



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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Fc γ RIII in mouse (Lux and Nimmerjahn 2013). In humans, Fc γ RIIa 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 Fc γ RIIa 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 Fc γ RIIa 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 Fc γ RIIa 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 Fc γ R composition and their interaction with human IgGs. However, to date, the knowledge on the binding properties of porcine Fc γ R to human antibodies is still scarce. In addition, the number of low-affinity Fc γ Rs 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 Fc γ Rs 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

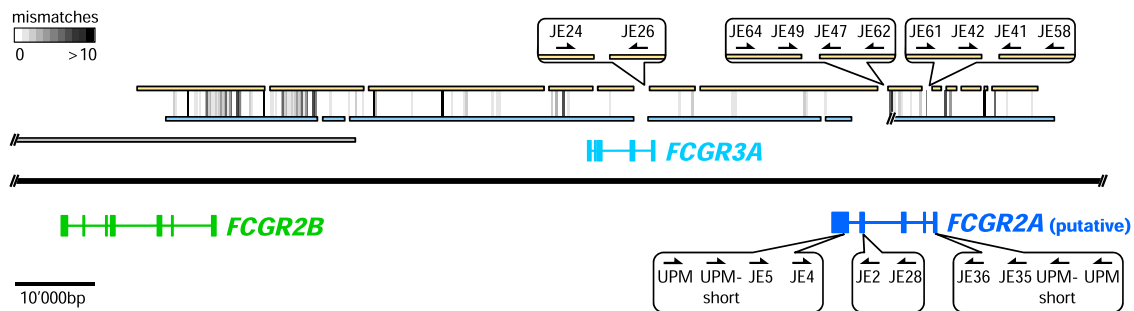


Fig. 1 Genomic organization of the minipig *FCGR* locus. The black line represents the genomic sequence scaled as indicated in the lower left corner. *FCGR* genes are shown as colored lines with boxes representing the exon structure. Genes above and below the black line are encoded at the forward strand 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

Wuzhishan 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 FcγRIIIa (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

