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Fcγ-receptors in systemic autoimmune conditions - lessons from murine mercury-induced autoimmunity.

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If the string breaks, try another string!

A. A. Milne

ABSTRACT

In this thesis we investigated the role of activating (FcγRI, FcγRIII) and inhibitory (FcγRIIB) Fcγ-receptors on systemic autoimmunity using two mouse strains, DBA/1 (H-2^q) and BALB/c mice (H-2^d), susceptible to induction of autoimmunity by mercury (Hg).

Fc-receptors for IgG (FcγR) link cellular and humoral immune responses, control the balance between activating and inhibitory immune responses and are important in the development of several autoimmune diseases. Mercury induces a T cell-dependent autoimmune condition, Hg-induced autoimmunity (HgIA) in genetically (H-$2^{s,q,f,t2}$) susceptible mice characterized in its fullblown type by lymphoproliferation, hypergammaglobulinemia, systemic immune-complex (IC) deposits and antinucleolar antibodies (ANoA). All manifestations in HgIA are dependent on the presence of IFN-γ.

Hg-treated BALB/c mice lacking activating FcγRs (FcγRI, FcγIII and FcεRI) showed significantly higher levels of both IgG1- and IgG2a-CIC whereas renal mesangial and vessel wall IC deposits were severely delayed and reduced/abolished, compared to mice without mutations (wild type, wt). Wt mice developed modest levels of IgG1- and IgG2a-CIC followed by a distinct formation of IC deposits in the renal glomerular mesangium, as well in renal and splenic vessel walls. Compared to wt mice, the mice lacking the inhibitory FcγRIIB showed similar titres of IC deposits in the renal mesangium, whereas vessel wall IC deposits were reduced.

DBA/1 mice deficient for the FcRγ-chain (lack of the activating receptors FcγRI, FcγIII and FcεRI) or FcγRIII and treated with Hg showed a delayed and attenuated IgG1, IgG2a and IgG2b ANoA response compared to wt mice.

Increasing the Hg dose or prolonging the treatment time could not override the attenuated ANoA response seen in FcγRIII mice. Female Hg-treated FcγRIIB mice showed a significant increase of IgG2b ANoA development compared to wt mice.

The total serum IgG1 response due to treatment with Hg was attenuated in both BALB/c mice lacking the Fcγ-chain, and in DBA/1 mice lacking either the Fcγ-chain or specifically the FcγRIII compared to wt mice. This indicates that FcγRIII is the receptor important for the in HgIA characteristic serum IgG1 response. On the other hand, Hg-treated FcγRIIB deficient BALB/c and DBA/1 mice showed an increase of both serum IgG1 and IgE compared to wt mice.

The cytokine profile in DBA/1 wt mice treated with Hg revealed a more marked Th1 profile compared to FcγRIII deficient mice. In contrast, the total Th2 and Th17 profile increased in both wt and FcγRIII deficient mice. However, during Hg treatment IL-21 mRNA expression was significantly reduced in FcγRIII deficient mice compared with wt mice. The increased Th1 profile in the wt mice could not be attributed to an increase of IFN-γ secretion from the major IFN-γ cell source, NK cells.

We conclude that FcγRIII are important for the formation of IC deposits as shown by the delayed and reduced formation of IC deposits and the high levels of CIC in mice lacking FcγRIII. The expression of FcγRIII is also of importance for the rapidity and final strength of the ANoA response probably due to a reduced expression of Th1 cytokines and inflammatory factors. The ANoA response is modestly counter-regulated by FcγRIIB. The increase of serum IgG1 in HgIA is dependent on FcγRIII which is likely to be mediated by the low expression of IL-21 in mice deficient for FcγRIII. In contrast, lack of FcγRIIB increases both the serum IgG1 and IgE response.

TABLE OF CONTENTS

POPULÄRVETENSKAPLIG SAMMANFATTNING 3

ORIGINAL PUBLICATIONS 5

ABBREVIATIONS 7

INTRODUCTION 9

Autoimmunity in general 9
Autoimmunity and gender 10
Autoimmunity, autoantibodies and immune-complexes 10
Autoimmunity and treatment 14
Autoimmunity and experimental models 14

Mercury 15
Toxicology of mercury 15
Mercury and autoimmunity 16

Fcγ-receptors 17
Fcγ-receptors and IgG immunoglobulins 17
Fcγ-receptor expression, regulation and actions 20
Fcγ-receptors and autoimmunity 23

Th1, Th2 and Th17 26

Mercury-induced autoimmunity - HgIA 28
ANoA/AFA and immune-complex in HgIA 28
Genetic susceptibility, gender and dose in HgIA 31
Cell types, cytokines and biological molecules in HgIA 32

AIM 35

MATERIALS AND METHODS 37

Mice and housing 37

Treatment 38

Blood and tissue sampling 38

Ethical consideration 38

Assessment of tissue immune-complex deposits 38

Light microscopy 39

Serum anti-nuclear antibodies assessed by indirect immunofluorescence 39

Serum anti-nuclear antibodies assessed by immunoblotting 40

Enzyme-linked immunosorbent assay (ELISA) 40

Assessment of circulating immune-complex 43

NK cell sorting 44

Isolation of mRNA, cDNA reverse transcription and Real-Time PCR 45

Statistical methods 46

RESULTS 47

Tissue immune-complex formation in wt mice, FcγR-, FcγRIII- and FcγRIIB-deficient mice (Paper I, II and III) 47

Development of circulating immune-complex in wt mice and FcγR deficient mice (Paper III) 53

ANoA and anti-chromatin antibodies in wt mice and FcγR-, FcγRIII- and FcγRIIB-deficient mice (Paper I, II, IV) 55

Immunoglobulin levels and polyclonal B cell markers in wt mice and FcγR-, FcγRIII- and FcγRIIB-deficient mice (Paper I, II and IV) 58

Cytokine profiles in in wt mice and FcγRIII-deficient mice (Paper IV) 62

Animal health (Paper I, II, III and IV) 63

DISCUSSION 65

Relation between FcγRs and manifestations of HgIA 65

Activating FcγRs and mRNA cytokine expression in HgIA 72

NK cells 73

CONCLUDING REMARKS 75

ACKNOWLEDGEMENTS 77

REFERENCES 79

POPULÄRVETENSKAPLIG SAMMANFATTNING

Systemiska autoimmuna sjukdomar utgörs hos människa av reumatiska sjukdomar som tex systemisk lupus erythematosus (SLE) och ledgångsreumatism (reumatoid artrit, RA). Vid autoimmuna sjukdomar uppfattar immunsystemet felaktigt kroppens egna celler, organ och vävnader som främmande, vilket kan leda till allvarlig inflammation med förlust av organfunktion. De bakomliggande mekanismerna är dock fortfarande till stor del okända.

Under de senaste åren har betydelsen av vissa yt-receptorer (Fcγ-receptorer) på immunceller som känner igen och binder den konstanta delen av immunglobuliner fått ökad uppmärksamhet. Fcγ-receptorer har visat sig vara inblandade i utveckling av autoimmune sjukdomar men även fungera som prognostisk faktor såväl hos människa som i experimentella djurmodeller. Två olika typer av Fcγ-receptorer existerar och balansen mellan aktiverande eller hämmande receptorer på cellytan bestämmer om en immunaktivering ska ske.

Hos människa kan betydelsen av polymorfi i genloci som styr uttrycket av Fcγ-receptorer studeras medan man i möss kan studera den patogenetiska betydelsen av olika typer av Fcγ-receptorer ännu tydligare genom att genloci för de olika receptortyperna förändras/muteras och därför inte uttrycks som proteiner på cellytan.

I detta doktorandprojekt använder vi oss av en autoimmun modell där möss som genom specifika förändringar i arvsmassan saknar Fcγ-receptorer, och utröner på så sätt hur aktiverande och inhiberande Fcγ-receptorer påverkar utvecklingen av systemisk autoimmunitet inducerad av kvicksilver.

De erhållna resultaten visar att aktiverande Fcγ-receptorer är viktiga för deponering av immunkomplex i njurens mesangium och mjältkärl, eliminering av immunkomplex i cirkulationen samt utvecklingen av autoantikroppar mot proteiner i cellkärnan. Den inflammationshämmande FcγRIIB visar på en tydlig roll vid regleraring av immunoglobulinnivåer av IgG1 och IgE i serum men även en mindre roll vid reglering av autoantikroppars utveckling mot kärnproteiner.

För att kunna utveckla läkemedel är det viktigt att förstå hur en sjukdom uppkommer. Detta avhandlingsarbete har visat att både aktiverande och inhiberande Fcγ-receptorerna är viktiga för utveckling av systemisk autoimmunitet inducerad av en miljöfaktor. Detta kan bli värdefull kunskap för att på sikt kunna utveckla mer skonsamma och specifika terapeutiska åtgärder.

ORIGINAL PUBLICATIONS

I

Martinsson K, Hultman P. The role of Fc-receptors in murine mercury-induced systemic autoimmunity. Clinical and Experimental Immunology. 2006 May:144(2):309-18

II

Martinsson K, Carlsson L, Kleinau S, Hultman P. The effect of activating and inhibiting Fc-receptors on murine mercury-induced autoimmunity. Journal of Autoimmunity. 2008 Aug: 31(1):22-9.

III

Martinsson K, Hultman P. Lack of Fcγ-receptors causes increased circulating immune-complexes but delays development of tissue immune-complex deposits. Manuscript.

IV

Martinsson K, Cederbrant K, Hultman P. Cytokines in induction of ANoA and hypergammaglobulinemia in mercury-induced autoimmunity – a lesson from FcγRIII deficient mice. Manuscript

ABBREVIATIONS

Ab, Abs	Antibody, Antibodies
ACA	Anti-Chromatin Autoantibody
ADCC	Antibody Dependent Cytotoxcity
AFA	Anti-Fibrillarin Autoantibody
AIHA	Autoimmune Haemolytic Anemia
ALP	Alkaline Phosphatase
ANoA	Antinucleolar Autoantibody
BCR	B Cell Receptor
BTK	Bruton´s tyrosine kinase
cDNA	complementary DNA
CD40L	CD40Ligand
CIA	Collagen Induced Arthritis
CIC	Circulating Immune-complex
CNS	Central Nervous System
CTLA-4	Cytotoxic T-lymphocyte Associated antigen-4
CV	Coefficient of Variation
Daf1	Decay accelerating factor 1
DC	Dendritic Cell
DNP	Dinitrophenly
dsDNA	double-stranded DNA
ΔC_T	Delta Cycle Threshold
ELISA	Enzyme-Linked Immunosorbent Assay
FAM	6-Carboxyfluorescein
FBS	Fetal Bovine Serum
FcγRs	Fc-gamma receptors
FITC	Fluorescein Isothiocyanate
GN	Glomerulonephritis
Hep-2 cell	Human epithelial-2 cell
Hg	Mercury
HgIA	Mercury-Induced Autoimmunity
Hmr1	Heavy Metal resistance 1 (gene locus)
HRP	Horseradish Peroxidase

IC	Immune-complexes
ICE	Interleukin Converting Enzyme
ICOS	Inducible T cell Co-Stimulator
Ig	Immunoglobulin
Igh6	Immunoglobulin heavy chain 6 (gene locus)
IFN-γ	Interferon-gamma
IRF-1	Interferon Response factor-1
ITAM	Immunoreceptor Tyrosine based Activating Motif
ITIM	Immunoreceptor Tyrosine based Inhibitory Motif
IVIg	Intravenous Immunoglobulin
JUN	JUN N-terminal Kinase
kDa	kilo Dalton
LPS	Lipopolysaccaride
IL	Interleukin
NK cell	Natural Killer Cell
NZB/W	New Zeeland Black/White
PBS	Phosphate Buffered Saline
PEG	Polyethylen Glycol
PKC	Protein Kinase C
PLCγ	Phospholipase C gamma
RA	Rheumatiod arthritis
RAS RHO	Small G protein
mRNA	messenger Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
RT-PCR	RealTime Polymerase Chain Reaction
sno RNA/RNPs	small nucleolar Ribonucleic Acid/ Ribonucleoproteins
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
SD	Standard Deviation
SECs	Sinusoidal Endothelial Cells
SLE	Systemic Lupus Erythematosus
STAT-4	Signal-transducing Activators of Transcription
TGFβ	Transforming growth factor-beta
Th	T helper
Wt	Wild type

INTRODUCTION

Autoimmunity in general

One of the major functions of the immune system is to discriminate between "self" and "non-self". The ability of the immune system to avoid reactivity to self-antigens is defined as "tolerance" and is achieved by central and peripheral mechanisms. The central tolerance occurs in the primary lymphoid organs (thymus and bone-marrow) and involves positive and negative selection. Positive selection yields functional T cells that have the potential to recognize both self and foreign antigens. Therefore, negative selection exists to manage potentially self-reactive cells. Cells recognising self-antigens may still escape and enter the periphery. These cells are kept under control with help from the mechanisms of peripheral tolerance, which include the suppression of autoreactive cells by regulatory T cells and the generation of hyporesponsiveness (anergy) in lymphocytes, which encounter antigen in the absence of the co-stimulatory signals that accompany inflammation [1].

However, this recognition system is not perfect and defects in genes controlling normal immune responses (like the major histocompatibility complex (MHC)), T cell receptors, and genes affecting antigen processing and presentation and lymphocyte proliferation are all linked to development of autoimmunity. The heritability for most of the autoimmune diseases in monozygotic twins is less than 50%, indicating that the induction of autoimmunity must be influenced by environmental factors as well [2]. This has been shown to be the case with environmental factors like infections, drugs, toxic oils, pristane and heavy metals in several cases [3-6]. "Antigen mimicry", "epitopic spreading" and altered proteins are mechanisms triggering autoimmune diseases [7]. Microbial molecules and proteins share regions of homology with mammalian proteins

and can induce a cross-reactive immune response, “antigen mimicry”. Initially, this may not induce an autoimmune response but could lead to subsequent exposure of other regions on the same self-antigen stimulating further antibodies, some potentially pathogenic, a process called “epitope spreading”. Mutations in proteins and altered expression of proteins can trigger development of autoimmune diseases by making the immune system recognize original self-antigens as non-self antigens [7].

