A field guide to human Fc-gamma receptors
Genetics, cellular expression and interaction with immunoglobulins
Nagelkerke, S.Q.

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Kroeskoppelikaan
*Pelecanus crispus*
Dalmatian pelican

In de broedtijd hebben kroeskoppelikanen een vlek op de borst, die de schijn opwekt dat daar een bloedvlek zit. Volgens een oude legende pikt de moeder kroeskoppelikaan haar borst open met de punt aan haar snavel, zodat haar jongen van het bloed kunnen drinken. Om deze reden staat de kroeskoppelikaan symbool voor het doneren van bloed, en heeft Sanquin deze vogel als logo gekozen.
1

General introduction and scope of the thesis

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

Sietse Q. Nagelkerke, Taco W. Kuijpers

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GENERAL INTRODUCTION

Autoimmune diseases are the cause of substantial morbidity and health care costs, with an estimated prevalence of ~5% in the population. In many autoimmune diseases, antibodies (immunoglobulins) play a central role in the pathophysiology. For instance, auto-antibodies directed against the patients’ own blood cells can cause destruction of platelets (in immune thrombocytopenia (ITP)) or erythrocytes (in autoimmune hemolytic anemia (AIHA)). Similarly, antibodies directed against blood cells are a major complicating factor in erythrocyte and platelet transfusions. Other autoimmune diseases in which antibodies play a role include Kawasaki disease (KD) and systemic lupus erythematosus (SLE). Interestingly, many autoimmune diseases (including ITP and KD) can also be treated by antibodies; by injection of intravenous immunoglobulins (IVIg) isolated from a large pool of healthy blood donors. High doses of IVIg are necessary for this immunomodulatory effect, and since IVIg is derived solely from plasma donations, this is a costly therapy, that imposes a great burden on the blood donation system. The mechanism by which IVIg alleviates autoimmune diseases is poorly understood.

The destructive effect of antibodies in (auto)immunity is exerted mainly via receptors for immunoglobulins, the Fc receptors, which are expressed on immune cells. Previous studies have shown that genetic variation in Fc-gamma receptors (FcγRs), which bind immunoglobulin G (IgG, the most abundant of the immunoglobulins), is associated with susceptibility to various autoimmune diseases. However, the genetic structure of FcγRs is complex, which hampers the genetic studies into these receptors.

In this thesis, we set out to further characterize the complex genetics of FcγRs and investigated their role in SLE, KD and the alloimmune platelet destruction that can take place during platelet transfusion, aiming to elucidate the pathophysiologic mechanisms that underlie these diseases. Furthermore, we investigated the interaction between FcγRs and IVIg in the context of IgG-mediated blood cell destruction, in order to understand the working mechanism, which will help to improve this therapy.

In this chapter, I will first provide some background information on the functioning of the immune system, IgG and FcγRs, followed by a description of the genetics of FcγRs, their role in the destruction of IgG-targeted blood cells, and the possible mechanisms by which IVIg may interfere with these processes.

THE IMMUNE SYSTEM

All animals, including humans, need to protect themselves against invading (micro)organisms that may have detrimental effects. In the first place, this is done simply by forming a barrier to the outside world (i.e. the skin, the lining of the gut) which prevents microorganisms from entering the body. However, this barrier cannot always prevent microorganisms from entering, and when
this happens, a highly specialized system comes in to defend the body that is infected (hereafter called ‘host’). This system is called the immune system, and it is specialized in recognizing and eliminating invading microorganisms (pathogens).

The immune system consists of a cellular and a humoral (non-cellular, i.e. soluble proteins and some other molecules) compartment, which collaborate to eradicate pathogens. Because invading pathogens can strike anywhere in the body, the immune system must be able to act throughout the body, and so both the cellular and humoral compartment are mobile, mainly travelling via blood and lymph to the site of infection.

In all vertebrate animals, the immune system can be further divided into the innate immune system and the adaptive immune system. The innate immune system can directly recognize and eliminate some invading microorganisms, but this is not always sufficient for an adequate protection of the host. All the proteins of the innate immune system that play a role in recognizing pathogens are directly encoded on the genome, and are constitutively expressed on the immune cells. The adaptive immune system, albeit a bit slower, is much more sophisticated; it can recognize a wide range of molecules on an invading pathogen. Molecules recognized by the adaptive immune system are termed ‘antigens’. Antigens induce an adaptive immune response when they bind to specific antigen receptors on the cells of the adaptive immune system, B and T cells. Antigen receptors are formed in an intricate process that involves random rearrangement of a set of different gene segments, which leads to a virtually endless repertoire of different antigen receptors. Each B and T cell carries only one particular antigen receptor. When this antigen receptor encounters its specific antigen, this leads to the activation of the cell expressing the receptor, which is the start of an adaptive immune response. Activation leads to multiplication of the cell, and this clonal expanse is necessary for the development of a potent adaptive immune response, as well as the formation of immunological memory. In the case of B cells, one major consequence of activation is that they start to secrete their antigen receptor as a soluble molecule, known as immunoglobulins or antibodies. These immunoglobulins form the humoral compartment of the adaptive immune system, and are very important for an adequate protection of the host, as exemplified by the fact that individuals with X-linked agammaglobulinemia, who do not have immunoglobulins, suffer from severe bacterial infections if untreated, and fail to develop a proper immune response upon vaccination.

In humans, immunoglobulins (Igs) can be categorized into five classes: IgM, IgD, IgG, IgA and IgE. In the initial phase of a B cell response, only IgM and IgD are formed. Along the course of a B cell response, several further genetic rearrangements can occur that lead to somatic hypermutation (an improvement of the binding affinity of the immunoglobulin for its antigen) and class switching (a process that enables the B cell to secrete either IgG, IgA or IgE, instead of the initial IgM and IgD, although in some instances the B cells keep secreting IgM and do not switch to IgG, IgA or IgE). In many cases, the B cells also differentiate into long-lived plasma cells that keep secreting immunoglobulins for the rest of the life of the host, offering long-term protection to the pathogen that carries the antigen that is recognized by the immunoglobulin. Obviously,
it is important that B cells that carry an antigen receptor that reacts with molecules on the hosts’ own cells or extracellular milieu do not become activated. In that case they would start to produce antibodies that would attack the own body. Such unwanted activation is prevented by a phenomenon called tolerance, which ensures that B cells with an antigen receptor that reacts with ‘self’ antigens are eliminated. However, in some cases, tolerance mechanisms fail, and so-called autoantibodies are formed.

Immunoglobulins mainly act in concert with the innate immune system to eliminate recognized pathogens, for instance by binding to Fc receptors for immunoglobulin on cells of the innate immune system. Specific Fc receptors exist for IgM, IgG, IgA and IgE (receptors for IgD have never been identified and probably do not exist). The typical Fc receptors for IgG are called Fc-gamma receptors (FcγRs).

**IMMUNOGLOBULIN G**

IgG is the most prevalent of the immunoglobulins and has a plasma concentration of 7-16 g/L, which makes it the second most abundant protein found in plasma, after albumin. IgG consists of 2 Fab (fragment, antigen-binding) regions that specifically bind the antigen, and an Fc (fragment, crystallizable) region (Figure 1a). IgG can exert its protective function in three ways (Figure 1b); by neutralizing the antigen that is bound, by activation of the humoral innate immune system (complement), or by activation of the cellular innate immune system, via receptors that bind to the Fc region of immunoglobulin, the Fc receptors.

Apart from the major differences in the Fab region that determine the specificity of an antibody, IgGs can also be different in the Fc region. First, and most importantly, IgG consists of four isotypes with differences in the Fc regions: IgG1-4. These isotypes differ greatly in their capacity to activate complement and bind to Fc receptors (Table 1). Additional differences between the Fc regions of IgG molecules derive from the carbohydrate side-chain linked to the conserved Asn297 residue in the C\(_{\text{H}2}\) domain of all isotypes (Figure 1c).

Several cellular Fc receptors for IgG have been described, of which the classical Fc gamma receptors are the best known; these are the receptors that exert most (if not all) of the cellular innate immune responses towards IgG-opsonized targets. Six different FcγRs exist in humans, and these are discussed in detail below.

Other receptors that bind IgG are the neonatal Fc receptor (FcRn), which is structurally very different from FcγRs, and binds IgG only at low pH (<6.5), which means that it cannot bind IgG in the extracellular milieu. Its main functions include the transport of IgG over the placenta, which enables the transmission of IgG from mother to fetus, and the extension of the half-life of IgG by recycling IgG that is taken up by endocytosis\(^7\). Another receptor that binds IgG-Fc regions is the tripartite motif-containing protein 21 (TRIM21), which is a structurally unrelated intracellular protein that resides in the cytosol. IgG is not normally found in the cytosol, but
Antigen binding site
Carbohydrate that is essential for binding to FcγRs
N-linked to Asn297 residue
Disulfide bonds connecting the four polypeptide chains
Carbohydrate that is essential for binding to FcγRs
N-linked to Asn297 residue

Fab region

Fc region

Direct neutralization of viruses, toxins
Activation of complement
Activation of cellular Fc receptors
(dependent on Fab region only)
(dependent on the combination of Fab and Fc regions)
(dependent on the combination of Fab and Fc regions)

N-Acetylgalcosamine (GlcNac)
Mannose
Galactose
Fucose
N-acetyleneuraminic acid (Sialic acid)

* Variably present structures

Figure 1. Immunoglobulin G
IgG has 4 isotypes: IgG1-4, encoded by separate gene segments present in the genome. These isotypes differ greatly in their capacity to activate complement and bind to Fc receptors; IgG1 and IgG3 are much more active than IgG2 and IgG4 in this respect. The serum half-life is also different: IgG3 has a much shorter half-life than the other isotypes. Additional differences between IgG molecules derive from the carbohydrate side-chain linked to the conserved Asn297 residue in the C\(_{\text{H}2}\) domain of all isotypes, to which varying sugar moieties can be added, which can dramatically affect binding affinity to FcγRs. Finally, allotypic variants of IgG1, IgG2 and IgG3 are present in the human population, which are involved in allo-immunization towards injected foreign allotypes of IgG. On a functional level, these allotypes may have an influence on the half-life of IgG molecules, but they do not seem to influence binding to FcγRs, although this has not been studied in detail.
A basic structure of an IgG molecule. Like all immunoglobulins, IgG molecules consist of four polypeptide chains; two identical heavy chains (in white) and two identical light chains (in grey), linked to each other by disulfide bonds. Both the heavy and the light chains contain one variable domain (VH and VL), which is different in each separate clone of IgG. The variable domains of the heavy and light chain together determine the specificity of the antibody at the antigen binding site; each IgG molecule has two identical antigen binding sites. Variability of the VH and VL domains is greatly increased in the region where the antibody binds, as indicated by the grey shading. In addition, the heavy chains of an IgG have three constant domains (CH1 – CH3), whereas the light chains have only one constant domain (CL). The light chain and the CH1 and CH2 domains of the heavy chain together form the Fab (fragment, antigen-binding) region, and the CH2 and CH3 domains of the heavy chain form the Fc (fragment, crystallizable) region. B different mechanisms by which IgG exerts its function. C overview of carbohydrate side-chain linked to the conserved Asn297 residue in the CH2 domain of all isotypes, to which varying sugar moieties can be added, which can dramatically affect binding affinity to FcγRs.

