Correction

IMMUNOLOGY

PNAS notes that a conflict of interest statement was omitted during publication. PNAS declares that “The editor, Jeffrey Ravetch, is a recent coauthor with an author (F.N.) of this publication, having last published a paper with him in 2010.”

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Fcγ receptor IIB (FcγRIIB) maintains humoral tolerance in the human immune system in vivo

Anne Baerenwaldt, Anja Lux, Heike Danzer, Bernd M. Spriewald, Evelyn Ulrich, Gordon Heidkamp, Diana Dudziak, and Falk Nimmerjahn

*Department of Biology, Institute of Genetics, University of Erlangen-Nürnberg, 91058 Erlangen, Germany; †Department of Hematology and Oncology, University Hospital of Erlangen, 91054 Erlangen, Germany; and ‡Department of Dermatology, Nikolaus-Fiebiger-Centre for Molecular Medicine, University Hospital of Erlangen, 91054 Erlangen, Germany

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Maintenance of immunological tolerance is crucial to prevent development of autoimmune disease. The production of autoantibodies is a hallmark of many autoimmune diseases and studies in mouse model systems suggest that inhibitory signaling molecules may be important checkpoints of humoral tolerance. By generating humanized mice with normal and functionally impaired Fcγ receptor IIB (FcγRIIB) variants, we show that the inhibitory Fcγ-receptor is a checkpoint of humoral tolerance in the human immune system in vivo. Impaired human FcγRIIB function resulted in the generation of higher levels of serum immunoglobulins, the production of different autoantibody specificities, and a higher proportion of human plasmablasts and plasma cells in vivo. Our results suggest that the inhibitory FcγRIIB may be an important checkpoint of humoral tolerance in the human immune system.

Results and Discussion

Humanized Mice Carrying the FcγRIIB-232T Variant Produce Higher Levels of Serum IgM and IgG. To study the function of human FcγRIIB and the functionally impaired FcγRIIB-232T variant, we injected human HSC carrying the respective fcgr2b alleles into newborn NSG mice. Transplantation of NSG mice with human HSCs resulted in the development of a human immune system consisting of different subsets of B cells, T cells, NK-cells, monocytes, and dendritic cells (18) (Fig. S1). Irrespective of the fcgr2b genotype, all groups of mice showed an equal level of engraftment with human cells in the peripheral blood (Fig. L4). As in humans, all B cells in the peripheral blood expressed FcγRIIB on their cell surface (Fig. L8). Despite comparable levels of expression, FcγRIIB function was impaired in the FcγRIIB-232 T/T group as simultaneous cross-linking of the B-cell receptor with the FcγRIIB-232T variant did not result in reduced calcium flux compared with cross-linking the B-cell receptor alone (Fig. 1C). Analysis of earlier B-cell developmental stages in the bone marrow of humanized mice and humans demonstrated that FcγRIIB starts to be expressed at the pre–B-cell stage and becomes up-regulated on germinal-center B cells, memory B cells, and plasma cells (Fig. 1 D–F and Fig. S2). Compared with the human spleen samples, a much lower percentage of B cells expressed memory markers, such as CD27, which may be explained by the maintenance of humanized mice under controlled environmental conditions versus the exposure to all types of infectious agents and vaccinations of the human adult population (Fig. 1F and Fig. S2D). Along the same lines, it has to be considered that the human immune system in humanized mice (age 4–6 mo) has not yet


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1To whom correspondence should be addressed. E-mail: fnimmerj@biologie.uni-erlangen.de

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reached the steady state with respect to the development of all hematopoetic cell lineages. This finding may be reflected by the higher amount of B cells compared with other cell types, such as T cells, in the spleen of humanized mice, which need more time to reach levels comparable with an adult human immune system (Fig. S1). When interpreting the results from the human samples, it should be noted that all bone marrow and spleen samples were obtained from patients with different malignant or autoimmune diseases (see Materials and Methods for further details) and therefore may not fully represent the healthy human situation.