Autoimmunity and gender

Autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) show an increased incidence in females. An effect that may be attributed to hormones and the fact that female have a stronger cell- and humorally mediated immune response compared to males [8, 9]. The concept that sex hormones alone could explain the dominance of autoimmunity found in females is however an oversimplification [10]. The role of X-chromosome linked autoimmune-related loci [11] and X-chromosome dosage, including differences in X-chromosome silencing [10], are regarded as more important. Still, sex hormones are important in immune responses as regulators of autosomal gene expression which influences the function of T cells [12, 13] and antigen presenting cells (APC) [14]. In males, autosomal genes entail a suppressed autoimmune phenotype, which may be attributed to a lower incidence of autoimmune development [15].

Autoimmunity, autoantibodies and immune-complexes

Autoimmune diseases are characterized by the generation of autoantibodies which can be used to classify the specific disease [16]. Autoimmune diseases can be divided into two different groups according to the distribution of autoantigens and the mechanism of tissue destruction. In **systemic autoimmune diseases**, like RA and SLE, the target tissues and molecules are

widely distributed in the body and result in inflammation and loss of functions in several organs [17]. In **organ-specific autoimmune diseases**, like type I diabetes and multiple sclerosis (MS), the immune attack and autoantigens are localized to a specific organ [17]. The presence of autoantibodies is extensive in autoimmune diseases and levels of autoantibodies are often associated with the disease severity.

The normal immune system is able to produce self-binding antibodies (abs) eg. natural autoantibodies which differ from pathogenic autoantibodies with regard to the B cells producing them and their immunoglobulin (Ig) class [16]. The relationship between natural and pathogenic autoantibodies is not proven. However, several data show that B cells producing pathogenic autoantibodies do exist in healthy individuals, and a plausible explanation is that exposure of environmental antigens may lead to a change in the physiological characteristics and make B cells secreting natural autoantibodies subsequently differentiate into B cells instead secreting pathological autoantibodies [16, 18]. A defect in clearance of apoptotic cells may serve as a microenvironment where autoreactive B cells can mature, proliferate and differentiate into autoantibody-producing plasma cells, leading to increases in autoantibodies and the formation of immune-complexes (IC) [19, 20]. Epitope spreading [21], somatic hypermutation [22] and citrullination [23] are other possible mechanism for autoantibody induction.

The pathological manifestations of autoantibodies to extracellular or membrane-bound structures include actions enhancing complement fixation [24], leading to cascades ending in lysis. In addition, autoantibodies can bind cell surface receptors leading to either inhibition or activation of cell activity [25]. The IgG class of the autoantibodies determine the pathogenic activity depending on the capacity to active Fcγ-receptors (FcγRs) and/or complement. In mice IgG2a and IgG2b show the highest pathogenic activity by effectively

binding both FcγRs and complement receptors whereas IgG1 only bind FcγRs and IgG3 only complement receptors [26]. Autoantibodies to intracellular antigens are useful as markers of disease [27, 28] but can also be associated with pathogenesis, as dsDNA in development of lupus nephritis [25] and the fact that antibodies from patients suffering from RA can induce arthritis in mice deficient in FcγRIIB [29]. Intracellular autoantibodies may also serve as markers of progression, as several anti-nuclear autoantibodies in systemic sclerosis do [27].

A central immunological disturbance in systemic autoimmune diseases is the production of autoantibodies. One of the pathological manifestations of autoantibodies is to form IC with antigens, subsequently leading to inflammation and tissue damage [30], as shown in connective tissue autoimmune diseases like RA and SLE [31]. In SLE renal involvement is frequent [30]. Formation of IC in order to eliminate antigens is also a normal function of the immune system [32]. IC are cleared in the liver via Kupffer cells [33] and sinusoidal endothelial cells (SECs) [34]. If these mechanisms fail or become overloaded deposition of IC may instead occur, which can cause inflammation and lead to reduced function of organs [35, 36].

The damage following tissue IC deposits depends on the mechanism and site of formation. The valence and the number of available combining sites influence the size of IC, which greatly affects the fate of the individual IC. The composition of the IC, eg affinity of Igs, and amount are other characteristics relevant for IC pathogenicity [35]. Formation of tissue IC deposits in SLE may be due to abnormal handling by FcγRs since clearance of IC via FcRs in the liver is less effective in patients suffering from SLE than in healthy individuals [37].

Circulating immune-complexes

Tissue IC deposits may be derived from circulating immune-complexes (CIC), as shown in murine models [38, 39], and in humans with diffuse proliferative lupus nephritis [40], or membranous glomerulonephritis (GN) [41]. CIC consist of antigens and antibodies and may also contain complement components [42].

The fate and biological effects of CIC are influenced by their size. Small IC can persist in the circulation and are inefficient at activating complement and attaching to FcRs. Large IC are effectively engulfed by macrophages since they show high affinity to FcRs and complement, whereas medium sized CIC are less well cleared and tend to form deposits. These medium sized CIC can initiate inflammation due to the persisting ability to bind FcRs and complement [36]. Since the capillary walls in the kidney are negatively charged, trapping of positively charged CIC in these tissues is favoured. Trapping of CIC occurs otherwise primarily in the mesangium and/or in the subendothelial space [36].

The amount of CIC correlates with disease severity in the prototype systemic autoimmune disease SLE, where patients with overt nephritis show higher levels of CIC than patients with silent nephritis [43, 44].

Immune-complex deposits

Tissue IC deposits may however also form *in situ*, either by the interaction of abs with antigens planted in the glomerulus, for example cationic histones, or by binding of abs to intrinsic glomerular antigens [45]. The size of the planted antigen affects the subsequent location of immune deposits. IC less than 550 kDa can pass the lamina densa of the glomerular basement membrane and be filtered with the urine [42].

The site where IC is formed *in situ* influences the effect of the deposits. IC formed in mesangial or subendothelial sites lead to inflammation and nephritis

due to the ability to recruit inflammatory cells [36]. Mesangial cells exhibit macrophage-like features and can bind IC via FcRs and release a number of inflammatory mediators [46]. Cells inducing inflammation resident in the circulation do not have access to subepithelial deposits, resulting in resident glomerular injury mediated by non-inflammatory complement mechanisms [36].

Autoimmunity and treatment

Since an autoimmune disease is self-sustained as long as autoantigens and autoreactive lymphocytes persist, therapy needs to eliminate the source of autoantigens and terminate the autoreactive lymphocyte population to be fully effective. Treatment of autoimmune diseases consists mainly of suppressive substances affecting the entire immune system. However, the use of more disease-specific treatments could overcome the negative effects seen with substances affecting the complete immune system. Blocking of cytokines increased in SLE may be a promising therapy as well as inactivating autoreactive B cells by the binding to- and cross-linking of autoantibodies to tolerogens [47]. Administration of intravenous immunogloblins (IVIg) can also be used to inactivate auto-reactive T cells, down-regulate B cell proliferation, by up-regulating FcγRIIB [48], and autoantibody production and restore the balance between Th1 and Th2 cytokines [49]. B cell depletion has also shown to be an effective treatment of RA [50] and SLE [51].

Autoimmunity and experimental models

To experimentally address the mechanism of autoimmune diseases, the use of animal models have been and continue to be very useful tools [52]. To study disease progression murine strains, that spontaneously develop autoimmunity are most useful, whereas direct immunization models are most relevant for organ-specific autoimmunity. Many genes influencing autoimmunity have been

revealed using specific gene mutations although the relevance in human autoimmune diseases can be discussed. The use of xenobiotic agents such as drugs, toxic oils, pristane and heavy metals is a reliable and controlled way to study induction of autoimmune diseases [3-6]. Exposure to heavy metals may exert toxic effects on the immune system, which can cause dysfunctional immune responsiveness but also elicit specific effects on antigen receptors expressed on T cells and B cells [53]. In this thesis we have used mercury (Hg) as inducer of autoimmunity.

Mercury

Hg shows a highly reactive property and binds efficiently to sulfhydryl-groups but also to a lesser degree to hydroxyl-, carboxyl-, and phosphoryl-groups [54]. This gives Hg the capacity to bind to a wide range of proteins and biological molecules and affect their functions. Three forms of inorganic mercury are found in the environment; metallic mercury (Hg^0), mercurous (Hg^{++}) and the very shortlived form mercuric (Hg^{++}). The different forms of organic mercury are derivatives of the latter [55].

Toxicology of mercury

Mercury occurs naturally in the crust and erosion of mineral deposits, volcanoes and human activities like metal smelting, coal production and chemical syntheses may lead to contamination of the environment [54]. Hg has been included in numerous products like germicidal soap, teething powders, fungicides, preservations in vaccines, electric devices and skin bleaching creams, many products used still today [56] [54, 57, 58]. Fish and seafood dominates the exposure sources of organic Hg in humans [58].

Metallic Hg vapor is easily absorbed and diffuses efficiently through the alveolar membrane but oxidises quickly to mercuric Hg. Upon acute exposure

of Hg vapor, lungs are the critical organ whereas the critical organ during long-time exposure is the central nervous system (CNS) [59]. About 10-20% of mercuric Hg is absorbed and the dominating pool of Hg is in the kidney followed by the liver, mucous membranes of the intestinal tract and spleen. Hg does not readily cross the blood-brain barrier. Upon accidental exposure the critical organs are the kidney and the intestinal tract and during chronic poisoning renal damage has been described [56]. Organic Hg shows greater distribution in the brain than metallic or mercuric Hg and the CNS is the critical organ during exposure [60]. The predominant excretion of Hg is via the urinary and fecal pathways [55].

Mercury and autoimmunity

Hg, gold and silver can modulate the immune system, not only suppress but also produce immunostimulatory signals which may induce autoimmunity in both humans and mice [61-63]. There are several case reports where Hg have been connected with autoimmunity [54, 64] and autoantibody formation in humans [53, 65]. Chronic exposure may lead to nephrotic syndromes due to membranous GN. [66].

Since the mechanisms of induction of autoimmune diseases are not fully known, Hg and other heavy metals constitutes a valid experimental model. One of the most intriguing findings in mice is the genetical susceptibility for induction of autoimmunity by Hg [67] and mice predisposed to develop autoimmune diseases can accelerate onset and increase severity following Hg treatment [68, 69]. The role of Hg as an inducer of autoimmunity is also found in the murine model of RA, collagen induced arthritis (CIA), where mice induced with both Hg and collagen show increased clinical scores compared to mice treated with collagen only [70]. Presuming that genetical susceptibility

exists in humans as well, information from studies in mice on heavy metal-induced autoimmunity can be very informative [71].

Fcγ-receptors

A well-balanced immune response is generated by an effective integration between activating and inhibiting signals. The family of FcγRs consists of receptors activating as well as inhibiting immune responses and are important in linking the cellular and the humoral immune response [72]. There are three activating FcγR gene families; in humans called FcγRI (A, B, C) FcγRII (A, C) and FcγRIII (A, B) and in mice called FcγRI, FcγRIII [48] and FcγRIV [73]. Only one FcγR, FcγRIIB, inhibit immune responses in both humans and mice [74]. The activating FcγRs contain an extracellularligand-binding α-chain and an intracellular signal-transducing γ-chain, which carry the immunoreceptor tyrosine based activating motif (ITAM) except for human FcγRIIA and FcγRIIC, which carries the ITAM in the intracellular α-chain. The human FcγRIIIB do not carry an ITAM but instead signals via a glycosylphosphatidylinositol-linked receptor [48]. The inhibitory FcγRIIB consist of only an α-chain and signals via an immunoreceptor tyrosine based inhibitory motif (ITIM) [48]. Two isoforms of FcγRIIB exist; FcγRIIBb1 and FcγRIIBb2 [75].

Fcγ-receptors and IgG immunoglobulins

FcγRs bind the constant part of the Ig IgG and the receptors have a varied affinity for the different subtypes, IgG1-IgG4 in humans and IgG1, IgG2a, IgG2b and IgG3 in mice (Figure 1), which can also be influenced by amino acid substitutions [76]. FcγRI is a high affinity receptor with specificity for binding of human IgG1 and IgG3, whereas in mice FcγRI preferably bind IgG2a. The FcγRII and FcγRIII are low affinity receptors and must bind IgG in the form of

immune-complex to elicit a signal. FcγRII and FcγRIII have specificity for binding of human IgG1 and IgG3 whereas in mice FcγRIII preferably bind IgG1 followed by IgG2a, IgG2b and IgG3 [77]. The medium affinity FcγRIV, only present in mice, selectively binds IgG2a and IgG2b [78]. The inhibitory FcγRIIB is also a low affinity receptor with specificity for IgG3, IgG1 and IgG4 in humans and in mice IgG1, IgG2a and IgG2b [77]. Binding efficiency of Igs to FcγRs is also determined by glycosylation where the presence of glycoproteins like fucose increase binding affinity [78] and the presence of terminal sialic acid decreases affinity to FcγRs [79].

The summarized response following engagement of activating and inhibitory FcγRs is regulated by the binding affinities of the IgG. IgG1 shows a low affinity ratio between activating and inhibitory FcγRs (A/I-ratio) and lack of FcγRIIB show strongest impact on the IgG1 isotypes. Under circumstances where activating FcγRs are up-regulated autoreactive IgG1 abs can induce severe damage. Under conditions when FcγRIIB is up-regulated, IgG isotypes with higher A/I-ratios like IgG2a can induce more damage than Igs with low A/I-ratio [73].

