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Research article

Neonatal Fc receptor FcRn is involved in intracellular transport of the Fc fusion protein aflibercept and its transition through retinal endothelial cells

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ABSTRACT

Retinal endothelial cells (REC) likely contribute to the clearance of intravitreally injected IgG. Because this is of high relevance to the pharmacokinetic assessment of the widely used therapeutic Fc fusion protein aflibercept, we studied its transport through immortalized bovine REC (iBREC) in detail. For shuttling of IgG or Fc fusion proteins like aflibercept, endothelial cells use the highly conserved neonatal Fc receptor (FcRn) also expressed in iBREC where it is down regulated by serum depletion. Therefore, we focused on studying intracellular localization and transport of aflibercept under conditions affecting its interaction with the FcRn. Intracellular localization of aflibercept was assessed by Western-blot analyses of subcellular protein fractions or by immunofluorescence staining. After uptake in a temperature-dependent process, aflibercept co-localized with early endosomes, which harbor FcRn. Similar amounts of aflibercept were co-extracted with proteins from membranes/organelles irrespectively of the amount of FBS in the culture medium. Lowering the concentration of FBS resulted in a strong, but reversible association with cytoskeletal proteins suggesting a block in intracellular transport. In accordance with this finding, aflibercept's transport through an iBREC monolayer grown on porous membrane inserts was markedly delayed in the absence of FBS in the culture medium indicating that aflibercept is taken up but not exocytosed under these conditions. Transcytosis of aflibercept was also strongly delayed by inhibition of phosphatidylinositol 3-kinase with LY294002, which affects FcRn-mediated IgG transport. A similar inhibition of aflibercept's transport was observed with IgG-binding proteins (i.e. protein A or protein G) that block interaction between FcRn and aflibercept. Interfering with aflibercept's binding to the FcRn with protein A (or protein G) or the inhibitory FcRn-specific monoclonal antibody 1G3 resulted in a reduced amount of intracellular aflibercept. Taken together, our results strongly suggest that FcRn is involved in transport of aflibercept through REC in vitro.

1. Introduction

Pathogeneses of diabetic retinopathy, retinal vein inclusion and age related macular edema are associated with de-regulated expression of various cytokines including the most relevant vascular endothelial growth factor A (VEGF-A). Accordingly, VEGF-binding proteins ranibizumab, aflibercept and bevacizumab are now widely used to treat these conditions, and their VEGF-inactivating activities were clearly confirmed by in vitro studies with retinal endothelial cells (REC) (Presta et al., 1997; Holash et al., 2002; Ferrara et al., 2006, Arevalo et al., 2011, Do et al., 2012; Lang et al., 2013; Deissler et al., 2008, 2011, 2012, 2014; Stewart et al., 2011). The pharmacokinetic profiles of therapeutic IgG, their derivatives and fragment crystalisable (Fc) fusion proteins are determined by their clearance from the eye, a process in which REC seem to play a role (Kim et al., 2009; Julien et al., 2014). We have recently shown that immortalized endothelial cells of the bovine retina (iBREC) can take up the humanized VEGF-binding antibody bevacizumab and transport it through the monolayer or release it from

Abbreviations: DMSO, dimethyl sulfoxide; EEA1, early endosome antigen 1; Fc, fragment crystalisable; FcRn, neonatal Fc receptor; FBS, fetal bovine serum; (i)BREC, (immortalized) bovine retinal endothelial cells; PI3-kinase, phosphatidylinositol 3-kinase; MAb, monoclonal antibody; PBSd, phosphate buffered saline without Ca^{2+} and without Mg^{2+} ions; REC, retinal endothelial cells; RPE, retinal pigment epithelium; TJ, tight junction; VEGF(-A), vascular endothelial growth factor (A).

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the uptake side (Deissler et al., 2016). Blocking the interaction of the IgG with the neonatal Fc receptor (FcRn) impaired transport of bevacizumab and affected its subcellular localization in these cells (Deissler et al., 2016). FcRn, which is expressed in various ocular structures including the retinal endothelium, is primarily involved in transcytosis and recycling of IgG and albumin in endothelial cells of various origins and responsible for maintaining homeostasis of these proteins in the serum (Kim et al., 2008; Powner et al., 2014; Junghans and Anderson, 1996; Ward et al., 2003). This receptor is a dimer consisting of two not covalently linked subunits: β_2-microglobulin and a larger polypeptide related to major histocompatibility complex class I proteins (Junghans and Anderson, 1996). In early endosomes, which are characterized by the expression of the early endosome antigen 1 (EEA1), the FcRn binds IgG and albumin independently from each other at pH 6. The complexes are then shuttled away from the lysosomes and both proteins are released by exocytosis at physiological pH (Junghans and Anderson, 1996; Ward et al., 2003, 2005; Ober et al., 2004; Goebl et al., 2008; Jerdeva et al., 2010, Sand et al., 2015). Interfering with vesicle formation by blocking phosphatidylinositol 3-kinase (PI3-kinase) with LY294002 or Wortmannin strongly abolished FcRn-mediated IgG transport in rat kidney epithelial cells expressing FcRn (McCarthy et al., 2000). The human and bovine homologues of FcRn are highly conserved, and it was confirmed in several studies that human IgG is indeed efficiently bound, transported and released in the transcytosis pathway by bovine FcRn (Ober et al., 2001; Kacskovics et al., 2000, 2006; Cui et al., 2014). Expression of the FcRn in iBREC is exclusively associated with proteins from the membrane/organelle fraction, is weaker in the absence of FBS but not affected by bevacizumab. Most interestingly, transport of bevacizumab through an iBREC monolayer is retarded in the absence of FBS accompanied by its accumulation in the fraction of cytoskeleton proteins (Deissler et al., 2016). iBREC also take up aflibercept, which consists of binding domains of VEGF receptors and the human IgG1 Fc domain, within a few hours after exposure, and the Fc fusion protein is then mainly localized in a perinuclear region (Holash et al., 2002; Deissler et al., 2014). These observations invited the hypothesis that binding to FcRn might also be involved in aflibercept's intra- and transcellular transport and we therefore studied uptake and transport of aflibercept under conditions affecting its potential interaction with FcRn.

2. Materials and methods

2.1. Aflibercept and antibodies

Eylea (40 mg/ml aflibercept) was a kind gift from Bayer Vital GmbH (Leverkusen, Germany) (Holash et al., 2002; Do et al., 2012). Goat polyclonal detection antibodies ($F(ab')_2$, labelled with Alexa Fluor 594/488) were from Thermo Scientific (Karlsruhe, Germany); other antibodies are listed in Table 1.

2.2. General information on cell culture, treatment of iBREC with effectors and transcytosis assay

Only general information is provided here, because the current study is based on an approach already described in great detail (Deissler et al., 2016). Confluent monolayers of telomerase-immortalized bovine microvascular REC (iBREC) established after cultivation for at least 4 d were used in all experiments, which were repeated at least twice (Deissler et al., 2005, 2008, 2011, 2016). In control experiments, cells were processed identically in medium only lacking the effector(s) under investigation. A final concentration of 250 μg/ml (~2 μM) aflibercept - achievable by intravitreal injection - was always used.