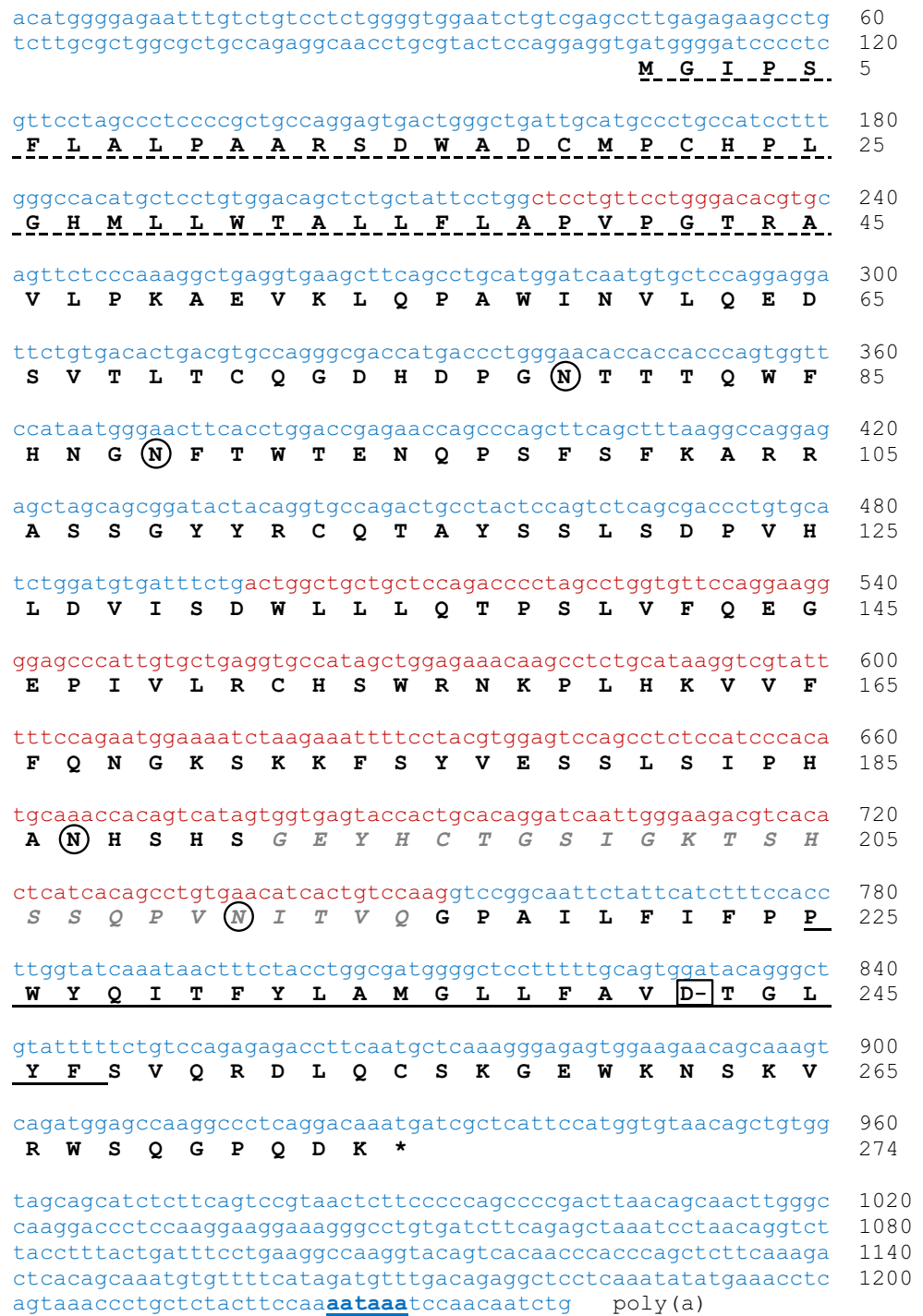
Localization of porcine *FCGR3A* and identification of putative *FCGR2A*

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 Wuzhishan 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 (Asn⁷⁹, Asn⁸⁹, Asn¹⁸⁷, and Asn²¹¹) 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

