Identification and characterization of FcγRs in Göttingen minipigs – implications for preclinical assessment of therapeutic antibodies

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1 Abstract

Purpose – Antibodies of the human (hu) Immunoglobulin G (IgG) isotype are used as therapeutics for patients with cancer, rheumatoid arthritis, asthma, and other diseases. Often, these therapeutic huIgG antibodies mediate effects by binding to human Fc gamma receptors (FcγRs) expressed on various cells of the patient's immune system. Three classes of huFcγRs comprising a total of six receptors are known in humans, namely FcγRIa (CD64), FcγRIIa/b/c (CD32a/b/c), and FcγRIIIa/b (CD16a/b). FcγR-mediated effector functions range from desired depletion of tumor cells via antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis, to unwanted toxic effects by exaggerated cytokine release, thrombosis, and infusion reactions. These functions depend on the FcγR, the binding strength, and the involved immune cells. Prior to human use, the safety and efficacy of therapeutics have to be demonstrated in animal studies where human antibodies interact with the immune system of the selected species. The Göttingen minipig is highly suitable for such mandatory preclinical studies. However, the relevance of such studies for assessing the safety and efficacy of therapeutic antibodies is limited due to unknown characteristics of porcine (po)FcγRs. Therefore, this thesis aims to characterize the poFcγRs, focusing on the expression on immune cells of the minipig and the binding to huIgG.

Methods – To study the set of poFcγRs in minipigs, we performed a detailed genome analysis of the locus coding for most FcγRs by polymerase chain reaction (PCR) and manual assembly of existing sequences. We used single cell ribonucleic acid (RNA) sequencing to determine the transcription, and flow cytometry to show the expression of different poFcγRs on various cells within blood, lymph node, and spleen. Cloning and expression of all poFcγRs as soluble proteins enabled the binding assessment of monomeric, as well as immune complexed huIgG1 therapeutic antibodies to poFcγRs by surface plasmon resonance (SPR; Biacore). Furthermore, we investigated the binding of monomeric antibodies and immune complexes to FcγR-expressing cell lines and immune cells of the minipig by flow cytometry.

Results – We used genome analysis to identify the missing poFcγRIIa and to map the gene coding for the known poFcγRIIIa, which had not been annotated to date. The genomic organization of poFcγRs resembles that of most mammals except humans, who have two additional genes coding for huFcγRIIc and IIIb. In general, the distribution of FcγRs on immune cells and the binding properties to free- and immune-complexed huIgG1, both prerequisites for effector functions mediated by huIgG1, are similar in minipigs and humans. However, we observed several key differences which may affect the use of minipigs in preclinical studies with therapeutic huIgG1 antibodies. Firstly, the binding of huIgG1 to FcγRIIa, which is expressed on blood platelets, was stronger in minipigs (poFcγRIIa) compared to humans (huFcγRIIa). Despite this, the minipig could be a valuable model to study IgG-mediated platelet activation, aggregation, and thrombosis. Secondly, for the inhibitory poFcγRIIb, we observed stronger

binding versus huFcγRIIb. In humans, FcγRIIb regulates the immune response and is expressed on B cells, dendritic cells, and tissue monocytes. In contrast, we reported expression of poFcγRIIb on blood monocytes in minipigs. We suggest that anti-inflammatory effects with therapeutic huIgG1 antibodies could be stronger in minipigs than in humans due to the divergent expression and the stronger binding to the inhibitory poFcγRIIb. Lastly, we observed a lack of binding of huIgG1 to poFcγRIIIa. In humans, cytotoxic huIgG1 antibodies mediate ADCC via binding to huFcγRIIIa expressed on natural killer (NK) cells and on a subset of monocytes in the blood. The lacking binding of huIgG1 to poFcγRIIIa excludes NK-mediated ADCC and additionally restricts functions of monocytes, thus limiting studies with certain huIgG1 therapeutics. However, we reported binding of endogenous poIgG1 enabling effector functions in tumor vaccination or infection studies.

Conclusion – The results compiled in this thesis generally recommend the use of minipigs for the assessment of therapeutic huIgG1 antibodies. However, the limitations of this animal model regarding differential binding of huIgG1 to poFcγRs and their expression pattern on immune cells in comparison to the human have to be considered. Therefore, functional studies are recommended to further assess the translatability of FcγR-mediated effector functions with various therapeutic antibodies from the minipig to the human. Nevertheless, this work delivers a foundation for species selection and allows the interpretation of results from preclinical safety and efficacy studies with Göttingen minipigs.

2 Abbreviations

aa	amino acid
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCP	Antibody-Dependent Cellular Phagocytosis
C1q	Complement component 1q
CD	Cluster of Differentiation
CDC	Complement Dependent Cytotoxicity
CEA-TCB	Carcinoembryonic Antigen- T Cell Bispecific
CHO	Chinese Hamster Ovary cell line
CpG-ODN	CytosinePphosphate–Guanosine Oligodeoxynucleotides
ConA	Concanavalin A
Cyno	Cynomolgus monkey
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DPBS	Dulbecco's Phosphate-Buffered Saline
ELISA	Enzyme-Linked Immunosorbent Assay
Fab	Fragment, antigen binding
Fab-A-FH	Format of HuCAL antibodies composed of Fab, alkaline phosphatase, FLAG and His-tag
Fc	Fragment, crystallizable
FCGR	Fc *gamma* Receptor (gene)
FcR-γ chain	Fc Receptor common *gamma* chain
FcRL	Fc Receptor-Like
FcRn	neonatal Fc Receptor
FcαR	IgA Fc Receptor
FcγR	Fc *gamma* Receptor (IgG Fc Receptor)
FcεR	IgE Fc Receptor
FcμR	IgM Fc Receptor
FDA	US Food and Drug Administration
GPI	glycosylphosphatidylinositol
HEK293F	Human Embryonic Kidney 293F
HER2	Human Epidermal growth factor Receptor 2
hu	Prefix for human
HuCAL	Human Combinatorial Antibody Libraries
IC	Immune Complex
ICH	International Council for Harmonisation
Ig	Immunoglobulin
IgG	Immunoglobulin G
IVIg	Intravenous Immunoglobulin
IL	Interleukin
IM	Interaction Map
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITAMi	inhibitory Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
KD	equilibrium dissociation constant
NK cell	Natural Killer cell

LC-MS	Liquid Chromatography – Mass Spectrometry
LPS	Lipopolysaccharide
MALS	Multi-Angle Light Scattering
MBL2	Mannose-Binding-Lectin 2
MBP	Maltose-Binding Protein
MFI	Median Fluorescence Intensity
MMR	Macrophage Mannose Receptor
mRNA	messenger Ribonucleic Acid
NFAT	Nuclear Factor of Activated T cells
NHP	Non-Human Primate
NK cell	Natural Killer cell
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
pIgR	polymeric Ig Receptor
PK	Pharmacokinetics
po	Prefix for porcine (of swine, pig, or pigs)
PGLALA	Mutations in Fc silent IgG (Pro329G, Leu234Ala, Leu235Ala)
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RU	Response Units
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SPR	Surface Plasmon Resonance
SUMO	Small Ubiquitin-like Modifier
TGF-β	Transforming Growth Factor *beta*
TNF-α	tumor necrosis factor *alpha*
VEGF	Vascular Endothelial Growth Factor

3 Preface

This thesis addresses the suitability of the Göttingen minipig for preclinical safety and efficacy studies with human therapeutic antibodies focusing on Fc gamma receptor (FcγRs) functions.

A background on therapeutic antibodies, FcγRs, and minipigs in biomedical research is given in the introduction part. The subsequent section is separated in two main chapters with two published manuscripts as a central part, as well as unpublished experiments in subsections. Manuscript 1 describes the genomic organization and expression pattern of FcγRs in the minipig whereas Manuscript 2 addresses the interaction of human therapeutic antibodies to porcine FcγRs. The discussion, conclusion, and an outlook sections combine and interpret the information gained in both previous chapters.

The data presented here were compiled at F. Hoffmann - La Roche Ltd and the release of this thesis has been approved.

4 Introduction

4.1 Therapeutic antibodies and effector functions

4.1.1 Antibodies have become important therapeutics

Antibodies are glycoproteins secreted by B cells to specifically bind to a variety of molecules (Fig. 4.1). A regular antibody is composed of two Fab (Fragment, antigen binding) arms that bind to antigens and thus determine its specificity. On the other hand, the antibody Fc (Fragment, crystallizable) part is important for the binding to Fc receptors and for activation of the complement system (Fig. 4.1) [2]. As an important part of the immune system, antibodies have been selected during evolution for high specificity, affinity, and long half-life for long-lasting protection from various pathogens via Fc-mediated functions. All these properties are also desired for therapeutics.

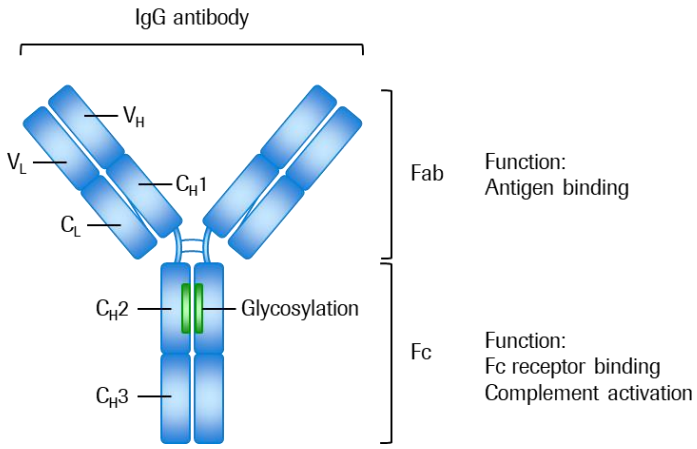

Fig. 4.1 Structure of Immunoglobulin G (IgG) antibodies. IgG antibodies consist of two heavy and two light chains linked by disulphide bonds (blue lines). The heavy chain comprises one variable (VH) and three constant domains (CH1, CH2, CH3) whereas the light chain only contains one variable (VL) and one constant (CL) domain. Together, the variable domains are responsible for antigen binding. Therefore, the fragment composed of VH, CH1, VL, and CL is named Fab (Fragment, antigen binding). The Fc (Fragment, crystallizable) part, interacting with Fc receptors, combines the CH2 and CH3 domains and is usually N-glycosylated (green box).

In 1986, the first therapeutic antibody was approved by the US (United States) Food and Drug Administration (FDA) [3]. However, significant drawbacks such as allergic reactions, anti-drug antibodies, and poor effector functions were observed with this monoclonal antibody based on mouse structures. Therefore, efforts have been made to produce fully human (hu) antibodies of the immunoglobulin G (IgG) isotype or derivatives thereof to overcome these limitations. As of the end of 2014, more than 45 mostly chimeric or human antibodies are on the market as specific treatments for an enormous number of patients suffering from cancer, rheumatoid arthritis, asthma, and other diseases [4]. With over 50 therapeutic antibodies in late-stage clinical studies and 10 novel approvals in the United States and the European Union in 2017, their development is still gaining importance [5]. The global market size for monoclonal antibodies is predicted to increase by 12.5% to USD 218.97 billion from 2017 to 2023 [6].

The mode of action of current therapeutic antibodies is diverse including inhibition, activation, cross-linking, target blocking, immune modulation, cargo delivery or depletion. Many of these effects rely on the involvement of the patient's immune system by interactions via antibody Fc receptors.

4.1.2 Fc receptors bind the Fc portion of antibodies

Fc receptors are a group of cell surface glycoproteins that bind to the Fc part of immunoglobulins. Most mammals have receptors for IgE (FcεR), IgA (FcαR), and IgM (FcμR). In addition, there are structurally unrelated receptors for immunoglobulins such as the neonatal Fc receptor (FcRn), Fc Receptor-Like (FcRL) proteins, polymeric Ig receptors (pIgR), and many more [7].

Fc gamma receptors (FcγR) are a family of receptors binding IgG, the most abundant Ig in the human body. The FcγR family consists of three functionally distinct classes based on their amino acid similarity: 1) The activating high affinity receptor FcγRI, 2) the low affinity FcγRII comprising of the activating FcγRIIa and the inhibitory FcγRIIb, and 3) the low affinity FcγRIII. Furthermore, FcγRs can be classified according to their affinity and activation properties (Fig. 4.2). For the major human huFcγRs, orthologue receptors with the same evolutionary ancestry were identified in most mammalian species [8].

In the human, huFcγRIa (cluster of differentiation 64 [CD64]) is the only high affinity activation receptor with three extracellular Ig-like domains (Fig. 4.2). In contrast to the other low affinity receptors it efficiently binds to monomeric IgG and is usually saturated under physiological conditions. However, huIgG readily dissociates from huFcγRIa with a half-life in the range of minutes that allows aggregation by binding to small immune complexes (IC) or sparsely opsonized large complexes [9]. In general, FcγRs require aggregation for the phosphorylation of downstream signaling molecules by associated immunoreceptor tyrosine-based activation (ITAM) or inhibition motifs (ITIM) domains and ultimately for signal transduction [10]. Humans constitutively express huFcγRIa on most myeloid cells including monocytes, and macrophages. Additionally, most dendritic cell (DC) subsets, except blood DCs, express huFcγRIa where it is regulated by the cytokine milieu (Fig. 4.3). The huFcγRIa expression on these cells is involved in antigen internalization, degradation and presentation to T cells. Furthermore, it initiates the production and release of pro-inflammatory cytokines [9, 11]. The protein structures and naming of FcγRIa is similar between different species (Fig. 2) whereby the human is the only species having additional pseudogenes for *FCGR1B* and *FCGR1C* [8].

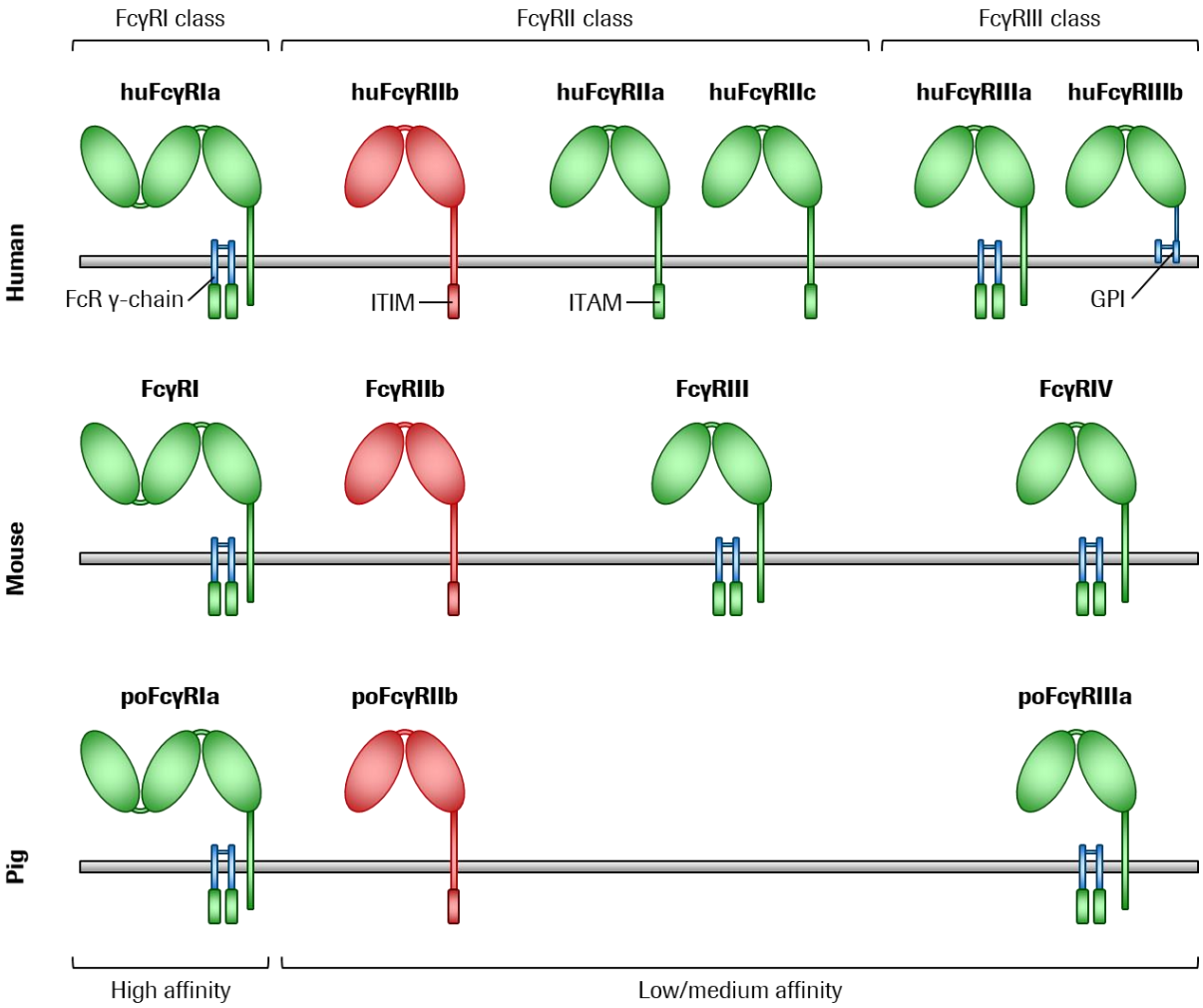

Fig. 4.2 Fc gamma Receptors (FcγR) in human (hu), mouse, and pig (po). The set of FcγRs is separated in three classes according to the amino acid similarity in humans (I, II, and III). Alternatively, it can be classified according to the high and low/medium affinity receptors due to IgG binding properties, or into activation (green) and inhibitory (red) receptors due to signaling via Immunoreceptor tyrosine-based activation (ITAM, green boxes) or inhibition (ITIM, red boxes), respectively. FcγRs are bind their ligands via Ig-like extracellular domains (filled ellipses). Often, signaling is transduced via association with the Fc receptor common gamma chain (FcR γ-chain). Human FcγRIIIb is anchored to the cell membrane (grey bar) via glycosylphosphatidylinositol (GPI)-linker. Orthologous receptors from the different species are displayed below each other. The orthologue of human FcγRIIa/c is named FcγRIII in the mouse and is so far unknown in the pig. Adapted from Nimmerjahn, Gordan [12].

The other two FcγR classes (II and III) have two extracellular Ig-like domains. Most IgG subclasses in human and mouse have a low affinity to these FcγRs (Fig. 4.2). IgG-antigen IC can efficiently bind to these low affinity receptors by avidity-based interactions. The low affinity FcγRs can be further separated by their activation or inhibition potential mediated by ITAM and ITIM, respectively (Fig. 4.2). HuFcγRIIa (CD32a) and huFcγRIIIa (CD16a) are both activation receptors expressed on various cell types, such as neutrophils, natural killer (NK) cells, monocytes, and dendritic cells in humans (Fig. 4.3). The orthologue of the ITAM bearing huFcγRIIa is named FcγRIII in the mouse and requires the association with the Fc receptor common gamma chain (FcR γ-chain). This transmembrane adaptor molecule then signals via an integrated ITAM and is required for cell surface expression of the receptor [13]. So far,

no orthologous receptor has been identified in pigs. However, the orthologue of huFcγRIIIa is also known as porcine (po)FcγRIIIa and named FcγRIV (CD16.2) in the mouse (Fig. 4.2). No orthologue to huFcγRIIc (CD32c) and the glycosylphosphatidylinositol (GPI)-anchored huFcγRIIIb (CD16b) was identified in the mouse, the pig, or any other mammal hitherto. HuFcγRIIb (CD32b) is a low affinity receptor that has an intracellular ITIM domain for inhibitory signaling. Its structure and name is highly conserved between different species and it plays an important role in antigen presentation and regulation of the immune response against pathogens. The effector functions mediated by activation receptors are balanced by the inhibitory receptor leading to tightly regulated immune reactions [8, 14].

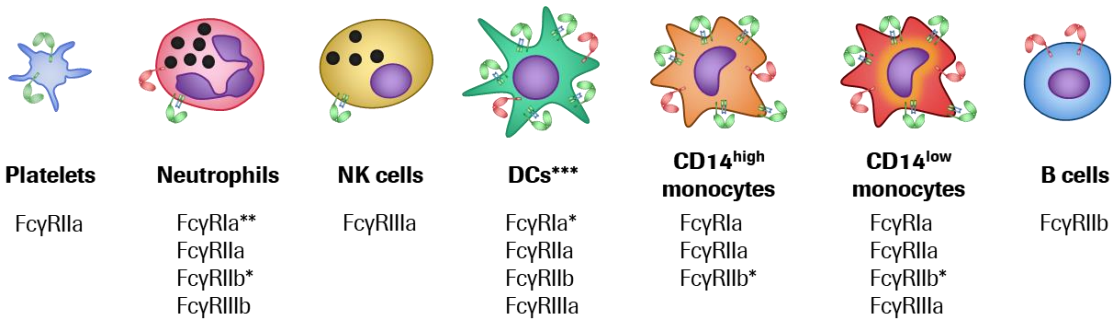

*Fig. 4.3 Expression of huFcγRs on immune cells of the human. Inhibitory (red) and activating (green) FcγRs are shown on cells involved in antibody-mediated effector functions. The CD14 expression separates human monocytes in classical ($CD14^{high}$), intermediate, and non-classical ($CD14^{low}$) monocytes. The hucγRIIc expression on 20% of the human population is not reflected in this figure. * Indicates absence of expression in the blood. ** Indicates the inducible expression of huFcγRIa on neutrophils. *** FcγR expression in human dendritic cells (DCs) refers to monocyte-derived DCs. Adapted from Nimmerjahn, Gordan [12].*

4.1.3 Therapeutic antibodies mediate functions via Fc receptor interactions

The ability to mobilize the innate immune system, the specificity, stability, and long serum half-life is what makes antibodies successful therapeutics. Besides the specificity, these properties are mediated by the Fc part of IgG antibodies in interaction with Fc receptors or complement component 1q (C1q). The latter mediates complement activation and thus enables complement-dependent cytotoxicity (CDC) as an important mode of action of cytotoxic antibodies [15]. The interactions of the Fc part with Fc receptors mediate a variety of functions depending on the antibody subclass, as well as on the binding strength to the particular Fc receptor and its cellular distribution. On one hand, the pH-dependent binding of the IgG antibody to FcRn is important for recycling of absorbed IgG and thus strongly influences the serum half-life. On the other hand, FcγR binding regulates the interaction with the innate immune system and contributes to efficacy and influences the safety profile [16, 17].

Different affinities of huIgG subclasses towards different huFcγRs influence the immune cell activation and ultimately control their effector functions. The mediated reactions largely depend on the immune cell expressing the receptor. NK cells, monocytes, and macrophages are potent mediators of antibody-dependent cellular cytotoxicity (ADCC) leading to destruction of target cells via release of cytotoxic

granules [18]. HuIgG-coated pathogens or particles are also eliminated by macrophages via huFcγR-mediated antibody-dependent cellular phagocytosis (ADCP) [19]. Additionally, huFcγR activation can lead to cytokine production and release by macrophages and DCs [20], to antigen uptake by DCs for subsequent cross-presentation to $CD8^+$ T cells [21] or to regulation of plasma cell persistence [22].

In particular, ADCC is a common mechanism of action of therapeutic cytotoxic antibodies, mediated mainly by huFcγRIIIa expressed on by NK cells, monocyte subsets, or macrophages. The engagement of these cells is an important mechanism for the elimination of human epidermal growth factor receptor 2 (HER2) positive tumor cells by the therapeutic antibody trastuzumab [23]. Two polymorphic variants of huFcγRIIIa with different affinities huIgG1 antibodies are known in human. The huFcγRIIIa polymorphism with the higher affinity was found to be associated with a better clinical outcome in anti-cancer treatment with the huIgG1 trastuzumab. However, this association is critically discussed and not found to be predictive for the outcome of the treatment [24].

Interestingly, the pharmacokinetic (PK) and pharmacodynamic (PD) properties of some antibodies depend not only on FcRn but also on FcγRs [25]. For an IgE-depleting therapeutic antibody it was shown that effector functions were important for the mode of action and thus the clearance of IgE-therapeutic antibody complexes. Decreased FcγR binding led to increased systemic exposure of the complexes and their distribution to the liver [26].

4.1.4 Fc receptor interactions can mediate toxicity

Effector cell activation via FcγRs upon treatment with therapeutic antibodies can lead to severe side-effects. Infusion reactions are a common adverse effect of therapeutic antibodies usually observed upon first administration [27]. These reactions are caused by activation of neutrophils by huFcγRIIIb binding to IC composed of the therapeutic antibody and its target [28]. Large IC can also be formed by bevacizumab binding to vascular endothelial growth factor (VEGF) resulting in huFcγRIIa-mediated platelet activation [29] and thrombosis in huFcγRIIa transgenic mice [30]. Similarly, antibodies against CD40 ligand build IC which activate huFcγRIIa on platelets *in vitro* and resulted in serious events of thromboembolism followed by myocardial infarction in clinical studies. Importantly, these toxicities were not predicted in mouse models lacking FcγR expression on platelets [31, 32]. The examples mentioned above highlight the importance of IC for the activation of low affinity FcγRs and the consequences of these interactions.

4.1.5 Antibody Fc engineering is used to alter Fc receptor binding

Fc engineering modulates the binding to Fc receptors and C1q, thus affecting a variety of functions discussed above. Significant efforts have been made to fine tune the interactions of IgG Fc parts with FcγRs, allowing modulation of the efficacy, PK/PD properties and safety profile of therapeutic

antibodies. Modifications of the Fc part are often performed by amino acid substitutions to influence the binding to FcγRs, FcRn or complement [33, 34]. Diverse mutations were studied to enhance FcγR binding for stronger functions, such as huFcγRIIa for ADCP, huFcγRIIIa for ADCC, or huFcγRIIb for inhibition [35]. Conversely, effector functions are not desired for many applications where target cell death or cytokine secretion is unwanted or could potentially lead to toxicity [36]. Therefore, Fc engineering is also applied to reduce or abolish FcγR binding [37]. Another way to manipulate FcγR binding is glycoengineering. Usually, IgG antibodies are N-glycosylated on the two heavy chains of the Fc part during post-translational modification (Fig. 4.1). The glycan composition of IgG affects effector functions directly via FcγR binding [38]. Glycoengineering have been successfully applied to therapeutic antibodies, such as obinutuzumab where afucosylated glycans improve huFcγRIIIa binding. This leads to increased ADCC and therefore to enhanced depletion of malignant B cells [39, 40]. Taken together, affinities to FcγRs are intentionally modulated to impact effector functions in humans and ultimately influence safety and efficacy of therapeutic antibodies.

4.2 Preclinical studies with therapeutic antibodies

4.2.1 Species selection for antibody development

All therapeutics have to be extensively tested to provide safe and efficacious medicine for human use. Prior to clinical studies involving the first dosing of a human being, a wide range of preclinical studies are required by regulatory agencies to demonstrate safety and efficacy of the drug. Apart from *in vitro* testing, pharmacologically relevant animal species are used as a proof-of-concept to translate pharmacology and efficacy data from the animal model into humans [41]. To assess the safety of biotechnological pharmaceuticals, including therapeutic antibodies, most regulatory agencies follow the S6(R1) guideline of the International Council for Harmonisation [42]. Therefore, preclinical safety testing also requires the use of a relevant animal species in which the therapeutic is pharmacologically active. When selecting a relevant species, drug exposure, half-live, bioavailability, activity and affinity, as well as cross-reactivity with the target have to be considered [43]. Most often a rodent and a non-rodent species is used for safety assessment. Historically, rats, dogs and NHP have been used for toxicity testing while mice were used for efficacy studies. Today, also pigs and especially minipig breeds are considered [44].

Therapeutic antibodies are mostly based on human IgG frameworks and interact with the cellular machinery of the animal models. Because FcγRs and cells expressing them are of high importance for many safety and efficacy related aspects, the cross-reactivity of huIgG to these huFcγRs can cause misleading readouts. Therefore, it is crucial to have good knowledge about the animal model and its interactions with human therapeutic antibodies [45].

4.2.2 Difficulties of animal testing with therapeutic antibodies

There are several difficulties associated with testing of therapeutic antibodies in animal models. Due to their high specificity, therapeutic antibodies do not necessarily cross-react in other species. The result is a lack of pharmacological relevance. Surrogate antibodies, binding to orthologous targets in other species, can be used in such cases. Alternatively, genetically engineered animal models are used in preclinical development [46].

Therapeutic antibodies are intended for human use and are therefore mostly composed of human or humanized backbone structures. The more distant an animal model is from the human, the more distinct are usually its proteins. According to the self/nonself concept, human proteins are therefore nonself with low similarity to self-proteins in distant animal species [47, 48]. This is often a reason for immunogenicity, such as anti-drug antibody production, limiting study duration and PK and influencing toxicology readouts. Additionally, human antibodies, distinct from self-antibodies in the animal model, might not fully interact with the effector functions system of the animal species. It was found that humans are the only species expressing huFcγRIIc and huFcγRIIIb [8]. Infusion reactions in the clinics with human therapeutic antibodies mediated by huFcγRIIIb are therefore hard to predict using standard animal models [28].

Due to the differences of FcγRs between humans and animals, many studies have been performed to investigate affinities of human antibodies to FcγRs of different animal models. Additionally, species differences regarding FcγR expression on different effector cells and resulting effector functions have been addressed. It was shown that mouse orthologues to human FcγRs are 60-70% identical. Nevertheless, remarkably similar binding strengths of human antibodies were reported to the FcγRs of the mouse [49]. It was also shown that mouse FcγRs can mediate similar effector functions as in humans although there are distinct differences [11]. HuIgG1 is the most widely used subclass for therapeutic antibodies. It shows identical FcγR interaction properties in cyno (cynomolgus monkey, *Macaca fascicularis*) and in humans, thus leading to similar effector functions. However, fundamental differences in binding and effector functions were observed for the less frequently used huIgG2 and huIgG4 subclasses. The main differences were the stronger binding to cyno FcγRIIb and the differential expression of FcγRIIb and FcγRIIIb on granulocytes leading to altered effector functions [50]. In pig-tailed macaques (*Macaca nemestrina*), however, FcγRIIb showed enhanced binding to huIgG1 whereas binding to FcγRIIa was markedly impaired [51]. These differences in affinity have implications for preclinical evaluation of human IgG1 antibodies in pig-tailed macaques, but not in cynomolgus monkey. Studies with rhesus macaques (*Macaca mulatta*) were performed to assess binding of human therapeutic antibodies to their FcγRs; many FcγR polymorphisms were identified, showing different affinities to different human IgG antibodies. Furthermore, in contrast to humans, no FcγRIIIa and

FcγRIIIb was found on neutrophils of rhesus macaques whereas FcγRIIa and FcγRIIb expression were barely studied. The differential expression is, together with the altered affinities, assumed to cause differential effector functions with human IgG in the rhesus macaque [52].

4.2.3 The minipig is a suitable animal model for preclinical studies

Due to the high anatomical and functional similarities to humans, pigs have been extensively used for biomedical research in the fields of dermatology, organ transplantation and cardiovascular diseases [53-55]. Many breeds of miniature pigs exist worldwide, but in particular the Göttingen minipig (Fig. 4.4) has become an important model for preclinical pharmacology and drug safety studies. The utility of the minipig for toxicology testing with human therapeutics has been thoroughly assessed in comparative studies with humans and other preclinical species [56]. Between pigs and minipigs, no major differences regarding the immune system have been reported so far but detailed studies are lacking [57]. In general, minipigs mainly differ from domestic pigs in their growth range and size at sexual maturity but not in anatomical structures [58]. Therefore, it can be assumed that pigs and minipigs share the sequences and functions of immune-related genes. Advantages of the Göttingen minipigs are the controlled health status, the ease of handling and low consumption of food, space, and pharmacological products in comparison to domestic pigs [59]. Additionally, their high similarity to humans in terms of genetics, physiology, and anatomy make the minipig a desired alternative to other non-rodent species [60].

Fig. 4.4 The Göttingen minipig provided by Ellegaard is used for biomedical research. Taken from [1]

In comparison to NHPs, breeding, handling, and housekeeping of minipigs is much easier, leading to reduced costs. Furthermore, genetic manipulation of minipigs is better feasible and accepted in comparison to manipulation of NHPs. For example, transgenic minipigs expressing the human Pro23His rhodopsin mutation as a model of retinitis pigmentosa were successfully generated by somatic cell nuclear transfer [61]. Analogous, the humanization for therapeutic antibody targets could make the minipig pharmacologically active and thus more useful for preclinical studies [62]. Additionally, the pressure of the public to stop animal experimentation on primates and their limited availability is forcing pharmaceutical companies to seek for alternatives. Even though minipigs are ethically of the same value as NHP, their use is less problematic as seen by the broad public due to the use of pigs as farm animals. In general, decisions about species selection for preclinical studies have to be scientifically sound and ethically justified [63].