Loss of Humoral Tolerance in the Absence of Functional Human FcγRIIB. Besides enhanced Ig production, FcγRIIB-deficient mice break humoral tolerance and produce anti-double-stranded DNA (anti-dsDNA) antibodies on the C57BL/6 background (8, 20–22). Consistent with this data from classic mouse models, human B cells in humanized mice that received stem cells from five different FcγRIIB-232T/T donors started to produce autoantibodies directed against dsDNA, glucose phosphate 6-isomerase (GPI), rheumatoid factor (RF), and antibodies directed against cyclic citrullinated peptides (anti-CCP) (Fig. 2C–F). Anti-CCP autoantibodies are rarely seen in classic animal models of autoimmune disease and represent a unique feature of the human autoreactive B-cell repertoire, and in combination with other autoantibodies have a highly predictive value of arthritis development in humans (23). To be able to distinguish how individual mice receiving stem

Fig. 1. FcγRIIB expression and function in humanized mice. (A) Presence of human cells in the blood of humanized mice 4 mo after transplantation with HSC carrying the FcγRIIB-232I/I (232I/I), -232I/T (232I/T), or -232T/T alleles. (B) Expression of FcγRIIB on CD19+ B cells of humans and humanized mice in the peripheral blood. (C) Assessment of FcγRIIB function in the peripheral blood of humanized mice with the indicated FcγRIIB genotypes by calcium flux analysis after cross-linking the B-cell receptor either alone (red line) or in combination with FcγRIIB (blue line). (D–F) Expression of FcγRIIB on different B-cell developmental stages in the bone marrow (D) and spleen (E and F) of humanized mice. Expression level of FcγRIIB is shown as mean fluorescence intensity (MFI). *P < 0.05, **P < 0.01, ***P < 0.001.
cells from the same or different donors behave with respect to serum Ig levels and autoantibody production, all datapoints stemming from the same HSC donor are depicted in the same color. As shown in Fig. 2, mice receiving stem cells from different FcγRIIB-232T/T donors produced higher levels of serum Ig and had detectable levels of autoantibodies in their serum. Interestingly, the autoantibody repertoire differed depending on the individual HSC donor. Whereas almost all FcγRIIB-232T/T-derived mouse groups produced autoantibodies to dsDNA (although at different levels), only select mice produced RF (two donors) or anti-CCP (three donors) autoantibodies. This finding may suggest that impaired FcγRIIB function lowers the threshold for autoantibody production, but that other factors, such as the genetic background or the environment, can influence the level or specificity of the resulting autoimmune response. Heterozygous FcγRIIB-232I/T carriers showed a more restricted autoantibody repertoire, including elevated levels of anti-DNA and anti-GPI antibodies but did not produce elevated IgG levels.

**Influence of HLA Haplotypes and Activating FcγR Alleles on Loss of Humoral Tolerance.** Besides inhibitory signaling molecules, certain haplotypes of the HLA complex (HLA) or allelic variants of activating Fcγ-receptors may be associated with the development or severity of autoimmune disease (24–28). Therefore, we genotyped all homozygous FcγRIIB-232I and FcγRIIB-232T carriers for selected HLA class II-haplotypes (HLA-DR*15/DQ*06 and HLA-DR*03/DQ*02) and Fcγ-receptor alleles. As shown in Fig. 3A, no enrichment of these HLA-haplotypes was seen in autoantibody-producing individuals. Of note, there was a trend toward lower autoantibody production in individuals carrying the HLA-DR*15/DQ*06 haplotype. With respect to different activating FcγR alleles, a similar picture emerged, with a trend toward a higher level of autoantibody production in homozygous carriers of the high-affinity FcγRIIA allele (FcγRIIA-131I/I) (Fig. 3B). The sole presence of this allele in humanized mice carrying the functionally intact FcγRIIB-232I variant was not sufficient to break humoral tolerance (Fig. 3B). Previous studies in mouse models support the notion that FcγRIIB deficiency leads to a higher number of autoantibody-producing B cells, including plasmablasts and plasma cells (20). To test if impaired function of human FcγRIIB results in a similar phenotype, we analyzed the proportion of human plasmablasts and plasma cells in the different humanized mouse cohorts. As shown in Fig. 4A and B, impaired FcγRIIB function resulted in higher levels of both IgM-positive and -negative plasmablasts and plasma cells. Consistently, ELISPOT analysis confirmed that the autoantibody-producing B cells could be detected in the bone marrow of humanized mice carrying the impaired FcγRIIB-232T but not the FcγRIIB-232I variant (Fig. 4C and D).