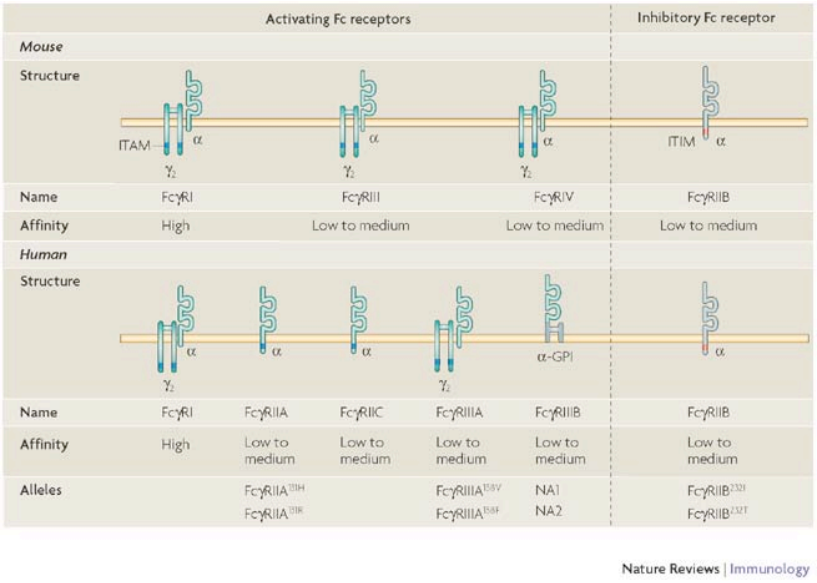


Figure 1

The family of human and mouse FcγRs for IgG, showing structure, name and affinity for different IgG isotypes (Picture from Nimmerjahn 2008, Nature reviews Immunology Jan 01, 2008 with permission to reprint).

Fcγ-receptor expression, regulation and actions

FcγRs are widely expressed on haematopoietic cells as shown in Table 1 [48, 72].

Table 1

Expression of FcγRs on haematopoietic cells.

Cell type	Monocytes and macrophages	Neutrophils	B cells	Dendtritic cells	Natural killer cells
Humans	FcγRI FcγRIIA and B FcγRIIIA	FcγRI FcγRIIA and B FcγRIIIB	FcγRIIB	FcγRI FcγRIIA and B FcγRIIIA	FcγRIIIA
Mice	FcγRI FcγRIIB FcγRIII FcγRIV	FcγRI FcγRIIB FcγRIII FcγRIV	FcγRIIB	FcγRI FcγRIIB FcγRIII	FcγRIII

The expression of activating and inhibitory FcγRs is regulated by cytokines and inflammatory mediators [73, 80]. Interferon-gamma (IFN-γ) up-regulates protein expression of activating FcγRs on monocytes [81] and glomerular mesangial cells [82], which is followed by IC binding and enhanced synthesis of chemoattractants inducing renal inflammation [82]. C5a/C5aR up-regulates FcγRIII on macrophages leading to increased inflammation [80, 83]. Transforming growth factor (TGF)-β, Interleukin (IL)-4 and IL-10 down-regulate expression of activating FcγRs [73, 81, 84]. TGFβ, IL-4 and IL-10 up-regulate FcγRIIB expression [73]. The effect of IL-4 is however dependent on cell type as this interleukin up-regulates FcγRIIB expression on myeloid cells [81] but down-regulates expression on activated B cells [85]. IFN-γ and C5a as well down-regulate expression of FcγRIIB [73, 80, 81].

The Fcγ-chain does not only harbour the signalling function but is also important for transport and the assembly of α-chains which upon ligand binding activate FcγRs. On monocytes and macrophages ligand binding of FcγRs activates phagocytosis and antibody-dependent cytotoxicity (ADCC) whereas on dendritic cells (DC) antigen presentation by IC induce transcriptional activation of cytokine genes. FcγRs activated natural killer (NK) cells also induce transcription of cytokine genes and ADCC and neutrophils induces ADCC and superoxide production [86]. All theses events lead to inflammatory cascades involving either of the following signalling pathways RAS, RHO, protein kinase C (PKC) and phospholipase Cγ (PLCγ), which trigger Ca^{2+}-influx (Figure 2) [48].

The activating FcγRs also have an important function in the uptake and internalization of IC. Macrophages expressing FcγRs efficiently take up and degrade IC which is important for elimination of foreign antigens and pieces of dead cells which is a central purpose of the immune system [87]. On the other hand DC are more specialized in directing internalized and degraded IC and present the antigenic peptides to T cells [87] which has been showed to be much more efficient if IC are internalized via FcγRs [88].

The expression of FcγRIIB down-regulates the effect of the different actions induced by activating FcγRs [86] and is demonstrated to be a late peripheral checkpoint of humoral tolerance regulating expansion of autoreactive B cells and plasma blasts [74]. The isoform FcγRIIBb1 is exclusively expressed on B cells and control the B cell activation by interacting with the Fc portion of antibodies. Crosslinking of the B cell receptor (BCR) and FcγRIIBb1 mediate signals which inhibit signalling pathways involving RAS, BTK and PLCγ leading to inhibited activation as well as inhibited expansion of the B cell

(Figure 2). In addition apoptosis can be induced by isolated ligand binding of the FcγRIIBb1 [48, 75]. The other isoform FcγRIIBb2 is expressed on all other cell types and in rodents this receptor is phagocytosis competent [89, 90] while in humans FcγRIIBb2 can not internalize opsonized particles but can to some extent mediate uptake of small soluble IC [90].

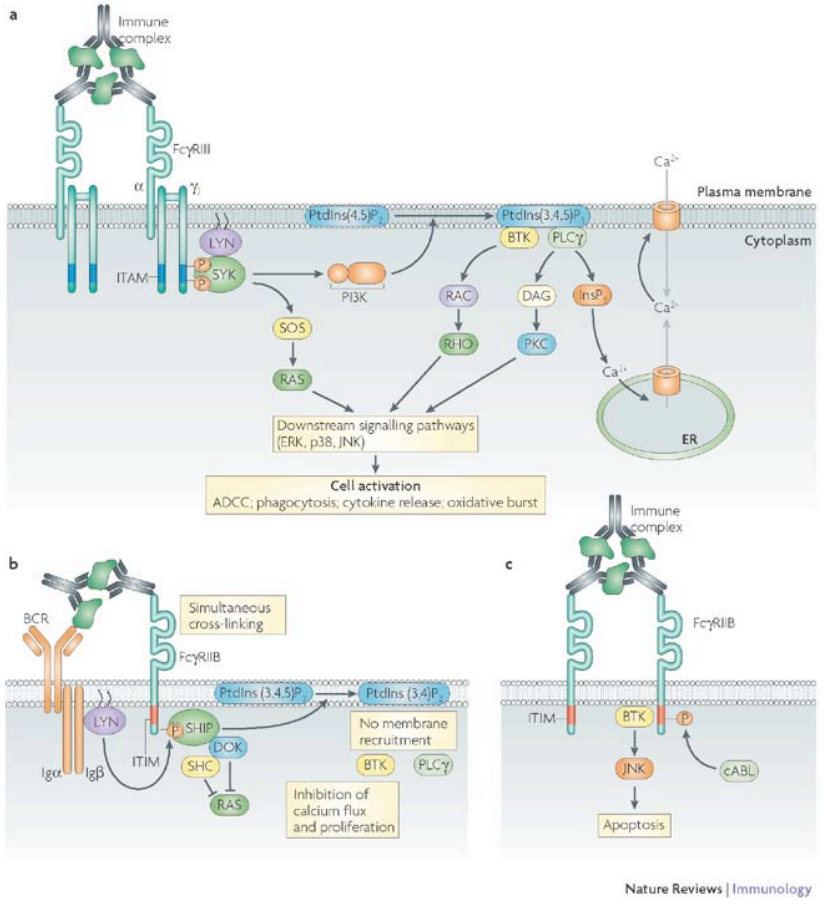


Figure 2

Signalling pathways following ligand binding of the activating FcγRIII (a) or the inhibitory FcγRIIB (b, c). (Picture from Nimmerjahn 2008, Nature reviews Immunology Jan 01, 2008 with permission to reprint).

Fcγ-receptors and autoimmunity

The use of mouse strains deficient or transgenic for FcγRs, have been and will be invaluable to describe the role of these receptors *in vivo*. Loss of or altered function in both activating and inhibiting FcγRs have shown to be of importance for development of autoimmune diseases as a consequence of an imbalanced immune system [48, 74]. Mice deficient for activating FcγRs show normal T cell development but have lost their ability to phagocytose antibody-coated particles via macrophages, and NK cells shows a defect in ADCC [91]. On the contrary, mice lacking FcγRIIB show defects in the signalling pathways of B cells leading to perturbations in IC-mediated feedback inhibition of antibody production as well as elevated Ig levels in respons both to thymus-dependent and thymus-independent antigens [92] (Table 2). Both strains however, develop normally and are fertile.

Fcγ-receptors and experimental models for autoimmune diseases

Activating FcγRs are important in systemic as well as in organ-specific autoimmune diseases (Table 2). For example, by affecting uptake of IC, delivery of IC to APC and IC-induced inflammation in mice [93]. In CIA, the murine model of RA, mice lacking FcγRIII do not develop CIA [94] and loss of FcγRI reduces cartilage destruction [95]. Mice lacking FcγRIII are resistant to autoimmune haemolytic anaemia (AIHA), a model for organ specific autoimmunity [96]. The morphological damage in anti-glomerular basement membrane GN is dependent on recruitment of neutrophils expressing FcγRIII [97]. Although some lupus-prone mouse strains in which the γ-chain has been deleted (no expression of FcγRI, FcγRIII and FcεRI) develop IC deposits, they are usually protected from the subsequent GN [98-100]. Mice deficient for the Fcγ-chain lack proteinuria in pristane-induced lupus [101]. Renal disease due to deposition of IgG2b IC deposits and neutophil infiltration due to IgG2a- and IgG2b-induced disease is accelerated in the presence of FcγRIV [102, 103] .

Table 2

Murine FcγRs for IgG in health and disease [72, 73, 75, 77, 86, 87, 102-105].

Receptor	Function	Involvement in disease
FcγRI	ADCC, Phagocytosis	Deficiency leads to impaired Phagocytosis, Cytokine release, ADCC, and Antigen presentation. Deficiency leads to Reduced arthritis, Enhanced antibody response, Reduced GN, Reduced IgG2a induced AIHA
FcγRIII	ADCC, Antigen presentation, Phagocytosis, Degranulation, Transcriptional activation of cytokine genes	Deficiency leads to impaired Phagocytosis, ADCC and Antigen presentation. Deficiency leads to Reduced arthritis, Reduced GN, Reduced IgG1 and IgG2a induced AIHA, Reduced antibody-mediated vasculitis
FcγRIV	Ligand binding induces calcium influx.	Ab blocking leads to reduced IgG2a- and IgG2b-induced IC deposition and neutophil infiltration.
FcγRIIB	Negative regulation of B cells and Antibody production. Induces Apoptosis, Endocytosis, Antigen presentation.	Deficiency leads to enhanced arthritis, EAE, GN and expansion of IgG plasma cells. Deficiency leads to spontaneous development of GN, dysregulation of B and T cell responses and Attenuated effect of IVIG

In contrast, mice deficient in the inhibitory FcγRIIB show an augmentation of experimental autoimmunity [79, 106, 107] (Table 2). Lack of FcγRIIB can even induce autoimmunity in C57BL/6 mice, a strain not prone to develop characteristic features of autoimmunity [108]. Loss of FcγRIIB does not induce either GN or autoantibody responses in BALB/c mice [108]. However, Clynes *et al* have shown spontaneously induced ACA and increase of total serum IgG in BALB/c mice lacking FcγRIIB [101]. Still, FcγRIIB deficiency probably results in autoimmunity and autoimmune disease only when modified by specific genetic backgrounds since susceptibility to induce AIHA is not affected by lack of FcγRIIB [109]. Survival in the lupus-prone strain NZB/WF1 can be prolonged by treatment with soluble FcγRIIB [110] and treatment with IVIg has been shown to up-regulate FcγRIIB [48].

Table 3

Human FcγRs in health and disease [48, 72, 75, 77, 87, 104, 105, 111, 112]

Receptor	**Function**	**Involvement in disease**
FcγRI	Endocytosis, Antigen presentation, ADCC, Phagocytosis, Respiratory burst, Transcriptional activation of cytokine genes	Increased expression in SLE patients.
FcγIIA	Endocytosis, Antigen presentation, Respiratory burst, Phagocytosis, Degranulation, Transcriptional activation of cytokine genes	Polymorphisms leading to a phenotype susceptible to SLE or MS induction.
FcγRIIIA	Endocytosis, ADCC, Phagocytosis, Respiratory burst, Transcriptional activation of cytokine genes	Polymorphism leading to a phenotype susceptible to SLE and RA induction.
FcγRIIB	Endocytosis, Negative regulation of B cells and antibody production	Impaired expression, up-regulation and recruitment to lipid raft due to different polymorphisms leading to SLE.

Genetical influences on Fcγ-receptors and the relation to autoimmune diseases

The affinity of FcγRs for Igs has been shown to influence development of autoimmune diseases. Murine strains prone to develop different autoimmune diseases display three different haplotypes of the FcγRIII, which could be linked to differences in the susceptibility to develop autoimmune diseases [113]. A polymorphism in the inhibitory FcγRIIB resulting in two alleles at the Ly-17 locus, Ly-17.1 and Ly-17.2, has been described [114]. Mouse strains that spontaneously develop autoimmune diseases usually bear the Ly-17.1 allele [115]. Polymorphisms resulting in reduced expression levels of FcγRIIB have been linked to autoimmune-prone mouse strains [116]. Mice genetically prone to develop autoimmune diseases [117] and patients with non-organ-specific disease [118] show the presence of anti-FcγR autoantibodies indicating an additional explanation for the reduced function in binding and efficiently degrading IC.

In humans, polymorphisms in the coding sequences of FcγRIII have been associated with lupus [119, 120] and RA [8, 121, 122], probably due to altered ligand binding and receptor-mediated effector functions [123] (Table 3). Genetically undefined impaired expression of FcγRIIB [124] and defined polymorphisms in the promotor region leading to either increased or decreased expression of FcγRIIB have been associated with SLE [125-127]. One polymorphism affecting recruitment of the FcγRIIB receptor to lipid raft, the site associated with consecutive signalling, has also been found [128] (Table 3).