Table 1. Characteristics of the IgG isotypes

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum half life</td>
<td>21 days</td>
<td>21 days</td>
<td>7 days</td>
<td>21 days</td>
</tr>
<tr>
<td>Serum levels (in adults)</td>
<td>3.4-8.9 g/L</td>
<td>1.7-6.3 g/L</td>
<td>0.2-1.1 g/L</td>
<td>0.0-1.2 g/L</td>
</tr>
<tr>
<td>Complement activation</td>
<td>yes*</td>
<td>no</td>
<td>yes*</td>
<td>no</td>
</tr>
<tr>
<td>Binding FcγRs</td>
<td>yes</td>
<td>little</td>
<td>yes</td>
<td>intermediate</td>
</tr>
<tr>
<td>FcγRI</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>+++</td>
<td>+/-/+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>FcγRIIb/c</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>FcγRIIIa</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>FcγRIIIb</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

Binding affinities adapted from Bruhns et al.34.  
++++: high affinity binding ($K_a 10^7 – 10^8 \text{ M}^{-1}$);  
+++: medium affinity binding ($K_a 10^6 – 10^7 \text{ M}^{-1}$);  
++: low affinity binding ($K_a 10^5 – 10^6 \text{ M}^{-1}$);  
+: very low affinity binding ($K_a 10^4 – 10^5 \text{ M}^{-1}$);  
- : no binding.  
*IgG1 and IgG3 can fix complement, but this depends also on the antigen; only if the IgGs are able to form hexamers, they will fix complement124.  
** binding affinity of IgG2 to FcγRIIa depends greatly on a polymorphism in FcγRIIa.

can come along when IgG-opsonized non-enveloped viruses or bacteria enter the cells. In these cases, TRIM21 can target these structures for intracellular proteasome degradation34.
**FC-GAMMA RECEPTORS**

*Function of FcyRs*

FcyRs are found on almost all immune cells and, upon binding of IgG, mediate a wide range of cellular responses that can destroy and eliminate the opsonized target. These responses are only initiated when multiple IgG molecules are bound simultaneously; a single IgG does not activate FcyRs. When multiple IgG molecules are fixed close to each other, as is the case in opsonized bacteria or in an immune complex, this results in cross-linking of several FcyRs in the cell membrane, which leads to their activation. This activation is transmitted to the cell by an intracellular signaling cascade (Figure 2), which instructs the cell to start cellular defense mechanisms against the IgG-opsonized target. Various cellular defense mechanisms of innate immune cells can be induced by FcyRs, depending on the target and the type of immune cell that is activated; different mechanisms can also act in concert.

These mechanisms include phagocytosis of IgG-opsonized microorganisms or immune complexes, leading to their destruction in intracellular phagolysosomes. Immune cells can also induce the death (usually by apoptosis) of other host cells (for instance cells that are infected with a virus, or cancer cells) when they are opsonized with IgG, in a process called antibody-dependent cellular cytotoxicity (ADCC), which can be performed by neutrophils, monocytes, macrophages or Natural Killer cells (NK cells). FcyRs on neutrophils can cause activation of the NADPH oxidase, which produces reactive oxygen species (ROS) that are toxic to cells and bacteria, and are used to kill bacteria in the extracellular space or in phagolysosomes. Furthermore, neutrophils can release several proteases that cleave bacterial proteins, upon stimulation of FcyRs. Finally, cellular activation by FcyRs can cause the release of cytokines, especially by monocytes and macrophages, which causes inflammation and attracts and activates other immune cells. In addition, FcyRs are expressed on dendritic cells (DCs), the cells that initiate adaptive immune responses, and activation of FcyRs can modulate these responses.

In humans, six isoforms of FcyR exist. Based on their affinity for monomeric IgG, FcyRs can be divided into one high affinity FcyR (FcyRI) and five low affinity FcyRs (the different isoforms of FcyRII and FcyRIII) (Figure 2). On a functional level, most of the FcyRs are activating receptors that can induce the cellular responses mentioned above, but one, FcyRIIb, is an inhibitory receptor. The various human FcyRs are differentially expressed on a range of immune cells. Table 1 gives an overview of the cellular expression of the different FcyRs, Figure 2 shows the structure of the FcyRs.

*FcyRI*

FcyRI (CD64) has three extracellular (EC) Ig-like domains, involved in binding of IgG, a transmembrane (TM) domain and a short intracellular (IC) domain of 61 amino acids. The TM domain associates with the FcRγ-chain, an adaptor protein containing an immunoreceptor tyrosine-based activating motif (ITAM), to induce signaling and maintain stable expression.
General introduction

6 SNPs determine NA1/NA2/SH

Linked to membrane by GPI anchor

Figure 2. Human FcγRs

Overview of the structure of human FcγRs. The oval shapes in the extracellular part of the FcγRs represent the different extracellular domains; the light grey domains are the domains where IgG molecules bind. All FcγRs except FcγRIIIb are linked to the plasma membrane by transmembrane (TM) domains indicated by small rectangles. FcγRIIIb is linked to the plasma membrane through a GPI anchor.

FcγRI and FcγRIIa have small intracellular domains that associate with adaptor molecules that can initiate an intracellular signaling cascade when multiple FcγRs are cross-linked, which ultimately leads to activation of the cell on which the FcγRs are expressed. FcγRII receptors have a much larger intracellular domain, and contain a signaling motif to start this cascade in their own polypeptide chain.

Signaling by activating FcγRs is mediated by immunoreceptor tyrosine-based activating motifs (ITAM) that are present either in the cytoplasmic tail of the receptor itself or in non-covalently associated signaling adaptor proteins, such as the common γ-chain (FcRγ). Aggregation of activating FcγR by binding of multivalent ligands, such as an opsonized pathogen or blood cell or an immune complex, results in the phosphorylation of ITAM tyrosine residues by Src family protein tyrosine kinases (PTKs), and ultimately leads to activation of cellular responses. Aggregation of the inhibitory FcγRIIb, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), also results in phosphorylation of tyrosine residues by Src family PTKs. In contrast to ITAMs, phosphorylated ITIMs serve as binding sites for phosphotyrosine phosphatases (PTPs) which dephosphorylate other proteins resulting in inhibition of activating pathways.

Approximate location of functional SNPs in the FcγRs are indicated by small grey circles, SNPs are indicated by 1-letter amino-acid codes, for each SNP the most common nomenclature is shown followed by alternative nomenclature in brackets, please refer to table 3 for details on the nomenclature.

ITAM: immunoreceptor tyrosine-based activating motif. ITIM immunoreceptor tyrosine-based inhibitory motif
FcγRI is constitutively expressed by monocytes, macrophages and dendritic cells and its expression can be induced on neutrophils by stimulation with IFN-γ and/or G-CSF and is induced on neutrophils in patients with bacterial infection, for which it may serve as a useful diagnostic marker. Since FcγRI has a high affinity for IgG, it binds monomeric IgG, which is always present in blood and extracellular fluid. Therefore, it will in general be fully occupied by its ligand, and it could be argued that this occupation makes it less available for multivalent IgG such as immune complexes or opsonized targets, rendering this high-affinity FcγR less potent than low-affinity FcγRs. However, it has been shown that FcγRI can bind multivalent targets also in the presence of monomeric IgG when the cells expressing the FcγRI are stimulated by cytokines, by an inside-out regulation that is not yet well characterized.

Table 2. Expression of FcγRs on different cell-types

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>FcγRI</th>
<th>FcγRIIa</th>
<th>FcγRIIb</th>
<th>FcγRIIc</th>
<th>FcγRIIIa</th>
<th>FcγRIIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>NK cells</td>
<td>-</td>
<td>-</td>
<td>genotype-dependent*</td>
<td>genotype-dependent**</td>
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<td>-</td>
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<tr>
<td>Dendritic cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>genotype-dependent??</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>genotype-dependent**</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Monocytes</td>
<td>-</td>
<td>+</td>
<td>subsets</td>
<td>genotype-dependent**</td>
<td>subsets</td>
<td>-</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>induced</td>
<td>+</td>
<td>genotype-dependent***</td>
<td>genotype-dependent**</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>induced****</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>induced****</td>
</tr>
<tr>
<td>Platelets</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

Expression of FcγRs on different cell types, derived from our own data as well as review of the literature. *Expression of FcγRIIb occurs in NK cells in individuals with a deletion of CNR1 (figure 2). **Expression of FcγRIIc is dependent on SNPs in exon3 and intron7 of the FCGR2C gene, which in most individuals is a non-expressed pseudogene. ***Expression of FcγRIIb on neutrophils strongly correlates with SNPs in the promoter of the FCGR2B gene (promoter haplotype 2B.4). ****Although FcγRI and FcγRIIIb are definitely absent from eosinophils in the resting state, and are often regarded not expressed by these cells, some reports have described inducible expression in vitro for FcγRI and FcγRIIIb and also in vivo for FcγRIII.

FcγRIIa

FcγRIIa (CD32a) consists of a single polypeptide chain which contains an ITAM signaling motif in the intracellular domain. FcγRIIa is the most widely expressed isoform of FcγRII and is found on monocytes, macrophages, dendritic cells, neutrophils and platelets. It can induce many dif-
ferent cellular defense mechanisms such as phagocytosis of IgG-opsonized targets, ADCC, ROS production and cytokine production.