To study the effect of FBS on internalization or transport of aflibercept, iBREC were incubated with culture medium containing 0%, 1% or 5% FBS for 24 h before they were exposed to aflibercept for up to 1 d (Deissler et al., 2014, 2016). Potential competitive effects of Fc-binding proteins (i.e. protein A and protein G; Thermo Scientific) on the internalization or transport of aflibercept were explored as described (Raghavan et al., 1994; Deissler et al., 2016). To prevent interaction of aflibercept with FcRn, iBREC were pre-treated with 10 μg/ml of mouse monoclonal antibody (MAb) 1G3 for 1 h before aflibercept was added for additional 4 h. Whole cell extracts and subcellular fractions, i.e. proteins localized in the cytoplasm, in membranes/organelles, and components of the cytoskeleton, were prepared from fresh or frozen cell pellets as described in detail elsewhere (Deissler et al., 2012, 2014, 2016).

Transcytosis assays to assess transport of aflibercept from the lower to the upper chamber through a confluent iBREC monolayer cultivated on membrane inserts (4.7 cm^2, pore size 0.4 μm; Corning) were performed as published (Antohe et al., 2001; Deissler et al., 2016). The effect of inhibition of PI3-kinase on aflibercept transport was evaluated by adding LY294002 (500 nM in 0.01% dimethyl sulfoxide (DMSO); Sigma-Aldrich, Dreieich, Germany) to both chambers. After pre-treating iBREC for 2 h, aflibercept was placed in the bottom chamber and samples were then taken from the upper chamber.

All Western blot analyses were performed under reducing conditions; equal amounts of protein were loaded per lane when cell extracts (~25 μg) or fractions thereof (~5 μg) were analyzed (Deissler et al., 2012, 2014). To determine the amount of transported aflibercept, the sample (75 μl) taken from the supernatant in upper chamber (total volume: 1.5 ml) was directly analyzed by Western blot without further processing; 0.1% of the supernatant was loaded per lane (Deissler et al., 2016). Western blot analyses were repeated at least twice; a typical image is shown. Observed differences

Table 1
Antibodies used in study.

Target	Specificity	Host	Source	Concentration
FcRn (large chain)	human, bovine	rabbit, polyclonal	bio-techne (Wiesbaden, Germany), NBP1-89127	Western blot analysis: 3 μg/ml immunofluorescence staining: unspecific
FcRn (heterodimer)	human	mouse, monoclonal	Genetex (Biozol, Eching, Germany), 1G3	functional assay: 10 μg/ml
EEA1	human, bovine	rabbit, polyclonal	abcam (Cambridge, UK), ab137403	Western blot analysis: 70 ng/ml, immunofluorescence staining: 20 μg/ml
claudin-1	human, bovine	rabbit, polyclonal	Thermo Scientific, JAY.8	Western blot analysis: 100 ng/ml JAY.8 does not cross react with claudin-3
actin	human, bovine	mouse, monoclonal	abcam, AC-40	Western blot analysis: 500 ng/ml
IgG, γ-chain	human	goat, polyclonal, coupled to HRP[a]	Thermo Scientific, 628420	Western blot analysis: 1:20000
whole IgG	rabbit	goat, polyclonal, coupled to HRP[a]	Biorad (Munich, Germany), 170-5046	Western blot analysis: 1:30000
whole IgG	mouse	goat, polyclonal, coupled to HRP[a]	Biorad, 170-5047	Western blot analysis: 1:20000

[a] HRP: horseradish peroxidase.

between differently treated iBREC were always very clear cut, reproducible and resulted in contrasting chemiluminescence signals. Subsequent quantification of chemiluminescence signals would not have gained any additional insight.

Immunofluorescence staining of confluent iBREC monolayers was carried out as previously described (Deissler et al., 2014, 2016). Aflibercept-positive cells were quantified by counting cells immunostained with the antibody binding to human IgG in four different microscopic fields divided by the number of cell nuclei therein.

2.3. Measurement of cell index

To assess cell viability or barrier function of iBREC in the presence of aflibercept under different serum conditions, electric cell-substrate impedance measurements were performed using the microelectronic biosensor system for cell-based assays xCELLigence RTCA DP (Acea, OLS, Bremen, Germany) (Sun et al., 2012). Impedance was measured between electrodes in an individual well of an E-Plate VIEW 16 PET (Acea) and calculated as the unit-free parameter cell index (CI) according to the formula $CI=(Zi-Z_0)/15\Omega$ where Zi is the impedance measured at an individual time point and Z_0 the impedance measured at start of the experiment. 100 μl culture medium were placed in each well of an E-Plate VIEW 16 PET (coated overnight with 50 μg/ml fibronectin at 4 °C) and plates were equilibrated at 37 °C in the incubator for at least 1 h before background measurements were performed. 1.2×10^4 iBREC in 100 μl were seeded per well and cell index was determined every 15 min for the first 24 h and afterwards every 1 h until a stable value of CI ≈ 20 was reached 3 d after seeding indicating a stable confluent monolayer. Culture medium was replaced by medium with or without FBS and CI was monitored for additional 24 h before aflibercept (final concentration: 250 μg/ml) was added. Subsequently, CI was measured every 15 min for up to 48 h and values were normalized in relation to those measured immediately before addition of aflibercept. Similar experiments were carried out in the presence of LY294002 (final concentration: 50, 500 and 5000 nM) to assess its influence on barrier stability.

3. Results

3.1. Subcellular localization and transcytosis of aflibercept strongly depend on presence of serum

Because expression of the FcRn is regulated by serum components in iBREC (see also Fig. 1A), we investigated the effects of FBS on aflibercept's subcellular localization, its transport through an iBREC monolayer (→ transcytosis), as well as its transporting back to the same cell side it came from (→ recycling).

Irrespective of the serum concentration, internalized aflibercept was found to be associated with proteins from membranes or organelles (Fig. 1A) as shown by Western blot analyses of subcellular fractions prepared 4 or 24 h after its addition to iBREC pre-incubated with 0–5% FBS for 1 d. A vast amount of aflibercept taken up by iBREC was co-extracted with cytoskeletal proteins (e.g. actin, vimentin) only when cells were exposed to lower FBS concentrations (0% or 1%; Fig. 1A). Aflibercept did not affect viability of iBREC - assessed by cell index measurements - at either concentration of FBS (data not shown). Immunofluorescence staining revealed that about 50% of iBREC had internalized aflibercept irrespectively of the FBS concentration in the culture medium (aflibercept-positive cells: 52% ± 12% after cultivation with 5% FBS versus 56% ± 10% without FBS, $p > 0.05$). Aflibercept was localized in small isolated clusters or spots close to the nucleus when iBREC had been cultivated without FBS (Fig. 3, red arrowheads) whereas it was more homogenously distributed in a perinuclear region after cultivation in the presence of 5% FBS (Fig. 3, yellow arrowheads). FBS-dependent accumulation of aflibercept in the subcellular fraction of cytoskeleton proteins was shown to be reversible: When iBREC were treated for 1 d with aflibercept in medium with 1% FBS before the serum concentration was increased to 5% for another day, the amount of aflibercept associated with cytoskeleton proteins decreased substantially during cultivation with more FBS (Fig. 1B).