Taken together, these results are unique in showing that impaired function of human FcγRIIB may result in the loss of humoral tolerance in the human immune system in vivo. Of note, we did not observe overt autoimmune disease, such as rheumatoid arthritis or SLE, within the observation period. Nonetheless, the
humanized mouse model allows studying developmental and functional differences of human genetic traits in a controlled environment, and may thus become a valuable experimental system to close the gap between data obtained in mouse knockout models and human genetic association studies.

Materials and Methods

Mice. NSG mice (The Jackson Laboratories) were held under specific pathogen-free conditions and in isolated ventilated cages in the animal facility of the Franz-Penzold-Zentrum of the University of Erlangen, according to institutional guidelines and the rules and regulations of the German animal welfare laws.

Reconstitution of NSG Mice with CD34+ HSC. Newborn NSG mice were irradiated with 1.3 Gy. After 4–6 h, 25,000–55,000 HSC were injected intravenously using a Hamilton syringe (gastight 1710; Hamilton Bonaduz). Twelve to 16 wk after transplantation, mice were tested for the presence of human cells in the peripheral blood by FACS analysis with anti-mouse CD45.1-PE and anti-human CD45-APC-H7 antibodies. For all of the studied genotypes, stem-cell preparations from several donors (232I/I: 7 donors; 232I/T: 2 donors; 232 T/T: 5 donors) were used. With the exception of the FcγRIIIB-232I/I group, different mouse litters were transplanted with individual stem-cell samples, and therefore could be distinguished from each other. To demonstrate how mice receiving stem cells from the same donor differ with respect to serum Ig levels and autoantibody production within the group, and to compare the data to mice receiving stem cells from a different donor, all datapoints stemming from one human stem-cell donor were labeled in the same color. Peripheral blood samples from three healthy donors (232I/I) served as controls.

Antibodies and Flow Cytometry. The following human antibodies were used for flow cytometry: CD3-PerCp, CD3-PE-Cy7, CD4-APC, CD8-PE, CD11c-PE-Cy7, CD14-PerCp, CD19-APC, CD19-PE-Cy7, CD27-APC, CD33-PE, CD34-PE, CD38-APC, CD45-APC-H7, CD56-PE, CD138-APC, IgM-PE, IgM-FITC (all from BD Biosciences). To stain CD20, we used the 1F5 hybridoma clone (ATCC) and labeled the purified antibody with FITC. To stain human FcγRI we used the 2B6 antibody (provided by Jeffrey Stavenhagen, Macrogenics, Rockville, MD) labeled with Alexa647. The human CD10-PerCp and the mouse CD45.1-PE antibody were purchased from Biolegend. Staining of cells was performed in PBS/2% FCS, 0.02% sodium acid on ice. Before staining, mouse Fc receptors were blocked with 2.4G2. Data were acquired on a FACS Canto II (BD Biosciences) and analyzed with FACS Diva software.

Isolation of CD34+ HSC. Mononuclear cells were isolated from cord blood samples by density gradient centrifugation on a Pancoll (PAN Biotech) gradient. CD34+ cells were enriched with the Direct human CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec) according to the instructions provided by the manufacturer. CD34+ HSC were stored until further usage at −80°C or in liquid nitrogen.

Generation of Single-Cell Suspensions from Human Spleen. Human spleen samples were obtained from patients (labeled patients 1–5) undergoing surgery at the University Hospital of Erlangen, Germany. All human samples used in this study were used with permission of the donors and according to the ethical guidelines of the hospitals of Erlangen and Fürth. The indications for