Th1, Th2 and Th17

Upon activation naïve $CD4^+$ cells divide into three different subsets, Th1, Th2 and the recently found Th17 subset, depending on the cytokine milieu present.

The Th1 subset is derived in the presence of IL-12 secreted from macrophages or DC. Activated Th1 cells secrete IFN-γ and further activate macrophages, NK cells and Th1 $CD8^+$ cells [129]. In addition Th1 cells induces B cells to production of IgG2a [130].

In the presence of IL-4 the Th2 subset emerges [129] and this cell type secretes interleukins like IL-4, IL-5, IL-13, IL-25. Th2 cells mediate humoral immunity and induce the immunoglobulin class switch to IgG1 and IgE [131] as well as activates mast cells and allergic type responses [129]. The cytokines IFN-γ and IL-4 are at the same time self-reinforcing but also antagonistic for their opposite Th cell subset [129].

Th1 cells have long been associated with defects leading to autoimmune diseases but there have been flaws in the different explanations [129-131]. This has lead to the discovery of a third Th subset, Th17. The Th17 subset is derived in the presence of TGF-β, IL-6 and IL-21, a process which is promoted by IL-1 [132]. Th17 expansion, survival and pathogenicity are dependent on IL-23 [129]. As Th1 and Th2 counteract one another the cytokine IFN-γ and IL-4 negatively regulate Th17 cells [129, 133]. IL-27, a promoter of Th1, negatively regulates Th17 development. Although the exact contribution of Th17 cells in autoimmune diseases remains to be elucidated, they have been suggested to be key effector T cells in MS, RA and SLE [134, 135].

Mercury-induced autoimmunity - HgIA

Induction of an autoimmune disease by metals, especially mercury, in rodents (HgIA) is a well-established and relevant model for systemic autoimmunity [61]. HgIA can be induced by subcutaneous injections [68], oral ingestion of $HgCl_2$ [136], inhalation of Hg vapor [137] and by implantation of dental amalgan [138]. In the most full-blown form of HgIA, susceptible mouse strains develop antinucleolar autoantibodies (ANoA) especially to the in HgIA major autoantigen, the 34-kDalton (Da) nucleolar protein fibrillarin (AFA) and to a minor autoantigen, chromatin (ACA) [67, 139, 140]. Hg also induces hypergammaglobulinemia with increases in serum total IgG1 and IgE, and IC deposits in the renal glomerular mesangium and systemically in vessel walls [141].

ANoA/AFA and immune-complex in HgIA

Fibrillarin, the antigen in AFA, is the core protein of small nucleolar ribonucleoproteins (snoRNPs) containing the very conserved and unique small nucleolar ribonucleic acid (snoRNA) sequence motif boxes C and D. Fibrillarin is involved in post-translation of ribosomal ribonucleic acid (rRNA) and catalyses site-specific ribosomal methylation, a reaction present in all cells possessing a nucleous [142]. The AFA response is present in a subset of scleroderma patients [143] but has also been found in SLE [144]. Autoantibodies against fibrillarin outline the nucleolar structures in a clumpy pattern, and with multiple nuclear dots in the nucleoplasm (Figure 3) [145].

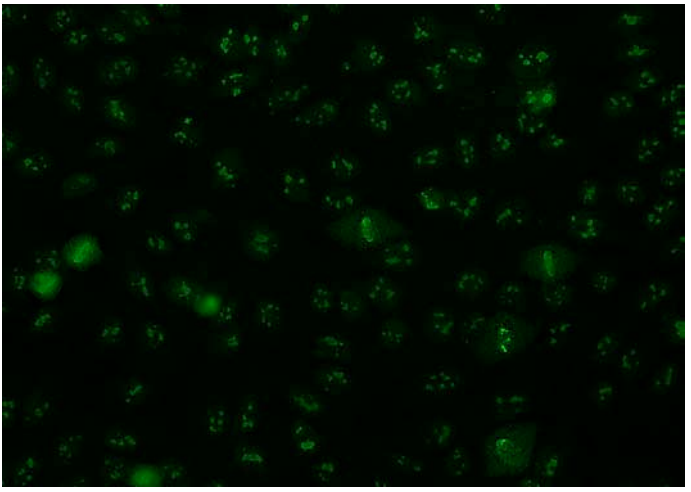


Figure 3 .
Serum from a DBA/1 male mouse treated with Hg for 5 weeks incubated on Hep-2 cells showing ANoA with the nucleoli stained in a clumpy pattern and bright dots seen in the nucleus. Detection by FITC-conjugated goat anti-mouse IgG abs.

The generation of autoantibodies to fibrillarin is believed to involve proteolytic cleavage of native fibrillarin to a 19-kDa fragment due to Hg treatment, which following immunization results in AFA production in mice [146]. The initial AFA response is restricted to Hg-modified fibrillarin but epitope spreading subsequently occurs and following long-term Hg treatment T cells respond both to Hg-modified fibrillarin and native fibrillarin [147]. AFA can also be found up to a year after termination of Hg treatment [148].

One of the hallmarks of HgIA is IC deposit in the renal mesangium as well as systemically in vessel walls (Figure 4) [141].

A B

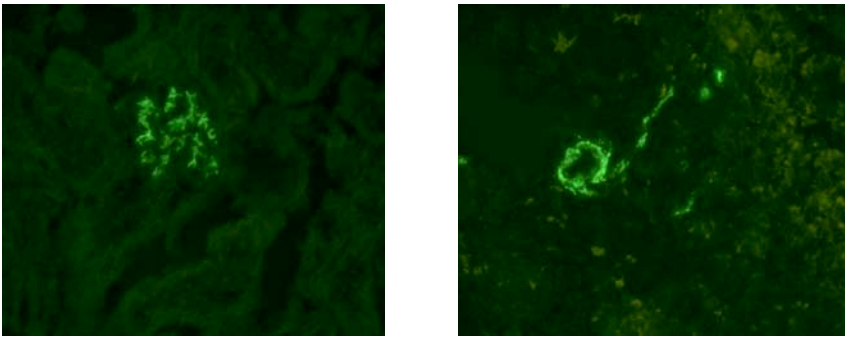


Figure 4.
Direct immunofluorescence on cryostate sections showing granular mesangial deposits (A) and granular splenic vessel walls (B) after incubation with FITC-conjugated goat anti-mouse IgG abs, from female BALB/c treated with Hg for 5 weeks.

Histological examination of kidneys from mice treated with Hg show a mild glomerular endocapillary cell proliferation accompanied by slight widening of the mesangium [149] and the IC are dominated by the Igs of the IgG1 isotype [150], an IgG which does not activate complement. Both these aspects lead to the minor histological damage [151] seen in HgIA. Development of GN is dependent on cell-cell interactions via CD40/CD40Ligand (CD40L) and influenced by increased IFN-γ secretion [46], shown to be true also in HgIA [152, 153]. IC deposits in tissues from mice treated with Hg have been shown to contain AFA [154]. However, the presence of AFA does not necessarily lead to formation of IC since mice treated with silver [155] and gold [156] develop AFA but not IC deposits [138, 155]. IC deposits can also develop in HgIA without AFA as shown in BALB/c mice [67, 150]. Hg not only induces IC deposits but Hg itself can deposit in glomerular cells and may interfere with phagocytosis [149].

Genetic susceptibility, gender and dose in HgIA

There is a strong genetical correlation in mice for development of ANoA/AFA, and the susceptibility is closely linked to the major histocompatibility complex (in mice H-2), and only strains of the H-2^s H-2^q H-2^f and H-2^{t2} haplotypes will develop ANoA/AFA during Hg treatment [67, 140]. Susceptibility to development of other HgIA parameters, such as lymphoproliferation, hypergammaglobulinemia, ACA and IC deposits, are not linked to H-2 [52], which is illustrated in the two H-2^d strains DBA/2 and BALB/c. The former is completely resistant to HgIA, while the latter shows hypergammaglobulinaemia and systemic IC deposits [67]. The resistance to develop HgIA in DBA/2 mice is linked to a major quantitative trait locus on the chromosome 1, designated heavy metal resistant 1 (*Hmr1*), a region also containing several lupus susceptibility loci [157]. The gene for Decay accelerating factor 1 (*Daf1*) is also found in this *Hmr1* region. *Daf1* shows greater expression in the HgIA resistant DBA/2 strain [158].

Gender and dose of Hg influences the induction of HgIA. Male mice tend to accumulate more mercury than female mice but female mice show higher titres of AFA in response to lower doses compared to male mice. This is in good agreement with human clinical experiences where gender has a clear effect and females are often more susceptible [159]. Both a short exposure of a high Hg dose and a prolonged exposure of a lower Hg dose can induce HgIA. It exist a minimum concentration for Hg to be able to induce autoimmune reactions and it is not possible to circumvent this with prolonged treatment time. There is also a maximum concentration where increasing the dose of Hg does not further increase the autoimmune response. The thresholds vary among the susceptible strains. Additionally, the prerequired genetical susceptibility to HgIA cannot be overruled by increased Hg dose or prolonged treatment [159]. A dose between

11 and 28μg Hg/kg/day can in the most susceptible mouse strain ASW induce HgIA [67, 160] but doses up to 1800μg Hg/kg/day induces HgIA without any signe of immunesuppression [161-164]. The safety level in humans is 0.1μg Hg/kg/day and a dose of 0.1-0.3mg/kg is normally present in humans [165]. Since mice show a greater metabolic turnover, the dose to induce immunresponses in mice contra humans is thought to be somewhat higher.

Cell types, cytokines and biological molecules in HgIA

HgIA is T cell ($CD4^+$) dependent, since mice otherwise genetically susceptible to Hg but either homozygous for the nude mutation (athymic) or treated with anti-CD4 monoclonal abs, develop neither ANoA nor IC deposits [166]. T cell proliferation and increased expression of activation markers like CD25, CD71 [164] and CD44 is shown following Hg treatment. HgIA resistant mice fail to up-regulate CD44, a mechanism correlated with a normal expression of *Daf1* [158]. Other cell types involved in HgIA are B cells, since genetically susceptible $Igh6^{-/-}$ mice (lacking B cells) are resistant to HgIA induction [167], but also monocytes/macrophages since Hg-induced T-cell proliferation *in vitro* requires adherent splenocytes producing IL-1 [168]. Mice lacking β2-microglobulin genes (no MHC-I expression) show reduced hypergammaglobulinemia and ANoA development following Hg treatment [52], indicating that the function of $CD8^+$ T cells affect HgIA induction although they are of less importance than $CD4^+$ T cells [166]. There are some indications that NK cells might be important for the induction of HgIA [52] as they show high prevalence to produce IFN-γ, a process influenced by CD28 and FcγRIII [169].

In HgIA the Th1 cytokine IFN-γ and its downstream factors IFN-γ receptor and interferon response factor-1 (IRF-1) are necessary for all manifestations of HgIA [167]. However, for full blown induction of HgIA only a modest increase

of IFN-γ is needed as seen by the 25% increase of IFN-γ mRNA expression [170] in the HgIA high-responder strain ASW [171]. Mediators that drive Th1 immunity and IFN-γ production like IL-12, interleukin converting enzyme (ICE) and STAT-4 do not affect induction of HgIA except for IL-12p35 where a deficiency leads to reduced ACA titres [52]. However, administration of IL-12 down-regulates ANoA and serum IgG1 but not serum IgE [172]. Mice deficient for IL-6 reveal suppression of polyclonal B cell responses (IgG and IgM) and abolish ACA development [52]. The Th2 cytokine IL-4 is important for hypergammaglobulinemia (IgG1 and IgE) induction only [152]. Mice resistant to HgIA show a stronger IL-10 mRNA expression than HgIA susceptible mice [170]. However, injection of IL-10 can not reduce HgIA manifestations [173].

The use of mice with a single gene deficiency has shown that the importance of T cells for induction of HgIA includes signalling via the co-stimulatory molecules CD40L, CD28 [153] and the inducible T-cell co-stimulator (ICOS) [174]. Loss of CD40L and CD28 reduces expression of all major disease parameters whereas loss of ICOS results in an almost complete suppression of ANoA and a significant reduction in serum IgE production. Blocking CTLA-4, a negative regulator of T cell activation, exacerbate HgIA [175]. Loss of the inhibitory FcγRIIB in mice not susceptible to HgIA, only induce an increase in serum IgE but do not induce ANoA developmet [175].

AIM

General aim

Determine the specific role of FcγRs in the development of systemic autoimmunity.

Specific aim

- Determine the importance of activating and inhibiting FcγRs for the development of IC deposits, ANoA and serum immunoglobulin.
- Further study the role of FcγRs for formation of IC deposit and circulating IC.
- Clarify the mechanism of FcγRs in ANoA development.

MATERIALS AND METHODS

Mice and housing

Female mice on the BALB/c background homozygous for a targeted mutation of the Fcγ-chain of the FcγR (FcRγ$^{-/-}$) (Paper I, III), or the FcγRIIB (FcγRIIB$^{-/-}$) (Paper I), were obtained from Taconic M&B, Georgtown, USA. BALB/c mice without mutations, (wild type-wt) (Paper I, III) were obtained from Taconic M&B, Ry, Denmark.

Breeding pairs of the three DBA/1 strains with a homozygous targeted mutation for (1) the Fcγ-chain (FcRγ$^{-/-}$) (Paper II), (2) the FcγRIII (FcγRIII$^{-/-}$) (Paper II, IV) or (3) the FcγRIIB (FcγRIIB$^{-/-}$) (Paper II) generated as previously described [107] were obtained from Sandra Kleinau (Department of Cell and Molecular Biology, Molecular Immunology, Uppsala University, Uppsala, Sweden) and the continuous breeding was performed at the Animal Department, Faculty of Health Sciences (Linköping, Sweden). The corresponding wt DBA/1 mice (Paper II, IV) were obtained from Taconic M&B, Denmark.