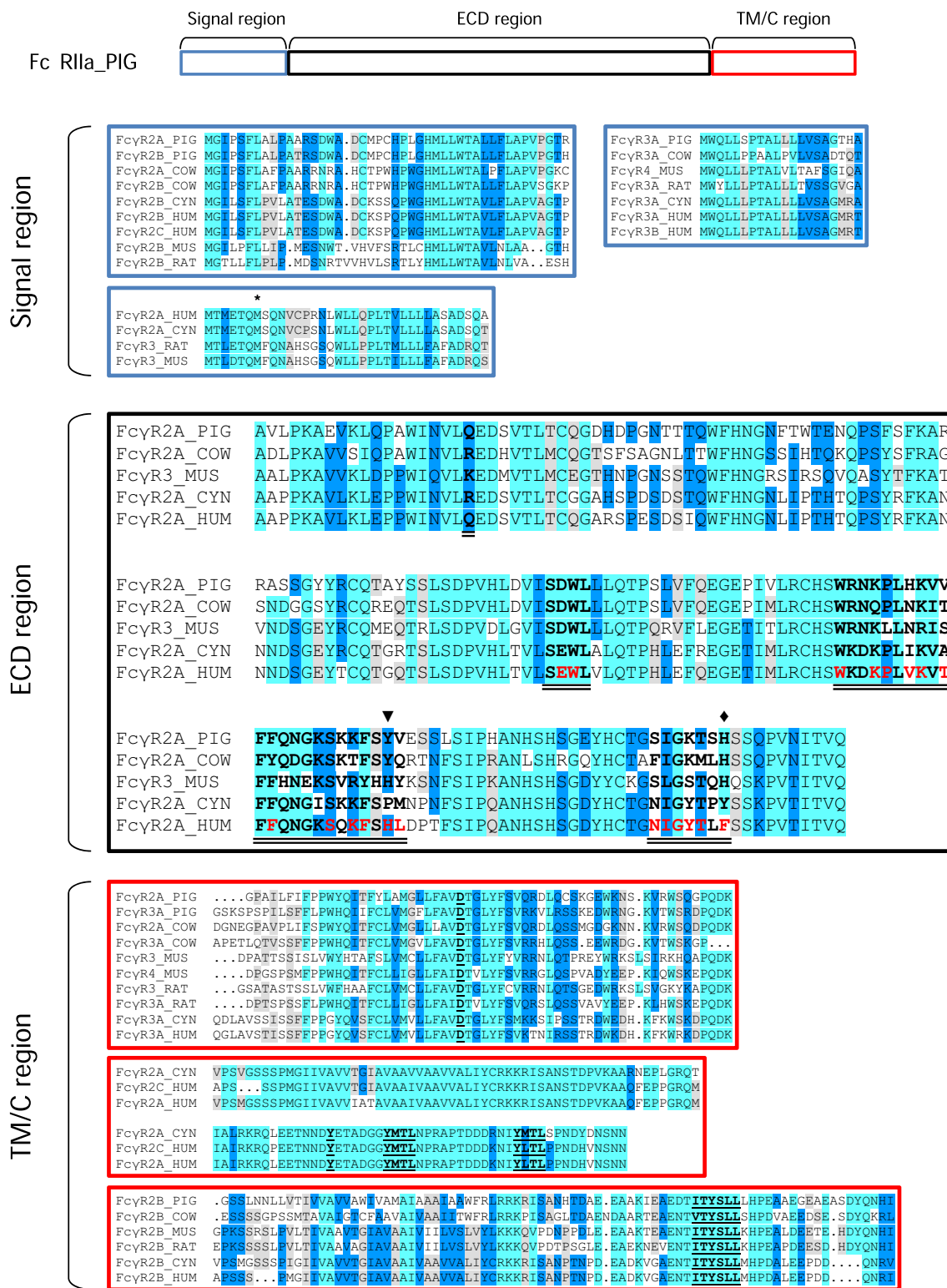


Fig. 3 Comparison of FcγR protein sequences. A schematic representation of the putative porcine (PIG) FcγRIIa transcript is shown at the top. The boxes within the transcript represent signal regions (blue boxes), extracellular domains (ECD, black box), and transmembrane/cytoplasmic regions (TM/C, red 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 Fc γ RIIa amino acid residues involved in IgG-Fc γ R 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-Fc γ R interactions showed strong conservation among species, including conserved tryptophan residues, thus indicating that the identified putative porcine Fc γ RIIa is capable of IgG binding (Fig. 3, black box). Extracellularly, the putative porcine Fc γ RIIa (aa 46–215) shares 75% similarity to mouse Fc γ RIII (Uniprot, P08508; aa 31–196), 79% to cattle Fc γ RIIa (Uniprot, A8DC37; aa 46–215), 80% to cyno (cynomolgus monkey, *Macaca fascicularis*) Fc γ RIIa (Uniprot, Q8SPW4; aa 30–199), and 79% to human Fc γ RIIa (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 Fc γ Rs 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 Fc γ Rs (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 Fc γ RIIa is required for direct activation signaling (Isakov 1997) (Fig. 3, red middle box). Mouse Fc γ RIII, cattle Fc γ RIIa, and putative porcine Fc γ RIIa, on the other hand, are lacking such an intracellular ITAM. Like human Fc γ RIIIa, 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 Fc γ RIIa 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 Fc γ RIIa. 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 Fc γ RIIa. However, both polymorphic variants were detected by sequencing of one Göttingen minipig.