To assess aflibercept's transport through an iBREC monolayer cultivated on porous membrane inserts (→ transcytosis), cells were exposed to 0 or 5% FBS for 24 h in both chambers before aflibercept was added to the lower chamber for up to 2 d (Fig. 1C). Aflibercept was always placed in contact to the basal cell membrane because after intravitreal injection the basal side of the REC layer of the vessel walls is primarily exposed to the drug. Samples were taken from the upper chamber (above the apical cell membrane) at various time points (Fig. 1C) to determine the amounts of aflibercept released into the medium by Western blot analyses. In accordance with aflibercept's accumulation in the fraction of cytoskeleton proteins in the absence of serum, its transport by cells in medium without FBS was much slower compared to 5% FBS (Fig. 1C) suggesting a block in intracellular transport under these conditions. In addition to transcytosis, internalized aflibercept might also be recycled to the compartment it came from. To study the relevance of this transport route, iBREC were first exposed to aflibercept in the lower chamber in medium with 5% FBS for 24 h after which a considerable amount of the protein was detected in the upper chamber as a consequence of uptake and transport through the monolayer. However, during subsequent cultivation of the thoroughly washed cells in aflibercept-free medium for additional 24 h, only a very small amount of aflibercept was recycled to the lower chamber (data not shown). When medium containing only 1% FBS was used, recycling of aflibercept was not observed at all.

3.2. Uptake of aflibercept by iBREC is temperature-dependent

Possible involvement of FcRn in uptake and intracellular transport of aflibercept by iBREC requires vesicle-based trafficking, which is known to be slowed down at lower temperature. Accordingly, the amount of internalized aflibercept was lower when the cells were temporarily cultivated at 4 °C (Fig. 2A). That the tight iBREC barrier was not affected by the temperature switch was confirmed by measuring the strong and stable expression of the tight junction (TJ)-protein claudin-1. Presence of claudin-1 strongly correlates with a stable barrier in iBREC (Deissler et al., 2013).

3.3. Inhibition of PI3-kinase delays transcytosis of aflibercept through an iBREC monolayer

To investigate whether blocking PI3-kinase activity affects aflibercept's transport, iBREC cultivated on porous membrane inserts were exposed to 500 nM LY294002 for 2 h before aflibercept was added to the lower chamber. Under these conditions, the iBREC barrier was stable over several days; higher concentrations resulted in barrier dysfunction (data not shown). Pre-treating of iBREC with LY294002 indeed strongly inhibited transcytosis of aflibercept (Fig. 2B), but changes in aflibercept's uptake - amount and intracellular localization - were not evident (data not shown).

