A field guide to human Fc-gamma receptors
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Al in de Griekse oudheid stonden uilen symbool voor wijsheid. De associatie met wijsheid zou te maken kunnen hebben met het uitzonderlijk goede waarnemingsvermogen van uilen en hun nachtelijke activiteit. Daardoor werd vroeger gedacht dat uilen kennis hebben van zaken waar mensen niets van weten. Overdag zitten uilen te rusten en ze kijken daarbij met een ogen-schijnlijk wijze blik rond. Het lijkt dan net alsof ze aan het filosoferen zijn, wat ook kan hebben bijgedragen aan hun wijze uitstraling. Om precies te achterhalen waar de symboliek vandaan komt, is echter meer onderzoek nodig.
General discussion and future perspectives

Parts of this discussion have been published as:
Immunomodulation by IVIg and the Role of Fc-Gamma Receptors: Classic Mechanisms of Action after all?

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INTRODUCTION

In this thesis, we have investigated the role of FcγRs in the immune system, in order to better understand the pathophysiology of IgG – FcγR interactions in various autoimmune diseases, as well as the poorly understood beneficial effects of IVIg. First, we have characterized the complex genetics of human FcγRs in great detail, and used this knowledge in association studies on systemic lupus erythematosus (SLE), Kawasaki disease (KD) and the alloimmune platelet destruction that can take place during platelet transfusion. Second, we studied the function and expression of FcγRs using several in vitro and ex vivo approaches, aiming to elucidate the role of FcγRs and IVIg in the context of IgG-mediated blood cell destruction.

Our findings and the strength and potential shortcomings of our methods have been discussed in detail in the individual chapters. In this chapter, we discuss the findings of this thesis in a broader context, focusing on the use of genetic association studies of FcγRs, and the immunomodulatory role of IVIg in autoimmune diseases. We will first discuss the meaning of our findings for association studies with FCGR2/3 genes, what we can learn from these association studies, the potential clinical application of the results of these studies and recommendations for the future. This is followed by a brief reflection on the evolution and function of FcγRIIc and FcγRIIIb, two FcγRs that appeared relatively recently in evolution. Finally, we will discuss the impact of our findings on the different theories that aim to understand the working mechanism of IVIg, which may help to improve this therapy for a broad range of autoimmune diseases.

GENETIC ASSOCIATION STUDIES WITH FCGR2/3 GENES

Implication of our findings for the interpretation of FCGR2/3 genotyping data

The FCGR2/3 locus is a complicated genetic locus with a high degree of homology between genes, copy number variation and multiple SNPs, and is therefore difficult to analyze. We have characterized the genetic variation at the FCGR2/3 locus in great detail in large cohorts of healthy individuals and patients, for the first time analyzing all known functionally relevant genetic variation together, in a much larger sample size (>4000) than has been done before. This revealed extensive linkage disequilibrium (LD) and ethnic variation (chapter 3), and a clear and distinct pattern of the copy number variation at the locus (chapter 5), which has to be considered in the interpretation of all genetic association studies with variants at this locus.

Strong LD is present between several of the genetic variants at the locus (chapter 3). This should have a great impact on all the association studies performed for variants at this locus, which often genotype one or two variants only (mostly the FCGR2A-His131Arg and FCGR3A-Val158Phe SNPs). Associated variants found in these studies may be the mere reflection of an even stronger association for a variant that is in LD with the identified variant, but was not genotyped in the
study (i.e. an increased prevalence of FCGR3A-158Val in a certain patient group may simply be the result of the classical FCGR2C-ORF haplotype being even more increased in the same patient group, because these two variants are in LD with each other). If only the FCGR3A-Val158Phe SNP is genotyped, this could lead to the wrong conclusion that FCGR3A-158Val causes an increased susceptibility to the disease. To be able to most accurately identify a potentially causative variant for an increased disease susceptibility, all the known functional genetic variants at the FCGR2/3 locus have to be genotyped, as we have done with the MLPA technique in our studies. A multiple logistic regression analysis can then identify independent risk markers. Even then, for variants that are in strong LD with other variants, it may be hard to identify independently associated variants, and large groups are needed. For instance, in chapter 3, four variants that are in strong LD with each other were all associated with an increased susceptibility to Kawasaki disease (KD) in single logistic regression analyses. It seems likely that only one of these variants (the classical FCGR2C-ORF haplotype) actually causes the increased risk, but to prove this as an independent marker in a multiple logistic regression analysis, many more patients would need to be included in the association study, which is not easily done for rare diseases, such as KD. On the other hand, for SNPs that are in less strong LD, multiple logistic regression analysis can still identify independent risk markers also in patient groups that are somewhat smaller (chapter 2).

