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IgG is transported across the mouse yolk sac independently of FcγRIIb

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Abstract

It is generally accepted that FcRn is the major IgG transporter in human syncytiotrophoblast and the mouse yolk sac endoderm, however the finding of a different Fc receptor FcγRIIb (RIIb) in the human placental endothelium has suggested the existence of an additional IgG transporter. To test this hypothesis in the mouse, we utilized wild-type (RIIb^{+/+}) mice and mice with a null mutation in the gene encoding RIIb (RIIb^{-/-} mice). We found that while RIIb is expressed in the placental yolk sac vasculature of RIIb^{+/+} mice, the IgG concentrations in fetuses of RIIb^{+/+} mice and RIIb^{-/-} mice are not different. This result refutes the hypothesis that yolk sac RIIb is required for transport of IgG *in utero* in the mouse. However, the capillary bed in the mouse yolk sac is structurally more complex than in human placenta, consisting of 3 types of cells: an RIIb-negative endothelium, a unique RIIb-bearing cell that also expresses two out of four macrophage markers but not endothelial cell or pericyte markers, and pericytes. As in the human placenta the b2 isoform of RIIb predominates in the mouse yolk sac. Remarkably only a single capillary channel rather than two channels with a loop is found in each yolk sac villus, which along with intracapillary erythrocytes, suggests that blood flow is peristaltic and mediated by pericytes. It is not clear whether RIIb in the human placental villus might contribute to IgG transport function in light of our finding that the mouse yolk sac equivalent is unnecessary in this role.

Keywords

Capillary; endothelium; macrophage; pericyte; quantitative microscopy; protein transport

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Contributions:

SM, JK, LPG, WLH, JMR and CLA planned and performed the experiments and analyzed the data; GSP, KH and DJ advised about the statistical analysis; and SM, JK and CLA wrote the paper.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflicts of interest.

1. INTRODUCTION

Babies are born with a full complement of maternal IgG antibodies and are thus immunized for the first 3 months of life while their immune systems mature. Underlying this phenomenon is a well-studied IgG transport mechanism is present in the placenta. Two cellular layers of the placenta separate the maternal and fetal circulations in human. IgG is moved across the first cellular layer, the syncytiotrophoblast, by FcRn, a pH-driven IgG transporter expressed mostly in intracellular vesicles (Brambell, 1970; Burmeister et al., 1994; Anderson et al., 2006; Ghetie and Ward, 2000). How IgG crosses the second cellular layer of endothelial cells (endothelial cells), to reach the fetal circulation is not known. While it was shown more than 3 decades ago that the endothelial cells of the human terminal villus expresses Fc receptors for IgG (reviewed by Faulk and Johnson, 1980), only recently has the molecule been identified and suggested as a potential IgG transporter. It is FcγRIIb2 (RIIb) (Lyden et al., 2001; Takizawa et al., 2005), the product of one member of the small FcγR gene family of 8 similar genes in human and 4 in mouse. The receptor is distinguished by the expression in its cytoplasmic tail of an ITIM which serves as a docking site for Src-homology 2 (SH2) domains integral to a group of phosphatases that down-regulate a variety of biological responses; two are inositol phosphatases (SHIP-1, SHIP-2), and one is a protein tyrosine phosphatase (SHP-1). This receptor is most conspicuous on B lymphocytes where in tetra-complex with IgG and the B cell receptor and antigen it down-regulates B cell activation. However it is also present in cells of myeloid and mononuclear phagocyte and dendritic cell lineages where it down modulates IgG immune complex-mediated functions (Daeron, 1997).

Only a single member of the diverse FcγR family, RIIb, is found to be expressed in endothelial cells and remarkably, it is the only inhibitory member of the family. Furthermore, RIIb is found in the endothelial cells of only 2 organs of the body, the placenta and the liver (Mousavi et al., 2007), and in both sites it appears expressed uniquely in the absence of a recognized opposing agonistic ITAM-bearing receptor. While this same receptor on immune cells has been studied in considerable detail, the function of RIIb at these endothelial cells sites is completely unknown.

We have hypothesized that RIIb in the human placenta may be transporting IgG across the endothelial cells, moving it from the abluminal surface of the villus capillary to the lumen. This suggestion is attractive because it might explain a paradox. FcRn, which binds and extends the half-lives of both IgG and albumin (Anderson et al., 2006), appears to transport only IgG and not albumin across the placenta. Postulating that RIIb transports IgG but not albumin across the villus endothelium would provide the exquisite specificity of dual receptors placed in tandem to assure that only IgG and not albumin is transported to the fetus. Further, early studies of the biology of RIIb, transfected into various hosts, indicated its capacity for ligand uptake and trafficking (Miettinen et al., 1989; Miettinen et al., 1992; Van Den Herik-Oudijk et al., 1994)

Since we were unable for practical reasons to test this hypothesis in human, we have moved to the mouse where the availability of strains lacking RIIb enables a direct assessment. However, the placental anatomy of the mouse is different from the human. While the human has a single chorioallantoic placenta that serves all functions, mouse has two placentas, one a chorioallantoic placenta which does not transport IgG, and the other a yolk sac placenta, which is the organ responsible for maternofetal IgG transport (Brambell, 1970; Kim et al., 2009) and perhaps other functions (Christensen and Verroust, 2002). The human yolk sac disappears at week 20 and is irrelevant to IgG transport to the mature fetus. As in the human placenta, two cell barriers of yolk sac placenta separate the maternal circulation from the fetal circulation. Endoderm is the first layer and like the syncytiotrophoblast in human, it

expresses FcRn and is virtually completely responsible for IgG transport to the fetus in that the IgG concentrations of both FcRn^{-/-} fetuses at 19–20 dpc and FcRn^{-/-} newborns are near to zero (Kim et al., 2009; Israel et al., 1995). Vitelline capillaries underlie the endoderm.

Extending further our study of IgG transport across the yolk sac, we hypothesized that mouse yolk sac RIIB transports IgG from the mother to the fetus during pregnancy, and we made two predictions: (1) that RIIB is expressed in the IgG transport pathway, most likely in the endothelial cells as in human, and (2) that IgG in RIIB^{-/-} fetuses will be very low compared to RIIB^{+/+} fetuses. We tested these predictions by multiple approaches.