All mice were 6 -25 weeks old at onset of the experiments.

The wt BALB/c mice were housed under 12 hour dark-12 hour light cycles in steel-wire cages and given pellets and sterilised water *ad libitum* (Paper I) or under specific pathogen-free conditions given sterilised pellets and water (Paper III). The BALB/c strains with targeted mutations were kept under specific pathogen-free conditions given sterilised pellets and water (Paper I and III).

The wt DBA/1 mice and DBA/1 strains with targeted mutations were housed under 12 hour dark-12 hour light cycles in steel-wire cages and given pellets and tap water *ad libitum* except for the FcγRIIB knockout which were kept under specific pathogen-free conditions given sterilised pellets and water (Paper II and IV).

Treatment

Hg treatment consisted of tap water (sterilised or not depending on the particularly animal housing) prepared weekly with 15 or 25 mg/L $HgCl_2$ (Fluka Chemie, Buchs, Germany) given *ad libitum* under a short period (2-14 days) (Paper IV) or up to 35-70 days (Paper I, II, III) maximum depending on the study. The control groups received tap water (sterilised or not depending on the particularly animal housing) without any additions.

Blood and tissue sampling

Blood was obtained from the retro-orbital vein plexa at different times depending on the four different studies specific treatment schedule, although well considered regarding immune activation. The blood was allowed to clot at 4°C over night and the sera were stored at –20°C. At sacrifice samples of the kidney and spleen were obtained for examination of IC deposits (Paper I-III). In Paper IV spleens and lymph nodes were collected for NK cell and mRNA isolation.

Ethical consideration

The local animal ethics committee have approved all four studies (Paper I-IV).

Assessment of tissue immune-complex deposits

Pieces of the left kidney and the spleen were examined for IC deposits with direct immunofluorescence [176]. Briefly, snap frozen tissue pieces were sectioned and incubated with either FITC-conjugated goat anti-mouse IgG abs against all IgG isotypes (total IgG) (Sigma) diluted 1/40-1/2,560 (Paper I, II), an IgM ab (Sigma) diluted 1/40 (Paper I, II), or an anti-C3c ab (Organon-Technica, West Chester, PA, USA) diluted 1/320-1/10,240 (Paper I- III).

Deposits of the IgG1, IgG2a, IgG2b and IgG3 isotypes were assessed using diluted FITC-conjugated goat anti-mouse abs to the different Ig isotypes (Southern Biotechnology, Birmingham, AL, USA) (Paper I, III). Kidneys from aged NZW/BF1 mice were used as a positive control and to evaluate the specific affinity of the different abs for IgG isotypes. The presence of IC deposits in the glomeruli, and renal and splenic vessel walls, was examined with a fluorescence microscope (Nikon Tokyo, Japan) and recorded. The endpoint titre for IgG, the IgG isotypes and C3c was defined as the highest dilution, which gave a specific staining. The amount of the IgM deposits in the glomeruli, as well as total IgG, the different IgG isotypes, and C3c in renal and splenic vessel walls, was scored from 0-4 (0, no specific staining; 1, slight staining; 2, moderate staining; 3, strong staining and 4, very strong staining).

Light microscopy

Renal and splenic tissues from three to four randomly selected BALB/c mice from wt and mice with targeted mutations treated with Hg or controls were obtained at the end of the study (Paper I) and examined by light microscopy using paraffin-embedded sections [149]. The type and degree of glomerular cell proliferation was assessed without knowledge of treatment or other data and graded as follows: 0, no difference compared with reference sections from young untreated mice; 1, slight proliferation; 2, moderate proliferation; 3, severe proliferation. The presence of histological alterations in the glomeruli or in other renal and splenic tissues was searched for.

Serum anti-nuclear antibodies assessed by indirect immunofluorescence

Antibodies to nuclear antigens were detected by indirect immunofluorescence [145] (Paper I, II, IV). HEp-2 slides (Binding Site Ltd, Birmingham, England) were used as the antigenic substrate. For screening, serum was diluted 1/40 and

bound antinuclear abs were detected with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG ab (Sigma, St Louis, Missouri, USA) diluted 1/50. The slides were analysed using a fluorescence microscope (Nikon). Sera showing a nuclear staining at a dilution of 1/40 were further diluted up to 1/2,560. A specific staining using the dilution 1/40 (Paper I) or 1/80 (Paper II, IV) was considered a positive result. The highest dilution of the serum, which resulted in a specific nuclear staining, was defined as the titre. The titre and the pattern of the serum antinuclear ab were documented.

Serum anti-nuclear antibodies assessed by immunoblotting

The specificity of the antinuclear abs in the serum was assessed by immunoblotting [145] (Paper I). Mouse liver nuclei were isolated [137], separated using SDS-PAGE and transferred to nitrocellulose membranes (BioRad Lab, Hercules, CA, USA). The nitrocellulose strips were blocked with 5% fat-free milk, incubated with diluted sera and the bound ab was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG abs (Southern Biotechnology) followed by enhanced chemiluminescence (ECL Western blotting detection reagents; Amersham, Stockholm, Sweden).

Enzyme-linked immunosorbent assay (ELISA)

Serum IgG1 concentration assessed by ELISA

Microtiter plates (Nunc, Copenhagen, Denmark) were coated with purified rat anti-mouse IgG1 monoclonal ab (Mab) (1mg/ml) (LO-IMEX, Brussels, Belgium) overnight at 4°C [176] (Paper I-III). The plates were washed, blocked and incubated with diluted serum. Bound IgG1 was detected using an HRP-conjugated rat anti-mouse IgG1 mab (LO-IMEX). After incubation and repeated washes the substrate was added. The reaction was stopped with 2M $H_2SO_{4,}$, the optical density measured at 450 nm (Multiscan, Thermolabsystems,

Helsinki, Finland), and the background values subtracted. To obtain the actual concentration standard curves using mouse myeloma proteins of the IgG1 (LO-IMEX) isotype were used.

Serum IgG2a, IgG2b and IgG3 concentration assessed by ELISA

The serum IgG2a, IgG2b and IgG3 concentrations were measured using kits from Bethyl Laboratories Inc (Montgomery, Texas, USA) (Paper I (all), II (IgG2a and IgG2b), III (IgG2a)). Microtiter plates (Nunc) were coated with capture ab, washed, blocked and diluted serum added. Following incubation the bound Igs were detected with a HRP-conjugated detection ab. The substrate was added, the reaction stopped with 2M H_2SO_4, the optical density was read at 450 nm, and the background values were subtracted. To obtain the actual IgG2a, IgG2b or IgG3 concentrations, standards supplied with the kits were used.

Serum IgE concentration assessed by ELISA

To measure serum IgE microtiter plates (Nunc) were coated with rat anti-mouse IgE ab (Southern Biothechnology), incubated overnight, washed, blocked and diluted serum added (Paper I, II) [176]. After incubation with the sera a HRP-conjugated goat anti-mouse ab (Nordic Immunological Lab, Tilburg, Netherlands) was added to the wells and used to detect bound IgE. Following washes the substrate was added and then the reaction was stopped with 0.5M citric acid. The optical density was measured at 450 nm and the backgrounds values were subtracted. A standard monoclonal anti-DNP ab of IgE type (SP-7) (Sigma) was used to derive the actual IgE concentration in the samples.

Serum anti-chromatin antibodies assessed by ELISA

ACA were measured using a method described by Burlingame and Rubin [177] with minor modifications [176] (Paper I). Mictrotiter plates (Nunc) were coated

overnight at 4°C with chromatin (1.25 mg/ml). The plates were blocked with gelatin (BioRad Laboratories, Richmond, CA, USA), and diluted serum added after overnight incubation. Pooled sera from aged MRL-*lpr/lpr* mice were used as highly and moderatly positive controls. Pooled sera from young mice in non-autoimmune mouse strains were used as negative controls. The plates were incubated with alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG ab (Sigma), washed, and the substrate added. The optical density was read at 405 nm and the background values were subtracted.

Serum anti-ssDNA antibodies assessed by ELISA

In Paper I anti-ssDNA abs were measured using microtiter plates (Nunc) coated overnight at 4°C with ssDNA, washed, blocked, and diluted serum added [164]. The positive control was a pool of sera from aged MRL *lpr/lpr*-mice and the negative control a pool of sera from non-autoimmune young mice. After incubation the plates were washed, and the ALP-conjugated goat anti-mouse Ig-ab reacting with IgG, IgA and IgM (Sigma) were added. Following incubation the substrate was added, and the reaction was stopped by adding NaOH (3M) when the controls reached their predetermined values. The optical density was measured at 405 nm and background values subtracted.

Serum anti-DNP antibodies assessed by ELISA

Microtiter plates (Nunc) were coated overnight at 4°C with 2µg/ml dinitrophenyl-substituted human albumin (DNP) to analyse anti-DNP abs in BALB/c mice (Paper I) [164]. The plates were washed and diluted serum was added. The same sera as in the anti-ssDNA ab assay were used as positive and negative controls. Following repeated washes the anti-DNP ab were detected with the ALP-conjugated goat anti-mouse Ig-ab (Sigma) as in the anti-ssDNA method. The substrate was added and the reaction was stopped with 3M NaOH

when the controls reached their predetermined values. The optical density was measured at 405 nm and the background values were subtracted.

Assessment of circulating immune-complex

In paper III two different methods were used for assessment of CIC.

Assessment with PEG-precipitation

CIC in serum was measured with polyethylene glycol (PEG)-induced precipitation of IC [38]. Equal volumes of serum and 8% PEG was incubated at 4°C and then centrifuged at 1000g for 1h. The pellet was washed, resuspended in PBS pH 7.4 and stored at -20°C. Detection of IgG1 content in CIC was measured with ELISA. Microtiter wells (Nunc) were coated overnight at 4 °C, diluted sera was added and the optical density measured at 450 nm, and the background values subtracted. A standard curve, using mouse myeloma protein of the IgG1 (LO-IMEX, Brussels, Belgium) isotype was used to obtain the actual concentration. The IgG2a and IgG2b content was measured using kits from Bethyl Laboratories Inc (Montgomery, Texas, USA) and the optical density was read at 450 nm, and the background values were subtracted. To obtain the actual IgG2a or IgG2b concentration a standard supplied with the kit was used. NZB/WF1 mice were used as a positive control of the method.

Assessment with C1q-binding assay

CIC in serum was measured using a kit from Alpa Diagnostic (San Antonio, TX, USA). Briefly, serum was added to C1q pre-coated wells and the wells were washed after incubation and HRP-conjuagted detection antibody added. The substrate was added followed by stop solution and the absorbance was measured at 450 nm. Background values were subtracted. Positive and negative controls were obtained within the kit.

NK cell sorting

In paper IV spleens were collected from the mice and finely grinded into a single-cell suspension between the frosted ends of glass-slides and placed in 6-wells culture plates (Costar, Corning, NY, USA) with RPMI-1640 (Invitrogen, Grand Island, NY, USA) + 0.01% NaN_3 (KeboLab, Spånga, Sweden) on ice. The single cell suspensions from the spleens were transferred to 14ml tubes and 5ml Ficoll Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was carefully underlayed the cell suspensions and then centrifuged for 30min, 400g, 4°C. The interphase cell layer was transferred to a new 14ml tube, washed two times with RPMI-1640 + 0.01% NaN_3 and the cells were then filtered using Macs Pre Separation Filters (Milteny Biotec, Bergisch Gladbach, Germany), counted and then $10*10^6$ cells/ml was transferred to a 5ml tube. 40% rabbit serum (Dako, Glosterup, Denmark) was used to block Fc-receptors on the cells, incubated for 20min, 4°C followed by washing. The abs used, CD49-PE, CD3-FITC and CD220-APC (BD Biosciences, Franklin Lakes, NJ, USA), were diluted 1/50, added to the cells and incubated 30min, 4°C. The cells were washed and finally resuspended in PBS pH 7.4 + 2% fetal bovine serum (FBS) (Invitrogen). The single cell suspension was run through a FACSAria flow cytometer (BD Biosciences) and the NK cell population ($CD49^+$ $CD3^-$ $CD220^-$) was sorted in to FBS (Invitrogen) precoated tubes with RPMI-1640 + 2% FBS, excluding cell expressing CD3 and CD220, centrifugated for 5min at 500g and then placed in -70°C. The mean purity of NK cells was shown to be 95% by reanalysis.

Isolation of mRNA, cDNA reverse transcription and Real-Time PCR

Lymph nodes

In paper IV lymph nodes were collected from the mice, placed in RNAlater (Qiagen) and stored in -70°C. Before mRNA isolation lymph nodes were thawed, the RNAlater (Qiagen) removed and the lymph nodes were homogenised in a Tissue Lyser Adapter (Qiagen) by adding a small steelbead and 1ml QIAzol Lysis Reagent (Qiagen) to each tube. mRNA was then isolated using the RNeasy Lipid Mini kit from Qiagen. The High Capacity cDNA Reverse Transcription kit (Applied BioSystems, Foster City, CA, USA) was used for cDNA transcription and a mRNA concentration of 100 ng was used. The Real Time-PCR (RT-PCR) was performed with duplicate samples using the Applied Biosystems 7900HT Fast RT-PCR System with Applied Biosystems Taqman Gene Expression kit (Applied BioSystems). In lymph nodes the expression of IFN-γ, IRF-1, IL-12p35, IL-15, IL-17, IL23R, IL-21 and IL-4 messenger ribonucleic acid mRNA) was measured with reporter dye FAM (6-carboxyfluorescein) labelled probes. The results are presented as relative transcription using the comparative C_T method. The delta cycle threshold, ΔC_T, was calculated for each of the target gene in every mouse by subtracting the endogenous control, 18S. The $\Delta\Delta C_T$ was determined by calculating the ΔC_T difference between Hg-treated and untreated mice. Relative quantification was calculated as $2^{-\Delta\Delta C_T}$ where untreated mice are given the value 1-fold [178]. The ΔC_T in Hg-treated mice showed an average coefficient of variation (CV) of 4.2 ± 2.4 (mean ± SD) and untreated mice of 4.8 ± 1.8 (mean ± SD) when the results from all target genes in lymph nodes were combined.

