w/v). Microtitre plates (Greiner Bio-One) were coated overnight at 37°C

277   with LPS at 5 μg/ml in carbonate buffer. Serum sample serial dilutions in PBS-Tween+1% BSA

278   were applied in duplicate and incubated. Plates were washed and total Ab detected with HRP-

279   conjugated goat anti-mouse Ab (Southern Biotech), detection was with SigmaFast OPD

280   substrate (Sigma-Aldrich) with absorbance read FLUOstar Omega (BMG Labtech)**.**

281

282   **Complement analysis**

283   Plasma samples were collected from CO_2 euthanized mice via heart puncture and put directly

284   on ice. EDTA-plasma was collected with syringes pre-treated with EDTA and tubes with final

285   EDTA concentration of 10mM. Blood was kept on ice for 30-120 min, centrifuged twice

286   at 3000-5000g for 10 minutes at 4°C. Samples were pooled and aliquoted to single use batches

287   and stored at -80°C.

288   Measurement of functional pathway activities was performed in plasma of mice as described

289   (32) In brief, complement activation was induced by incubation of serial dilutions in ELISA

290   plates (Nunc Maxisorp plates, Thermo Fisher Scientific) coated with human IgM, mannan and

291   LPS to induce Classical Pathway, Lectin Pathway and Alternative Pathway respectively.

292   Activation of complement was either quantified at the level of C3 deposition, using an Ab

293   directed against mouse C3b/C3c/iC3b, or at the level of C9 deposition using a rabbit anti-mouse

294   C9 polyclonal Ab. Complement activity in the experimental samples was calculated using CD1

295   serum as a standard which was put at 100 AU/ml.

13

296   Complement factors in plasma were quantified using specific sandwich ELISAs. C3 was

297   quantified in the form of C3b/C3c/iC3b as previously described. C1q was quantified using

298   rabbit anti-mouse C1q pAb (33). Mouse properdin was measured using coating with an anti-

299   mouse properdin mAb, and detection with Rabbit anti-mouse properdin pAb-DIG, whereas C6

300   and C9 were quantified using rabbit polyclonal anti-mouse C6 and rabbit polyclonal anti-mouse

301   C9 (34,34).

302

Histology

304   Complete necropsy was performed following standard procedures. Tissues were fixed in 4%

305   neutral buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with

306   haematoxylin and eosin and evaluated by light microscopy. Histopathological analysis was

307   performed by a European board certified veterinary pathologist. All main organs were analysed.

308   Light microscopy pictures were taken with a DP26 Olympus camera.

309

Metabolic parameters

311   In 12 weeks-old mice, body weight was measured and lean and fat mass was assessed by MRI-

312   based body composition analysis (Echo MRI, Echo Medical Systems, Houston, TX, USA).

313   Blood was drawn from overnight fasted mice via tail vein into paraoxon (Sigma-Aldrich, St.

314   Louis, MO) coated capillary tubes. After centrifugation, plasma was collected and triglyceride

315   (TG), total cholesterol (TC), free fatty acid (FA), glucose and insulin levels were determined

316   using commercially available kits (11488872 and 236691, Roche Molecular Biochemicals,

317   Indianapolis; NEFA-C Wako Chemicals GmbH, Neuss, Germany; ab83390, Abcam,

318   Cambrigde, UK; Instruchemie, Delfzijl, The Netherlands and Crystal Chem Inc., IL, USA,

319   respectively). Indirect calorimetry measurements were performed using metabolic cages

320 (LabMaster System, TSE Systems, Bad Homburg, Germany) as previously described (35).

321

FcγR expression during embryonic development

323 Total RNA was extracted using QIAzol (5346994; Qiagen). 1 µg of total RNA was used for

324 reverse transcription with the RevertAid H Minus First Strand cDNA Synthesis Kit (K1632;

325 Thermo). qRT-PCR was performed in triplicate on a C1000TM Thermal cycler (Bio-Rad) with

326 SYBR Green (170-8887; Bio-Rad). Data was normalized to β-actin. The following primers were

327 used:

328 *β-Actin*_F: GGCTGTATTCCCCTCCATCG; *β-Actin*_R: CCAGTTGGTAACAATGCCATGT;

329 FcγRI_F: AAGTGCTTGGTCCCCAGTC; FcγRI_R: CTGCAGCCTGTGTATTTTCA;

330 FcγRIIb_F: AATTGTGGCTGCTGTCACTG; FcγRIIb_R: GTTTCCTGGGAGAGCTGGA;

331 FcγRIII_F: TGGGGACTACTACTGCAAAGG;

332 FcγRIII_R:AGAAATAAAGGCCCGTGTCC

333 FcγRIV_F: TGGAATGTACAGGTGCCAGA; FcγRIV_R:

334 TTCCGTACAGGTCTGTTTTGC

335

336

337

338

339

340

341

342

343

344 **RESULTS**

345

346 **Generation of the FcγRI/II/III/IV quadruple KO mouse model**

347 To overcome the drawbacks of the existing FcγR KO mouse models we generated a novel

348 mouse model in C57BL/6 background deficient for all four FcγR ligand binding chains while

349 maintaining the promiscuous FcR γ signal transduction subunit. To this end we crossed a newly

350 generated mouse model with a 90.4 Kb deletion on chromosome 1 deficient for the

351 FcγRII/III/IV gene cluster (Fig.1) with our previously generated mouse model with a deletion of

352 the FcγRI gene (25) located on chromosome 3 (Fig.1 and Fig.S1). The FcγRI/II/III/IV quadruple

353 KO (FcγRI/II/III/IV$^{-/-}$) offspring developed normally and showed normal breeding

354 characteristics. The phenotype of the FcγRI/II/III/IV$^{-/-}$ mouse was analyzed in a series of *in vitro*

355 and *in vivo* assays

356

357 **IgG downstream effector functions are impaired in FcγRI/II/III/IV$^{-/-}$ mice**

358 In order to confirm that the novel FcγRI/II/III/IV$^{-/-}$ mouse model had impaired known FcγR

359 dependent IgG downstream effector functions, we studied IgG collagen Ab induced arthritis

360 (CAIA), IgG induced passive systemic anaphylaxis and IgG induced antibody dependent cell

361 mediated phagocytosis (ADCP) in these mice. As expected, FcγRI/II/III/IV$^{-/-}$ mice were almost

362 completely resistant to CAIA initiated by intravenous (i.v.) injection of a cocktail of four

363 different anti-collagen IgG Abs (Fig.2.a). CAIA cannot be induced easily in WT C57BL/6 mice,

364 whereas FcγRIIb$^{-/-}$ mice are more sensitive (36). We therefore compared FcγRI/II/III/IV$^{-/-}$ mice,

365 which lack FcγRIIb, with FcγRIIb$^{-/-}$ mice. In contrast to FcγRIIb$^{-/-}$ mice, FcγRI/II/III/IV$^{-/-}$ mice

366 showed little footpad swelling. This confirms our previous results with K/BxN serum induced

367 arthritis (37).