The gene family of Fc γ Rs displays a similar genomic organization as in most mammals (Fig. 4). Low-affinity Fc γ Rs 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 Fc γ RIIb 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 Fc γ RIa 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 Fc γ RIa 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 Fc γ RIIa and inhibitory Fc γ RIIb including the orthologues in mouse and rat (Fig. 5). Fc γ RIIIa proteins, including mouse Fc γ RIV, form a separate group with high interspecies similarity. Full-length porcine Fc γ RIIa, for example, shows an amino acid sequence similarity of 88% to porcine Fc γ RIIb (Uniprot, Q461P7), and only 61% to porcine Fc γ RIIIa (Uniprot, Q28942) whereas the ECD region of porcine Fc γ RIIa and Fc γ RIIb are highly similar to each other (95.3%).

Cellular distribution of Fc γ Rs

Understanding the functional impact of Fc γ Rs requires a thorough characterization of their expression pattern in different cell types. Hereto, the expression of the different Fc γ Rs 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

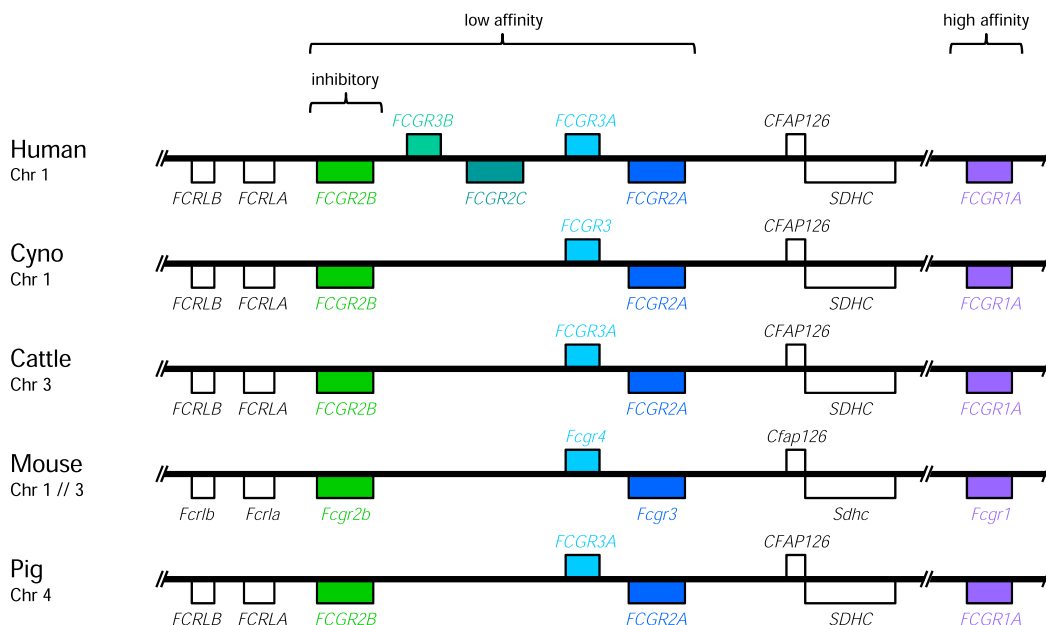


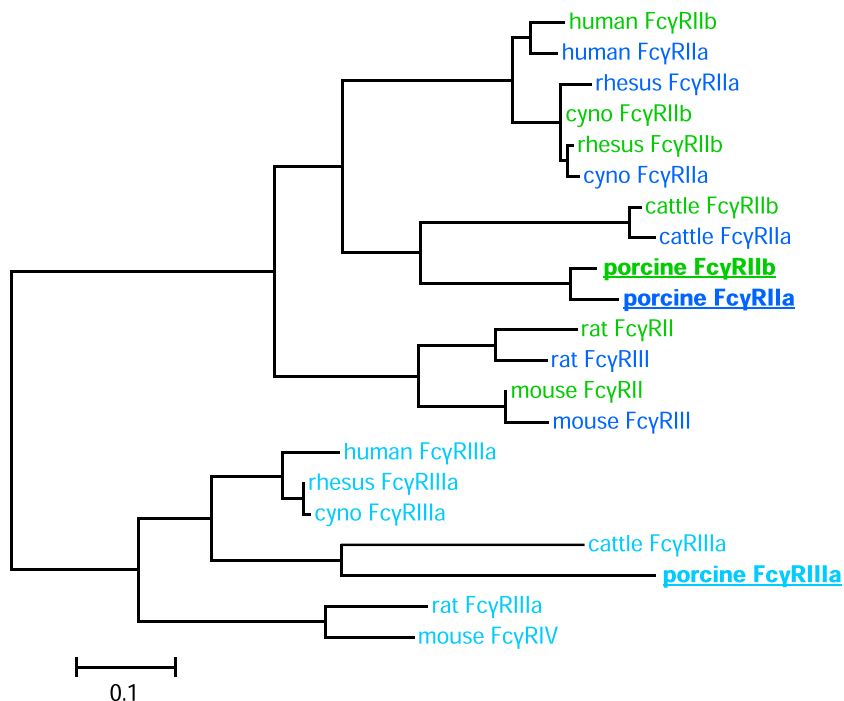
Fig. 4 Genomic organization of the *FCGR* locus in human, cyno, cattle, mouse, and pig according to the Ensembl database. The black lines represent a stretch of genomic DNA interrupted by lines indicating a gap of diverse length. All species shown here, except the mouse, carry the gene coding for the 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