Table 1. Primer sets used to identify FcγR alleles

| FcγR | Primer set | Orientation | Primer sequence (5′–3′)
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<tbody>
<tr>
<td>1st PCR</td>
<td>Sense</td>
<td>gagaacacatatgcgtcag</td>
<td>gtagctgctgcagcttc</td>
</tr>
<tr>
<td>2nd PCR, allele specific</td>
<td>Sense</td>
<td>gaaatrccagaaaaatttcca</td>
<td>gaaatrccagaaaaatttcca</td>
</tr>
<tr>
<td>2nd PCR, allele specific</td>
<td>Sense</td>
<td>caattttctgtcagtggg</td>
<td>caattttctgtcagtggg</td>
</tr>
<tr>
<td>2nd PCR</td>
<td>Antisense</td>
<td>gtagcttccagctgctg</td>
<td>gtagcttccagctgctg</td>
</tr>
<tr>
<td>1st PCR</td>
<td>Sense</td>
<td>tccatatttcagatggcaaaag</td>
<td>tccatatttcagatggcaaaag</td>
</tr>
<tr>
<td>2nd PCR, allele specific</td>
<td>Antisense</td>
<td>tttgggagttataaaattgcttctagaga</td>
<td>tttgggagttataaaattgcttctagaga</td>
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removal of the spleen were carcinoma (patient 3), polytrauma (patient 4), and immunothrombocytopenia (patient 5). The indications for bone marrow biopsies were diagnosis of lymphoma (patient 1 and 2). The spleen specimens were kept at 4 °C in RPMI medium until the start of processing. The samples were initially disrupted in C-tubes (Miltenyi), containing 5-mL buffer (PBS, 2% FCS), using the gentleMACS dissociator (Miltenyi) in the presence of Collagenase NB 4G (Serva) and DNase I (Roche). After 45 min at 37 °C, 100 μL 0.5 M EDTA were added and the samples were incubated for a further 5 min. The cell suspension was then filtered through 100-μm cell strainers (Becton Dickinson). The falcon tubes were filled with PBS, 2% FCS and centrifuged at 470 × g and 4 °C for 5 min. Cell pellets were resuspended in 10 mL PBS, 2% FCS and applied to 70-μm cell strainers. After resuspension in 20 mL of PBS the cells were loaded onto 14 mL Pancoll (PAN Biotech; density 1.077 g/mL), and centrifuged for 45 min at 470 × g. The interphase was recovered and washed twice with PBS, 2% FCS (5 min, 4 °C, 470 × g). The cells were either used immediately for further analysis or stored in aliquots at −80 °C for future experiments.

Isolation of Genomic DNA and Genotyping. For genotyping of CD34+ HSC, 250 μL of umbilical cord blood samples were taken and stored at −20 °C before proceeding to the isolation of HSC. Genomic DNA was isolated with the QiAamp DSP Blood Mini Kit (Qiagen) following the instructions of the distributor. Low-resolution genotyping for HLA-A, -B, DRB1, and DOB1 was carried out using the respective commercial LABType SSO typing kit (One Lambda) according to the manufacturer’s instructions. FcγRIIB genotyping was carried out with a two-step PCR protocol as previously described (17). Briefly, a 15-kb product was amplified using the Qiagen LongRange PCR Kit (Qiagen) followed by purification via gel electrophoresis and by using the Qiagen Gel purification Kit. This PCR product was used as a template for the nested PCR to amplify either the promoter region or the transmembrane region. Subsequently, both PCR products were gel-purified and sequenced. To identify the FcγRIIa-131HR and FcγRIIa-158FV allelic variants, allele-specific nested PCRs were used according to published protocols and with the primer sets listed in Table 1 (29–31).

ELISA. Sera of humanized mice were collected once per month to analyze the immune status of mice. Serum samples were generally stored at −80 °C until further use. For quantification of total serum IgM and IgG in humanized mice, the Bethyl Human IgM ELISA Quantitation Kit and the Human IgG ELISA Quantitation Kit (Biomol) were used according to the manufacturer’s instructions. OD was measured with VersaMax tunable microplate reader (Molecular Devices) at 450 and 650 nm. Anti-dsDNA. For the detection of anti-DNA antibodies, ELISA plates were coated with 10 μg/mL methylated BSA (Sigma) in PBS for 2 h at room temperature. After washing, the plates were coated with 50 μg/mL calf thymus DNA (Sigma) in PBS at 4 °C overnight. Blocking of unspecific binding was performed with PBS0.1% Gelatin/3% BSA/1% EDTA for 2 h at room temperature. Sera were diluted 1:100 in the blocking solution and incubated for 1 h at room temperature. As a detection antibody, the HRP-conjugated antibody of the human IgM Quantitation Kit (Bethyl) was used and diluted 1:10,000 in blocking solution followed by incubation for 1 h at room temperature. PBS was used for

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.1111810108)