16

368 FcγRI/II/III/IV$^{-/-}$ mice were also resistant to passive systemic anaphylaxis, induced by

369 challenging mice, sensitized by i.v. injection of IgG2a anti-TNP, with DNP-HSA, whereas WT

370 C57BL/6 mice were not (Fig 2.b). The *in vivo* phagocytosis of CD8$^+$ T cells by ADCP after

371 intraperitoneal (i.p.) injection of rat IgG2b anti-CD8 Ab was completely abrogated in

372 FcγRI/II/III/IV$^{-/-}$ mice (Fig.2.c) Together these results confirm that in our FcγRI/II/III/IV$^{-/-}$

373 mouse model IgG downstream effector functions are strongly impaired in a variety of in vivo

374 experimental IgG induced inflammation models.

375

376 ***In vivo* Cross-presentation of soluble IgG-IC derived Ag is normal in FcγRI/II/III/IV$^{-/-}$**

377 **mice**

378 It has been demonstrated with a variety of *in vitro* and *in vivo* experiments that FcγR on Ag

379 presenting cells facilitate the presentation of soluble IC-derived Ag to cytotoxic T cells, the

380 process of cross presentation (12,14,15). In accordance with these observations the uptake of

381 fluorescent labeled OVA-IgG IC by dendritic cells from FcγRI/II/III/IV$^{-/-}$ mice was strongly

382 impaired *in vitro* compared to the uptake by dendritic cells from WT C57BL/6 mice (Fig.3.a).

383 Moreover, as shown in Fig.3.b, the *in vitro* presentation of SIINFEKL peptide processed from

384 OVA-ICs to B3Z hybridoma cells by FcγRI/II/III/IV$^{-/-}$ BMDCs was strongly inhibited.

385 SIINFEKL synthetic peptide was used as control, and was presented by BMDCs from

386 FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 control mice with similar efficiency indicating that MHC

387 Class I expression was comparable between genotypes. These data show that FcγR are required for

388 the *in vitro* IC uptake and subsequent MHC class I-restricted presentation of IC-derived

389 peptides.

390 Surprisingly, proliferation of adoptively transferred OT-1 CD8$^+$ T cells was indistinguishable

391 between FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 control mice. No difference in CFSE division was

392   observed after activation by IC, which were formed in situ by i.v. administration of OVA and

393   subsequently anti-OVA Abs (Fig.3.c and d). Compared to WT C57BL/6 mice FcγRI/II/III/IV^{-/-}

394   mice showed a delay in clearance of IgG-IC from circulation (Fig.3.e and f) while the uptake of

395   IgG-IC by the liver appeared to be decreased (Fig.3.g). These observations imply that *in vivo*,

396   FcγR are dispensable for cross presentation of IgG-IC-derived Ag, but involved in clearance of

397   IgG-IC from circulation.

398

399   **Adaptive immunity is not impaired in FcγRI/II/III/IV^{-/-} mice**

400   In order to further characterize the adaptive immune system in the FcγRI/II/III/IV^{-/-} mice the B

401   and T lymphocyte compartments were analyzed by flowcytometry using a panel of fluorescent

402   labeled Abs specific for B and T lymphocyte surface markers. No differences in percentages of

403   CD8^{+} and CD4^{+} T cells in the thymus, lymph nodes or spleen were found between WT

404   C57BL/6 and FcγRI/II/III/IV^{-/-} mice (fig.4.a). Also, CD19^{+} B220^{+} B cell numbers in the spleen

405   and bone marrow were comparable between WT C57BL/6 and FcγRI/II/III/IV^{-/-} mice.

406   After vaccination with BSA-TNP in CFA no gross differences were observed in BSA Ag

407   specific Ab titers between WT C57BL/6 and FcγRI/II/III/IV^{-/-} mice except a small increase in

408   IgG1 in the FcγRI/II/III/IV^{-/-} mice compared to WT C57BL/6 mice (Fig. 4.b), which can be

409   explained by the absence of FcγRIIb on B cells in FcγRI/II/III/IV^{-/-} mice. These results are in

410   agreement with the results of a previous experiment using a milder immunisation protocol (long

411   synthetic peptide in saline with CpG) in our FcγRI/II/III/IV [-/-] mice (38) indicating that FcγRs

412   are dispensable for T cell dependent B cell responses against a protein Ag.

413   In order to test further the functionality of the adaptive immune response in FcγRI/II/III/IV^{-/-}

414   mice, the ability of these mice to respond to a bacterial infection was analyzed. We inoculated

415   FcγRI/II/III/IV^{-/-} and WT C57BL/6 mice with a live vaccine consisting of the non-virulent

416 SL3261 attenuated *aroA Salmonella* Typhimurium strain, and analyzed the T and B cell

417 responses (Fig. 5. a and b). We did not observe significant differences in the induction of T cell

418 responses between the groups. B cell responses were not hampered in FcγRI/II/III/IV^{-/-} mice.

419 The higher *Salmonella*-specific Ab responses detected in these mice are in keeping with the

420 absence of FcγRIIb on their B cells (Fig.5.b).

421

422 **Normal Ab levels and no auto-Ab formation in aging FcγRI/II/III/IV^{-/-} mice**

423 Serum titers of IgG in 10 months old naïve mice were comparable between FcγRI/II/III/IV^{-/-} and

424 WT C57BL/6 mice (Fig.6.a). FcγRIIb^{-/-} mice on mixed 129/C57BL/6 background develop high

425 anti-nuclear Ab (ANA) titers with age (17) whereas FcγRIIb^{-/-} mice on pure C57BL/6

426 background hardly develop ANA (20). The FcγRII/III/IV deletion (on Chr1) has been generated

427 in C57BL/6 derived ES cells whereas the FcγRI^{-/-} mice were generated by gene targeting of the

428 FcγRI gene (on Chr3) in 129 derived ES cells (25) and subsequent backcrossing into C57BL/6

429 background (n>12). We compared ANA titres in the serum of old FcγRI/II/III/IV^{-/-} mice with

430 ANA titres in old FcγRIIb^{-/-} mice on mixed 129/C57Bl/6 background (n≥ 8) and FcγRIIb^{-/-} mice

431 on pure C57BL/6 background. Only FcγRIIb^{-/-} mice generated by gene targeting in 129 derived

432 ES cells and backcrossed into C57BL/6 background developed high ANA titres (Fig. 6.b, c and

433 d) confirming that 129 derived FcγRIIb-flanking sequences (*SLE16* on Chr1) (39) determine the

434 development of ANA. The absence of FcγRIIb does not lower the threshold for the development

435 of autoimmunity in FcγRI/II/III/IV^{-/-} mice.