**FcγRIIb**

FcγRIIb (CD32b) is the only FcγR that results in an inhibitory signal to the cell, which is transferred by the immunoreceptor tyrosine-based inhibitory motif (ITIM) on its intracellular signaling domain. FcγRIIb is found in two isoforms deriving from two different transcripts (Figure 2), FcγRIIb-1 and FcγRIIb-2, with FcγRIIb-1 having an additional intracellular exon in between the transmembrane and signaling domains. FcγRIIb-1 is highly expressed on B cells, where it constitutes the only surface-expressed FcγR, and co-crosslinking of FcγRIIb-1 with the antigen receptor on B cells (also called B cell receptor (BCR)) inhibits activating signals induced by the BCR. Other cell types also express FcγRIIb, albeit at much lower levels, and on these cells FcγRIIb-2 is the main transcript expressed. These cells include a subset of monocytes, macrophages, and dendritic cells. Expression of FcγRIIb can also be detected on neutrophils and NK cells, but only in individuals with certain genotypes. FcγRIIb on phagocytic cells can inhibit pro-phagocytic signals induced by activating FcγRs, balancing the immune response against IgG-opsonized targets.

**FcγRIIc**

FcγRIIc (CD32c) has long been considered not to be expressed at all, as its gene (FCGR2C) was thought to be a pseudogene, and therefore relatively little was known about the expression pattern of this receptor. In 1998, FcγRIIc was first found on NK cells of individuals that carry an open reading frame (ORF) of this receptor (p.57Gln, FCGR2C-ORF), as opposed to the majority of individuals in which this receptor is indeed a pseudogene and cannot be expressed as a result of a stop codon in exon3 (p.57Ter, FCGR2C-Stop). Determining the cellular expression pattern of FcγRIIc has long been complicated, but we now know that FcγRIIc can be expressed on NK cells, neutrophils, monocytes and macrophages (Figure 4). Obviously, FcγRIIc can only be functional in individuals with an FCGR2C-ORF. Although expression on NK cells is relatively low, it has been shown to be capable of inducing killing of target cells in a redirected ADCC assay, functioning as an activating receptor.

**FcγRIIIa**

FcγRIIIa (CD16a) is similar to FcγRI in its TM and IC domains. In monocytes and macrophages, this receptor associates with the FcRγ-chain, while in NK cells it associates with the CD3ζ-chain to induce signaling. In contrast to FcγRI, association with these adaptor proteins is not only essential for maintaining stable expression, but also for targeting the receptor to the cell membrane. FcγRIIIa expressed on NK cells can induce ADCC by these cells, and on phagocytes it can induce phagocytosis.
FcγRIIIb (CD16b) is a GPI-anchored protein, expressed in high numbers on neutrophils, and sometimes on eosinophils. As it does not have a transmembrane domain, it cannot associate with FcRγ or the ζ-chain. FcγRIIIb is not capable of IgG-induced production of ROS25. However, it does contribute in in vitro experiments to the exocytosis of neutrophil granule proteins 26 and Ca²⁺ influx27, and may also cooperate with FcγRIIa on the same neutrophil to induce such responses28. Because FcγRIIIb can induce these responses, FcγRIIIb is usually classified as an activating receptor, although the exact mechanism(s) by which FcγRIIIb activates cells are still unclear29,30. It has also been proposed that FcγRIIIb functions mainly as a decoy receptor31.

Genetics of FcγRs

FCGR genes

All human FcyRs are encoded by genes on chromosome 1. The high-affinity FcyRI is encoded by the FCGR1A gene at 1q21.2. FCGR1A has two homologues, the pseudogenes FCGR1B and FCGR1C, located at 1p11.2 and 1q21.1 respectively. The low-affinity FcyRs; FcyRIIa, FcyRIIb, FcyRIIC, FcyRIIla and FcyRIIlb are encoded respectively by FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B. All these genes are located in a cluster at 1q23.3 in the FCGR2/3 locus (Figure 3a). The locus consists of two 82 kb paralogous repeats with >98% sequence homology, that were formed as the result of an unequal crossover event17. This unequal crossover event between FCGR2A and FCGR2B, the two genes that flank the region, has led to a segmental duplication in which FCGR2C was formed17, with the resulting FCGR2C gene being highly homologous to FCGR2B in the first six exons and highly homologous to FCGR2A in the last 2 exons. Figure 5 provides an overview of the differences between the three FCGR2 genes. Furthermore, the segmental duplication created the two different FCGR3 genes, FCGR3A and FCGR3B, which are also highly homologous in
sequence (Figure 6). The genes encoding the classical FcγRs are highly polymorphic and functionally relevant genetic variations have been described for all low-affinity FcγRs. An overview of the functionally relevant SNPs is given in Table 3, and the approximate locations within the FcγRs are shown in Figure 2. Inconsistencies in the nomenclature of these SNPs exist, because some SNPs are commonly indicated by the amino acid position in the mature protein (from which the signal peptides have been cleaved off), whereas others are indicated by the amino acid position in the full protein. Please use Table 3 as the standard for the nomenclature, which is used throughout the thesis. The functional consequences of the SNPs are discussed below. Besides being polymorphic, some of the low-affinity FCGR genes are subject to gene copy number variation (CNV) (Figure 3b).

Genetic analysis of the FCGR2/3 locus
As a result of the high sequence homology between the genes, genotyping of this locus is very complicated, and it is important to realize that commonly used genome databases such as Ensembl or ncbi BLAST are not in all cases accurate in the distinction between a SNP in one of the FCGR genes and a genuine difference between two homologous FCGR genes (paralogous sequence variant, PSV). Detailed knowledge of the organization of the locus is essential for a proper genetic analysis. Perhaps the best source for this is Supplementary Table 1 in the recent paper by Mueller et al. 32.

Having said this, when great care is taken to ensure gene-specific analysis, all genes can be adequately genotyped. This can be performed by designing long-range polymerase chain reaction (PCR) experiments in which large (>10 kb) fragments of genomic DNA are amplified, which can ensure specificity for each FCGR gene, when adequate primers for the PCR are chosen. However, this technique is very elaborate. An easier way of genotyping FCGR genes specifically is by performing a quantitative analysis of the genomic DNA, as it is done with the multiplex ligation-dependent probe amplification (MLPA) assay, which can adequately detect almost all of the functional SNPs, as well as CNV 19. MLPA is the standard technique used throughout this thesis for genotyping the FCGR2/3 locus, supported by gene-specific long-range PCR when necessary.

Functional consequences of SNPs in the FCGR2/3 genes
In FCGR2A, encoding for FcγRIIa, a single nucleotide polymorphism (SNP) was first noticed, which results in either a histidine or an arginine at position 131 (p.His131Arg) in the IgG binding domain (EC2) 33. FcγRIIa-His131 has a higher binding affinity for IgG1 and especially IgG2, as compared to FcγRIIa-Arg131, but binding to IgG3 and IgG4 is similar for both variants 34. Functionally, mononuclear cells from FcγRIIa-131HH individuals produce more IL-1β when stimulated with IgG2 than FcγRIIa-131HR and -131RR individuals 35. Similarly, neutrophils from individuals homozygous for H131 (FcγRIIa-131HH) have been shown to have increased phagocytosis and degranulation in response to serum-opsonized bacteria and increased rosette formation and phagocytosis in presence of IgG3 anti-D sensitized erythrocytes when compared to FcγRIIa-131RR individuals 36,37.
Figure 4. FcγRIIC expression on various cell types in healthy human subjects
**Figure 4.** Determining the cellular expression pattern of FcyRIIc has long been difficult, because of the high sequence homology with FcyRIia, and the complete sequence homology of the extracellular domains with FcyRIib. However, since monoclonal antibody (MoAb) 2B6 (which recognizes both FcyRIib and FcyRIIc but not FcyRIia) became available, the expression pattern could be clarified. Although flow cytometry data with this MoAb are not straightforward to interpret because of the (varying) presence of FcyRIib on immune cells, expression of FcyRIIc can easily be deduced by comparing the stainings from individuals who cannot express FcyRIIc (FCGR2C-Stop) with individuals who can (FCGR2C-ORF).

A Expression of FcyRIIc and FcyRIib on circulating leukocytes. Figure adapted from van der Heijden et al.\(^\text{13}\), including measurements from additional individuals. MoAb 2B6 recognizes an extracellular epitope of both FcyRIib and FcyRIIc, but since FCGR2C-Stop individuals cannot express FcyRIIc, the difference in MFI between FCGR2C-Stop and FCGR2C-ORF individuals can be assumed to derive from expression of FcyRIIc. FCGR2C-Stop (individuals with zero copies of FCGR2C-ORF) n=105; FCGR2C-ORF (including individuals with one and two copies of FCGR2C-ORF) n=31. Means + s.e.m. are shown.

B Recently, it was proposed that FcyRIIc may also be expressed on B cells\(^\text{120}\). However, detailed analysis of MoAb 2B6 staining on circulating B cells in our cohort, showing individual measurements, reveals no evidence of expression of FcyRIIc on B cells. FCGR2C-Stop n=105; FCGR2C-ORF(1x), individuals with one copy of FCGR2C-ORF, n=24; FCGR2C-ORF(2x), individuals with two copies of FCGR2C-ORF, n=7.

C Expression of FcyRIIc on macrophages. Upper panel: MoAb 2B6 staining on monocyte-derived macrophages cultured for 9 days from 36 healthy individuals, performed as described\(^\text{121}\). FCGR2C-Stop: n=24; FCGR2C-ORF, individuals with one (n=11) or two (n=2) copies of the FCGR2C-ORF allele. Data are shown for M-CSF (left) and GM-CSF (right) cultured macrophages. Lower left panel: Detection of FCGR2C mRNA in monocyte-derived macrophages cultured for 9 days. qPCR with FCGR2C1-specific primers was performed as previously described\(^\text{19}\), using cDNA from M-CSF monocyte-derived macrophages as a calibration curve, as described in\(^\text{13}\). FCGR2C-ORF, individuals with 1 FCGR2C-ORF allele, n=3; FCGR2C-Stop, individuals with zero copies of FCGR2C-ORF, n=4. Means + s.e.m. are shown. Lower right panel: Immunoprecipitation of FcyRIIc from M-CSF monocyte-derived macrophages confirms expression of FcyRIIc. Experiment was performed essentially as described in\(^\text{13}\), in this case using MoAb 2B6 to capture FcyRIIc (and FcyRIib), followed by a specific staining for the intracellular part of FcyRIIc with a rabbit polyclonal antibody against the cytoplasmic tail shared by FcyRIIc and FcyRIib\(^\text{103}\), in macrophages from an individual with zero copies of FCGR2C-ORF (Stop), and an individual with one copy of FCGR2C-ORF (ORF). Data are representative of 3 independent experiments with different individuals.