2. MATERIALS AND METHODS

2.1. Reagents

Affinity purified rabbit IgG anti-NG2 and rabbit IgG anti-PDGFβR were provided by William Stallcup (Burnham Institute, San Diego, CA, USA) (Ozerdem et al., 2001). A goat anti-mouse RIIB serum (unpublished) was provided by Mark Coggeshall (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) and a rabbit IgG anti-mouse RIIB (Nakamura and Cambier, 1998) by John Cambier (University of Colorado Health Science Center and National Jewish Medical Research Center, Denver, CO, USA). A chicken IgY anti-caveolin 1 (chicken IgY anti-CAV1_(aa3-14)) was produced in house (Lyden et al., 2002; Takizawa et al., 2005; Kim et al., 2009). Several antibodies were purchased from various commercial sources; rat IgG anti-mouse RIIB/RIII (MAb 2.4G2), rat IgG anti-mouse CD31 (anti-CD31), rat IgG anti-mouse Flk1 (anti-Flk1) and rabbit IgG anti-caveolin1 (rabbit IgG anti-CAV1_(aa68-75)) all from BD Bioscience (San Jose, CA, USA); rat IgG anti-mouse CD34 (anti-CD34) from Abcam (Cambridge, MA, USA); rat IgG anti-mouse F4/80 (anti-F4/80), biotin-conjugated rat IgG anti-F4/80 (biotin-F4/80) and rat IgG anti-mouse CD68 (anti-CD68) from AbD Serotec (Raleigh, NC, USA); and FITC-conjugated mouse IgG anti-αSMA (anti-αSMA) from Sigma-Aldrich (St. Louis, MO, USA). The Alexa dye-conjugated secondary antibodies, namely goat IgG anti-rat IgG, goat IgG anti-rabbit IgG and goat IgG anti-chicken IgY, were purchased from Invitrogen (Carlsbad, CA, USA). Purified rat IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa 594 dye-conjugated streptavidin (594-Streptavidin) and ProLong anti-fade mounting media were from Invitrogen, DAPI from Sigma-Aldrich (St. Louis, MO, USA) and SuperfrostPlus slides were purchased from Fisher Scientific (Pittsburgh, PA, USA). A cDNA expressing mouse FcγRIIB1 was provided by Mark Hogarth (Hogarth et al., 1992).

2.2. Mice and breeding

The Ohio State University Institutional Laboratory Animal Care and Use Committee approved all animal studies. Mice with a null mutation in RIIB (C.129S4(B6)-*Fcgr2* b^{tm1TrK} RIIB^{-/-}) and the wild-type control strain (BALB/c; RIIB^{+/+}) were purchased from Taconic. Mice heterozygous for RIIB (RIIB^{+/-}) were produced by crossing RIIB^{+/+} x RIIB^{-/-} mice. Subsequently, RIIB^{+/-} heterozygotes were crossed to produce fetuses with three different receptor genotypes: RIIB^{+/+}, RIIB^{+/-}, and RIIB^{-/-}. Mice were 4–8 months old and provided litter sizes of 5–10 pups per litter.

2.3. Genotyping

The animals were genotyped by PCR using primers designed to distinguish the targeted allele from the wild-type by the sizes of the reaction products. The DNA was isolated from fetal tail tips and maternal liver. Each 50 μL PCR reaction contained 100 ng sample template DNA, 200 μM dNTPs, 1× PCR buffer with 1.5 mM MgCl₂, and 1 unit of Taq. Primer pair FcγRIIup2-CACTCCTTGATTTCCCTGG vs OL4-080-

TTGACTGTGGCCTTAAACGTGTAG generated a 371-bp wild-type allele; the oligo OL4143-CTCGTGCTTTACGGTATCGCC vs OL4-080 generated a 161-bp targeted allele. Thermocycler conditions were one cycle of 95°C for 10 min, 35 cycles of 94°C for 45 sec, 60°C for 1 min, 72°C for 1 min; and a final one cycle of 72°C for 5 min. The PCR products were resolved on agarose gels and stained with ethidium bromide.

2.4. Immunoblotting

Placental and yolk sac tissues lysates were prepared as described earlier (Kim et al., 2009). Lysates were incubated on ice for 30 min and centrifuged at 23,000 ×g for 10 min at 4°C. Post nuclear lysates containing 1 mg of protein for yolk sac and placenta and 250 µg of protein for the cell lines were incubated overnight with goat anti-mouse RIIb serum and protein G-agarose beads (Invitrogen). The mixtures were boiled in SDS sample buffer (60mM Tris pH 6.8, 2.3% SDS, 10% glycerol, and 0.01% bromophenolblue) for 5 min. The proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Piscataway, NJ, USA) that were blocked in 5% nonfat milk at room temperature for 1 h and then probed with rabbit anti-mouse RIIb antibody overnight on a rocker at 4°C. Membranes were washed and incubated in peroxidase-conjugated secondary antibodies for 1 h at room temperature, and developed and imaged by chemiluminescence.

2.5. RT-PCR to distinguish RIIb isoforms

Placenta and yolk sac were lysed in Trizol and stored at -80°C until use. Total RNA was extracted using a modified procedure (Gavrilin et al., 2006). Thermo script RNase H⁻ Reverse transcriptase (Invitrogen) was used to transcribe 1 µg of RNA into cDNA. Based on the mouse RIIb sequence obtained from Pubmed (accession number NM_010187), forward primer (5'-GATTGCTGTCGCAGCCATTGTTA-3') and reverse primer (5'-AGCCCTGGATGAAGAAACAGAGCA-3') were designed and synthesized. PCR of RIIb cDNA was performed using 1 µg of cDNA and 200 nM primers under the following conditions: 95°C for 3 min, 35 cycles of 94°C for 1 min, 67°C for 1 min, and 72°C for 1 min. The PCR products were resolved on 1.5% agarose gel. Band sizes of 313 and 172bp correspond to FcγRIIb1 and FcγRIIb2, respectively.

2.6. Preparation of yolk sac sections and immunofluorescence

At the gestational age of 19–20 days the Cesarean-delivered placentas were bisected such that each half retained its associated yolk sac, and were fixed and processed for immunofluorescence analysis (blocking, antibody treatment and washing, etc.) as described earlier (Kim et al., 2009). For immunolocalization of RIIb, the sections were incubated with MAb 2.4G2 (20 µg/ml), and MAb binding was localized indirectly by Alexa 594 dye-conjugated goat IgG anti-rat IgG diluted 1/200. All secondary antibodies used in the present studies were used at 1/200 dilutions unless stated otherwise. A section labeled with goat IgG anti-rat IgG alone controlled for auto- and nonspecific fluorescence.