436

437 **FcγR deficiency does not affect the development and homeostasis of the myeloid cell**

438 **compartment**

439 Since the adaptive immune system was not impaired in FcγRI/II/III/IV^{-/-} mice, we focused on

440   the innate immune system. Considering the extensive expression of FcγR on myeloid cells, we

441   envisaged that FcγR mediated interactions might influence the development or differentiation of

442   myeloid cells, and that the absence of these receptors in FcγRI/II/III/IV$^{-/-}$ mice could cause

443   alterations in the innate immune compartment. We therefore evaluated the relative numbers of

444   CD11c^{+}/CD8a^{+} and CD11c^{+}/CD8a^{-} dendritic cells in bone-marrow and spleen (Fig.7.a) and the

445   relative numbers of the different myeloid subsets in spleen, using a gating strategy described by

446   Shawn Rose et al. (40) (Fig 7.b). We found no variances in percentages of either subset of cells

447   between FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 mice. These results indicate that FcγR deficiency

448   does not influence the development, differentiation or homeostasis of the cells on which they

449   are normally most prominently expressed.

450

451   **No difference in complement, overall organ architecture and metabolic homeostasis**

452   **between FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 control mice**

453   Since a direct connection between complement and FcγR effector pathway activation has been

454   reported (41) we analyzed the complement system in FcγRI/II/III/IV$^{-/-}$ mice. We quantified the

455   complement activity in an ELISA based system (32), upon initiation of the three pathways of

456   complement activation. Both at the level of C3 and C9 deposition, there were no differences in

457   complement activity between FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 mice (Fig. 8a). In line with

458   this, also circulating levels of individual components, including C1q, properdin, activated C3 or

459   C9 were not different between FcγRI/II/III/IV$^{-/-}$ mice and WT C57BL/6 control mice (Fig.8b).

460   Circulating plasma levels of C6 were somewhat higher in FcγRI/II/III/IV$^{-/-}$ mice (Fig.8b),

461   however this did not result in an increased terminal pathway complement activity (Fig.8a).

462   Similarly, also complement activity measured at the level of C3 deposition was comparable

463   between both groups.

464   Furthermore, we examined histological sections of several organs, including kidney, liver, lung,

465   and spleen of 47 weeks old female mice. In keeping with the flow cytometry data, there were no

466   abnormalities in overall architecture of these organs detectable in the FcγRI/II/III/IV$^{-/-}$ mice

467   compared to WT C57BL/6 control mice (Fig.S2). There were no differences also regarding

468   bronchus-associated lymphoid tissue (BALT) composition in representative lung sections

469   between the 2 groups. Glomerular and kidney pathology was also absent. In representative liver

470   sections from both groups lymphoid aggregates were absent.

471   It has been postulated that the adaptive immune system, the intestine and microbiota govern a

472   homeostatic metabolic function (42). B cells and pathogenic IgG promote insulin resistance in

473   mice fed a high-fat diet (43). Moreover, we have recently shown that mice deficient for the FcR

474   γ chain are protected against diet-induced obesity and insulin resistance (35), suggesting a role

475   of activating FcR in intestinal and systemic metabolic homeostasis. Therefore, we measured a

476   series of metabolic parameters in the FcγRI/II/III/IV$^{-/-}$ mice. Statistical analysis using unpaired

477   t-tests did not reveal significant differences between measured parameters of FcγRI/II/III/IV$^{-/-}$

478   and WT C57BL/6 control mice. (Table S1).

479 **DISCUSSION**

480

481 The novel mouse model presented here is the first C57BL/6 model exclusively and completely

482 deficient for all four FcγR. This enabled us for the first time to study the consequences of

483 complete FcγR deficiency without confounding factors. The phenotype of the FcγRI/II/III/IV$^{-/-}$

484 mouse demonstrates the dominant role of FcγR in IgG downstream effector pathways whereas

485 complement is dispensable. These results confirm older studies with single, double or triple

486 FcγR KO mouse strains or FcR γ$^{-/-}$ mice, lacking all three activating receptors. However, the

487 overall immune system of the FcγRI/II/III/IV$^{-/-}$ mice was surprisingly normal. Lymphoid

488 organs, subsets of lymphoid and myeloid cells, complement and metabolic homeostasis were

489 comparable to WT mice.

490 Mammals are exposed to the Abs of their mother before birth. Also, before birth they have

491 developed immune cells expressing a variety of FcγR (Fig.S3) which can directly interact with

492 the Fc part of these Abs. The high affinity FcγRI binds monomeric IgG whereas the other FcγR

493 are low affinity receptors which bind immune complexes. After birth the animals develop their

494 own Abs in response to threats from the outside world. The lack of aberration in the phenotype

495 of the FcγRI/II/III/IV$^{-/-}$ mouse suggests that the absence of all FcγR has little impact on the

496 ontogeny and functionality of the immune system of these mice except their downstream Ab-

497 mediated inflammatory effector functions.

498 Biological systems have a strong tendency to bypass a blockade in development and

499 functionality by adaptation (44). The complement system and FcγR are redundant in the

500 downstream effector pathways of IgG. However, the loss of FcγR was not compensated by

501 increased activity of the complement system. We did not find indications for other

502 compensation mechanisms in FcγRI/II/III/IV$^{-/-}$ mice.

22

503   Surprisingly, *in vivo* cross presentation of IgG-IC derived protein Ag, the adaptive immune

504   response against Salmonella and the antibody response against a model antigen were almost

505   indistinguishable between FcγRI/II/III/IV$^{-/-}$ and WT mice. This suggests strong redundancy in

506   the involved adaptive immune pathways.