For reasons of simplicity, in this figure, individuals with the non-classical FCGR2C-ORF allele that is not expressed\(^\text{13}\) (n=6 for A and B), were grouped with FCGR2C-stop individuals. Individuals with a deletion of CNR1 (FCGR2C and FCGR3B genes), which leads to ectopic expression of FcyRIib on NK cells\(^\text{13}\) (n=14), were left out of the analysis of NK cells in a. Statistical significance was tested by Mann Whitney test. ns (p>0.05); * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001. Δ MFI: median fluorescence intensity of MoAb 2B6 minus median fluorescence intensity of isotype control. Some individuals were analyzed more than once at different time points with similar results, means are shown for these individuals.

FCGR2B, encoding for FcyRIIb, also exists in two allelic variants, containing either an isoleucine or a threonine at position 232 in the TM domain\(^\text{38}\). As this SNP (p.Ile232Thr) does not affect the IgG-binding EC domains, it has no influence on the binding affinity. However, its localization at the TM domain results in differences in downstream signaling and subsequent inhibition of FcyRII signaling in macrophages and BCR signaling in B cells. In particular, I232 provides stronger inhibitory signaling than T232, and this is caused by the exclusion from lipid rafts of FcyRIIb-T232\(^\text{39,40}\). Other genetic variations influence the expression of FcyRIIb. For instance, in individuals with a deletion in the FCGR locus that includes FCGR2C, FCGR3B and is called CNR1, FcyRIIb can surprisingly also be expressed on the surface of NK cells, where it is capable
to inhibit killing of target cells in ADCC\textsuperscript{13}. Expression of FcγRIIb in other cells is hardly affected by this deletion. Furthermore, two SNPs in the promoter of \textit{FCGR2B} and \textit{FCGR2C}, a guanine or cytosine at position -386 and a thymine or adenine at position -120, form four haplotypes of which one (-386G, -120A; 2B.3) has never been found in any individual thus far. In case of \textit{FCGR2B}, the rare 2B.4 promoter haplotype (-386C, -120A) appeared to have higher transcriptional activity than the wild-type promoter 2B.1 (-386G, -120T)\textsuperscript{41}, resulting in increased expression on neutrophils and B cells\textsuperscript{14,41}, although another research group has found that this 2B.4 promoter led to a decreased expression of FcγRIIb on B cells\textsuperscript{42}.

In \textit{FCGR2C}, the previously mentioned p.Gln57Ter SNP determines whether or not individuals can express FcγRIIc at all. This mutation results in either an open reading frame (\textit{FCGR2C}-ORF, allele frequency ~10-15\% in Caucasians) or a stop codon (\textit{FCGR2C}-Stop)\textsuperscript{19}. Classically, ORF/Stop genotyping of individuals is done based on this SNP alone. However, we have recently found that some individuals carry splice site mutations in intron7, which leads to alternative transcripts, causing a frameshift in exon8 and the introduction of novel stop codons, leading to a loss of FcγRIIc expression\textsuperscript{15}. Genotyping of \textit{FCGR2C} should therefore include these novel mutations to provide an accurate prediction for FcγRIIc expression.

In \textit{FCGR2C}, the promoter haplotypes of \textit{FCGR2B} as mentioned above, can also be found. In general, only the wildtype and one other promoter haplotype (-386C, -120T; 2B.2) are found. The 2B.2 haplotype is linked to p.57Gln\textsuperscript{19}.

The FcγRIIIa-encoding \textit{FCGR3A} gene contains a SNP that results in either a valine or a phenylalanine at position 158 (p.Val158Phe), located in the EC2 domain\textsuperscript{43}. FcγRIIIa-158Val has a higher binding affinity for all human IgG classes compared to FcγRIIIa-158Phe\textsuperscript{34}. In ADCC assays, NK cells from FcγRIIIa-158Val donors show increased killing of target cells that are opsonized with sub-saturating levels of the human anti-CD20 MoAb Rituximab\textsuperscript{23}.

The FcγRIIIb-encoding \textit{FCGR3B} gene exists in three polymorphic variant proteins, best known as the NA1, NA2 and SH haplotypes. These haplotype consist of a set of 6 SNPs in exon3 of \textit{FCGR3B} (Table 2 and Figure 6). The \textit{FCGR3B} variants encoded by these haplotypes determine the allotypic variants of the Human Neutrophil Antigen1 (HNA1), which is involved in allo-immunization against neutrophilic granulocytes (See Figure 6 for a detailed description). Apart from determining allo-immunization against neutrophils, these haplotypes are known to have functional differences. Compared to NA1, the NA2 and SH variants have two additional N-linked glycosylation sites (Figure 5). The SH variant differs from NA1 and NA2 by a p.Ala78Asp amino acid change that predicts a change in the tertiary structure of the protein\textsuperscript{44}, although the actual functional consequences of this SNP are not well-characterized. While the binding affinities for IgG1 and IgG3 appear similar between NA1, NA2 and SH\textsuperscript{34}, neutrophils from FcγRIIIb-NA1NA1 individuals bind and phagocytize IgG-opsonized bacteria and red blood cells more
**General introduction**

The **FCGR2C** gene is the crossover product from an unequal crossover between **FCGR2A** and **FCGR2B**. Coloring of **FCGR2C** matches the color of the other **FCGR2** genes in the parts where it is highly homologous to that gene. Exons are shown by boxes, white exons are included in all transcripts, red exons are always spliced out, red-shaded exons are spliced out in some transcripts but retained in others. Exon names are below and followed by the number of coding base pairs in that exon. S1, S2: signal peptides; EC1, EC2: extracellular domains; TM: transmembrane domain; C1, C2, C3: cytoplasmic domains. The C3 exons contain an immunoreceptor tyrosine-based activation motif (ITAM) in **FCGR2A** and **FCGR2C**, and contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in **FCGR2B**.

There is a potential confusion with regard to exon numbering in **FCGR2** genes: In the **FCGR2B** gene, transcripts exist that do (FCGR2B1) or do not (FCGR2B2) retain the 57 bp exon6, dependent on the cell type in which the receptor is expressed. In **FCGR2A** and **FCGR2C** a very homologous exon6 is present on genomic level, but this exon is always spliced out in **FCGR2A** and **FCGR2C** transcripts. Only in some rare cases exon6 is retained in **FCGR2A**, which we have previously reported to result in a gain-of-function FcγRIIa isoform103. The final two exons of **FCGR2A** and -2C are often designated exon6 and 7, but to reflect the homology between the 3 **FCGR2** genes, we chose to include the potential exon6 in the nomenclature of all **FCGR2** genes, designating the final 2 exons exon7 and 8. However, we do not include the basepairs of this exon6 when indicating the nucleotide positions in the **FCGR2A** and **FCGR2C** transcripts, because this is not normally done in the literature.

In **FCGR2A** transcripts, further inconsistencies exist as a result of alternative splicing at the beginning of exon3 because of two adjacent splice acceptor sites that can both be used. The most commonly used nucleotide and amino acid numbering is derived from the shorter transcript in which the 3’ splice acceptor site is used, so we chose to use this transcript for nucleotide numbering throughout this thesis.

Several SNPs are indicated in the figure, all are indicated by their amino-acid position excluding signal peptides, followed by the amino acid position including signal peptides between brackets, bold variants reflect the nomenclature used most often in the literature (which is also used in this thesis).

*the p.Gln57Ter SNP in **FCGR2C** is part of a haplotype of 8 SNPs in intron2 and exon3 in **FCGR2C**, this whole haplotype is identical to **FCGR2B** in the case of p.57Gln122. p57Gln is the only nonsynonymous coding SNP in this haplotype.
efficiently than those from FcγRIIIb-NA1NA2 and -NA2NA2 individuals\textsuperscript{36,45}. It is not known whether the SH is functionally different from the otherwise similar NA2 variant.

**FCGR3A**

(254 amino acids)

\[
\begin{array}{c}
5' \text{ UTR} \\
1 \quad 2 \quad 3 \quad 4 \quad 5 \\
S1(40) \quad S2(21) \quad EC1(258) \quad EC2(258) \quad TM(188)
\end{array}
\]

Figure 6. FCGR3 exons

Exons are shown by boxes. Exon names are below and followed by the number of coding base pairs in that exon. S1, S2: signal peptides; EC1, EC2: extracellular domains; TM: transmembrane domain. Several SNPs are indicated in the figure, all are indicated by their amino-acid position excluding signal peptides, followed by the amino acid position including signal peptides between brackets, bold variants reflect the nomenclature used most often in the literature (which is also used in this thesis). Sequences for FCGR3A and FCGR3B are very similar, but four nonsynonymous differences in the coding sequence exist, most notably the stop codon at p.234 in FCGR3B as indicated in the figure, which truncates the transmembrane domain of this receptor. Other amino acid differences between FCGR3A and FCGR3B include p.147 (Gly in FCGR3A, Asp in FCGR3B), p.158 (Tyr in FCGR3A, His in FCGR3B) and p.203 (Phe in FCGR3A, Ser in FCGR3B)\textsuperscript{123}. A set of 6 SNPs in exon3 of FCGR3B form three rather well-defined haplotypes, the FCGR3B-NA1, -NA2 and -SH. FCGR3A is identical to NA1 at some sites, but to NA2 at others\textsuperscript{123}.