Dual immunofluorescence labeling of RIIb with MAb 2.4G2 and the endothelial cells marker caveolin1 with chicken IgY anti-CAV1_(aa3-14) was done to determine if RIIb is expressed in endothelium. Additional dual labeling compared chicken IgY anti-CAV1_(aa3-14) with rabbit IgG anti-CAV1_(aa68-75), rat IgG anti-CD31 and rat IgG anti-CD34 to validate chicken IgY anti-CAV1_(aa3-14) as an endothelial marker and to explore the yolk sac endothelium phenotype. The sections were incubated with 1/400 chicken IgY anti-CAV1_(aa3-14) antibody in combination with 20 µg/ml MAb 2.4G2, 2.5 µg/ml rabbit IgG anti-CAV1_(aa68-75), 1.5 µg/ml rat IgG anti-CD31 or 4 µg/ml rat IgG anti-CD34. Chicken IgY anti-CAV1_(aa3-14) binding was localized indirectly by Alexa 488 dye-conjugated goat IgG anti-chicken IgY. Rat IgG MAbs 2.4G2, anti-CD31 and anti-CD34 were localized indirectly

by Alexa 594 dye-conjugated goat IgG anti-rat IgG. Rabbit IgG anti-CAV1_(aa68-75) was localized indirectly by Alexa 594 dye-conjugated goat IgG anti-rabbit IgG. Specificity of antibodies were tested by incubating the yolk sac section without primary antibodies that included goat IgG anti-chicken IgY plus goat IgG anti-rat IgG and goat IgG anti-chicken IgY plus goat IgG anti-rabbit IgG. The green dye (Alexa 488) and red dye (Alexa 594) were switched for each dual labeling combination to ensure that the labeling pattern was not dye dependent.

Dual immunofluorescence labeling of RIIb with macrophage marker F4/80 and pericyte markers α SMA, NG2 or PDGF β R was done to determine if the RIIb+ cell expressed macrophage or pericyte markers. To dual label RIIb with macrophage marker F4/80 we modified our method because both antibodies are of rat origin. Briefly, the sections were incubated with 20 μ g/ml MAb 2.4G2, washed, incubated with Alexa 594 dye-conjugated goat IgG anti-rat IgG for 1 hr to indirectly label MAb 2.4G2; the sections were washed and then incubated with 10 μ g/ml rat IgG for 30 minutes to block antigen binding sites on Alexa 594 dye-conjugated goat IgG anti-rat IgG. These sections were incubated with 10 μ g/ml biotin-F4/80 for 1 hr, washed, and incubated with 488-Streptavidin for 30 minutes to indirectly label biotin-F4/80, and then washed to remove excess reagents. The control sections received the same incubations except without MAb 2.4G2 and anti-F4/80 antibodies. To test the specificity of reaction we dual labeled mouse liver sections with 2.4G2 and biotin-F4/80 and did not see signal overlap (not shown). For RIIb in combination with pericyte markers the sections were incubated with 20 μ g/ml MAb 2.4G2 in combination with 1/400 dilutions of anti-NG2, anti-PDGF β R or FITC anti- α SMA. Mab 2.4G2 was localized indirectly by Alexa 594 dye-conjugated goat IgG anti-rat IgG. Anti-NG2 and Anti-PDGF β R were localized indirectly by Alexa 488 dye-conjugated goat IgG anti-rabbit IgG. FITC-conjugated anti- α SMA was visualized by direct immunofluorescence. Controls for dual labeling of 2.4G2 and α SMA included incubating the sections with FITC-conjugated mouse IgG and Alexa 594 dye-conjugated goat IgG anti-rat IgG. Controls for RIIb dual labeling with anti-NG2 and anti-PDGF β R included incubating the section with Alexa 594 dye-conjugated goat IgG anti-rat IgG and Alexa 488 dye-conjugated goat IgG anti-rabbit IgG.

To determine if F4/80 positive cells express CD68, another macrophage marker, the sections were dual labeled for immunofluorescence, first applying MAb anti-CD68 (4 μ g/ml), then Alexa 488 dye-conjugated goat IgG anti-rat IgG, then an excess of rat IgG (10 μ g/ml) to block the 488 dye-conjugated goat IgG anti-rat IgG. Biotin-F4/80 (10 μ g/ml) was added and was visualized indirectly with 594-Streptavidin (1/200). Control sections were processed in the same manner without CD68 and biotin-F4/80.

Nuclei were then labeled with DAPI for 10 min; the cells were washed five times in PBS and were mounted under coverslips in ProLong antifade mounting medium. Fluorescence and differential interference contrast (DIC) images were collected with a Nikon Optiphot microscope and a Photometrics Cool Snap *fx* camera (Roper Scientific, Germany) and were captured with the MetaMorph image analysis system (Universal Imaging/Molecular Device, Downingtown, PA, USA). Confocal images were captured on the Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss, Germany). Image analysis was done with LSM 510 META software.

2.7. Quantitative microscopy

To localize and quantify RIIb positive cells in the yolk sac sections, we first examined DIC images and DAPI stained nuclei to identify morphologically the three major yolk sac cell types, i.e., mesenchymal, endodermal, and mesothelial cells. We next examined the corresponding fluorescence images above an intensity threshold selected to optimize the

ratio of MAb 2.4G2 labeling intensity to labeling intensity of the secondary antibody alone (signal-to-noise ratio). We then studied 8–10 villi from each animal (RIIb^{+/+}, n=3; RIIb^{-/-}, n=3), examining ~500 mesenchymal cells, ~1000 endodermal cells, and ~50 mesothelial cells, each cell identified by morphology and DAPI stained nuclei, scoring the percentage of cells positive for (above threshold) MAb 2.4G2 fluorescence staining, taking this value as a function of the magnitude of RIIb expression.