Another important implication for genetic association studies on the FCGR2/3 locus is that we have found that all copy number variation (CNV) at the locus always occurs in well-defined combinations of several genes (copy number regions, CNRs). We did not identify any deletions or duplications of single genes (chapter 5). This is important to realize when interpreting genotyping data from this locus; in fact, it greatly facilitates the correct interpretation of raw MLPA data, which is easier to understand with precise knowledge of the different CNRs. We want to note here that our finding that CNV at the FCGR2/3 locus always involves CNRs of ~82 kb is in contrast with an earlier report by our laboratory, which did describe rare duplications and deletions of the FCGR3A gene alone, as well as a duplication of the all the CNV genes (FCGR3A, FCGR2C and FCGR3B) in one stretch1. However, upon retesting of the same samples with newer and improved MLPA protocols (which involve more adequate buffering of the sample DNA with a TRIS buffer, please refer to www.mlpa.com for detailed protocols), we found that these were misinterpretations, and all singular deletions/duplications of FCGR3A could be classified as CNR2, whereas the duplication of all the CNV genes was in fact a combination of CNR1 and CNR2 as described in chapter 5 (Nagelkerke & Breunis, data not shown).

Considering the strong LD and well-defined structure of CNRs at the locus, it may be better to analyze-and report on-some of the genetic variations at the locus as haplotypes, instead of analyzing single variations. This has already been the standard for the SNPs in FCGR3B, which are usually reported as the haplotypes NA1, NA2 and SH. Now, since we know that CNV at the FCGR2/3 locus always occurs in CNRs, we suggest that CNV should be analyzed in the form of these CNRs, which basically form haplotypes. For instance, CNV in FCGR3B never
occurs alone, but is always accompanied by CNV of FCGR2C and HSPA7 (in essence, CNV in FCGR3B is in perfect LD with CNV in FCGR2C and HSPA7). Thus, an independent association of FCGR3B CNV with disease is impossible to prove, although in this case seems very likely because both the FCGR2C and HSPA7 genes are pseudogenes in the majority of individuals (chapter 2). Similarly, a decreased copy number of FCGR3A, which most often occurs as part of the copy number region CNR2, is in these cases always accompanied by the newly described FCGR2A/2C chimeric gene (chapter 5) (in essence, decreased copy number of FCGR3A is in very strong LD with the presence of an FCGR2A/2C chimeric gene). Concluding, the presence of CNV at the FCGR2/3 locus cannot be analyzed separately for single genes, and therefore we think it is better to report the CNV as haplotypes in the form of the different CNRs at the locus, as we have done in chapters 2-4.

From a functional point of view, FCGR2C variations should also be reported as haplotypes (classical FCGR2C-ORF, nonclassical FCGR2C-ORF and FCGR2C-Stop). These consist of two (possibly three) SNPs that together determine expression of FcγRIIc, as we have shown in chapter 3. Analyzing the FCGR2C-Gln57Ter alone would include individuals with the non-expressed nonclassical FCGR2C-ORF haplotype in the group of the classical FCGR2C-ORF haplotype, whereas we show that on a functional level, the nonclassical FCGR2C-ORF haplotype is similar to FCGR2C-Stop. Therefore, these FCGR2C variations should always be genotyped together and reported as haplotypes. FCGR2C-Gln57Ter should never be genotyped alone.

In view of the LD at the locus, and because the FcγR proteins encoded by the genes are functionally related and could thus act together in pathophysiologic mechanisms, it could even be attempted to report extended haplotypes, including all the different SNPs across the whole locus. However, the LD is not absolute for any of the SNPs, and thus the list of possible haplotypes is very long. Therefore, such an approach seems unpractical and confusing. When analyzing the locus as extended haplotypes, disease associations of a single gene encoding one of the FcγRs will not be obvious anymore, which will obscure valuable information on pathophysiology of the disease that is studied. On the other hand, an approach that analyzes extended haplotypes of the whole locus may prove a useful strategy in risk stratification studies (discussed later).

Finally, the extensive ethnic variation we have found at the FCGR2/3 locus, especially for the FCGR2C haplotypes (chapter 3) is relevant for future genetic association studies, as it emphasizes the importance of carefully selecting ethnicity-matched control groups for genetic association studies. If patient and control groups are not matched for ethnicity, false-positive associations at this locus are very likely to occur, as we have actually shown to be the case in chapter 4.
**The importance of FCGR2/3 genotyping**

*FCGR2/3 genetic variation in association studies*

*FCGR2/3* polymorphisms are useful when investigating the role of FcγRs in human disease by means of genetic association studies. In general, such studies can suggest that FcγRs are involved in the pathophysiology of a certain disease. More specifically, association with specific FcγR genetic variations can give a more precise clue, as they may incriminate a certain cell type in the pathophysiology. The best example is the association of FcγRIIIb CNV with SLE (chapter 2). Since FcγRIIIb is expressed almost exclusively in neutrophils, this is a strong suggestion that neutrophils are involved in the pathophysiology of SLE. All the other FcγRs are expressed on multiple cell types, and thus, associations of genetic variation in other genes than *FCGR3B* are less indicative of a specific cellular involvement in a disease. In some cases, it may be only possible to determine whether, in very general terms, the *more activating* or the *less activating* FCGR variants are associated with the disease studied, and thus gain insight on the general role of FcγRs in the pathophysiology of the disease. An overview of the more and less activating variants at the *FCGR2/3* locus is given in Table 1. Interestingly, several variants that are more activating are in LD with each other: *FCGR2A*-131His, *FCGR3A*-158Val and the classical *FCGR2C*-ORF, although this may be ‘balanced’ by the LD of the classical *FCGR2C*-ORF with the 2B.4 promoter haplotype in *FCGR2B*. (chapter 3).

