



# RSV neutralization by palivizumab, but not by monoclonal antibodies targeting other epitopes, is augmented by Fc gamma receptors



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## ABSTRACT

Palivizumab efficiently blocks respiratory syncytial virus (RSV) infection *in vitro*. However, virus neutralization assays generally omit Fc region-mediated effects. We investigated the neutralization activity of RSV-specific monoclonal antibodies on cells with Fc receptors. Subneutralizing concentrations of antibodies resulted in antibody-dependent enhancement of RSV infection in monocytic cells. Contrary to antibodies targeting other epitopes, the neutralization by palivizumab was augmented in cells with Fc receptors. This unrecognized characteristic of palivizumab may be relevant for its performance *in vivo*.

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## 1. Introduction

Respiratory syncytial virus (RSV) is the major cause of severe respiratory infections during infancy (Nair et al., 2010). The only approved antiviral intervention is palivizumab, a monoclonal, humanized, immunoglobulin G1 (IgG1) directed against the fusion glycoprotein (F) (Groothuis et al., 1993). Prophylactic administration of palivizumab to neonates at high-risk for severe RSV infection reduces hospitalization rates by 50% (Group, 1998). Currently, novel monoclonal antibodies (mAb) and more than 50 RSV vaccine candidates are in development (Modjarrad et al., 2015).

Serum antibodies against RSV correlate poorly with probability of protection in challenge studies and severe RSV infection in neonates can occur despite the presence of maternal antibodies in serum (Habibi et al., 2015; Eick et al., 2008). Human sera and RSV-specific murine monoclonal antibodies can also enhance RSV infection (Gimenez et al., 1989; Krilov et al., 1989; Gimenez et al., 1996) by interacting with Fc gamma receptors (FCGR). RSV-antibody immune complexes also modulate the response of immune cells (Gomez et al., 2016; Jans et al., 2014). Thus, antibodies may play an ambivalent role during RSV infection, like in dengue virus infection (van der Schaar et al., 2009).

Novel therapeutic antiviral mAbs are primarily selected for

better virus neutralization as compared to palivizumab. This is assessed by virus neutralization assays *in vitro*. Antibody-mediated effector functions such as antibody-dependent cell cytotoxicity, immunomodulation, and complement activation act beyond neutralization, but are generally omitted from analysis (Pelegrin et al., 2015). In the present study we analyzed to what extent the neutralizing capacity of humanized RSV-specific monoclonal antibodies in general, and of palivizumab in particular, is affected in cells with FCGRs. The presence of FCGR generally weakened RSV neutralization. In contrast, palivizumab showed augmented neutralization. Additionally, all tested humanized mAbs demonstrated antibody-dependent enhancement (ADE) of RSV infection in THP-1 cells, even at very low concentrations.

## 2. RSV infection of THP-1 cells is enhanced by mAbs

The interaction of endogenous FCGRs on murine P388D1 cells with complexes of RSV and murine mAbs facilitates ADE (Krilov et al., 1989). We asked whether humanized mAbs, in particular the therapeutic mAb palivizumab or the next generation, high-affinity mAb D25, cause ADE. To assess the dilution range where mAbs cause ADE, a recombinant RSV expressing green fluorescent protein (E7-rRSV\_X (Widjojoatmodjo et al., 2010)), was pre-incubated with serial diluted mAbs (overview in Table 1), a human IgG1 isotype control (Mab901, Eureka therapeutics), or purified, human, serum-derived immunoglobulin (IVIG, KIOVIG, Baxter) for 1 h at 37 °C. THP-1 cells or Vero-E6 cells were inoculated at a

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**Table 1**  
Characteristics of monoclonal antibodies.