Fig. 4. Identification of autoantibody producing B cells in humanized mice with different FcγRIIB alleles. (A) Identification and quantification of IgM* or IgM− plasmablasts in the spleen of 6-mo-old humanized mice with the FcγRIIB-232I/I, -232T/T, or -232T/T genotype. B cells were identified by gating on CD19. (B) Identification and quantification of CD138+ plasma cells in the spleen of 6-mo-old humanized mice with the FcγRIIB-232I/I or -232T/T genotype. B cells were identified by gating on CD19. Bars indicate the median of the group and statistical significance was evaluated with the Student’s T-test. *(C and D) Identification (C) and quantification (D) of IgM-producing B cells, IgM anti-GPI, and IgM anti-DNA–producing B cells obtained from the bone marrow by ELISPot analysis of 6-mo-old humanized mice with the FcγRIIB-232I/I or -232T/T genotype.
ELISA plates were coated with 1 ng/mL GPI in PBS at 4 °C overnight. Blocking was performed with PBS/3% BSA for 1 h at room temperature. Sera were diluted 1:100 in PBS/3% BSA and incubated for 1 h at room temperature. Detection antibody was diluted 1:10,000 in PBS/3% BSA for 1 h at room temperature. Detection of bound human antibodies was performed as described for the anti-DNA ELISA.

**Bf (anti-IgG antibodies).** One-hundred nanograms human IgG1 was coated on ELISA plates overnight at 4 °C. Unspecific binding were blocked with PBS/3% BSA for 1 h at room temperature. Sera were diluted 1:100 in PBS/3% BSA and incubated for 1 h at room temperature. Detection antibody and detection was according to the anti-DNA ELISA.

**Anti-CCP.** For the detection of anti-CCP antibodies, the IMTEC-CCP Antibodies Kit (Human Diagnostic, Wiesbaden, Germany) was used. Sera of humanized mice were diluted 1:50 to 1:10,000 in the provided dilution buffer and incubated for 1 h at room temperature. For the detection of anti-CCP IgM, the anti-IgM-HRP antibody from the Bethyl Quantitation Kit was diluted 1:10,000 in the provided dilution buffer of the kit and incubated at room temperature for 1 h. TMB substrate was added and incubated for 30 min. The reaction was stopped with the stop solution included in the kit.

**ELISpot.** ELISpot assays were performed with the ELISpotPLUS for Human IgM Kit (Mabtech) according to the manufacturer’s instructions. Membranes of ELISpot plates were activated with 70% ethanol for 1.5 min. After washing, membranes were coated with the provided anti-IgM antibody (for detection of total amount of IgM producing cells) or with 1 µg/mL GPI (Sigma) (for detection of anti-GPI IgM producing cells) at 4 °C overnight. For detection of anti-DNA IgM-producing cells, membranes were coated with 5 mg/mL poly-L-Lysin (Sigma) in TE buffer overnight at 4 °C and subsequently with 20 µg/mL calf thymus-DNA (Sigma) in TE buffer at 4 °C overnight. Membranes were washed with PBS and blocked with PBS/2% FCS for 2 h at room temperature followed by 1 h at 37 °C. To evaluate antibody production by human B cells, mCD45+ cells, and mCD3+ cells were depleted from single-cell suspensions of spleen and bone marrow of humanized mice using magnetic cell sorting (Miltenyi Biotech). Cells were counted and the amount of CD19+ cells was analyzed by flow cytometry. Next, 10,000, 50,000, or 100,000 CD19+ cells were seeded in duplicates in RPMI/10% FCS/1% Penicillin/Streptomycin/1% non-essential amino acids/1% sodium pyruvate/1% glutamate on ELISpot membranes and incubated overnight at 37 °C and at 5% CO2. Detection of spots was done as described in the protocol of the kit. Membranes were measured with the AID ELISpot Reader ELR 03 and analyzed with ELISpot 5.0 software.

**Measurement of Calcium Influx.** Pooled peripheral blood mononuclear cells from at least six humanized mice were loaded with Indo-1-AM (Molecular Probes) by resuspending 5 × 10⁶ cells in RPMI/5% FCS and adding 7 µL of Indo-1 at 30 °C with permanent shaking. After addition of 700 µL RPMI/10% FCS, cells were incubated at 37 °C for 10 min. After washing with buffer, surface staining with mCD45.1-FITC, mCD45-APC (BD Biosciences) and CD19-PE (BD Biosciences) was performed. Cells were washed in ice cold Krebs-Ringer solution [10 mM Hepes (7.0), 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose]. For detection of calcium influx, cells were resuspended in prewarmed Krebs-Ringer solution to a final volume of 500 µL. For stimulation of cells, 13 µg anti-IgM F(ab), or 27 µg anti-IgG IgG (both from Jackson Laboratories) were added and emission of Indo-1 was measured at 405 and 485 nm with a BD-LSR II FACS. Analysis was done with the FlowJo software.

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