4.2.4 Studies with therapeutic antibodies in the minipig

Today, Göttingen minipigs are regularly used for preclinical general toxicology studies with various routes of administration, and have gained a wide acceptance for safety pharmacology [64]. The Göttingen minipig has been used in immunogenicity studies with adalimumab and infliximab, whereby it was found that adalimumab, but not infliximab, triggered anti-drug antibody responses leading to decreased plasma levels of the drug. The authors concluded that, for the prediction of immunogenicity in humans, minipig and NHP seem to be comparable [65]. Zheng, Tesar [66] assessed the PK translatability to humans upon administration of therapeutic antibodies. It was found that the clearance was predictive for humans, but distinct differences in absorption and bioavailability were observed. Binding of therapeutic antibodies to FcRn was comparable between humans, NHPs, and minipigs resulting in similar clearance. Only few other studies have been performed with therapeutic antibodies due to lack of knowledge about minipig pharmacology [56, 67]. From another perspective, the advances in veterinary medicine led to the broad use of various types of antibodies for immunoprophylaxis or therapeutic purposes in the pig [68]. Occasionally, antibodies based on human sequences are used for therapy of pigs allowing learnings about their interactions with the porcine immune system [69, 70].

4.2.5 Porcine FcγRs are poorly studied

Because antibody pharmacology and toxicology is often mediated by FcγRs, it is important to understand this component in the animal model. Knowledge about porcine FcγRs is still sparse although the porcine immune system is the best characterized after the murine and primate immune systems [44].

The presence of FcγRI (CD64) is conserved in most mammalian species, including pigs [8, 71]. Nevertheless, the huFcγRI gene family comprises a total of three FcγRI variants named *FCGR1A*, *FCGR1B*, and *FCGR1C,* but only *FCGR1A* is expressed as a full length cell surface receptor [72]. Most other species, including pigs, only express FcγRIa. PoFcγRIa was recently cloned and its function has been demonstrated by binding to chicken erythrocytes sensitized with porcine total poIgG [73]. No antibodies specific for poFcγRIa have been described so far, complicating expression studies. However, poFcγRIa messenger ribonucleic acid (mRNA) was identified mainly in the $CD163^{high}$ DC subset and in alveolar macrophages [74]. No further cellular distribution studies of poFcγRIa have been performed in pigs or minipigs.

The low affinity FcγRII (CD32) family is composed of an inhibitory and at least one activation receptor. The structure and function of the inhibitory FcγRIIb (CD32b) is highly conserved in humans, pigs, mice and other mammalian species [8]. PoFcγRIIb was cloned and found to bind chicken erythrocytes with porcine total IgG [75]. Another sub-isoform named poFcγRIIb1 was isolated from porcine peripheral

blood leucocytes ribonucleic acid (RNA) and identified to be generated by alternative splicing. It shows significant homology to huFcγRIIb1 whereas the previously described poFcγRIIb is thought to be orthologous to the huFcγRIIb2 sub-isoform [76, 77]. The cellular distribution of poFcγRIIb has not yet been thoroughly analyzed, also due to a lack of commercially available specific antibodies. A transcriptomic analysis, however, found high level expression of poFcγRIIb on the conventional DC subset 2 in the blood [78]. Despite the importance of the human orthologue, the activation poFcγRIIa (CD32a) and its gene *FCGR2A* have not been identified yet in pigs (Fig. 4.2).

FcγRIIIa (CD16) is an activating low affinity FcγR that requires association with the FcR γ-chain for signaling [13]. In the pig, poFcγRIIIa was first cloned and characterized by Halloran, Sweeney [79]. In addition to poFcγRIIIa expressed on the cell surface, a soluble poFcγRIIIa isoform generated by alternative splicing was identified, possibly regulating FcγR-mediated immune responses [80]. A unique association of poFcγRIIIa with a 15 kDa molecule was detected that shows significant homology to porcine cathelin. This complex was hypothesized to further link the innate and acquired immune responses and therefore indicate further functions of this receptor [81]. The availability of monoclonal antibodies directed against poFcγRIIIa (CD16) facilitated the research on its cellular distribution and function. PoFcγRIIIa shows the highest expression of all FcγRs in the pig, and is known to be expressed on all blood monocytes, NK cells, and neutrophils, as well as on most DC subsets including monocyte-derived DCs and blood DCs [82, 83]. Even though all porcine monocytes express poFcγRIIIa, individual pig breeds differ regarding the expression level of poFcγRIIIa on $CD14^{high}$ and $CD14^{low}$ monocytes. In contrast, human $CD14^{high}$ classical monocytes completely lack huFcγRIIIa [84]. Although poFcγRIIIa is the earliest and most widely analyzed Fc receptor in pigs, its gene structure and genetic localization have not yet been determined [8].

As mentioned before, studies in pigs with human antibodies were used to draw conclusions about Fc-mediated effector functions based on interactions with poFcγRs. Treatment of pigs with a mouse IgG2b antibody led to platelet activation, cytokine release, and subsequent toxicity. These effects were mediated by poFcγR and complement interactions. Replacing the constant region of the antibody with a human IgG2/IgG4 framework abolished poFcγR and complement binding and related toxicities [70]. Another study investigated the therapeutic effect of a hemagglutinin-specific antibody that is anticipated to be mediated by FcγR-interaction [85]. However, this antibody of the huIgG1 subclass lacked the expected efficacy in the pig and no poFcγR interaction and ADCC induction was observed. Therefore, the authors concluded that huIgG1 antibodies do not interact with poFcγRs [69]. To conclude, the expression of FcγRs in minipigs is not thoroughly analyzed and studies with human antibodies have questioned the binding of huIgG1 to poFcγRs without looking at individual receptors. This limits a justified species selection for preclinical studies with human therapeutic antibodies and hinders the subsequent interpretation and translation of responses from minipigs to humans.

5 Aims of the thesis

The main goal of this thesis is to assess the utility of the Göttingen minipig for preclinical safety and efficacy studies with human therapeutic antibodies in order to enable a justified species selection. This includes studies of the genomic organization and expression pattern of the poFcγRs in the minipig to allow an estimation of possible effector functions with antibodies. Furthermore, this thesis aims to measure the binding properties of human therapeutic antibodies to all poFcγRs in minipigs in order to highlight similarities and differences to huFcγRs in humans. Therefore, we defined the following milestones:

- Exploration of the porcine FcγR family by screening of the low affinity *FCGR* locus in a novel genome draft of the Göttingen minipig
- Characterization of the FcγR expression in immune cells of the Göttingen minipig
- Cloning and expression of all porcine FcγRs
- Interaction studies of human therapeutic antibodies with porcine FcγRs

Additional aims and hypotheses arose during the term of the thesis. Upon the identification of a gap within the low affinity *FCGR* locus, we planned to identify the missing sequences by bioinformatics tools and sequencing. After the subsequent identification of a hitherto unknown porcine FcγR, its characterization became an additional aim. Upon initial binding studies with free IgG, we intended to further test binding of IC to poFcγRs that was hypothesized to be stronger due to higher avidity.

6 Genomic organization and expression pattern of porcine FcγRs

6.1 Purpose

Most mammalian species express three classes of FcγRs: 1) The activating high affinity receptor FcγRI, 2) the low affinity FcγRII comprising of the activating FcγRIIa and the inhibitory FcγRIIb, and 3) the low affinity FcγRIII. In humans, duplications of these four different receptors have led to an extended repertoire. Minipigs represent an animal model of high interest for preclinical studies with human therapeutic antibodies, which are potential ligands of poFcγRs. In pigs, however, no low affinity activating poFcγRIIa was described so far and the situation of possible duplications was unclear. Furthermore, poFcγRIIIa was not genetically characterized and the cellular expression of poFcγRIa and poFcγRIIb was unclear. We aimed to address the abovementioned gaps to identify potential effector cells and estimate the effector functions of human therapeutic antibodies.

6.2 Main results

We assembled the complete low affinity *FCGR* locus of the minipig, localized the gene coding for poFcγRIIIa, and identified the missing poFcγRIIa. The expression of all poFcγRs in the minipig was described on transcription and protein level and found to be comparable to the human expression pattern.

6.3 Manuscript 1

The genomic organization and expression pattern of the low affinity Fc *gamma* Receptors (FcγR) in the Göttingen minipig

Jerome Egli, Roland Schmucki, Benjamin Loos, Stephan Reichl, Nils Grabole, Andreas Roller, Martin Ebeling, Alex Odermatt, Antonio Iglesias

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Contribution – I assembled the minipig low affinity *FCGR* locus, amplified the missing parts by PCR, identified and cloned the novel poFcγR from RNA, and compared it to orthologous receptors. However, phylogenetic analysis, single cell RNA sequencing, and related data processing were performed by co-authors. Nevertheless, I was responsible for data analysis and the generation of the figures. Using fresh blood cells provided by colleagues, I performed flow cytometry and processed the data. Finally, I drafted and wrote the manuscript.

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ORIGINAL ARTICLE

The genomic organization and expression pattern of the low-affinity Fc gamma receptors (FcγR) in the Göttingen minipig

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Abstract

Safety and efficacy of therapeutic antibodies are often dependent on their interaction with Fc receptors for IgG (FcγRs). The Göttingen minipig represents a valuable species for biomedical research but its use in preclinical studies with therapeutic antibodies is hampered by the lack of knowledge about the porcine FcγRs. Genome analysis and sequencing now enabled the localization of the previously described FcγRIIIa in the orthologous location to human *FCGR3A*. In addition, we identified nearby the gene coding for the hitherto undescribed putative porcine FcγRIIa. The 1′241 bp long *FCGR2A* cDNA translates to a 274aa transmembrane protein containing an extracellular region with high similarity to human and cattle FcγRIIa. Like in cattle, the intracellular part does not contain an immunoreceptor tyrosine-based activation motif (ITAM) as in human FcγRIIa. Flow cytometry of the whole blood and single-cell RNA sequencing of peripheral blood mononuclear cells (PBMCs) of Göttingen minipigs revealed the expression profile of all porcine FcγRs which is compared to human and mouse. The new FcγRIIa is mainly expressed on platelets making the minipig a good model to study IgG-mediated platelet activation and aggregation. In contrast to humans, minipig blood monocytes were found to express inhibitory FcγRIIb that could lead to the underestimation of FcγR-mediated effects of monocytes observed in minipig studies with therapeutic antibodies.

Keywords CD32 · FcγRIIa · *FCGR* locus · Flow cytometry · Single-cell RNA sequencing · *Sus scrofa*

Introduction

Therapeutic antibodies of the IgG (immunoglobulin G) isotype represent an important group of new medical entities and interactions of Fc gamma receptors (FcγRs) with the Fc part of IgG antibodies are crucial in the antibody-based immunotherapy. Most mammals were shown to have three functionally distinct classes of FcγRs with different affinities and properties. FcγRIa (CD64) is capable of binding to free IgG antibodies and is hence considered as a high-affinity receptor. Its expression and function are conserved in most mammalian species, including pigs (Akula et al. 2014; van der Poel et al. 2011). Low-affinity receptors efficiently bind immune complexes and are divided into inhibitory and activating FcγRs. The structure and function of FcγRIIb (CD32b), the inhibitory low-affinity receptor, is also highly conserved in humans, pigs, mice, and other mammalian species (Akula et al. 2014). FcγRIIIa (CD16a) is an activating low-affinity FcγR that requires the association with FcR γ-chain (Fc receptor common gamma chain) for signaling (Kim et al. 2003). Different affinities to IgG were observed for the human FcγRIIIa V158F polymorphism within the extracellular domain (ECD). It was shown to be associated with differential response to therapeutic antibodies and disease progression (Mellor et al. 2013). Although FcγRIIIa is the most widely analyzed Fc receptor in pigs (Halloran et al. 1994), its gene structure and genetic localization has not yet been determined. In mouse, the orthologous receptor of FcγRIIIa is known as FcγRIV (Nimmerjahn and Ravetch 2006). FcγRIIa (CD32a) is another activating low-affinity receptor present in humans, non-human primates (NHPs), cattle, and rat and named as

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FcγRIII in mouse (Lux and Nimmerjahn 2013). In humans, FcγRIIa is expressed on the cell surface of monocytes, neutrophils, macrophages, eosinophils, basophils, dendritic cells, and platelets. It is involved in the process of phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and cytokine release (Powell and Hogarth 2008). The FcγRIIa R131H polymorphism is associated with severity and progression of idiopathic pulmonary fibrosis and with response to rituximab therapy (Bournazos et al. 2010; Ziakas et al. 2016). Immune complexes binding to FcγRIIa on human platelets can lead to thrombus formation (Zhi et al. 2015) and ultimately to heparin-induced thrombocytopenia (Greinacher 2009). Despite its importance, the minipig FcγRIIa and its gene *FCGR2A* could not be identified yet.

The Göttingen minipig is increasingly used as a valuable animal model for preclinical pharmacology and drug safety studies. The high similarity to humans in terms of genetics, genomics, physiology, and anatomy makes the minipig a desired alternative to NHPs (Ganderup et al. 2012). Additionally, Göttingen minipigs have a controlled health status, are easy to handle, and need less food, space, and pharmacological products compared to domestic pigs and other non-rodent species (McAnulty et al. 2011). Minipigs mainly differ from domestic pigs in their growth range and size at sexual maturity but not in anatomical structures (Swindle et al. 2012). Regarding the immune system, no major differences between pigs and minipig have been reported so far but detailed studies are lacking (Descotes et al. 2018). The use of the minipig as an adequate species for toxicity and efficacy evaluation of therapeutic antibodies requires a detailed knowledge of the FcγR composition and their interaction with human IgGs. However, to date, the knowledge on the binding properties of porcine FcγR to human antibodies is still scarce. In addition, the number of low-affinity FcγRs existing in the minipig and the allocation of the *FCGR* genes in the corresponding locus of the Göttingen minipig genome was not conclusively determined. The latest version of the Göttingen minipig genome was generated by Heckel et al. by mapping of the whole genome-sequencing data on the Duroc pig genome *Sus scrofa* 10.2 (Heckel et al. 2015). There, *FCGR2B* was the only gene annotated in the low-affinity *FCGR* locus. Recently, the new assembly *Sus scrofa* 11.1 was released containing a more accurate view of the pig genome including this particular locus (Li et al. 2017).

In this paper, we describe the complete assembly of the genetic *FCGR* locus of the Göttingen minipig including the exact mapping of *FCGR3*. Additionally, we demonstrate the identification, sequence characterization, and genomic location of *FCGR2A*, and the expression of low- and high-affinity FcγRs in the Göttingen minipig across blood cell types.

Materials and methods

FCGR locus assembly and *FCGR* mapping

The Göttingen minipig genome draft generated by Heckel et al. (2015) based on *Sus scrofa* 10.2 was used as a reference genome. Known sequences of *FCGR2B* and *FCGR3A* were blasted (Altschul et al. 1990) against whole genome shotgun-sequencing data of the Göttingen minipig (accession: AOCR01000000) and the Wuzishan minipig (accession: AJKK01000000) to identify overlapping contigs (contiguous sequences). A minimum of 95% identity over 200 base pairs was considered as sequence identity. The ends of each newly identified contig and exon sequences from known porcine *FCGR* genes were again blasted against the data from both minipig breeds to form longer contiguous sequences (Fig. 1). All sequences were continuously screened for potential *FCGR* genes by pairwise alignment (EMBOSS Water) to published porcine, human, and mouse *FCGR* exons.

Genomic DNA was isolated from the frozen spleen of a Göttingen minipig using the DNeasy Blood and Tissue Kit (Qiagen). PCR on genomic DNA with primers JE24/JE26 (see Fig. 1 for primer positions and Online Resource 1 for primer sequences) allowed sequencing of the gap within an intron of *FCGR3A* (Fig. 1) (GenBank ID: MH574548). The two remaining gaps in the putative *FCGR2A* introns were amplified by nested PCR using primers JE62/JE64 followed by JE47/JE49 and JE58/JE61 followed by JE41/JE42, respectively (Fig. 1, Online Resource 1). The obtained products were cloned using the TOPO TA cloning kit and sequenced (GenBank ID: MH574549, and MH574550). All sequencing reactions were performed by Microsynth.

Identification and sequencing of putative porcine *FCGR2A*

Total RNA was isolated from blood cells of Göttingen minipigs and RNA integrity was determined on the Agilent 2100 Bioanalyzer System (Agilent Technologies). Then, putative *FCGR2A* cDNA ends were amplified in a nested PCR approach using SMARTer RACE 5′/3′ kit (Clontech). Rapid amplification of cDNA ends (RACE) PCR was performed by generation of 5′- and 3′-RACE-ready cDNA and subsequent PCR reactions using SMARTer RACE 5′/3′ kit (Clontech). More precisely, 5′- and 3′-RACE-ready cDNA was generated from total RNA serving as a template. In the first round of PCR, the supplied universal primer mix (UPM) was used together with primer JE5 or JE28, designed on predicted putative *FCGR2A* sequences. In a second round, nested UPM-short was used with primers JE4 or JE2 to generate 5′ or 3′ cDNA ends, respectively (Fig. 1, Online Resource 1). The products were analyzed on a 0.8% agarose gel and purified using the QIAquick gel extraction kit. Sanger sequencing was

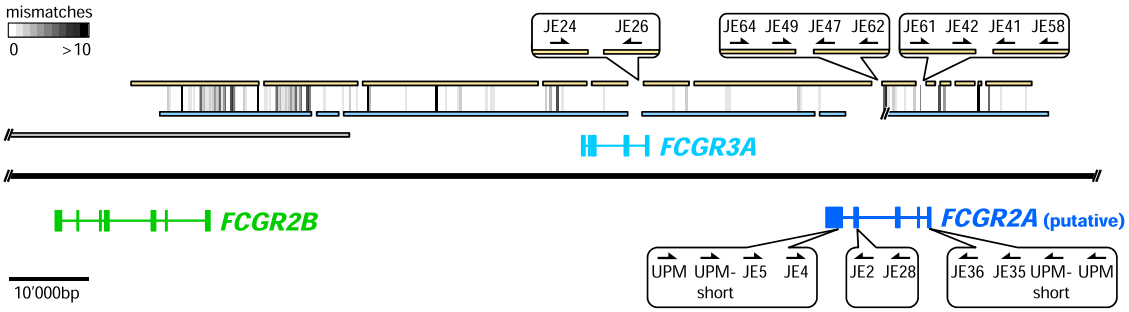

Fig. 1 Genomic organization of the minipig *FCGR* locus. The black line represents the genomic sequence scaled as indicated in the lower left corner. *FCGR* genes are shown as colored lines with boxes representing the exon structure. Genes above and below the black line are encoded at the forward strand and the reverse strand, respectively. The sequence from the initial minipig genome draft containing *FCGR2B* (Heckel et al. 2015) is represented by a gray line. Yellow and blue lines represent whole genome shotgun contigs of the Göttingen minipig and the Wuzishan minipig, respectively. Vertical lines between the contigs of the two minipig breeds highlight regions with mismatches. The grayscale in the upper left corner indicates the number of mismatches found in intervals of 300 bp, from white (0 mismatches) to black (10 or more mismatches). Callouts enlarge the gaps now closed by sequencing using the primers indicated by the arrows. Refer to Online Resource 1 for primer sequences

performed using several primers designed on predicted putative *FCGR2A* exons to identify the cDNA ends.

A final nested RT-PCR was performed on total RNA from minipig blood using first strand cDNA synthesis (New England Biolabs), the outer primers JE35/JE5, and the inner primers JE36/JE4 (Fig. 1, Online Resource 1). The product was cloned using the TOPO TA cloning kit and 30 colonies were sequenced from both sides using M13 and M13r primer. RACE PCR and RT-PCR sequences were assembled to generate the full-length transcript of the putative porcine *FCGR2A*.

Sequence analysis and comparison

Signal sequences were predicted by similarity to porcine *FCGR2B* (Qiao et al. 2006) by signalP 4.1 Server (Nielsen 2017), SMART (Letunic and Bork 2018), and Sigcleave (von Heijne 1986). SMART also predicted the extracellular structures. Transmembrane (TM) helices were predicted from similarity to human FcγRIIa (Moi et al. 2010) and by the average result from the following prediction tools: TMpred (Hofmann and Stoffel 1993), DAS (Cserzo et al. 1997), SOSUI (Hirokawa et al. 1998), PredictProtein (Yachdav et al. 2014), Phobius (Kall et al. 2004), SMART, and ALOM (a program implemented at Roche according to Klein et al. (1985)).

For the phylogenetic tree, protein sequences were first aligned with MUSCLE (Edgar 2004) then poorly aligned positions and divergent regions were filtered with GBLOCKS (Castresana 2000) so that only the conserved ECD region remained. PHYLIP software package was used to calculate a protein sequence distance matrix followed by bootstrapping with 1000 replicates (Felsenstein 2005). Data was graphically displayed with the TreeExplorer software V2.12 (Jie 2017).

Single-cell RNA sequencing

PBMCs were isolated using Ficoll-Paque Plus (GE Healthcare) and Leucosep tubes (Greiner bio-one, 12 mL) from K2 EDTA–treated whole blood of three different healthy human donors, Göttingen minipigs, or mice. Lysis buffer (BD Pharm Lyse) was used for subsequent removal of erythrocytes. Cell count and viability were determined using the Countess Automated Cell Counter (Invitrogen).

Single-cell capture was performed using the microfluidic chromium instrument (10x Genomics) capturing single cells in microdroplets. Cell suspensions containing approximately 4000 cells per sample from three different individuals were loaded together with the provided enzyme mix, beads, and oil. According to the manufacturer's protocol, cDNA was generated, purified, and quality was checked on the Agilent 2100 Bioanalyzer System (Agilent Technologies). In a second step, a sequencing library was prepared by attaching Illumina Indices to fragmented cDNA strands. After size selection for approximately 500 bp fragments, library concentration was measured by a Qubit fluorometer (ThermoFisher). Every sample was adjusted to a final concentration of 2.5 nM, by dilution with buffer EB (Qiagen). All samples were pooled in same amounts. A PhiX solution was added, resulting in a spike-in amount of 1% in the final pool. Pooled fragments were denatured and mixed with a master mix consisting of EPX reagents 1–3 (Illumina), resulting in a final volume of 50 μL and a final concentration of 225 pM. After cluster generation, the flow cell was inserted into a HighSeq4000 instrument (Illumina). The sequencer cycle program consisted of 27 cycles for read one, 8 cycles for the index read and 99 cycles for read two.

Sequencing data were further processed using cell ranger version 2.0.0. First, fastq files were generated using the mkfastq function. Second, count files were generated using the count function. Human sequences were mapped against the genome

assembly hg19, mouse sequences against the mm10, and minipig sequences against the RefSeq (reference sequence) (Pruitt et al. 2012) genome assembly *Sus scrofa* 11.1 containing all *FCGR* gene entries. Raw counts were further processed using an R (version 3.3.2) based in an in-house pipeline. First, data were imported using scater::read10XResults (version 1.6.3) function and QC parameters were calculated. The human raw cells were filtered using a minimum of 1.000 and a maximum of 50.000 umi (unique molecular identifier) counts in total. Second, cells having less than 300 genes expressed or more than 5% mitochondrial gene counts were filtered out. Mouse raw counts were filtered using a minimum of 700 and a maximum of 20.000 umi counts in total and at least 200 genes expressed. Finally, minipig raw counts were filtered using a minimum of 800 and a maximum of 20.000 umi counts in total and at least 200 genes expressed. Next, data were processed using the scater::normaliseExprs function using the 99th percentile for normalization. Confounding factors were determined based on their correlation to the first ten principle components of the normalized data. For human, we identified pct_counts_top_100_endogenous_features, log10_total_features, and donor; for mouse, we identified pct_counts_top_500_features and total_counts; and for minipig, we identified pct_counts_top_50_features, log10_total_counts, and donor as independent confounding factors. We applied a linear regression model to remove the effects of the identified confounders on the normalized data. Finally, we used the Seurat:: FindClusters function (version 1.4.0.16) and Seurat::RunTSNE function to run the t-SNE (t-distributed stochastic neighbor embedding) dimensionality reduction on selected features. Clusters were summarized according to the differential expression of various genes (Online Resource 2).

Flow cytometry

Antibodies directed against porcine FcγRIIa (AbD29332.1) and FcγRIIa/b (AbD32591.1) were generated by Bio-Rad using the HuCAL technology. Generation and specificity of the HuCAL antibodies used here will be published elsewhere. Whole blood from three different Göttingen minipigs was collected in K2 EDTA–coated vacutainer tubes (BD). Erythrocytes were removed with the lysing buffer (BD Pharm Lyse) prior to staining of dead cells with amine-reactive dye Zombie Aqua (BioLegend). Leukocytes were then incubated in separate stainings with antibodies against porcine FcγRIIa (AbD29332.1), FcγRIIa/b (AbD32591.1), FcγRIIIa (CD16-PE, clone G7, Bio-Rad), and HuCAL Fab-A-FH-negative control antibody (AbD05930). Unlabeled HuCAL antibodies were then stained with a secondary PE-conjugated goat $F(ab')_2$ fragment anti-human IgG, $F(ab')_2$ fragment specific polyclonal antibody from Jackson ImmunoResearch. Cell events were acquired on BD LSRFortessa with BD FACSDiva and analyzed using FlowJo software.

Results

Localization of porcine *FCGR3A* and identification of putative *FCGR2A*

The low-affinity *FCGR* locus on chromosome 4 in the minipig genome draft based on *Sus scrofa* 10.2 was successfully supplemented with contigs from the Göttingen and the Wuzishan minipig and completed by PCR, cloning, and sequencing (Fig. 1, Online Resource 3). Sequences from the two minipig breeds differ in 0.31% mismatches and 1.25% indels spread over the total alignment comprising 115,000 nucleotides. The new assembly enabled the identification of exon sequences of *FCGR3A* in a forward orientation. Additionally, exon sequences were detected with high similarity to the porcine *FCGR2B* extracellular domain (ECD) and to porcine *FCGR3A* transmembrane/cytoplasmic (TM/C) region. These sequences belong to the putative porcine *FCGR2A* gene that is located in reverse orientation where the orthologue to human *FCGR2A* was expected (Fig. 1 and Fig. 4). Thus, the obtained sequence of the minipig low-affinity *FCGR* locus is completed and entirely contiguous. The newly characterized locus is highly similar to the most recent reference sequence (RefSeq) genome assembly of *Sus scrofa* 11.1 (Li et al. 2017).

Exon sequences of the putative porcine *FCGR2A* gene were disclosed from the low-affinity *FCGR* locus of the minipig by alignment of the sequences to porcine, human, and mouse *FCGR* exons. This enabled the design of gene-specific primers used for RACE PCR to identify cDNA ends. In combination with RT-PCR, we determined the complete sequence of the putative porcine *FCGR2A* transcript. The expected transcript, two potential polymorphisms, and three splice variants were identified in the total RNA preparation of one Göttingen minipig (Fig. 2) by Sanger sequencing of 30 clones.

The putative porcine *FCGR2A* cDNA is 1′241 bp long, contains an 822 bp open reading frame (ORF) translating to a 274 amino acids (aa) long protein (RefSeq No. XM_021089520.1). Bioinformatic analysis revealed a 45aa long signal peptide followed by an ECD region containing two immunoglobulin-like parts (Ig1, 74aa; Ig2, 78aa). Like porcine FcγRIIb, the ECD contains four potential *N*-glycosylation sites (Asn^{79}, Asn^{89}, Asn^{187}, and Asn^{211}) identified by the common motif (N-X-S/T) (Aebi 2013). The receptor sequence predicts a 23aa hydrophobic TM part with a negatively charged aspartic acid residue allowing interaction with the FcR γ-chain (Kim et al. 2003). In the 27aa long intracellular part, no immunoreceptor tyrosine-based activation motif (ITAM; Y-X-X-L/I) or immunoreceptor tyrosine-based inhibition motif (ITIM; S/I/V/L-X-Y-X-X-I/V/L) was found in contrast to human FcγRIIa or FcγRIIb, respectively (Isakov 1997; Ravetch and Lanier 2000) (Fig. 2).

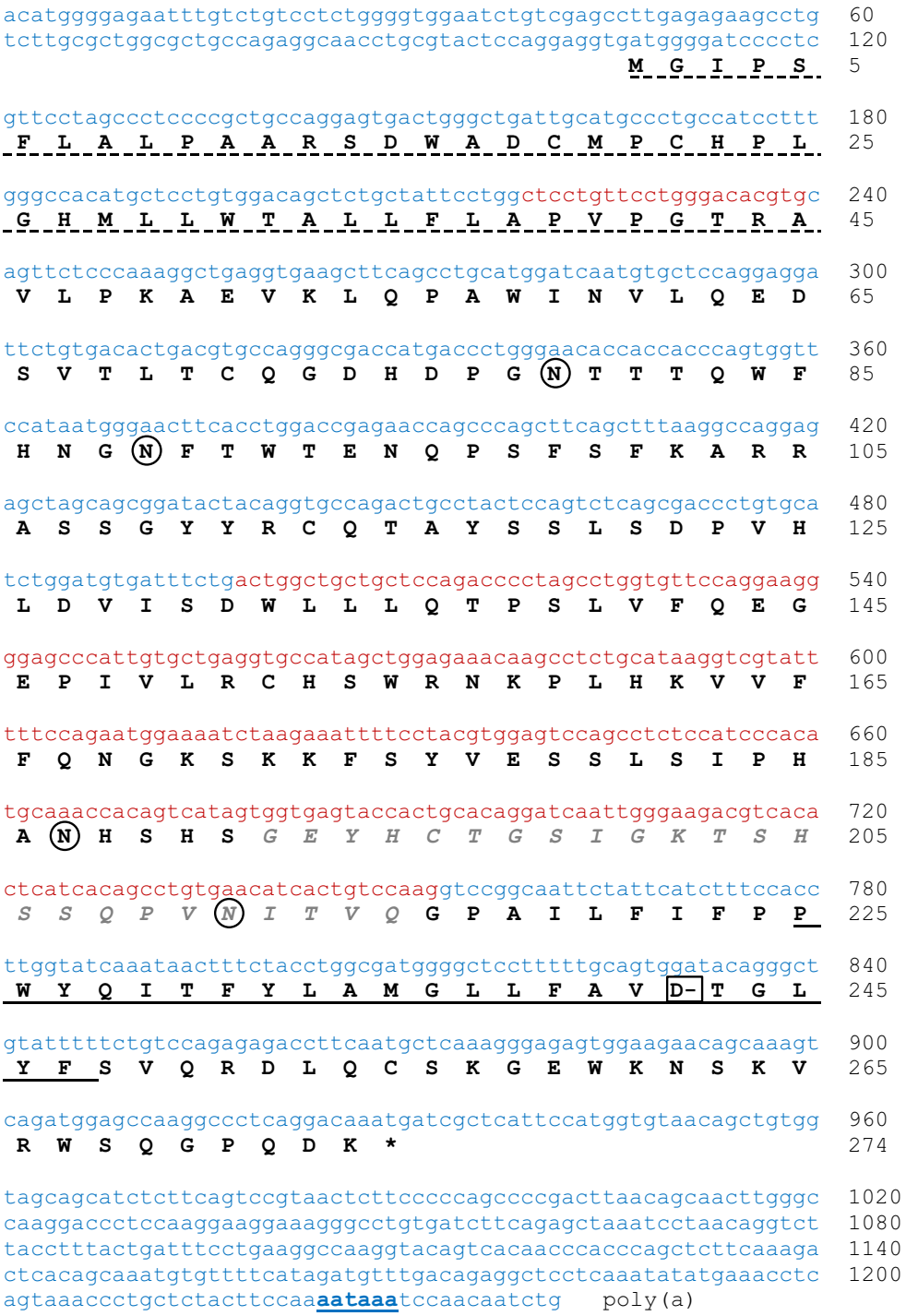

Fig. 2 The sequence of putative porcine FcγRIIa mRNA is written in lower case letters with colors indicating alternating exons. In the 3′ untranslated region, the poly adenylation signal (aataaa) is underlined and bold. The amino acid sequence deduced from the ORF is written in capital letters below the nucleotide sequence. The predicted signal sequence is marked with a broken underline and the transmembrane (TM) spanning part is underlined. Letters in gray and italic mark the missing 24 amino acids observed in variant FcγRIIa1 and FcγRIIa3. All four potential N-glycosylation sites (N-X-S/T) are circled and the negatively charged aspartic acid residue in the TM domain, required for FcR γ-chain interaction, is indicated as "D-" in a box

The putative porcine FcγRIIa.1 variant revealed a 24aa deletion within the Ig2-like part of the ECD (Gly192_Gln215del) (Fig. 2). Further variants include FcγRIIa.2 lacking the whole Ig2-like part of the ECD (Asp131_Gln215del) and FcγRIIa.3 lacking the whole Ig1-like part of the ECD (Ala45_Ser130del) and bearing the 24aa deletion of FcγRIIa.1. Furthermore, four single nucleotide polymorphisms were detected, two of them affecting the coding sequence and thus representing potential polymorphisms. The A11S polymorphism is located in the signal sequence and the H205Y polymorphism in the Ig2-like part of the ECD.