Splenic NK cells

The FACSAria sorted NK cells were thawed, centrifugated 5min, at 500g, the supernatant removed and NK cell mRNA was then isolated using RNeasy Micro kit (Qiagen). The rest of the procedure was done as for the lymph nodes above except that only the expression of IFN-γ mRNA was measured. When the results from the target gene in NK cells were combined we received an average CV of 10.6 ± 5.1 (mean ± SD) in Hg-treated mice and in untreated mice an average CV of 15.2 ± 11.3 (mean ± SD).

Statistical methods

The statistically analyses were performed using Prism Graphpad (Software Inc.) Since our data are considered not to show a nominal frequency non-parametric tests have been used.

Throughout the studies (Paper I-IV) the differences between the groups were analysed with Mann-Whitney's test except for the presence or absence of IC deposits in the vessel walls and ANoA where Fisher's exact test were used. $P < 0.05$ was considered statistically significant and checked to be correct by the Bonferroni method to avoid gaining significant results due to multiple comparisons.

RESULTS

Tissue immune-complex formation in wt mice, FcγR-, FcγRIII- and FcγRIIB-deficient mice (Paper I, II and III)

BALB/c

Renal glomerular immune-complex deposits and histological alterations

IgG deposits

Wt mice

Renal mesangial IgG1 deposits with distinct titres began to develop after 16 days of Hg treatment in wt mice (Table 4), and the fraction and/or titre (magnitude) of wt mice with IC deposits continued to increase until after 35 days of treatment (Table 5). The order of increase of the different IgG isotypes in titre was IgG1 >> IgG2b ≈ IgG3 > IgG2a (data not shown).

FcγR$^{-/-}$ mice

In the Hg-treated FcγR$^{-/-}$ mice the magnitude of renal mesangial IgG deposits was significantly different compared to wt mice (Table 5). High titres of IgG deposits (< 320) were not seen until after 26 days of treatment and not until day 35 was the titre of IgG deposits higher and significantly different from the strain-specific untreated mice (data not shown). Titres were in addition substantially lower than in Hg-treated wt mice. The Hg-treated FcRγ$^{-/-}$ mice showed increased mesangial deposits of IgG1, IgG2b and IgG3 but the titre was significantly lower than in Hg-treated wt mice (data not shown).

Table 4

Development of tissue IgG1 immune-complex deposits during Hg treatment for 35 days (data pooled from Paper I and III).

Strain	Renal mesangium IgG1 deposits						Splenic vessel wall IgG1 deposits					
Day	12	14	16	18	26	35	12	14	16	18	26	35
Wt	1/4	0/4	3/4	4/4	4/4	14/14	0/4	0/4	1/4	2/4	3/4	12/14
FcR$\gamma^{-/-}$	2/5	ND	ND	4/5	5/5	8/8	1/5	ND	ND	0/5	4/5	4/8
FcγRIIB$^{-/-}$	ND	ND	ND	ND	ND	7/7	ND	ND	ND	ND	ND	7/7

ND = not determined. No statistically significant differences were observed (Fisher's exact test).

Table 5

Tissue immune-complex deposits after 35 days of Hg treatment (Paper I).

Strain	Renal mesangium				Renal vessel walls		Splenic vessel walls	
	IgG	IgM	C3c	Glomerular proliferation	IgG	C3c	IgG	C3c
Wt	1920±675 [A]	2.1±0.3 [B]	4608±1079 [A]	1.5±0.6 [B]	0.6±0.4 [B]	0 [B]	2.3±0.5 [B]	1±0.4 [B]
FcR$\gamma^{-/-}$	550±507***	1.8±0.5	800±296***	1.3±0.6	0**	0	0.3±0.7**	0**
FcγRIIB$^{-/-}$	1920±684	1.4±0.5	3200±1185	1.5±0.6	0.3±0.4	0.1±0.2	1.4±0.8	0.5±0.5

[A] Reciprocal titre ± SD, [B] Scoring 1-4 ± SD. *** = significantly different from wt mice $p > 0.001$ and ** = significantly different from wt mice $p < 0.01$. Statistical calculations performed with Mann-Whitney's test.

FcγRIIB$^{-/-}$ mice

The FcγRIIB$^{-/-}$ mice showed an increase in mesangial IgG deposits after 35 days of Hg treatment; the increase was identical in magnitude compared with wt mice (Table 4, Table 5). The order of increase in titre of the different IgG isotypes were the same as in wt mice (data not shown).

IgM deposits

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice

Development of IgM in renal mesangium differed in the three strains. It was significantly higher in wt mice treated with Hg compared to untreated mice but was present in both Hg-treated and untreated FcRγ$^{-/-}$ and FcγRIIB$^{-/-}$ mice (Table 5).

C3c deposits

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice

C3c deposits were seen after 12 days of treatment in the renal mesangium of both Hg-treated wt mice and FcγR$^{-/-}$ mice and with significantly lower titres in FcγR$^{-/-}$ mice than in wt mice (Table 5). A significant increase in mesangial C3c titre was also seen in Hg-treated FcγRIIB$^{-/-}$ mice with a similar magnitude as in wt mice (Table 5).

Histological examinations

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice

The histological examination showed a mild glomerular endocapillary cell proliferation accompanied by slight widening of the mesangium in all three Hg-treated strains and the proliferation varied only slightly (Table 5). Neither extracapillary cell proliferation nor inflammation was present.

Renal vessel wall immune-complex deposits

IgG deposits

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice

Wt mice treated with Hg developed a significantly higher titre of total IgG deposits in the renal vessel walls compared to untreated mice (Table 5). The IC contained IgG1 deposits only, except for a single mouse which showed slight IgG2a deposits. There were no or low titres of the different IgG isotypes in the renal vessel walls of Hg-treated FcRγ$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice.

C3c deposits

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice

None of the Hg-treated mice showed C3c deposits in the renal vessel walls, and light microscopy revealed no signs of vasculitis (Table 5).

Splenic vessel wall immune-complex deposits

IgG deposits

Wt mice

Splenic vessel walls IgG1 deposits began to develop after 16 days of Hg treatment in wt mice (Table 4), and the fraction continued to increase until almost all wt mice showed IC deposits with a high titre of IgG after 35 days of treatment (Table 5). The IgG deposits consisted mainly of IgG1, but some mice also showed IgG3 deposits, and slight deposits of IgG2a and IgG2b (data not shown).

FcγR$^{-/-}$ mice

Splenic vessel wall IgG1 deposits were seen after 12 days but higher titres of IgG1 deposits (< 1.5) were not seen until after 26 days of treatment and still after 35 days of Hg treatment only 4 out of 8 FcRγ$^{-/-}$ mice showed deposits

consisting of IgG1 (Table 4). The titre was moderate and significantly lower compared to Hg-treated wt mice (Table 5).

FcγRIIB$^{-/-}$ mice

All Hg-treated FcγRIIB$^{-/-}$ mice had developed splenic vessel wall IC deposits after 35 days consisting of IgG1 but some also showed IgG3 deposits (Table 4, Table 5).

C3c deposits

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice

C3c deposits in splenic vessel walls were not seen until after 35 days of Hg treatment in wt mice. In FcγR$^{-/-}$ mice C3c deposits were seen in the splenic vessel wall after 26 days and were significantly higher in titre than in wt mice (data not shown). After 35 days of Hg treatment both the fraction of mice with C3c deposits (data not shown) and the amount of deposits were significantly lower in Hg-treated FcγR$^{-/-}$ mice as compared to Hg-treated wt mice (Table 5). Hg-treated FcγRIIB$^{-/-}$ mice developed splenic vessel wall IC deposits consisting of C3c deposits (Table 5).

DBA/1

Renal glomerular, renal vessel wall and splenic vessel wall immune-complex deposits

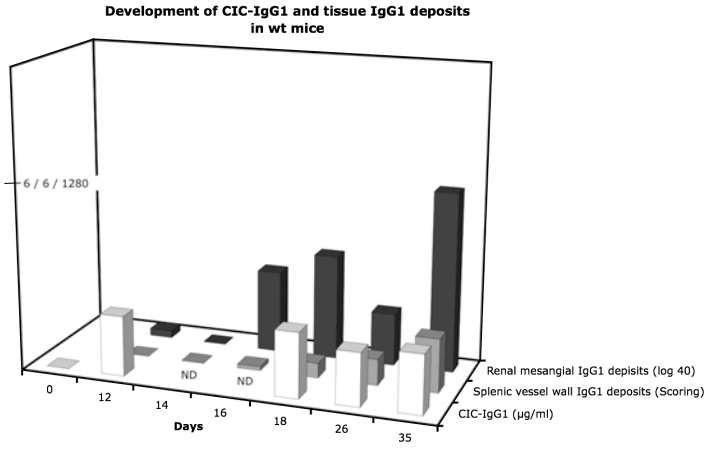
All strains developed some IgG, IgM and C3c immune deposits in the glomerular mesangium but the deposits were generally of low titre and bore no similarity to the deposits observed in HgIA. Furthermore, no deposits were present in renal or splenic vessel walls (data not shown).

Development of circulating immune-complex in wt mice and FcγR deficient mice (Paper III)

Wt mice and FcγR$^{-/-}$ mice

In wt mice Hg treatment induced aggravated formation of CIC containing IgG1 and IgG2a. The CIC-IgG1 in wt mice were subsequently followed by IC deposits in tissues (Figure 5A). FcγR$^{-/-}$ mice showed significantly higher concentrations of IgG1-CIC compared to wt mice during the entire treatment time. The IgG-CIC concentration gradually declined significantly in FcγR$^{-/-}$ mice during the treatment. The development of of tissue IC in FcγR$^{-/-}$ mice was delayed compared to wt mice (Figure 5B). In contrast, the IgG2a-CIC levels increased significantly during Hg treatment in FcγR$^{-/-}$ mice and were as the levels of IgG1-CIC significantly higher in FcγR$^{-/-}$ mice compared to wt mice (data not shown).

A



B

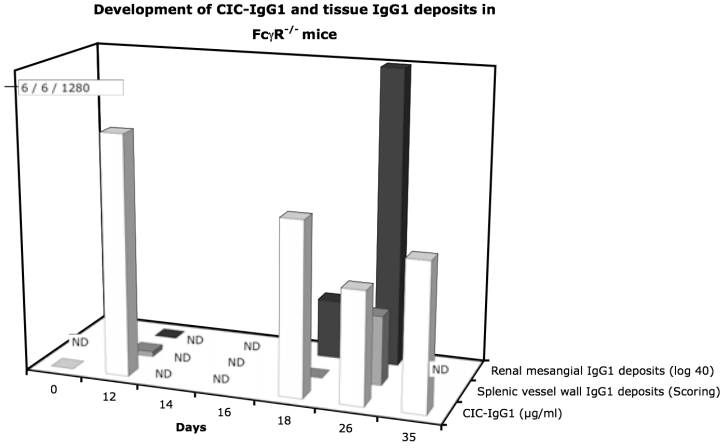


Figure 5

Development of circulating IgG1-containing immune-complex (IC) (clear bars), splenic vessel wall IgG1 IC deposits (grey bars) and renal mesangial IgG1 IC deposits (black bars) in (A) wt mice and (B) FcγR deficient mice following Hg treatment for 35 days. Bars denote net mean. The y-axel shows the highest score from the respective analyses (µg/ml / scoring / titre).

ANoA and anti-chromatin antibodies in wt mice and FcγR-, FcγRIII- and FcγRIIB-deficient mice (Paper I, II, IV)

DBA/1

Effect of deleted FcγR genes on ANoA development in mice treated for 35 or 70 days with 15 or 25 mg/L Hg

Wt mice

Both male and female DBA/1 wt mice treated with 15 mg/L Hg developed ANoA (Table 6), which outlined the nucleolar structures in a clumpy pattern, and with multiple nuclear dots in the nucleoplasm. The Hg-treated wt mice started to develop IgG ANoA after 10 days. After 35 days 90% of male wt mice and 80% of female wt mice showed IgG ANoA. The order of the different IgG isotypes was IgG1 >>> IgG2a = IgG2b = IgG3 (data not shown). Increasing the Hg dose from 15 mg/L to 25 mg/L actually delayed ANoA development in male wt mice until after 35 days of treatment but did not alter the ANoA response in female wt mice (data not shown).

FcγR$^{-/-}$ mice and FcγRIII$^{-/-}$ mice

FcγR$^{-/-}$ mice (lacking FcγRI, FcγRIII and FceRI) and FcRγ$^{-/-}$ mice and FcγRIII$^{-/-}$ mice showed similare development of ANoA (Table 6).

Neither male nor female FcγRIII$^{-/-}$ mice showed IgG ANoA at 14 days of Hg treatment. After 35 days of treatment 70% male and 30% female FcγRIII$^{-/-}$ mice showed IgG ANoA . The IgG isotype order was IgG1 >>> IgG2a = IgG2b (data not shown). Even if Hg treatment was prolonged up to 70 days or increased from 15 mg/L to 25 mg/L female FcγRIII$^{-/-}$ mice still showed a significantly lower titre of ANoA compared with wt mice (data not shown).

Table 6

Development of ANoA during treatment for up to 70 days in DBA/1 mice using 15mg/L Hg (data pooled from Paper II and IV).

Strain	Gender	ANoA IgG								
		2 days	4 days	6 days	8 days	10 days	14 days	35 days	49 days	70 days
Wt										
	Male	0/4	0/4	0/4	0/3	2/4	11/20	14/15	6/6	6/6
	Female	ND	ND	ND	ND	ND	1/14**	11/14	6/6	6/6
FcγRIII$^{-/-}$										
	Male	0/4	0/4	0/4	0/4	ND	0/14***	7/10	ND	ND
	Female	ND	ND	ND	ND	ND	0/12	4/12*	4/5	3/5
FcγRIIB$^{-/-}$										
	Male	ND	ND	ND	ND	ND	3/12	9/11	3/4	3/4
	Female	ND	ND	ND	ND	ND	4/8*	6/8	ND	ND

** = significantly different from male wt mice, *** = significantly different from male wt mice $p < 0.001$, * = significantly different from female wt mice $p < 0.05$. ND = not determined. Statistical calculations performed with Fisher's exact test.