Antibody	IVIg <sup>b</sup>	D25 <sup>c</sup>	Palivizumab <sup>b,c</sup>	47F <sup>d</sup>	101F <sup>e</sup>	131-2A <sup>b</sup>	131-2G <sup>b</sup>	3D3 <sup>f</sup>	Isotype <sup>b</sup>
Species	Human	Human	Human	Murine	Human	Murine	Murine	Human	Human
Fc subclass	diverse	IgG1	IgG1	IgG1	IgG1	IgG2a	IgG1	IgG1	IgG1
Target RSV protein	Total RSV	F, prefusion	F, pre/postfusion II	F, pre <sup>a</sup> /postfusion II	F, pre/postfusion IV	F, postfusion I	G <sup>g</sup>	G <sup>g</sup>	Unspecified
Antigenic site in F	Polyclonal	Ø	II	II	IV	I	n.a.	n.a.	n.a.
IC50 in Vero cells (ng/ml) (95% CI)	11,857 (11,010–12,761)	2.99 (2.79–3.18)	478 (443–519)	1068 (975–1176)	37.8 (33.4–42.5)	n.a.	n.a.	n.a.	n.a.
IC50 in THP-1 cells (ng/ml) (95% CI)	56,717 (41,147–80,992)	125 (8.94–17.26)	179 (139–267)	809 (501–1351)	150 (105–223)	n.a.	n.a.	n.a.	n.a.

CI = 95% confidence interval, n.a. Not applicable.

<sup>a</sup> Predicted.

<sup>b</sup> Commercial.

<sup>c</sup> Rigter et al., 2013.

<sup>d</sup> Garcia-Barreno et al., 1989.

<sup>e</sup> Produced similarly as described previously for D25 and Palivizumab (Rigter et al., 2013) based on sequences in patent US 20060159695 A1.

<sup>f</sup> Collarini et al., 2009.

<sup>g</sup> Binding to central conserved region in RSV-G.

multiplicity of infection corresponding to 5% infection or 300 plaques per well, respectively. THP-1 cells display Fc gamma receptor 1a (FCGR1A) and Fc gamma receptor 2a (FCGR2A) on the plasma-membrane whereas Vero cells lack FCGRs (Fleit and Kobasiuk, 1991). THP-1 cells were infected by spinoculation at 700×g for 1 h at room temperature, after which the inoculum was removed, cells were washed and fresh medium was added. After overnight incubation, RSV infection was quantified by assessing GFP-positive cells using flow cytometry (FACS Canto II, BD). Titration curves were defined normalized to samples without antibody (100% infection) (Fig. 1A). Inhibition of RSV infection of Vero cells was determined by plaque reduction neutralization assays as described earlier (van Remmerden et al., 2012). In short, RSV-antibody complexes were spinoculated, followed by 1 h incubation at 37 °C. Next, the inoculum was replenished with medium containing 1% methylcellulose. 48 h post infection, GFP-positive foci were counted. Similar to IVIG, all RSV-specific mAbs increased infection of THP-1 cells to >200% at sub-neutralizing concentrations (Fig. 1C–K). Treatment of RSV with various antibodies that do not recognize RSV antigens (data not shown) as well as isotype control antibody (Fig. 1D) resulted in similar infection at all tested dilutions. RSV-neutralizing mAbs blocked infection at high concentrations and enhanced infection at low concentrations (Fig. 1E–H). For D25, the maximum infection was observed at a dilution corresponding to a concentration as low as 1 ng/ml. Therefore, our experiments proved to be an extremely sensitive tool to detect antibody-FCGR interactions. Non-neutralizing antibodies 131-2A, 131-2G, and 3D3 increased infection up to 350% at all tested concentrations until diluted out (Fig. 1I–K). ADE occurred independent of neutralization titer, epitope specificity, species, or any other known attribute of the antibody (Table 1). To exclude interference by the Fc region subclass, all antibodies with human Fc regions were chosen to be of subclass IgG1 that efficiently interacts with FCGR1A and FCGR2a. Murine IgG2a, present in mAb 131-2A, interacts strongly with human monocytic Fc receptors, whereas murine IgG1 Fc regions of 47F and 131-2G interact less (Lubeck et al., 1985). However, all murine Abs sufficiently bound human FCGR to cause ADE in THP-1 cells.