After translation of the ORF, we compared the newly identified putative porcine FcγRIIa to orthologous FcγRs from different species by multiple sequence alignment (Fig. 3). All human FcγRIIa orthologs share high sequence similarity

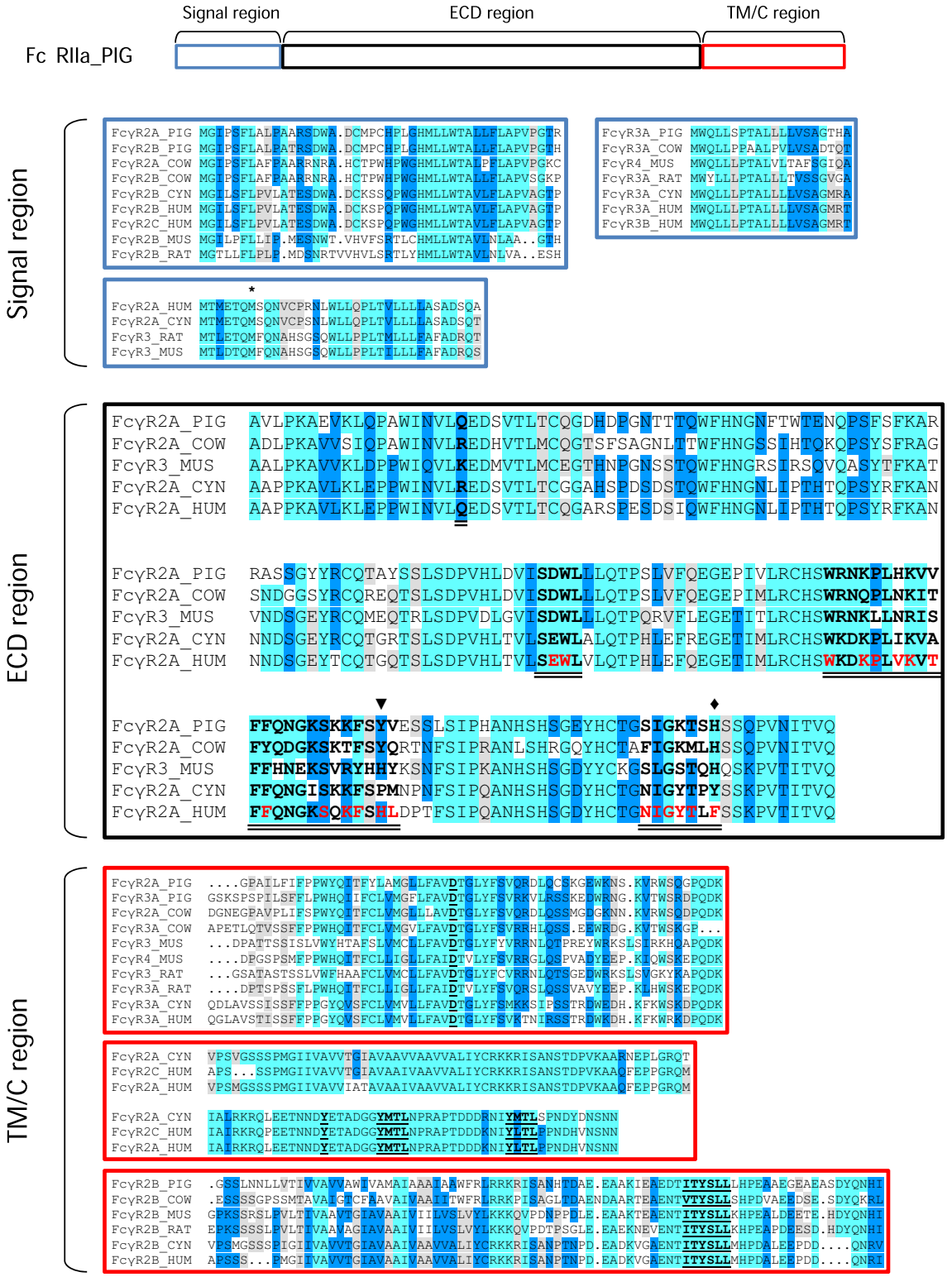

Fig. 3 Comparison of FcγR protein sequences. A schematic representation of the putative porcine (PIG) FcγRIIa transcript is shown at the top. The boxes within the transcript represent signal regions (blue boxes), extracellular domains (ECD, black box), and transmembrane/cytoplasmic regions (TM/C, red boxes) of cattle (COW), mouse (MUS), rat (RAT), cyno (CYN), and human (HUM). Human FcγRIIa amino acid residues in the ECD involved in IgG-FcγR contact are marked in red and deduced areas of contact are bold and double underlined. Human polymorphism R131H in FcγRIIa and the minipig polymorphism H205Y in FcγRIIa are indicated as arrowhead and diamond, respectively. Above and below the ECD alignment are shorter alignments of the three different versions of the signal region and the TM/C regions, respectively. These alignments are enhanced with sequences from other related FcγRs to demonstrate the homology within each cluster. Note that, in the signal region, some protein sequences are annotated as starting with the methionine indicated by an asterisk. The conserved aspartic acid residue (D) for FcR γ-chain interaction, the ITAM (Y-X-X-L/I) and ITIM (S/I/V/L-X-Y-X-X-I/V/L) motifs are bold and underlined

having a conserved extracellular structure including four cysteine residues required for disulfide bonds to form Ig-like domains (black box in Fig. 3). Human FcγRIIa amino acid residues involved in IgG-FcγR contact (Caaveiro et al. 2015) are marked in red and the deduced areas of IgG contact including residues predicted by other publications are indicated in bold and double underlined (Hulett et al. 1995; Radaev et al. 2001) in Fig. 3. In general, ECD regions involved in the IgG-FcγR interactions showed strong conservation among species, including conserved tryptophan residues, thus indicating that the identified putative porcine FcγRIIa is capable of IgG binding (Fig. 3, black box). Extracellularly, the putative porcine FcγRIIa (aa 46–215) shares 75% similarity to mouse FcγRIII (Uniprot, P08508; aa 31–196), 79% to cattle FcγRIIa (Uniprot, A8DC37; aa 46–215), 80% to cyno (cynomolgus monkey, *Macaca fascicularis*) FcγRIIa (Uniprot, Q8SPW4; aa 30–199), and 79% to human FcγRIIa (Uniprot, P12318; aa 37–206). However, striking differences between the species are observed in the signal region and the TM/C region of the Fc receptors. A closer inspection and comparison to other FcγRs revealed three different non-related signal regions and three different non-related TM/C regions (shown in Fig. 3 as blue and red boxes, respectively). These regions are well conserved between species and combined in different ways with the ECD region of FcγRs (Fig. 3). This suggests a gene "mosaicism" that is very likely the result of duplication and rearrangement of events in the complex *FCGR* locus. We note that this mosaicism implies that the concept of "orthology" should only be applied to the ECD region of the receptors. The intracellular ITAM of human and NHP FcγRIIa is required for direct activation signaling (Isakov 1997) (Fig. 3, red middle box). Mouse FcγRIII, cattle FcγRIIa, and putative porcine FcγRIIa, on the other hand, are lacking such an intracellular ITAM. Like human FcγRIIIa, these receptors signal through associated adaptor proteins including FcR γ-chain (Lux and Nimmerjahn 2013). Charged residues in TM domains are thought to be important for protein-protein interactions in the cell membrane (Cosson et al. 1991). Especially, aspartic acid residues in TM helices are thought to be required for stable surface expression and interaction with the FcR γ-chain (Kim et al. 2003). These residues are also present in the predicted transmembrane domain of the newly identified gene, suggesting that also the putative porcine FcγRIIa signals through the FcR γ-chain (Fig. 3 red upper box).

A complete picture of the genomic organization of the porcine *FCGR* locus

The new RefSeq assembly contains genes and curated transcripts of *FCGR1A* (gene ID, 613130; transcript ID, NM_001033011.1.1), *FCGR2B* (gene ID, 613131; transcript ID, NM_001033013.2.1), and recently also *FCGR3A* (gene ID, 397684; transcript ID, NM_214391.1.1). The predicted transcript (transcript ID: XM_021089520.1) from the RefSeq gene LOC110260307 (gene ID, 110260307) codes for the 11A 205H polymorphism of putative porcine FcγRIIa. In contrast, the transcript identified from sequences of the Göttingen and the Wuzishan minipig (Fig. 1, Online Resources 3) codes for the 11S 205Y polymorphism of putative porcine FcγRIIa. However, both polymorphic variants were detected by sequencing of one Göttingen minipig.

The gene family of FcγRs displays a similar genomic organization as in most mammals (Fig. 4). Low-affinity FcγRs are organized in one locus flanked by *FCRLB* and *FCRLA* on one side, and *CFAP126* and *SDHC* on the other side. The gene coding for the inhibitory FcγRIIb is highly conserved in mammalian species. *FCGR3A* in humans and pigs is also known as *FCGR3* in macaque and sheep and as *FCGR4* in the mouse (Nimmerjahn and Ravetch 2006). Similarly, *FCGR2A* in humans, NHP, and cattle is referred to as *Fcgr3* in the mouse (Fig. 4). The human genome was found to have species-specific duplications of the low-affinity *FCGR2A* and *FCGR3A* and the high-affinity *FCGR1A* resulting in *FCGR2C* and *FCGR3B* as well as pseudogenes *FCGR1B* and *FCGR1C*, respectively (Machado et al. 2012; Warmerdam et al. 1993). Human and NHP have the gene-encoding high-affinity FcγRIa located distant to the low-affinity *FCGR* locus on chromosome 1. The same organization was found in pig and cattle on chromosomes 4 and 3, respectively. Dogs, mice, and rats, on the other hand, have lost the chromosomal cohesion of FcγRIa and the low-affinity *FCGR* locus. We assume that the ECD region of the newly identified porcine *FCGR* gene is orthologous to human *FCGR2A* and mouse *FCGR3* due to their sequence similarities (Fig. 3) and the orientation within the *FCGR* locus (Fig. 4).

The phylogenetic tree shows a high intraspecies similarity between ECD region of activating FcγRIIa and inhibitory FcγRIIb including the orthologues in mouse and rat (Fig. 5). FcγRIIIa proteins, including mouse FcγRIV, form a separate group with high interspecies similarity. Full-length porcine FcγRIIa, for example, shows an amino acid sequence similarity of 88% to porcine FcγRIIb (Uniprot, Q461P7), and only 61% to porcine FcγRIIIa (Uniprot, Q28942) whereas the ECD region of porcine FcγRIIa and FcγRIIb are highly similar to each other (95.3%).

Cellular distribution of FcγRs

Understanding the functional impact of FcγRs requires a thorough characterization of their expression pattern in different cell types. Hereto, the expression of the different FcγRs in minipig PBMCs was addressed by single-cell RNA sequencing in comparison to human and mouse (Fig. 6). This technology was previously used to identify novel immune cell subtypes and monitor responses after immune activation (Jaitin

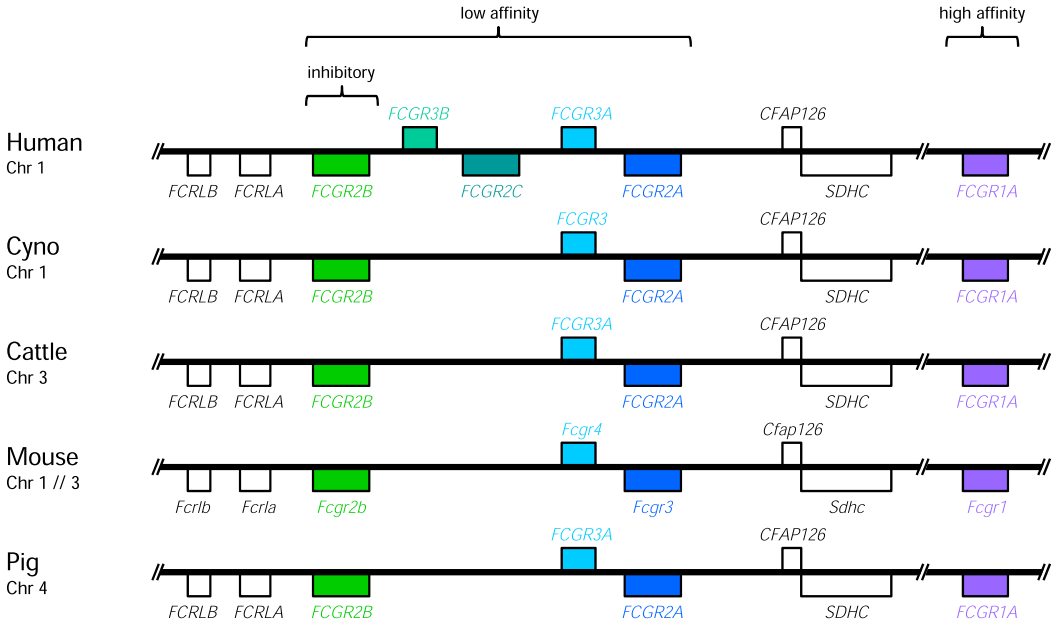

Fig. 4 Genomic organization of the *FCGR* locus in human, cyno, cattle, mouse, and pig according to the Ensembl database. The black lines represent a stretch of genomic DNA interrupted by lines indicating a gap of diverse length. All species shown here, except the mouse, carry the gene coding for the low-affinity receptors on the same chromosome. Boxes above and below the black line indicate genes oriented in forward and reverse orientation, respectively. Open boxes represent conserved genes flanking the *FCGR* locus, whereas colored boxes represent various *FCGR* genes found in the species indicated on the left

et al. 2014; Villani et al. 2017); however, the cross-species comparison was not performed yet. First, cells of every species were clustered according to their expression profile and displayed by dimensionality reduction on the t-SNE plots (Fig. 6). Then, we identified clusters composed of NK cells, cytotoxic T lymphocytes, T cells, and B cells in all species by their characteristic expression profiles (Online Resource 2). Such an approach enables to enumerate the expression levels of any gene of interest in all cell types in an antibody-independent manner. It was striking to see that minipigs have a considerably larger part of PBMCs assigned to the monocytic lineage. At the same time, the number of B cells identified in minipig PBMCs is smaller than in humans and significantly smaller than in mouse PBMCs. Subsequently, the

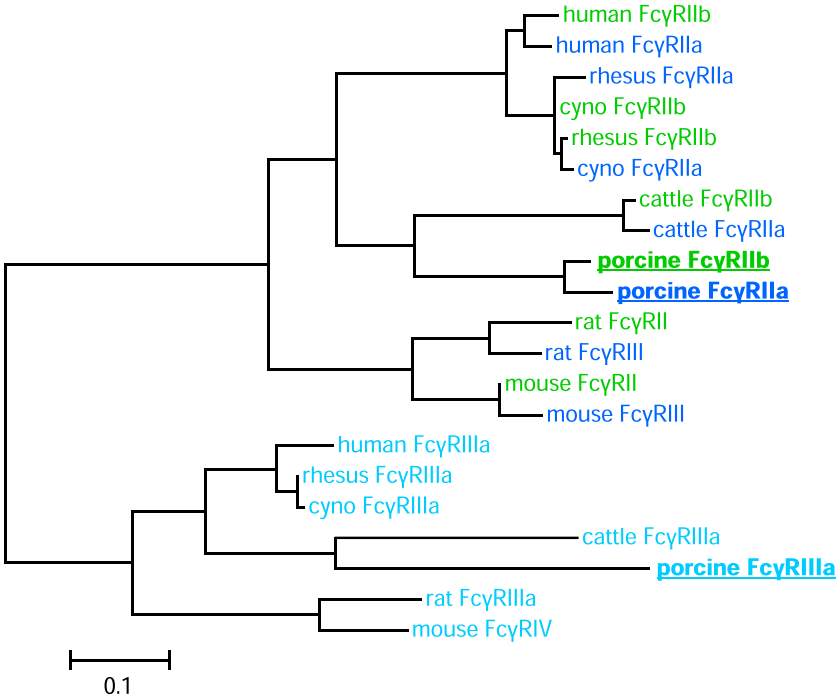

Fig. 5 Phylogenetic tree of FcγR proteins in different species. Inhibitory human FcγRIIb and its orthologues are colored in green, whereas low-affinity human FcγRIIa and its orthologues are shown in dark blue. All human FcγRIIIa orthologues are colored in light blue. Porcine FcγRs are displayed in bold and underlined

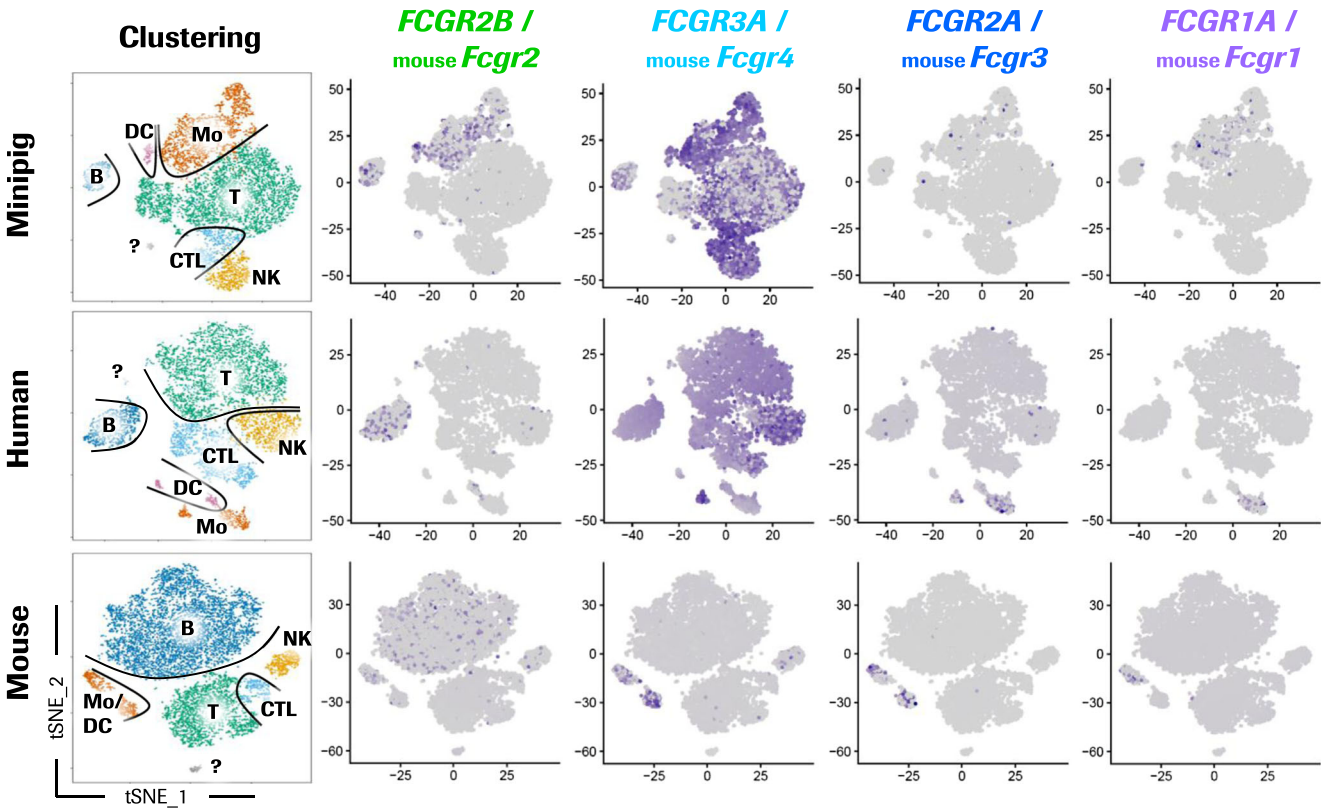

Fig. 6 Single-cell RNA sequencing analysis of *FCGR* expression in minipig, human, and mouse PBMCs. For every species, the cells were clustered individually according to their gene expression pattern and displayed as dot plots by dimensionality reduction using t-SNE. The clustering for every species is shown on the left with outlines for better separation. Individual clusters are labeled with "Mo" for monocytes, "DC" for dendritic cells, "NK" for NK cells, "CTL" for cytotoxic T lymphocytes, "T" for T cells, "B" for B cells, and "?" for mixture cell types. In mouse PBMCs, monocytes and dendritic cells are summarized in the "Mo/DC" cluster. The visualization shows the expression of the *FCGR* indicated above where positive cells are labeled in blue and negative cells in gray

mRNA expression of the different FcγRs was then analyzed in every species (Fig. 6).

The activating low-affinity FcγRIIIa is most strongly expressed among the FcγRs in all the species studied here. Minipig PBMCs revealed a strong and relatively homogeneous FcγRIIIa expression on all monocytes, DCs, NK cells, and cytotoxic T lymphocytes. Interestingly, T cells and B cells showed heterogeneous expression suggesting either different cell subsets or activation states. Human monocytes are often separated in classical, intermediate, and non-classical monocytes according to the CD14 and CD16 (FcγRIIIa) expression (Ziegler-Heitbrock 2015). As expected, the larger $CD14^{high}$ classical monocyte subset did not express FcγRIIIa, whereas the minor non-classical $CD14^{low}$ subset was strongly positive for FcγRIIIa. Also, in mice, it is the cluster containing the monocytes that shows expression for FcγRIIIa, while the other immune cell types, in contrast to the other species, show no expression. The inhibitory low-affinity FcγRIIb was found to be expressed mainly on monocytes, B cells, and DCs of the minipig. Human monocytes were not found to express FcγRIIb, while mouse FcγRII was weaker expressed in the monocyte and DC cluster as compared to the minipig. Expression of FcγRIIb in human and mouse PBMCs was mainly found in B cells. FcγRIIa, the activating low-affinity receptor we identified with our mapping strategy, is expressed at lowest levels in minipigs and humans. In the minipig, FcγRIIa mRNA was only detected in very few cells of the monocyte cluster. More monocytes were positive in the human and expression levels are slightly higher. Mouse FcγRIII, the orthologue of FcγRIIa, is expressed on most cells of the monocyte/DC cluster at highest levels compared to the other species. Similar expression levels and patterns were observed for FcγRIa. In the minipig, the expression is at low levels and restricted to monocytes. In humans, $CD14^{high}$ CD16- classical monocytes express FcγRIa, in contrast to $CD14^{low}$ CD16+ non-classical monocytes. Mice show a similar FcγRI expression pattern on a subset of the monocyte/DC cluster.

As gene expression studies only measure the mRNA, which may not fully reflect surface protein expression, we performed flow cytometry to assess the FcγR expression in the blood of three Göttingen minipigs. Cell types were identified according to the forward and side scatter properties, and their identity was confirmed using specific antibodies (Online Resource 4). Figure 7 shows a strong

staining with the FcγRIIa-specific HuCAL antibody on platelets (P1) and a weak staining on a subpopulation of eosinophils (P5). The FcγRIIa/b cross-reactive HuCAL antibody stains platelets, most monocytes (P3), and some eosinophils as well. FcγRIIIa staining was observed with varying intensities on monocytes, neutrophils, and eosinophils. Only a few cells were positive in lymphocyte population (P2).

Discussion

The three different classes of FcγRs form a finely tuned system required for efficient immune reactions in mammals. Minipigs represent a valuable alternative to NHP in preclinical studies. Thus, it is of particular importance to know all FcγR components in a preclinical animal model intended for testing of therapeutic antibodies. The characterization of the low-affinity FcγR proteins and genes in minipigs should provide a basis for preclinical studies with therapeutic antibodies.

While the inhibitory receptor is widely described as FcγRIIb (in the mouse known as FcγRII), the nomenclature of the low-affinity-activating FcγRs has evolved in a far more divergent manner. The low-affinity FcγRIIa is well-known in humans and has been described in the NHP, cattle, and other mammals, such as rabbits and sheep (Akula et al. 2014). The orthologue in the mouse, however, was named FcγRIII at its discovery (Nimmerjahn and Ravetch 2006). This receptor was initially not known in pigs due to an incomplete genome characterization and therefore was not described by Akula et al. (2014). In the present study, we were able to identify the putative porcine FcγRIIa located on chromosome 4 of the Göttingen minipig. The orthologue to human FcγRIIIa is known in NHPs, cattle, and other mammals, including the mouse, where it was designated as FcγRIV (Nimmerjahn et al. 2005). The orthologous FcγRIIIa cDNA and protein were also described in the pig but the corresponding gene

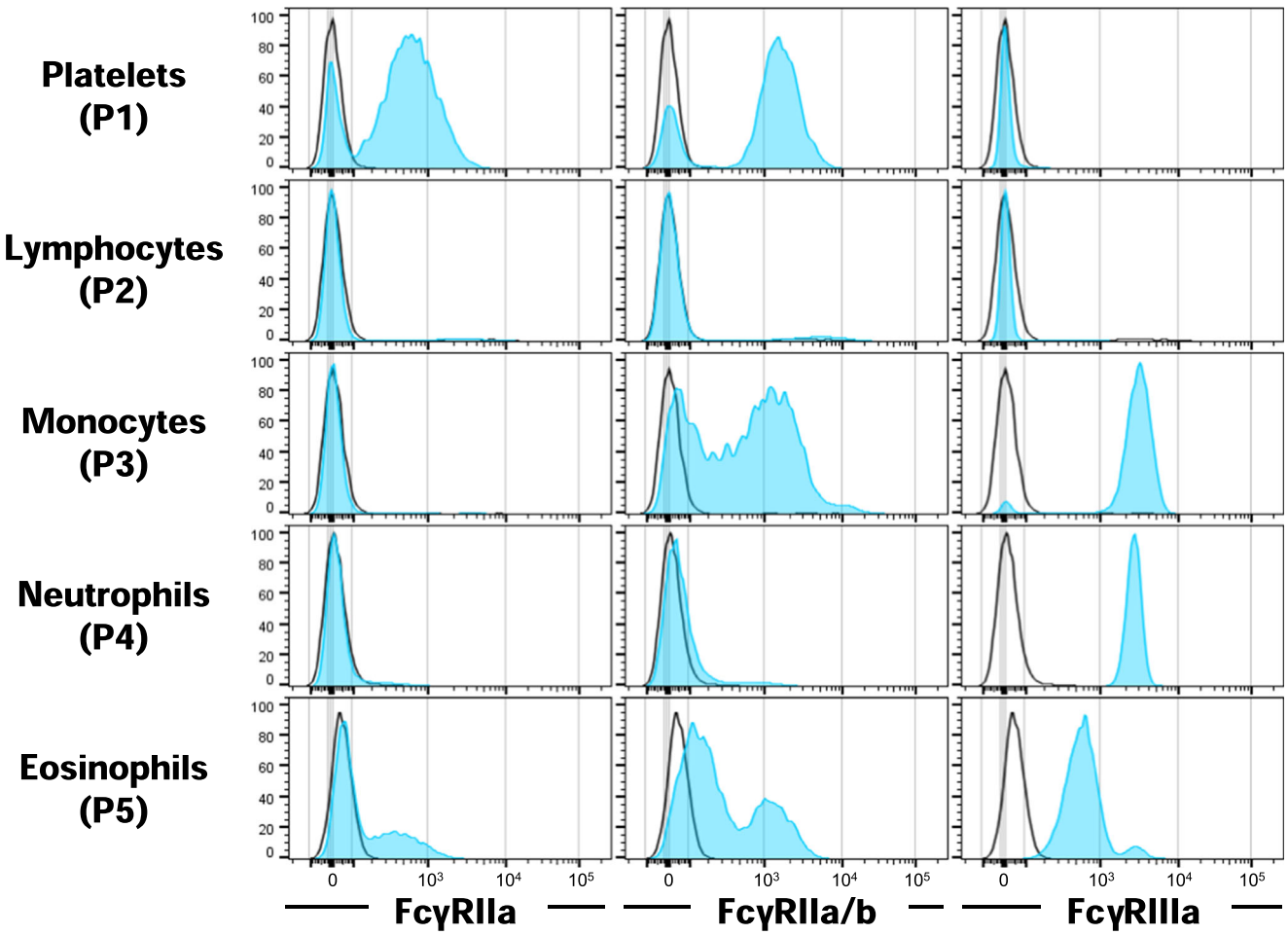

Fig. 7 Flow cytometry analysis of FcγR distribution on minipig blood leukocytes. Gating strategy is shown in Online Resource 4. Histograms normalized to mode show stainings observed using an FcγRIIa-specific HuCAL antibody, an FcγRIIa/b cross-reactive HuCAL antibody, and an anti-CD16 (FcγRIIIa) antibody in blue. Stainings with a HuCAL control antibody are shown as an overlay with a black line representing the background. A representative analysis of one out of three experiments with different Göttingnen minipigs is shown

and its genomic localization was unknown (Akula et al. 2014; Halloran et al. 1994). Here, we describe the localization of the gene *FCGR3A* encoding the minipig FcγRIIIa between *FCGR2B* and the putative *FCGR2A* on chromosome 4 of the Göttingen minipig on the forward strand. The identification of the putative *FCGR2A* and the localization of *FCGR3A* in pigs allow the comparison of the low-affinity *FCGR* locus to other species. We found that this locus of the minipig is organized similarly as in NHP, cattle, rat, and mouse with the position of the putative porcine *FCGR2A* gene coinciding with the other species. Nevertheless, significant differences to the human *FCGR* locus were observed. Thus, the complete characterization of the low-affinity *FCGR* locus of the minipig presented here confirms the absence of genes coding for homologs of the human FcγRIIIb and FcγRIIc, as is the case for all other animal species studied so far.

Sequence similarity displayed in the phylogenetic tree in Fig. 5 shows that FcγRIIa and FcγRIIb of the same species usually cluster together, probably originating from a duplication event early in speciation (Akula et al. 2014). The high similarity of the ECD region of porcine FcγRIIb to the newly identified porcine FcγRIIa fits in the pattern observed with the corresponding receptors from other species. Therefore, we suggest naming the transcript FcγRIIa. However, as detailed in Fig. 3, exons coding for the signal region and the TM/C region of the FcγRs appear to be shuffled during gene duplications and rearrangements leading to a mosaic structure that is characteristic for primates, rodents, and artiodactyls, respectively. Predictions suggest intracellular signaling by porcine FcγRIIa via interaction with the FcR γ-chain as it is described for cattle FcγRIIa and mouse FcγRIII (Lux and Nimmerjahn 2013). This similarity strengthens the hypothesis of the orthology among these receptors. On the other hand, FcγRIIa in primates is known to signal via integrated intracellular ITAM. It should be considered that differences in ITAMs potentially lead to functional differences between Fc receptors (Herik et al. 1995).

Two potential polymorphisms, A11S and H205Y, were identified in the main FcγRIIa transcript. The first located in the signal region and the latter was identified in the Ig2-like part of the ECD involved in the interaction with IgG antibodies (Fig. 3). Due to its location, the H205Y polymorphism could potentially influence binding affinities to certain IgG subclasses. Apart from that, we found three potential isoforms of porcine FcγRIIa with unknown functions and significance, probably generated by alternative splicing. Similar splice variants were already described for porcine FcγRIIb (Xia et al. 2012; Xia et al. 2011) and FcγRIIIa (Jie et al. 2009). In particular, humans were shown to have splice variants and polymorphisms with significant functional consequences. Altered binding affinities are associated with the outcome of therapeutic antibody treatments and with disease progression (Bournazos et al. 2010; Ziakas et al. 2016). Studies with more minipigs are required in order to assess the potential incidence of polymorphisms, splice variants, and sub-isoforms. Additionally, their biological relevance remains to be assessed.

Biological responses triggered by FcγRs do not only depend on the affinity of IgG interaction but also on their cellular distribution (Albanesi and Daeron 2012). Knowing the expression of FcγRs on immune cells facilitates the estimation of effects triggered by IgG interaction. We performed single-cell RNA sequencing on minipig, human, and mouse PBMCs to study the FcγR expression profile on various cell types.