Functional consequences of CNV in the FCGR2/3 genes

Besides being polymorphic, some of the low-affinity FCGR genes are subject to gene copy number variation (CNV). Although several large-scale studies on CNV have suggested that human FCGR2A and FCGR2B are candidate genes for CNV\textsuperscript{46-49}, our group has shown previously that this is not the case. In fact, CNV in the FCGR locus is restricted to FCGR2C, FCGR3A and FCGR3B\textsuperscript{50}. It occurs in three different combinations: FCGR3A/FCGR2C, with two possibilities with slightly different borders to the CNV region (CNRs), and FCGR2C/FCGR3B, and these
three CNV regions have been named CNR1, CNR2 and CNR3\textsuperscript{51} (Figure 3). CNV results in differences in expression levels of FcγRIIc (in case of \textit{FCGR2C}-ORF), FcγRIIIa and FcγRIIIb, with more gene copies leading to a higher receptor expression\textsuperscript{50,52,53}. In case of FcγRIIIa, the level of expression on NK cells is, at least for 1 versus 2 copies, related to the level of killing of target cells in (redirected) ADCC assays\textsuperscript{50,54}. Increased expression of FcγRIIIb leads to higher binding and uptake of immune complexes by neutrophils\textsuperscript{54}.

\textit{Linkage disequilibrium at the FCGR2/3 locus}

With all the \textit{FCGR2} and \textit{FCGR3} genes so closely associated in the \textit{FCGR2/3} locus, the different SNPs and CNRs are prone to have a high degree of linkage disequilibrium (LD). Linkage disequilibrium refers to the situation when SNPs at two (or more) locations in the genome do not co-occur completely independently in the population. When one of the variants of a certain SNP co-occurs with one of the variants of a certain other SNP more often than would be expected, these SNPs are said to be in LD.

This has already been shown for some of the SNPs at the \textit{FCGR2/3} locus. For instance, the \textit{FCGR2A}-131His variant is in significant albeit rather weak LD with \textit{FCGR3A}-158Val\textsuperscript{55}. Similarly, the \textit{FCGR3A}-66Leu/His/Arg SNP has been shown to be in LD with \textit{FCGR3A}-158Val/Phe\textsuperscript{56}, and this linkage was responsible for an initially observed difference in binding affinity for IgG in \textit{FCGR3A}-66Leu/His/Arg\textsuperscript{57}, which was later shown to be solely the result of the linkage with the \textit{FCGR3A}-158Val/Phe\textsuperscript{56}, which conferred the actual difference in binding affinity. Thus, knowledge of LD is very important for a correct interpretation of genotyping results, but nevertheless, little is known about the LD between most of the SNPs at the \textit{FCGR2/3} locus. This is one of the topics that will be addressed in this thesis.

\textit{Genetic variation in FCGR2/3 genes: associations with disease}

Both SNPs and CNV in \textit{FCGR} genes have been associated with susceptibility to several autoimmune and infectious diseases. Table 3 provides an overview for a selection of these associations, using data from meta-analyses whenever possible. In general, most of the studies focus on only one or two SNPs; the \textit{FCGR2A} His131Arg and \textit{FCGR3A} Val158Phe are the most studied. In this thesis, we studied all functional \textit{FCGR2/3} SNPs and CNV in a comprehensive manner in the context of three diseases; systemic lupus erythematosus (SLE), Kawasaki disease (KD), and platelet transfusion refractoriness.

SLE is a complex autoimmune disease that involves deposition of immune complexes in tissues. It has been extensively studied for associations with \textit{FCGR2/3} genetic variation, which has revealed various associations (Table 3), although these were never investigated in an integrated way. KD is an acute systemic vasculitis in children. A genome-wide association study (GWAS) has revealed \textit{FCGR2A} His131 to be strongly associated with disease susceptibility\textsuperscript{58}, but this GWAS did not take into account other genetic variation at the \textit{FCGR2/3} locus. Recently, also CNV of \textit{FCGR2C} and \textit{FCGR3B} was shown to be associated with KD\textsuperscript{59}. 
### Table 3. Overview of single nucleotide polymorphisms (SNPs) and copy number variation (CNV) at the FCGR2/3 locus

<table>
<thead>
<tr>
<th>Rs #</th>
<th>Nucleotide*</th>
<th>amino acid position**</th>
<th>amino acid</th>
<th>Functional change</th>
<th>Associations with disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FCGR2A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs201218628</td>
<td>c.184C&gt;c.185A</td>
<td>27(62)</td>
<td>Gln</td>
<td>no functional changes known</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c.184T&gt;c.185G</td>
<td></td>
<td>Trp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1801274</td>
<td>c.497A</td>
<td>131(166)</td>
<td>His</td>
<td>higher affinity for human IgG</td>
<td>KD&lt;sup&gt;97&lt;/sup&gt;, possibly childhood ITP&lt;sup&gt;98&lt;/sup&gt;, possibly GBS&lt;sup&gt;99&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>c.497G</td>
<td></td>
<td>Arg</td>
<td></td>
<td>SLE&lt;sup&gt;100&lt;/sup&gt;, meningococcal sepsis&lt;sup&gt;101&lt;/sup&gt;</td>
</tr>
<tr>
<td>rs150311303</td>
<td>c.612 + ins CTT</td>
<td>170(205)</td>
<td>Leu</td>
<td>higher affinity for human IgG&lt;sup&gt;102&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>rs72717038</td>
<td>c.739 +871 A&gt;G</td>
<td></td>
<td></td>
<td>G retains exon 6, increased signaling&lt;sup&gt;103&lt;/sup&gt;</td>
<td>Anaphylaxis in patients with hypogammaglobulinemia&lt;sup&gt;103&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>FCGR2B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs143796418</td>
<td>-386 C&gt;G ***</td>
<td></td>
<td></td>
<td>Promoter haplotypes 2B.1, 2B.2 and 2B.4 influences expression&lt;sup&gt;41,42&lt;/sup&gt;</td>
<td>2B.4 haplotype associated with susceptibility to SLE&lt;sup&gt;41,42&lt;/sup&gt;</td>
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<tr>
<td>rs780467580</td>
<td>-120 T&gt;A ***</td>
<td></td>
<td></td>
<td>Promoter haplotypes 2B.1, 2B.2 (2B.4?) functional change unknown</td>
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</tr>
<tr>
<td>rs1050501</td>
<td>c.695T</td>
<td>188(232)</td>
<td>Ile</td>
<td></td>
<td>Susceptibility to SLE, protection against malaria&lt;sup&gt;104&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>c.695C</td>
<td></td>
<td>Thr</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FCGR2C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Expression levels (only in FCGR2C-ORF)&lt;sup&gt;59&lt;/sup&gt; KD (unexplained mechanism)&lt;sup&gt;59&lt;/sup&gt;</td>
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<td>rs149754834</td>
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<td>-</td>
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<tr>
<td>rs759550223</td>
<td>c.169T</td>
<td>13(57)</td>
<td>Ter</td>
<td>Stop codon, no expression of FcγRIc</td>
<td>ITP&lt;sup&gt;19&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>c.169C</td>
<td></td>
<td>Gln</td>
<td>Results in an open reading frame (ORF) and expression of FcγRIc&lt;sup&gt;13,19&lt;/sup&gt;</td>
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</tr>
<tr>
<td>rs76277413</td>
<td>c.798 +1 A&gt;G</td>
<td>-</td>
<td>-</td>
<td>A causes exon7 to be spliced out&lt;sup&gt;13&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>rs430178</td>
<td>c.799 -1 C&gt;G</td>
<td>-</td>
<td>-</td>
<td>C leads to retention of 62 intronic base pairs&lt;sup&gt;13&lt;/sup&gt;</td>
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Table 3. Overview of single nucleotide polymorphisms (SNPs) and copy number variation (CNV) at the FCGR2/3 locus (continued)

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<th>Rs #</th>
<th>Nucleotide*</th>
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<th>amino acid</th>
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<th>Associations with disease</th>
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<td><strong>FCGR3A</strong></td>
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<tr>
<td>CNV</td>
<td></td>
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<td></td>
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<tr>
<td>rs10127939</td>
<td>c.197T</td>
<td>48(66)</td>
<td>Leu</td>
<td>Expression levels</td>
<td>SLE (&lt;2 copies)\textsuperscript{109}, Sjögren syndrome (&lt;2 copies)\textsuperscript{110}, systemic sclerosis (&lt;2 copies)\textsuperscript{111}, possibly RA (&lt;2 copies)\textsuperscript{109,112}</td>
</tr>
<tr>
<td></td>
<td>c.197A</td>
<td></td>
<td>His</td>
<td>Decreased ADCC (1 copy vs 2 copies)</td>
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</tr>
<tr>
<td></td>
<td>c.197G</td>
<td></td>
<td>Arg</td>
<td>no great functional changes known, linked to rs396991\textsuperscript{105} may influence ligand binding\textsuperscript{107}</td>
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<tr>
<td><strong>rs396991</strong></td>
<td>c.526G</td>
<td>158(176)</td>
<td>Val</td>
<td>Expression levels</td>
<td>SLE (both &lt;2 and &gt;2 copies)\textsuperscript{108}, anti-GBM disease (&gt;2 copies)\textsuperscript{106}</td>
</tr>
<tr>
<td></td>
<td>c.526T</td>
<td></td>
<td>Phe</td>
<td>higher affinity for human IgG\textsuperscript{2456}</td>
<td>Susceptibility to ITP\textsuperscript{19,66}, susceptibility to RA\textsuperscript{108}</td>
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<td><strong>FCGR3B</strong></td>
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<td>CNV</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>rs200688856</td>
<td>c.108G</td>
<td>18(36)</td>
<td>Arg</td>
<td>Expression levels</td>
<td>SLE (&lt;2 copies)\textsuperscript{109}, Sjögren syndrome (&lt;2 copies)\textsuperscript{110}, systemic sclerosis (&lt;2 copies)\textsuperscript{111}, possibly RA (&lt;2 copies)\textsuperscript{109,112}</td>
</tr>
<tr>
<td></td>
<td>c.108C</td>
<td></td>
<td>Ser</td>
<td>Uptake of immune complexes</td>
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<tr>
<td>rs527909462</td>
<td>c.114C</td>
<td>20(38)</td>
<td>Leu</td>
<td>NA1****</td>
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<tr>
<td></td>
<td>c.114T</td>
<td></td>
<td>Leu</td>
<td>NA2 and SH****</td>
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<tr>
<td>rs448740</td>
<td>c.194A</td>
<td>47(65)</td>
<td>Asn</td>
<td>NA1</td>
<td></td>
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<tr>
<td></td>
<td>c.194G</td>
<td></td>
<td>Ser</td>
<td>NA2 and SH</td>
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</tr>
<tr>
<td>rs5030738</td>
<td>c.233C</td>
<td>60(78)</td>
<td>Ala</td>
<td>NA1 and NA2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c.233A</td>
<td></td>
<td>Asp</td>
<td>SH</td>
<td></td>
</tr>
<tr>
<td>rs147574249</td>
<td>c.244G</td>
<td>64(82)</td>
<td>Asp</td>
<td>NA1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c.244A</td>
<td></td>
<td>Asn</td>
<td>NA2 and SH</td>
<td></td>
</tr>
<tr>
<td>rs2290834</td>
<td>c.316G</td>
<td>88(106)</td>
<td>Val</td>
<td>NA1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c.316A</td>
<td></td>
<td>Ile</td>
<td>NA2 and SH</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Overview of all the currently known functional SNPs at the FCGR2/3 locus. For each SNP, Rs numbers (Reference SNP cluster ID, the common identification method of SNPs as included in the dbSNP database), nucleotide and amino acid positions, functional changes, and associations with disease are shown. Rs numbers shown in bold are included in the MLPA assay used in this thesis.