### 3. RSV neutralization by palivizumab is augmented on THP-1 cells

Next, we asked how virus neutralization is affected by FCGR interactions. We expected that ADE mitigates neutralization by increasing the 50% inhibitory concentration (IC50). IC50s of IVIG

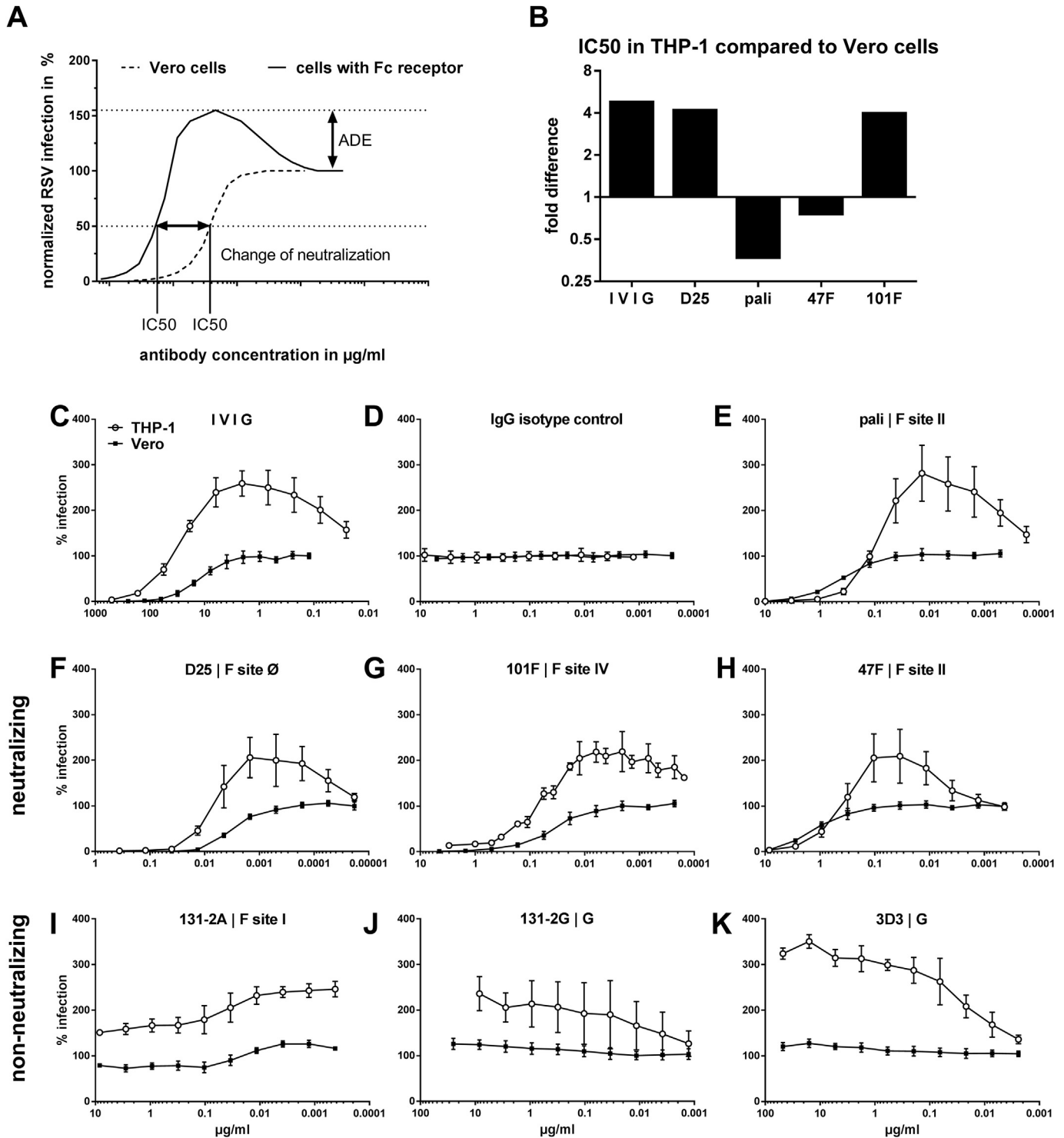
and the different mAbs were calculated by non-linear regression (Graphpad Prism 7). Intriguingly, the IC50 of palivizumab (Medimmune) was lower in THP-1 cells than in Vero cells (Fig. 1B). Identical results were obtained with in-house produced palivizumab (Rigter et al., 2013). We could find a similar, but weaker augmentation of neutralization for 47F (Fig. 1H) that also binds to antigenic site II in RSV F (Garcia-Barreno et al., 1989). In contrast, other mAbs and IVIG had a higher IC50 in THP-1 cells.