507   The role of FcγR in cross presentation is still puzzling. We and others (11-14) have shown

508   that the cross presentation by DCs, in vitro loaded with IgG-IC, is FcR γ chain dependent. In

509   contrast, more recently we demonstrated that the enhanced *in vivo* cross presentation of

510   protein Ag derived from injected pre-formed IgG-IC is partially but not crucially dependent on

511   the FcR γ chain (22) which is in line with previous data. Den Haan and Bevan showed that in

512   the absence of FcR γ chain the uptake of i.v. injected IgG-IC and enhanced cross presentation

513   by DC was not impaired (45). An obvious explanation for this discrepancy is that an in vivo

514   dominant FcR γ chain independent - most likely complement dependent - pathway is bypassed

515   by loading DCs with IC in vitro. Our cross-presentation experiments with the FcγRI/II/III/IV$^{-/-}$

516   mice directly demonstrated that the IgG mediated enhanced cross presentation by DC in

517   vitro loaded with IgG-IC is exclusively dependent on FcγR and not on other FcR γ chain

518   associated receptor molecules. In contrast *in vivo*, FcγRs are not required, neither the

519   inhibiting FcγRIIB nor the activating FcγRs. For the uptake of exogenous Ag DCs display

520   besides FcγR a large variety of receptors such as c-type lectin receptors, TLR and complement

521   receptors. We have found a pivotal role for C1q in the presentation of Ag derived from i.v.

522   administered IgG-IC to CD8$^+$ T cells *in vivo* (22) indicating that the complement system

523   provides alternative pathways in IgG-dependent cross presentation.

524   Remarkably, in comparison to WT control mice, FcγRI/II/III/IV$^{-/-}$ mice were not hampered in B

525   cell responses but rather developed somewhat higher Ag-specific Ab titres upon immunization.

526   This is not in agreement with the previous observation that FcR γ chain KO mice, lacking

527   functional expression of all three activating receptors, develop lower Ag specific Ab titers

528   compared to WT mice (10). The discrepancy between these results might be explained by the

529   use of different FcγR deficient mouse strains. The FcR γ chain is associated with at least nine

530   other receptor complexes (18). Therefore, FcR γ chain deficiency might cause a more

531   pleiotropic effect in immunity than FcγRI/II/III/IV deficiency. On the other hand, the

532   FcγRI/II/III/IV$^{-/-}$ mouse lacks not only the activating FcγR but also the regulatory inhibiting

533   FcγRIIb. The higher IgG titres in FcγRI/II/III/IV$^{-/-}$ mice suggest that the Ab response in these

534   mice is affected by the deficiency of FcγRIIb on B cells, but not the deficiency of the three

535   activating FcγR expressed on APCs. These data indicate that activating FcγRs on antigen

536   presenting cells (APCs) are not required for the development of a full- blown antibody response

537   by facilitating presentation of IC derived Ag resulting in efficient priming of Th cell responses

538   as was suggested by previous results with FcR γ chain KO mice (10).

539   Combined, our data suggest that *in vivo* the role of activating FcγR in the regulation of adaptive

540   immunity by facilitating APC mediated presentation of IgG-IC derived Ag is dispensable. The

541   role of FcR has been implicated in enhancing an anti-tumor response by facilitating antigen-

542   presentation of IC derived tumor antigen after anti-tumor antibody therapy (46,47). In light of

543   our findings, indicating that other FcγR-independent mechanisms play a dominant role, most

544   likely complement associated, we propose to study IgG-IC mediated immune modulation in

545   more detail in our FcγRI/II/III/IV$^{-/-}$ mice, as it is the first C57BL/6 model in which these

546   questions can be answered without confounding factors. The use of antibodies with a mutation

547   in their Fc domain, destroying FcγR binding without affecting interactions with complement, is

548   limited to passive models (3), whereas our FcγRI/II/III/IV$^{-/-}$ mouse enables to study active

549   models such as vaccination and infection.

550

557   **Reference List**

558

559   1.   **Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch. 1994. FcR gamma chain deletion results
560   in pleiotrophic effector cell defects.** *Cell* **76: 519-529.**

561   2.   **Bruhns, P., and F. Jonsson. 2015. Mouse and human FcR effector functions.** *Immunol. Rev.* **268:
562   25-51.**

563   3.   **Lee, C. H., G. Romain, W. Yan, M. Watanabe, W. Charab, B. Todorova, J. Lee, K. Triplett, M.
564   Donkor, O. I. Lungu, A. Lux, N. Marshall, M. A. Lindorfer, O. R. Goff, B. Balbino, T. H. Kang, H.
565   Tanno, G. Delidakis, C. Alford, R. P. Taylor, F. Nimmerjahn, N. Varadarajan, P. Bruhns, Y. J. Zhang,
566   and G. Georgiou. 2017. IgG Fc domains that bind C1q but not effector Fcgamma receptors
567   delineate the importance of complement-mediated effector functions.** *Nat. Immunol.* **18: 889-
568   898.**

569   4.   **Fearon, D. T., and M. C. Carroll. 2000. Regulation of B lymphocyte responses to foreign and self-
570   antigens by the CD19/CD21 complex.** *Annu. Rev. Immunol.* **18: 393-422.**

571   5.   **Ahearn, J. M., M. B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R. G. Howard, T. L.
572   Rothstein, and M. C. Carroll. 1996. Disruption of the Cr2 locus results in a reduction in B-1a cells
573   and in an impaired B cell response to T-dependent antigen.** *Immunity.* **4: 251-262.**

574   6.   **Molina, H., V. M. Holers, B. Li, Y. Fung, S. Mariathasan, J. Goellner, J. Strauss-Schoenberger, R. W.
575   Karr, and D. D. Chaplin. 1996. Markedly impaired humoral immune response in mice deficient in
576   complement receptors 1 and 2.** *Proc. Natl. Acad. Sci. U. S. A* **93: 3357-3361.**

577   7.   **Fearon, D. T., and R. H. Carter. 1995. The CD19/CR2/TAPA-1 complex of B lymphocytes: linking
578   natural to acquired immunity.** *Annu. Rev. Immunol.* **13: 127-149.**

579   8.   **Heyman, B. 2014. Antibodies as natural adjuvants.** *Curr. Top. Microbiol. Immunol.* **382: 201-219.**

580   9.   **Hjelm, F., F. Carlsson, S. Verbeek, and B. Heyman. 2005. IgG3-mediated enhancement of the
581   antibody response is normal in Fc gammaRI-deficient mice.** *Scand. J. Immunol.* **62: 453-461.**

