Supporting Information

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SI Text

Cloning, Expression, and Purification of Recombinant Human FcRn. By using the described mammalian expression vector PIGG (1), α-chain and β2 microglobulin of heterodimeric human FcRn were expressed by an engineered bidirectional CMV promoter cassette. A PCR fragment encoding human β2 microglobulin was amplified from a full-length cDNA plasmid (OriGene) by PCR using primers beta-5′ and beta-3′ and cloned into PIGG by SacI/SalI ligation. A PCR fragment encoding the extracellular part of the human FcRn α-chain (nucleotides 70–1095) was amplified from a full-length cDNA plasmid (OriGene) with overlap extension PCR by using primer pairs alpha-5′/HindIII-mut3′ and HindIII-mut5′/alpha-3′ and cloned into PIGG by HindIII/XbaI ligation. Both expression cassettes were verified by DNA sequencing.

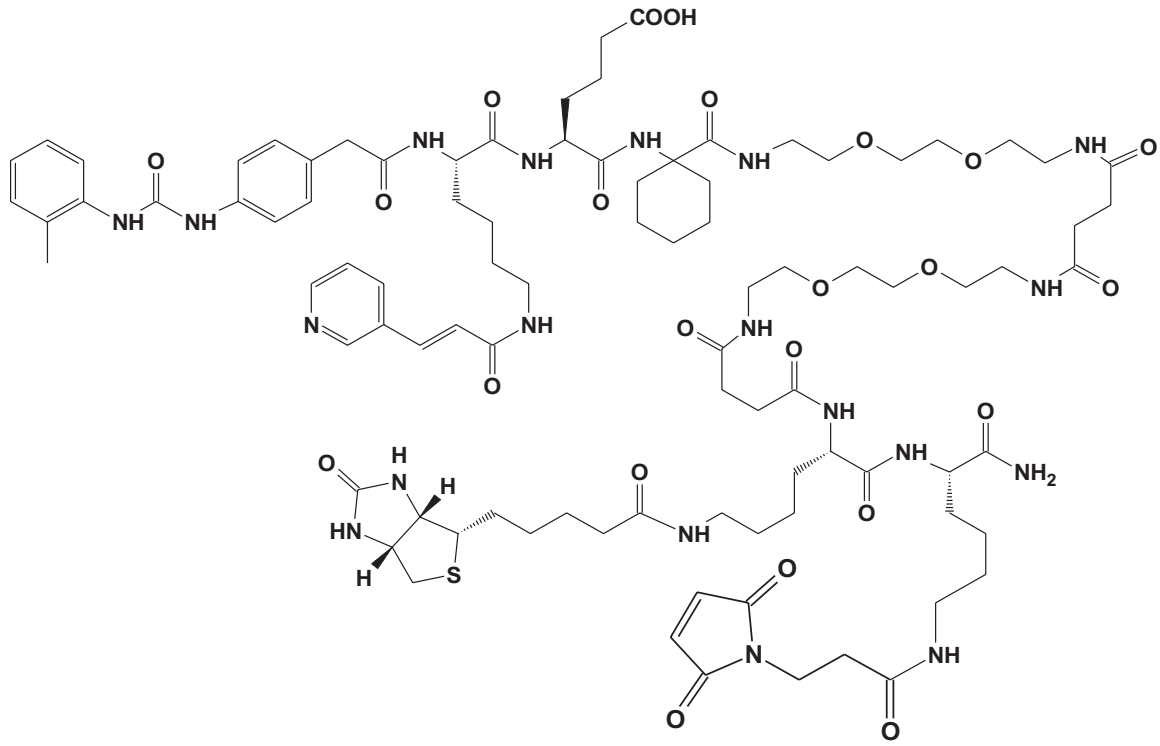
Primer Sequences. beta-5′: gaggaggagctcactccatccagcgtactccaaagattca; beta-3′: agctgtcgacatgatttatttaacttgttacatgtctcgatcccactta; alpha-5′: gagaagcttgttgctctggatctctggtgcctacggggcagaaagccacctctccct; HindIII-mut3′: ccaaagccttgaaagcctccagaaaga; HindIII-mut5′: tctttctggaggctttcaaggctttgg; alpha-3′: ggagtctagattacagctccaccctgaggggctgcgcca.

Transient transfection of the recombinant human FcRn expression vector into HEK 293F cells, culturing of the cells, and concentration of the supernatant was carried out as described for Fc protein expression. The concentrated supernatant was subsequently brought into acidic PBS (pH 6.0). For purification, Fc-stop protein was immobilized to an NHS HisTrap column (GE Healthcare) using the manufacturer's protocol. After loading the concentrated supernatant in acidic PBS (pH 6.0), the column was washed with 30-ml acidic PBS (pH 6.0) and bound recombinant human FcRn was eluted with neutral PBS (pH 7.4). Purified recombinant human FcRn (5 μg) was analyzed by electrophoresis on a NuPage 4% to 12% gradient gel (Invitrogen) followed by staining with Simply Blue SafeStain (Invitrogen).