### 4. RSV neutralization by palivizumab is augmented on Vero cells with Fc gamma receptors

To gain more insight into the augmented neutralizing capacity of palivizumab and to exclude cell line-specific differences, we developed Vero cells with either of the two FCGR present on THP-1 cells. FCGR1A has a >10-fold higher affinity for IgG1 and both FCGRs contain an intracellular immunoreceptor tyrosine-based activation motif. Vero cells expressing FCGR2A (designated Vero2a) or co-expressing the murine common gamma chain (FCER1G) and FCGR1A (designated Vero1a) were generated by retroviral transduction (Bruhns and Jonsson, 2015). Stable, polyclonal cell lines were selected by antibiotics and surface display of FCGRs was confirmed by flow cytometry (data not shown). Vero1a, Vero2a, and the parental Vero cells produced similar amounts of plaques after infection with RSV (data not shown). The presence of sub-neutralizing mAb concentrations enhanced RSV infection (Fig. 2A–I) for IVIG, D25, 101F, 131-2A (MAB8599, Millipore), and 131-2G (MAB858-2, Millipore) on Vero1a and IVIG, 131-2A, and D25 (University Utrecht (Rigter et al., 2013)) on Vero2a. This ADE was not as pronounced as on THP-1 cells. Furthermore, IC50 values of neutralizing antibodies were altered in presence of FCGRs as demonstrated by shifting of the titration curves when using Vero1a and Vero2a cells instead of Vero cells (Fig. 2J). Palivizumab had a 14.2-fold and 1.7-fold lower IC50 using Vero1a and Vero2a, respectively (Fig. 2K). Slightly better neutralization was also observed for 47F using Vero2a (2.6-fold) and D25 in Vero1a (1.3-fold). All other RSV-specific antibodies and IVIG required a higher concentration for neutralization in Vero cells with FCGRs. Shifts of IC50s in FCGR-bearing Vero cells were statistically significant in all cases (Fig. 2J).