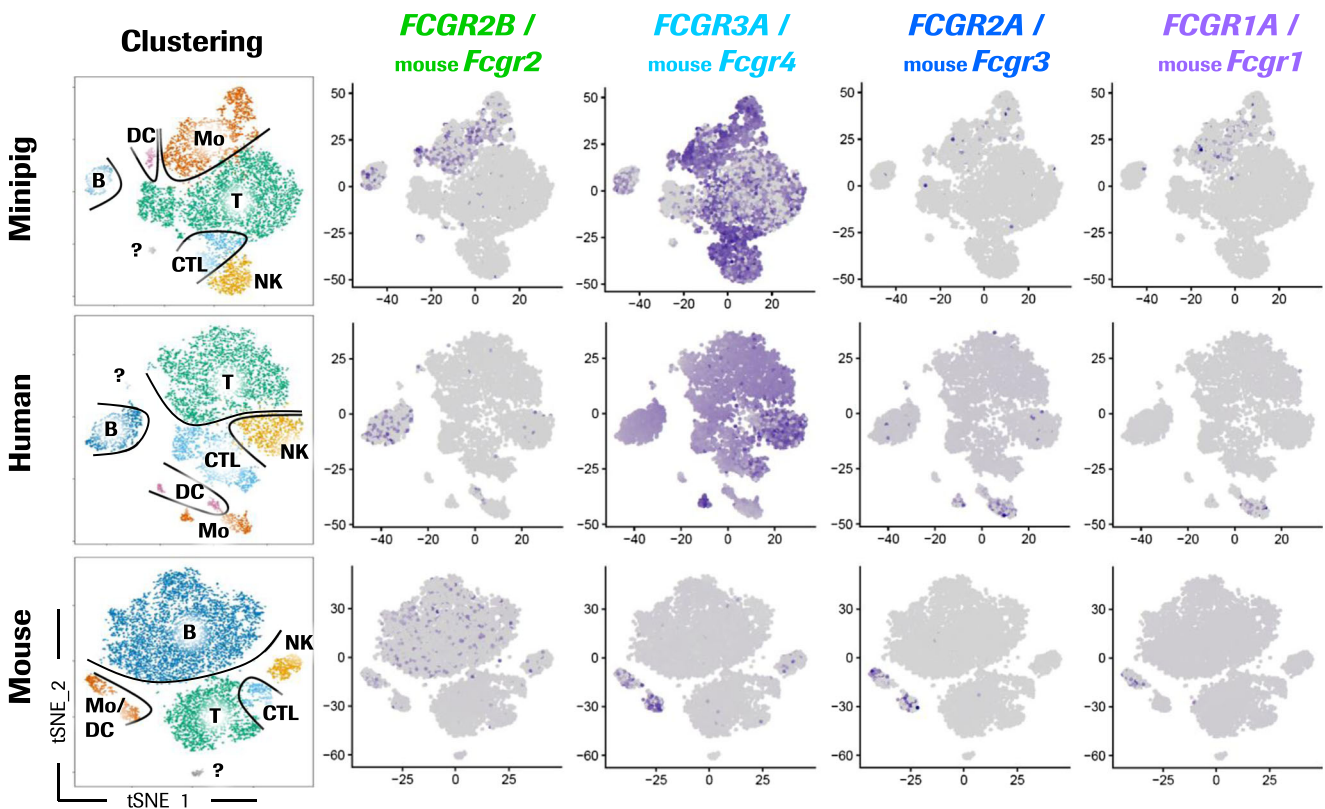


Fig. 6 Single-cell RNA sequencing analysis of *FCGR* expression in minipig, human, and mouse PBMCs. For every species, the cells were clustered individually according to their gene expression pattern and displayed as dot plots by dimensionality reduction using t-SNE. The clustering for every species 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 $Fc\gamma R$ s was then analyzed in every species (Fig. 6).

The activating low-affinity $Fc\gamma RIIIa$ is most strongly expressed among the $Fc\gamma R$ s in all the species studied here. Minipig PBMCs revealed a strong and relatively homogeneous $Fc\gamma RIIIa$ 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 ($Fc\gamma RIIIa$) expression (Ziegler-Heitbrock 2015). As expected, the larger CD14^{high} classical monocyte subset did not express $Fc\gamma RIIIa$, whereas the minor non-classical CD14^{low} subset was strongly positive for $Fc\gamma RIIIa$. Also, in mice, it is the cluster containing the monocytes that shows expression for $Fc\gamma RIIIa$, while the other immune cell types, in contrast to the other species, show no expression. The inhibitory low-affinity $Fc\gamma RIIb$ was found to be expressed mainly on monocytes, B cells, and DCs of the minipig. Human monocytes were not found to express $Fc\gamma RIIb$, while mouse $Fc\gamma RII$ was weaker expressed in the monocyte and DC cluster as compared to the minipig.

Expression of $Fc\gamma RIIb$ in human and mouse PBMCs was mainly found in B cells. $Fc\gamma RIIa$, the activating low-affinity receptor we identified with our mapping strategy, is expressed at lowest levels in minipigs and humans. In the minipig, $Fc\gamma RIIa$ 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 $Fc\gamma RIII$, the orthologue of $Fc\gamma RIIa$, 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 $Fc\gamma RIa$. In the minipig, the expression is at low levels and restricted to monocytes. In humans, CD14^{high} CD16- classical monocytes express $Fc\gamma RIa$, in contrast to CD14^{low} CD16+ non-classical monocytes. Mice show a similar $Fc\gamma RI$ 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 $Fc\gamma R$ 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 Fc γ RIIa-specific HuCAL antibody on platelets (P1) and a weak staining on a subpopulation of eosinophils (P5). The Fc γ RIIa/b cross-reactive HuCAL antibody stains platelets, most monocytes (P3), and some eosinophils as well. Fc γ RIIIa 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 Fc γ Rs 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 Fc γ R components in a preclinical animal model intended for testing of therapeutic antibodies. The characterization of the low-

affinity Fc γ R proteins and genes in minipigs should provide a basis for preclinical studies with therapeutic antibodies.