3.4. Intracellular aflibercept is associated with EEA1-positive vesicles

The above-mentioned results suggested that aflibercept's

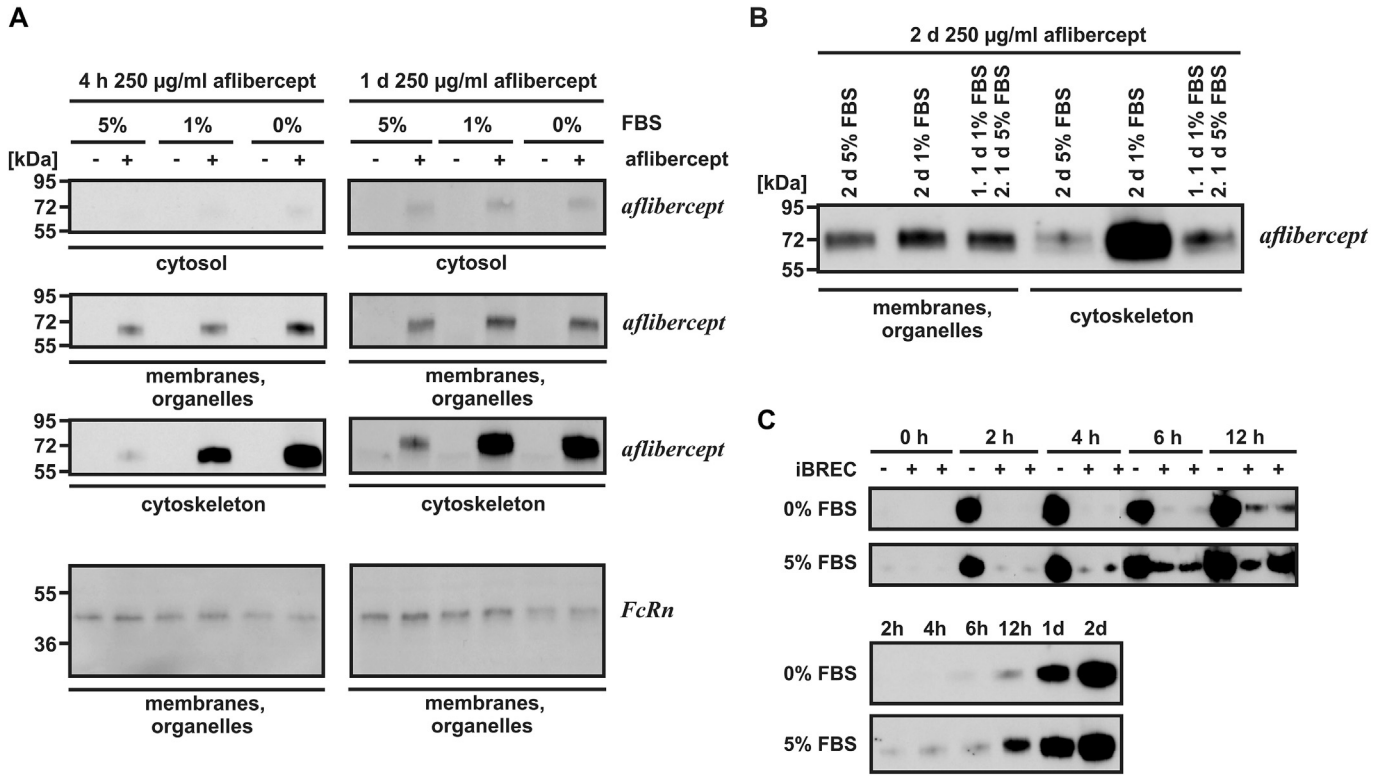

Fig. 1. Subcellular localization and transcytosis of aflibercept are serum-dependent. **(A)** Confluent iBREC pre-incubated for 24 h with medium containing 0–5% FBS were treated with aflibercept before subcellular fractions were prepared 4 or 24 h later. These were analyzed by Western blot to determine internalized aflibercept and presence of FcRn. Similar amounts of aflibercept were co-extracted with membrane proteins, irrespectively of the FBS concentration in the culture medium. In contrast, very strong aflibercept-specific bands assigned to the fraction consisting of cytoskeleton proteins were observed only after cultivation in serum-reduced media. iBREC expressed FcRn when cultivated in medium with serum, but only weak FcRn-specific bands were seen when membrane/organelle fractions of cells kept without serum for some time were analyzed. Aflibercept did not affect FcRn expression. **(B)** Accumulation of aflibercept in the cytoskeleton fraction is reversible: Confluent iBREC were treated with aflibercept for 2 d in medium with 1% or 5% FBS, or in medium with 1% FBS for 1 d before FBS was increased to 5% for another day. After incubation with 1% FBS, most of the aflibercept was found in the fraction containing cytoskeleton proteins and this portion strongly decreased when the FBS concentration was brought to 5%. **(C)** Confluent iBREC cultivated on porous membrane inserts were treated as described in (A) before aflibercept was added to the lower chamber. Subsequently its transport through the iBREC monolayer (+iBREC) or its diffusion through cell-free membrane inserts (-iBREC) were monitored by Western blot analyses of samples from the top chamber. Transport of aflibercept through the iBREC monolayer was strongly promoted by FBS whereas its diffusion through membranes without cells was not affected.

uptake and transport is mediated by vesicles. Therefore, presence of aflibercept in EEA1-positive early endosomes was studied. iBREC treated with aflibercept for 4 h (with or without FBS) were stained with antibodies against EEA1 in combination with visualization of aflibercept. The uniform EEA1-specific staining seen after cultivation in medium with 5% FBS clearly co-appeared with signals indicating internalized aflibercept, particularly in areas close to the nucleus (Fig. 3, yellow arrow). However, a mosaic pattern rather than overlapping staining of aflibercept and EEA1 was observed in these regions of cells cultivated without FBS (Fig. 3, red arrows). Western blot analyses of subcellular fractions confirmed that exposure to aflibercept did not alter expression of EEA1 (data not shown).

3.5. Protein A and protein G retard aflibercepts's transcytosis and affect its intracellular localization

If binding of aflibercept to FcRn was involved at some stage of its uptake and intracellular trafficking, preventing the interaction of the Fc domain of the fusion protein with FcRn should alter its transport through an iBREC monolayer and its subcellular localization. Protein A or protein G were considered useful tools because they compete with the FcRn for binding of the IgG1 Fc part (Raghavan et al., 1994; Wines et al., 2000). In transcytosis assays performed in the presence of an excess of one of the IgG-binding proteins for 6 h, protein A (5 Fc terminus-binding sites, 10 µM) or protein G (2 Fc terminus-binding sites, 25 µM) markedly delayed transport of aflibercept (Fig. 4A). Western blot analyses of subcellular fractions prepared 4 h after addition of aflibercept to iBREC showed that aflibercept was localized in the fractions of membrane and cytoskeletal proteins (Fig. 4B). However, when binding of aflibercept to FcRn was impaired by protein A or protein G, the Fc fusion protein was no longer co-extracted with proteins from membranes and organelles. Their moderate excess (≈10 Fc-binding sites) resulted in an overall reduced amount of intracellular aflibercept; in the presence of a larger excess (≈50 Fc-binding sites) aflibercept was co-extracted only with cytoskeleton proteins (Fig. 4B). FcRn expression was not altered by protein A/G (data not shown).

3.6. FcRn-specific antibody 1G3 reduces amount of internalized aflibercept

Binding of MAb 1G3 to the FcRn heterodimer blocks interaction of other IgG with this receptor (Raghavan et al., 1994). As expected, less aflibercept was present in iBREC pre-treated with 1G3 for 1 h before addition of the Fc fusion protein (Fig. 4C).

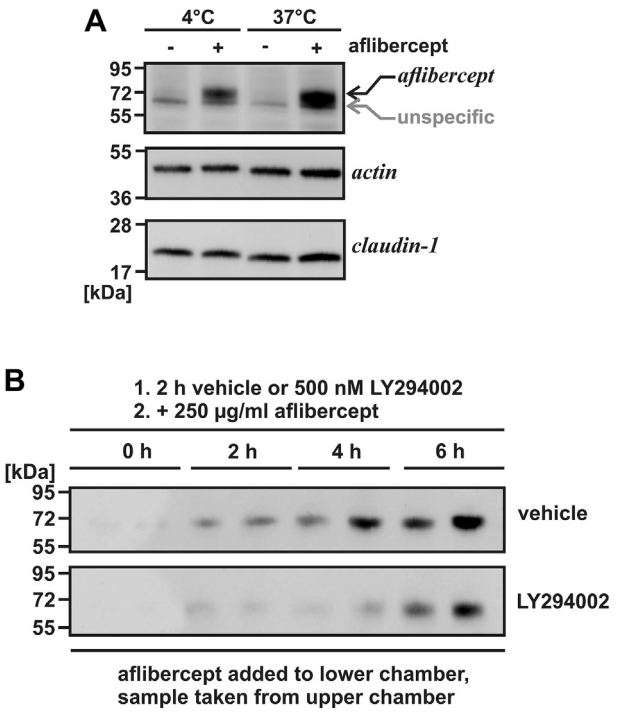

Fig. 2. Temperature-dependent internalization and retardation of its transport by PI3-kinase inhibition is indicative of an active uptake of aflibercept by iBREC. (A) Confluent iBREC were incubated with aflibercept for 4 h at 4 °C or 37 °C before cellular extracts were prepared and analyzed. More aflibercept was taken up by iBREC kept at 37 °C. Actin was assessed as loading control and strong expression of the TJ-protein claudin-1 confirmed stability of the tight barrier. **(B)** iBREC grown on porous membrane inserts were pre-treated with LY294002 or 0.02% DMSO (vehicle) for 2 h present in both chambers. Aflibercept was then added to the lower chamber and samples taken from the upper chamber were analyzed by Western blot. Inhibition of PI3-kinase delayed transcytosis of aflibercept.