### 5. Implications of antibody-FCGR interaction

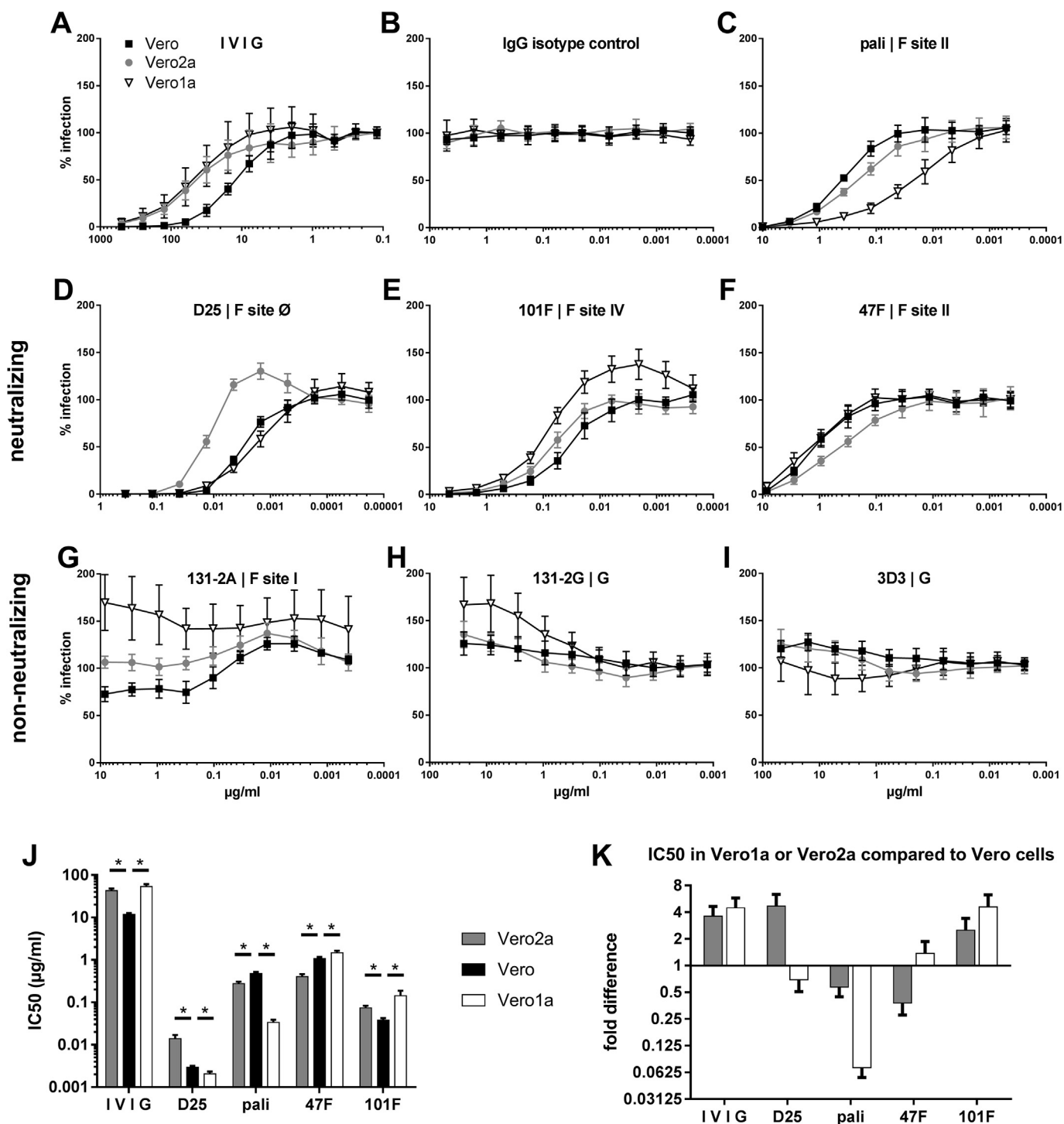
We studied the impact of interactions between FCGRs and humanized mAb on RSV infection by RSV neutralization assays. Higher



**Fig. 1.** Neutralization activity of palivizumab was augmented on THP-1 cells compared to Vero cells. Infection of Fc gamma receptor carrying, monocytic THP-1 cells by RSV in the presence of monoclonal antibodies. (A) Schematic of dose-dependent RSV neutralization normalized to infection in the absence of antibodies. Neutralizing antibodies block RSV infection at high concentrations. Antibody-dependent enhancement (ADE) of infection can occur at low concentrations or by non-neutralizing antibodies. Furthermore, the half maximal inhibitory concentration (IC50) is changed when using cells with Fc gamma receptors, illustrated by shifting titration curves. (B) To compare neutralization, IC50 derived from THP-1 cells was divided by IC50 on Vero cells. Titration curves of human plasma immunoglobulin (IVIg, panel C) and individual antibodies are shown (panels D–K). The RSV epitope that is recognized by mAbs is indicated. Graphs report means and standard deviations based on  $\geq 3$  independent experiments measured in duplicate or triplicate for THP-1 or Vero cells, respectively.

concentrations of most mAbs and IVIg were required to neutralize RSV when FCGRs are present in target cells. In contrast, the presence of FCGRs, especially high-affinity receptor FCGR1A, greatly

augmented the neutralization activity of palivizumab. This previously unrecognized characteristic of palivizumab should be taken into account when using palivizumab as a reference standard for