| Table 1. Effects of genetic variants at the FCGR2/3 locus on immune function |
|---------------------------------|---------------------------------|-----------------|---------------|
| *FCGR2A*-p.His131Arg            | Affinity for IgG               | More immune activation | Less immune activation |
| *FCGR3A* CNV                    | Expression of activating receptor | CNV > 2         | CNV < 2       |
| *FCGR3A*-p.Val158Phe            | Affinity for IgG               | Val              | Phe           |
| *FCGR2C* haplotype              | Expression of activating receptor | Classical ORF    | Nonclassical ORF |
| *FCGR2B*-p.Ile232Thr            | Strength of inhibitory signal  | Thr              | Ile           |
| *FCGR2B* promoter haplotype     | Expression of inhibitory receptor | 2B.1             | 2B.4          |
| *FCGR3B* CNV                    | Expression of receptor         | ?                | ?             |
| *FCGR3B* haplotype              | Phagocytosis (unknown mechanism) | NA1              | NA2           |

Traditionally, it has been thought that activating and inhibitory FcγRs constitute an immunological balance that ensures adequate protection against pathogens, but on the other hand does not result in auto-immunity. Simply speaking, *FCGR2/3* genetic variation may tip this balance to either side, leading to auto-immunity when the balance is tipped towards the activating side, or leading to decreased immunity against pathogens or cancer cells when the balance is tipped towards the inhibitory side. However, our results show that this is an over-simplification of the
matter, and marked differences in FCGR2/3 genetic variations occur between several autoimmune and autoinflammatory diseases. In Kawasaki disease (KD, chapter 3) and in immune thrombocytopenia (ITP), it is indeed the case that the more activating variants (FCGR2C-ORF, FCGR3A-158V, FCGR2A-131H) are associated with disease susceptibility. However, in other autoinflammatory diseases, the less activating variants, which would be expected to tip the balance towards the inhibitory side, actually predispose to disease. This is for instance the case in SLE, which is associated with the less activating variants FCGR2A-131R, and the FCGR2B promoter haplotype 2B.4, which causes increased expression of the inhibitory FcγRIIb (chapter 2). Another difference between SLE on the one hand and KD and ITP on the other hand is that SLE is associated with low copy number of FCGR3B (as is Sjögren syndrome, systemic sclerosis and possibly RA). Low copy number of FCGR3B is not associated with KD or ITP.

Clearly, autoimmunity is not necessarily associated with more activating FCGR2/3 genetic variations, and FCGR2/3 variants have different, sometimes opposite, effects on different autoimmune and inflammatory diseases, suggesting different pathophysiologic contributions of IgG and FcγRs between the diseases. Possibly, activating FcγRs actually protect against SLE by enabling ‘waste disposal’ of pathogenic immune complexes involved in the disease. On the other hand, in the diseases in which activating variants are associated (KD and ITP), damage done by IgG may be exerted directly by cellular activation via FcγRs, which is enhanced in individuals with more activating variants. In the other diseases (SLE and RA), IgG does not seem to cause harm via cellular activation via FcγRs. Interestingly, the diseases in which the more activating variants are associated with susceptibility, are also the diseases in which IVIg is an effective therapy (KD and ITP), whereas IVIg is of no value in RA, Sjögren syndrome, systemic sclerosis or SLE, also suggesting a difference in the pathophysiological contribution of FcγRs in these diseases. An explanation for this finding may be that IVIg blocks activating FcγRs, which is beneficial in diseases in which these activating FcγRs are directly involved in pathophysiology, whereas in diseases in which activation of FcγRs does not play a role, blockade of FcγRs is not important.

Altogether, FCGR2/3 association studies from this thesis and previous literature suggest that the pathophysiological mechanisms leading to SLE, RA, and Sjögren syndrome may be fundamentally different from the mechanisms leading to KD and ITP, a fact that is supported by the observation that SLE, RA and Sjögren syndrome occur much more frequently in women than in men, whereas in KD and ITP there is a slight predisposition in males.

Concluding, knowledge of FCGR2/3 genetic variation in autoinflammatory and autoimmune diseases may increase our knowledge on the pathophysiology of these complicated and multifactorial diseases, and may be related to effectiveness of IVIg therapy.

FCGR2/3 genetic variation and personalized medicine
Perhaps the most clinically useful application of genotyping FCGR2/3 genetic variation could be predicting response to therapy, and FCGR2/3 polymorphisms could be of potential value in
personalized medicine. Predicting treatment responses could for instance be useful in KD. Most KD patients respond well to IVIg therapy, but a minority (~20%) does not respond well, and these are more at risk for developing coronary aneurysms. Recent evidence suggests that these non-responsive patients may benefit from corticosteroid therapy in addition to IVIg. Clinical scores have been developed to predict IVIg response in KD, but these are not very precise. Adding genetic information to the risk scores could help to improve these predictions. In fact, one research group reported an association of FCGR3B-NA1 with failed treatment response, and a correlation of the 2B.4 promoter haplotype in FCGR2B with a good IVIg response, suggesting that FCGR2/3 genetic variation may be useful for predicting treatment response. However, in a preliminary analysis of the patients for which detailed clinical information was available in our cohort of KD patients (chapter 3), we could not find any association with IVIg response, or coronary aneurysms (Nagelkerke & Tacke, unpublished results).