In the Göttingen minipig, FcγRIa transcripts were only identified in monocytes at similar levels as observed in human and mouse. Like in humans, no FcγRIa expression was detected in minipig blood DCs although FcγRIa expression was often reported in human DCs (Nimmerjahn et al. 2015; Tamoutounour et al. 2012). FcγRIa expression, however, was usually analyzed in tissue resident or induced DCs and not found in blood DCs (Langlet et al. 2012). Devriendt et al. (2013) showed that the FcγRIa expression profile on porcine DCs depends on the activation stimulus, and similar findings were observed for human DCs. Therefore, FcγRIa expression can neither be excluded from minipig blood DCs nor from tissue-resident subsets. Varying expression levels of FcγRIa between minipig and human DCs could, however, result in varying capacity for antigen presentation by immune complexes and cytokine production (Cohen-Solal et al. 2004; van der Poel et al. 2011).

Only a few monocytes of the minipig showed weak staining for FcγRIIa. Generally, the FcγRIIa expression in PBMCs seems to be lower in the minipig as compared to humans and mice. This low expression was also observed in porcine gene expression data from NCBI (Li et al. 2017). Low expression of FcγRIIa in monocytes could theoretically be upregulated upon inflammatory stimuli similar to other activating Fc receptors (Nimmerjahn et al. 2005; Pricop et al. 2001). Like humans, minipigs express FcγRIIa on platelets as detected by flow cytometry (Rosenfeld et al. 1985). Platelets are mediators of immune responses upon binding of IgG immune complexes via FcγRIIa. This interaction can lead to platelet activation, phagocytosis, and ultimately to thrombus formation with pathological consequences (Worth et al. 2006; Zhi et al. 2015). The minipig might thus be a good model to study platelet-mediated functions and side effects of therapeutic antibodies, such as bevacizumab-induced retinal vein thrombosis, in contrast to mice that do not express FcγRIIa on platelets (Meyer et al. 2009). Gene expression data from NCBI Gene show that FcγRIIa is mainly expressed in the liver and the lung of pigs. Generally, the porcine FcγR expression is mainly detected in the liver, lung, and spleen tissue. This expression profile suggests that FcγRIIa mediates important immune functions in tissue-resident cells other than platelets in the blood.

Single-cell RNA sequencing of minipig PBMCs shows FcγRIIb expression on B cells, DCs, and monocytes. FcγRIIb expression on monocytes correlated with flow cytometry data using FcγRIIa specific and FcγRIIa/b cross-reactive HuCAL antibodies. Presently, the exact cellular distribution of FcγRIIb cannot be evaluated due to the lack of specific antibodies. A previous study postulates cross-reactivity of anti-human CD32 antibody (AT10) without showing data (Balmelli et al. 2005), a finding that could not be confirmed in our hands (not shown). The expression of FcγRIIb on minipig B cells and DCs reflects the situation in humans. On the other hand, minipig and mouse blood monocytes were found to express FcγRIIb as well, whereas human blood monocytes do not (Nimmerjahn et al. 2015). Low levels of FcγRIIa together with high levels of FcγRIIb on minipig monocytes could result in enhanced inhibitory signaling compared to humans. Hence, this could lead to an underestimation of effects or toxicity observed in minipig studies with therapeutic antibodies with FcγR-mediated effector functions.

Porcine FcγRIIIa was so far the best studied Fc receptor due to its high expression and the availability of specific antibodies. Its expression pattern was closely reflected in our single-cell RNA sequencing and flow cytometry analysis (Piriou-Guzylack and Salmon 2008). Minipig and human FcγRIIIa was found to be the highest expressed FcγR in PBMCs. In both species, T cells and B cells were found to express FcγRIIIa mRNA. Whereas FcγRIIIa expression on human T cells is controversially discussed in the literature (Nimmerjahn and Ravetch 2008), it can be excluded on B cells. Therefore, the FcγRIIIa expression in T cells and B cells of both species is considered as unspecific or represents different subsets or activation states. The difference between minipig and human is that FcγRIIIa is only expressed on monocyte subpopulations in humans, whereas it is expressed in all monocytes in the pig (Rubic-Schneider et al. 2016). The ubiquitous expression of activating FcγRIIIa on minipig monocytes could possibly counteract the inhibitory effects of FcγRIIb and the low levels of FcγRIIa. In therapeutic antibody research, a careful evaluation of the interaction to the various FcγRs would be needed to estimate the activation or inhibition potential of the antibody on minipig monocytes. Altogether, the human expression pattern of these FcγRs is more concordant with porcine than with murine monocytes (Fairbairn et al. 2013). The expression pattern of FcγRs is known to vary not only between species but also between individuals. As mentioned before, it can also be influenced by different stimuli, the immune status, or upon treatment. Therefore, further studies with more minipigs under different conditions are required to make a precise statement about the FcγR distribution in health and disease.

Our work allowed the localization of FcγRIIIa and the identification of the hitherto undescribed FcγRIIa on chromosome 4 of the Göttingen minipig. The newly identified FcγRIIa described here is considered as an orthologue to human, NHP, and cattle FcγRIIa as well as to mouse FcγRIII due to the highly conserved extracellular structures. The identification of FcγRIIa completes the picture of FcγRs in the pig and provides the genetic foundation for further studies. Our expression studies are the first to describe the expression of FcγRIa in monocytes and FcγRIIa on platelets of the Göttingen minipig. Additionally, FcγRIIb was found in monocytes, DCs, and B cells. The higher expression of FcγRIIIa and FcγRIIb and the lacking expression of FcγRIIa on monocytes are different to humans. Therefore, effects on monocytes should be carefully evaluated before using the minipig in preclinical studies with therapeutic antibodies. Nevertheless, FcγRIIa expression on platelets makes the minipig a valuable model to study platelet-mediated effects of therapeutic antibodies which are hard to evaluate in mice.

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Online Resource 1 List of primers used for amplification of FCGR sequences and the identification of the putative porcine FCGR2A transcript. Refer to Fig. 1 for an overview of the primer location.

Primer	Region	Orientation	Sequence
JE2	*FCGR2A* Ig2	forward	CCAGCCTCTCCATCCCACATGCAAACC
JE4	*FCGR2A* TM/Cyt	reverse	GCAAAAAGGAGCCCCATCGCCAGGTAG
JE5	*FCGR2A* 3'UTR	reverse	GGCCCAAGTTGCTGTTAAGTCGGGGCTG
JE24	*FCGR3* Ig2	forward	CTTCGGAGGCTGTGAAAGTC
JE26	*FCGR3* TM	reverse	TGATGGGATAGGTGATGGAC
JE28	*FCGR2A* Ig2	forward	ACCCCTAGCCTGGTGTTCC
JE35	*FCGR2A* 5'UTR	forward	TGCGTACTCCAGGAGGTGATGG
JE36	*FCGR2A* 5'UTR	forward	TGCTATTCCTGGCTCCTGTTCC
JE41	*FCGR2A* intron	forward	GGTCAGTCTCTTGGGTCAGC
JE42	*FCGR2A* intron	reverse	CCACCTAAGATGTGGTCCCAG
JE47	*FCGR2A* intron	forward	GGGCTCAATGACTGTTTGCTG
JE49	*FCGR2A* intron	reverse	CTGATCCTCCAGGGCAGTATCC
JE58	*FCGR2A* intron	forward	TCCAGGGGCCTTCTTATACTC
JE61	*FCGR2A* intron	reverse	AGCCCTCGGATGTATGAAAAG
JE62	*FCGR2A* intron	forward	TTGCTGGCCTGTTAGTACCTG
JE64	*FCGR2A* intron	reverse	GAGGAGCCTACGTTTGGAATC
UPM	5' or 3' RACE		CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPM-short	nested primer		CTAATACGACTCACTATAGGGC

Online Resource 2 List of differentially expressed genes used to summarize clusters to the indicated cell types. In the mouse, it was not possible to separate monocytes and dendritic cells.

	Minipig	Human	Mouse
Monocytes	*SIRPA*	*CD14*	*Cd14*
	CD14	*CD16*	*Cd68*
	CD163	*CX3CR1*	*Adgre1*
	SLA-DRB1	*ITGAM*	*Lgals3*
	SLA-DRA	*CD163*	*Apoe*
	FCN1	*CD68*	*Mafb*
	LGMN	*CD86*	*Fcgr3*
	TREM1	*CSF1R*	*Ly6e*
	CLEC4E	*CCR2*	*H2-Aa*
	CLEC7A	*SELL*	*H2-Eb1*
	CCR2		
	CYP1B1		

	Minipig	Human	Mouse
DC	*SIRPA*	*ITGAX*	*Itgax*
	CD14	*PLAC8*	*Thbd*
	FLT3	*FCER1A*	*Cd38*
	ITGAX	*IL3RA*	*Cd209a*
	PLAC8	*CD1C*	*Cd74*
	FCER1A	*CD33*	*Flt3*
	CD74	*CD1E*	*H2-Aa*
	SLA-DRB1	*HLA-DRB1*	*H2-Eb1*
	SLA-DRA	*CLEC4C*	*Ifi30*
	CD33	*NRP1*	*Napsa*
	IFI30	*LY75*	*Itgb7*
	ITGB7	*ANPEP*	*Syngr2*
	SYNGR2		*Clec10a*
			Ahr
			Tlr13
			CD24a

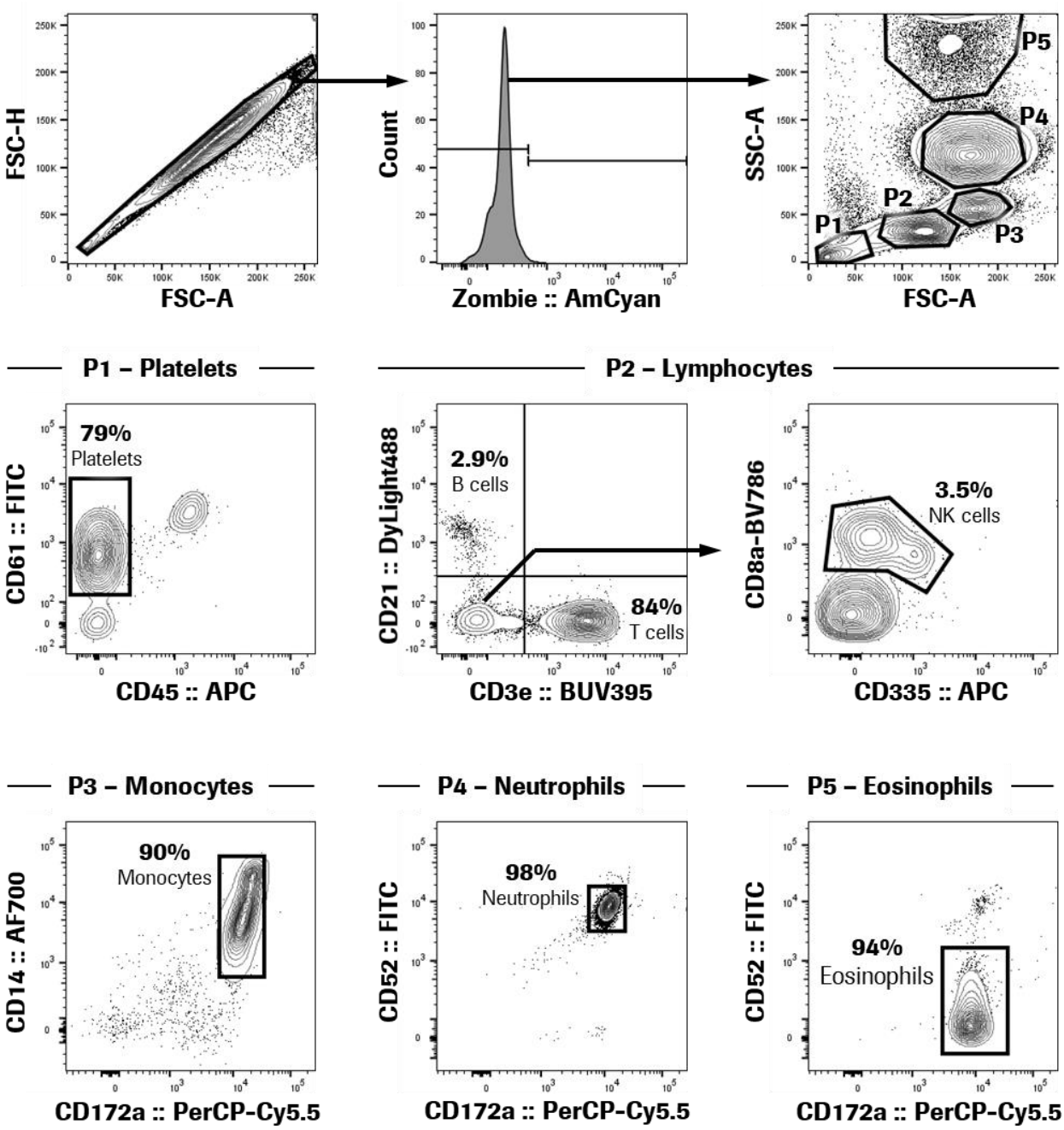
	Minipig	Human	Mouse
B cells	*CD79A*	*MS4A1*	*Cd79a*
	CD19	*CD19*	*Cd19*
	MS4A1	*CD79A*	*Cr2*
	SLA-DRB1	*CD40*	*Fcer2a*
	SLA-DRA	*CD86*	*CD22*
	CD86	*HLA-DRB1*	*Ms4a1*
	CD40		*CD86*
			H2-Ab1
			Cd24a
			Cd38
			CD40

	Minipig	Human	Mouse
T cells	*CD3*	*CD3E*	*Cd3e*
	CD4	*CD4*	*Cd3d*
	CD8	*CD5*	*CD40*
	ITGB1	*IL2RA*	*CD8a*
	CD5	*CCR7*	*CD8b1*
	FOXP3	*MAL*	*CD5*
	IL2RA		*Ms4a4b*
	CCR7		*Cd28*
	Cd28		*Il7r*
	Il7r		*Lef1*
	Lef1		*Dapl1*
	Dapl1		

	Minipig	Human	Mouse
Cytotoxic T cells	*CD3E*	*CD3D*	*Cd3e*
	CD8A	*CD8A*	*Cd8a*
	GZMK	*CD8B*	*Gzmk*
	GZMB	*GZMK*	*Gzmm*
	GZMA	*GZMH*	*Ccl5*
	GNLY	*GZMA*	*S100a6*
	CCL5	*CCL5*	*Lgals1*
	Klrk1		*Klrk1*
	Lgals1		*Crtam*
	Crtam		*Eomes*
	Eomes		
	GZMH		
	Gzmm		
	S100a6		

	Minipig	Human	Mouse
NK cells	*KLRB1*	*NCR1*	*Ncr1*
	NCR1	*NCAM1*	*Klrb1c*
	PRF1	*NCR3*	*Klrd1*
	GZMA	*KLRC1*	*Klrc1*
	GZMB	*KLRD1*	*Klrk1*
	GNLY	*B3GAT1*	*Itga2*
	KLRD1	*NKG7*	*Gzma*
	NKG7	*KLRB1*	*Gzmb*
	CCL5	*CCL5*	*Prf1*
	KLRK1	*GNLY*	
		GZMB	

Online Resource 3 Nucleotide sequence of the low affinity FCGR locus of the minipig including FCGR3A in forward orientation and FCGR2B and FCGR2A in reverse orientation. Exon sequences from FCGR2B, FCGR3A, and FCG2A are highlighted in green, light blue, and dark blue, respectively. Adjacent 5' and 3' untranslated regions are marked in grey. Splice acceptor (AG or CT) and donor (GT or AC) sites are bold and underlined. Start and stop codons are marked with an open box.
Available online in the published version or on request



Online Resource 4 Gating strategy for flow cytometry analysis of minipig blood. Whole blood from Göttingen minipigs was stained with the indicated fluorochrome-labeled antibodies. From single and live cells, gates P1-P5 were selected using forward (FSC) and side scatter (SSC) and cell types were identified using the following antibody clones: CD45 (K252.1E4), CD61 (JM2E5), CD3e (BB23-8E6-8C8), CD21 (BB6-11C9.6), CD335 (VIV-KM1), CD8a (76-2-11), CD172a (74-22-15A), CD14 (MIL2), and CD52 (11/305/44). Numbers indicate the percentage of cells within the respective population (P1-P5).

6.4 Supplementary experiments

This section describes and discusses further results collected during the characterization of poFcγRs, beyond the results shown in Manuscript 1.

To recap, Fig. 6.1 summarizes the expression of poFcγRs on cells of the immune system to provide a general overview. This summary reflects compiled data from Manuscript 1 and previously published studies on FcγRs in pigs [84, 86, 87].

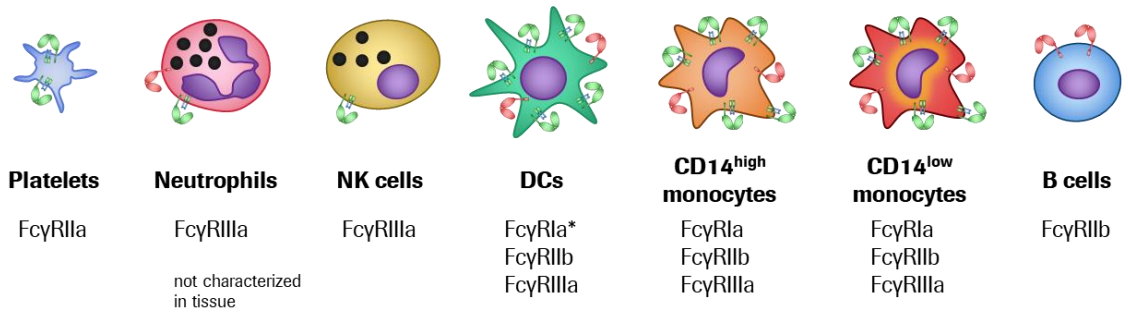


*Fig. 6.1 Expression of poFcγRs on immune cells of the minipig. Inhibitory (red) and activating (green) FcγRs are shown on cells involved in antibody-mediated effector functions analogous to Fig. 4.3. The CD14 expression separates human and porcine monocytes in classical ($CD14^{high}$), intermediate, and non-classical ($CD14^{low}$) monocytes with varying poFcγRIIIa levels [84]. The inducible expression of poFcγR is so far not studied and therefore not reflected in this figure. * Indicates the absence of poFcγRIa on DCs in the blood.*

6.4.1 Characterization of the genomic *FCGR* locus and its genes in the minipig

Manuscript 1 describes the complete assembly of the low affinity *FCGR* locus from next generation sequencing data of minipigs. We found that this locus was not correctly assembled in the chromosome draft of the Göttingen minipig and the pig genome *Sus scrofa* 10.2 probably due to repetitive sequences within *FCGR* genes. Furthermore, we identified problems with the integrity of the whole genome shotgun contig AJKK01167168 of the Wuzishan minipig (Manuscript 1 Fig. 1 blue line on the far right). This contig included the beginning of *FCGR2A* next to other sequences of *FCGR2B* suggesting a wrong assembly and questioning the integrity of the other whole genome shotgun contigs. However, the product length of PCR screenings confirmed their integrity and the correct transitions between neighboring contigs.

We also tried to sequence the locus around the newly described *FCGR2A* at the contract research organization Cergentis. For this investigation a technology called Targeted Locus Amplification with *FCGR2A* specific primers was utilized. This technology amplifies and sequences nucleotides in close physical proximity to the primers [88]. From this approach, we obtained many sequencing reads mapping to different regions within the *FCGR2A* gene. Nevertheless, it was not possible to use these reads for any assembly due to their process-related fragmentation.

6.4.2 Distribution of FcγRs on immune cell subsets and in organs of the minipig

The distribution of poFcγRs on the cell surface of various immune cells in the blood was studied in Manuscript 1. Expression patterns of huFcγRs, however, have been characterized more thoroughly for example in minor immune cell subsets in blood and immune organs. For example, NK cells that are important mediators of ADCC via huFcγRIIIa only represent a small fraction of lymphocytes [89]. Their detection by flow cytometry requires a combination of different cell surface markers to be distinguished from other blood lymphocytes. Moreover, huFcγRIIb is largely absent on monocytes and neutrophils in the blood, but present in spleen and lymph nodes [12]. Therefore, it is important to further analyze immune cell subsets in various tissues of the minipig for the expression of different poFcγRs.

To get a more detailed view on the poFcγR distribution on specific cell subsets in different organs of minipigs, we performed multicolor flow cytometry stainings including diverse cell type-specific surface markers. Blood, lymph nodes, and spleen of two minipigs were collected by pathologists. The organs were passed through a 70 µm cell strainer to generate single cell suspensions. Then, cells were stained with different cocktails of cell surface markers together with one FcγR antibody at the time. PoFcγRIIa and poFcγRIIa/b were stained using unlabeled HuCAL antibodies (clones AbD29332.1 and AbD32591.1, respectively) and poFcγRIIIa was detected using the commercially available PE labeled anti-pig CD16 (clone G7) antibody. The lymphoid staining includes CD3e (BUV395, BB23-8E6-8C8), CD8a (BV786, 76-2-11), CD4a (PE-Cy7, 74-12-4), CD335 (APC, VIV-KM1), and CD21 (DyLight488, BB6-11C9.6) enabling the identification of B cells, T helper and T effector cells, and NK cells. Platelets were identified in another staining with CD61 (FITC, JM2E5) and CD45 (AF647, K252.1E4). A myeloid staining was also included using CD14 (AF700, MIL2), CD172a (PerCP-Cy5.5, 74-22-15A), CD4 (PE-Cy7, 74-12-4), and CD52 (FITC, 11/305/44) for the identification of monocytes, DCs, neutrophils, eosinophils, and basophils. In this experiment, we used the secondary PE labeled goat F(ab')2 anti-human IgG antibody (Jackson) to detect the unlabeled HuCAL antibodies. The use of other secondary antibodies or direct labeling with different antibody labeling kits did not improve the results.

Fig. 6.2A shows the gating of the lymphoid cell subsets in the different organs obtained with the lymphoid staining after gating on single and live cells. The poFcγRIIa and poFcγRIIa/b expression on B cells, T cell subsets, and NK cells in blood, lymph node, and spleen is shown in Fig. 6.2B. The detailed analysis of minipig whole blood revealed poFcγRIIIa staining of NK cells and neutrophils, but not of B cells and T cell subsets, as previously described (not shown [87]). However, flow cytometry analysis of lymphoid cells within the blood, lymph node, and spleen did not reveal further expression of poFcγRIIa and poFcγRIIb (Fig. 6.2B). As described in Manuscript 1, we found poFcγRIIa on blood platelets and poFcγRIIb on blood monocytes of the minipig (not shown). The results from single cell RNA sequencing

of minipig peripheral blood mononuclear cells (PBMCs) indicate the expression of poFcγRIIb in B cells (Manuscript 1 Fig. 6). Interestingly, we did not detect expression of poFcγRIIb in CD3- CD21+ B cells by flow cytometry (Fig. 6.2B). In general, a high background was observed with the HuCAL control antibody in combination with the secondary anti-human IgG antibody masking a possible specific staining for poFcγRIIb (Fig. 6.2B). Importantly, the flow cytometry analysis excluded the expression of poFcγRIIa and poFcγRIIb on NK cells and T cell subsets in blood, lymph nodes and spleen.

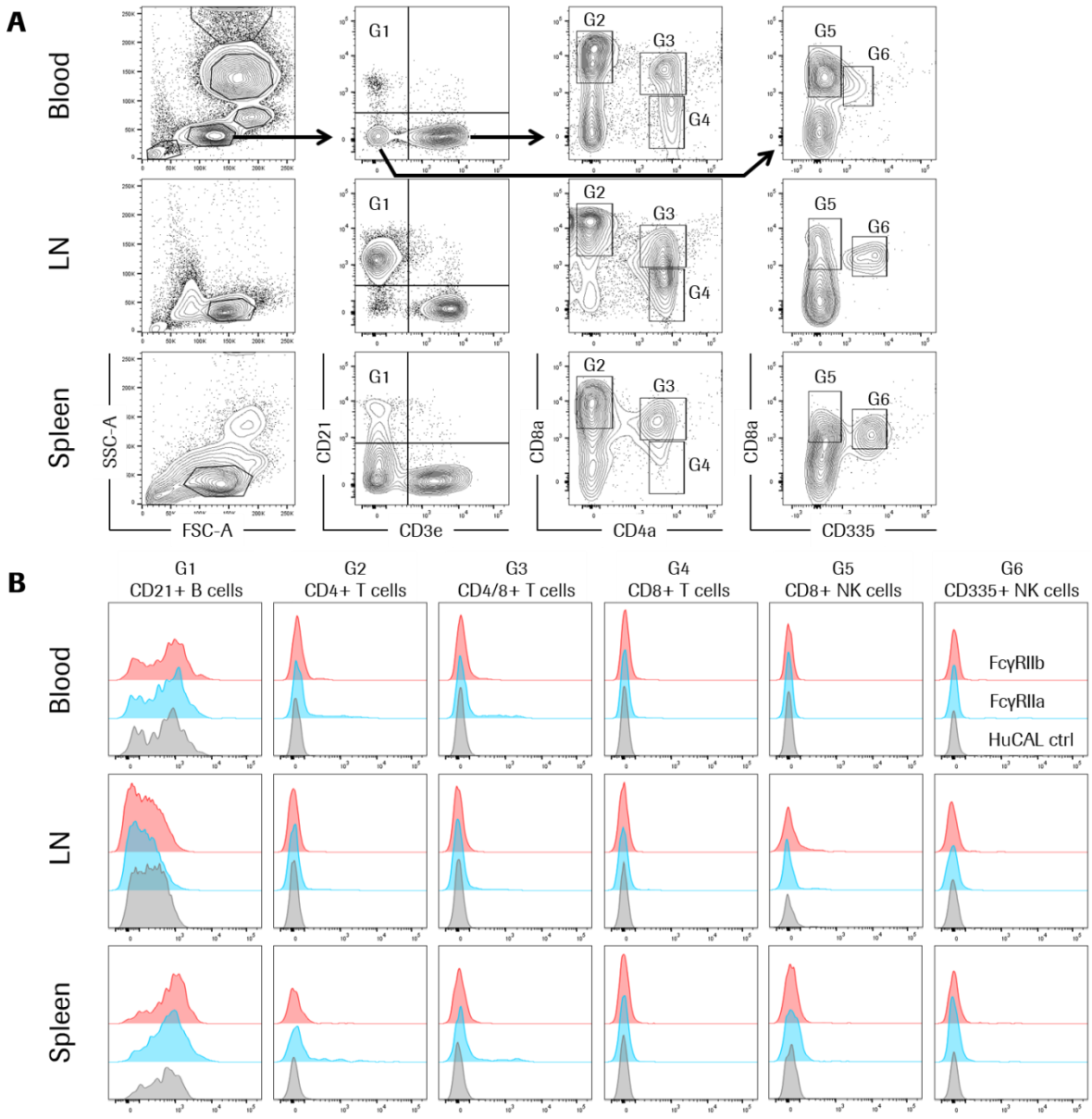


Fig. 6.2 Detailed flow cytometry analysis of the poFcγR distribution on blood, lymph nodes and spleen of a Göttingen minipig. ***(A)*** *Gating strategy for identification of specific lymphocyte subsets. T cells were separated from B cells (G1) by staining with CD3e (BUV395) and CD21 (DyLight488). CD3e positive T cells (G2-G4) were further characterized by CD4a (PE-Cy7) and CD8a (BV786). CD3e negative cells were further divided in two NK cell subsets (G5 and G6) using CD335/NKp46 (APC) and CD8a (BV786).* ***(B)*** *Histograms show stainings for poFcγRIIa (blue), poFcγRIIa/b (red), and HuCAL control antibodies (grey). Data from one out of two minipigs is shown.*

The myeloid staining for monocytes, DCs, and granulocyte subsets was also tested in all organs but has not worked together with the HuCAL antibodies due to high background signals. Further analyses revealed that the anti-human IgG antibody yielding best results for HuCAL antibody detection cross-reacted with many antibodies required for the myeloid staining (not shown). We only applied the platelet staining in the blood compartment, because CD45- CD61+ platelets were not expected in the other organs. This staining allowed the identification of poFcγRIIa on all platelets using HuCAL antibodies (not shown).

In another experiment, we stained minipig blood cells with different anti-human and anti-mouse FcγR antibodies to identify cross-reactive clones. Furthermore, lymph nodes and spleen cells were also analyzed with anti-human CD32 (FcγRIIa/b, clone AT-10) and CD64 (FcγRIa, clone 10.1) antibodies that were previously shown to be cross-reactive [90]. However, the results did not identify cross-reactive clones. The lacking cross-reactivity was also confirmed by enzyme-linked immunosorbent assay (ELISA) with recombinant porcine FcγRs (not shown).

FcγR expression studies in minipigs using HuCAL or human FcγR antibodies remain difficult due to low expression levels of certain FcγRs, lack of cross-reactivity, and assay-dependent limitations. The set of experiments presented here was omitted from the Manuscript 1 because it did not give further insights in the expression pattern of FcγRs in minipigs. The Fab domains of the HuCAL antibodies used here are dimerized via alkaline phosphatase and contain a FLAG and His6 protein tag (Fab-A-FH). This special format possibly causes high background in flow cytometry experiments due to unspecific interactions. The conversion into a fully human antibody and subsequent labeling with fluorochromes would eliminate the alkaline phosphatase and the need of secondary antibodies, thus preventing related background signals. Furthermore, the readily available soluble poFcγRs technically also allow the generation further antibodies specific for poFcγRIa or poFcγRIIb. Such tools would enable further cell surface expression studies in minipigs.

Apart from experiments with further HuCAL antibodies, we have observed poor poFcγRIIa expression in minipigs by single cell RNA sequencing compared to other species (Manuscript 1 Fig. 5). PoFcγRIIa could reflect the situation of huFcγRIIc that is expressed only in 7 to 15% of healthy individuals [91]. In this case, more minipigs and possibly also other breeds would have to be analyzed to cover a larger population. Alternatively, poFcγRIIa could be upregulated in inflammatory conditions. Upregulation of activating FcγR expression, such as huFcγRIa or mouse FcγRIV, was shown to be induced by lipopolysaccharide (LPS) or interferon gamma (IFN-γ). Similarly, other cytokines like interleukin (IL)-4, IL-10, or transforming growth factor beta (TGF-β) are known to upregulate the inhibitory FcγRIIb while downregulating activating FcγRs [14, 92]. In one experiment, we tried to stimulate different cell types by various stimulants and to detect poFcγRIIa upregulation on transcription and expression level.

Therefore, whole blood of one Göttingen minipig was stimulated for 48h with three concentrations of four different stimulants. We have chosen 1) LPS as a general inflammatory stimulus for monocytes and DCs via pattern recognition receptors [93]; 2) human tumor necrosis factor *alpha* (TNF-α) as an inflammatory mediator stimulating the differentiation of various cell types [94]; 3) Concanavalin A (ConA), a plant mitogen stimulating T cells in mice and humans [95]; and 4) cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) mediating immunostimulatory effects on B cells, NK cells and monocytic cells and known to enhance huFcγRIa-mediated cross-presentation of DCs [96, 97]. Following stimulation, the cells were analyzed for poFcγRIIa and poFcγRIIIa expression by flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR). The results did not show upregulation of the analyzed activation poFcγRs in the tested culture conditions (not shown). Reasons for the negative result could be manifold including the choice of stimulants, their concentrations, incubation times, and culture conditions as well as the detection methods. Although human TNF-α can directly stimulate porcine endothelial cells leading to upregulation of inflammatory markers, its porcine orthologue was shown to be more effective [98]. Additionally, the assay was not optimized resulting in a low viability of granulocytes. Further assay optimization and a broader range of stimuli would be necessary to investigate the induction of poFcγRIIa. Due to the negative result and the required assay optimization, we did not further assess the stimulation-induced upregulation of poFcγRIIa.

7 Interaction of human IgG with porcine FcγRs

7.1 Purpose

FcγR binding is crucial for effector functions of many therapeutic antibodies and hence for their mode of action and safety profile. So far, it was unknown how human antibodies interact with the poFcγRs of the minipig which represents a preclinical animal model of high interest. Cross-reactivity of human therapeutics with the immune system of the animal model is a prerequisite for species selection for preclinical studies. Therefore, we aimed to assess the binding of huIgG to poFcγRs in the minipig, as it was assessed for FcγRs in the mouse and the NHP (Fig. 7.1).

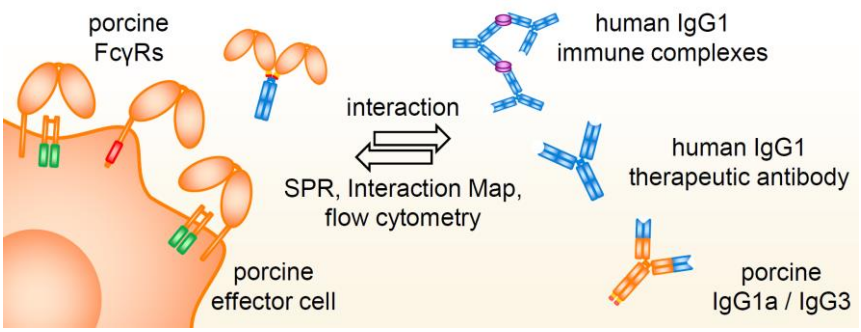


Fig. 7.1 Graphical abstract illustrating interaction studies assessing the binding of IgG antibodies to soluble and membrane-bound poFcγRs.