582   10.   **Hamano, Y., H. Arase, H. Saisho, and T. Saito. 2000. Immune complex and Fc receptor-mediated
583   augmentation of antigen presentation for in vivo Th cell responses.** *J. Immunol.* **164: 6113-6119.**

584   11.   **Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C.
585   Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Fcgamma receptor-mediated induction
586   of dendritic cell maturation and major histocompatibility complex class I-restricted antigen
587   presentation after immune complex internalization.** *J. Exp. Med.* **189: 371-380.**

588   12.   **Schuurhuis, D. H., A. Ioan-Facsinay, B. Nagelkerken, J. J. van Schip, C. Sedlik, C. J. Melief, J. S.
589   Verbeek, and F. Ossendorp. 2002. Antigen-antibody immune complexes empower dendritic cells
590   to efficiently prime specific CD8+ CTL responses in vivo.** *J. Immunol.* **168: 2240-2246.**

591   13.   **Yada, A., S. Ebihara, K. Matsumura, S. Endo, T. Maeda, A. Nakamura, K. Akiyama, S. Aiba, and T.
592   Takai. 2003. Accelerated antigen presentation and elicitation of humoral response in vivo by
593   Fcgamma.** *Cell Immunol.* **225: 21-32.**

26

594   14. Schuurhuis, D. H., N. van Montfoort, A. Ioan-Facsinay, R. Jiawan, M. Camps, J. Nouta, C. J. Melief,
595   J. S. Verbeek, and F. Ossendorp. 2006. Immune complex-loaded dendritic cells are superior to
596   soluble immune complexes as antitumor vaccine. *J. Immunol.* 176: 4573-4580.

597   15. Platzer, B., M. Stout, and E. Fiebiger. 2014. Antigen cross-presentation of immune complexes.
598   *Front Immunol.* 5: 140.

599   16. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J. V. Ravetch. 1996. Augmented humoral and
600   anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 379: 346-349.

601   17. Bolland, S., and J. V. Ravetch. 2000. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient
602   mice results from strain-specific epistasis. *Immunity.* 13: 277-285.

603   18. Fodor, S., Z. Jakus, and A. Mocsai. 2006. ITAM-based signaling beyond the adaptive immune
604   response. *Immunol. Lett.* 104: 29-37.

605   19. Wandstrat, A. E., C. Nguyen, N. Limaye, A. Y. Chan, S. Subramanian, X. H. Tian, Y. S. Yim, A.
606   Pertsemlidis, H. R. Garner, Jr., L. Morel, and E. K. Wakeland. 2004. Association of extensive
607   polymorphisms in the SLAM/CD2 gene cluster with murine lupus. *Immunity.* 21: 769-780.

608   20. Boross, P., V. L. Arandhara, J. Martin-Ramirez, M. L. Santiago-Raber, F. Carlucci, R. Flierman, J. van
609   der Kaa, C. Breukel, J. W. Claassens, M. Camps, E. Lubberts, D. Salvatori, M. P. Rastaldi, F.
610   Ossendorp, M. R. Daha, H. T. Cook, S. Izui, M. Botto, and J. S. Verbeek. 2011. The inhibiting Fc
611   receptor for IgG, FcgammaRIIB, is a modifier of autoimmune susceptibility. *J. Immunol.* 187: 1304-
612   1313.

613   21. van Montfoort, N., S. M. Mangsbo, M. G. Camps, W. W. van Maren, I. E. Verhaart, A. Waisman, J.
614   W. Drijfhout, C. J. Melief, J. S. Verbeek, and F. Ossendorp. 2012. Circulating specific antibodies
615   enhance systemic cross-priming by delivery of complexed antigen to dendritic cells in vivo. *Eur. J.*
616   *Immunol.* 42: 598-606.

617   22. van Montfoort, N., J. M. de Jong, D. H. Schuurhuis, E. I. van der Voort, M. G. Camps, T. W.
618   Huizinga, C. van Kooten, M. R. Daha, J. S. Verbeek, F. Ossendorp, and R. E. Toes. 2007. A novel
619   role of complement factor C1q in augmenting the presentation of antigen captured in immune
620   complexes to CD8+ T lymphocytes. *J. Immunol.* 178: 7581-7586.

621   23. Mahler, C. M., M. Berard, R. Feinstein, A. Gallagher, B. Illgen-Wilcke, K. Pritchett-Corning, and M.
622   Raspa. 2014. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea
623   pig and rabbit colonies in breeding and experimental units. *Lab Anim* 48: 178-192.

624   24. Boross, P., P. L. van Lent, J. Martin-Ramirez, J. van der Kaa, M. H. Mulder, J. W. Claassens, W. B.
625   van den Berg, V. L. Arandhara, and J. S. Verbeek. 2008. Destructive arthritis in the absence of both
626   FcgammaRI and FcgammaRIII. *J. Immunol.* 180: 5083-5091.

627   25. Ioan-Facsinay, A., S. J. de Kimpe, S. M. Hellwig, P. L. van Lent, F. M. Hofhuis, H. H. van Ojik, C.
628   Sedlik, S. A. da Silveira, J. Gerber, Y. F. de Jong, R. Roozendaal, L. A. Aarden, W. B. van den Berg, T.
629   Saito, D. Mosser, S. Amigorena, S. Izui, G. J. van Ommen, M. van Vugt, J. G. van de Winkel, and J.
630   S. Verbeek. 2002. FcgammaRI (CD64) contributes substantially to severity of arthritis,
631   hypersensitivity responses, and protection from bacterial infection. *Immunity.* 16: 391-402.

632   26. Dahan, R., E. Sega, J. Engelhardt, M. Selby, A. J. Korman, and J. V. Ravetch. 2015. FcgammaRs
633   Modulate the Anti-tumor Activity of Antibodies Targeting the PD-1/PD-L1 Axis. *Cancer Cell* 28:
634   543.

635   27. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V. S. Zimmermann, J.
636   Davoust, and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth
637   factor-dependent long-term cultures. *J. Exp. Med.* 185: 317-328.

638   28. Shashidharamurthy R., E. Bozeman, J. Patel, R. Kaur, J. Meganathan and P. Selvaraj Analysis of
639   cross-species IgG binding to human and mouse Fcgamma receptors (FcγRs) (138.29 J Immunol
640   April 1, 2010, 184 (1 Supplement) 138.29

641   29. Sanderson, S., and N. Shastri. 1994. LacZ inducible, antigen/MHC-specific T cell hybrids. *Int.*
642   *Immunol.* 6: 369-376.