* Nucleotide numbering excludes exon6 in FCGR2A and FCGR2C transcripts, because this exon is spliced out from these transcripts, but includes exon 6 in FCGR2B, in which it is retained in many transcripts (splice variant known as FCGR2B1).

In FCGR2A transcripts, inconsistencies exist as a result of alternative splicing at the beginning of exon3 because of two adjacent splice acceptor sites that can both be used. The most commonly used amino-acid numbering is derived from the shorter transcript in which the 3’ splice acceptor site is used, so we chose to use this transcript for nucleotide numbering throughout this thesis.

** Inconsistencies exist in the amino-acid numbering used in the literature, because some SNPs are named by the position when including the signal peptides, and others are named by their position in the mature protein, excluding the signal peptides. In this table, position in the mature protein is shown first, followed by the position in the full protein between brackets. For the rest of this thesis, we chose for each SNP to use the amino-acid position most often used, as indicated in bold in the table.

*** relative to the start of translation. Three haplotypes have been described: 2B.1 (-386C, -120T); 2B.2 (-386C, -120T) and 2B.4 (-386C, -120A). -386C, -120A has never been found to date.

**** The set of 6 SNPs in FCGR3B determines the NA1, NA2 and SH haplotypes. These are the three major haplotypes that exist, although rare additional variants have been reported128. The term 'NA' is derived from 'Neutrophil Antigen'. The term 'SH' derives from the fact that an alloantibody recognizing this antigen was first found in serum "h" among several different investigated sera (Jürgen Bux, personal communication). FCGR3B-NA1 and -NA2 nucleotide sequences differ at five positions (c.108G>C, c.114C>T, c.194A>G, c.244G>A and c.316G>A), with four predicted amino acid differences (p.Arg36Ser, p.Asn65Ser, p.Asp82Asn and p.Val106Ile for NA1 and NA2, respectively). As a consequence, the NA2 variant has two additional N-linked glycosylation sites compared to NA1 (the p.65Ser of NA2 completes a consensus sequence for N-linked glycosylation with the nonpolymorphic p.63Asn residue, and the p.82Asn of NA2 forms a consensus sequence with the nonpolymorphic p.84Ser)129. The SH variant is identical to NA2 at the five positions that distinguish NA1 from NA2, but differs from both variants at one additional position (c.233C>A), resulting in p.Ala78Asp amino acid change that predicts a change in the tertiary structure of the protein44. Additional complexity is added by the discovery of rare individuals carrying other mutations within this gene or different combinations of these nucleotide polymorphisms128,130, indicating that the NA1/NA2/SH typing is incomplete. Sometimes, the NA1/NA2/SH haplotypes are indicated respectively as FCGR3B*01, FCGR3B*02 and FCGR3B*03, to prevent confusion with the nomenclature for antigenic epitopes determined by these haplotypes. These haplotypes determine the allotypic variants of the Human Neutrophil Antigen1 (HNA1), which is involved in allo-immunization against neutrophilic granulocytes. The HNA classification system recognizes HNA1a (encoded by FCGR3B-NA1), HNA1b (encoded by FCGR3B-NA2 and FCGR3B-SH) and HNA1c (encoded by FCGR3B-SH)44,123,128,131. Recently, a fourth antigenic epitope was described (HNA1d, also encoded by FCGR3B-SH)131.

Note that the nucleotide positions as indicated here are indicating the position in the coding sequence, which differs from nucleotide positions often used in the literature for these haplotypes, as derived from Ravetch and Perussia132 who used a nucleotide numbering not related to the coding sequence of FCGR3B, which includes 33 additional nucleotides of the 5'UTR.
Platelet transfusion refractoriness has never been studied in the context of FCGR2/3 genetic variation. This clinical entity is discussed in greater detail below.

THE ROLE OF FcγRs IN THE CLEARANCE OF IgG-OPSONIZED BLOOD CELLS

Antibodies directed against blood cells are responsible for morbidity (and sometimes mortality) in various clinical situations, such as autoimmune diseases and transfusion medicine. These antibodies consist of autoantibodies, directed against various structures on the patients’ own blood cells, or alloantibodies, which are directed against polymorphic antigens (i.e. blood groups) that differ between different individuals. Since alloantibodies do not react with the own blood cells of an individual, they do not cause problems in the normal situation. However, they can cause great problems when a person comes into contact with blood cells of another individual, which is the case in blood transfusion (and in pregnancy and organ transplantation, but this is beyond the scope of this thesis). The blood cells most often targeted by antibodies are platelets, red blood cells and neutrophils, and an overview of the clinical situations in which antibodies against these cells play a role is given in Table 4. Some of these situations are discussed in detail below.

Immune thrombocytopenia

Patients with immune thrombocytopenia (ITP) have a low platelet number as a result of immune-mediated destruction of the circulating platelets (sometimes accompanied by a decrease in platelet production by immune-mediated effects on megakaryocytes, the cells that produce platelets in the bone marrow). In most cases, platelet destruction is caused by antibodies.

The insight that a factor in plasma could be singly responsible for the thrombocytopenia in ITP started in 1951, from the famous experiments of Harrington et al. who injected plasma from ITP patients into healthy recipients (including himself). This plasma immediately caused a deep thrombocytopenia in the recipients, indicating that a soluble factor in plasma was causing platelet destruction. This factor was later identified to be IgG. It is not known exactly why such IgG auto-antibodies against platelets are formed, and different immunologic events that can lead to auto-antibody formation have been described, reviewed elsewhere. These auto-antibodies opsonize platelets, which causes their destruction via FcγRs on phagocytes, predominantly in the spleen, although the destruction can also, in a minority of cases, take place in the liver. Phagocytosis of the IgG-opsonized platelets presumably takes place in the tissue-resident macrophages in these organs, but the exact mechanism by which this occurs, and which FcγRs are involved in this process, is not known. The phagocytosis of platelets may be influenced by several factors. For instance, it was recently shown that C-reactive protein (CRP) can enhance phagocytosis of IgG-opsonized platelets. Furthermore, SNPs in the low-affinity FcγRs are associated with ITP susceptibility (Table 3), underlining their important role in ITP.
<table>
<thead>
<tr>
<th></th>
<th>Autoimmune</th>
<th>Transfusion-related</th>
<th>Alloimmune</th>
<th>Pregnancy-related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>ITP (immune causes)</td>
<td>PLT refractoriness</td>
<td>FNAT (immune causes)</td>
<td>Placental alloantibodies from the</td>
</tr>
<tr>
<td>Mechanism</td>
<td>Autoantibodies cause destruction of platelets via FcγR-bearing phagocytes in the spleen</td>
<td>Alloantibodies cause destruction of transfused platelets</td>
<td>Platelet-reactive alloantibodies from the</td>
<td>mother cross the placenta</td>
</tr>
<tr>
<td>Clinical presentation</td>
<td>purpura, bleeding, sometimes intracranial hemorrhage</td>
<td>Failure to adequately raise platelet counts with platelet transfusion</td>
<td>Mild bleeding, intracranial hemorrhage in 10-20%</td>
<td></td>
</tr>
<tr>
<td>Antigens</td>
<td>GPIIb/IIa and GPIb/IX</td>
<td>HLA (majority) and HPA (rare)</td>
<td>HPA1a (85%)</td>
<td></td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>Mainly IgG</td>
<td>Mainly IgG</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Watchful waiting, IVlg, Corticosteroids, splenectomy</td>
<td>HLA/HPA Matched transfusion, Cross matching</td>
<td>Platelet transfusion, IVlg</td>
<td></td>
</tr>
<tr>
<td>Prevention</td>
<td>General measures (leukoreduction of blood products), no individual prevention</td>
<td>IVlg in subsequent pregnancies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cells</td>
<td>wAIHA / cAIHA</td>
<td>Blood group incompatibility</td>
<td>HDFN</td>
<td></td>
</tr>
<tr>
<td>Mechanism</td>
<td>Autoantibodies cause destruction of red blood cells via FcγR-bearing phagocytes in the spleen (wAIHA), or by fixing complement (cAIHA) leading to phagocytosis in the liver</td>
<td>Alloantibodies cause destruction of transfused blood cells by direct complement lysis or extravascular phagocytosis</td>
<td>Red blood cell-reactive alloantibodies from the mother cross the placenta</td>
<td></td>
</tr>
<tr>
<td>Clinical presentation</td>
<td>signs of anemia</td>
<td>Acute or delayed hemolytic transfusion reactions</td>
<td>Intrauterine anemia (hydrops foetalis)</td>
<td>Neonatal anemia</td>
</tr>
<tr>
<td>Antigens</td>
<td>Glycophorin antigens, Rh antigens</td>
<td>ABO, RhD and many other blood groups</td>
<td>RhD (most severe), other blood groups</td>
<td></td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>IgG (wAIHA), IgM (cAIHA)</td>
<td>IgM (ABO), IgG (other blood groups)</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Corticosteroids, Splenectomy, IVlg</td>
<td>Stop transfusion, Supportive care</td>
<td>Red cell transfusion (intrauterine if necessary)</td>
<td>Exchange transfusion in severe hyperbilirubinemia</td>
</tr>
<tr>
<td>Prevention</td>
<td>General measures (leukoreduction of blood products), no individual prevention</td>
<td>ABO and RhD compatible blood pre-transfusion cross-matching</td>
<td>screening for RhD negative women during pregnancy, RhD prophylaxis</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Autoimmune</td>
<td>Transfusion-related</td>
<td>Alloimmune</td>
<td>Pregnancy-related</td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
<td>---------------------</td>
<td>------------</td>
<td>-------------------</td>
</tr>
<tr>
<td><strong>Mechanism</strong></td>
<td>Autoantibodies cause destruction of neutrophils via FcγR-bearing phagocytes in the spleen</td>
<td>Alloantibodies in a transfused product cause activation of neutrophils in the lung of the recipient</td>
<td>Neutrophil-reactive alloantibodies from the mother cross the placenta</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical presentation</strong></td>
<td>Increased risk of bacterial and fungal infections</td>
<td>Respiratory insufficiency with lung infiltrates</td>
<td>Can be asymptomatic Associated with neonatal sepsis</td>
<td></td>
</tr>
<tr>
<td><strong>Antigens</strong></td>
<td>FcγRIIb*, CR3 (CD11b/CD18)</td>
<td>Allotypic variants of HLA or HNA*</td>
<td>HNA*, FcγRIIb* in FCGR3B deficient mothers</td>
<td></td>
</tr>
<tr>
<td><strong>Immunoglobulin</strong></td>
<td>Mainly IgG</td>
<td>IgG/IgM?</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>G-CSF, IVIg, corticosteroids</td>
<td>Supportive care</td>
<td>Antibiotics when signs of infection present Self-limited disease</td>
<td></td>
</tr>
<tr>
<td><strong>Prevention</strong></td>
<td>-</td>
<td>Test for anti HLA and HNA antibodies in donor of the product that induced the TRALI</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>