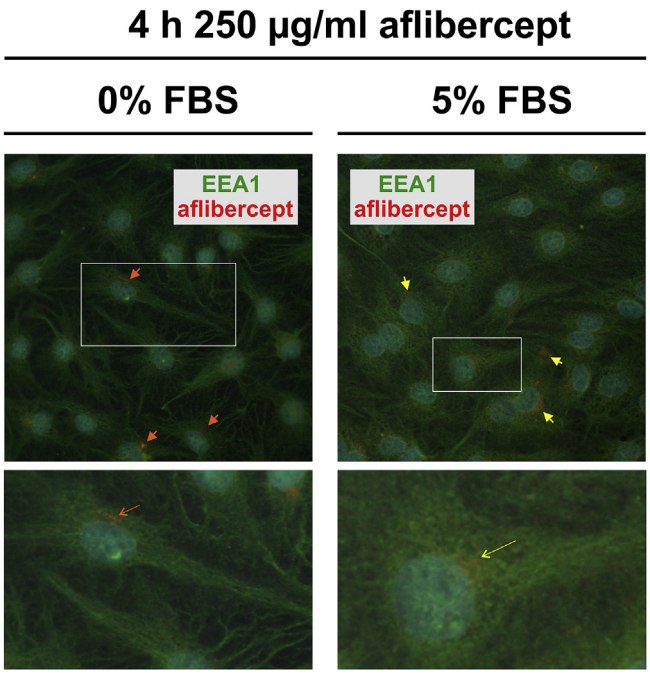

Fig. 3. Aflibercept is associated with early endosomes. iBREC cultivated for 24 h in media containing 0% or 5% FBS were treated with aflibercept for additional 4 h, and aflibercept (red) or early endosome-specific EEA1 (green) were visualized by immunofluorescence staining. Variation of the FBS concentration resulted in different patterns of aflibercept- and EEA1-staining: After cultivation of iBREC without FBS, aflibercept localized to small clusters or spots close to the nucleus (red arrowheads) and these did not overlap with the EEA1-positive endosomes (red arrows). In cells cultivated with 5% FBS, the perinuclear aflibercept-specific staining as well as that of EEA1 were more homogenous (yellow arrowheads), and co-localization of aflibercept and EEA1 was evident (yellow arrow).

4. Discussion

Involvement of the Fc-binding transporter/receptor FcRn in uptake of aflibercept by iBREC and its transition through these cells was investigated because of solid evidence indicating that intravitreally injected therapeutic IgG or related proteins are – at least to some extent - cleared from the eye via retinal capillaries (Kim et al., 2009). Accordingly, aflibercept was detected in retinal vessels in the monkey eye one day after intravitreal injection (Julien et al., 2014).

Although smaller than an IgG, aflibercept is still too large to pass through the very small intercellular gaps between individual cells of the unchallenged retinal endothelium (Hofman et al., 2000). Therefore, aflibercept's transition is most likely a consequence of an active transport mediated by receptors and vesicles. An obvious candidate receptor, typically involved in such processes and binding to the Fc domain of an IgG or Fc fusion protein, is the transporter/receptor FcRn (Junghans and Anderson, 1996; Ward et al., 2003; Ober et al., 2004; Goebl et al., 2008; Kim et al., 2008; Powner et al., 2014).

To study transcytosis and uptake of aflibercept under conditions potentially affecting its interaction with the FcRn, we used the established iBREC cell culture model. In accordance with our previous findings that aflibercept is taken up by iBREC without affecting their barrier function, it is efficiently transported through an iBREC monolayer (Deissler et al., 2014). Internalized amounts of aflibercept were markedly lower when cells were cultivated at 4 °C instead of 37 °C, supporting our hypothesis that non-specific endocytosis and active vesicle-based transport are involved, whereas paracellular passage might play no more than a minor role. Accordingly, most of the internalized aflibercept was co-extracted with proteins from membranes and organelles, and it was also detected in EEA1-positive early endosomes, the organelles in which the FcRn is mainly localized (Ward et al., 2003, 2005; Jerdeva et al., 2010). However, FcRn could not directly be immunostained with the available specific antibody directed against the heavy chain of the receptor; visualization of a putative interaction between aflibercept and FcRn was therefore not possible (Powner et al., 2014; Deissler et al., 2016). Interfering with vesicle formation by inhibiting PI3-kinase also markedly affected transport of aflibercept whereas intracellular amount and localization were not obviously altered. These findings suggest that aflibercept is taken up and then degraded under these conditions. In epithelial cells of the rat kidney, PI3-kinase blocking only affected FcRn-mediated transport, supporting our hypothesis that FcRn is indeed involved in aflibercept transport in iBREC (McCarthy et al., 2000).

Transcytosis of aflibercept was most efficient in the presence of substantial amounts of FBS when amongst other things the FcRn is strongly expressed by iBREC. A similar serum-dependent transport was observed in analogous experiments with the IgG bevacizumab, suggesting that the Fc domain plays a crucial role in intracellular transport of such macromolecules (Deissler et al., 2016). The importance of an aflibercept-FcRn interaction for intracellular transport was further supported by our observation that uptake and transcytosis of aflibercept was inhibited by IgG-binding proteins A and G, again in complete accordance with similar effects reported for bevacizumab (Deissler et al., 2016). Binding sites for