Association of FCGR2/3 genetic variation has also been extensively evaluated in monoclonal antibody therapy in cancer patients. Antibodies directed against specific tumor antigens may help in eradicating cancer cells, and this takes place in part by cellular effector mechanisms mediated by FcγRs, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Thus, the efficacy of the antibody may be related to FCGR2/3 genetic variation. MoAb therapy is costly, and since not all patients seem to benefit from it, predicting an individual patients’ response could help to identify the patients likely to respond to this therapy. Initial studies showed increased response rates in breast cancer patients carrying the FCGR2A-131His variant when treated with trastuzumab, which binds to a specific breast cancer antigen. However, later studies failed to reproduce such an association. Similarly, a meta-analysis of FCGR3A association studies with response to Rituximab therapy in lymphoma patients did not find an association with FCGR3A-158Val that had been reported earlier. Concluding, there is no clear difference that would justify treatments to be individualized on the basis of FCGR2/3 polymorphisms. A major drawback of these studies is the fact that only two SNPs are analyzed, whereas other SNPs at the locus also potentially influence treatment response rates, and the SNPs are in LD with each other. Possibly, an analysis of all the SNPs and CNV, and analysis as extended haplotypes across the locus may be more useful.

Concluding, currently there is not yet a role for FCGR2/3 genotyping in personalized medicine.

FCGR2/3 genetic variation and laboratory findings

Information on FCGR2/3 polymorphisms is essential for correct interpretation of certain in vitro experiments. For instance, when determining whether FcγRIIb is expressed on a certain cell type, this must be done in individuals that cannot express FcγRIIc, because otherwise one could misinterpret a signal given by FcγRIIc as expression of FcγRIIb. Therefore, individuals with an FCGR2C-ORF must be excluded from expression analyses of FcγRIIb, as we have done
in chapter 7 and chapter 8. In addition, assessing mRNA transcript levels of FCGR2B allows for specific detection of FcγRIIb expression (chapter 7).

The future of FCGR2/3 genotyping

The FCGR MLPA used throughout our research offers a comprehensive method to analyze both CNV and relevant SNPs at the same time. However, MLPA is rather laborious and expensive in comparison with the high-throughput PCR-based arrays that are used to determine SNPs in non-copy number variable genomic areas. With techniques in genotyping evolving fast, novel techniques may ultimately replace MLPA as the most suitable technique for genotyping the FCGR2/3 locus. Recently, an attempt was made to determine FCGR3A and FCGR3B CNV from intensity values derived from an Immunochip platform, allowing the authors to determine CNV in >18,000 individuals\textsuperscript{11}. The authors claim that they can reliably identify cases with 0, 1, 2 or more copies, although 3 copies could not be reliably be distinguished from higher copy numbers. The authors did however not validate their findings with standard techniques to determine CNV at the FCGR2/3 locus, such as MLPA or the paralogue ratio test (PRT)\textsuperscript{12}. The fact that in this study the authors could not find any relation of FCGR3B CNV with expression of FcγRIIib on neutrophils puts serious doubts on the reliability of these data, as CNV of FCGR3B clearly correlates with expression of FcγRIIib as shown by us (chapter 2) and others\textsuperscript{13}. Thus, whether this newly described technique can reliably be used as a high-throughput method to determine CNV remains to be seen.

Similarly, other ‘next generation’ sequencing techniques are currently insufficient to determine FCGCR2/3 genetic variations, as multiple variants were mistyped in subjects that were genotyped by whole-exome sequencing, in this case on an Illumina platform (chapter 5). Thus, it appears that next generation sequencing techniques will have to be improved greatly before such methods can be used to adequately genotype the FCGR2/3 locus (as well as other complicated loci with duplications with high homology), and it is not sure whether such methods will be present for high-throughput analysis in the near future.

Until then, it seems more reasonable to use the currently available and reliable methods for genotyping the FCGR2/3 locus. The major advantage of MLPA is that it determines both CNV and the currently known relevant SNPs. One disadvantage is that SNPs not included in the probe-mix will not be genotyped (and thus, novel potentially interesting SNPs will not be found). Recently, several novel SNPs at the locus have been described that are not included in the current probe-mixes for the FCGR2/3 MLPA. Table 2 provides an overview of these SNPs. Including these SNPs to the MLPA may lead to even more accurate genotyping and may reveal novel associations. However, a third separate probe-mix would probably have to be used to incorporate all these SNPs.
Table 2. Potentially relevant SNPs at the FCGR2/3 locus not currently included in the FCGR MLPA.