FcγRIIB$^{-/-}$ mice

Among the Hg-treated FcγRIIB$^{-/-}$ mice, 25% and 80% of the males developed IgG ANoA after 14 and 35 days of treatment, respectively (Table 6). The IgG isotype order was IgG1 >> IgG2a >> IgG2b = IgG3. The fraction of IgG2a ANoA positive male FcγRIIB$^{-/-}$ mice was significantly lower compared to wt mice after 35 days Hg treatment (data not shown). After 14 and 35 days of Hg treatment 50% and 75% of the female mice showed IgG ANoA, respectively (Table 6). After 35 days the ANoA were dominated by the IgG1 isotype with a 9-fold increase in mean titre compared to female wt mice. ANoA of especially the IgG2a and IgG2b isotypes were also present and the fraction of mice with IgG2b ANoA was significantly higher compared with wt mice (data not shown). The fraction of ANoA positive male FcγRIIB$^{-/-}$ or the titre did not change significantly during extended treatment time (70 days) or increased Hg dose (25mg/L) (data not shown).

BALB/c

ANA development

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$ mice

ANA with a finely speckled pattern, including distinct staining of the nucleolar membrane and the condensed chromosomes in dividing cells, was seen after 14 days of Hg treatment in all three strains with a significantly higher titre in FcγR$^{-/-}$ mice compared to wt mice. After 35 days of Hg treatment the IgG ANA titre in FcγR$^{-/-}$ mice had increased similar to the titre in wt mice. The IgG ANA consisted of a significantly higher titre of IgG1 than of IgG2a (data not shown). No consistent reactivity to mouse liver nuclear proteins was detected in finely speckled positive sera using immunoblotting.

Serum anti-chromatin antibodies

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$

No statistically significant increase in ACA was shown in response to Hg treatment in wt mice and FcγR$^{-/-}$ mice whereas FcγRIIB$^{-/-}$ mice showed a significant increase in ACA titre due to Hg treatment, but the increase from 0.011 ± 0.002 to 0.019 ± 0.005 after 14 days and from 0.012 ± 0.003 to 0.023 ± 0.012 after 35 days (mean ± SD, OD405) is unlikely to be biologically significant (data not shown).

Immunoglobulin levels and polyclonal B cell markers in wt mice and FcγR-, FcγRIII- and FcγRIIB-deficient mice (Paper I, II and IV)

BALB/c

Serum immunoglobulins

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$

In wt mice the serum IgG1 and IgE concentrations increased in response to Hg treatment, whereas Hg treatment showed no effect on serum IgG2a, IgG2b, or IgG3 concentrations. In contrast, the IgG1 concentration decreased after 35 days in FcγR$^{-/-}$ mice during Hg treatment but serum IgE increased with the same magnitude as in wt mice. FcγR$^{-/-}$ mice did not show any significant differences in serum IgG2a, IgG2b, or IgG3 concentrations following Hg treatment. The FcγRIIB$^{-/-}$ mice responded to Hg treatment with an increase in both serum IgG1 and IgE concentrations, which both also were significantly higher compared to wt mice. In addition, FcγRIIB$^{-/-}$ mice treated with Hg showed a significant increase in IgG2a and IgG2b (data not shown).

Serum markers of polyclonal B cell activation

Wt mice, FcγR$^{-/-}$ mice and FcγRIIB$^{-/-}$

Wt mice showed no increase in anti-DNP abs, but a significant increase in anti-ssDNA abs following Hg treatment. However, the increase from 0.141 ± 0.021 to 0.157 ± 0.022 (mean ± SD, OD405) is unlikely to be biologically significant. The FcγR$^{-/-}$ strain showed no significant increase in anti-ssDNA or anti-DNP abs during Hg treatment compared to untreated mice. The FcγRIIB$^{-/-}$ mice showed a statistically but unlikely biological significant anti-DNP ab response, while there was no significant increase in anti-ssDNA abs during Hg treatment (data not shown).

DBA/1

Serum immunoglobulins

Wt mice

Serum IgG1 increase could be detected 8 days following onset of Hg treatment and continued to increase during treatment up to 70 days in male wt mice (Figure 6). Serum IgG1 also increased in female wt mice due to Hg treatment. No significant increase in serum IgG2a could be detected in either male or female wt mice.

Male wt mice treated with Hg showed a significant increase in serum IgE after 6 days and the maximum mean concentration was reached after 14 days as seen also in female wt mice (data not shown).

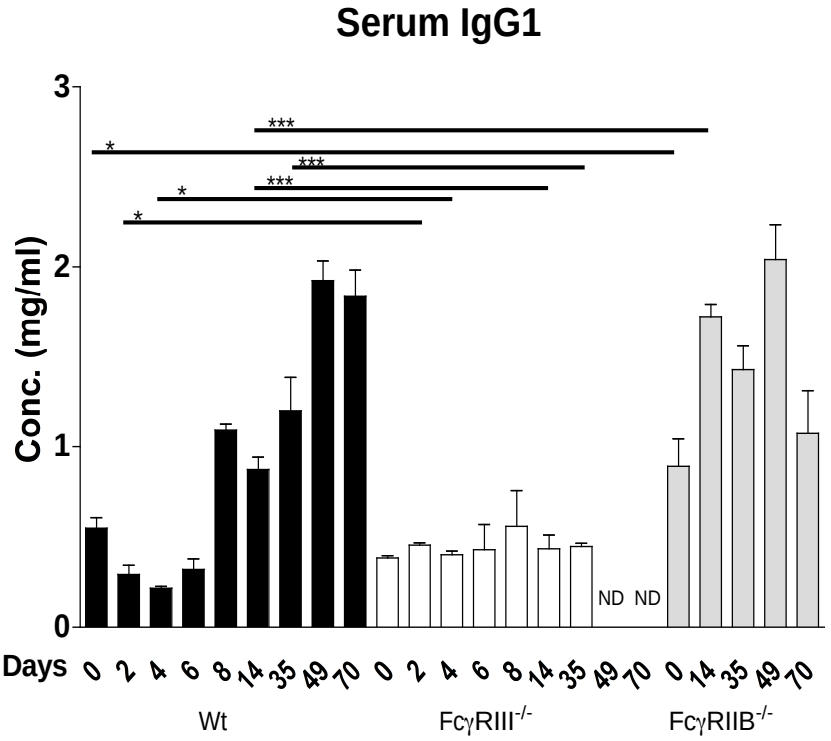


Figure 6

Serum concentration of IgG1 in male mice treated with Hg up to 70 days (data pooled from Paper II and IV). ND = not determined. Bars denote mean ± SD. Statistical calculations performed with Mann-Whitney's test.

FcγR$^{-/-}$ mice and FcγRIII$^{-/-}$ mice

FcγR$^{-/-}$ mice and FcγRIII$^{-/-}$ mice showed the same pattern of serum Igs.

The serum IgG1 increase in Hg-treated male wt mice was not seen in Hg-treated FcγRIII$^{-/-}$ mice (Figure 6). Female FcγRIII$^{-/-}$ mice showed a statistically significant increase in serum IgG1 concentration due to Hg treatment compared to untreated mice. However the serum IgG1 level was significantly lower

compared with Hg-treated female wt mice. Prolonged treatment with 15 mg/L for 70 days or increasing the Hg dose from from 15 mg/L to 25 mg/L did not result in increased serum IgG1 in female FcγRIII$^{-/-}$ mice (data not shown).

Male but not female FcγRIII$^{-/-}$ mice responded with an increase in serum IgG2a after 14 days Hg treatment compared to untreated mice. However, this difference was small and probably not biologically significant (data not shown).

In Paper II no statistically significant effect of Hg on serum IgE was seen in either male or female FcγRIII$^{-/-}$ mice. However, the increase in mean serum IgE was at least 2-fold higher in Hg-treated male and female FcγRIII$^{-/-}$ mice compared to untreated mice. In Paper IV a significant increase in serum IgE was seen after 6 and 14 days due to Hg treatment in FcγRIII$^{-/-}$ male mice (data not shown).

FcγRIIB$^{-/-}$ mice

Male FcγRIIB$^{-/-}$ mice showed an increase in serum IgG1 (Figure 6) but not in serum IgG2a (data not shown) after 35 days of Hg treatment. The serum IgG1 increase was significantly higher than in male wt mice. In female FcγRIIB$^{-/-}$ mice no difference was observed in serum IgG1, but was significant increased in serum IgG2a due to Hg treatment (data not shown).

In male FcγRIIB$^{-/-}$ mice serum IgE increased due to Hg treatment and showed a mean maximum after 14 days, which was also significantly higher than in male wt mice. However, already before treatment these mice showed a 2 –fold higher concentration of mean serum IgE than untreated mice. Hg treatment did not increase serum IgE in female FcγRIIB$^{-/-}$ (data not shown).

Cytokine profiles in in wt mice and FcγRIII-deficient mice (Paper IV)

Cytokine mRNA in lymph nodes

Th1-profile

Wt mice and FcγRIII$^{-/-}$ mice

Data from IFN-γ, IRF-1 and IL-12p35 lymph node mRNA were merged to establish a Th1-profile. A significant increase was found in wt mice between day 2 and 6 due to Hg treatment but not in FcγRIII$^{-/-}$ mice. Individually in wt mice the mean expression of IFN-γ, IRF-1 and IL-12p35 reached a maximum after 6 days of Hg treatment whereas in FcγRIII$^{-/-}$ mice IFN-γ and IL-12p35 also reached a maximum after 6 days while the mean expression of IRF-1 decreased. IFN-γ showed a 3.9 fold increase in wt mice compared to a 2.1 fold increase in FcγRIII$^{-/-}$ mice (data not shown).

Th2-profile

Wt mice and FcγRIII$^{-/-}$ mice

The IL-4 mRNA expression increased in lymph nodes during Hg treatment both in wt mice and FcγRIII$^{-/-}$ mice. The maximum increase was seen following 4-6 days of treatment. In addition, wt mice showed a significantly higher increase in IL-4 mRNA after 4 days of Hg treatment compared to FcγRIII$^{-/-}$ mice (data not shown).

Th17-profile

Wt mice and FcγRIII$^{-/-}$ mice

Establishing a Th17-profile by merging IL-6, IL23R, IL-21and IL-17 data from the lymph nodes showed an increase of Th17-associated cytokine mRNA in

both wt mice and FcγRIII$^{-/-}$ mice due to Hg treatment. After 8 days of Hg treatment wt mice still showed significantly higher mRNA expression of Th17 cytokines compared to FcγRIII$^{-/-}$ mice. The individual IL-6, IL-23R, IL-17 mRNA expression in lymph nodes reached a maximum after 6 days of Hg treatment in both strains (data not shown).

IL-21

Wt mice and FcγRIII$^{-/-}$ mice

The IL-21 mRNA expression increased during Hg treatment in wt and was significantly higher compared to FcγRIII$^{-/-}$ mice (data not shown).

Cytokine mRNA in splenic NK cells

Wt mice and FcγRIII$^{-/-}$ mice

The expression of IFN-γ mRNA in NK cells increased in wt mice and FcγRIII$^{-/-}$ mice due to Hg treatment. However, the increase was not significant during Hg treatment neither within the respective mouse strain nor between the mouse strains. However, while NK cell-generated IFN-γ mRNA from wt mice showed the same expression during that treatment period, IFN-γ mRNA expression in NK cells from FcγRIII$^{-/-}$ mice decreased 6.2-fold between day 4 and 6 (data not shown).

Animal health (Paper I, II, III and IV)

Although the mice developed an autoimmune condition, no signs of disease were observed during the experiment. However in Paper I a single FcγRIIB$^{-/-}$ mouse showed at pre-treatment serum anti-DNP ab and IgE values which were higher than mean + 3 SD in all mice. Data from this mouse were therefore not further included in the results. In Paper II 3.5% mice died during the blood sampling at day 14 unrelated to disease.

DISCUSSION

Relation between FcγRs and manifestations of HgIA

Role of activating and inhibitory FcγRs for CIC and tissue IC deposits

Mice genetically susceptible for Hg-induced autoimmune manifestations and lacking the Fcγ-chain showed a delayed development of IC deposits in the renal mesangium and splenic vessel walls compared to wt mice following Hg treatment. The IC were mostly composed of IgG, particularly IgG1, but C3c deposits were also found. In paper I we speculated that the reduced IC formation could be due either to a global lack of IgG1 response in FcγR deficient mice or a role of activating FcγRs for formation of IC. In paper III we could demonstrate that Hg treatment induced increased levels of CIC, a response linked to either increased formation or reduced elimination of CIC. Hg *per se* does not affect the elimination pathway in BALB/c mice [38] leaving increased formation as the expected mechanism. Mice deficient for the activating FcγRs showed increased levels of CIC composed of both IgG1 and IgG2a compared to Hg-treated wt mice. Therefore the lack or even decreased secretion of serum IgG1 was not responsible for the reduced IC deposits in the tissues. However, FcγRs are necessary for the efficient transfer of IC to phagocytic cells as shown using specific inhibitors [179]. The mechanism suggested is that recognition and binding of the erythrocyte-bound IC substrates by FcRs allows close contact between the erythrocyte and the acceptor cell. The reduction of IC deposits in tissue seen in activating FcγR deficient mice is therefore thought to be dependent on the binding of IC to FcγRs followed by IC clearance [87]. In wt mice treated with Hg, CIC development is followed by IC tissue deposits. The CIC level is, as shown in

this thesis, constant whereas the IC deposits increases. This indicates an overload of FcγRs IC clearance resulting in persistent IC tissue formation since Hg *per se* does not affect clearance [38].