Interestingly, the antigen that is most often involved in antibodies directed against neutrophils is itself an FcγR, FcγRIIb. In these cases, FcγRIIb is specifically bound by the Fab fragment of the antibody, and the Fc fragment of the antibody binds to other FcγRs, presumably on other cells, leading to destruction of the neutrophil. Antibodies against FcγRIIb include autoantibodies and alloantibodies. The alloantibodies are reactive with the different variants of the HNA1 antigen, which are encoded by the FCGR3B-NA1, -NA2 and –SH haplotypes mentioned previously.
The majority of ITP is caused by IgG against glycoprotein IIb/IIIa (GPIIb/IIIa) and glycoprotein Ib/IX (GPIb/IX), which are specifically expressed on platelets, although other molecules on the platelet surface can also be targeted\(^6\). Most, but not all ITP is caused by antibodies, and rare cases of ITP that are not associated with antibodies have also been described, for instance by T cell mediated lysis\(^6\).

Treatment of ITP consists of watchful waiting when there are no symptoms, but when needed (for instance when a patient is bleeding or needs to undergo surgery), platelet numbers can be increased with medication, such as IVIg or corticosteroids. When an immediate rise in platelet numbers is warranted, IVIg is the first choice, and results in rapid responses in most of the patients\(^7\),\(^8\). However, it is not exactly known how IVIg alleviates ITP, and different theories exist to explain this phenomenon, discussed in more detail below. In children, the disease is often self-limited, but it can be chronic, which is most often the case in adults. In severe and chronic cases that do not respond well to therapy, a splenectomy can be performed as a last resort therapy\(^2\). 

**Platelet transfusion refractoriness**

In many patients receiving platelet transfusions, an inadequate increment of platelet number is obtained, a phenomenon known as platelet transfusion refractoriness (PLT refractoriness), with an estimated incidence of 7-34%\(^7\). Although most cases of PLT refractoriness have other, non-immune causes, up to 20% of cases are attributed to platelet-reactive allo-antibodies alone\(^7\),\(^8\). The presence of platelet-reactive allo-antibodies is known as allo-immunization, and these antibodies can be directed towards polymorphic human leukocyte antigens (HLA), or, less frequently, polymorphic human platelet antigens (HPA) and/or platelet glycoproteins. HLA allo-antibodies are mainly formed when foreign leukocytes enter the circulation of a patient, i.e. with transfusion of blood products (erythrocyte and/or platelet concentrates) that contain trace amounts of leukocytes, with transplantation, or during pregnancy. HPA allo-antibodies are predominantly formed in response to foreign platelets (PLT transfusion or pregnancy). Unlike the situation with red blood cell transfusions, in which donor and recipient are always matched for the major blood group antigens (ABO and RhD) and an *in vitro* cross-match between donor and recipient blood is performed to screen for other antibodies, platelet transfusions are only matched for ABO, and are not regularly cross-matched. Furthermore, one adult platelet transfusion consists of a pool of platelets from 5 donors, which would be expected to increase the propensity for mismatches and the consequent development of allo-immunity.

Clinically relevant platelet-reactive allo-antibodies are mainly of the IgG isotype, and reasoning to the analogy with ITP, presumably lead to rapid destruction of the transfused platelets by phagocytes in the spleen via FcγRs. Allo-immunization negatively influences the yield of PLT transfusion, and thus contributes to PLT refractoriness, although patients with broad allo-immunization still can have good platelet yields. It is not known whether the degree of platelet destruction in allo-immunized patients with PLT refractoriness is associated with genetic varia-
Autoimmune hemolytic anemia (AIHA) can be divided into two categories: cold AIHA (cAIHA) and warm AIHA (wAIHA). cAIHA is usually caused by IgM antibodies, and FcγRs are not involved in this condition. In wAIHA, the antibodies against red blood cells are IgG antibodies, and, similar to the situation in ITP, these lead to destruction of the red blood cells by phagocytes in the spleen, which is often enlarged in wAIHA. FcγR polymorphisms were found to predispose to a certain form of wAIHA, and were also associated with the clearance rate of transfused IgG-opsonized red blood cells in an experimental setting. Clearance of the red blood cells leads to anemia, which can be more or less severe, and usually requires treatment. Red blood cell transfusions are of limited help, since the antibodies in AIHA are directed towards universal antigens on red blood cells, and will also target the transfused blood cells. Initial treatment of wAIHA is by corticosteroids. In severe and prolonged cases, a splenectomy is usually performed. IVIg can also be used as a resort treatment for patients with wAIHA, although it is not usually as successful as it is in ITP.

**IVIG THERAPY**

Intravenous IgG (IVIg) is a blood product containing polyclonal IgG isolated and pooled from thousands of donors. As such, IVIg typically contains IgGs with many different specificities, covering the majority of pathogens encountered by the population. In theory it does not contain high levels of clinically relevant auto-antibodies, because only plasma from healthy individuals is used, and even if in rare cases these healthy individuals have circulating auto-antibodies, these would be diluted in such a way that they cannot do harm to the recipient. In general, IVIg has little side effects.

IVIg is mainly used in two clinical situations. It is administered at a low dose at regular intervals as antibody replacement therapy in patients with low plasma IgG concentrations. In this context, the therapy provides the recipient with a repertoire of protective antibodies against a range of predominantly bacterial infections. On the other hand, IVIg can also be used at a high dose as an immunomodulatory treatment in auto-immune or auto-inflammatory diseases, including KD and ITP. A list of indications for treatment with IVIg is provided in Table 5. Many of these diseases involve (auto)-antibody responses. Even though IVIg is widely used and has proven to be an effective treatment for many diseases, the exact immunomodulatory mechanism(s) have remained elusive. Several mechanisms by which IVIg may exert its anti-inflammatory effects have been proposed, which are described below. These mechanisms are not necessarily mutually exclusive, and may act in concert to modulate the immune system. Furthermore, different mechanisms may be at work in the different diseases for which IVIg is administered. An overview of these mechanisms is given in Figure 7 and Table 6.
Table 5. Overview of indications for treatment with intravenous immunoglobulins (IVIg)

<table>
<thead>
<tr>
<th>IVIg as substitution therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary immunodeficiency disease</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>Pediatric HIV infection</td>
</tr>
<tr>
<td>Common variable immunodeficiency</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IVIg as immunomodulatory therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory disorders</td>
</tr>
<tr>
<td>Kawasaki’s disease</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney transplantation involving a recipient with a high antibody titer or an ABO-incompatible donor</td>
</tr>
<tr>
<td>Allogeneic bone marrow transplantation</td>
</tr>
<tr>
<td>Graft-versus-host disease</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hematologic disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune thrombocytopenia</td>
</tr>
<tr>
<td>Autoimmune hemolytic anemia</td>
</tr>
<tr>
<td>Autoimmune neutropenia</td>
</tr>
<tr>
<td>HIV-associated thrombocytopenia</td>
</tr>
<tr>
<td>Foetal and Neonatal alloimmune thrombocytopenia</td>
</tr>
<tr>
<td>Severe anemia associated with parvovirus B19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dermatologic disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bullous pemphigoid</td>
</tr>
<tr>
<td>Epidermolysis bullosa acquisita</td>
</tr>
<tr>
<td>Mucous-membrane (cicatricial) pemphigoid</td>
</tr>
<tr>
<td>Pemphigus vulgaris</td>
</tr>
<tr>
<td>Toxic epidermal necrolysis or Stevens–Johnson syndrome</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neuromuscular disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birdshot retinopathy</td>
</tr>
<tr>
<td>Chronic inflammatory demyelinating polyneuropathy</td>
</tr>
<tr>
<td>Multifocal motor neuropathy</td>
</tr>
<tr>
<td>Guillain–Barré syndrome</td>
</tr>
<tr>
<td>Lambert–Eaton myasthenic syndrome</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
</tr>
<tr>
<td>Opsoclonus–myoclonus</td>
</tr>
<tr>
<td>Polyradiculoneuropathy</td>
</tr>
<tr>
<td>Refractory dermatomyositis</td>
</tr>
<tr>
<td>Refractory polymyositis</td>
</tr>
<tr>
<td>Relapsing–remitting multiple sclerosis</td>
</tr>
</tbody>
</table>

Overview of different indications for which IVIg treatment has been used with success, adapted from Gelfand et al., divided into indications in which the IVIg is given to substitute decreased autologous IgG levels and indications in which IVIg is given for an immunomodulatory effect.
Potential working mechanisms for the immunomodulatory effect of IVIg

Potential mechanisms can be divided into two categories, being dependent on either the Fc part or the Fab part of the IgG molecule. For some indications, such as ITP, clinical studies with human subjects have in fact already revealed what part of the IgG molecule is effective, as preparations with only Fab fragments of IVIg were not effective\(^79\), whereas purified Fc fragments did have a good clinical effect\(^80\). Thus, we know that at least for ITP, the immunomodulatory effect is Fc-mediated, although this may be different for other indications. Table 6 gives an overview of proposed potential working mechanisms of IVIg, which are discussed in detail below.