**Fig. 2. Neutralization activity of palivizumab is augmented on Vero cells in presence of Fc gamma receptors.** IC50 of human plasma immunoglobulin (IVIg, (A)) and monoclonal antibodies (B–I) were determined in Vero cells and Vero cells that stably express Fc gamma receptor 1a (Vero1a) or Vero cells with Fc gamma receptor 2a (Vero2a). The RSV epitope that is recognized by mAbs is indicated. Graphs report means and standard deviations based on  $\geq 3$  independent experiments measured in triplicate. (J) Mean IC50 (with 95% confidence interval) of  $\geq 4$  independent experiments were calculated. IC50 on Vero2a and Vero1a are significantly different from Vero cells using Dunnett's test ( $P < 0.01$ ). (K) To illustrate the change of IC50 in the presence of FCGRs, logIC50 in Vero1a or Vero2a were compared to that in Vero cells using a two-way ANOVA and plotted with 99% confidence interval after linear transformation.

novel antibodies and vaccine concepts (Kwakkenbos et al., 2010; Collarini et al., 2009).

This study elucidates some aspects, but cannot fully comprehend how IC50s are modulated by antibody-FCGR interactions. The F protein antigenic site II that is recognized by palivizumab and 47F

seems to support FCGR-augmented neutralization independent of the origin of the Fc region and the engaged FCGR. Nevertheless, direct comparison with other mAbs is challenging, since variable phenotypes may occur when different in Fc regions are present. How individual FCGRs operate could not be concluded from this

study due to mixed results for FCGR1A or FCGR2A. In general, the Fc-FCGR interactions depend on the immunoglobulin species, (sub-)class, and modifications of the N-linked glycan in the Fc region (Vidarsson et al., 2014).

Besides altering the IC50, FCGRs facilitated antibody-dependent enhancement of RSV infection (Krillov et al., 1989). For human sera and murine mAbs, ADE was dependent on the balance between neutralization and enhancement, whereas non-neutralizing mAbs generally enhanced infection (Gimenez et al., 1996). We report similar effects for humanized mAbs that are in clinical use or in development. ADE even occurs when the mAbs was designed for high potency, binding to prefusion F epitope Ø, and present at concentrations as low as 0.1 ng/ml (Fig. 2F). All tested RSV-neutralizing mAbs could enhance infection of THP-1 cells, hence we could not address whether ADE was determined of Fc region, affinity, and neutralization capacity.

Mechanism that causes ADE of RSV infection remains elusive. (Antibody-mediated) endocytosis may traffic RSV into a favorable endosomal compartment where proteases unleash the fusion machinery of RSV to enable viral release in to the cytoplasm (Krzyszaniak et al., 2013). ADE was generally more pronounced in Vero1a than Vero2a. This might originate either from the higher affinity with which FCGR1A binds IgG, relate to the increased trafficking of FCGR1A-bound immunocomplexes to the late endosomal/lysosomal compartment (Dai et al., 2009), and is consistent with the enhanced uptake of and infection by Dengue virus immune complexes via FCGR1A (Chawla et al., 2013). Augmented neutralization of RSV, however, is unique to palivizumab in our study. The generally weak ADE in FCGR-bearing Vero cells may occur due to cell type specific differences, incompatibility between simian Vero cells and human FCGRs, or the lack of synergy between FCGR1A and FCGR2A.

Antibody-Fc gamma receptor interactions result in altered neutralization and ADE *in vitro*. *In vivo*, FCGR-bearing immune cells are crucial for the antiviral response. Stimulation of FCGR-dependent immune responses may contribute to RSV-mediated pathogenesis (Ponnuraj et al., 2003; Gomez et al., 2016; Kruijsen et al., 2013). Like in dengue virus infection (van der Schaar et al., 2009), antibodies may thus play an ambivalent role during RSV infection. Hence, Fc receptor-mediated effects should be considered when evaluating novel antibodies and vaccines (Bournazos and Ravetch, 2015).

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