| Rs #           | Nucleotide* | amino acid position** | amino acid | Functional change                                         |
|----------------|-------------|------------------------|------------|----------------------------------------------------------|
| **FCGR2A**     |             |                        |            |                                                          |
| rs150311303*   | c.612 + ins CTT | 170(205) | Leu | higher affinity for human IgG49                          |
| rs72717038**   | c.739 +871 A>G | - | - | G retains exon 6, increased signalling50                 |
| rs382627****   | c.818T       | 238(273) | Leu | Wild type                                                |
|                | c.818C       |            | Pro | introduced to FCGR2A in chimeric FCGR2A/2C gene (chapter 5) |
| **FCGR2C**     |             |                        |            |                                                          |
| rs430178****   | c.799 -1 C>G | - | - | C leads to retention of 62 intronic base pairs51         |
| not listed***  | c.839C       | 236(280) | Pro | Wild type                                                |
|                | c.839T       |            | Leu | introduced to FCGR2C in chimeric FCGR2C/2A gene (chapter 5) |
| **FCGR3A**     |             |                        |            |                                                          |
| rs10127939     | c.197T       | 48(66) | Leu | may influence ligand binding52                           |
|                | c.197A       |            | His |                                                          |
|                | c.197G       |            | Arg |                                                          |
| **FCGR3B**     |             |                        |            |                                                          |
| rs448740****** | c.194A       | 47(65) | Asn | NA1                                                      |
|                | c.194G       |            | Ser | NA2 and SH                                               |
| rs147574249***** | c.244G     | 64(82) | Asp | NA1                                                      |
|                | c.244A       |            | Asn | NA2 and SH                                               |
| rs2290834****** | c.316G       | 88(106) | Val | NA1                                                      |
|                | c.316A       |            | Ile | NA2 and SH                                               |

*synthetic MLPA probes for this insertion of 3 nucleotides have been included in some of our more recent MLPA and appear to give a good signal in a some individuals of African descent, although these samples still await verification by gene-specific PCR.

**synthetic probes for this variation have been tried in the past, but gave non-specific signals because of the similarity of this sequence to FCGR2C and FCGR2B. Possibly, this SNP can still be determined by MLPA, by instead designing a probe for the wild-type sequence in FCGR2A, which does not occur in FCGR2B and FCGR2C, and detect this SNP by a decrease of the wild-type sequence.

***The amino acid changes introduced to FCGR2A and FCGR2C genes in the chimeric variants caused by nonallelic homologous recombination (chapter 5) can in fact also be viewed as a SNPs and in the case of FCGR2A have indeed been described as such53. This study did not determine copy number of the FCGR genes, and possibly, the individual described as having the SNP actually has a deletion of CNR2 and a chimeric FCGR2A/2C gene. Probes for these variants are actually included in the MLPA, but have to be interpreted with caution because a probe detecting an abnormal variant in one of the genes will by definition also detect the wild-type version of the other gene. To complicate matters, the probe recognizing the Leucine variant is not designed well – it actually appears to bind both the Leucine and Proline variants equally well. This is the result of the base at the ligation site on the 3’ end of the left probe, which is a Thymine, which not only binds the complementary Adenine that
encodes the Leucine variant, but can also form weak bonds with Guanine (which is the nucleotide encoding the Proline variant). This probe may be improved by designing the probe on the complementary DNA strand (see www.mlpa.com for protocols on probe design).

****We have actually designed and tested a probe for this SNP in the MLPA, but it did not give good results, probably because of competition with the probes that detect the previous variant, which lies in close proximity. A separate FCGR MLPA probe-mix may be required to include this variant.

*****These SNPs are part of the haplotypes NA1 and NA2 of FCGR3B, but are not actually included in the MLPA. Haplotypes are deduced from other typed SNPs in the haplotypes, which is a reasonable approach, although in some African individuals different variants of these haplotypes have been described. Another SNP that may affect N-linked glycosylation has been described in two Japanese subjects.

**EVOLUTION OF THE FCGR2/3 LOCUS**

**On the origin of the FCGR2C and FCGR3B genes**

The FCGR2C and FCGR3B genes that occur in humans appear to have evolved recently. They were formed in a segmental duplication of the FCGR2/3 locus. This segmental duplication is not present in all primates, for instance, *Macaca* (macaque) species do not have an FCGR3B gene. According to a recent publication that compared Fc receptor genes in genomes of multiple species, not even non-human primates as closely related to *Homo sapiens* as *Pan troglodytes* (chimpanzees) or *Pongo abelii* (orangutans) do have FCGR2C and FCGR3B genes, suggesting that *Homo sapiens* may be the only species in which the segmental duplication of the FCGR2/3 locus has occurred. However, the complexity of the FCGR2/3 locus may have precluded the authors from finding FCGR2C and FCGR3B genes in genomes that are not so well characterized. In fact, Machado et al. looked in more detail and did find evidence of the duplication of the FCGR3 gene in *Pan troglodytes* and in members of the Hylobatidae family (gibbons). In any case, the segmental duplication that created the FCGR2C and FCGR3B genes appears to have occurred relatively recently in evolution. Therefore, it is not so surprising that null variants of these genes are compatible with life; FCGR2C is a pseudogene in >80% of the population, and healthy human individuals lacking the FCGR3B gene have been described. Indeed, a complete lack of the FCGR3B gene was found in about 0.2% of individuals we have tested. However, FCGR3B clearly has an important role in the human immune system, given the fact that low copy number increases the risk of developing SLE. Apparently, the emergence of FCGR3B was beneficial to the species, and this resulted in the fact that the segmental duplication was maintained, and the FCGR3B gene has evolved to be very different from the FCGR3A gene in expression pattern and function.