ELISA and Surface Plasmon Resonance with Recombinant Human FcRn.
ELISA. All steps were carried out side-by-side in acidic PBS (pH 6.0) or neutral PBS (pH 7.4) for 1 h at 37°C. First, 500 ng of recombinant human FcRn in 25-μl PBS was coated on a 96-well Costar 3690 plate (Corning). After blocking with 3% (wt/vol) BSA/PBS, the plate was incubated with Fc-Sec-His/LLP2A-biotin or Fc*-Sec-His/LLP2A-biotin at 4 μg/ml (200 ng per well), followed by washing with acidic or neutral PBS (10 $\times$ 200 μl per well), and incubation with HRP-coupled streptavidin (50 ng per well) in 1% (wt/vol) BSA/PBS. The plate was washed with acidic or neutral PBS as before, and colorimetric detection was performed using 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (Roche) as substrate according to the manufacturer's directions.

Surface plasmon resonance. A comparison for the interaction of conjugated and unconjugated Fc proteins with human FcRn was performed by surface plasmon resonance using a Biacore X100 instrument (GE Healthcare). A CM5 sensor chip (GE Healthcare) was activated for immobilization with *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide. Recombinant human FcRn in 10-mM sodium acetate (pH 4) was immobilized at a density of 2,000 RU. Subsequently, the sensor chip was deactivated with 1 M ethanolamine hydrochloride (pH 8.5). The real time kinetics of rituximab, Fc-Stop, Fc-Sec-His, Fc-Sec-His/LLP2A-biotin, and Fc*-Sec-His/LLP2A-biotin in acidic PBS (pH 6) or neutral PBS (pH 7.4) were analyzed at five different concentrations in the range of 400 to 600 nM. A single 1-min pulse of PBS (pH 7.4) was used for the regeneration after each measurement without any loss of binding capacity. Parallel blank runs were used for instantaneous subtraction by using Biacore X100 evaluation software (GE Healthcare).

1. Rader C, Popkov M, Neves JA, Barbas CF III (2002) Integrin alpha-(v) beta3 targeted therapy for Kaposi's sarcoma with an *in vitro* evolved antibody. *FASEB J* 16:2000–2002.



LLP2A-biotin-maleimide

Fig. S1. Trifunctional LLP2A derivative with the integrin $\alpha_4\beta_1$-binding core linked to a biotin group for detection and an electrophilic maleimide moiety for conjugation.