7.2 Main results

Surface plasmon resonance (SPR) analysis and cellular binding assays revealed that poFcγRIa, IIa, and IIb bind free- and immune-complexed therapeutic huIgG1 antibodies suggesting possible triggering of effector functions. PoFcγRIIIa in minipigs, however did not bind huIgG1 possibly leading to an underestimation of NK cell mediated efficacy or toxicity.

7.3 Manuscript 2

The interaction of minipig FcγRs with human IgG – implications for preclinical assessment of therapeutic antibodies

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Pharmaceutical Research 2019 36:47 doi: 10.1007/s11095-019-2574-y

Contribution – First, I designed, cloned and expressed all soluble and full-length poFcγRs, as well as HER2 specific poIgGs in the laboratory of Stefan Seeber. Subsequently, I purified and analyzed the soluble proteins, including IC, and interpreted the SPR data generated by Christian Spick. Furthermore, I conducted all phenotyping and cell-based assays and finally, drafted and wrote the manuscript.

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RESEARCH PAPER

The Binding of Human IgG to Minipig FcγRs – Implications for Preclinical Assessment of Therapeutic Antibodies

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ABSTRACT

Purpose The Göttingen minipig is a relevant non-rodent species for regulatory toxicological studies. Yet, its use with therapeutic antibodies has been limited by the unknown binding properties of human immunoglobulins (huIgG) to porcine Fc *gamma* receptors (poFcγR) influencing safety and efficacy readouts. Therefore, knowing IgG-FcγR interactions in the animal model is a prerequisite for the use of minipigs in preclinical safety and efficacy studies with therapeutic antibodies.

Methods Here, we describe the cloning and expression of poFcγRs and their interactions with free and complexed human therapeutic IgG1 by surface plasmon resonance and flow cytometry.

Results We show here that poFcγRIa, poFcγRIIa, and poFcγRIIb bind huIgG1 antibodies with comparable affinities as corresponding huFcγRs. Importantly, poFcγRs bind huIgG immune complexes with high avidity, thus probably allowing human-like effector functions. However, poFcγRIIIa binds poIgG1a but not to huIgG1.

Conclusions The lack of binding of poFcγRIIIa to huIgG1 might cause underestimation of FcγRIIIa-mediated efficacy or toxicity as mediated by porcine natural killer cells. Therefore, the suitability of minipigs in preclinical studies with human therapeutic antibodies has to be assessed case by case. Our results facilitate the use of Göttingen minipigs for assessment of human therapeutic antibodies in preclinical studies.

KEY WORDS antibody effector function · FcγR · Göttingen minipig · IgG · interaction map

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
FcγR	Fc *gamma* receptor protein
FCGR	Fc *gamma* receptor gene
FcR-γ chain	Fc receptor common *gamma* chain
HuCAL	Human combinatorial antibody library
IC	Immune complex
ITAM	Immunoreceptor tyrosine-based activation motif
NHP	Non-human primate
SPR	Surface plasmon resonance

INTRODUCTION

Fc *gamma* Receptors (FcγRs) are a family of gylcoproteins expressed on the surface of leukocytes. They interact with the fragment crystallizable (Fc) part of immunoglobulin G (IgG) antibodies and trigger a variety of effector functions including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), antigen internalization and presentation, or inflammatory cytokine release (1). The set of FcγRs of most mammalian species consists of the high affinity FcγRIa (CD64), low affinity FcγRIIa (CD32a) and FcγRIIIa (CD16), and the inhibitory FcγRIIb (CD32b) (2). Their cellular distribution and distinct affinities

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towards different IgG subclasses influence immune cell activation and control their effector functions upon IgG binding. Many novel therapeutic antibodies are IgG Fc engineered to alter the FcγR binding in order to achieve enhanced activity via ADCC or ADCP or to reduce effector function-mediated toxicity (3,4). Often, antibody effector functions are mediated upon interactions of low affinity FcγRs with immune complexes (IC). For example, IC formed by bevacizumab binding to vascular endothelial growth factor (VEGF) can lead to FcγRIIa-mediated platelet activation (5) and thrombosis in FcγRIIa transgenic mice (6). Thus, it is very important to characterize the binding of free- and immune-complexed IgG to different FcγRs as this can dramatically influence safety and efficacy.

The porcine species (*Sus scrofa*) is an increasingly used animal model for biomedical research. In particular the Göttingen minipig has gained importance for preclinical safety and efficacy studies due to its high similarity to the human (7,8). Also, the regulatory acceptance of the minipig as a relevant animal model for toxicological studies with biotherapeutics is growing (9). Furthermore, handling, housekeeping, and breeding of minipigs are much easier and cheaper than of non-human primates (NHP). So far, the Göttingen minipig has already been used for immunogenicity studies with infliximab and adalimumab (10). Presently, only few other minipig studies are performed with therapeutic antibodies (11) due to lacking knowledge about their pharmacology (12). Therefore, the importance of an adequate immunological characterization of the Göttingen minipig as a non-rodent species is widely recognized and promoted (13). The evaluation of the interactions of human therapeutic antibodies with porcine FcγRs (poFcγRs) is a basic requirement for the use of the minipig in preclinical studies. So far, only functional binding studies of poFcγRIa and variants of poFcγRIIb to porcine total IgG have been reported confirming the conserved function of these receptors in pigs (14,15). We have recently annotated the complete low affinity *FCGR* locus of the minipig including the localization of all poFcγR genes and the description of the hitherto unknown poFcγRIIa (16). Binding and function of NHP or mouse FcγRs interacting with human IgG (huIgG) were studied to assess cross-reactivity and to estimate the translation potential of this preclinical species (17–19). To our knowledge, no extensive studies investigating the interactions of huIgG to poFcγRs were performed for any porcine species. Thus, the lacking knowledge of the binding properties of huIgG to poFcγRs is still limiting the use of the minipig as a preclinical species with human therapeutic antibodies.

In the present work we hypothesized minipigs as a useful alternative for preclinical studies with therapeutic antibodies. Therefore, we qualitatively characterize the binding of human therapeutic antibodies to all FcγRs in the minipig. Furthermore, we assessed the binding of free- and immune-complexed huIgG1 antibodies to poFcγRs in comparison to huFcγRs. The data provide first insights into possible effector functionalities of human immunoglobulins in preclinical studies in minipigs.

MATERIALS AND METHODS

Recombinant FcγRs and Antibodies

Cloning

Soluble FcγRs were designed as dimeric IgG Fc fusion proteins. Extracellular domains of poFcγRIa (UniProtKB: Q461Q0), poFcγRIIa (XM_021089520.1; 205Y), poFcγRIIb (UniProtKB: Q461P7), poFcγRIIb1 (UniProtKB: B9VVN4), poFcγRIIIa (UniProtKB: Q28942) as well as huFcγRIa (UniProtKB: P12314), huFcγRIIa (UniProtKB: P12318 R131), huFcγRIIb (UniProtKB: P31994), huFcγRIIIa (UniProtKB: P08637 V158) were used. The sequences were back translated, codon optimized, and ordered as gene syntheses from GeneArt (Invitrogen). Subsequently they were cloned into an expression vector containing the signal peptide from mouse Ig heavy chain variable region, an Avi biotinylation tag (GLNDIFEAQKIEWHE, Avidity), a His6 tag, and an IgA protease cleavage site (VVAPP'AP). The vector also contained inert huIgG1 (PGLALA) Fc parts allowing the dimerization of the FcγR extracellular domains by the expression as Fc fusion proteins (20). These constructs are referred to as soluble FcγRs hereafter.

Full-length poFcγRIa (amino acids (aa) 16-346), poFcγRIIa (205Y; aa 46-274), poFcγRIIb (aa 46-297), poFcγRIIb1 (aa 46-316), and poFcγRIIIa (aa 20-257) contained the human CD33 signal peptide (MPLLLLLPLLWAGALA) and a FLAG-tag (DYKDDDDK) at the N-terminus. Full-length huFcγRs, the human Fc receptor common *gamma* chain (FcR-γ chain), and the poFcR-γ chain (UniProtKB: Q9XSZ6) were designed without the FLAG-tag.

PoIgG1a (GenBank: U03781.1) and poIgG3 (GenBank: EU372658.1) heavy chain and Ig-*kappa* light chain (21) constant regions were coupled to the variable regions of the anti-human epidermal growth factor receptor 2 (HER2) antibody trastuzumab heavy chain (DrugBank: DB00072; aa 1-120) and Ig-*kappa* light chain (DrugBank: DB00072; aa 1-108), respectively. The correct transitions between the variable and the constant region of both antibodies were confirmed by molecular modeling. The recombinant antibodies contained the mouse Ig heavy chain V region 3 signal peptide (MGWSCIILFLVATATGVHS) and a C-terminal Avi biotinylation tag (GLNDIFEAQKIEWHE, Avidity). The resulting HER2 specific poIgG constructs are named poIgG1a-HER2 and poIgG3-HER2 hereafter.

The sequences of all constructs were verified prior to expression by DNA-sequencing (SequiServe and Microsynth).

Expression

Soluble FcγRs and poIgGs were expressed in human embryonic kidney 293F (HEK293F) suspension cells cultured in shaker flasks (120 rpm, 37°C, 5% CO_2, 85% humidity) using F17 expression medium supplemented with Pluronic and GlutaMAX (Gibco). Plasmids coding for FcγRs were transfected alone and poIgG heavy chains were co-transfected in equimolar ratio with plasmids coding for poIg-*kappa* light chain. Transient transfection was performed using 293free (Merck Millipore) premixed with OptiMEM (Gibco) and expression was enhanced by feeding and addition of valproic acid. The fed-batch culture was harvested by centrifugation 7 days after transfection and the supernatant was cleared by filtration.

Full length FcγRs were transiently expressed using the Expi293 system (Thermofisher). Suspension cells were seeded in 6 well-plates (120 rpm, 37°C, 5% CO_2, 85% humidity) and co-transfected with porcine or human FcγRs together with the related FcR-γ chain in an equimolar ratio. The transfected cells were used 48 h post transfection.

Purification and Analysis

Soluble FcγRs and poIgGs were purified by protein A (MabSelect SuRe, GE Healthcare) or, in the case of soluble FcγRIa, by nickel (HisTrap HP, GE Healthcare) affinity chromatography using the ÄKTAexplorer 100 Air system (GE Healthcare). Soluble FcγRs were further purified by preparative size exclusion chromatography (SEC) using a HiLoad 26/600 Superdex prep grade column (GE Healthcare) with 20 mM MOPS, 150 mM NaCl, pH 6.0 as a running buffer.

Purified proteins were quantified on a Nanodrop spectrophotometer (Thermo Scientific) and analyzed under reducing and non-reducing conditions by capillary gel electrophoresis using Caliper LabChip (Perkin Elmer) or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with NuPAGE 4–12% Bis-Tris gels in MES buffer followed by Coomassie staining (SimplyBlue, ThermoFisher). Aggregation and molecular weight of the FcγR products were determined by SEC coupled to Multi-Angle Light Scattering (MALS) using a Superdex 200 increase 10/300 GL column (GE Healthcare).

Biotinylation

Soluble poFcγRs were biotinylated via the Avi-tag using the BirA Biotin-Protein Ligase standard reaction kit (Avidity). The biotinylation efficacy was assessed by liquid chromatography – mass spectrometry (LC-MS) after deglycosylation with PNGase F.

Generation of FcγRIIa and FcγRIIa/b Specific Antibodies

Purified soluble poFcγRIIa, poFcγRIIb, and poFcγRIII were sent to BioRad for the generation of bivalent Fab antibodies dimerized via alkaline phosphatase containing FLAG and His6 epitope tags (Fab-A-FH). Binders were selected via phage display method (CysDisplay®) on BioRads Human Combinatorial Antibody Libraries (HuCAL). PoFcγRIIa/FcγRIIb cross-reactive HuCAL antibodies were generated by using poFcγRIIa as an antigen and poFcγRIII as a closely related antigen to prevent further cross reactivity. Similarly, poFcγRIIa specific antibodies were generated by using poFcγRIIb as a closely related antigen. All binders (HuCAL clones) were tested for their specificity by enzyme-linked immunosorbent assay (ELISA) coated with porcine FcγRIIa, FcγRIIb, and FcγRIII.

Immune Complex Generation

IC were generated by overnight incubation at room temperature of the huIgG1 therapeutic antibody bevacizumab (149 kDa; Roche) and its dimerized target VEGF165 (38 kDa, BioLegend), as described earlier (5). The antibody to target ratio of 1:2.5 was generated using 4 μM bevacizumab and 10 μM dimerized VEGF165, whereas the ratio of 1:0.5 was generated using 20 μM bevacizumab and 10 μM VEGF165, and the ratio of 1:0.1 using 20 μM bevacizumab and 2 μM VEGF165. IC formation was analyzed by SEC-MALS using a HPLC system equipped with a Superdex 200 increase 10/300 GL column (GE Healthcare), a TREOS laser light scattering detector, and a T-rEX differential refractometer (Wyatt Technology).

Flow Cytometry

Phenotyping of FcγR Expression

FcγR expression was assessed in whole blood of a Göttingen minipig sampled in K2EDTA Vacutainer tubes (BD) followed by treatment with lysing buffer (BD PharmLyse) to remove erythrocytes. Minipig blood cells were stained with PE-conjugated antibodies against porcine CD16 (clone G7, BioRad) or unconjugated HuCAL antibodies against poFcγRIIa (clone AbD29332.1 "HuCAL32"), FcγRIIa/b (clone AbD32591.1 "HuCAL91"), or the isotype control Fab-A-FH (clone AbD05930). The FcγR expression of transfected HEK293F cells was assessed by staining using the abovementioned antibodies or the PE-conjugated antibodies against human CD64 (clone 10.1, BioLegend), human CD32

(clone 3D3, BD), human CD16 (clone 3G8, BD), or FLAG tag (clone L5, BioLegend). Unconjugated antibodies were detected using the secondary PE conjugated goat F(ab')2 anti-huIgG after washing with FACS buffer (Dulbecco's phosphate-buffered saline (DPBS, Gibco) containing 2% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma)). After washing with DPBS, dead cells were stained by the amine reactive Zombie Aqua dye (BioLegend) and the preparations were fixed using BD CellFIX. Events were acquired on BD LSRFortessa with BD FACSDiva software and data was further analyzed using FlowJo.

Immune Complex Binding

Binding of IC was assessed by flow cytometry analysis on whole blood of three Göttingen minipigs. Fresh blood was collected in Vacutainer tubes coated with K2EDTA (BD) and subsequently treated with erythrocyte lysis buffer (PharmLyse, BD) and washed with DPBS. The remaining blood cells were incubated with the amine reactive dye Zombie Aqua (BioLegend). After washing with FACS buffer, the blood cells were incubated in 96 well plates for 1 h at 4°C with different concentrations of bevacizumab, or bevacizumab-VEGF165 IC diluted in FACS buffer. Bevacizumab to VEGF165 ratios of 1:2.5, 1:0.5, and 1:0.1 were used. Unbound antibodies or complexes were removed by intensive washing with FACS buffer. PE-conjugated secondary goat F(ab')2 antibodies against huIg-*kappa* (Biorad) were used to detect membrane-bound bevacizumab or IC. After another two washes with FACS buffer, 100′000 events were recorded on BD LSRFortessa with and the software BD FACSDiva. Data was further analyzed using FlowJo.

SPR Experiments

IgG Capturing Setup

The interaction of porcine or human FcγR variants to porcine or human IgG anti-HER2 was analyzed using surface plasmon resonance (SPR) on a Biacore T200 system (GE Healthcare). First, the extracellular domain of HER2 was immobilized at pH 4.5 to >3000 response units (RU) on a CM5 chip using the amine coupling kit (GE Healthcare). Then, the HER2 specific antibodies trastuzumab (huIgG1, Roche), poIgG1a-HER2 and poIgG3-HER2 were injected at a concentration of 100 nM in PBS-P+ buffer (GE Healthcare) with a pulse of 30s at a flow rate of 10 µl/min reaching capturing levels of 1000RU. Soluble porcine or human FcγRs were prepared in solutions of 600, 200 nM and 66.7 nM in PBS-P+ and applied at a flow rate of 30 µl/min for 90s. The dissociation phase was monitored for 600 s followed by regeneration of the surface by a 60s and 20s washing step with a 10 mM Glycine pH 2.1 at a flow rate of 10 µl/min. All experiments were performed in PBS-P+ pH 7,4 running buffer.

FcγR Capturing Setup

An alternative setup was used to compare binding of poFcγRs to free- and immune-complexed huIgG1. Biotinylated soluble poFcγRs were reversibly captured on a CAP chip using the standard Biotin CAPture reagent kit (GE Healthcare) at pH 7.4 PBS-P+. The capturing level of FcγR variants reached 940–2543 RU. Porcine or human biotinylated FcγR variants were prepared as solution of 200 nM in PBS-P+ and captured with a pulse of 180 s at a flow rate of 5 µl/min. Subsequently, human free- or immune-complexed IgG1 were applied at a concentration of 600, 200 and 66.7 nM in PBS-P+ at a flow rate of 30 µl/min for 120 s. The dissociation phase was monitored for 600 s. Then, the surface was regenerated by a 120 s washing step with the regeneration solution for the CAP chip (GE Healthcare) at a flow rate of 10 µl/min.

SPR Data Analysis

The Biacore T200 software (GE Healthcare) was used to evaluate data from SPR experiments and to display binding curves. Interaction Map was used to separate heterogeneous binding into its individual 1:1 interactions with different kinetics. For this, data from SPR experiments were imported into TraceDrawer software (Ridgeview Instruments AB) and further processed with the Interaction Map program (Ridgeview Instruments AB).

RESULTS

Interactions between IgG antibodies and their Fc receptors are of high complexity. To obtain a thorough characterization, we studied poFcγRs as recombinant soluble proteins and expressed on the cell surface, as well as minipig blood cells that natively express FcγRs. Interactions of poFcγRs were assessed with different free- or immune-complexed IgG antibodies and therapeutics.

Binding of huIgG to poFcγRs

The purpose of this experiment was to show qualitative binding of poFcγRs to huIgG1, the most commonly used therapeutic human antibody isotype, by SPR. A highly sensitive assay is needed to detect weak interactions because low affinity FcγRs (FcγRIIa, FcγRIIb, and FcγRIIIa) generally interact only weakly with free IgG. Therefore, soluble porcine and human FcγRs were designed and used here as dimers of FcγR extracellular domains expressed as inert Fc fusion proteins. The dimeric structure provides an avidity effect and

increases the molecular mass leading to higher sensitivity and therefore allowing a qualitative binding analysis (20). Transient expression in HEK293F cells and subsequent purification yielded soluble FcγRs of >98% purity as determined by capillary gel electrophoresis or SDS-PAGE (not shown). PoFcγRIIb is exclusively composed of dimers, whereas poFcγRIa, poFcγRIIa, and poFcγRIIIa preparations additionally contained 32%, 74%, and 77% aggregates, respectively, even after SEC purification (not shown). N-linked glycosylation of FcγRs and Fc fusion was effective in HEK293F cells as observed by PNGase F digestion followed by SDS-PAGE (Fig. S1).

For the SPR binding analysis, the recombinant HER2 antigen was coated on a CM5 sensor chip and then allowed to capture trastuzumab, a HER2 specific huIgG1 therapeutic antibody, or HER2 specific poIgGs (Fig. 1a). To this purpose, the two most abundant isotypes in porcine blood, IgG1a and IgG3 (22), were recombinantly expressed with HER2 specificity, and named poIgG1a-HER2 and poIgG3-HER2, respectively. The soluble poFcγRs were then allowed to bind human and porcine HER2-specific IgG (Fig. 1a).

Figure 1b shows the maximum responses observed with huIgG1 in interaction with 600 nM of porcine and human FcγRs. From this analysis we conclude that trastuzumab binds to most poFcγRs in a similar magnitude as to huFcγRs (Fig. 1b). A closer analysis of the sensorgrams generated using three concentrations of the soluble FcγRs permits a ranking of the binding strength among the different FcγRs (Fig. 1c). The sensorgrams show the response (RU) during association of the soluble FcγRs to antigen bound IgG until the steady state in the first 100 s followed by their dissociation. Among poFcγRs, we identified poFcγRIa as the strongest binder for huIgG1 based on the quicker association and the slower dissociation, followed by poFcγRIIa. PoFcγRIIb is the weakest binder with the quickest dissociation whereas poFcγRIIIa did not bind huIgG1. However, the functionality of poFcγRIIIa was demonstrated through its binding to poIgG1 (Fig. 1c). A similar binding pattern was observed for huFcγRs with huFcγRIa as the strongest binder of huIgG1 followed by huFcγRIIa, FcγRIIb, and huFcγRIIIa in a similar range. Comparing the orthologous porcine and human FcγRs, huIgG1 bound stronger to poFcγRIIa and poFcγRIIb but weaker to poFcγRIa and poFcγRIIIa as compared to the human orthologue (Fig. 1c). For all poFcγRs, except poFcγRIIIa, we observed a similar binding pattern of huIgG1 and poIgG1a-HER2. In contrast, poIgG3-HER2 showed only weak interactions to poFcγRIa and poFcγRIIa and no binding to poFcγRIIb and poFcγRIIIa. Vice versa, huFcγRs did not notably bind to poIgGs (Fig. 1c). In sum, we found that poIgG1 binds to poFcγRIa > poFcγRIIa > poFcγRIIb and poFcγRIIIa in a similar range, whereas huIgG1 binds to poFcγRIa > poFcγRIIa > poFcγRIIb > > poFcγRIIIa.

The shape of the sensorgrams in Fig. 1c suggested complex multiple interactions contributing to IgG-FcγR bindings. Such heterogeneous interactions probably originate from different qualities of the individual FcγRs based on their integrity and the presence of aggregates. To assess the contribution of quality issues leading to heterogeneous interactions, we also analyzed the huIgG1 binding data using the Interaction Map method (Fig. 1d). It allows the decomposition of time-resolved binding curves into separate interactions with unique combinations of association rates k_a [M^{-1}, s^{-1}] and dissociation rates k_d [s^{-1}], contributing to the total binding (23). Therefore, the Interaction Map analysis allows addressing the heterogeneity of IgG-FcγR interactions. The resulting on-off plots display single interactions by their dissociation ($\log(k_d)$, x-axis) and association ($\log(k_a)$, y-axis) values colored according to their contribution to the total binding (Fig. 1d). Because no interaction of trastuzumab was observed with poFcγRIIIa, this data could not be analyzed by Interaction Map. For the other FcγRs, this analysis disclosed multiple interactions involved in the binding of huIgG1 to poFcγRIa, poFcγRIIa, and huFcγRIIIa (Fig. 1d). Interestingly, the FcγRs with the most obvious multivalent binding properties were the preparations with the highest proportion of aggregates. Therefore, one spot originates from the bivalent functional binding, whereas the other spot reflects the binding to aggregates contained in the preparation. Because aggregates reformed after SEC purification, it was not possible to identify which interaction was responsible for the functional binding. The correct binding kinetics of these FcγRs must be a mixture of the observed interactions. Therefore, we refrain from reporting affinities based on one 1:1 kinetic. Additionally, it was shown by other authors that IgG-FcγR interactions do not depend on only one 1:1 kinetic and are strongly influenced by the experimental setup and other factors, such as FcγR glycosylation (24).

In addition to poFcγRIIb, another isoform named poFcγRIIb1 has been reported having a 19 amino acid in-frame insertion in the cytoplasmic domain. Apart from the signal sequence, these variants also differ by one polymorphism in the extracellular domain 1 and two polymorphisms in the extracellular domain 2 (the latter are marked in yellow in Fig. S2) (25). We directly compared these two polymorphic variants in SPR regarding binding to porcine or human IgG and found no differences in IgG isotype selectivity and negligible stronger binding of the poFcγRIIb1 variant (Fig. S3).

Binding of huIgG1 to poFcγRs on Cells

Next, we addressed binding of free huIgG1 to poFcγRs in a more biological system with transfected HEK293F cells expressing surface-anchored FcγRs. Due to the lack of available antibodies specific for poFcγRs, we also generated phage-display based recombinant antibodies with specificity for poFcγRs using the HuCAL technology. The specificity of

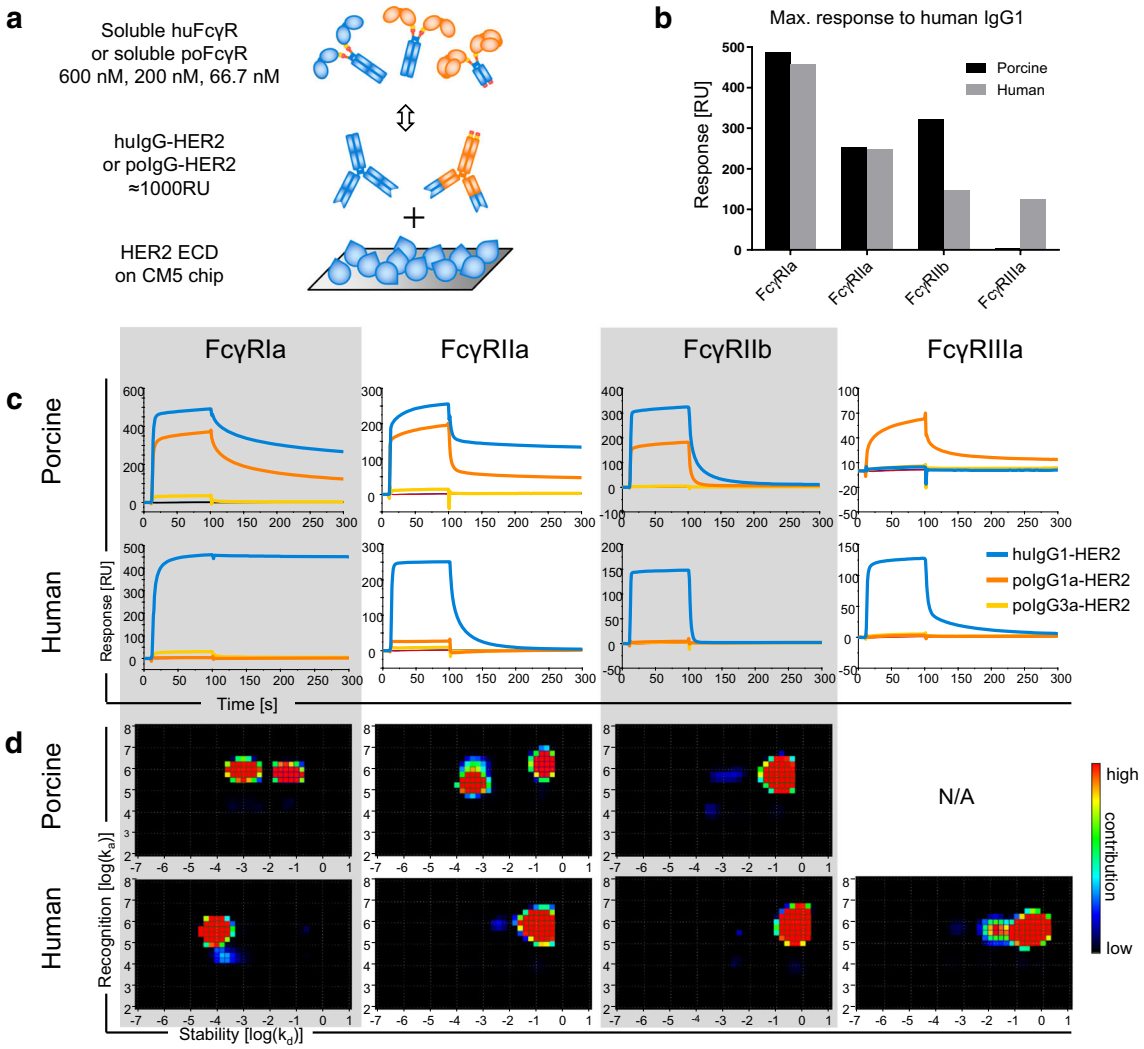

Fig. 1 SPR binding analysis of soluble FcγRs to IgG. (**a**) Scheme depicting the assay setup. First, extracellular domains of HER2 were coated on a CM5 sensor chip. Then, HER2 specific human (blue structures) and porcine (orange structures) antibodies were captured on different flow cells. Their interactions with soluble porcine or human FcγRs were measured. The drawing shows low affinity FcγRs with two and the high affinity FcγRIa with three extracellular domains (oval shapes). (**b**) The graph shows the maximum response of 600 nM porcine (black bars) and human (grey bars) FcγRs obtained with huIgG1. (**c**) Real-time sensorgrams from SPR analysis. Interaction of IgG to poFcγRs is shown in the upper row and to huFcγRs in the lower row whereas the respective FcγRs are named above. Binding of 600 nM soluble FcγRs to trastuzumab (huIgG1, blue line), poIgG1a-HER2 (orange line), and poIgG3-HER2 (yellow line) is shown. Only the highest concentration of the titration with 600, 200, and 66.7 nM of soluble FcγRs is shown for clarity. (**d**) Interaction Map analysis resulting from trastuzumab binding to all concentrations of porcine and human FcγRs is shown in the upper and lower row, respectively. The binding is separated in several parallel interactions with unique kinetics, as displayed by spots on a graph with k_d on the x-axis and k_a on the y-axis. The heat map is a measure of the contribution from red = high to blue = low of each interaction to the total binding. No interaction was detected with poFcγRIIIa; therefore, it could not be analyzed (N/A).

these HuCAL antibodies was also assessed using cell surface-anchored FcγRs.

Full length poFcγRs with extracellular FLAG tags encoded at the N-terminus were transiently expressed on HEK293F cells. However, full-length huFcγRs were expressed without FLAG tags. The data shown in Fig. 2a demonstrate expression of all porcine and human FcγRs on the cell surface of HEK293F cells. The expression of huFcγRs and of poFcγRIIIa was characterized via commercial FcγR-specific antibodies whereas a FLAG tag specific antibody was used to characterize the expression of all poFcγRs. The expression of poFcγRIIa was further demonstrated with the antibody clone HuCAL32 that binds specifically to this FcγR in contrast to the antibody clone HuCAL91 that is cross-reactive to the closely similar FcγRIIb (Fig. 2a).