On the other hand, FCGR2C seems to be not so beneficial, and this result of the segmental duplication has been modified by evolution to reduce its function. We assume that with the formation of FCGR2C by the segmental duplication of the locus, this gene must have been created as a bona fide receptor with an open reading frame. Subsequently, evolution seems to have selected variants that cause reduced function in this receptor through multiple ways (the stop codon in exon3, splice variants in intron7 that abrogate expression, and a 3’UTR...
that does not favor expression when compared with the similar 3'UTR of FCGR2A (chapter 5). All these changes indicate that having an active FcγRIIc has been selected against, and the classical FCGR2C-ORF haplotype may be the last functional remnant of the original FCGR2C formed in the segmental duplication. No benefits for having an active FcγRIIc are known, but it does predispose for certain autoimmune diseases (chapter 3). The fact that it does occur at higher frequencies in the European population is intriguing, since it is extremely rare to absent in African populations (chapter 3), which constitute the ancestors of the human race. One possibility is that the few classical FCGR2C-ORF alleles that may have been present in the European population after the migration out of Africa, were positively selected (or at least selection against this variant was less strong) and now represent the increased prevalence when compared to African populations. Another possibility is that the classical FCGR2C-ORF allele has been newly created in the European population by subsequent recombinations of CNR1 and CNR4, a theory which is supported by the fact that the classical FCGR2C-ORF alleles are actually a haplotype of multiple SNPs in intron2 and exon3 that completely resemble FCGR2B (chapter 5). Considering this possibility, one could predict that FCGR2B-Stop alleles (chapter 5) were also formed in this way, but the rarity of FCGR2B-Stop alleles suggests great evolutionary pressure on this variant, which may be associated with severe autoimmunity.

The function of FcyRIIib

The function of the recently evolved FcyRIIib is still debated today. Traditionally, it has been seen as an activating receptor, although it is unclear how the receptor causes cellular activation, as it does not contain or associate with an activating intracellular motif. Currently, some researchers believe it functions more as a decoy receptor that neutralizes immune complexes, and thus in essence may decrease the immune response (chapter 5). Previous well-designed studies have shown that FcyRIIib is capable of triggering Ca2+ influx and neutrophil granule exocytosis, but that it does not induce the production of reactive oxygen species (ROS) by neutrophils, showing that FcyRIIib can induce some, but not all, of the activating functions known for FcyRs. Recently, a paper has been published in which the authors argued that FcyRIIib also contributes to neutrophil ROS production in response to IgG immune complexes. However, this study has serious methodological errors, since the author have used intact MoAb 3G8 to block FcyRIIib. Intact 3G8 induces tremendous clustering of neutrophils in vitro, which it does by binding to cells via FcyRIIa on adjacent cells (Nagelkerke, data not shown). This clustering or agglutination will obviously have a tremendous negative impact on the activating response of neutrophils in in vitro assays, thus explaining the decreased ROS production that was falsely attributed to the inhibition of FcyRIIib. In contrast, when F(ab)2 fragments of 3G8 are used, neutrophil ROS production is not inhibited at all (chapter 6), and furthermore, ROS production in response to immune complexes was not diminished in an individual completely deficient of FcyRIIib (Nagelkerke, data not shown). This confirms that FcyRIIib does not contribute to neutrophil
ROS production, although FCGR3B polymorphisms do somehow influence this response (chapter 6), in a way that is still unclear.

In ADCC responses by neutrophils however, the situation is very different, as increasing the binding of a tumor-directed IgG to FcγRIIIb has been shown to actually hinder the activating function that IgG in a cytotoxicity assay with neutrophils\textsuperscript{25}. In support of this view, ADCC of tumor cells by neutrophils appears to be increased when blocking FcγRIIIb by F(ab’)2 fragments of 3G8 (Nagelkerke & Matlung \textit{et al.}, unpublished data). On the other hand, FcγRIIIb does appear to contribute to phagocytosis of smaller lymphoid tumor cells by neutrophils, as this phagocytosis was blocked by Fab fragments of 3G8\textsuperscript{26}. Concluding, the effects of FcγRIIIb vary for different immune responses. Possibly, the size of the immune target partly determines the role of FcγRIIIb in immune responses. Altogether, FcγRIIIb appears to have a dual role in the immune system, sometimes causing activating effects, but in other cases has a decoy function or even exerts directly inhibiting effects. The net effect may actually be more towards the decoy or inhibiting effects, as disease association studies show that decreased copy number of the FCGR3B gene predisposes to the autoinflammatory disorder SLE (chapter 2).