643   30. Zhang, D., K. Fujio, Y. Jiang, J. Zhao, N. Tada, K. Sudo, H. Tsurui, K. Nakamura, K. Yamamoto, H.
644   Nishimura, T. Shira, and S. Hirose. 2004. Dissection of the role of MHC class II A and E genes in
645   autoimmune susceptibility in murine lupus models with intragenic recombination. *Proc. Natl.*
646   *Acad. Sci. U. S. A* 101: 13838-13843.

647   31. Hoiseth, S. K., and B. A. Stocker. 1981. Aromatic-dependent Salmonella typhimurium are non-
648   virulent and effective as live vaccines. *Nature* 291: 238-239.

649   32. Kotimaa, J. P., M. B. van Werkhoven, J. O'Flynn, N. Klar-Mohamad, G. J. van, G. Schilders, H.
650   Rutjes, M. R. Daha, M. A. Seelen, and C. van Kooten. 2015. Functional assessment of mouse
651   complement pathway activities and quantification of C3b/C3c/iC3b in an experimental model of
652   mouse renal ischaemia/reperfusion injury. *J. Immunol. Methods* 419: 25-34.

653   33. Trouw, L. A., M. A. Seelen, J. M. Duijs, H. Benediktsson, C. van Kooten, and M. R. Daha. 2003.
654   Glomerular deposition of C1q and anti-C1q antibodies in mice following injection of antimouse
655   C1q antibodies. *Clin. Exp. Immunol.* 132: 32-39.

656   34. Kotimaa, J., N. Klar-Mohammad, F. Gueler, G. Schilders, A. Jansen, H. Rutjes, M. R. Daha, and C.
657   van Kooten. 2016. Sex matters: Systemic complement activity of female C57BL/6J and BALB/cJ
658   mice is limited by serum terminal pathway components. *Mol. Immunol.* 76: 13-21.

659   35. van Beek, L., I. O. Vroegrijk, S. Katiraei, M. M. Heemskerk, A. D. van Dam, S. Kooijman, P. C.
660   Rensen, F. Koning, J. S. Verbeek, K. Willems van Dijk, and V. van Harmelen. 2015. FcRgamma-
661   chain deficiency reduces the development of diet-induced obesity. *Obesity. (Silver. Spring)* 23:
662   2435-2444.

663   36. Nandakumar, K. S., M. Andren, P. Martinsson, E. Bajtner, S. Hellstrom, R. Holmdahl, and S.
664   Kleinau. 2003. Induction of arthritis by single monoclonal IgG anti-collagen type II antibodies and
665   enhancement of arthritis in mice lacking inhibitory FcgammaRIIB. *Eur. J. Immunol.* 33: 2269-2277.

666   37. Hobday, P. M., J. L. Auger, G. R. Schuneman, S. Haasken, J. S. Verbeek, and B. A. Binstadt. 2014.
667   Fcgamma receptor III and Fcgamma receptor IV on macrophages drive autoimmune valvular
668   carditis in mice. *Arthritis Rheumatol.* 66: 852-862.

669

670 38. Quakkelaar, E. D., M. F. Fransen, W. W. van Maren, J. Vaneman, N. M. Loof, S. H. van Heiningen, J. S.
671   Verbeek, F. Ossendorp, and C. J. Melief. 2014. IgG-mediated anaphylaxis to a synthetic long
672   peptide vaccine containing a B cell epitope can be avoided by slow-release formulation. *J.*
673   *Immunol.* 192: 5813-5820.

674 39. Bygrave, A. E., K. L. Rose, J. Cortes-Hernandez, J. Warren, R. J. Rigby, H. T. Cook, M. J. Walport, T.
675   J. Vyse, and M. Botto. 2004. Spontaneous autoimmunity in 129 and C57BL/6 mice-implications for
676   autoimmunity described in gene-targeted mice. *PLoS. Biol.* 2: E243.

677 40. Rose, S., A. Misharin, and H. Perlman. 2012. A novel Ly6C/Ly6G-based strategy to analyze the
678   mouse splenic myeloid compartment. *Cytometry A* 81: 343-350.

679 41. Schmidt, R. E., and J. E. Gessner. 2005. Fc receptors and their interaction with complement in
680   autoimmunity. *Immunol. Lett.* 100: 56-67.

681 42. Shulzhenko, N., A. Morgun, W. Hsiao, M. Battle, M. Yao, O. Gavrilova, M. Orandle, L. Mayer, A. J.
682   Macpherson, K. D. McCoy, C. Fraser-Liggett, and P. Matzinger. 2011. Crosstalk between B
683   lymphocytes, microbiota and the intestinal epithelium governs immunity versus metabolism in
684   the gut. *Nat. Med.* 17: 1585-1593.

685 43. Winer, D. A., S. Winer, L. Shen, P. P. Wadia, J. Yantha, G. Paltser, H. Tsui, P. Wu, M. G. Davidson,
686   M. N. Alonso, H. X. Leong, A. Glassford, M. Caimol, J. A. Kenkel, T. F. Tedder, T. McLaughlin, D. B.
687   Miklos, H. M. Dosch, and E. G. Engleman. 2011. B cells promote insulin resistance through
688   modulation of T cells and production of pathogenic IgG antibodies. *Nat. Med.* 17: 610-617.

689 44. Rudmann, D. G. 2013. On-target and off-target-based toxicologic effects. *Toxicol. Pathol.* 41: 310-
690   314.

691 45. den Haan, J. M., and M. J. Bevan. 2002. Constitutive versus activation-dependent cross-
692   presentation of immune complexes by CD8(+) and CD8(-) dendritic cells in vivo. *J. Exp. Med.* 196:
693   817-827.

694 46. Dhodapkar, K. M., J. Krasovsky, B. Williamson, and M. V. Dhodapkar. 2002. Antitumor
695   monoclonal antibodies enhance cross-presentation ofcCellular antigens and the generation of
696   myeloma-specific killer T cells by dendritic cells. *J. Exp. Med.* 195: 125-133.