Binding to FcγRs expressed on HEK293F cells was then assessed using different concentrations of bevacizumab, a huIgG1 anti-VEGF therapeutic antibody displaying similar SPR binding to poFcγR as trastuzumab (not shown). Cell bound bevacizumab was detected via flow cytometry using goat F(ab')2 anti-huIg-*kappa* secondary antibody. The results show a concentration-dependent binding of bevacizumab to

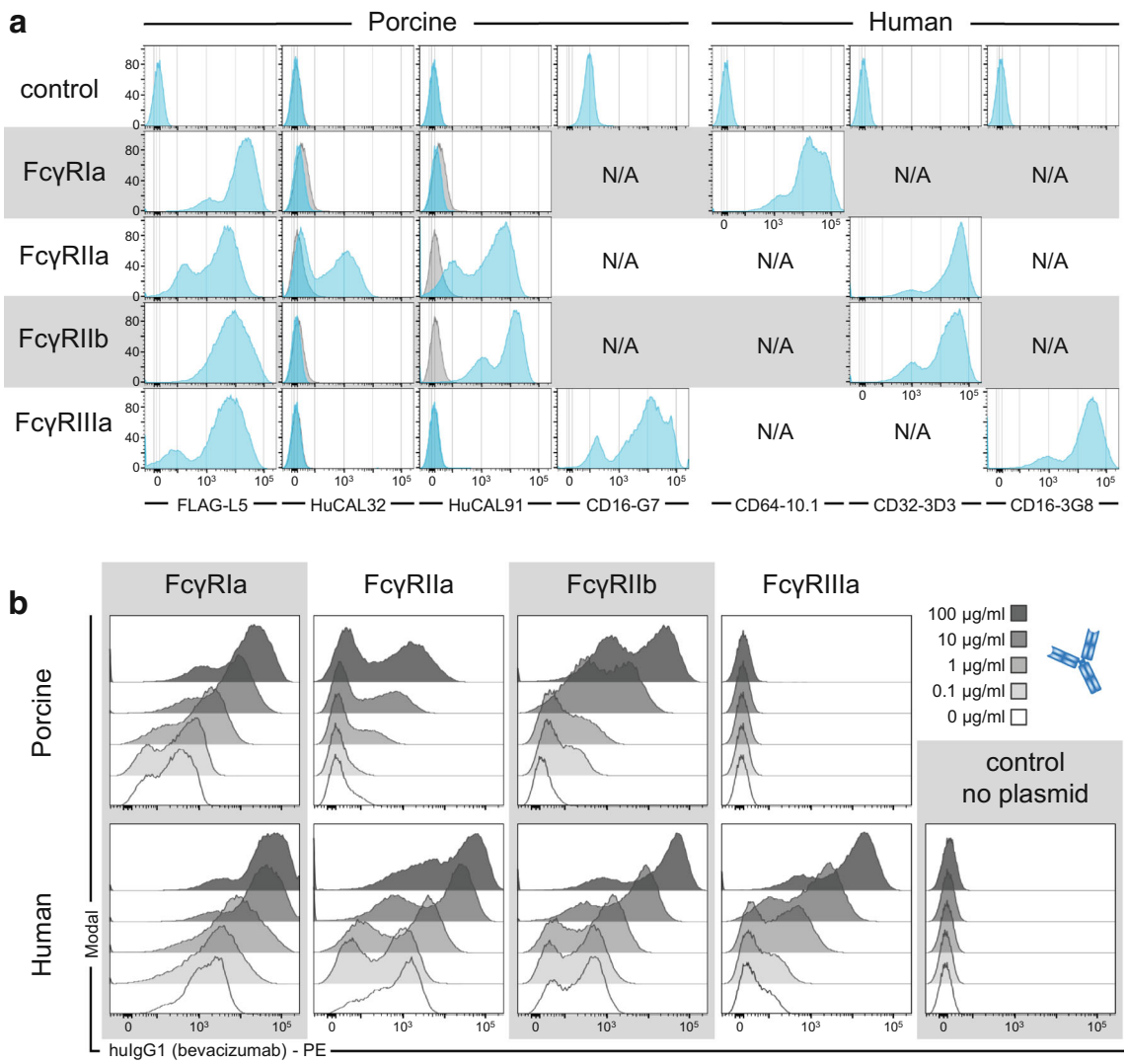

Fig. 2 Binding of bevacizumab (huIgG1) to FcγRs transiently expressed on HEK293F cells. (**a**) HEK293F cells expressing the indicated porcine (left panel) and human (right panel) FcγRs were analyzed by flow cytometry using the antibodies indicated below each column. HEK293F cells transfected without plasmid served as a negative control (first row). Blue histograms show binding of the antibody to the respective cells whereas N/A indicates combinations of antibodies and FcγRs that were not analyzed. Overlaid grey histograms display the staining with the HuCAL control antibody. (**b**) Bevacizumab was titrated and incubated with HEK293F cells expressing the indicated FcγR. After intense washing, FcγR-bound IgG was stained with PE-conjugated goat F(ab')2 anti-hulg-*kappa* secondary antibody and analyzed by flow cytometry. Stacked histograms show binding of increasing concentrations with increasing intensity: no bevacizumab (open histogram), 0.1 μg/ml (shaded in light grey), 1, 10, and 100 μg/ml (shaded in dark grey).

porcine (except for poFcγRIIIa) and human FcγRs (Fig. 2b). From these data we conclude that surface-anchored poFcγRIa, IIa and IIb, but not poFcγRIIIa can bind by free huIgG1.

Binding of huIgG1 Immune Complexes to poFcγRs

Human low affinity FcγRs mediate their functions rather via interaction with IC in contrast to free IgG (26). The increase in avidity compensates for the low affinity and allows stable binding to the IC ultimately leading to activation of the FcγRs. In order to assess binding of poFcγRs to huIgG1 IC we performed SPR experiments with pre-formed IC of bevacizumab and its dimeric target antigen VEGF.

To generate physiological IC, bevacizumab was co-incubated with VEGF and the resulting complexes were studied by SEC-MALS. The stoichiometric ratio of one antibody together with an excess of five VEGF dimers resulted in large IC without remaining free IgG where the majority of complexes is composed of three or more antibodies (Fig. 3a). For the measurement of their binding profiles in comparison to free IgG, all poFcγRs were biotinylated and coated on the sensor chip (Fig. 3b). For every FcγR, two different capturing densities were assayed. The densities of FcγRIa (940RU), FcγRIIa (1020RU), and FcγRIIb (2543RU) were found to give best results probably reflecting their different affinities to huIgG1. We, however, did not achieve sufficient biotinylation of poFcγRIIIa to increase its capturing density above

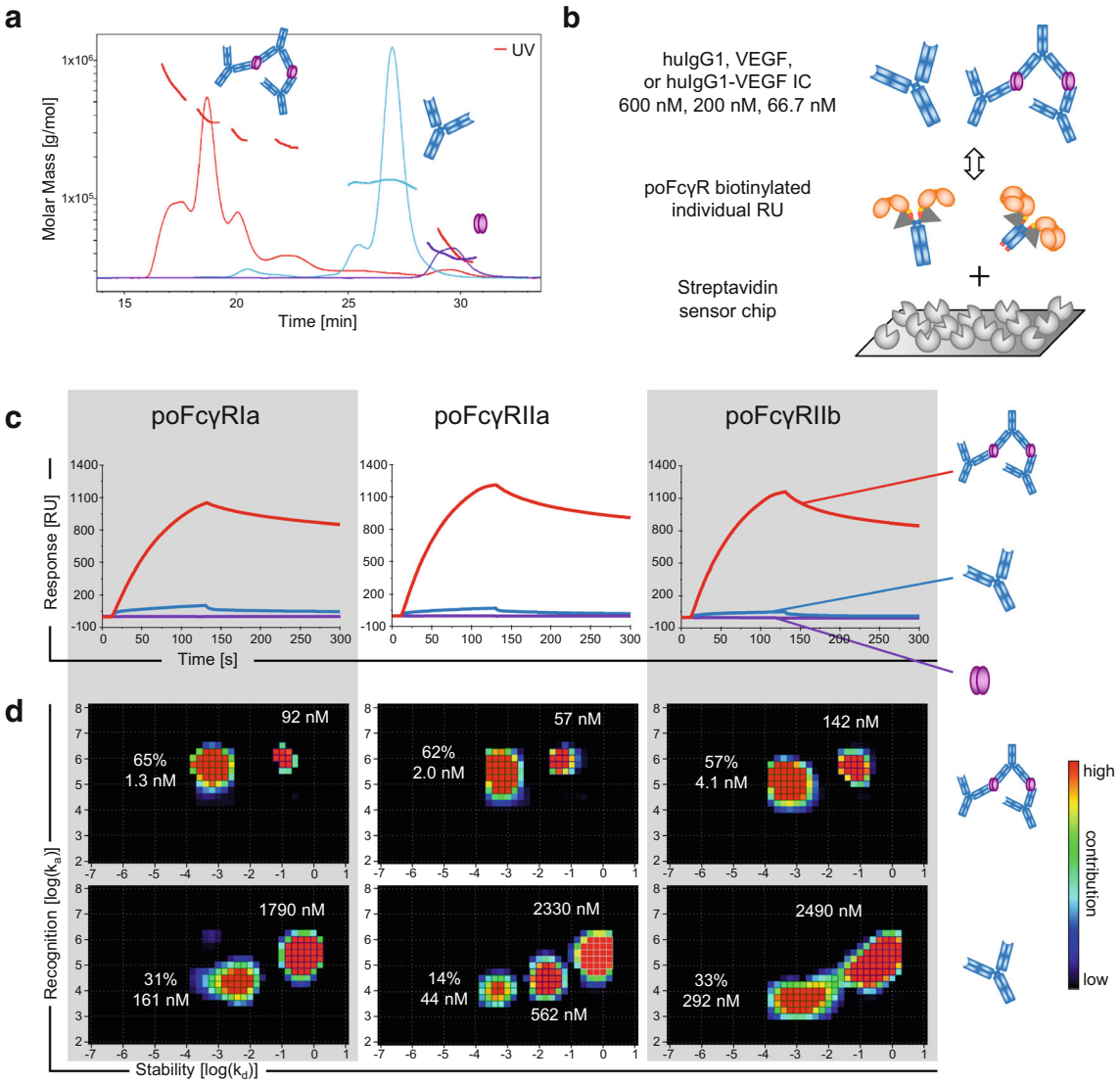

Fig. 3 Comparison of free bevacizumab and IC by binding to poFcγRs using SPR. (**a**) SEC-MALS analysis shows the molecular weight of free bevacizumab (blue line), VEGF (purple line), and complexes formed by bevacizumab and VEGF in a molar ratio of 1:5 (red line). (**b**) Biotinylated (grey triangles) soluble poFcγRs were captured with different densities on a streptavidin senor chip. Interactions with free bevacizumab (blue) and IC with VEGF dimers (purple ovals) were measured by SPR. Three different concentrations of IC and free huIgG1 with the same amount of IgG were assayed (600, 200, 66.7 nM). PoFcγRIIIa was excluded from this experiment due to insufficient biotinylation. (**c**) SPR sensorgrams resulting from binding of the above indicated FcγR to the highest concentration of VEGF (purple line), bevacizumab (blue line), and IC (red line) are shown. (**d**) Binding curves from panel C were resolved by the Interaction Map method. Binding of huIgG1 IC to the FcγR indicated above each column is shown in the upper row and binding of free huIgG1 is shown below. The equilibrium binding constant K_D [nM] is indicated next to each major interaction spot and its contribution [%] to the total binding is indicated for the spot with the highest affinity.

54RU, and was therefore excluded from the experiment. Subsequently, 600, 200, and 66.7 nM of free huIgG1 or IC formed with the same amount of huIgG1 were used to assess the binding strength (Fig. 3b).

The sensorgrams in Fig. 3c show a strong increase in the maximum response and a more stable interaction with IC compared to free huIgG1 in all poFcγRs. We again analyzed the SPR binding data with the Interaction Map method (Fig. 3d) first, because the observed maximum response largely depends on the size of the bound complex and second, because we expect avidity based heterogeneous IC-FcγR interactions. Using this setup, we observed two to three interactions contributing to the binding of poFcγRs to free huIgG1, probably resulting from partial activity of the soluble FcγRs (Fig. 3d). The contribution of all interactions shifted towards lower k_d and higher k_a values and ultimately towards a stronger binding comparing free bevacizumab to IC. Additionally, Fig. 3d shows a shift of the individual interactions towards a higher affinity. For poFcγRIa, for example, the higher affinity interaction shifts the center of the spot to a 5x longer half-life (shift towards lower k_d) as seen in Fig. 3d and evaluated by the interaction map software, a 10x quicker

association (shift towards higher k_a), and therefore a 100x enhanced affinity ($K_D = k_d/k_a$) comparing free huIgG1 to IC.

Additionally, its contribution increases from 31% to 65%. On the other hand, the low affinity interaction decreases its contribution from 50% to 16% (Fig. 3d, left plots). For poFcγRIIa and poFcγRIIb the changes in affinity from free huIgG1 to IC are comparable to those for poFcγRIa. This data clearly demonstrates a stronger and more stable interaction of huIgG1 IC with poFcγRs than with free huIgG1 based on avidity effects. IC binding is a prerequisite for effector functions triggered by huIgG in minipigs.

Binding of huIgG1 IC to Minipig Blood Cells

Next, we studied interactions of free huIgG1 (bevacizumab) to blood cells of Göttingen minipigs that natively express poFcγRs. Free huIgG1 and different preparations of IC were titrated and co-incubated with minipig whole blood for 1 h at 4°C in FACS buffer containing sodium azide to prevent internalization. Bound antibodies or IC were stained using goat F(ab')2 anti-huIg-*kappa* secondary antibody and analyzed by flow cytometry. The different blood cell subsets were gated from forward and side scatter (FSC/SSC) of viable single cells without including specific cell surface markers due to limited availability of specific antibodies, fluorochromes, and cross-reactivity. The gating strategy and identity of the different cell types of the minipig blood is shown in Fig. S4. The FcγR expression in the respective minipig blood cells was assessed in separate stainings and shown in Fig. 4a. PoFcγRIIa (stained by HuCAL32) was found to be expressed on platelets and a sub-population of eosinophils. The poFcγRIIa/b cross-reactive antibody (HuCAL91) additionally stained a large proportion of monocytes that are thus thought to express poFcγRIIb. Monocytes, neutrophils, and eosinophils all express poFcγRIIIa. Furthermore, small lymphocyte subsets, such as B cells and NK cells are known to express poFcγRIIb and poFcγRIIIa, respectively and monocytes are known to express poFcγRIa (16,27). The poFcγRIa expression beyond lymphocytes and monocytes is largely unknown and can thus not be excluded on platelets, neutrophils, and eosinophils. Histograms in Fig. 4b show the binding of 0.1 μg/ml free huIgG1 and the same amount of huIgG1 complexed using different ratios of VEGF165 to the different minipig blood cell subsets. The antibody (bevacizumab, Bev) to target (VEGF) ratio of 1:2.5 yielded the largest IC without free huIgG1 whereas IC generated in the ratio of 1:0.5 and 1:0.1 were smaller and contained more free huIgG1 (Fig. S5). Here, we observed that large IC showed enhanced binding to all platelets and most monocytes *versus* smaller IC and free huIgG1. Furthermore, large IC resulted in the strongest shift of neutrophils and eosinophils, even though the MFI was lower than in platelets and monocytes. A small subpopulation of lymphocytes also bound large IC better than small IC and free huIgG1 (Fig. 4b). As in the histograms, it is apparent from the titration of all IC preparations in the blood of three Göttingen minipigs that free- and immune-complexed huIgG1 exhibit the strongest binding to platelets, followed by monocytes, eosinophils, neutrophils and lastly lymphocytes (Fig. 4c). The titration shows that in particular the largest IC strongly bind to poFcγR-expressing cell types at the lowest concentrations translating to the highest affinity. Vice versa, preparations with limited VEGF165 or without VEGF165 (huIgG1 alone) require higher concentrations to bind to poFcγR-expressing cell types, translating to lower affinities. VEGF165 did not bind to minipig blood cells at the concentration used to generate the largest IC (ratio 1:2.5) containing 10 μg/ml huIgG1. The strongest differences between free-and immune-complexed huIgG1 were observed in platelets and monocytes. Neutrophils and eosinophils also bound IC stronger than free huIgG1, however the maximum percentage of positive cells in these cell types were lower and the individual differences were more pronounced leading to a higher standard deviation (Fig. 4c).

DISCUSSION

The use of the Göttingen minipig in preclinical studies with therapeutic antibodies is limited by the lack of knowledge on the expected pharmacology for the translatability of corresponding findings to the human. The pharmacology of antibodies with active Fc parts often depends on effector mechanisms mediated by interaction with FcγRs. The aim of this study was to assess the binding properties of huIgG1 therapeutic antibodies to poFcγRs which is a prerequisite for the consideration of the minipig for preclinical safety and efficacy studies with therapeutic antibodies.

The present study demonstrates that poFcγRs bind human therapeutic antibodies of the IgG1 isotype. The binding properties of the poFcγRIa, poFcγRIIa, and poFcγRIIb closely resemble those of the human orthologues albeit some differences were identified. Importantly, poFcγRIIIa was shown not to bind huIgG1 antibodies. Similar to huFcγRs, all poFcγRs except poFcγRIIIa were shown to bind IC composed of huIgG1 with a higher affinity than free huIgG1. Especially, monocytes, eosinophils, platelets and a subset of lymphocytes of minipig blood showed enhanced binding to human IC.

The poFcγRIa was cloned by Zhang, Qiao (15) and shown to bind poIgG. Here we also demonstrate that poFcγRIa, similar to its human orthologue, strongly binds huIgG1 (28). The high affinity interaction of huFcγRIa is supposed to be mediated by a hydrophobic pocket for Leu235 within the Fc part of huIgG (29). The same pocket was also identified in poFcγRIa supporting its high affinity for huIgG1 (Fig. S2). Nevertheless, we found differences between the two species in residues forming H bonds (Lys128, Ala143 in Fig. S2). This could explain the weaker

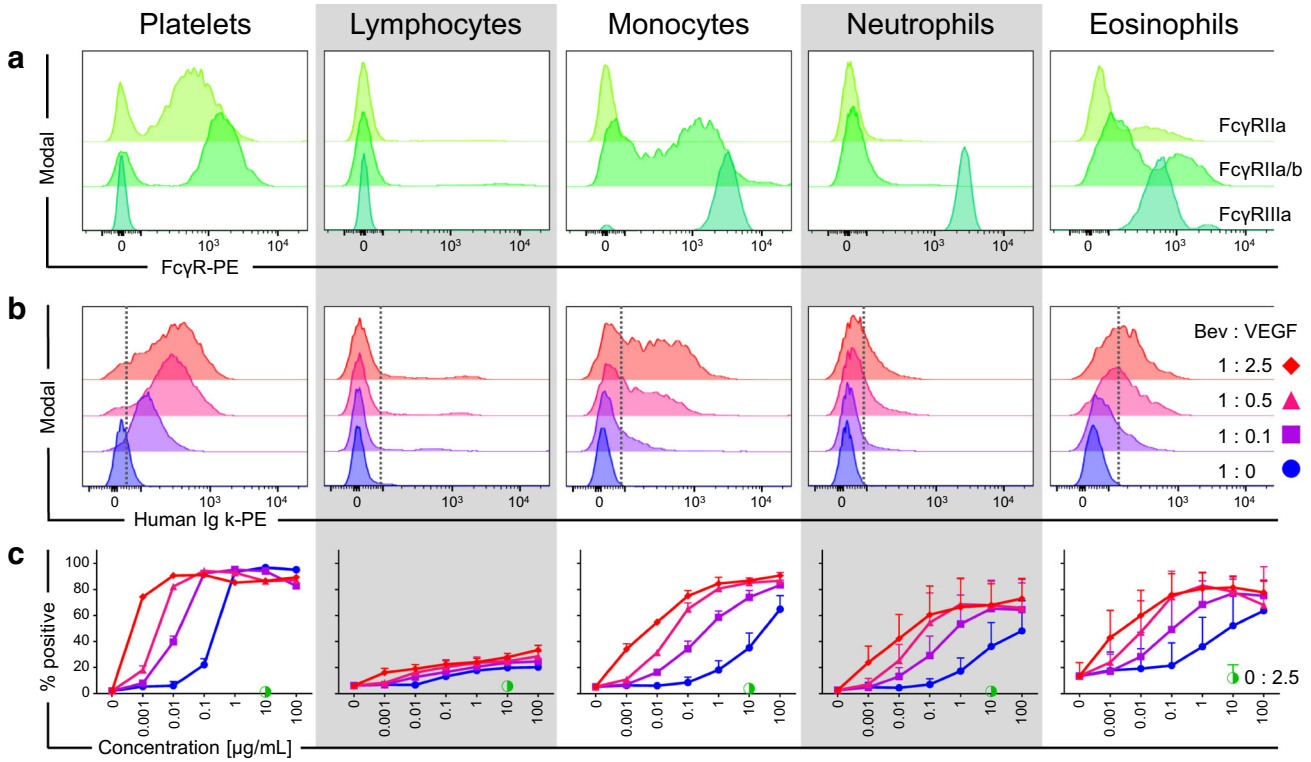

Fig. 4 Binding of free huIgG1 (bevacizumab) and IC to minipig whole blood in comparison to the FcγR expression. FcγR expression and huIgG binding was assessed by flow cytometry in whole blood of Göttingen minipigs. The cell types were gated from single live cells by their FSC and SSC properties as described in detail in Fig. S4. (**a**) Histograms show the expression of poFcγRIIa (HuCAL32, light green histogram), poFcγRIIa/b (cross reactive HuCAL91, green histogram) and poFcγRIIIa (clone G7, dark green histogram) in platelets, lymphocytes, monocytes, neutrophils and eosinophils (from left to right). (**b**) Stacked histograms show the binding of 0.1 µg/ml free huIgG1 (blue) and the same amount of huIgG1 complexed using 0.1 parts of VEGF165 (purple), 0.5 parts of VEGF165 (magenta), or 2.5 parts of VEGF165 to the different minipig blood cell subsets. The dotted line represents the gate separating PE-negative (left) from PE-positive (right) events. (**c**) Graphs show the percentage of PE-positive cells with increasing concentrations of free- (blue circles) and immune-complexed bevacizumab with concentrations ranging from 100 to 0.001 µg/ml of huIgG1 and a control containing 0 µg/ml bevacizumab or IC. IC generated by the following antibody to target ratios are displayed: 1:0.1 (purple squares), 1:0.5 (magenta triangles), 1:2.5 (red diamonds), and VEGF alone (half-filled green circle). Error bars represent the standard deviation within one representative experiment using three minipigs. Multiple experiments with IC (ratio 1:2.5) using a total of seven minipigs led to similar results.

binding of huIgG1 to poFcγRIa compared to huFcγRIa. The observed difference concerning the heterogeneity of interactions probably results from avidity effects caused by FcγR aggregation. PoFcγRIa is, like its human orthologue, expressed on monocytes in peripheral blood of minipigs (16). A fraction of minipig monocytes binds huIgG1 IC already at low concentrations, possibly mediated by poFcγRIa, although poFcγRIIb and poFcγRIIIa cannot be excluded since these FcγRs are also expressed on monocytes. The enhanced binding of complexed *versus* free huIgG1 to poFcγRIa was confirmed by SPR. Notably, we did not observe a strong staining with free huIgG1 as it could be expected for binding to poFcγRIa. A gradual dissociation of free IgG1 from huFcγRIa is believed to allow capturing small IC or sparsely opsonized large complexes (30). Our results suggest a similar role of poFcγRIa by the observation of the strong IC binding (Fig. 3c and d) and weak staining of poFcγRIa expressing monocytes with low concentrations of free huIgG (Fig. 4b).

FcγRIIa is known as a low affinity receptor signaling through an integrated intracellular immunoreceptor tyrosine-based activation motif (ITAM) in the human. However, orthologues to FcγRIIa in the mouse, cattle and pig, for example, are lacking this integrated ITAM and require FcR γ-chain interactions for signaling (16,31). In terms of binding, we found that FcγRIIa of both species bind huIgG1 (Fig. 1). Conserved tryptophan residues Trp104 and Trp127 forming the "Trp sandwich" of FcγRs that interacts with Pro329 of IgG Fc parts could enable such cross-species interactions (Fig. S2) (32). Interestingly, trastuzumab bound to poFcγRIIa with an increased stability compared to huFcγRIIa (Fig. 1). HEK293F cells expressing porcine and human FcγRIIa showed similarities in binding properties to bevacizumab as observed by the concentration-dependent increase of binding (Fig. 2b). The differences in background and the intensity of the positive population possibly originate from the lower expression of poFcγRIIa compared to huFcγRIIa on HEK293F cells (Fig. 2). A high avidity binding of IC to

poFcγRIIa was observed by SPR as described for human low affinity receptors (Fig. 3c and d) (33). This was also reflected in the strong binding of IC to minipig platelets expressing highest levels of FcγRIIa (Fig. 4a). Yet, platelets were also the strongest binders of free bevacizumab. This could be explained by the increased affinity of poFcγRIIa in relation to huFcγRIIa. The increased affinity could further lead to an enhanced sensitivity of the minipig for FcγRIIa binding and ultimately to an overprediction of FcγRIIa-mediated toxicities in preclinical studies. Lau, Gunnarsen (34) observed platelet aggregation and toxicities in domestic pigs treated with mouse IgG2b anti-porcine CD14 (clone MIL2) possibly due to FcγR activation and complement binding. A recombinant huIgG2/4 anti-porcine CD14 antibody (rMIL2) however, did not induce aggregation probably due to abolished FcγR or complement binding in pigs.

The inhibitory low affinity FcγRIIb is mainly expressed on human B cells, dendritic cells (DC), and tissue macrophages and is an important regulator of immune responses (1). Here, we report enhanced binding of poFcγRIIb to trastuzumab in comparison with huFcγRIIb (Fig. 1). The Interaction Map analysis shows a homogeneous interaction for the porcine and human FcγRIIb. Therefore, we conclude that the three times increased affinity is not assay dependent. This finding is also in concordance with *Macaca nemestrina* FcγRIIb showing enhanced huIgG1 binding (32). FcγRIIb of macaques and cyno have residues His131 at the location of the huFcγRIIa His131Arg polymorphism and Met132 nearby. These residues were shown to account for the increased binding while huFcγRIIb has Arg131 and Ser132 in these positions. In poFcγRIIb, however, we identified residues Tyr and Val at the corresponding positions probably influencing the binding in another way (Fig. S2). Triggering of the inhibitory huFcγRIIb in macrophages and dendritic cells can counteract the effects mediated by activating FcγRs (35). Enhanced binding of huIgG1 to poFcγRIIb could therefore enhance the threshold for cell activation and result in a more tolerogenic milieu in inflamed tissue, thus leading to an overestimated efficiency of immunosuppressive therapeutic antibodies in minipigs. Simultaneously, treatment with therapeutic huIgG1 antibodies could lead to enhanced risk for pneumococcal peritonitis while reducing pathological immune stimulation due to reduced reactivity of macrophages (36,37). Furthermore, FcγRIIb expressed on B cells plays an important role in maintenance of peripheral tolerance (38). Thus, the stronger binding of huIgG1 antibodies to poFcγRIIb on B cells could lead to enhanced tolerance and hence to underestimation of immunogenicity concerns.

From all studied receptors, the most pronounced differences between minipig and human were observed for FcγRIIIa. In humans, FcγRIIIa is a low affinity activating receptor binding huIgG1 IC with high avidity and mediating important functions such as ADCC of monocytes and natural killer (NK) cells. PoFcγRIIIa, in contrast, binds neither free- nor immune-complexed huIgG1, and poIgG1a only with low affinity. This binding pattern was observed with recombinant soluble poFcγRIIIa in SPR assays with trastuzumab and with HEK293F cells and neutrophils expressing poFcγRIIIa in interaction with bevacizumab (Figs. 1, 2 and 4). The nature of the poor binding properties of poFcγRIIIa is unknown. However, we cannot exclude binding to other porcine or human IgG subclasses. Similarly, it is known that huIgG isotypes bind differently to mouse FcγRs than mouse IgG isotypes (39). The strong surface expression of FcγRIIIa on porcine monocytes, eosinophils, neutrophils and NK cells suggests important roles for effector functions involving these cell types. Possibly, poFcγRIIIa mediates such functions in with poIgG1a IC or in association with other poIgG isotypes. Indeed, 11 Ig heavy constant *gamma* (IGHG) genes coding for six different IgG subclasses exist in pigs whose specific functions are still unknown (40).

Interestingly, an influenza virus study in landrace cross pigs by Morgan, Holzer (41) reported a lack of efficacy of a hemagglutinin-specific huIgG1 antibody that was expected to reduce the viral load via FcγR-interaction. The mechanistic investigation by flow cytometry revealed no significant binding of free- and immune-complexed huIgG1 to porcine peripheral blood mononuclear cells including lymphocytes and monocytes, even though a slight elevation of positive cells was observed with IC. However, the results from the present study show that large IC, but not free huIgG1 below 10 μg/ml bind to monocytes and weakly to a lymphocyte subset (Fig. 4). These results are difficult to compare to our study due to the unknown huIgG1 concentration, unreported gating, and uncharacterized IC in the publication. Importantly, Morgan, Holzer (41) have shown that the therapeutic huIgG1 antibody does not elicit ADCC by porcine PBMCs and thus concluded a lacking interaction between huIgG1 and all poFcγRs. The present study confirms the lacking interaction between huIgG1 and poFcγRIIIa, that is an important mediator of ADCC in monocytes and NK cells. Nevertheless, we found that huIgG1 antibodies bind to all other poFcγRs. Even though no reduction of the viral load was observed due to lacking ADCC, the said study reported reduced gross pathology (decreased surface of lung lesion) with the hemagglutinin antibody and the huIgG1 control. As proposed before, this finding could be explained by the strong binding huIgG1 to poFcγRIIb and the expression of this receptor on porcine monocytes. The inhibitory function of poFcγRIIb could thus lead to a monocyte-mediated anti-inflammatory effect in interaction with huIgG1 complexes and therefore to reduced tissue damage. On the other hand, the inhibition could be another reason for the unaffected viral load in addition to the lack of NK cell-mediated ADCC.

CONCLUSION

In this study, we identified similarities and differences between porcine and human FcγRs regarding binding to huIgG. Taken together, we inferred proper FcγR-mediated effector functions upon treatment of minipigs with human therapeutic antibodies. Due to the similar binding properties of FcγRIa, FcγRIIa, and FcγRIIb we suggest the minipig as a valuable species for assessment of IC-mediated toxicities such as bevacizumab induced platelet activation. The limitations of the minipig relate to the failure of poFcγRIIIa to bind huIgG1 antibodies to mediate effects such as ADCC as demonstrated by the influenza study in pigs with a huIgG1 antibody discussed before (41). Because minipig NK cells express poFcγRIIIa as the only FcγR, we conclude that this cell type cannot mediate ADCC and other effector functions via huIgG1. However, monocyte-mediated effector functions cannot be excluded with huIgG1 because this cell type expresses other FcγRs in addition to poFcγRIIIa. Nevertheless, a reduced or lacking efficacy of huIgG1 antibodies is expected in the minipig. Furthermore, as in most animal species for preclinical studies, also FcγRIIIb-mediated effects of neutrophils, such as acute infusion reactions, cannot be predicted in the minipig due to the unique expression of FcγRIIIb in the human (42). However, the minipig is well suited for pharmacodynamic (PD) studies with therapeutic antibodies as comparable binding strengths of huIgGs were observed to the neonatal Fc receptor (FcRn) between minipigs and humans (43). Nevertheless, it has to be mentioned that the selection of the Göttingen minipig for preclinical studies is dependent on the pharmacological activity of the therapeutic antibody and thus cross-reactivity with the porcine target is required. Furthermore, *in vitro* functional studies and activity assays should be performed to assess the pharmacology of a particular therapeutic antibody prior to the selection of the minipig for preclinical studies.

Here we have described for the first time the cloning and expression of poFcγRIIa, as well as the binding pattern of human therapeutic antibodies to all poFcγRs. The Interaction Map analysis used in this study is a tool to understand complex binding mechanisms *in vitro* and highlights the complexity of FcγR-IgG interactions. Furthermore, it relativizes statements about FcγR affinities in interaction with IgG. Additionally, many novel special formats of therapeutic antibodies are often Fc engineered for altered FcγR binding influencing their mode of action. The binding properties of these novel antibody formats to minipig FcγRs can thus not easily be predicted from our data and will have to be established in a case by case evaluation. The experimental systems described here provide a suitable basis of tools for such evaluation.

