The neonatal Fc receptor does not modulate hepatitis C virus neutralization

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The neonatal Fc receptor (FcRn) is the only receptor known to be able to transport IgG across cell barriers and may therefore modulate virus infection. FcRn is expressed efficiently in hepatocytes. We therefore investigated the possible involvement of an FcRn-dependent mechanism in hepatitis C virus (HCV) neutralization. Our study, in both HCV pseudoparticles and HCV in cell-culture models, showed that FcRn was not involved in the intracellular neutralization of HCV, in contrast to the situation observed for influenza A virus.

The human neonatal Fc receptor (FcRn) is the only receptor known to be able to transport IgG across cell barriers (Roopenian & Akilesh, 2007). During the perinatal period, it provides the neonate with humoral immunity, by transporting maternal IgG across the placenta. Throughout life, it mediates transcytosis, thereby ensuring the extravascular biodistribution of IgG. In addition to this transcytotic function, FcRn plays a critical role in serum IgG homeostasis, through the recycling of IgG away from a catabolic pathway in the vascular endothelium, thereby extending the lifespan of IgG in the bloodstream. FcRn binds IgG in acidic conditions and releases IgG in neutral or basic conditions (Raghavan et al., 1995). In most cell types, FcRn resides principally in early acidic endosomal vesicles, in which it binds to IgG entering the cell by endocytosis and then recycles IgG back to the plasma membrane, where the neutral pH of the extracellular compartment causes the IgG to be released from the FcRn (Tesar et al., 2006).

It has recently been demonstrated that influenza A virus (IAV) neutralization is modulated by the FcRn-dependent transport of antibodies to the endosomal compartment (Bai et al., 2011). The application of a pH-dependent mAb (clone Y8) to the basolateral surface of Madin–Darby canine kidney (MDCK) cells expressing rat FcRn significantly decreased virus replication rates following apical exposure of the cell monolayer to influenza virus. More importantly, prophylactic administration of the Y8 mAb before viral challenge resulted in significantly lower pulmonary virus titres in WT mice but not in FcRn-knockout (FcRn-KO) mice. It has been suggested that the Y8 mAb neutralizes the virus intracellularly, as it can block fusion and egress from endosomes, resulting in the transport of virions to the lysosome for degradation. It has been suggested that other IgG antibodies with a broad spectrum of action or directed against the viral envelope regions containing the fusion domain might work in a similar manner, or even more effectively, through FcRn-dependent intracellular neutralization mechanisms.

FcRn is produced in hepatocytes (Schilling et al., 2003), and the most efficient neutralizing mAbs against hepatitis C virus (HCV) have been shown to inhibit infection at a post-attachment step (Sabo et al., 2011). We therefore investigated the possible involvement of an FcRn-dependent mechanism in HCV neutralization. Studies of patients with acute HCV infection have suggested that the production of large amounts of neutralizing antibodies contributes to control of the virus during the acute phase of HCV infection and facilitates viral elimination by the cellular immune response (Lavillette et al., 2005; Pestka et al., 2007). An understanding of the mechanisms leading to HCV neutralization by antibodies might therefore be important for the design of vaccine candidates or therapeutic antibodies capable of eliciting a protective humoral response.