697 47. Kalergis, A. M., and J. V. Ravetch. 2002. Inducing tumor immunity through the selective
698   engagement of activating Fcgamma receptors on dendritic cells. *J. Exp. Med.* 195: 1653-1659.
699
700

701
702

703

704

705

Additional footnotes

Legends:

732 **Fig.1. Generation of the FcγRI/FcγRII/FcγRIII/FcγRIV quadruple KO (FcγRI/II/III/IV$^{-/-}$)**

733 **mouse strain.**

734 FcγRIIb$^{fl/fl}$ mice (13) were crossed with FcγRIII$^{fl/fl}$ mice (supplemental figure S1). Offspring was

735 selected for crossover between both floxed alleles. By crossing FcγRIIb$^{fl/fl}$ / FcγRII$^{fl/fl}$ mice with

736 the EIIaCre deleter strain a 90.4 kb fragment between the two most distant loxP sites, containing

737 the main part of the FcγRIIb and FcγRIII gene and the complete FcγRIV gene, was removed

738 resulting in a FcγRII/III/IV KO allele. The presence of the deletion was confirmed by PCR and

739 DNA sequencing. The FcγRII/III/IV$^{-/-}$ mice were crossed with our previously generated FcγRI$^{-/-}$

740 mice (16) and FcγRI/II/III/IV$^{-/-}$ offspring was selected. The absence of all four FcγR was

741 confirmed by FACS analysis.

742 a. From top to bottom are depicted the genomic structure of the WT FcγRIIb/FcγRIV/FcγRIII

743 gene cluster on chromosome 1, the gene targeting strategy for the generation of the floxed FcγRIIb

744 and FcγRIII genes, the genomic structure after the crossover between the two floxed genes and

745 subsequently after Cre mediated recombination. The locus is shown in reverse orientation in

746 relation to the chromosomal nucleotide numbering. The exact location of the borders of the

747 deletion (NC_000067.6:g 171054449_170964079del according to HGVS nomenclature) on

748 chromosome 1 are depicted based on the mouse reference genome build GRCm38.p3 (C57BL/6J)

749 provided by the Genome Reference Consortium.

750 b. Core sequence flanking the remaining LoxP site within the 437 bp PCR fragment.

751 c. Flow cytometry of thioglycolate elicited peritoneal cells from FcγRI/II/III/IV$^{-/-}$ (black lines) and

752 WT C57BL/6 mice (grey lines) stained with fluorescent labeled antibodies specific for F4.80 and

753 CD11b and antibodies specific for the different FcγR as indicated. d. Agarose gel electrophoresis

754   of the unique PCR fragment bridging the 90.4 kb deletion. By using a FcγRIII specific 'Geno Fw':

755   GAGGGCATCCGATTTCATTA and a FcγRIIb specific 'Null B Rev'

756   GCTTCCATTGACCTGCCTAC primer, and genomic DNA from a FcγRII/III/IV$^{-/-}$mouse as a

757   template, a unique 437 bp fragment with the remaining LoxP site was synthesized. M: 100bp

758   ladder, KO: FcγRII/III/IV$^{-/-}$ mouse; WT: WT control mouse.

759

760   **Fig.2. IgG downstream effector functions are impaired in FcγRI/II/III/IV$^{-/-}$ mice**

761   **a**. CAIA. From each phenotype footpad swelling was measured using a caliper. The average of

762   combined left and right footpad swelling of the forepaws was plotted and expressed as Mean and

763   SEM of increase in footpad thickness in mm. The area under the curve was calculated per mouse

764   from day 7 until day 28 and a Mann-Witney test was performed for statistics. The response of the

765   FcγRI/II/III/IV$^{-/-}$ mice was significantly lower compared to the response of FcγRIIb$^{-/-}$ mice

766   (P=0.0159). Five mice per group. One representative experiment out of two performed is shown.

767   **b**. Passive systemic anaphylaxis. Time course of blood pressure, expressed in mm HG, as mean

768   arterial pressure (MAP) plus SEM, in FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 mice passively

769   sensitized by i.v. injection of mouse anti-TNP IgG2a, and challenged 4 hours later with DNP-

770   HSA. Six mice per group. Each time point is analyzed by a separate T test, and the curves are

771   significantly different (p<0.01) from 5 minutes onwards, indicated with *.

772   **c.** ADCP. Mice were injected with CD8^{+} depleting Ab (2.43). Before and after Ab injection, the

773   number of CD8^{+} T cell in blood was determined by flow cytometry and depicted as percentage of

774   CD8^{+}/CD3^{+} cells of total lymphocyte population. Data shown are from one out of two experiments

775   with similar results. Four FcγRI/II/III/IV$^{-/-}$ and two WT mice per group.

776   Data was statistically analyzed with a T test at each time point, p=0.15 at day 0, and 0.0001 at day

777   6 as compared to WT C57BL/6 mice.

Fig.3. FcγR involvement in the cross-presentation of IgG-IC derived Ag

a. Uptake of IgG-IC derived Alexa488 labeled Ag by bone-marrow derived dendritic cells (BM-DC) from WT and FcγRI/II/III/IV$^{-/-}$ mice measured by flow cytometry, presented as mean plus SD of three samples. Extracellular binding was quenched by the addition of trypan blue. Depicted is percentage of Alexa488 positive cells out of total cell count. One representative experiment of two experiments performed is shown, statistical analysis was performed with T test. Asterisks indicate significant differences (***$p < 0.001$) as compared to WT C57BL/6.

b. BM-DC from FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 mice were incubated with OVA-IgG IC and subsequently co-cultured with T cell hybridoma B3Z which recognized an OVA-CTL epitope in MHC class I. Recognition leads to activation of the LacZ reporter gene which was measured with a β-galactosidase assay, and analyzed as absorption of light at OD590 nm. Minimal SIINFEKL OVA epitope was included as an MHC class I loaded positive control in both DC types. Presented as mean plus SEM, 4 samples per group. Statistical analysis was performed with a T test, (***$p < 0.001$ for the mice that received immune complexes as compared to WT C57BL/6 mice

c and d. WT C57BL/6 and FcγRI/II/III/IV$^{-/-}$ mice were injected with CFSE labeled OT-I T cells and subsequently injected with OVA with or without anti-OVA IgG. *In vivo* cross presentation was determined by analyzing the CFSE dilution of OT-I cells using flow-cytometry. Depicted are percentage of proliferating OT-I cells (CFSE fluorescence is diluted at least once) of total OT-I gated cells as mean of group plus SD (c) and representative CFSE plots (d). Data shown are from one out of two experiments with similar results. Five mice per group. Statistical analysis with T test per condition revealed no differences, p=0.63, 0.15, 0.73 respectively for OVA+Ab, OVA alone and naïve mice compared to WT C57BL/6 mice.

e and f. Western blot analysis of the presence of Alexa488 labeled OVA in serum of mice at

802   different time-points after i.v. injection of the OVA anti-OVA IgG IC (e) and quantification of

803   fluorescent OVA in Western Blot samples (f). Data shown are representative samples from three

804   mice per experiment. Three experiments with similar results were performed.