To identify the phenotype of RIIb positive cells, we quantified the distribution of RIIb and endothelium markers, relative to each other, in the following way: A scanning line 1 pixel wide and ~5–10 μ m long was drawn and placed on the mesenchymal area at a right angle to the long axis of a villus near the tip. The number of positive pixels of each of the two colors (i.e., green chicken IgY anti-CAV1_(aa3-14) and red rat IgG anti-RII) and number of pixels of both colors above threshold value were recorded. The scanning line was then placed at a new location ~2 μ m below and data were recorded again. This process was repeated until the scanning line reached the base of the villus, recording ~30 scans from each villus. A threshold value for each dual labeling combination was determined based on pixel intensities of the sections labeled with secondary antibodies alone. Percent colocalization was calculated for each dual labeling combination and averaged from ~1000 scanning lines from a total of 3 animals. The data were plotted as mean $\pm$ SD.

To determine if F4/80 positive cells expressed another macrophage marker, CD68, we manually counted the number of green (CD68 label) or red (F4/80 label) blobs appearing isolated and appearing overlapped in >100 dual labeled villi from a total of 3 animals. The data were plotted as mean $\pm$ SD.

2.8. Serum concentrations of IgG, albumin and transferrin

At the gestational age of day 19–20, pregnant heterozygous female mice mated to heterozygous male mice were anesthetized with isoflurane and subjected to Cesarean section to obtain fetuses. Fetal blood (~10 μ L) was taken by cardiac puncture with 28-gauge needles attached to 0.5-ml insulin syringes. Maternal blood (~30 μ L) was collected before section from the retroorbital sinus. Sera were harvested and stored at -80°C was used for sandwich ELISA determination of concentrations of IgG, albumin, and transferrin as earlier described (Chaudhury et al., 2003; Chaudhury et al., 2006; Kim et al., 2006). Individual serum concentrations of IgG or albumin in the fetuses were normalized to their transferrin concentrations, which did not differ among strains.

2.9. Immunization

To boost maternal IgG concentrations, female RIIb-heterozygous mice were immunized subcutaneously by repeated inoculations weekly for 6 weeks of keyhole limpet hemocyanin (KLH; 100 μ g/mouse, Sigma-Aldrich) in Freund's incomplete adjuvant (1:1 volume ratio, Sigma-Aldrich). Heterozygous breeding started one week after the last inoculation. Maternal plasma IgG concentrations were raised to 25.8 ± 8.9 mg/ml (mean $\pm$ SD) by the time of conception.

2.10. Statistical analyses

In the experiments to determine fetal steady-state concentrations of IgG and albumin, mixed linear models were fitted to assess the fixed effects of RIIb^{+/+}, RIIb^{+/-} and RIIb^{-/-} fetuses on the concentrations after controlling maternal concentrations. Differences in protein concentration among three strains were considered significant at $P < 0.05$. Analyses were performed using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Differences in RIIb in the IgG transport pathway were tested by a random-effects negative binomial regression (Liang and Zeger, 1986). A random-effects linear regression was used to identify

differences in RIIb in the IgG transport pathway (Rabe-Hesketh S. and Skrondal A., 2005). Random-effects regressions were used to account for the correlation due to the nesting of the results within mice. Analyses were performed using Stata (version 10.1, Stata Corporation, College Station, Texas). Data were presented as mean $\pm$ SD.

3. RESULTS

3.1. RIIb is expressed in the IgG transport pathway in yolk sac

The first prediction of our hypothesis is that RIIb should be expressed in the yolk sac vasculature. Evaluating histologic sections of 19–20dpc yolk sac by indirect immunofluorescence with MAb 2.4G2 directed against Fc γ RIIb and Fc γ RIII and identifying cells by DIC imaging and by association with stained nuclei, we indeed saw fluorescence displayed solely in the mesenchymal layer, appearing morphologically along the course of the vitelline capillaries and larger vessels. The immunofluorescence signal was seen only in RIIb^{+/+} animals and not in RIIb^{-/-} animals (Fig. 1A and 1B, respectively). This pattern suggests that RIIb is expressed in the yolk sac vasculature and, further, that Fc γ RIII is not expressed. Quantifying the RIIb immunofluorescence signal from 6 yolk sacs we found that approximately half of RIIb^{+/+} mesenchymal cells examined expressed RIIb ($48 \pm 3\%$) whereas RIIb^{-/-} mesenchymal cells were barely positive ($<1\%$, $P < 0.001$). Relatively few endoderm and mesothelial cells of RIIb^{+/+} and RIIb^{-/-} animals showed MAb 2.4G2-mediated fluorescence ($<2\%$ cells, $P < 0.001$) (Fig. 1 histogram). Since a section of a cell may not always include its nucleus, this strategy of quantification may lead to an overestimation of the proportions of RIIb positive cells in the mesenchyme.

3.2. Yolk sac expresses the b2 isoform of RIIb

Extending the immunofluorescence data above, we assayed for RIIb expression in mouse yolk sac by biochemical methods. Using 2 different anti-RIIb antibodies, one for immunoadsorption and the other for immunoblotting, we evaluated the presence of RIIb in lysates of yolk sac, placenta, and of cultured cells well-known either to express RIIb (RAW 264.7 and A20) or to fail to express RIIb (COS-7) (Fig. 2A). Comparing band densities, the recovery of RIIb from starting material of equal protein concentration was greater from yolk sac than from placenta. However, occasionally, we found equal RIIb expression in placental lysates. Note that while it is easy to dissect the conceptus yolk sac away from maternal tissue, often placental tissue is contaminated by maternal cells, thus rendering it difficult to define the origin of placental RIIb as maternal or conceptus. In the immunoadsorption step, substituting normal goat serum for antiserum yielded no bands of the appropriate RIIb size, affirming that the identified band is RIIb (not shown).

Further, our hypothesis concerning the homology of human and mouse prenatal IgG transport predicted that of the two RIIb isoforms, b2 would be expressed preferentially to b1 in the mouse yolk sac, as it is in the human placenta (Lyden et al., 2001; Takizawa et al., 2005). Analyzing tissue lysates of yolk sac by RT-PCR, we found that the band for isoform b2 is much denser than that for b1, which is barely visible (Fig. 2B). The placenta expresses both isoforms. Confirming the reliability of our assay, the b1/b2 patterns seen in the 5 controls are characteristic of their reported phenotypes, with the B cell showing only b1, COS cells showing neither, RAW cells showing both, bone marrow macrophages showing a dense b2, and the b1 plasmid showing only b1 (Fig. 2B).