To this end, we established a model of FcRn-depleted hepatocytes derived from the HuH7.5 cell line. This clone of the HuH7 hepatoma cell line supports a high-level HCV RNA replication and is widely used to study HCV entry and infection. These cells are known to be poorly or not polarized, but evidence for FcRn functionality has been demonstrated previously in different non-polarized cellular types (Zhu et al., 2001; Akilesh et al., 2007). We then used a specific hairpin (sh)RNA targeting FCGRT encoding the FcRn α-chain to screen transfected HuH7.5 cells. We identified one subclone displaying a stable knockdown of FcRn production. At least 90% inhibition of FcRn expression...
was observed in this clone in Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) experiments (Fig. 1a) and on Western blot analysis (Fig. 1b). This FcRn-depleted Huh7.5 clone was compared with Huh7.5 cells expressing a control shRNA in HCV neutralization assays in vitro. We first investigated the impact of FcRn depletion on HCV neutralization with the HCV pseudoparticle model (HCVpp), which constitutes a relevant model for studies of the early phases of HCV entry (Bartosch et al., 2003). The activity of an AR3A mAb, targeting a discontinuous epitope in the envelope glycoprotein E2 and known to inhibit HCV at both pre- and post-attachment stages (Giang et al., 2012), was assessed, together with that of a serum previously shown to neutralize HCV (from a patient chronically infected with HCV genotype 1b; data not shown). The interaction between AR3A mAb and the FcRn at an acidic pH was checked using flow cytometry as shown in the Fig. S1 (available in the online Supplementary Material). Activity was assessed against viral particles pseudotyped with HCV envelope glycoproteins derived from genotypes 1a (H77) and 2a (JFH-1). No difference between the FcRn-depleted and control cells was observed. The AR3A mAb and the HCV-1b-infected serum decreased HCVpp entry by 90 and 60%, respectively, in both cell types (Fig. 2). We explored the potential role of FcRn in HCV neutralization further, by performing additional assays in the HCV cell-culture (HCVcc) (JFH-1) model, mimicking all the steps of the HCV infectious cycle. Previous studies have reported that the most potent HCV neutralizing antibodies inhibit infection at a post-attachment step (Sabo et al., 2011), so the HCVcc model may be a more relevant system for addressing the question posed here, as it reconstitutes the various stages of HCV entry, including envelope fusion and virion uncoating (Lohmann & Bartenschlager, 2014). We therefore used the AR3A mAb and two serum samples from chronically infected patients (with genotypes 1b and 4, respectively) to determine whether the FcRn exerted neutralizing effects preferentially during the late phases of HCVcc entry. In tests with HCVcc used at an m.o.i. of 0.01, no difference was observed between the FcRn-depleted and control cells, with the AR3A mAb and human serum samples decreasing HCVcc infection by 100 and 70–80%, respectively, in both cell types (Fig. 3a). To avoid a bias induced by a possible antibody-mediated neutralization at a very early entry step(s), similar experiments were conducted after virions were bound to the cells at 4°C to check the neutralization of the infection at a post-attachment step (Fig. 3b). The results displayed the same tendency as for the experiments conducted pre-attachment. No difference in terms of neutralizing activity could be observed between the FcRn-depleted and control cells (Fig. 3).

In the IAV model, the critical step at which the Y8 mAb interferes with the virus life cycle during its trafficking by FcRn is unknown (Bai et al., 2011). It is not clear whether FcRn organizes the antibodies in the endosome into an

![Graph](a) FcRn mRNA relative to control shRNA

![Graph](b) Western blot analysis of FcRn expression in control and FcRn-depleted cells

**Fig. 1.** Selection of a stable Huh7.5 cell clone silenced for FcRn. Huh7.5 cells were transfected with shRNA targeting the FCGRT coding sequence or a control shRNA (SA Biosciences), according to the manufacturer’s recommendations. Cells were then selected with puromycin (1.5 µg ml⁻¹) in a conditioned medium containing 15% fetal bovine serum (FBS). Cellular clones were isolated with cloning cylinders and amplified by conventional culture methods. (a) The clone with the highest FcRn extinction was selected after broad screening by RT-qPCR. Amounts of FCGRT mRNA in either cell pool were quantified by RT-qPCR and normalized to β-actin mRNA amounts. The histogram represents the mean values from five independent experiments ± SD. FcRn expression levels in the selected clone were 90% lower than those in the clone expressing the control shRNA. (b) FcRn depletion in this clone was analysed by Western blotting with a polyclonal anti-FcRn antibody (Novus Biological) at a dilution of 1:250 in 0.5% Tween 20 and 5% non-fat milk in PBS and an anti-rabbit secondary antibody coupled to HRP, used as a dilution of 1:10 000, for detection. We loaded 50 µg cell lysate from Huh7.5 cells expressing the control shRNA or the shRNA targeting FcRn in each lane. We used an anti-α-tubulin mAb (clone DM1A; Abcam) to control for equal protein loading.
orientation facilitating interactions with the viral envelope or whether it simply increases the endosomal concentration of the antibodies to levels more effective at blocking virus fusion. However, FcRn is involved in intracellular virus neutralization, as demonstrated by the findings for cell and KO mouse models (Bai et al., 2011). Unfortunately, it is not possible to use an FcRn-KO mouse model for investigations of the potential FcRn-dependent effect on