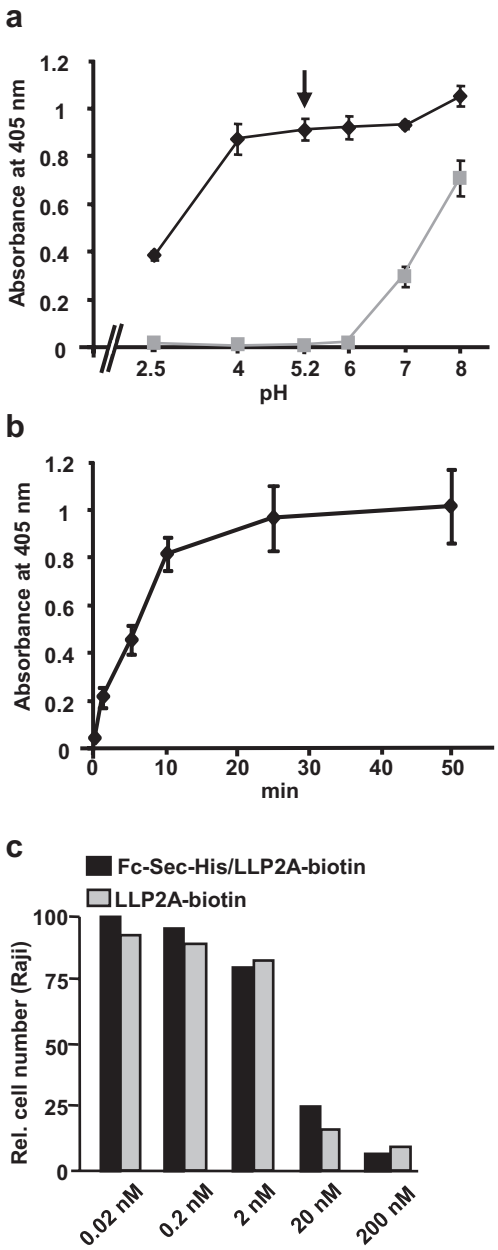


Fig. S2. (*a*) Analysis of the efficacy and specificity of conjugation at the Sec interface in relation to pH. The conjugation of Fc-Sec-His (black diamonds) and Fc-stop (gray squares) to (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine was performed in several buffers with pH values ranging from 2.5 to 8.0 and then analyzed by ELISA. The experiment demonstrated that the pH value (5.2) chosen for all conjugations at the Sec interface in this study is within a range (4–6) that permits highly efficient and specific conjugation of Fc-Sec-His, whereas pH values above 6 lead to an unspecific coconjugation of Cys present in both Fc-Sec-His and Fc-stop, and pH values below 4 only result in partial conjugation of Fc-Sec-His. Shown are mean ±SD of triplicates. (*b*) Analysis of the efficacy of conjugation at the Sec interface in relation to time. The conjugation of Fc-Sec-His to LLP2A-biotin-maleimide was analyzed after 1, 5, 10, 25, and 50 min by ELISA. The experiment demonstrated that the reaction is fast in the first 10 min and then decelerates without significant difference between the 25-min and the 50-min time points. Shown are mean ±SD of triplicates. (*c*) Using the same cell adhesion assay as in Fig. 2*D*, Fc-Sec-His/LLP2A-biotin and free LLP2A-biotin revealed equal potency when compared over a concentration range from 0.02 to 200 nM. A typical of three experiments is shown.

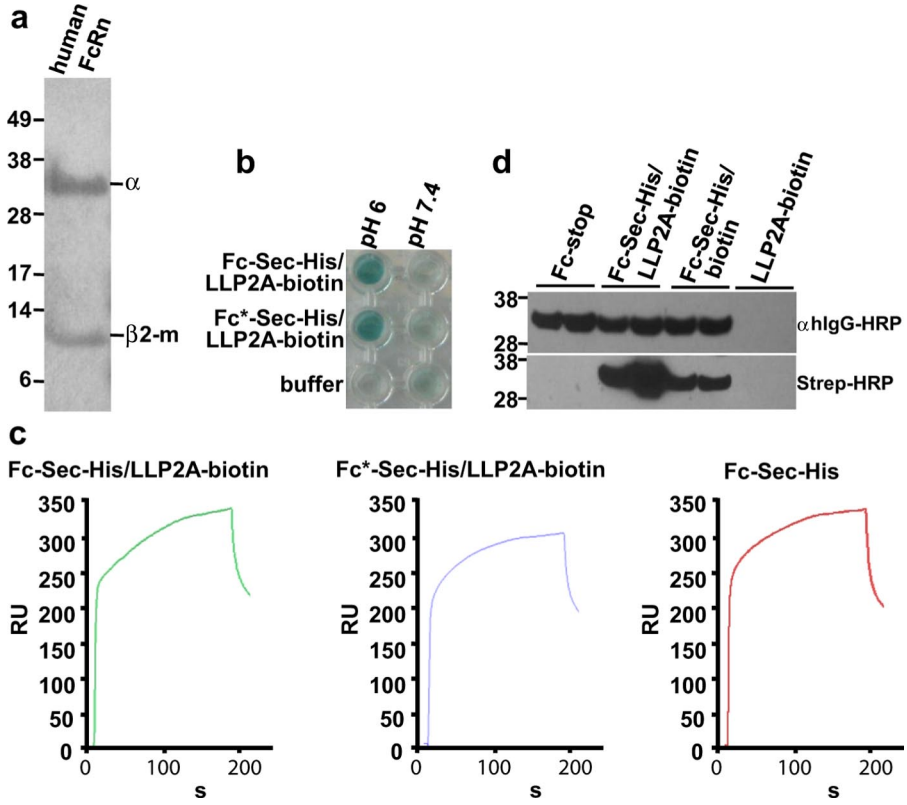


Fig. S3. (*a*) Recombinant human FcRn consisting of α chain and β2 microglobulin was purified from the supernatant of transiently transfected HEK 293F cells by using affinity chromatography on immobilized human Fc-stop protein at pH 6.0 and analyzed by reducing SDS/PAGE. (*b*) Analysis of Fc-Sec-His/LLP2A-biotin and Fc*-Sec-His/LLP2A-biotin for binding to immobilized recombinant human FcRn at pH 6.0 and pH 7.4 by ELISA. (*c*) Analysis of Fc-Sec-His/LLP2A-biotin, Fc*-Sec-His/LLP2A-biotin, and Fc-Sec-His for binding to immobilized recombinant human FcRn by surface plasmon resonance. Shown are Biacore X100 sensorgrams obtained at pH 6 for the binding of 500 nM of the indicated Fc proteins to recombinant human FcRn immobilized on the sensor chip. No significant difference between the Fc proteins was detected, demonstrating that FcRn binding is not influenced by conjugation at the Sec interface. No binding was detected at pH 7.4 (data not shown). Parallel blank runs were substracted instantenously. RU, resonance units. (*d*) Western blotting data for sera from eight neonatal mice confirming the preserved ability of Fc conjugates to enter the blood through FcRn-mediated transcytosis.