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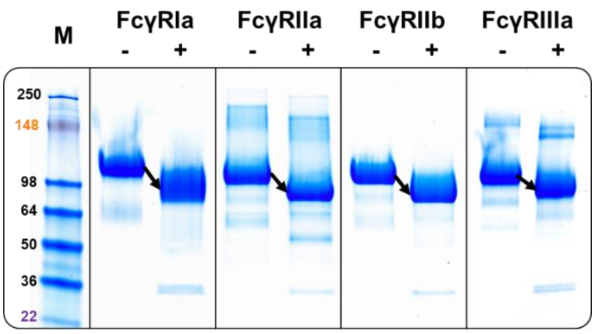

Fig. S1 Deglycosylation analysis of soluble poFcγRs. Purified soluble poFcγRs were treated with (+) or without (-) PNGase F and analyzed by SDS-PAGE. The molecular weight marker (M) is labeled with the corresponding sizes in kDa on the left of each band. Arrows highlight the reduction of the estimated size after deglycosylation. cytometry

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                  ▼                        ▼◊      ◊         ◊ ◊◊ *                        ●    ◊ ◊
Human   FcgRIa   GWLLLQVSSR VFTEGEPLAL RCHAWKDKLV YNVLYYRNGK AFKFFHWNSN LTILKTNISH NGTYHCSG.M GKHRYTSAGI SVT  184
Cyno    FcgRI    DWLLLQVSSR VFTEGEPLAL RCHAWKDKLV YNVLYYQNGK AFKFFYRNSQ LTILKTNISH NGAYHCSG.M GKHRYTSAGV SVT  184
Porcine FcgRIa   DWLLLQVSSR VFTEGEPLAL RCHGWRNKLM YNILFYQNGK TFKFSPRNSE FTILKTNLSH NGIYHCSG.M RVHRYTSAGV SIT  184
Cattle  FcgRIa   DWLLLQVTSR VFTEGDPLAL RCHAWKNMPV YKMLFYKDGK PFRFSSQDSE FTILQTNLSH NGIYHCSG.E RRRRYTSAGV SIT  184
Mouse   FcgRIa   DWLLLQASRR VLTEGEPLAL RCHGWKNKLV YNVVFYRNGK SFQFSS.DSE VAILKTNLSH SGIYHCSG.T GRHRYTSAGV SIT  183

Human   FcgRIIa  EWLVLQTPHL EFQEGETIML RCHSWKDKPL VKVTFFQNGK SQKFSHLDPT FSIPQANHSH SGDYHCTGNI GYTLFSSKPV TIT  204
Human   FcgRIIb  EWLVLQTPHL EFQEGETIVL RCHSWKDKPL VKVTFFQNGK SKKFSRSDPN FSIPQANHSH SGDYHCTGNI GYTLYSSKPV TIT  213
Cyno    FcgRIIa  EWLALQTPHL EFREGETIML RCHSWKDKPL IKVTFFQNGI AKKFSHMDPN FSIPQANHSH SGDYHCTGNI GYTPYSSKPV TIT  197
Cyno    FcgRIIb  EWLALQTPHL EFREGETILL RCHSWKDKPL IKVTFFQNGI SKKFSHMNPN FSIPQANHSH SGDYHCTGNI GYTPYSSKPV TIT  213
Porcine FcgRIIa  DWLLLQTPSL VFQEGEPIVL RCHSWRNKPL HKVVFFQNGK SKKFSYVESS LSIPHANHSH SGEYHCTGSI GKTSHSSQPV NIT  213
Porcine FcgRIIb  DWLLLQTSSL VFQEGEPILL RCHSWRNKPL HKVAFFQNGK SKKFSYVDSN FSIPQANQSH SGEYHCTGFI GKMSYSSQSV NIT  213
Cattle  FcgRIIa  DWLLLQTPSL VFQEGEPIML RCHSWRNQPL NKITFYQDGK SKTFSYQRTN FSIPRANLSH RGQYHCTAFI GKMLHSSQPV NIT  213
Cattle  FcgRIIb  DWLLLQTPSL VFQEGEPIML RCHSWRNQPL NKITFYQDRK SKIFSYQRTN FSIPRANLSH SGQYHCTAFI GKMLHSSQPV NIT  212
Mouse   FcgRIII  DWLLLQTPQR VFLEGETITL RCHSWRNKLL NRISFFHNEK SVRYHHYKSN FSIPKANHSH SGDYYCKGSL GSTQHQSKPV TIT  198
Mouse   FcgRII   DWLLLQTPQL VFLEGETITL RCHSWRNKLL NRISFFHNEK SVRYHHYSSN FSIPKANHSH SGDYYCKGSL GRTLHQSKPV TIT  199

Human   FcgRIIIa GWLLLQAPRW VFKEEDPIHL RCHSWKNTAL HKVTYLQNGK GRKYFHHNSD FYIPKATLKD SGSYFCRGLF GSKNVSSETV NIT  189
Cyno    FcgRIII  GWLLLQAPRW VFKEEESIHL RCHSWKNTLL HKVTYLQNGK GRKYFHQNSD FYIPKATLKD SGSYFCRGLI GSKNVSSETV NIT  189
Porcine FcgRIII  GWLLLQAPRW VVQEGESIRL RCHTWKNITI QKVQYFQNGM GKKFSHQNFE YHIPNATLKD GGSYFCRGII KNYDLSSEPV KVT  190
Cattle  FcgRIII  GWLLLQVAQR VVNVGKPIRL KCHSWKKTPV AKVQYFRNGR GKKYSHGNSD FHIPEAKLEH SGSYFCRGII GSKNESSESV QIT  189
Mouse   FcgRIV   GWLLLQTTKW LFQEGDPIHL RCHSWQNRPV RKVTYLQNGK GKKYFHENSE LLIPKATHND SGSYFCRGLI GHNNKSSASF RIS  188
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Fig. S2 Alignment of the Ig-like C2-type 2 domain (extracellular domain 2) of human, cyno, porcine, cattle and mouse FcγRs. Conserved Trp residues (Trp104 and Trp127 in huFcγRIa) found to be important for interaction with Pro293 of huIgG antibodies are indicated by arrowheads (▼). Residues marked with a diamond shape (◊) form hydrogen bonds between huFcγRIa and huIgG1 and the black circle (●) indicates the hydrophobic pocket huFcγRIa for Leu235 of IgG Fc (27). The asterisk (*) marks the position of the R131H polymorphism in huFcγRIIa influencing its affinity. The poFcγRIIb1 isoform differs from the displayed poFcγRIIb in the two amino acid residues highlighted in yellow (His153Asn and Asn168Asp). Sequences used for this MUSCLE alignment are: Human FcγRIa (Uniprot: P12314), FcγRIIa (Uniprot: P12318), FcγRIIb (Uniprot: P31994), FcγRIIIa (Uniprot: P08637); cyno FcγRI (Uniprot; Q8SPW5), FcγRIIa (Uniprot; Q8SPW4), FcγRIIb (Uniprot; Q8SPW3), FcγRIII (Uniprot; Q8SPW2); porcine FcγRIa (Uniprot; Q461Q0), FcγRIIa (Transcript XM_021089520), FcγRIIb (Uniprot; B9VVN4), FcγRIIIa (Uniprot; Q28942); cattle FcγRIa (Uniprot: Q9MZT0), FcγRIIa (Uniprot: A8DC37), FcγRIIb (Uniprot: Q28110), FcγRIII (Uniprot: P79107); mouse FcγRIa (Uniprot: P26151), FcγRIII (Uniprot: P08508), FcγRII (Uniprot: P08101), FcγRIV (Uniprot: Q3TC44).

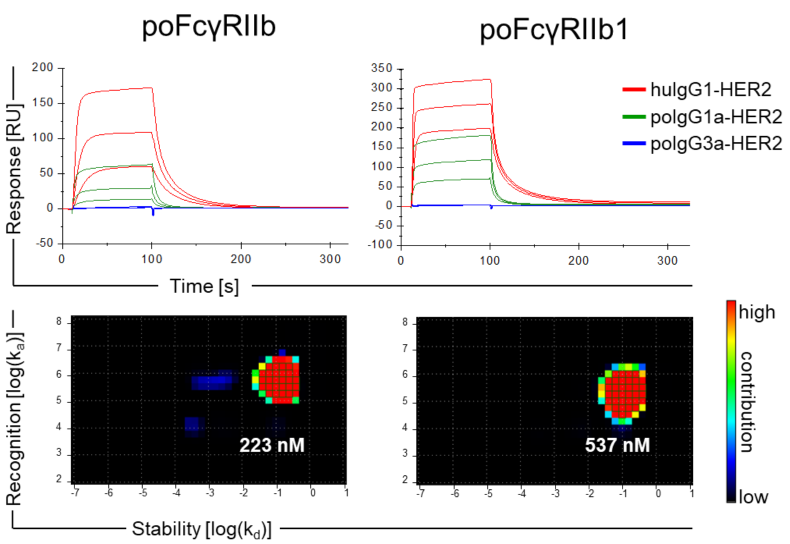

Fig. S3 SPR binding analysis comparing porcine FcγRIIb and its sub-isoform FcγRIIb1. This figure is analogous to Fig. 1b and c. The real-time sensorgrams from SPR analysis in the upper row show interaction of HER2-specific huIgG1 (trastuzumab, red), poIgG1a-HER2 (green), and poIgG3-HER2 (blue) with the respective FcγR named above. A titration with 600 nM, 200 nM, and 66.7 nM of soluble FcγR is shown binding the antigen-bound IgG on the chip surface. Interaction Map analysis resulting from trastuzumab binding to all concentrations of porcine FcγRs is shown in the lower row. The binding is separated in its parallel interactions with unique kinetics, as displayed by spots on a graph with kd on the x-axis and ka on the y-axis. The heatmap is a measure of the contribution (red = high, blue = low) of each interaction to the total binding.

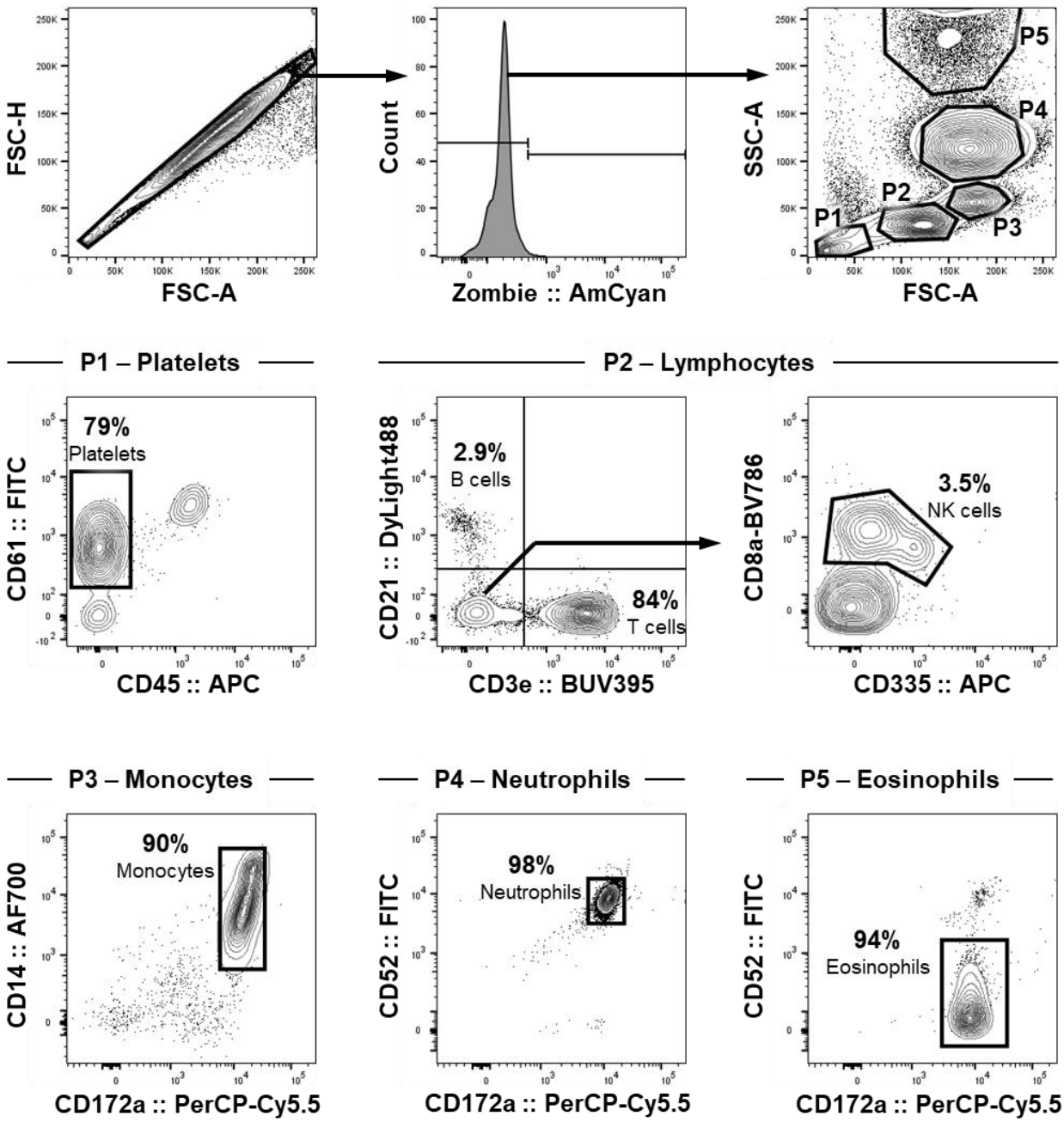

Fig. S4 Gating strategy for flow cytometry analysis of minipig blood. Whole blood from Göttingen minipigs was stained with the indicated fluorochrome-labeled antibodies. From singe and live cells, gates P1-P5 were selected using forward (FSC) and side scatter (SSC) and cell types were identified using the following antibody clones: CD45 (K252.1E4), CD61 (JM2E5), CD3e (BB23-8E6-8C8), CD21 (BB6-11C9.6), CD335 (VIV-KM1), CD8a (76-2-11), CD172a (74-22-15A), CD14 (MIL2), and CD52 (11/305/44). Numbers indicate the percentage of cells within the respective population (P1-P5).

7.4 Supplementary experiments

This section provides supporting information such as further experiments, approaches, and discussions regarding the binding characterization of human therapeutic antibodies to poFcγRs.

Table 7.1 shows a recap on the IgG-FcγR binding properties resulting from Manuscript 2. The binding strength was categorized according to the dissociation curves from SPR sensorgrams and affinity calculations from Interaction Map analysis (Manuscript 2 Fig. 1). Importantly, smaller differences in binding strength are not reflected in Table 7.1. Therefore, it should be noted that the huIgG1 interaction is stronger to poFcγRIIb and weaker to poFcγRIa than to the human homologs.

Table 7.1 Summary of interactions between IgG and FcγRs generated from Manuscript 1 Fig.1.

		Human IgG1	Porcine IgG1a	Porcine IgG3
Porcine	FcγRIa	+++	+++	+/-
	FcγRIIa	++	+	+/-
	FcγRIIb	+	+	-
	FcγRIIIa	-	+	-
Human	FcγRIa	+++	-	+/-
	FcγRIIa	+	+/-	-
	FcγRIIb	+	-	-
	FcγRIIIa	+	-	-

+++	stable interaction (slow dissociation, KD <10 nM)
++	heterogeneous stability (one quick and one slow dissociation)
+	low stability (quick dissociation, KD 100-800 nM)
+/-	trace interaction (KD in the range of 1 µM)
-	no interaction detected

7.4.1 Recombinant expression of soluble porcine FcγRs

SPR binding studies presented in Manuscript 2 are based on the recombinant expression of poFcγR as Fc fusion proteins, termed soluble poFcγRs. The generation of Fc fusions is a frequently employed method for better soluble expression and allowed the dimerization of the FcγR extracellular domains for higher assay sensitivity. Because regular Fc parts of IgG antibodies interact with FcγRs, they were modified by PGLALA (Pro329G, Leu234Ala, Leu235Ala) mutations to abolish huFcγR interactions [37]. After transient expression of soluble poFcγRs in human embryonic kidney 293F (HEK293F) cells, we observed strong aggregation of poFcγRIa, IIa, and IIIa but not of poFcγRIIb and IIb1. Not all aggregates could be excluded after size exclusion chromatography (SEC) purification and collection of the monomeric fraction. We systematically tested different buffer conditions to reduce the aggregate formation of soluble poFcγRs during purification. The results showed that acidic buffers (pH < 5.5) reduced the formation of aggregates whereas common stabilizers like arginine and potassium L-glutamate had no effect. Moreover, high concentrations of sodium chloride (500 mM), were found to be useful during SEC for a better separation of aggregates from the monomeric fraction. The reason

for the high content of aggregates and the dynamic equilibrium is still unknown. The aggregation could possibly be process-related initiated by the low pH during protein A purification or by protein concentration. Fine-tuning of the expression system and the purification process are thought to allow a higher yield of aggregate-free preparations. Alternatively, aggregates could result from binding of the respective poFcγR extracellular domain to the IgG Fc fusion tag of neighboring proteins. The PGLALA mutations included in the Fc fusion tag is known to abolish the binding to all huFcγRs. However, no data about poFcγRs are available describing PGLALA binding and we did not specifically test for that. Hence, testing of alternative fusion proteins for dimerization is recommended.

7.4.2 IC binding to FcγRs on HEK293F cells

In Manuscript 2, full length FcγRs expressed on the cell surface of HEK293F cells were tested for binding to free huIgG1 (bevacizumab). The results from Manuscript 2 indicated a concentration dependent binding of huIgG1 to all huFcγRs and to poFcγRIa, IIa, and IIb. PoFcγRIIIa, however, did not interact with free huIgG1. HEK293F cells expressing surface huFcγRs were also found useful to investigate binding to huIgG1 and huIgG4 hexameric-Fc fusion proteins representing immune complexes [99]. To study possible interactions of poFcγRIIIa with complexed huIgG1, we also used this system for binding studies with IC composed of bevacizumab and its target VEGF. In parallel to free huIgG1, we therefore incubated FcγR expressing cells with huIgG1 IC as described for studies in minipig blood (Manuscript 2). The cells were analyzed by flow cytometry after detection of surface bound IC with a PE-conjugated goat F(ab')2 antibodies against huIg-*kappa*.

Similar to the experiment with free huIgG1, we observed concentration dependent binding of huIgG1 IC (Fig. 7.2A). In contrast to the results with free huIgG1, complexed huIgG1 bound to poFcγRIIIa, albeit weaker than all tested human (not shown) and porcine FcγRs. The stronger binding of all porcine and human FcγRs to IC than to free huIgG1 is evident by elevated median fluorescence intensity (MFI) even though the fluorescence intensity is not normally distributed (Fig. 7.2B). Unexpectedly, non-transfected (not shown) or HEK293F cells, as well as HEK293F cells transfected without plasmid, showed high background signal by binding complexed huIgG1 in a concentration dependent manner (Fig. 7.2A and B). Therefore, data generated with IC could not be properly interpreted and were not included in the manuscript.

The reason for the strong IC binding and clean background with free IgG could be due to native expression of IgG receptors. However, the native expression of FcγRs was excluded on HEK293F cells by staining with anti-human CD64, CD32, and CD16 antibodies (see control in Fig. 2A of Manuscript 2). A diverse range of other, structurally unrelated, Fc binding proteins could be responsible for interactions with huIgG1 IC. Apart from Ig-specific proteins like the neonatal Fc receptor (FcRn), poly Ig receptors (pIgR), or Fc receptor-like (FcRL) proteins; other proteins such as mannose-binding lectins

(e.g. MBL2), macrophage mannose receptor (MMR), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), Dectin-1, or other C-type lectins could bind to glycostructures or repetitive patterns within IgG IC [7].

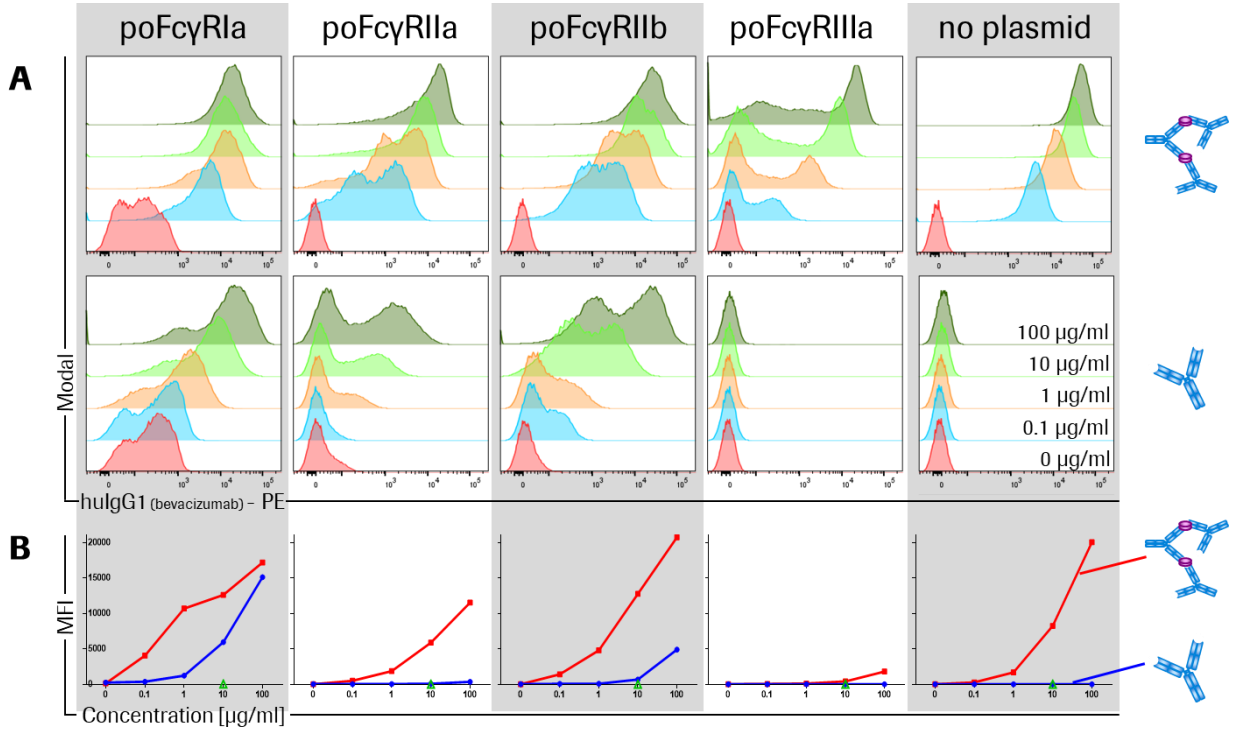

Fig. 7.2 Binding of free huIgG1 and IC to membrane-anchored porcine FcγRs expressed HEK293F cells. ***(A)*** *IC composed of huIgG1 and VEGF (upper panel, indicated by the IC drawing) or free huIgG1 (lower panel, indicated with the free antibody drawing) were incubated with HEK293F cells expressing the above indicated FcγR. After intense washing, FcγR bound IgG was stained with PE-conjugated goat F(ab')2 anti-huIg-*kappa *secondary antibody and analyzed by flow cytometry. Stacked histograms show binding of the following IgG concentrations in the according preparations: 100 µg/ml (dark green), 10 µg/ml (light green), 1 µg/ml (orange), 0.1 µg/ml (blue), and no IgG (red).* ***(B)*** *Graphs show the median fluorescence intensity (MFI, y-axis) measured with increasing concentration (x-axis) of IC (red curve) or free huIgG1 (blue curve). Green open triangles represent 10 µg/ml VEGF used as a negative control.*

7.4.3 IgG binding to blood and lymph nodes with cell surface markers

The binding of free huIgG1 and IC to the major blood cell subsets of the Göttingen minipig was studied in Manuscript 2. Thereby, we observed a minor lymphocyte subset binding to huIgG1 IC (Manuscript 2 Fig. 4). The aim of the following experiments described here was to closer identify this cell population and to assess the IC binding capacity of cells derived from lymph nodes and blood. The lymph node was in a special focus due to its active function during immune reactions and the abundance of B and T lymphocytes. Therefore, we performed further flow cytometry binding studies with free- and immune-complexed huIgG1 in blood and lymph nodes, as well as with huIgG1, 2, 3, 4 and poIgG1a, 3 subclasses in blood.

Therefore, we isolated the cells from blood and lymph nodes of two Göttingen minipig, as described earlier. Then, the isolated cells were co-incubated with free huIgG1 (bevacizumab) and huIgG1 IC (bevacizumab-VEGF) followed by staining with cell-type specific surface markers. Subsequently, cell-bound free- and immune-complexed huIgG1 was stained with PE-conjugated goat F(ab')2 antibodies against huIg-*kappa* and assessed by flow cytometry (experimental conditions described in Manuscript 1 and paragraph 6.4.2 above). Analogous, we co-incubated cells isolated from minipig blood with 10 µg/ml of human IgG1-4 *kappa* from human myeloma plasma (Sigma Aldrich), as well as with 10 µg/ml recombinant poIgG1a-HER2 and poIgG3-HER2 (Manuscript 2). Cell-bound free human and porcine IgG subclasses were stained with PE-conjugated goat F(ab')2 antibodies against huIg-*kappa* and assessed by flow cytometry.

The results in Fig. 7.3A show that lymphocyte subsets from minipig blood and lymph nodes only marginally bind free- and immune-complexed huIgG1, as previously observed without cell-type specific surface markers (Manuscript 1 Fig. 4). Nevertheless, the strongest binding of huIgG1 IC was identified on CD8+ NK cells (Fig. 7.3A; gating shown in Fig. 6.2A). Yet, the results varied in intensity among the two analyzed minipigs. CD335+ NK cells show reduced IC binding compared to the CD8+ subset, whereas both subsets did not bind free huIgG1. Here, NK cells from the blood are shown but similar results were observed in the lymph node where the abundance of NK cells is relatively low. The positive lymphocyte subset observed in Manuscript 2 Fig. 4 could be attributed to these NK cell subsets. NK cells express high levels of poFcγRIIIa as the only FcγR [82] (Fig. 6.1; Manuscript 1). Interestingly, highly sensitive SPR assays and cellular binding studies presented in Manuscript 2 indicate that huIgG1 does not interact with poFcγRIIIa while interactions with huFcγRIIIa were well detectable. Therefore, the binding of huIgG1 IC to porcine NK cells is unlikely to be mediated via poFcγRIIIa. Nevertheless, weakest interactions with poFcγRIIIa with an equilibrium dissociation constant (KD) of >10 µM cannot be excluded [37]. Thus, it could be speculated that a very weak, almost undetectable, interaction of huIgG1 with poFcγRIIIa remains, which allows avidity-based binding of high IC concentrations. As hypothesized for HEK293F cells, also NK cells express a broad range of receptors that could contribute to IC binding. In addition to the receptors mentioned before in the context of HEK293F cells, NK cells express e.g. killer cell lectin receptors (KCLR) containing C-type lectin structures, that could potentially bind structures of complexed IgG [100].

Interestingly, T cells mildly bound huIgG1 IC in concentrations above 10 µg/ml. Among them, CD8+ T effector cells showed the most pronounced shift of fluorescence intensity with high concentrations of IC. Interactions with CD4+ T helper cells and CD4/CD8 double positive T cells however were weaker (Fig. 7.3A). Older studies have already detected binding of IC, but not of free IgG to activated T lymphocytes in mice and postulated the presence of receptors for aggregated IgG in these cells [101, 102]. A subset of IC binding T cells was found to express Fc receptors [103]. Our single cell RNA

sequencing results suggest the presence of FcγRIIIa mRNA in T cells of minipigs and humans (Manuscript 1 Fig. 6). Nevertheless, the huFcγR expression on human T cells is controversially discussed in the literature. As previously indicated, the expression of huFcγRs is not excluded due to the difficulty to examine all possible T cell subsets and activation states [104]. Apart from poFcγRs and other IgG-binding receptors, the IC interactions with T cells, NK cells, and others could be influenced by charge-mediated interactions. In general, antibodies are positively charged at a neutral pH of 7.4. Bevacizumab used for these experiments is no exception with an isoelectric point of 8.3 [105]. The resulting positive charge at lower pH allows antibodies to interact with negatively charged cells resulting in uptake via fluid phase pinocytosis [106]. This suggests possible charge-mediated interactions with cells of the minipig that are enhanced by avidity effects in the case of large IC. However, B cells of minipigs and humans are known to express FcγRIIb (Manuscript 1; [12]). Even though poFcγRIIb can bind huIgG1, we did not observe IC binding to B cells probably due to the high background with the detection antibody (Fig. 7.3A, Fig. 6.2). Fig. 7.3A shows B cells and T cells from the lymph nodes due to their high abundance, but similar results were observed in the blood.

Apart from the huIgG1 therapeutic antibody bevacizumab, we also assessed the binding of further free porcine and human IgG subclasses to minipig blood (Fig. 7.3B). While both tested huIgG1 antibodies showed similar results (data not shown), also huIgG3 and huIgG4, but not huIgG2 interacted with platelets. Additionally, huIgG4 also showed the strongest binding to monocytes, neutrophils, and eosinophils. Similarly, huIgG1 and huIgG3, followed by huIgG4 are the strongest binders to most huFcγRs, [107]. HuIgG2 that does not bind to minipig blood cells also shows the weakest binding to huFcγRs. Analogous to huIgG1, also poIgG1a bound to minipig platelets. Additionally, poIgG1a also bound to minipig monocytes, neutrophils, and eosinophils in descending order of strength whereas huIgG1 did not bind these cell types. This difference is most likely mediated by poFcγRIIIa that binds poIgG1a but not huIgG1. Comparable to huIgG3, also poIgG3 did not bind any blood cell subsets in the minipig. However, these results do not suggest the orthology of the different IgG subclasses between the species. In contrast, poIgG3 was predicted by sequence analysis to show the strongest FcγR-binding among all porcine IgG subclasses [108].

Furthermore, we performed pilot studies with ICs composed of the different porcine and human IgG subclasses. The complexes were generated by cross-linking of the IgG subclasses via PE conjugated goat F(ab')2 anti-human Ig-*kappa* antibody as previously published [107]. However, these experiments did not yield acceptable results for all IgG subclasses. Either because of the lacking signal amplification obtained when a secondary antibody is used or because of an incomplete IC formation.

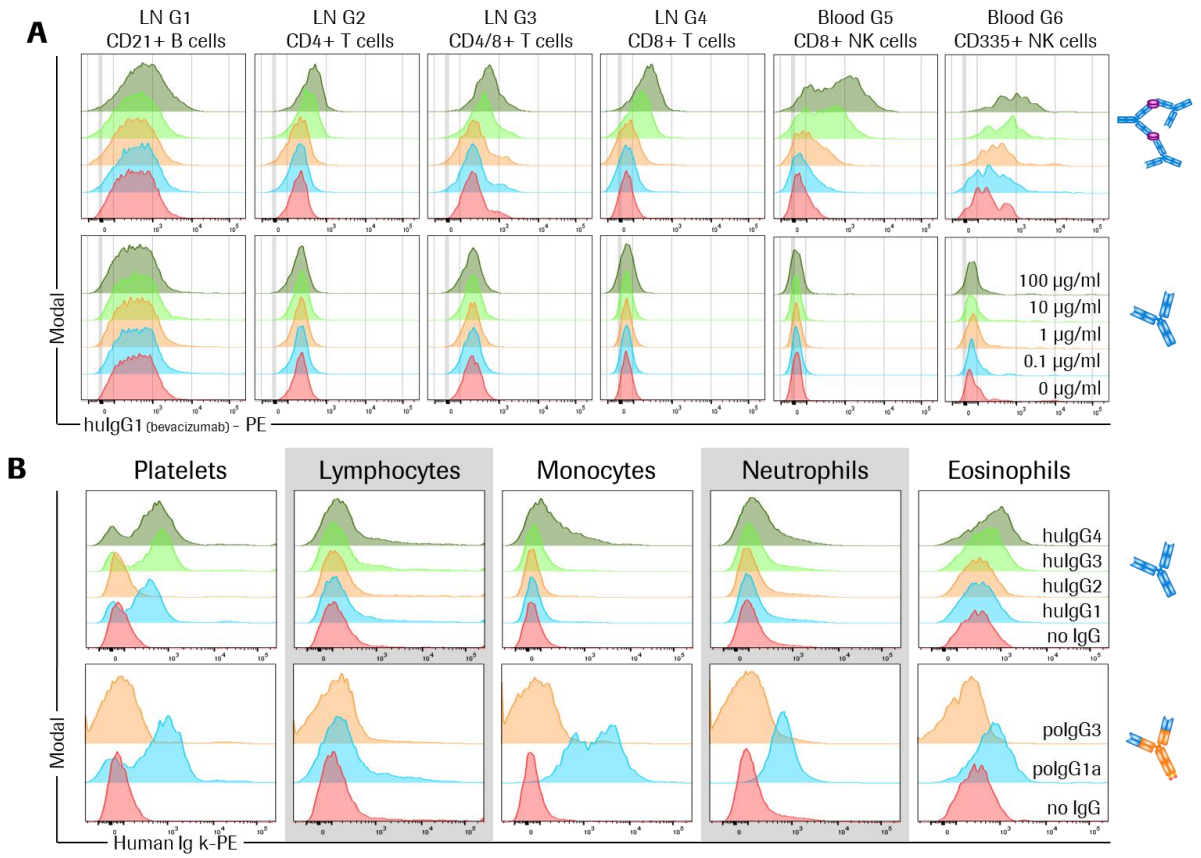

Fig. 7.3 Human and porcine IgG and IC binding to minipig cells. ***(A)*** *B cell and T cell subsets (G1-G4) from lymph nodes were investigated (LN) whereas NK cell subsets (G5 and G6) were analyzed in the blood (gating shown in Fig. 6.2A). IC (upper row) and free bevacizumab (lower row) indicated by the drawing on the right were titrated using preparations containing IgG concentrations of 100 µg/ml (dark green), 10 µg/ml (light green), 1 µg/ml (orange), 0.1 µg/ml (blue), and no IgG (red). After intense washing, cell bound IgG was stained with PE-conjugated goat F(ab')2 anti-huIg-kappa secondary antibody and analyzed by flow cytometry.* ***(B)*** *Platelets, lymphocytes, monocytes, neutrophils, and eosinophils (from left to right) were gated from forward and sidescatter as described in both manuscripts. Histograms in the upper row (huIgG, blue antibody drawing) show the binding of 10 µg/ml of free huIgG4 (dark green), huIgG3 (light green), huIgG2 (orange), huIgG1 (blue), and no IgG (red), whereas the lower row (poIgG, orange antibody drawing) shows binding of 10 µg/ml of free poIgG3 (orange), poIgG1a (blue), and no IgG (red).*

7.4.4 IgG binding after blocking of porcine FcγRIIIa with anti-pig CD16 antibody

The flow cytometry studies on minipig whole blood shown before indicate a possible role of poFcγRIIIa expressed on NK cells as a potential binder of huIgG1 IC. Blocking of poFcγRIIIa was supposed to clarify its contribution to the binding of IC as observed in poFcγRIIIa expressing NK cells.