805   **g**. At different time-points after injection, mice were sacrificed and a single lobe of liver was

806   isolated and imaged. Signal quantification of Alexa488 labeled IgG IC was performed. The

807   fluorescent signal is shown as the total radiant efficiency (TRE), expressed in

808   (photons/second)/(μW/cm²). The TRE/g liver in the time is shown. Data shown are from one out

809   of two experiments with similar results. Three mice per group.

810

811   **Fig.4. Adaptive immune system is normal in FcγRI/II/III/IV$^{-/-}$ mice**

812   **a**. Lymphoid organs were harvested from two months old FcγRI/II/III/IV$^{-/-}$ and WT mice. Single

813   cell suspensions were labeled with fluorescent Abs and analyzed using flow-cytometry. Each

814   symbol represents an individual mouse. Data shown are from one out of two experiments with

815   similar results. **b.** Mice were immunized three times with TNP-BSA. Serum samples were taken 8

816   days after first boost and 7 and 12 days after second boost. Titres of anti-BSA antibodies were

817   determined with ELISA. Data of day 12 are shown. Other time points showed similar results.

818   Eight mice per group, shown is mean plus SEM.

819

820   **Fig.5. T and B cell responses to *Salmonella* infection were similar in FcγRI/II/III/IV$^{-/-}$ and**

821   **WT control mice.**

822   **a**. CD4^{+} T cells were positively enriched from splenocytes of groups of seven WT C57BL/6 and

823   seven FcγRI/II/III/IV$^{-/-}$ mice infected 10 weeks earlier with STm SL3261. Groups of four WT

824   C57BL/6 and four FcγRI/II/III/IV$^{-/-}$ naïve mice were also included in the experiment. The cells

825   from individual mice were exposed to *Salmonella* Ag (salm) or anti-CD3 and anti-CD28 (pos) as a

826 positive control, or medium (neg), as negative control. IFNγ (left panel) and IL2 (right panel) were

827 measured in the supernatants by ELISA after 72 and 24 hours respectively. Data of one

828 representative experiment out of two performed are shown. Statistical analysis using ANOVA did

829 not show significant differences between WT C67BL/6 and FcγRI/II/III/IV$^{-/-}$mice.

830 **b**. Anti-STm LPS Abs were measured by ELISA in the sera of groups of five WT C57BL/6 and

831 five FcγRI/II/III/IV$^{-/-}$ mice infected as in fig 5a. Groups of four naïve mice were included as

832 controls. Ab titers are expressed as the reciprocal of the dilutions giving a reading equal to half of

833 the maximal absorbance. Data of one representative experiment out of two performed are shown.

834 Statistical analysis was performed with T-test. Asterisks indicate significant difference. (* p<

835 0.05) as compared to WT C57BL/6.

836

837 **Fig.6. IgG titres in older FcγRI/II/III/IV$^{-/-}$ and WT control mice**

838 **a.** IgG1, IgG2a and IgG2b titres were determined in sera of FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6

839 mice with ELISA, three mice per group. One representative experiment out of two performed is

840 shown.

841 **b, c and d.** IgG anti-dsDNA Ab titers (b) IgG anti-Chromatin titers (c) and IgG anti Histone titers

842 (d), were determined by ELISA using anti-mouse γ chain-specific secondary Abs. Each symbol

843 represents a mouse. Mean and SEM are shown with respective p-values.

844

845 **Fig.7. No differences in myeloid cell compartments between FcγRI/II/III/IV$^{-/-}$ and WT**

846 **C57BL/6 control mice.**

847 **a**. Spleens of three months old FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 mice were incubated with

848 Liberase, and single cell suspensions were labeled and analyzed by flow-cytometry. Graphs show

849 the percentage CD8 positive or negative cells of CD11c^{+}/CD19^{-}/ B220^{-}/7-AAD^{-} cells. Three mice

850   per group. Data shown are from one out of two experiments with similar results.

851   **b**. Spleens of three months old FcγRI/II/III/IV$^{-/-}$ and WT C57BL/6 mice were incubated with

852   Liberase, and single cell suspensions were labeled and analyzed by flow-cytometry. Gating

853   strategy was according to Shawn Rose et al (40), in short, gated on 7-aad$^-$/CD19$^-$/CD3$^-$ and

854   followed by CD11c$^+$ for CD11c$^+$ group, CD11b$^+$ for CD11b$^+$ group, F4-80 $^{+/-}$/ Ly6G$^+$ for

855   neutrophils, F4-80$^+$/ Ly6G$^-$/Ly6C$^{+/-}$, SSC high for eosinophils, F4-80$^+$/ Ly6G$^-$/Ly6C$^+$, SSC low

856   monocyte/macrophage type I, F4-80$^+$ /Ly6G$^-$/Ly6C$^-$, SSC low for monocyte/macrophage type II.

857   Four mice per group. Data shown are from one out of two experiments with similar results.

858   Statistical analysis performed with Sidak's multiple comparisons test, showed that all groups were

859   not significantly different from each other.

860

861   **Fig. 8. No differences in plasma concentrations and activity of complement factors between**

862   **FcγRI/II/III/IV$^{-/-}$ mice and WT C57BL/6 control mice.**

863   a. Functional complement activity in plasma of WT C57BL/6 and FcγRI/II/III/IV$^{-/-}$ mice was

864   determined for all three pathways (classical (CP), alternative (AP) and lectin pathway (LP), both

865   at the level of C3 deposition and at the level of C9 deposition. Samples were tested in serial

866   dilutions, quantified in comparison with a standard CD1 serum and depicted as AU/ml. Five mice

867   per group. Statistical analysis with Sidak's multiple comparison's test revealed no significant

868   difference between FcγRI/II/III/IV$^{-/-}$.and WT C57/BL/6 mice.

869   b. Individual complement factors were quantified in plasma of five mice per genotype using

870   specific ELISAs. This includes C1q as inducer of the classical pathway, properdin as stabilizer of

871   the alternative pathway, the central component C3 and C6 and C9 as part of the terminal pathway.

872   Data are shown as the amounts per ml serum of the indicated factors either in µg (properdin, C6

873   and C9) or in (AU) arbitrary units (C1q and C3 fragment).