3.3. RIIb is not expressed in yolk sac endothelium

Our hypothesis predicted that RIIb would be expressed not only in the wall of the yolk sac vitelline vessels but more specifically in the endothelium of the vessels, paralleling its expression in the human villus. We tested this prediction by two-color indirect

immunofluorescence of yolk sac tissue sections, labeling RIIb with red-tagged MAb 2.4G2 and endothelium with green-tagged chicken IgY anti-CAV1_(aa3-14) (C-CAV1) that identifies caveolin1 in the endothelium. We saw that the two signals RIIb and caveolin1 did not colocalize, as illustrated by the merged image (Fig. 3A, first row). Employing additional endothelium markers, we noted that chicken IgY anti-CAV1_(aa3-14) signals colocalized with signals from 4 other endothelium marker antibodies including a rabbit IgG anti-CAV1_(aa68-75) (R-CAV1), anti-CD31, anti-CD34 as illustrated by the merged images (illustrated in Fig. 3A rows 2–4), and anti-Flk1 (not shown).

We infer then that RIIb+ cells are not typical endothelium. However, they were morphologically indistinguishable from endothelium, appearing fusiform and sinuous, as judged by corresponding DIC and DAPI stained images at this resolution, but they were not as a rule found to line the vessel lumen (data not shown). Rather, they appeared immediately abluminal to the endothelium (data not shown). We quantified the 2-color immunofluorescence images illustrated in Fig. 3A by examining ~1000 scanning lines for each color-pair from villi of 3 yolk sac. We found very few CAV1_(aa3-14) (C-CAV1) pixels colocalized with RIIb pixels (<5%), but a high percentage (59–76%) colocalized with the pixels of the other endothelium markers ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 3B, upper panel). Considering the same data reciprocally, we saw that very few (<3%) RIIb pixels colocalized with CAV1_(aa3-14) (C-CAV1) pixels, but a high percentage (52–65%) of CAV1_(aa68-75) (R-CAV1), CD31, and CD34 pixels colocalized with CAV1_(aa3-14) (C-CAV1) pixels ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 3B, lower panel). These quantitative data of Fig. 3B substantiate our visual impression illustrated in Fig. 3A to indicate that RIIb is not expressed in endothelium. Acknowledging a limitation of our approach, we note that the CAV1_(aa3-14) (C-CAV1) overlap with CAV1_(aa68-75) (R-CAV1) was 75% and 50% ($P > 0.05$) reciprocally, less than the theoretical maximum of 100%. Similarly, the minimum overlap detectable was ~5–10 %, which was still greater than the theoretical 0% (Fig. 3B).

3.4. RIIb positive cells express 2 of 4 macrophage markers but 0 of 3 pericyte markers

Others have shown cells expressing pericyte and macrophage markers in yolk sac mesenchyme (Morris et al., 1991; Lichanska and Hume, 2000; Li et al., 2003). To characterize further the RIIb+ cell, we assessed yolk sac villi by 2-color immunofluorescence using red-tagged antibodies to RIIb and green-tagged antibodies to macrophage and pericyte markers. We found in the merged images that the RIIb marker colocalizes with the macrophage marker F4/80 (Fig. 4A row 1). Further, F4/80 positive cells expressed CD68 as well, as illustrated in the merged color image (Fig. 4C). Remarkably, CD68, known to identify a macrophage lysosomal component, also labeled vesicular structures of endoderm cells (likely lysosomes). However, Mac-1 was not detected on F4/80+ cells while macrophages in spleen and Kupffer cells in liver were positive (data not shown). The absence of a MAb 2.4G2 fluorescence signal in the mesenchyme of RIIb^{-/-} yolk sac in Fig. 1B would indicate that FcγRIII as well was not expressed in the yolk sac RIIb+ cell. Labeling yolk sac sections with green-tagged antibodies to 3 pericyte markers (αSMA, NG2, and PDGFβR) indicated the presence of an abundance of sinuous and fusiform cells adjoining both endothelium and RIIb+ cells (Fig. 4A). The merged 2-color immunofluorescence images of green-tagged pericyte markers with red-tagged MAb 2.4G2 showed little or no colocalization (Fig. 4A, rows 2–4).

Our visual impression from the color images was validated by a quantitative analysis of ~1000 scanning lines from a total of 3 yolk sacs for each color pair. Assessing colocalization of the two colors, we found that the number of RIIb pixels that colocalized with F4/80 pixels was very high, ~70% (Fig. 4B top), and nearly equal to the reciprocal colocalization of F4/80 pixels with RIIb pixels, ~70% (Fig. 4B bottom). In contrast, the percentage of RIIb

pixels that colocalized with the pixels of pericyte markers was comparatively low, averaging about 15% (Fig. 4B). It is clear that RIIb-positive cells express macrophage markers but not pericyte markers ($P < 0.001$) (Fig. 4B).

To quantify the expression by F4/80+ cells (red) of another macrophage marker (Holness et al., 1993), CD68 (green), we counted the red and green blobs from ~100 villi from a total of 3 animals and plotted the mean $\pm$ SD (Fig. 4C, histogram). Colocalization based on either of the two colors is close to 100% (Fig. 4C, histogram). We note that endodermal cells, which express abundant lysosomes, also are labeled with anti-CD68 (Fig. 4C thick arrows in paneled images). We demonstrate that the RIIb+ cells expressed macrophage markers F4/80 and CD68 but not Mac1 or Fc γ RIII, and not pericyte markers.

3.5. RIIb is not required for IgG transport to fetus

If RIIb were required for IgG transport to the fetus, then RIIb^{-/-} fetuses should have little or no plasma IgG, as we recently described for FcRn^{-/-} fetuses (Kim et al., 2009). To test this prediction we analyzed matings of heterozygote parents carrying a single RIIb-null allele on a BALB/c background, taking fetuses at day 19–20 by Cesarean section from 10 mothers, bleeding the fetuses by cardiocentesis for the determination of IgG concentrations by ELISA, and genotyping the fetuses. We found that RIIb^{-/-} fetuses (0.752 ± 0.211 mg/ml, $n = 18$) have modestly lower IgG concentrations (17%; $P < 0.03$; Fig. 5A) than RIIb^{+/+} fetuses (0.882 ± 0.375 , $n = 24$). As expected, since RIIb is not a receptor for albumin, the fetal albumin concentrations were not different among the two genotypes (data not shown). The analysis took into consideration that RIIb^{-/-} and RIIb^{+/+} fetuses were littermates by allowing for correlated observations within mothers. We used a linear mixed model of the correlated observations and also addressed whether maternal IgG affected differences between RIIb^{+/+} and RIIb^{-/-} fetuses.