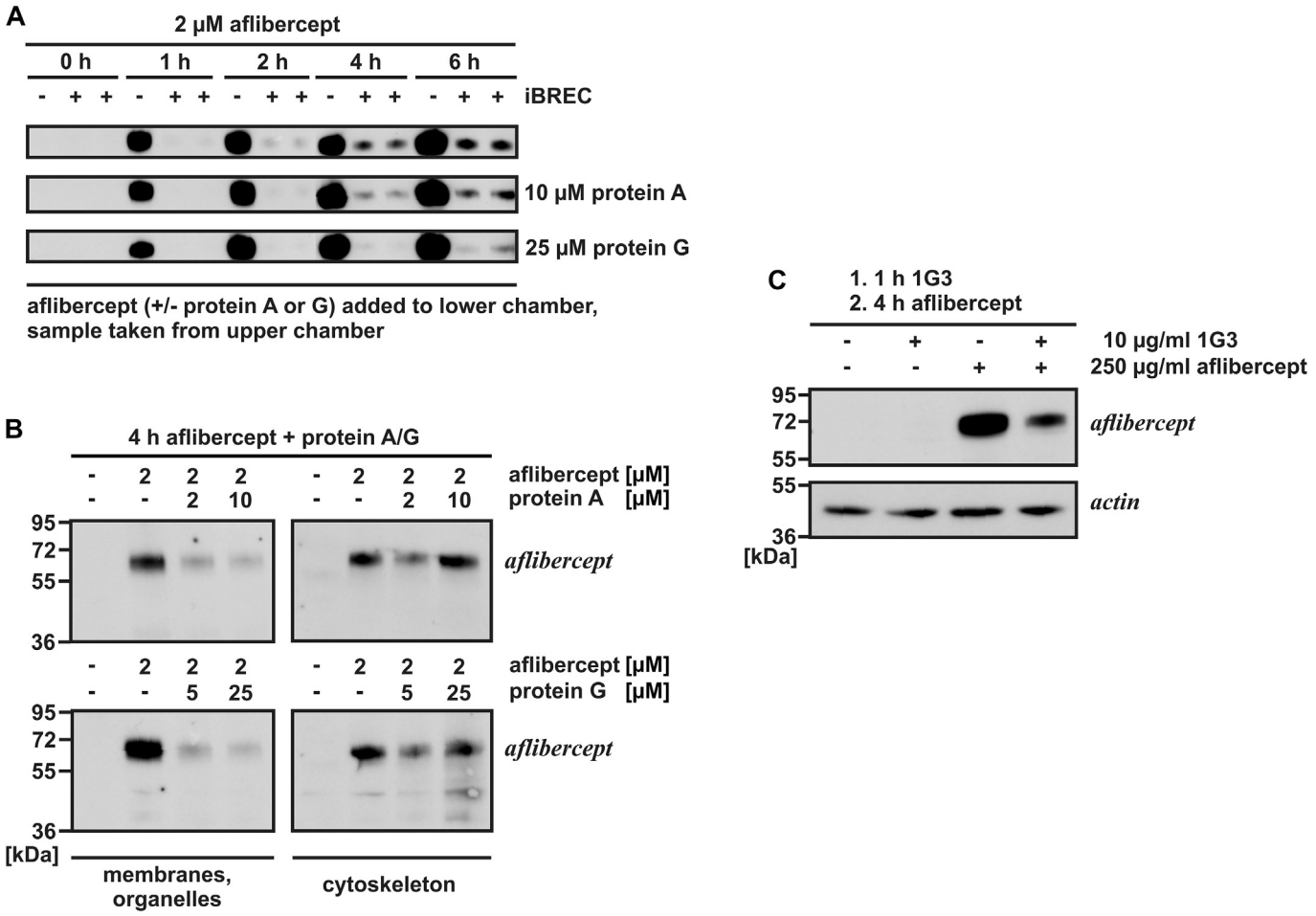

Fig. 4. Transcytosis, subcellular localization and internalization of aflibercept are affected when its binding to FcRn is blocked. **(A)** Aflibercept with or without an IgG-binding protein was added to the lower chamber and its transport through the iBREC monolayer (+iBREC) or its diffusion through cell-free membrane inserts (-iBREC) was monitored by Western blot analyses of samples from the top chamber. Transport of aflibercept (+iBREC) was markedly slowed down by an excess of protein A or protein G whereas its free diffusion (- iBREC) was not. **(B)** Subcellular fractions of confluent iBREC treated as described in (A) for 4 h were analyzed by Western blot. When protein A or protein G had been present in the culture medium, aflibercept was no longer co-extracted with membrane proteins but detected in the fraction of cytoskeleton proteins. **(C)** iBREC pre-treated with FcRn-specific MAb 1G3 were exposed to aflibercept for 4 h before cellular extracts were prepared. Western blotting revealed that less aflibercept is present in iBREC when binding to FcRn is blocked by 1G3.

protein A and protein G at the border between the C_H2 and C_H3 regions of the Fc terminus of human IgG1 (or aflibercept) overlap with those of the FcRn, whereas other known Fc receptors bind to different regions of a human IgG1 (Deisenhofer, 1981; Raghavan et al., 1994; Burmeister et al., 1994; Sauer-Eriksson et al., 1995; Wines et al., 2000). Moreover, pre-treating iBREC with the inhibitory MAb 1G3 strongly diminished the amount of intracellular aflibercept. Taken together, preventing the interaction between FcRn and aflibercept (i.e. in the presence of IgG-binding proteins or inhibitory MAb 1G3) likely resulted in degradation of the Fc fusion protein, as it would be expected. However, aflibercept as well as bevacizumab are still internalized by iBREC when interaction with FcRn is inhibited. Therefore, FcRn is likely not required in the process leading to uptake of aflibercept (or bevacizumab) by iBREC behaving like other sorts of endothelial cells.

Similar to the behavior of the therapeutic antibody bevacizumab in such experiments, the slower transcytosis of aflibercept induced by serum depletion was associated with a reversible accumulation of the Fc fusion protein in the fraction of cytoskeleton components, and aflibercept was no longer present in EEA1-positive early endosomes, suggesting a block of intracellular transport (Deissler et al., 2016). This might be due to reduced expression of the FcRn or other components of the transport machinery, e.g. the small Ras-like GTPases Rab11 and Rab4 (Ward et al., 2005). Exosomes staining positive for FcRn and Rab11, but not for Rab4, are involved in exocytosis of FcRn-bound IgG in human microvascular endothelial cells (Ward et al., 2003, 2005): These $Rab11^+/Rab4^-$-vesicles are transported to the plasma membrane where FcRn-bound IgG can be released by exocytosis at neutral pH. Preliminary data suggest that expression of Rab11 in iBREC exposed to serum-free culture medium is indeed reduced (unpublished observation). Block in intracellular transport of aflibercept (and bevacizumab) - likely bound to residual FcRn - might be due to lack of formation of $Rab11^+$-vesicles under these conditions. Proper exocytosis of the IgG or Fc fusion protein is then hindered. Both Fc-containing proteins are still taken-up by iBREC in the absence of FBS supporting our assumption that FcRn is likely not involved in their endocytosis. It seems possible that lack of serum components also prevents fusion of the early endosomes with lysosomes thereby blocking degradation of aflibercept or bevacizumab, which would be expected under these conditions. Interestingly, overloading the transport system of human microvascular endothelial cells with high amounts of IgG (2.5 mg/ml) resulted in an accumulation of IgG in the cells; the IgG was localized in EEA1-positive endosomes as

well as in lysosomes under these conditions (Ward et al., 2003). Similar results were obtained with lower amounts (250 μg/ml) of a mutated IgG that was not able to bind to FcRn, suggesting an important role of the receptor in this process. The ratio of FcRn to internalized aflibercept (or bevacizumab) present in iBREC under conditions with low serum may simply be still high enough to protect both proteins from degradation but too low to allow for further transport. This hypothesis is in accordance with the observation that human endothelial cells of the lung expressing high amounts of FcRn internalized more IgG (Goebl et al., 2008).

In addition to transport through a cell monolayer, an IgG might also be recycled to be released from the same cell side it had entered. Indeed, at least part of internalized bevacizumab or aflibercept seem to leave iBREC at the side of its uptake under certain conditions. Because data including exact quantification are not available, it cannot be elucidated whether transcytosis or recycling is the favored route of transport. One can assume that considerable amounts of an exogenous protein of a type not even present in the healthy retina need to be cleared rather than recycled under normal physiological conditions.

IgGs are usually not present in the healthy retina, but the strongly increased use of intravitreally applied antibodies or Fc fusion proteins heightens the interest in their intraocular fate and pharmacokinetics associated with relevant clearance pathways (Ziemssen et al., 2016). In addition to our findings concerning REC, *in vitro*-studies with retinal pigment epithelium (RPE)-choroid organ cultures revealed that these cells also transport Fc-carrying proteins bevacizumab and aflibercept through their intracellular space (Dithmer et al., 2016). Internalization of bevacizumab by RPE cells was markedly decreased when interaction with the FcRn was impeded, though blocking of the other Fc receptors had no effect (Dithmer et al., 2016). In addition, both proteins were detected in vessels of the retina and choroid as well as in the RPE after their intravitreal injection into the monkey eye, suggesting that all these ocular structures might be involved in the transport of Fc-containing proteins out of the eye (Heiduschka et al., 2007; Schraermeyer and Julien, 2012; Julien et al., 2014). Further studies however are required to elucidate the relative relevance of clearance of these proteins via posterior routes compared to the presumably more important release from the vitreous into the aqueous humor. In these investigations, clearance from the targeted compartments retina and choroid should be distinguished from overall clearance from the vitreous.