\textbf{IVIG THERAPY}

Here, we discuss the impact of our findings on the understanding of the working mechanism for the immunomodulatory effects of IVIg. To date, no conclusive theory exists for the immunomodulatory effect of IVIg, and several theories, as listed in the introduction chapter of this thesis, try to explain this phenomenon. Probably, some of these mechanisms act in concert, and the immunomodulatory effects of IVIg may differ between different diseases. Since we have focused in this thesis on the role of IVIg in the prevention of clearance of IgG-opsonized blood cells, and it is known from clinical studies that this clearance is Fc-mediated (preparations with only Fab fragments of IVIg were not effective\textsuperscript{27}, whereas purified Fc fragments did have a good clinical effect\textsuperscript{28}), we only review the major proposed Fc-mediated mechanisms here. Finally, we discuss the future of IVIg therapy.

\textit{Evaluation of potential working mechanisms for the immunomodulatory effects of IVIg}

\textit{Blockade of activating FcγR by saturation as a result of high IgG concentrations.}

The ‘classic’ mechanism of saturation of activating FcγRs has for a long time been assumed as the most plausible explanation for the effect of IVIg in immune thrombocytopenia\textsuperscript{29,30}, and in fact, our data support this theory. As shown in chapter 7, IVIg can block IgG-mediated phagocytosis of blood cells directly. Furthermore, IgG preparations with increased affinity for FcγRs appear to have an increased effect \textit{in vitro} (chapter 7) and also \textit{in vivo}\textsuperscript{31}, which indicates that the ability to bind to FcγRs is an important characteristic of IVIg. The idea that especially the
low-affinity FcγRs can be blocked by their monomeric ligand in vivo may at first hand seem surprising, but it has been shown in the past that also low-affinity receptors bind monomeric IgG\(^{32}\), indicating that some 'low-affinity' FcγRs are not so low-affinity, and maybe should be better named 'medium-affinity', especially in the case of FcγRIIa and FcγRIIIa\(^{33}\). Greatly increasing the concentration of monomeric IgG above the normal plasma levels may shift the equilibrium towards a situation in which too many FcγRs are occupied for proper functioning. A limitation of our study was that the phagocytosis assays were performed in the absence of human plasma, whereas human plasma is always present in phagocytosis of IgG-opsonized blood cells in vivo. In fact, phagocytosis of blood cells was very high in our in vitro system. If phagocytosis of red blood cells would occur at the same rate in vivo in the case of AIHA, this would lead to an unprecedented massive hemolysis in a short time-span, which is not usually the case. So, the in vitro system does not completely reflect the situation in vivo. However, a high rate of phagocytosis is needed in in vitro experiments, as it offers the opportunity to detect phagocytosis, and this is an inherent problem of in vitro experiments. Another limitation of the studies that we performed is that it used monocyte-derived macrophages, instead of the cells that are primarily responsible for clearing IgG-opsonized blood cells in vivo (splenic macrophages). We have shown that splenic macrophages are very different in FcγR expression as compared to monocyte-derived macrophages, having little expression of the high-affinity FcγRI, but high expression of the low/medium affinity FcγRIIIa (chapter 8). We would expect that these splenic macrophages may be even more sensitive to IVIg preparations with increased avidity, and these studies are currently being performed.

Concluding, the concept of saturation of activating FcγRs as the working mechanism of IVIg has, to our knowledge, never been disproved, and in our opinion remains the most plausible explanation, in particular in the case of disease entities where the balance is clearly poised toward activating FcγR effects. However, it will be hard to formally proof this concept in vivo.

Upregulation of the inhibitory FcγRIIb as a result of sialylated IgG-Fc.

Our results do not support the concept of sialylated IgG-Fc leading to upregulation of the inhibitory FcγRIIb on effector cells as an explanation for IVIg effects ITP and AIHA in humans (chapter 7). For instance, we have found that FcγRIIb is not upregulated in human macrophages in response to IVIg, but nevertheless, these macrophages respond very well to IVIg treatment, being inhibited in phagocytosis (chapter 7). In fact, a bunch of evidence arguing against this concept is appearing in the literature. The role of FcγRIIb in ITP treatment by IVIg was also questioned in mouse studies\(^{34}\). Similarly, we found that IgG-Fc sialylation was not important for the effect of IVIg on human macrophages (chapter 7), and many groups have recently published evidence that IgG-Fc sialylation of IVIg is not required for the immunomodulatory effects\(^{35-38}\). Even the binding of sialylated IgG-Fc to DC-SIGN could not be reproduced\(^{39}\). Furthermore, essentially all the evidence supporting this theory derives from murine studies, which may not be translated to the human situation, as mice and humans extensively differ in FcγR expression.
Many of the murine studies describing this theory for instance use a model for arthritis, but IVIg has never proven to be a useful therapy in treating arthritic patients\(^{40-42}\). Concluding, there is little evidence that sialylated IgG-Fc may be a fraction of IVIg with increased effectiveness, and in our opinion clinical trials that would use this fraction specifically in humans, are not justified.