While the inhibitory receptor is widely described as Fc γ RIIb (in the mouse known as Fc γ RII), the nomenclature of the low-affinity-activating Fc γ Rs has evolved in a far more divergent manner. The low-affinity Fc γ RIIa 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 Fc γ RIII 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 Fc γ RIIa located on chromosome 4 of the Göttingen minipig. The orthologue to human Fc γ RIIIa is known in NHPs, cattle, and other mammals, including the mouse, where it was designated as Fc γ RIV (Nimmerjahn et al. 2005). The orthologous Fc γ RIIIa cDNA and protein were also described in the pig but the corresponding gene

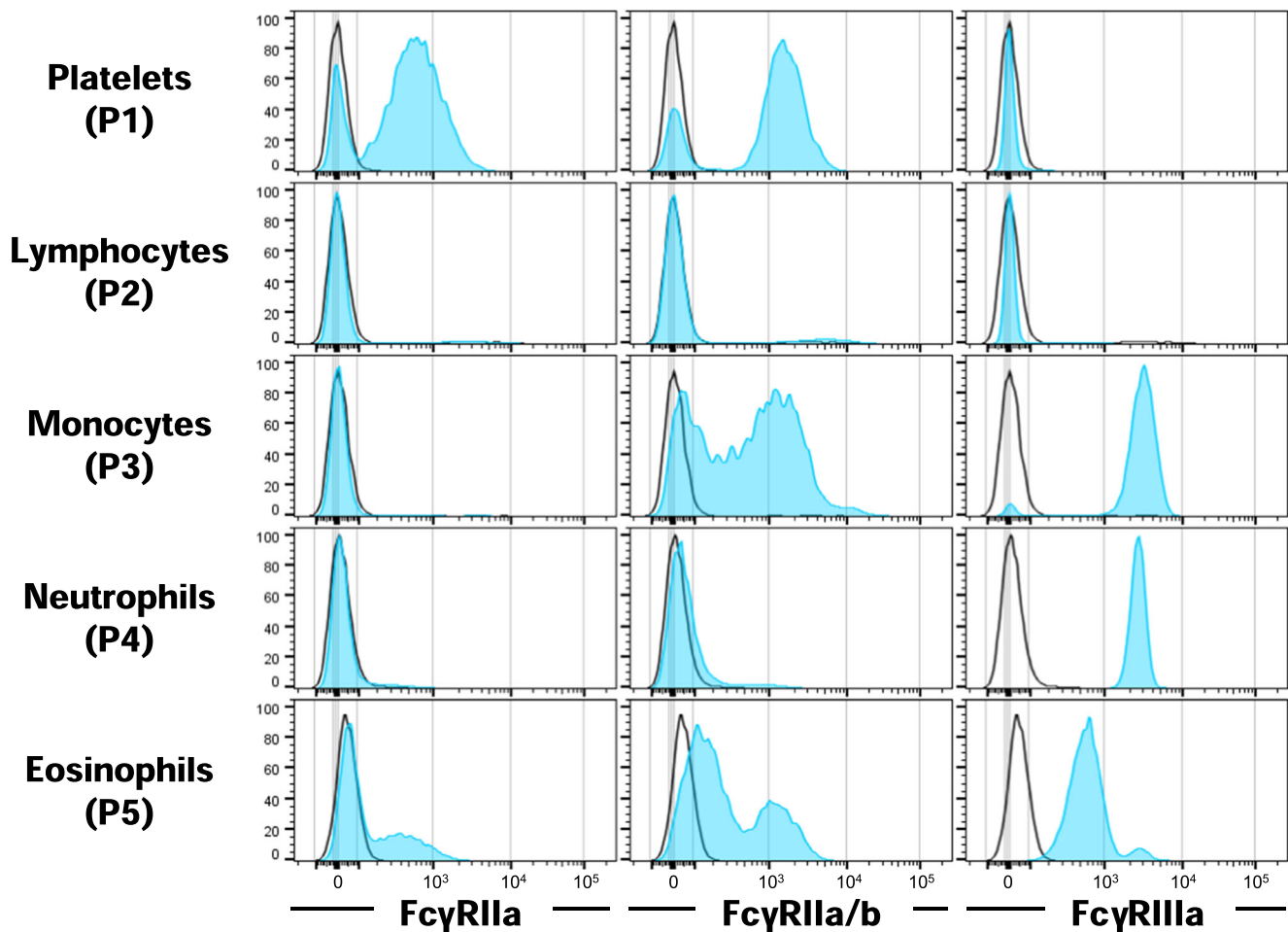


Fig. 7 Flow cytometry analysis of Fc γ R distribution on minipig blood leukocytes. Gating strategy is shown in Online Resource 4. Histograms normalized to mode show stainings observed using an Fc γ RIIa-specific HuCAL antibody, an Fc γ RIIa/b cross-reactive HuCAL antibody, and an

anti-CD16 (Fc γ RIIIa) 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 Göttingen 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 Fc γ RIIIa 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 Fc γ RIIb and Fc γ RIIc, as is the case for all other animal species studied so far.

Sequence similarity displayed in the phylogenetic tree in Fig. 5 shows that Fc γ RIIa and Fc γ RIIb 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 Fc γ RIIb to the newly identified porcine Fc γ RIIa fits in the pattern observed with the corresponding receptors from other species. Therefore, we suggest naming the transcript Fc γ RIIa. However, as detailed in Fig. 3, exons coding for the signal region and the TM/C region of the Fc γ Rs 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 Fc γ RIIa via interaction with the FcR γ -chain as it is described for cattle Fc γ RIIa and mouse Fc γ RIII (Lux and Nimmerjahn 2013). This similarity strengthens the hypothesis of the orthology among these receptors. On the other hand, Fc γ RIIa 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 Fc γ RIIa 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 Fc γ RIIa with unknown functions and significance, probably generated by alternative splicing. Similar splice variants were already described for porcine Fc γ RIIb (Xia et al. 2012; Xia et al. 2011) and Fc γ RIIIa (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 Fc γ Rs do not only depend on the affinity of IgG interaction but also on their cellular distribution (Albanesi and Daeron 2012). Knowing the expression of Fc γ Rs 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 Fc γ R expression profile on various cell types.