5. Conclusion

In accordance with our conclusions from experiments with bevacizumab, all results of our current study strongly suggest that binding of aflibercept to the neonatal Fc receptor is crucially involved at least at some stage in its intracellular trafficking in REC *in vitro*: (1) transport is most efficient when FcRn is strongly expressed and binding of aflibercept to FcRn is not inhibited, (2) uptake is temperature-sensitive and (3) intracellular aflibercept is localized in EEA1-positive early endosomes which harbor the FcRn.

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Contributor statement

HLD: design and analyses of experiments, supervision of technicians performing experiments, analyses of data, writing of manuscript, approval of final version.

GKL: supervision of research personal, approval of final version.

GEL: analyses of data, supervision of research personal, writing of manuscript, approval of final version.

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References

Antohe, F., Rădulescu, L., Gafencu, A., Gheţie, V., Simionescu, M., 2001. Expression of functionally active FcRn and the differentiated bidirectional transport of IgG in human placental endothelial cells. Hum. Immunol. 62, 93–105. http://dx.doi.org/10.1016/S0198-8859(00)00244-5.

Arevalo, J.F., Sanchez, J.G., Lasave, A.F., Wu, L., Maia, M., Bonafonte, S., Brito, M., Alezzandrini, A.A., Restrepo, N., Berrocal, M.H., Saravia, M., Farah, M.E., Fromow-Guerra, J., Morales-Canton, V., 2011. Intravitreal bevacizumab (avastin) for diabetic retinopathy: the 2010 GLADAOF lecture. J. Ophthalmol. 2011, 1–13. http://dx.doi.org/10.1155/2011/584238. Article ID 584238.

Burmeister, W.P., Huber, A.H., Bjorkman, P.J., 1994. Crystal structure of the complex of rat neonatal Fc receptor with Fc. Nature 372, 379–383.

Cui, D., Zhang, L., Li, J., Zhao, Y., Hu, X., Dai, Y., Zhang, R., Li, N., 2014. Bovine FcRn-mediated human immunoglobulin G transfer across the milk-blood barrier in transgenic mice. PLoS One 9, e115972. http://dx.doi.org/10.1371/journal.pone.0115972.

Deisenhofer, J., 1981. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochemistry 20, 2361–2370.

Deissler, H., Deissler, H., Lang, G.K., Lang, G.E., 2005. Generation and characterization of iBREC: novel hTERT-immortalized bovine retinal endothelial cells. Int. J. Mol. Med. 15, 65–70. http://dx.doi.org/10.3892/ijmm.16.1.65.

Deissler, H., Deissler, H., Lang, S., Lang, G.E., 2008. VEGF-induced effects on proliferation, migration and tight junctions are restored by ranibizumab (Lucentis®) in microvascular retinal endothelial cells. Br. J. Ophthalmol. 92, 839–843. http://dx.doi.org/10.1136/bjo.2007.135640.

Deissler, H.L., Deissler, H., Lang, G.E., 2011. Inhibition of VEGF is sufficient to completely restore barrier malfunction induced by growth factors in microvascular retinal endothelial cells. Br. J. Ophthalmol. 95, 1151–1156. http://dx.doi.org/10.1136/bjo.2010.192229.

Deissler, H.L., Deissler, H., Lang, G.E., 2012. Actions of bevacizumab and ranibizumab on microvascular retinal endothelial cells: similarities and differences. Br. J. Ophthalmol. 96, 1023–1028. http://dx.doi.org/10.1136/bjophthalmol-2012-301677.

Deissler, H.L., Lang, G.K., Lang, G.E., 2013. VEGF but not PlGF disturbs the barrier of retinal endothelial cells. Exp. Eye Res. 115, 162–171. http://dx.doi.org/10.1016/j.exer.2013.07.018.

Deissler, H.L., Lang, G.K., Lang, G.E., 2014. Capacity of aflibercept to counteract VEGF-stimulated abnormal behavior of retinal microvascular endothelial cells. Exp. Eye Res. 122, 20–31. http://dx.doi.org/10.1016/j.exer.2014.02.024.

Deissler, H.L., Lang, G.K., Lang, G.E., 2016. Internalization of bevacizumab by retinal endothelial cells and its intracellular fate: evidence for an involvement of the neonatal Fc receptor. Exp. Eye Res. 143, 49–59. http://dx.doi.org/10.1016/j.exer.2015.10.007.

Dithmer, M., Hattermann, K., Pomarius, P., Aboul Naga, S.H., Meyer, T., Mentlein, R., Roider, J., Klettner, A., 2016. The role of Fc-receptors in the uptake and transport of therapeutic antibodies in the retinal pigment epithelium. Exp. Eye Res. 145, 187–205. http://dx.doi.org/10.1016/j.exer.2015.12.013.

Do, D.V., Nguyen, Q.D., Boyer, D., Schmidt-Erfurth, U., Brown, D.M., Vitti, R., Berliner, A.J., Gao, B., Zeitz, O., Ruckert, R., Schmelter, T., Sandbrink, R., Heier, J.S., DA VINCI Study Group, 2012. One-year outcomes of the DA VINCI Study of VEGF Trap-Eye in eyes with diabetic macular edema. Ophthalmology 119, 1658–1665. http://dx.doi.org/10.1016/j.ophtha.2012.02.010.

Ferrara, N., Damico, L., Shams, N., Lowman, H., Kim, R., 2006. Development of

ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. Retina 26, 859–870. http://dx.doi.org/10.1097/01.iae.0000242842.14624.e7.

Goebl, N.A., Babbey, C.M., Datta-Mannan, A., Witcher, D.R., Wroblewski, V.J., Dunn, K.W., 2008. Neonatal Fc receptor mediates internalization of Fc in transfected human endothelial cells. Mol. Biol. Cell. 19, 5490–5505. http://dx.doi.org/10.1091/mbc.E07-02-0101.

Heiduschka, P., Fietz, H., Hofmeister, S., Schultheiss, S., Mack, A.F., Peters, S., Ziemssen, F., Niggemann, B., Julien, S., Bartz-Schmidt, K.U., Schraermeyer, U., Tübingen Bevacizumab Study Group, 2007. Penetration of bevacizumab through the retina after intravitreal injection in the monkey. Invest. Ophthalmol. Vis. Sci. 48, 2814–2823.

Hofman, P., Blaauwgeers, H.G., Tolentino, M.J., Adamis, A.P., Nunes Cardozo, B.J., Vrensen, G.F., Schlingemann, R.O., 2000. VEGF-A induced hyperpermeability of blood-retinal barrier endothelium in vivo is predominantly associated with pinocytotic vesicular transport and not with formation of fenestrations. Curr. Eye Res. 21, 637–645.