**Increased clearance of pathogenic antibodies by saturation of the neonatal FcR (FcRn)**

We have not investigated the effects of IVIg on FcRn in the sense that IVIg may saturate this ‘rescue-receptor’, shortening the half-life of all IgG including harmful auto-antibodies. In our opinion this theory is not likely to be an explanation for the immunomodulatory effects of IVIg in ITP, since plasmapheresis, aiming to remove pathogenic auto-antibodies by replacing the patients’ plasma with donor plasma is not effective in this disease\(^{43}\). Thus, apparently, rapid removal of auto-antibodies is not effective in ITP, suggesting that the effect of IVIg in ITP must be exerted in a different way. On the other hand, for a number of diseases in which IVIg therapy is beneficial, plasmapheresis is also a good option. This is for instance the case in Guillain-Barre syndrome\(^{44,45}\). Thus, saturation of FcRn may contribute to the immunomodulatory effects of IVIg in a selection of indications in which it is administered. It would be interesting to investigate genetic variation in the FCGRT gene, which encodes FcRn, for such indications. For instance, a variation in tandem repeats in the promoter region has been described to influence expression of FcRn\(^{46}\), and studying this variation may contribute to our understanding of the role of FcRn in these diseases.

**The future of IVIg therapy**

IVIg is used for many indications, varying from primary immunodeficiencies to autoimmune diseases. The list of indications may be growing, as IVIg is studied for a large number of additional indications (around 1250 clinical trials on intravenous immunoglobulins are currently listed in the clinical trial registry at https://clinicaltrials.gov/ct2/home). IVIg is a costly therapy that that imposes a great burden on the blood donation system; the demand for human plasma, from which several medications can be isolated (IVIg, coagulation factors, albumin, other plasma proteins) is dictated by the demand for IVIg. Shortages may even occur if this demand increases substantially.

If new preparations of IVIg, possibly as recombinant proteins, become available, such problems can be overcome. In addition, recombinant preparations eliminate the (very small) risk of viral transmission that will always be associated with transfusion of human blood products. Understanding of the working mechanism is crucial for the development of recombinant IVIg. In addition, detailed understanding of the working mechanisms may ultimately lead to the development of specialized IVIg preparations optimized for the various indications.

The working mechanism of IVIg as a suppletion therapy in immunodeficiencies is clear, as it is needed to supply these patients with IgG against a range of pathogens. For this indication, an IVIg preparation with a broad repertoire of antibodies against the pathogens that are frequently
encountered is required, and this can in fact best be obtained by pooling plasma from a large number of donors that encounter the same pathogens. Thus, for this indication, plasma donations will probably always remain the best source of IVIg.

However, in all the inflammatory diseases in which IVIg has an immunomodulatory effect, the repertoire of the IVIg presumably does not influence its efficacy much, and in some cases the Fab parts of the antibodies are not even required. Knowledge on whether the immunomodulatory effect of IVIg for a given indication is Fc- or Fab-mediated may become very important if alternative (i.e. not donor-derived) sources of IgG are to be used in the future. However, the results of the ITP studies preclude further clinical trials with Fab-only or Fc-only preparations for other indications, as this may withhold patients a currently effective therapy - which clearly is unethical. Hence, it will remain difficult to determine the relative importance of Fab and Fc for indications other than ITP in the human situation. Murine models may be used as an alternative, but should be interpreted with great caution, as the FcγRs of mice and men differ in many aspects.

When the effects of IVIg are Fc-mediated, the polyclonal aspect of IVIg is clearly not important, and recombinant IgG preparations or even recombinant IgG-Fc fragments may suffice. However, the dosage required for a good effect is high, and this may result in high costs for the production of such fragments. If such recombinant products would be modified, this may greatly increase their efficacy. A preparation of IgG-molecules enriched in IgG-dimers may be more effective (chapter 7), but the dimeric nature of the IgG molecules may increase side-effects as a result of unwanted stimulation of immune cells (in theory, a dimeric IgG molecule can crosslink two FcγRs, leading to cellular activation). An alternative product that may be more suitable is one of single IgG-Fc fragments modulated to bind FcγRs with higher affinity. In this respect, glycosylation may be important, because it can influence the binding affinity of IgG molecules to the various FcγRs. For instance, the binding affinity of FcγRIIIa is influenced by the level of fucosylation of the Fc-domain of IgG, a notion that may help to develop new, afucosylated IgG treatment options. Our finding that FcγRIIIa is highly expressed on splenic macrophages (chapter 8) suggests that this may be an attractive option in reducing phagocytosis of blood cells in ITP or AIHA.

Fab-mediated IVIg actions may be relevant for some indications other than ITP. For such indications, the polyclonality is likely to be very important, and recombinant preparations can only be successful if the relevant clones can be identified and expanded for therapeutic IgG production. These could then be produced as monoclonal antibodies (MoAbs), similar to some currently used MoAbs such as infliximab, which neutralizes the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α).

Hopefully, the research into IVIg working mechanisms will provide more insight that is likely to result in better, more suitable treatment options tailored for specific indications.
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