In the Göttingen minipig, Fc γ RIa transcripts were only identified in monocytes at similar levels as observed in human and mouse. Like in humans, no Fc γ RIa expression was detected in minipig blood DCs although Fc γ RIa expression was often reported in human DCs (Nimmerjahn et al. 2015; Tamoutounour et al. 2012). Fc γ RIa 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 Fc γ RIa expression profile on porcine DCs depends on the activation stimulus, and similar findings were observed for human DCs. Therefore, Fc γ RIa expression can neither be excluded from minipig blood DCs nor from tissue-resident subsets. Varying expression levels of Fc γ RIa 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 Fc γ RIIa. Generally, the Fc γ RIIa 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 Fc γ RIIa 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 Fc γ RIIa on platelets as detected by flow cytometry (Rosenfeld et al. 1985). Platelets are mediators of immune responses upon binding of IgG immune complexes via Fc γ RIIa. 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 Fc γ RIIa on platelets (Meyer et al. 2009). Gene expression data from NCBI Gene show that Fc γ RIIa is mainly expressed in the liver and the lung of pigs. Generally, the porcine Fc γ R expression is mainly detected in the liver, lung, and spleen tissue. This expression profile suggests that Fc γ RIIa 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 FcγRIIIa on chromosome 4 of the Göttingen minipig. The newly identified

FcγRIIIa described here is considered as an orthologue to human, NHP, and cattle FcγRIIIa as well as to mouse FcγRIII due to the highly conserved extracellular structures. The identification of FcγRIIIa 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 FcγRIIIa 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 FcγRIIIa 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, FcγRIIIa 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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