Holash, J., Davis, S., Papadopoulos, N., Croll, S.D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E., Ioffe, E., Huang, T., Radziejewski, C., Bailey, K., Fandl, J.P., Daly, T., Wiegand, S.J., Yancopoulos, G.D., Rudge, J.S., 2002. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc. Natl. Acad. Sci. U. S. A. 99, 11393–11398. http://dx.doi.org/10.1073/pnas.172398299.

Jerdeva, G.V., Tesar, D.B., Huey-Tubman, K.E., Ladinsky, M.S., Fraser, S.E., Bjorkman, P.J., 2010. Comparison of FcRn- and pIgR-mediated transport in MDCK cells by fluorescence confocal microscopy. Traffic 11, 1205–1220. http://dx.doi.org/10.1111/j.1600-0854.2010.01083.x.

Julien, S., Biesemeier, A., Taubitz, T., Schraermeyer, U., 2014. Different effects of intravitreally injected ranibizumab and aflibercept on retinal and choroidal tissues of monkey eyes. Br. J. Ophthalmol. 98, 813–825. http://dx.doi.org/10.1136/bjophthalmol-2013-304019.

Junghans, R.P., Anderson, C.L., 1996. The protection receptor for IgG catabolism is the β_2-microglobulin-containing neonatal intestinal transport receptor. Proc. Natl. Acad. Sci. U. S. A. 93, 5512–5516.

Kacskovics, I., Wu, Z., Simister, N.E., Frenyó, L.V., Hammarström, L., 2000. Cloning and characterization of the bovine MHC class I-like Fc receptor. J. Immunol. 164, 1889–1897.

Kacskovics, I., Kis, Z., Mayer, B., West Jr., A.P., Tiangco, N.E., Tilahun, M., Cervenak, L., Bjorkman, P.J., Goldsby, R.A., Szenci, O., Hammarström, L., 2006. FcRn mediates elongated serum half-life of human IgG in cattle. Int. Immunol. 18, 525–536. http://dx.doi.org/10.1093/intimm/dxh393.

Kim, H., Fariss, R.N., Zhang, C., Robinson, S.B., Thill, M., Csaky, K.G., 2008. Mapping of the neonatal Fc receptor in the rodent eye. Invest. Ophthalmol. Vis. Sci. 49, 2025–2029. http://dx.doi.org/10.1167/iovs.07-0871.

Kim, H., Robinson, S.B., Csaky, K.G., 2009. FcRn receptor-mediated pharmacokinetics of therapeutic IgG in the eye. Mol. Vis. 15, 2803–2812.

Lang, G.E., Berta, A., Eldem, B.M., Simader, C., Sharp, D., Holz, F.G., Sutter, F., Gerstner, O., Mitchell, P., RESTORE Extension Study Group, 2013. Two-year safety and efficacy of ranibizumab 0.5 mg in diabetic macular edema: interim analysis of the RESTORE extension study. Ophthalmology 120, 2004–2012. http://dx.doi.org/10.1016/j.ophtha.2013.02.019.

McCarthy, K.M., Yoong, Y., Simister, N.E., 2000. Bidirectional transcytosis of IgG by the rat neonatal Fc receptor expressed in a rat kidney cell line: a system to study protein transport across epithelia. J. Cell Sci. 113, 1277–1285.

Ober, R.J., Radu, C.G., Ghetie, V., Ward, E.S., 2001. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. Int. Immunol. 13, 1551–1559. http://dx.doi.org/10.1093/intimm/13.12.1551.

Ober, R.J., Martinez, C., Lai, X., Zhou, J., Ward, E.S., 2004. Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. Proc. Natl. Acad. Sci. U. S. A. 101, 11076–11081. http://dx.doi.org/10.1073/pnas.0402970101.

Presta, L.G., Chen, H., O'Connor, S.J., Chisholm, V., Meng, Y.G., Krummen, L., Winkler, M., Ferrara, N., 1997. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 57, 4593–4599.

Powner, M.B., McKenzie, J.A.G., Christianson, G.J., Derry, C., Roopenian, D.C., Fruttiger, M., 2014. Expression of neonatal Fc receptor in the eye. Invest. Ophthalmol. Vis. Sci. 55, 1607–1615. http://dx.doi.org/10.1167/iovs.13-12574.

Raghavan, M., Chen, M.Y., Gastinel, L.N., Bjorkman, P.J., 1994. Investigation of the interaction between the class I MHC-related Fc receptor and its immunoglobulin G ligand. Immunity 1, 303–315. http://dx.doi.org/10.1016/1074-7613(94)90082-5.

Sand, K.M., Bern, M., Nilsen, J., Noordzij, H.T., Sandlie, I., Andersen, J.T., 2015. Unraveling the interaction between FcRn and albumin: opportunities for design of albumin-based therapeutics. Front. Immunol. 5, 682. http://dx.doi.org/10.3389/fimmu.2014.00682.

Sauer-Eriksson, A.E., Kleywegt, G.J., Uhlen, M., Jones, T.A., 1995. Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. Structure 3, 265–278.

Schraermeyer, U., Julien, S., 2012. Formation of immune complexes and thrombotic microangiopathy after intravitreal injection of bevacizumab in the primate eye. Graefes Arch. Clin. Exp. Ophthalmol. 250, 1303–1313. http://dx.doi.org/10.1007/s00417-012-2055-z.

Stewart, E.A., Samaranayake, G.J., Browning, A.C., Hopkinson, A., Amoaku, W.M., 2011. Comparison of choroidal and retinal endothelial cells: characteristics and response to VEGF-isoforms and anti-VEGF treatment. Exp. Eye Res. 93, 761–766. http://dx.doi.org/10.1016/j.exer.2011.09.010.

Sun, M., Fu, H., Cheng, H., Cao, Q., Zhao, Y., Mou, X., Zhang, X., Liu, X., Ke, Y., 2012. A dynamic real-time method for monitoring epithelial barrier function in vitro. Anal. Biochem. 425, 96–103. http://dx.doi.org/10.1016/j.ab.2012.03.010.

Ward, E.S., Zhou, J., Ghetie, V., Ober, R.J., 2003. Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans. Int. Immunol. 15, 187–195.

Ward, E.S., Martinez, C., Vaccaro, C., Zhou, J., Tang, Q., Ober, R.J., 2005. From sorting endosomes to exocytosis: association of Rab4 and Rab11 GTPases with the Fc receptor, FcRn, during recycling. Mol. Biol. Cell. 16, 2028–2038. http://dx.doi.org/10.1091/mbc.E04-08-0735.

Wines, B.D., Powell, M.S., Parren, P.W., Barnes, N., Hogarth, P.M., 2000. The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors Fc gamma RI and Fc gamma RIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. J. Immunol. 164, 5313–5318. http://dx.doi.org/10.4049/jimmunol.164.10.5313.

Ziemssen, F., Sobolewska, B., Deissler, H.L., Deissler, H., 2016. Safety of monoclonal antibodies and related therapeutic proteins for the treatment of neovascular macular degeneration: addressing outstanding issues. Exp. Opion. Drug Saf. 15, 75–87. http://dx.doi.org/10.1517/14740338.2016.1121232.