**Fig. 2.** FcRn depletion in Huh7.5 cells has no influence on HCVpp entry. HCVpp harbouring envelope glycoproteins derived from genotype 1a (H77) or 2a (JFH-1) were generated (Bartosch et al., 2003) to assess the influence of FcRn in the process of HCV neutralization. Open bars represent the impact of neutralizing antibodies in cells transfected with control shRNA, whereas filled bars represent neutralization in the Huh7.5 clone depleted of FcRn. Pseudoparticles harbouring amphotropic envelope glycoprotein from amphotropic murine leukemia virus were also generated as controls. Pseudotyped virus were subjected to pre-incubation for 30 min at 37 °C with the anti-E2 mAb AR3A (5 μg ml⁻¹) (Law et al., 2008), serum from an infected patient (genotype 1b; 1 : 200) or heparin (500 μg ml⁻¹) under an atmosphere containing 5% CO₂. An isotype of the AR3A antibody (5 μg ml⁻¹) and a pool of serum samples from uninfected patients (1 : 200) were used as negative controls. As a positive control, cells were subjected to pre-incubation with an anti-hCD81 mAb (5 μg ml⁻¹; BD Pharmingen) to inhibit HCVpp entry. They were then incubated with pseudotyped virus and antibodies in 50 μl complete medium (with 3% FCS). After 6 h, inoculum was removed and fresh medium was added. After 72 h, entry levels were determined by measuring the luciferase activity of cell lysates with a Luciferase Assay System (Promega) and a Centro LB 960 luminometer (Berthold Technologies). Results are expressed as mean percentages of entry relative to the mock condition ± sd. Each graph represents three independent experiments.
FcRn does not modulate HCV neutralization, as these mice are not susceptible to HCV infection. Alternative approaches based on the genetically humanized mouse model for HCV infection could be established (Dorner et al., 2011), but this would require a back-cross of this mouse model with FcRn-KO mice. However, our results for a clone of the HCV-susceptible Huh7.5 cell line displaying strong FcRn depletion demonstrated that FcRn plays no role in HCV neutralization in vitro.

In conclusion, despite expression of the FcRn in hepatocytes and the occurrence of FcRn-dependent neutralization mechanisms in other viral models (Bai et al., 2011), we have shown here that FcRn does not modulate HCV neutralization in vitro. These results indicate the relative impact of intracellular FcRn-dependent neutralization in different viral models, and may be useful for the future development of antibody-based treatments for HCV infection (Sautto et al., 2012).

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Fig. 3. FcRn depletion in Huh7.5 cells does not affect HCVcc (JFH-1) infection. Open bars represent the impact of neutralizing antibodies in cells transfected with control shRNA, whereas filled bars represent neutralization in the Huh7.5 cell clone depleted of FcRn. (a) Pre-attachment protocol: HCVcc virions were subjected to pre-incubation for 30 min at 37 °C. Closed bars represent neutralization in the Huh7.5 cell clone depleted of antibodies in cells transfected with control shRNA, whereas filled bars represent neutralization in cells transfected with FcRn shRNA. (b) Post-attachment protocol: pre-chilled cells were incubated with viruses for 1 h at 4 °C. After 4 h, the medium was replaced and the cells were grown for 72 h before HCV core staining with C7.50 mAb. For both protocols, fluorescence was visualized with an anti-mouse secondary antibody coupled to Alexa Fluor 488. Results are expressed as percentages relative to the mock condition ± SD. The data shown are representative of two different experiments, each conducted in triplicate.

References