Therefore, we blocked the poFcγRIIIa in the blood of two different minipigs using 0.1, 1, and 10 µg/ml unlabeled anti-pig CD16 antibody (clone G7) prior to the incubation with 10 µg/ml free- or immune-complexed huIgG1 (bevacizumab). Apart from poFcγRIIIa blocking, all conditions were the same as in the previous experiment (Fig. 7.3A).

The anti-pig CD16 antibody clone G7 was shown to almost completely block ADCC in peripheral blood leucocytes and reducing ADCC in polymorphonuclear leukocytes [109]. Additionally, this antibody inhibits the poFcγRIIIa-mediated antibody-dependent enhancement of PRRSV infection [110]. In our hands binding of free- and immune-complexed huIgG1 to NK cells, monocytes, neutrophils, and eosinophils could not be reduced after blocking with this CD16 antibody (not shown). As expected, also cell types lacking poFcγRIIIa, such as platelets and T cells were not affected (not shown). This result suggests that poFcγRIIIa is not involved in the IC binding of NK cells and confirms its inability to bind huIgG1. However, due to the lack of a positive control confirming poFcγRIIIa blocking, we cannot confirm complete blocking by anti-pig CD16 antibody clone G7. Therefore, we excluded this data from the manuscript.

8 Discussion

The scarce knowledge about poFcγRs in the minipig limit the translatability of preclinical studies with human therapeutic antibodies in this animal model. Therefore, we investigated the poFcγRs on a gene, transcript, protein, and functional level in the scope of two comprehensive manuscripts and unpublished supplementary results. The results from Manuscript 1 about the FcγR expression pattern is graphically displayed in Fig. 6.1 whereas the results from Manuscript 2 about the interactions of IgG antibodies with porcine and human FcγRs is summarized in Table 7.1. All similarities and differences between porcine and human FcγRs found in this study are compiled in Table 8.1. The combination of data on poFcγR expression and their interactions with huIgG enable an estimation about the predictivity of the minipig in preclinical studies with human therapeutic antibodies. Apart from therapeutic antibody research, understanding of IgG-FcγR interactions is also important for therapeutic purposes and immunoprophylaxis in the pig [68], as well as for vaccine studies, PRRSV infection studies [110-112], and porcine inflammation models [70].

8.1 Similarities and differences of porcine and human FcγRs

In general, our results show that porcine and human FcγRs share a rather conserved genomic organization and similar protein structures. However, distinct differences between minipig and human were found concerning FcγR expression and binding (summarized in Table 8.1). Different expression patterns, protein domains, or binding affinities can impact the function of the respective FcγR.

8.1.1 Binding mechanisms of FcγRIa

In human, free huIgG1, 3, and 4 bind huFcγRIa with high affinity [107]. This binding was shown to mediate the internalization and recycling of the IgG-FcγRIa complex [113] suggesting a role as scavenger receptor [71] which allows constant sampling of extracellular antigens. Interestingly, the strong binding of huIgG1 to huFcγRIa (nanomolar dissociation constant) suggests a constant occupation of the receptor with IgG present in high concentrations in serum and body fluids [9]. But like for all activating FcγRs, IC-mediated aggregation of the FcγR on the cell surface is a prerequisite for activation signaling leading to effector functions. However, free IgG readily dissociates from huFcγRIa with a half-life in the range of minutes causing a constant turnover which allows the binding of IC. This can lead to receptor aggregation and activation and thus promoting inflammation and anaphylaxis. In addition to the function of huFcγRIa in anti-tumor immunotherapy, an efficient role is suggested in binding of sparsely opsonized antigen or large complexes as they appear early in immune responses [9, 114]. Similar mechanisms could be assumed in the minipig due to the strong binding of free poIgG1a and huIgG1 as reported in SPR experiments (Manuscript 2, Fig. 1). Even though huIgG1 binds to poFcγRIa with high affinity, its interaction was less stable as compared to the human orthologue. The

resulting consequences for the minipig as a preclinical species with therapeutic antibodies are unclear. On one hand, the lower stability could lead to a quicker dissociation of free huIgG and thus to a better availability for IC binding. On the other hand, the weaker binding could lead to less potent activation by huIgG1 IC. Nevertheless, poFcγRIa is anticipated to be of the right stability to allow receptor occupancy by circulating free IgG and binding of IC, as it is suggested for huFcγRIa. Furthermore, our results with poFcγRIa showed a stronger binding stability with IC compared to free IgG thus supporting the suggested similarity to huFcγRIa (Manuscript 2). We found a similar FcγRIa cellular distribution in the blood of both species further suggesting analogous functions (Manuscript 1).

8.1.2 FcγRIIa orthology and signaling

Together with other low affinity activating FcγRs, FcγRIIa is involved in ADCC, ADCP, endocytosis, cytokine release, and antigen presentation upon aggregation via IC [115, 116]. Orthology between porcine and human low affinity FcγRs is assumed based on sequence similarities of the extracellular domains (displayed as percentage in Table 8.1) and the structure of the low affinity *FCGR* locus (Manuscript 1, Fig. 4). In Manuscript 1, we describe the identification of a hitherto unknown porcine receptor with high extracellular similarity to FcγRIIa of primates and therefore named it poFcγRIIa. Despite the high extracellular similarity, no intracellular ITAM for transmission of activation signals was detected in the newly identified poFcγRIIa as it is described for human and NHP FcγRIIa [117]. Due to the presence of a conserved charged aspartic acid residue in the transmembrane domain we expect the association of poFcγRIIa with adaptor proteins, such as the FcR γ-chain for activation signaling (Manuscript 1) [13, 104]. Transcripts similar to poFcγRIIa lacking intracellular ITAM and requiring adaptor protein association were also found in cattle (Uniprot accession: A8DC37), sheep (W5PK06), and rat (M0R4F7) and known as FcγRIII in the mouse [118]. Interestingly, swapping of ITAMs between huFcγRIIa and the endogenous FcR γ-chain revealed qualitatively different responses mediated by the individual ITAMs [119]. Therefore, different intracellular domains and interactions with adaptor proteins between porcine and human FcγRIIa indicate different signaling mechanisms. Such differences have to be further studied and considered when using therapeutic antibodies binding to FcγRIIa of minipigs, cattle, and (to FcγRIIIa of) mice. Due to these pronounced intracellular differences, mouse FcγRIII is occasionally not considered as an orthologue to huFcγRIIa [120]. In general, the previously described mosaicism (Manuscript 1 Fig. 3) suggests a more complex picture about the relation between the low affinity FcγRs in mammals. Recent studies describe gene copy number variations within the human low affinity *FCGR* locus in association with disease [121]. Similar mechanisms during evolution possibly led to this mosaicism and the unique appearance of FcγRIIc and FcγRIIIb in humans [122, 123]. Even though the term “orthology” usually applies to a whole gene with a common ancestor, we would therefore suggest to limit the concept of orthology in low affinity FcγRs only to the extracellular domains.

8.1.3 IgG binding and function of FcγRIIIa

The huFcγRIIIa is known to mediate important effector functions such as ADCC upon interaction with huIgG1 opsonized cells. Human and porcine FcγRIIIa share similar protein structures for IgG binding, associate with the FcR γ-chain for activation signaling, and are expressed on NK cells and $CD14^{low}$ monocytes important for ADCC. Interestingly, the binding of huIgG1 to poFcγRIIIa is lacking, while endogenous poIgG1a binds to poFcγRIIIa with low affinity. Taken together with its strong expression in minipig blood these findings suggest important roles of this receptor that require further investigation. Six different IgG subclasses and five allotypes have been described in the pig that could differently interact with poFcγRIIIa to mediate effects similar to ADCC in the human [108]. The involvement of poFcγRIIIa in antibody-dependent enhancement of PRRSV infection suggests a role in endocytosis and cytokine production [110, 124]. Additionally, poFcγRIIIa associates with a protein similar to the antimicrobial cathelin, suggesting unique unknown functions in porcine immunity [81].

8.1.4 Inhibitory signaling via ITAM (ITAMi)

An interesting function of huFcγRIIa and huFcγRIIIa is the ability to transmit inhibitory signals via ITAM (ITAMi) upon binding of free huIgG having anti-inflammatory effects [125]. The inhibitory signaling via FcR γ-chain of huFcγRIIIa is therefore a potential mechanism of the anti-inflammatory treatment with intravenous immunoglobulin (IVIg) [126]. ITAMi signaling remains to be demonstrated for the pig. However, the inhibitory potential of huIgG1 via ITAMi is expected to be negligible in monocytes due to the lacking expression of poFcγRIIa and the inability of poFcγRIIIa to bind huIgG1. However, ITAMi signaling could be a possible mechanism of platelet homeostasis due to the strong expression of poFcγRIIa on this cell type.

8.1.1 Inhibitory signaling of FcγRIIb

Porcine and human FcγRIIb both contain an intracellular ITIM for inhibitory signaling [75, 117]. As the only inhibitory Fc receptor, FcγRIIb balances the signals of the activating FcγRs and thus inhibits their functions upon co-aggregation [127]. Differences in the expression level and IgG binding strength between the activating and inhibitory FcγRs are therefore thought to influence effector functions. Contrasting the human expression, we found poFcγRIIb on blood monocytes. Analogous to macaques [45, 51], we also reported a stronger binding of huIgG1 to porcine than to human FcγRIIb (Table 7.1; Table 8.1; Manuscript 2).

Table 8.1 Similarities and differences between minipigs and humans as identified in this thesis.

	Similarities	Differences
All FcγRs	◦ Rather conserved genomic organization ◦ FcγRs are glycosylated IgG binding proteins expressed on the cell surface of diverse immune cells [128] ◦ Conserved Trp residues interacting with Pro residues of IgG [51, 129] ◦ Enhanced binding to IC compared to free IgG [107]	◦ Orthology of individual receptors is not necessarily given
FcγRIa	◦ Extracellular amino acid (aa) similarity of 87% ◦ Contain three extracellular Ig-like domains and a hydrophobic pocket for interaction with IgG [130] ◦ Expressed on blood monocytes but not on blood DC [12] ◦ Regulated expression on human and porcine DCs [86, 131] ◦ High affinity FcγR for huIgG1 [107]	◦ *FCGR1B* and *FCGR1C* pseudogenes are known in humans but not in pigs [8] ◦ HuIgG1 binds more stable to human than to porcine FcγRIa
FcγRIIa	◦ Extracellular aa similarity of 79% ◦ Polymorphisms identified in both species [132] ◦ Expressed on blood platelets [120]	◦ Human but not porcine FcγRIIa contains an intracellular ITAM [118] ◦ Porcine but not human FcγRIIa interacts with FcR γ-chain via charged aspartic acid [13] ◦ Human but not porcine FcγRIIa is expressed on monocytes, neutrophils, and eosinophils [133] ◦ HuIgG1 binds stronger to porcine than to human FcγRIIa
FcγRIIb	◦ Extracellular aa similarity of 77% ◦ Expressed on B cells and DCs in the blood and on monocytes from lymph nodes and spleen [12] ◦ Low affinity FcγR for huIgG1 [107]	◦ Porcine but not human FcγRIIb is highly expressed on blood monocytes [12] ◦ HuIgG1 binds stronger to porcine than to human FcγRIIb
FcγRIIIa	◦ Extracellular aa similarity of 74% ◦ Expressed on NK cells [12]	◦ Porcine FcγRIIIa is expressed on all monocytes, and human FcγRIIIa on $CD14^{low}$ monocytes only [44] ◦ Porcine but not human FcγRIIIa is expressed on granulocytes [133] ◦ HuIgG1 binds to human but not to porcine FcγRIIIa [107]
FcγRIIc		◦ Not found in pigs
FcγRIIIb		◦ No GPI linked FcγR is found in pigs

8.1.1 Absence of huFcγRIIc and huFcγRIIIb in the minipig

So far, the human is the only species known to express the activating huFcγRIIc and the GPI-anchored huFcγRIIIb. Manuscript 1 shows the analysis of the complete low affinity *FCGR* locus in the minipig without the identification of potential presence of porcine *FCGR2C* and *FCGR3B* genes. If organized similar to humans, these genes should be located between *FCGR2B* and *FCGR3A* in the minipig, where we did not find any sequences associated with FcγRs. Therefore, we and others concluded that these duplications are exclusively found in humans [8].

8.2 Consequences for the evaluation of minipig in preclinical studies

Similar to poFcγRs; NHP and mouse FcγRs share similar structures and functions with huFcγRs. However, characteristic differences have been observed in terms of their expression and interactions with human antibodies [45, 118]. In general, such differences are likely to impact antibody-mediated effector functions and therefore also effects of human therapeutic antibodies tested in animal models [118]. It is assumed that the low transition rate of therapeutic antibodies from preclinical trials to approval may be influenced by misleading readouts in the preclinical species due to diverging FcγR properties [45]. For example, the CD28 superagonist TGN1412 triggered severe side effects in healthy volunteers that were not predicted from NHP studies [134]. Besides a divergent expression of CD28 in cyno and human [135] it is hypothesized that the cytokine storm was not predicted due to differences in FcγR interactions with huIgG4 [27]. More recent data emphasize the involvement of FcγRs in the toxic activity of this therapeutic antibody [136, 137].

8.2.1 FcγR-mediated platelet activation and toxicity

FcγRIIa is mainly expressed on platelets in pigs, whereas humans additionally express this receptor on neutrophils, DCs, monocytes, and macrophages (Fig. 6.1; Manuscript 1) [12]. IC-mediated aggregation of huFcγRIIa on platelets leads to the release of pro-inflammatory modulators attracting neutrophils to the site of infection. Furthermore, it also results in platelet activation and aggregation that can have pathologic consequences, such as thrombosis formation followed by stroke or and myocardial infarction [120]. Indeed, clinical trials with antibodies against CD40 ligand resulted in severe thromboembolic complications, such as myocardial infarction [31], that were not predicted in mice. Mechanistic studies indicated that IC (therapeutic antibody and CD40 ligand) lead to platelet activation through huFcγRIIa [32]. Similarly, treatment with bevacizumab forms large IC with VEGF, its dimeric target [29], which activate platelets in the presence of heparin. In huFcγRIIa transgenic mice but not in wild type mice, platelet activation leads to adverse events resembling heparin-induced thrombocytopenia [30]. In contrast to mice, minipigs expresses high levels of endogenous poFcγRIIa that interacts with free huIgG1 and strongly binds IC formed by bevacizumab and VEGF (Manuscript 2

Fig. 4). Therefore, the minipig could be a relevant model to study FcγRIIa-mediated activation of platelets and the subsequent toxic or therapeutic effects. Because huIgG1 binds stronger to porcine than to human FcγRIIa, there is the possibility of stronger huIgG1 mediated effects on platelets in minipigs. This could potentially lead to adverse events related to platelet aggregation in minipigs upon treatment with huIgG1 that would not be observed in humans at similar doses. In minipigs, also endogenous poIgG1a strongly binds to poFcγRIIa leaving platelet homeostasis unaffected. Therefore, human therapeutic antibodies first have to compete with endogenous levels of around 20 µg/ml poIgG for poFcγRIIa binding [138]. Nevertheless, IC-mediated platelet activation can still be expected since IC binding to poFcγRIIa is substantially enhanced compared to free IgG. Indeed, FcγR-mediated platelet activation and subsequent toxicities were observed upon treatment of pigs with a mouse IgG2b antibody against porcine CD14. The recombinant version, where mouse IgG2b is replaced by the human IgG2/IgG4 hybrid constant region, however, lacked the undesired effects probably due to abolished FcγR and complement binding [70]. This suggests the possibility of poFcγRIIa to bind mouse IgG2b, but not huIgG2/4 to trigger platelet-mediated toxicities.

8.2.2 ADCC in the minipig by NK cells and monocytes

Target cell killing via ADCC is of high importance for several cytotoxic therapeutic antibodies. Main drivers for ADCC with cytotoxic huIgG1 therapeutic antibodies in the human are interactions with huFcγRIIIa expressed on NK cells [139, 140]. NK cells in the minipig fully reflect the human situation by expressing FcγRIIIa as the only FcγR. Therefore, NK-mediated ADCC in minipigs would be expected due to the expression of the orthologous receptor. However, poFcγRIIIa does not bind huIgG1 and is therefore excluded as a mediator of ADCC with most human therapeutic antibodies. This finding is of high importance because it limits the use of minipigs for preclinical studies with cytotoxic antibodies of the huIgG1 subclass. On the other hand, our results suggest that NK cells could potentially mediate ADCC upon vaccination of minipigs because endogenous poIgG1a, generated in a regular immune response, interacts with poFcγRIIIa. This could be beneficial for tumor vaccination or infection studies where active immunization is desired.

Importantly, huFcγRIIIa was also found to be crucial for ADCC elicited by monocytes [141]. FcγRIIIa is only expressed on a subset of monocytes in the human, whereas it is expressed on all monocytes in the minipig. Again, the inability of huIgG1 to bind poFcγRIIIa on minipig monocytes excludes this receptor as a mediator of ADCC with huIgG1. Nevertheless, also huFcγRIa and huFcγRIIa on monocytes were identified to contribute to ADCC [18, 142, 143]. Single cell RNA sequencing identified the expression of poFcγRIa, but we detected only negligible RNA levels of poFcγRIIa (Manuscript 1 Fig. 6). Therefore, alternative mechanisms in monocytes involving poFcγRIa could trigger ADCC in the minipig [142, 143]. However, in contrast to the human, minipig blood monocytes additionally express the inhibitory poFcγRIIb representing a further mechanism suppressing ADCC with huIgG1 in the minipig [144].

Recently, an influenza virus study in landrace cross pigs by Morgan, Holzer [69] reported lack of efficacy of a huIgG1 antibody that was expected to reduce the viral load via FcγR-interaction. The mechanistic investigation by flow cytometry revealed no significant binding of free- and immune-complexed huIgG1 to porcine PBMCs and CD3- CD8a+ NK cells, even though a slight elevation of positive cells were observed with IC. The results from this thesis show that large IC, but not free huIgG1 below 10 µg/ml bind to monocytes and weakly to NK cells (Manuscript 2 Fig. 4; Fig. 7.3A). These results are difficult to compare to our study due to the unknown huIgG1 concentration, unreported gating, and uncharacterized IC in the publication. Furthermore, Morgan, Holzer [69] have shown that the therapeutic huIgG1 antibody does not elicit ADCC by porcine PBMCs and thus concluded a lacking interaction between huIgG1 and all poFcγRs. Interestingly, human monocytes and macrophages are less efficient and potent than NK cells in mediating ADCC *in vitro* [145] leading only to a cytotoxicity of 5-30% after 24h [40]. The shorter incubation time of 4h chosen by Morgan, Holzer [69] may therefore reflect ADCC elicited by NK cells but not by monocytes. This finding confirms the assumption of absent ADCC by NK cells due to absent poFcγRIIIa binding. However, the role of monocytes and macrophages in ADCC and also ADCP has to be further studied.

8.2.3 FcγR-mediated functions of neutrophils

Human neutrophils are important for the protection against pathogens and express high levels of huFcγRIIa and huFcγRIIIb, inducible expression of huFcγRIa but no huFcγRIIIa [133]. Among many functions of tissue-resident neutrophils, FcγRs were shown to be involved in the phagocytosis of antibody opsonized microbes, as well as release of reactive oxygen species and cytokines upon interactions with IC [133]. Neutrophils play a role in IgG1-mediated passive systemic anaphylaxis in the blood by interactions with huFcγRIIa, whereas huFcγRIa is involved in active systemic anaphylaxis [114, 146]. In relation to preclinical studies, huFcγRIIIb on neutrophils was shown to mediate first infusion reactions upon injection with huIgG1 antibodies forming IC in the blood [28]. Even though murine

neutrophils express FcγRIII and FcγRIV, both binding huIgG1, they failed to mediate similar infusion reactions suggesting a specific role of huFcγRIIIb [28, 133]. So far, poFcγRIIIa is the only FcγR detected on blood neutrophils in the minipig and therefore the only potential FcγR mediating antibody effector functions on this cell type. The absence of an orthologue to huFcγRIIIb suggests the incapability of porcine neutrophils to trigger first infusion reactions. Furthermore, the inability of huIgG1 binding to poFcγRIIIa also excludes alternative FcγR-mediated mechanisms to trigger such reactions. However, a potential expression of poFcγRIa was not be excluded on blood and tissue resident neutrophils in our studies. As in the human, its expression could depend on the activation status of the cell and therefore remains to be determined [147, 148]. The potential poFcγRIa expression in neutrophils could theoretically enable phagocytosis and cytokine release in neutrophils [133].

8.2.4 FcγR-mediated immunoregulatory functions in dendritic cells and monocytes

Different subsets of monocytes and DCs co-express activating and inhibitory FcγR influencing the uptake, processing, and presentation of antigens [149]. Mainly huFcγRIa on DCs enhances cross-presentation of extracellular antigen via major histocompatibility complex (MHC) class I to activate naïve CD8+ T cells to become cytotoxic T lymphocytes (CTL) [97, 150]. Besides the defense against intracellular viruses and bacteria, CTL are important for responses against tumor antigens. FcγR-mediated cross-presentation and priming of CTL is therefore wanted in cancer immunotherapy [151], with immunomodulatory antibodies [152], and DC-based immunotherapy [153]. We found that DCs in the blood of minipigs do not express poFcγRIa (Manuscript 1). However, the expression on porcine monocyte-derived DCs was shown to be regulated by inflammatory stimuli, fully reflecting the human situation. Furthermore, poFcγRIa was shown to be efficient in the uptake of IC into stimulated porcine DCs [86, 149]. Therefore, it can be anticipated that poFcγRIa exerts similar functions as its human counterpart, allowing the use of minipigs for immunotherapy involving IC uptake and cross-presentation by DCs. Knowing the poFcγR expression profile on DCs is important for vaccine research and immunogenicity studies due to the role in antigen presentation. Interestingly, poFcγRIIIa-mediated internalization was identified as the primary mechanism of DC maturation in pig [86]. While this mechanism is expected to be functional with poIgG1a in immune pigs, it is probably not reflected with huIgG1 in antibody therapy. In general, due to the incomplete characterization of poFcγRs on DC subsets, we cannot conclude an identical FcγR distribution compared to the human. Different expression levels between the two species might potentially affect antigen presentation and cytokine production of the affected DC subsets [71].

8.2.5 Consequences of inhibition by FcγRIIb

The regulating roles of FcγRIIb were mainly studied upon infection of mice with *Streptococcus pneumoniae*. While the absence of FcγRIIb results in increased pathogen clearance, it also leads to an overshooting immune reaction upon secondary infection [154]. Vice versa, the strong binging of huIgG1 and expression of poFcγRIIb on porcine blood monocytes could therefore render minipigs susceptible to concomitant infections during preclinical trials while reducing pathological immune stimulation [155]. The study of Morgan, Holzer [69] discussed before (on page 70) assessed the therapeutic potential of the huIgG1 antibody directed against hemagglutinin, the target of influenza A virus [85]. Interestingly, reduced gross pathology (decreased surface of lung lesion) but no reduction of the viral load was observed with hemagglutinin antibody and the huIgG1 control. As proposed before, this finding could be explained by the strong binding huIgG1 to poFcγRIIb and the expression of this receptor on pig monocytes. The inhibitory function of poFcγRIIb could thus lead to a monocyte-mediated anti-inflammatory effect in interaction with huIgG1 complexes and therefore to reduced tissue damage. On the other hand, the inhibition could be another reason for the unaffected viral load in addition to the lack of NK cell-mediated ADCC.

As concluded for the macaque, the increased binding to FcγRIIb could mask effects of therapeutic antibodies that would have been observed in humans where binding to huFcγRIIb is weaker [51]. Such effects could include overlooked toxicities mediated by exaggerated pro-inflammatory cytokine release and reduced efficacy related to inhibited phagocytosis or cytotoxicity [156].

8.2.6 Fc engineering

Antibody Fc engineering has become a widely used tool to modulate and fine-tune FcγR binding to enhance therapeutic effects and to reduce associated toxicities [35]. Enhanced huFcγRIIIa binding for increased ADCC can be achieved by several mutations of the IgG Fc part or by glycoengineering. [34]. Fc engineering for specific huFcγRIIa binding to increase phagocytosis [157] or for huFcγRIIb binding to suppress humoral immunity [158] is more challenging because the extracellular domains of these receptors differ by only 14 amino acids (94.5% similarity). This similarity requires highly specific adaptations of the antibody Fc part to discriminate the activation receptor from its inhibitory relative and to obtain the desired effects. The mechanisms for such adaptations are therefore highly specific for the human and not necessarily translatable to other species, such as the mouse, cyno, or the pig-tailed macaque [50, 51, 159]. Analogous, a similar failure of human translatability is expected from preclinical studies with Fc engineered antibodies in minipigs.

FcγR binding and complement activation are often not required for immunomodulatory therapeutic antibodies with a mode of action dependent on target binding via Fab arms. FcγR-mediated effector functions can be adverse as they may lead to toxicities by exaggerated cytokine release or to depletion

of target expressing cells [27, 37]. The carcinoembryonic antigen- (CEA) and T cell bispecific (TCB) therapeutic antibody CEA-TCB is currently in clinical trials. This antibody is based on an Fc silenced format that does not interact with FcγRs and complement component 1q (C1q) to avoid depletion of endogenous T cells. Simultaneously, binding to FcRn is not affected, significantly extending the half-life of the drug due to FcRn-dependent antibody recycling [160, 161]. In this case, the PGLALA mutations within the backbone of the huIgG1 antibody affect FcγR- but not FcRn-binding by disturbing interactions of Pro residues on IgG with Trp residues on FcγRs [37]. Because this Trp structure is conserved in mammals, including pigs, it can be assumed that this mutation is also devoid of effector functions in minipigs. Therefore, the minipig should be considered as a relevant animal model for preclinical testing with Fc silenced antibodies. The lack of effector functions, however, remains to be demonstrated by *in vitro* functional assays with minipig cells. Furthermore, it must be considered that interactions with FcγRs are not the only driver of effector functions. Often also complement-dependent cytotoxicity (CDC) is mediated by the Fc part of therapeutic antibodies in interaction with C1q [162]. Also these interactions have to be tested prior to the use of minipigs for toxicity studies with therapeutic antibodies.

8.3 Conclusion

This thesis describes the characteristics of the FcγRs in the Göttingen minipigs and their interaction with human and porcine IgG. Screening of the low affinity *FCGR* locus of the minipig revealed the hitherto unknown gene coding for poFcγRIIa on platelets. In general, the distribution of FcγRs on immune cells and the binding properties to free- and immune-complexed huIgG1 are similar in minipigs and humans. However, we observed several key differences between both species, as summarized in Table 8.1. The expression of poFcγRs and the binding to huIgG were used to assess the Göttingen minipig as a species for preclinical safety and efficacy studies with human therapeutic antibodies.

Previous studies identified the minipig as a valuable species for immunogenicity, tolerability, and PK studies with therapeutic antibodies [65, 66]. Due to the comparable expression pattern and similar binding properties of most FcγRs it can be generally concluded that the minipig is suitable as for the assessment of IC-mediated toxicity and efficacy. Translatable effector functions include FcγRIIa-mediated platelet activation, FcγRIa-mediated cytokine release and antigen sampling, and FcγRIIb mediated inhibition of activation signals. However, differences between minipig and human concerning the FcγR expression on NK cells and monocytes have to be considered. The highly sensitive SPR data that shows a lack of huIgG1 binding to poFcγRIIIa is of major concern for studies with ADCC-inducing antibodies of this particular IgG subclass. This lack of interaction is reflected in the publication by Morgan, Holzer [69] that investigates effects of a huIgG1 antibody in pig. Due to the unique mode of action and the individual characteristics of every engineered therapeutic antibody, we recommend case by case assessments of the suitability of the minipig. The tools presented in this work represent a possibility to investigate a therapeutic antibody *in vitro* for its translatability potential prior to the start of *in vivo* studies. Importantly, also functional studies are suggested to address the differences in effector functions to therapeutic antibodies between the species. FcγR humanization of minipigs, analogous to several mouse models, could circumvent the previously discussed caveats by replacement of the endogenous poFcγRs with the set of huFcγRs [118, 163, 164]. The description of the low affinity *FCGR* locus provides the basis for gene targeting. However, it has to be noted that effector functions of any preclinical species are restricted by the cross-reactivity of the therapeutic antibody with the antigen in the animal model.

The gained knowledge described in this thesis is of critical significance for the pharmaceutical development of therapeutic human antibodies because pharmacology, PK, PD, as well as possible toxicity issues are often dependent on FcγR-mediated effector functions. Taken together, this data enables the prediction of the relevance of Göttingen minipigs to assess certain effector functions of interest triggered with a therapeutic huIgG1 antibody. Therefore, this work delivers a basis for species selection and allows the interpretation of results from preclinical safety and efficacy studies with minipigs.

8.4 Outlook

This thesis presents a comprehensive set of data investigating the characteristics of poFcγRs and the interaction with huIgG1. However, further studies are suggested to gather more data about the suitability of the minipig for studies with therapeutic antibodies. The cellular FcγR expression studies presented in this thesis were studied by single cell RNA sequencing including all FcγRs in PBMCs or by flow cytometry including poFcγRIIa, poFcγRIIa/b, and poFcγRIIIa in the blood, lymph nodes and spleen. Generating antibodies specific for poFcγRIa and poFcγRIIb are required to further assess their expression in various tissues and on immune-related cell subsets. Such specific antibodies can again be generated using the HuCAL technology. The conversion of the current Fab-A-FH format to a regular huIgG isotype followed by direct fluorescent labeling is recommended to reduce background signals.

Further binding studies remain to be performed with other huIgG subclasses, glycoforms, or Fc engineered forms of huIgG antibodies to assess their potential use in preclinical studies with minipigs. This requires the modification of the SPR setup or the recombinant generation of antibodies with the same specificity, such as HER2 or VEGF as presented here. Biotin coupling of FcγRs to the sensor chip is recommended since capturing via His tag, PGLALA Fc, and direct crosslinking were found to be inefficient (not shown). However, biotin coupling was impossible for poFcγRIIIa and the binding assessment of free IgG with the other FcγRs resulted in multiple interactions. These issues can be addressed by the expression of the extracellular domain without Fc fusion, as described in most other SPR studies [45, 50, 52, 107]. Alternatively, common fusion tags, such as small ubiquitin-like modifier (SUMO), glutathione S-transferase (GST), or maltose-binding protein (MBP) can be used to enhance solubility and reduce aggregation [165]. Further binding studies with FcγR expressing HEK293F cells are not recommended due to the unexpected binding of huIgG1 IC. Stable Chinese hamster ovary (CHO) cell lines expressing porcine and human FcγRs would be a considerable alternative. The co-transfection with nuclear factor of activated T cells (NFAT) response element (plasmids available by Promega) would additionally enable the detection of FcγR activation and signal transduction [166]. Nevertheless, binding studies with free- and immune-complexed IgG in minipig blood were found useful and would be best supplemented by the direct comparison to human blood.

Most importantly, functional assays have to be performed to assess the FcγR- and complement-mediated effector functions of therapeutic antibodies in minipigs. A variety of different assays are described for the human that can be adapted to assess the ADCC potential of minipig PBMCs or cells isolated form the minipig [18]. For example the ADCC assay described by Morgan, Holzer [69] was found to be useful for porcine and human PBMCs with huIgG1 antibodies and serum from immune pig as a positive control, even though a longer incubation time is recommended to detect monocyte-mediated ADCC. Different human IgG subclasses could be used as comparators because it is laborious to generate immune serum or porcine surrogates as positive controls. Additionally, bone-marrow derived macrophages or other effector cells can be co-incubated with target cells and therapeutic antibodies to study ADCP, as described by Shi, Fan [167]. The hypothesis, that the release of pro-inflammatory cytokines and their inhibition via poFcγRIIb leads to reduced pathology can be studied by cytokine release assays in a whole blood setting, with cultured monocytes/macrophages, or with sorted cells with and without blocking of poFcγRIIb [168, 169]. Also the highly important C1q binding and subsequent complement-dependent mechanisms remain to be assessed *in vitro* by SPR or by functional complement assays as previously described in pigs [170]. Ultimately, minipig *in vivo* studies using approved human therapeutic antibodies with known effector mechanisms can be used as a validation of studies with minipigs.

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