Since the pregnant mothers, raised under pathogen-free conditions, were IgG-deficient (0.2–1.2 mg/ml), we repeated the experiment with mothers at physiologic IgG concentrations. We immunized mice prior to pregnancy to increase maternal IgG concentrations near the typical concentrations that have been reported in mice and humans (2–10 mg/ml) (Fahey and Robinson, 1963; Waldmann and Strober, 1969). We found that the immunized mothers at term had IgG concentrations about 5 fold greater than non-immunized mothers and values near mice in-the-wild, indicating that our study criteria were fulfilled. Quite clearly, we saw no difference in IgG concentrations between RIIb^{+/+} and RIIb^{-/-} fetuses from the immunized mothers (Fig. 5B) and from the two groups of mothers together (1.329 ± 0.618 mg/ml, $n = 46$ and 1.416 ± 0.782 , $n = 42$, in RIIb^{+/+} and RIIb^{-/-} fetuses, respectively; $P > 0.5$).

Analyzing the data further we identified 18 litters that contained $\geq$ one each RIIb^{+/+} and RIIb^{-/-} fetuses and plotted the ratio of averaged IgG concentrations of RIIb^{-/-} to RIIb^{+/+} vs the respective maternal IgG concentration. We found that mean $\pm$ SD of the ratios from all 18 mothers was 0.98 ± 0.19 suggesting essentially no difference in IgG concentrations between the fetuses of the two different genotypes ($P = 0.858$).

3.6. Finding a single lumen in the yolk sac capillary

Expecting to see looped afferent and efferent capillaries running through the villi, instead we were puzzled to find in all our histological preparations a single blind-ended lumen. An extensive search of the published literature yielded no evidence of multiple capillary lumens in yolk sac villi. Concluding that our observation was unique, we initiated a systematic study, defining a capillary as a lumen-like space in the mesenchyme area of villi bounded by endothelium markers such as caveolin1, CD31 and CD34; and containing RBCs.

Scrutinizing 192 villi from 10 yolk sac from a total of 6 mothers by immunofluorescence and histological examination we measured 10 cm of single channel lumen that fulfilled our criteria, but we found no evidence (zero distance) of a double lumen. How blood moves through these channels we can only speculate (see below).

4. DISCUSSION

Following our earlier finding that maternal IgG is transported across the yolk sac endoderm by FcRn (Kim et al., 2009), we tested the hypothesis that RIIb expressed in the wall of the yolk sac vasculature would function as a second, tandem-situated IgG transporter that would move maternal IgG from the basolateral surface of the endoderm across the vessel wall to fetal circulation.

Our strategy was to evaluate by multiple methods mouse fetuses and yolk sac taken at the end of pregnancy from RIIb^{+/-} mothers crossed with RIIb^{+/-} fathers. The yolk sac of the 2 homozygous genotypes were studied biochemically and by quantitative microscopic imaging to localize the expression of RIIb with respect to the vitelline vasculature, and the IgG concentrations of fetuses of all 3 genotypes were compared with their respective maternal IgG concentrations to test whether RIIb was required for IgG transport.

We find that, indeed, RIIb is expressed in the wall of the vitelline vasculature, but it is not expressed in the endothelium *per se* as was anticipated from its whereabouts in the human placenta. The vasculature of the yolk sac is more complex than that of the human villus. In human we find continuous RIIb-expressing endothelium of the non-fenestrated type with frequent tight junctions surrounded by occasional pericytes (Takizawa et al., 2005). Afferent and efferent capillary limbs loop to the villus tip. In contrast, the yolk sac vasculature appears to be made up of three distinctive types of cells. Facing the fetal lumen is a fenestrated, fusiform endothelium expressing 5 endothelial cells markers identified by immunofluorescence, but expressing no RIIb nor any macrophage or pericyte markers. The capillaries do not loop; they are blind-ended single channels, characteristic perhaps of their recent genesis. To our knowledge, these blind-ended capillaries have never before been commented upon in the scientific literature.

Adjacent to the endothelium in the vessel wall we find a second cell type that expresses RIIb. This RIIb⁺ cell also expresses 2 of our 4 macrophage markers (F4/80 and CD68 but not FcγRIII or Mac-1) but it does not have the appearance of a typical macrophage, nor does it appear laden with lysosomes (data not shown). Rather it has the appearance of endothelium in that it is long and thin, but it expresses none of our 5 endothelial cells markers nor any pericyte markers. Macrophages positive for F4/80 had earlier been described in the mesenchyme of the mouse yolk sac but were noted to disappear by 14dpc after peaking in density early after the development of the yolk sac (Morris et al., 1991). Our finding of F4/80⁺ cells at 19–20 days gestation is distinctly conflicting, but it likely represents a different set of cells that was overlooked by earlier workers, perhaps because of detection sensitivities. It seems also important to keep in mind that these cells are developmentally only days old and may not show marker patterns typical of adult cells. Reluctant to refer to these RIIb⁺ cells as macrophages, we await new data regarding their lineage.

The third cell type is almost certainly a pericyte. They are long, thin, and sinuous, intercalating with the RIIb⁺ cells and the endothelium, and they express all of the pericyte markers utilized. Although we have not quantified their presence in the vessel wall, they appear adequately abundant and extensive for us to postulate that they create a peristaltic force that drives blood to and fro in the single-channel vitelline capillaries, propelling

erythrocytes from the fetal circulation to the villus tips, and sending IgG from the villus tips to the larger vitelline vessels that are indeed composed of afferent and efferent channels.

How exactly these three types of cells are spatially arranged to form the wall of the vessel will have to await an extensive 3D evaluation of the tissue, but already it appears clear that IgG moving from endoderm to the fetal vessel lumen would have the opportunity to interact with the RIIb⁺ cell. However, whether all IgG obligatorily needs to move through a RIIb⁺ cell to reach the fetus, is not at all clear and will require detailed 3D studies.