### Table 6. Potential working mechanism of intravenous immunoglobulins (IVIg)

<table>
<thead>
<tr>
<th>Fc-mediated mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. blockade of activating FcγR by saturation via high-dosed IVIg making them less available for autoantibodies in oligo- or polymeric complex with their (auto)antigen(^70)</td>
</tr>
<tr>
<td>2. upregulation of the inhibitory FcγRIIb by sialylated IgG Fc(^85)</td>
</tr>
<tr>
<td>3. increased clearance of pathogenic antibodies by saturation of the neonatal FcR (FcRn)(^89,90)</td>
</tr>
<tr>
<td>4. tipping the cellular balance from pro- to anti-inflammatory reactivity by modulating dendritic cells (DCs)(^113)</td>
</tr>
<tr>
<td>5. reducing responses to IFN(^114)</td>
</tr>
<tr>
<td>6. inhibition of the complement cascade by sequestering complement away from the deposited autoantibodies(^115)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fab-mediated mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>7. neutralization of various agents (similar to monoclonal antibodies), including chemokines, inflammatory cytokines and apoptosis-inducing molecules, including FasL(^92,96)</td>
</tr>
<tr>
<td>8. neutralization of autoantibodies by anti-idiotype Abs(^116) - often claimed but never proven to effectively explain the anti-inflammatory potential</td>
</tr>
</tbody>
</table>

Overview of potential working mechanisms of IVIg that have been proposed in the literature, divided into mechanisms that require an intact Fc part of the IgG molecules, and mechanisms that theoretically only require the Fab part of IgG molecules.

**Fc-mediated working mechanisms**

*Blockade of activating FcγR by saturation as a result of high IgG concentrations.*

Administration of IVIg greatly increases the total concentration of IgG in the recipients’ plasma and extracellular fluid, and with such an increase, more FcγRs may be bound by circulating non-complexed IgG, thereby saturating the FcγRs and making them less available for autoantibodies in oligo- or polymeric complex with their (auto)antigen. Greatly increasing the concentration of monomeric IgG above the normal plasma levels may shift the equilibrium of bound vs unbound FcγRs towards a situation in which too many FcγRs are occupied for proper functioning (Figure 7a) – which may in part explain the immunomodulatory actions of IVIg under some of the conditions for which IVIg is used. Saturation of activating FcγRs was one of the first theories that was formulated to explain the working mechanism of IVIg\(^70\), and this ‘classic’ mechanism
has for a long time been assumed as the most plausible explanation for the effect of IVIg in ITP\textsuperscript{81,82} (Figure 7a). Circumstantial evidence for this theory derives from observations that IgG preparations with increased affinity for FcγRs appear to have an increased effect\textsuperscript{83}, and that in all diseases in which an immunomodulatory effect is wanted, high doses of IVIg are needed. Nevertheless, there is no formal proof for this concept, and although it has never been disproven, focus has shifted away from this theory as other explaining theories arose.

**Upregulation of the inhibitory FcγRIIb as a result of sialylated IgG-Fc.**
Over the past decade, the prevailing theory for the working mechanism of IVIg in most immunomodulatory situations has become that IVIg induces an upregulation of the inhibitory FcγRIIb on effector cells (Figure 7b). This upregulation led to a decrease of phagocytosis of circulating platelets in a mouse model of ITP\textsuperscript{84}. More specifically, a fraction of IVIg, i.e. the IgGs containing a sialic acid sugar residue at the end of the N-linked glycosylation site at Asn297, would be responsible for this effect by binding to SIGNR1 (mouse), or its human orthologue DC-SIGN, inducing various signaling cascades ultimately leading to the upregulation FcγRIIb. This theory has recently been reviewed extensively. Essentially all the evidence supporting this theory derives from murine studies\textsuperscript{85}, many of which use a model for arthritis. However, IVIg has never proven to be a useful therapy in treating arthritic patients\textsuperscript{86-88}. Furthermore, the interaction of DC-SIGN with human IgG-Fc could not even be demonstrated by another group\textsuperscript{7}. It is not known whether sialylated IVIg also induces upregulation of FcγRIIb in humans and if this mechanism may be an explanation for the effects of IVIg. This is investigated further in this thesis.

**Increased clearance of pathogenic antibodies by saturation of the neonatal FcR (FcRn)**
As mentioned before, FcRn is a receptor expressed by human endothelial cells to recycle plasma IgG, extending its half-life in the circulation\textsuperscript{89,90}; saturating this ‘rescue-receptor’ with a high dose of IVIg may shorten the half-life of all IgG including harmful auto-antibodies. This would lead to a lower serum concentration of the auto-antibodies, and less detrimental effects to the host (Figure 7c).

**Other potential Fc-mediated mechanisms**
Other proposed mechanisms, which have been less intensively studied, include shifting the balance from pro- to anti-inflammatory reactivity by modulating dendritic cells (DCs), reducing DC responses to IFN, and inhibition of the complement cascade, discussed in more detail elsewhere\textsuperscript{91}.

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**Figure 7. Different potential working mechanisms of IVIg (legend)**
Overview of the most prominent potential working mechanisms of IVIg, as shown for ITP. **A** saturation of activating FcγRs on effector cells. **B** upregulation of the inhibitory FcγRIIb on effector cells. This upregulation would be the result of IgG sialylated at the Asn297 carbohydrate chain (Figure 1c), which, after binding to DC-SIGN, would lead to upregulation of FcγRIIb (not illustrated). **C** Saturation of FcRn leads to increased clearance of the auto-antibody.
**General introduction**

**Without IVIg**

**A** Saturation of FcγRs
IgG opsonized platelet binds to FcγRs, initiating crosslinking and phagocytosis

**B** Upregulation of FcγRIIb
FcγRIIb cannot overcome signals by activating FcγRs

**C** Saturation of FcRn
IgGs enter endothelial cells by nonspecific pinocytosis

**With IVIg**

FcγRs saturated by high concentration IgG in IVIg

IVIg causes upregulation of inhibitory FcγRIIb, crosslinking does not lead to phagocytosis due to negative signal by FcγRIIb, platelet eventually escapes

**Legend**

- FcγRI (high-affinity)
- FcγRIIa (low-affinity)
- FcγRIIla (low-affinity)
- FcγRIIb (inhibitory)
- Pathogenic platelet-specific auto-IgG
- Normal (non-pathogenic) IgG
- Neonatal Fc Receptor (FcRn)

**Figure 7. Different potential working mechanisms of IVIg**
**Fab-mediated working mechanisms**

*Neutralization of autoantibodies by anti-idiotype Abs.*

One of the first explanations for the anti-inflammatory effect of IVIg was that there are anti-idiotypic antibodies present in the IVIg that neutralize the pathogenic auto-antibodies. This theory is often claimed but to our knowledge has not been proven to effectively explain the anti-inflammatory potential of IVIg for any of the indications in Table 5.

*Neutralization of endogenous inflammatory cytokines*

Apart from the known microbial antigen-specific binding properties, IgG preparations may also contain neutralizing and clearance-enhancing antibodies that neutralize proinflammatory cytokines such as interferon, granulocyte-monocyte-colony stimulating factor and other cytokines, which may switch a proinflammatory trigger into an anti-inflammatory condition. This suggests that healthy individuals from which plasma is collected and pooled for therapeutic IgG preparations contain autoreactive ‘natural’ antibodies at low levels in their blood. The infusion of such natural antibodies into the patient may be sufficient to reset certain diseases by the cross-reactive capacity of such natural ‘auto’antibodies.

**OUTLINE OF THE THESIS**

In chapter 2, we investigate the role of FCGR2/3 genetic variation in association with the autoinflammatory disorder SLE, and we further elucidate the functional consequences of the promoter haplotype 2B.4 in FCGR2B on the expression of FcyRIIb. In chapter 3, we first provide a comprehensive overview of the FCGR2/3 locus, describing linkage disequilibrium between the functional FCGR2/3 SNPs that are studied for associations with disease, and also describe ethnic variation in FCGR2/3 SNPs and CNV. Furthermore, we test all these FCGR2/3 genetic variations for associations with susceptibility to Kawasaki Disease. In chapter 4, we investigate whether the FCGR2/3 genetic variation, which has previously been shown to be associated with susceptibility to ITP, is also associated with its allo-immune counterpart; immune-mediated platelet refractoriness. In chapter 5, we go deeper into the origin of the copy number variation at the FCGR2/3 locus, describing how this copy number variation is formed, and that it leads to previously uncharacterized chimeric genes at the locus. These chimeric genes are highly different in expression and function. In chapter 6, we investigate the role of SNPs in FcyRIIa and FcyRIIIb, the major FcyRs on neutrophils, and how SNPs together influence the responses of neutrophils against IgG. In chapter 7, we investigate the role of IVIg in an *in vitro* model that investigates the phagocytosis of IgG-opsonized blood cells, and different fractions of IVIg (i.e. sialylated IgG vs non-sialylated IgG) are compared in order to obtain more knowledge on the working mechanism of IVIg in the setting of IgG-opsonized blood cell clearance. In chapter 8, we describe the development of novel methods to isolate the cell type that is responsible for the
clearance of IgG-opsonized blood cells in vivo, i.e. splenic macrophages, and characterize these cells for expression of multiple immunologic molecules on these cells, including FcγRs. Finally, in chapter 9, the results are discussed and recommendations for future research are given.
REFERENCE LIST


General introduction

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