Testing the possibility that the yolk sac RIIb serves as a second IgG transporter we predicted that the IgG concentrations in RIIb^{-/-} fetuses would be very low compared with RIIb^{+/+} fetuses. We took fetuses from heterozygote matings. The experiment was done using 2 groups of mother mice, one IgG-deficient having been raised in pathogen free conditions and the other immunized to produce normal in-the-wild IgG concentrations. None of the RIIb^{-/-} fetuses showed strikingly low IgG concentrations, effectively rejecting our hypothesis that RIIb is required for IgG transport. However, those RIIb^{-/-} fetuses from IgG-deficient mothers showed a modest (17%) diminution in IgG concentration compared with RIIb^{+/+} fetuses for reasons that we have not been able to discern. Nevertheless, RIIb^{-/-} fetuses from the mothers with normal IgG concentrations had IgG concentrations no different than those of the RIIb^{+/+} fetuses. We therefore conclude that IgG crosses the capillary wall to the fetal lumen by transcellular trafficking or intercellular diffusion down a concentration gradient established by FcRn expressed in the endoderm.

What function then might RIIb serve? An old hypothesis suggested that the Fc receptor expressed in human placental villus endothelium, later identified as FcγRIIb2, might function as an immune complex trap to protect the fetus from maternal alloantibody directed against paternally encoded fetal antigens (Faulk and Johnson, 1980). This possibility is being considered in our study of IgG transport across the mouse yolk sac.

Our measurements of fetal/maternal IgG concentrations yielded further observations that seem to have received no earlier systematic study. It has long been thought that relatively little IgG passes to the fetal mouse during pregnancy compared with the robust transport occurring postpartum *via* the neonatal gut. For example, Brambell described fetal/maternal IgG concentrations as modest (0.25), quite different from human ratios (1.25) (Brambell, 1970). On the contrary, we found that the mouse fetus can have an IgG concentration 2–3 times that of the mother, substantially in excess of the maternal blood. Perhaps the confusion has developed because of the dramatic drop in plasma IgG concentration that occurs in the pregnant female during pregnancy. Our group of female BALB/c mice that were immunized with KLH developed IgG concentrations of 26 ± 9 mg/ml ($n = 31$), whereas a subgroup of these females that became pregnant showed at parturition an IgG concentration of 4 ± 4 mg/ml ($n = 13$), a >5 fold reduction. Obviously, fetal/maternal ratios will be very different from fetal/adult female ratios. The mechanism of this drop in maternal IgG concentration is likely multifactorial. Certainly, it seems quite possible that some of the drop is due to the hemodilution of late pregnancy, which has been well studied in humans. Further, it would appear from our data that fetuses sequester IgG from the mother to meet a requisite IgG concentration of ~0.7 mg/ml at birth despite a maternal concentration at parturition of half that value. Considering that maternal IgG loss was divided among the progeny, we noted an inverse trend between litter size and maternal IgG concentration at parturition, but the correlation is not statistically significant (data not shown).

Is the mouse yolk sac a suitable surrogate for inferring functional details about IgG transport across the human placenta? We earlier found a strict parallel between the expression of FcRn in the epithelial layer of the two organs, the syncytiotrophoblast of the terminal villus

and the endoderm of the yolk sac (Leach et al., 1996; Kim et al., 2009). The virtual absence of IgG in the blood of term FcRn^{-/-} fetuses would suggest that yolk sac FcRn is necessary for transport. So it seems likely that further study of the yolk sac endoderm would yield, by analogy, valuable information about human IgG transport across the syncytiotrophoblast. But the parallel is definitely not as strict at the level of the capillary where the fetal vasculature of the two species shows distinctive anatomy. Not only are the two endothelial layers very different in terms of tight junctions, gaps, fenestrae, loops, and lumens; but the number of cell types constituting the vessel wall is different in the two species (Cattellino et al., 2003; Mohanty et al., 2009). In the human terminal villus we find a RIIb-expressing endothelium surrounded by pericytes, while in the mouse yolk sac capillary we find endothelial cells and pericytes and a third distinct RIIb+ cell not seen in human. Thus, while we can conclude with confidence that RIIb in the yolk sac placenta is not needed for transport of IgG to the fetus, we cannot infer that the same would be true of RIIb in the villus endothelium of human. On the other hand, perhaps over the course of evolution the function of RIIb has been conserved, such that RIIb has the same function in both mouse and human while being expressed in different cell types along the IgG-transport pathway. We currently have no strategy for resolving this question.

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ABBREVIATIONS

RIIb	FcγRIIb
placenta	mouse chorioallantoic placenta or the human placenta
YS	mouse yolk sac placenta
DIC	differential interference contrast
dpc	day post coitum
immunofluorescence	immunofluorescence

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Figure 1.

Detection of RIIb expression in yolk sac by quantitative immunofluorescence microscopy. (A) The image shows a RIIb^{+/+} yolk sac placenta in which RIIb, localized by indirect immunofluorescence with MAb 2.4G2, was found in mesenchymal (MC) cells of yolk sac villi (thick arrows) and in the larger vitelline blood vessel walls (thin arrows), but not in endoderm or mesothelium (endoderm or MS). Asterisks label the vessel lumina. A higher magnification view of another vitelline vessel labeled with MAb 2.4G2 is shown in the inset. Note the positive fluorescence signal associated with the vessel wall. Three nuclei are indicated (arrows). (B) The image shows a RIIb^{-/-} yolk sac placenta where immunofluorescence staining with FcγRIII-reactive MAb 2.4G2 in villus MC is negative (arrows). A higher magnification view of a vitelline vessel showing no 2.4G2 labeling is shown in the inset. Three vessel cell nuclei are indicated by arrows. For orientation, panels (C) RIIb^{+/+} and (D) RIIb^{-/-} show the same sections visualized with combined DAPI staining of nuclei and DIC image. The endoderm and MS are indicated by thick and thin arrows, respectively. Bar = 20 μm. The histogram shows quantitative estimation of RIIb positive cells in yolk sac cellular areas. Sections labeled with secondary antibody alone were used to estimate nonspecific- and auto-fluorescence. Fluorescent and non-fluorescent cells were counted from representative sites of the three major cellular areas of the yolk sac; i.e., endoderm, MC, and MS. The numbers were averaged for each area, corrected for nonspecific-and auto-fluorescence, and plotted as a percentage of total cells that were RIIb positive. Approximately one-half of yolk sac MC of the RIIb^{+/+} yolk sac showed MAb 2.4G2 fluorescence signals above threshold values whereas in the RIIb^{-/-} <1% of yolk sac MC cells were above threshold value, significantly lower compared with RIIb^{+/+} ($P < 0.001$). The intensity of fluorescence signals in endoderm and MS cells of both RIIb^{+/+} and RIIb^{-/-} was near background as compared to RIIb^{+/+} MC ($P < 0.001$). Data are from tissue samples from 3 RIIb^{+/+} yolk sac and 5 RIIb^{-/-} yolk sac.

Figure 2.

RIIb protein and mRNA expression in yolk sac. (A) RIIb was immunoprecipitated using goat anti-mouse RIIb antiserum from lysates containing equal protein concentration of placenta, yolk sac, COS-7 cells, A-20 cells, and RAW 264.7 cells; separated by SDS-PAGE and analyzed by immunoblotting with a different antibody, affinity purified rabbit anti-mouse RIIb antibody. The control is yolk sac lysate immunoprecipitated with normal goat serum. The identity of the 70 kD band in the COS lane is unknown. The bands along the buffer front are IgG light chains. Numbers are MW in kDa. RIIb is marked with an arrowhead. (B) RT-PCR analysis of mRNA expression from samples of placenta, yolk sac, A20, COS-7, RAW 264.7, bone marrow macrophages (BMM) using primers specific for RIIb. PCR products of size 313 and 172 bp (arrowheads) represent transcripts for RIIb1 and RIIb2, respectively. The last lane represents the PCR amplification of a plasmid expressing mouse RIIb1. The gels are representative of three different experiments, each using yolk sacs and placentas (3, 4, and 8 of each) from a different mother.

Figure 3.

(A) RIIb is absent from mouse yolk sac endothelium by two-color immunofluorescence. Twelve panels arranged in 3 columns and 4 rows of two-color immunofluorescence images of yolk sac villi. The left column shows green CAV1_(aa3-14) (C-CAV1) labeling in all 4 panels. The middle column, from top to bottom, shows red images of labeling with antibodies toward RIIb, CAV1_(aa68-75) (R-CAV1), CD31 and CD34. The images in the right column are merged images of the left and middle columns. C-CAV1 and RIIb do not colocalize as illustrated by merged image in the top row, whereas C-CAV1 colocalizes with other endothelial cells markers as illustrated by merged images of C-CAV1 with R-CAV1, CD31, and CD34 respectively in merged images in rows 2–4. (B) Quantification of data from Panel A experiments. Two-color images like the representative ones shown in Panel A were quantified by scanning ~1000 lines from ~30 villi from a total of 3 yolk sac, assessing red and green intensity in the third dimension in each of the pixels. Upper panel shows the percentage of green C-CAV1 pixels that colocalize with red pixels representing R-CAV1, CD31, CD34, and RIIb. RIIb colocalization is significantly lower compared to the endothelial cells markers ($P < 0.001$). Lower panel shows the percentage of R-CAV1, CD31, CD34, and RIIb positive pixels that colocalize with C-CAV1. Again, RIIb is lower than the endothelial cells markers ($P < 0.001$). Note the colocalization of C-CAV1 and R-CAV1, using 2 antibodies recognizing the same molecule gives practical maxima of these quantitative measurements (~50–75% colocalization; not significantly different; $P > 0.05$).

Figure 4.

(A) RIIb marker colocalizes with macrophage, not pericyte, markers. Twelve panels arranged in 3 columns and 4 rows of two-color immunofluorescence images of yolk sac villi. The green images in the left column, from top to bottom, illustrate labeling with antibodies toward F4/80, α SMA, NG2 and PDGF β R. The middle column shows red RIIb labeling in all 4 panels. The images in the right column are merged images of the left and middle columns. The merged images show that RIIb colocalizes with the macrophage marker F4/80 but not with any of the pericyte markers. Colocalizing images of the first row are marked with thin arrows. (B) Quantification of data from Panel A experiments. Two-color images like the representative ones in Panel A were quantified by scanning ~1000

lines from a total of 3 yolk sac and recording the percentage of total pixels having both red and green colors. Upper panel shows the percentage of red RIIb pixels that colocalize with green pixels representing F4/80, α SMA, NG2, and PDGF β R. RIIb colocalization with F4/80 is significantly higher compared to the pericyte markers ($P < 0.001$). Lower panel shows the percentage of F4/80, α SMA, NG2 and PDGF β R positive pixels that colocalize with RIIb. Again, F4/80 colocalization with RIIb is higher than the pericyte markers ($P < 0.001$). (C) F4/80 marker colocalizes with CD68 marker. A single tissue section is labeled with MAbs against CD68 (left) and F4/80 (middle), and the two images are merged (right). The colocalizing colors are identified by thin arrows in all images. Note that anti-CD68 also labels endodermal cells (thick arrow in left and right images). The histogram below shows the quantitative measurement of F4/80 and CD68 colocalization. CD68 signal and F4/80 signals in the mesenchyme area were counted in a total of 100 villi from a total of 3 wild type yolk sac placentae. Areas of colocalization of green with red staining were calculated and expressed as percent colocalization of these two signals with respect to one another. More than 95% F4/80 positive cells expressed the second macrophage marker, CD68, whereas a significantly smaller number ($< 5\%$) of F4/80 cells did not express CD68 ($P < 0.002$); and reciprocally, $> 95\%$ CD68 positive cells expressed the F4/80 marker as compared to a small $< 5\%$ CD68 positive cells that did not express F4/80 ($P < 0.001$).

Figure 5.

RIIb is not required for IgG transport from mother to fetus. Each point in scattered plots represents the serum IgG concentration of an individual term fetus, produced from parents heterozygous for the RIIb-null allele (RII^{+/-}), plotted against its maternal serum concentration. The dashed line for reference represents the same serum IgG concentrations between the mother and fetuses (i.e., fetus-to-mother ratio of IgG concentration = 1.0). The number of fetuses in each respective genotype is indicated in parenthesis. The bar graph shows mean fetal IgG concentrations and bars represent one SD. * $P < 0.05$ between RIIb^{+/+} and RIIb^{-/-} fetuses. (A) Fetuses from 10 non-immunized (normal) mothers. The mean fetal IgG concentration was modestly lower (17% lower, $P < 0.03$) in RIIb^{-/-} ($\times$) fetuses compared with RIIb^{+/+} ($\bullet$) fetuses. Panel B. Fetuses are from 10 immunized mothers. $P > 0.5$.