The receptor among the activating FcγRs most important for the formation of IC deposits and other manifestations in HgIA (see below) is likely to be FcγRIII since this receptor preferentially binds IgG1, the IgG isotype mainly found in IC in HgIA. Although FcγRIII *in vitro* can bind several isotypes there is a more restricted IgG affinity for IgG1 *in vivo* [180] supported by other model systems. For example, in the K/BxN mice serum transfer arthritis model IgG1 is the Ig isotype dominating the response and FcγRIII deficient K/BxN mice show abrogated formation of cartilage IC deposits [181]. Another example is that FcγRIII deficient mice are only resistant to experimental AIHA if the disorder is induced by pathogenic IgG1 anti-murine red blood cell monoclonal abs [96].

In NZB/WF1 mice reduced expression of FcγRs on sinusoidal liver cells tallies with spontaneous development of IC-mediated GN [182]. Mice immunized with acetylcholine receptors develop a condition resembling myasthenia gravis with destruction of the neuromuscular junction. FcγRIII deficient mice treated with acetylcholine receptors show a reduction of IC deposits in the neuromuscular junctions [183]. These animal models, HgIA included, show that the involvement of activating FcγRs in the development of autoimmunity can include two different mechanisms. The receptors can fail to bind ligand properly followed by reduced clearance rate and instead tissue deposits, demonstrated in humans where polymorphisms leading to expression of low-affinity FcγRIII results in a phenotype susceptible for SLE and RA [72]. The receptors can also act as activators to induce autoimmune conditions.

The damage in glomerular structures following IC deposits depends on the composition of the IC and the site of formation [35]. Subeithelial deposition leads to non-inflammatory complement–mediated damage whereas mesangial and especially subendothelial depositions are associated with increased inflammation due to the recruitment of inflammatory cells and cell proliferation [184]. In contrast to our finding, Fcγ-chain deficient mice prone to develop lupus-like disorders spontaneously develop IC deposits. However, they are protected from the following inflammation since this is dependent on recruitment of neutrophils expressing FcγRIII [97, 98, 100]. In HgIA, only a mild glomerular endocapillary cell proliferation and slight widening of the mesangium are seen in wt mice. This response is not affected by lack of the Fcγ-chain. Although the IC in HgIA contain C3c they are dominated by IgG1, which does not activate complement. In addition, the IC deposits are strictly localized in the mesangium [149] and mesangial deposits leads to less histological damage [151] explaining the low inflammation in HgIA. In fact, treatment with organic mercury even re-localizes spontaneously developing glomerular IC deposits in NZB/WF1 mice from the capillary loops to the mesangium [185].

The inhibitory FcγRIIB is not important for IC development neither in BALB/c nor in DBA/1 mice. Lack of FcγRIIB in DBA/1 mice could not override the restriction not to develop IC deposits in response to Hg, which is probably genetically determined. DBA/2 mice do not develop HgIA although expressing a susceptible H-2. This is explained by the region *Hmr1* on chromosome 1 found in DBA/2 mice which is linked to the inhibited formation of glomerular IC deposits [157]. The genetic background has previously been shown to influence the outcome of FcγRIIB deficiency since C57BL/6 but not BALB/c mice spontaneously develop IC deposits [108].

Role of activating and inhibitory FcγRs for ANoA induction

In paper II and IV mice deficient for the Fcγ-chain or the FcγRIII both showed delayed development and reduced titres of ANoA compared to wt mice. This indicates that FcγRIII is the receptor among the FcγRs important for ANoA formation in HgIA. The development of ANoA is time- and dose-dependent [159]. However, the delayed and reduced development of ANoA in FcγRs deficient mice could not be overcome with prolonging the treatment time or increasing the Hg dose. Hg-induced ANoA resembles induction of an adaptive immune response with the dependency of T cells [166], T cell co-stimulatory molecules as CD28/B7 and CD40/CD40L [153]. The susceptibility is linked to H-2 [67] and the IgG isotypes dominate the response [186]. In an adaptive immune response the presentation of antigens via DC and the following activation of $CD4^+$ and $CD8^+$ are important. Studies with Fcγ-chain deficient mice have shown that engagement of activating FcγRs induced increased expression of MHC and co-stimulatory molecules such as CD40, CD80 and CD86 on DC, giving them an activated phenotype. Both *in vitro* and *in vivo* studies have shown that the capacity of internalization and presentation of IC to $CD4^+$ and $CD8^+$ cells via DC is more efficient in the presence of activating FcγRs [88, 187, 188]. In HgIA, β2-microglobulin deficient mice, with no expression of MHC-I, showed reduced ANoA development [52]. This indicates that the attenuated ANoA response in FcγRs deficient mice is likely to be caused by a suboptimal presentation of peptides in DC.

The ANoA response was in general greater in males compared to females (paper I). An opposite finding compared to previous studies, which showed that females responded to lower doses of Hg compared to male mice. However, male mice accumulate more Hg than female mice [159]. Loss of the FcγRIII in female mice further weakened the ANoA response in wt mice, which might be linked to estrogen. Absence of estrogen induces an increase in the expression of FcγRIII [189] indicating that females show a lower expression of FcγRIII

and therefore a less efficient presentation of antigens. There is also a role for estrogen in RA, where women are less prone to develop RA although possessing a polymorphism in the FcγRIII 158VV locus which makes them susceptible to RA induction [8].

The inhibitory FcγR, FcγRIIB, acts as a modest regulator of ANoA induction since ANoA of the IgG2b and IgG1 isotypes showed a significant and a tendency to significantly enhanced response, respectively, in FcγRIIB deficient mice. Augmentation of $CD4^+$ T cell responses [190] and the role of FcγRIIB to act as a distal check point in antibody responses [191] have been indicated as possible mechanisms for the increase of autoimmune conditions in mice lacking FcγRIIB. In the bone marrow and the spleen B cell development is controlled by several mechanisms including receptor editing, clonal deletion and anergy. FcγRIIB regulate B cell development during later stages. Simultaneous cross-linking of FcγRIIB and the BCR inhibits downstream events otherwise leading to B cell activation and expansion [48]. If B cells show low affinity to antigens during germinal-centre reactions co-engagement of the BCR and FcγRIIB or homo-oligomerization of FcγRIIB can induce apoptosis [192]. Terminally differentiated plasma cells need anti-apoptotic signals from stromal cells to survive in the bone marrow [193]. Lack of these signals lead to apoptosis possibly induced by cross-linking of FcγRIIB and IC [48, 191]. The presentation of antigens via MHC I and II and the following activation of $CD4^+$ and $CD8^+$ cells is also regulated by FcγRIIB. FcγRIIB deficient mice show increased and prolonged immune responses when exposed to T cell dependent antigens whereas blocking of FcγRIIB in humans induces spontaneous maturation of DC [194, 195].

Induction of ANoA in HgIA is dependent on the expression of certain specific H-2 haplotypes [139], and loss of FcγRIIB could not override this restriction as shown in BALB/c mice (paper I) and in B6 mice [175].

Role of activating and inhibitory FcγRs for serum IgG1 and IgE induction

Both BALB/c (H-2^d) and DBA/1 (H-2^q) mice deficient for the Fcγ-chain or the FcγRIII, respectively, lacked the serum IgG1 increase characteristic for HgIA seen in strains with different H-2 haplotypes. This shows that lack of serum IgG1 increase in FcγRIII deficient mice is linked to the genetic background and not H-2. Previous studies showed that IL-12 treatment inhibits Hg-induced serum IgG1 increase [172]. We therefore hypothesised that lack of a serum IgG1 response in Fcγ-chain- or FcγRIII-deficient mice was due to enhanced IL-12 expression. However, we could not find any difference in the expression of IL-12p35 mRNA expression between wt mice and FcγRIII deficient mice. IL-12p35 has previously shown to excert only minor effects on HgIA development [153]. We did, however, find a significant difference in the expression of IL-21 mRNA between wt mice and FcγRIII deficient mice. One of the essential activities of IL-21 is to regulate B cell responses and it is thought to directly or indirectly contribute to disease manifestations in several murine lupus models by inducing both hypergammaglobulinemia and autoantibody production [153, 196, 197]. IL-21 specifically influences Th2 immunoglobulin regulation and enhances IgG1 secretion while down-regulates serum IgE [198, 199]. Therefore, a likely explanation for the lack of serum IgG1 increase in Hg-treated FcγRIII deficient mice is reduced IL-21 expression.

Another explanation for the lack of a distinct serum IgG1 increase following Hg treatment could be the restricted binding of IgG1 to FcγRIII [48]. This

might cause suboptimal presentation of IC containing IgG1 abs in FcγRIII deficient mice, as DC are more efficient antigen-presenting cells if they express activating FcγRs [88, 187, 188], which is followed by reduced serum IgG1 concentrations. Although reduced secretion of antigen-specific IgG have been reported previously in FcγR deficient mice [88] in CIA, FcγRIII deficient mice show collagen-specific serum IgG concentrations comparable with wt mice [107].

These two explanations for the lack of serum IgG1 in mice deficient for activating FcγRs are somewhat contradicting. IL-21 inhibits DC maturation as well as proliferation [200] but IL-21 can also modify the ability of DC to enhance Natural Killer T cell production of IFN-γ [201], the cytokine necessary for development of all manifestaions in HgIA [152].

In contrast to the differences in serum IgG1 response in wt mice and FcγRIII deficient mice, the serum IgE response is similar in the two strains indicating differences in the signalling mechanism between the two responses. This hypothesis is supported by the fact that serum IgE but not serum IgG1 is inhibited by blocking the ICOS signalling pathway [174]. This indicates that direct activation of T cells is necessary for Hg-induced IgE secretion.

The inhibitory FcγRIIB regulates the serum levels of IgG1 preferentially but also suppresses IgG2a, IgG2b and IgE in HgIA. This is in agreement with previous findings showing both a general up-regulation of Igs [92], a specific up-regulation of anti-collagen IgG abs in CIA [107] and serum IgG1 in pristane-induced lupus [101] following loss of FcγRIIB. The presence of FcγRIIB on plasma cells and the role of FcγRIIB to act as a distal check point in the ab response by inducing apoptosis through binding of IC, discussed above, is likely the mechanism [191] behind the enhanced hypergammaglobulinemia response shown in mice lacking FcγRIIB [87].

The fact that FcγRIIB show greatest effects on serum IgG1 in autoimmune disorders (see above) is in agreement with the low A/I-ratio for IgG1 and that lack of FcγRIIB therefore impacts most strongly on the IgG1 isotype [73].

Activating FcγRs and mRNA cytokine expression in HgIA

Th1

Previous studies by other [152, 167] as well as our group [170] have shown that IFN-γ is necessary for the induction of ANoA although presence, more than a marked increase of IFN-γ, seems to be important. We speculated that the IFN-γ secretion could be diminished in mice lacking FcγRIII. However, we were not able to detect any differences specifically in IFN-γ when mRNA expression was compared between wt mice and FcγRIII deficient mice. A limited number of mice were available for the studies, which might have influenced the ability to show a significant difference. We could, on the other hand, detect a reduced expression of Th1 mRNA cytokine and inflammatory mediators in FcγRIII deficient mice compared to wt mice. This might therefore be the mechanism, or a contributing mechanism, behind the delayed and attenuated ANoA response in Hg-treated FcγRIII deficient mice.

Th2

Both wt mice and FcγRIII deficient mice showed increased IL-4 mRNA expression in lymph nodes after 4 days of Hg treatment, which tallies with previous findings [170] including the narrow peak of serum IgE also present in both wt mice and FcγRIII deficient mice. The increase of IL-4 in wt mice can explain the presence of IgE despite the increase seen in IL-21 (see below), which in general is associated with serum IgG1 increase and serum IgE

decrease. The outcome of B cell proliferation in response to IL-4 and IL-21 is influenced by CD40 [197], a co-stimulatory pathway also important in HgIA [153]. This is also influenced by the fact that IL-4 secretion shows a stronger stimulatory effect on serum IgE secretion contra IgG1 secretion [202]. Presence of IL-4 and IL-21 together with CD40 engagement directs the B cell to a more IgE secreting phenotype. This may explain the presence of both an increase in serum IgG1 and IgE response seen in wt mice treated with Hg.

Th17

Th17 cells have emerged as a very interesting cell type for induction of autoimmunity [129, 135]. The expression of Th17-related cytokines and inflammatory markers have not been studied previously in HgIA.

Only IL-21 mRNA expression, among the Th17 cytokines and inflammatory mediators analysed, increased during Hg treatment in wt mice. This response was not seen in FcγRIII deficient mice and might have influenced the difference in serum IgG1 production in wt mice and FcγRIII deficient mice (see above). IL-6 and IL-23R mRNA increased in FcγRIII deficient mice during Hg treatment although not significantly compared to wt mice, which indicates a complex influence of FcγRIII on the Th17 response in HgIA. The Th17 response due to Hg seems to be dependent on dose as IL-6 deficient mice given a low dose did not develop ANoA [156] whereas a higher dose allowed ANoA development in this strain [52].

NK cells

NK cells are reduced and express a low affinity FcγRIII variant in some SLE patients [203]. NK cells are the major initial sources of IFN-γ in immune responses [204]. The absolute need for of IFN-γ in ANoA development [152] and the indication that NK cells may be responsible for this secretion of IFN-γ [52], coupled with the fact that IFN-γ is optimally secreted in the presence of FcγRIII [169], made us interested in evaluating the levels of IFN-γ in FcγRIII deficient mice. However, although the level of IFN-γ mRNA expression decreased during the Hg treatment in FcγRIII deficient mice in contrast to wt mice, where the levels of IFN-γ were stable, the decrease was not statistically significant. Therefore the attenuated ANoA response seen in FcγRIII deficient mice could not be shown to be associated with reduced expression of IFN-γ from NK cells.

CONCLUDING REMARKS

The immune system has evolved to defend the organism by discriminating between self and non-self. Multiple checkpoints have been found to regulate the induction and progression of an immune response. One of the checkpoints is the expression of activating and inhibitory FcγRs [73]. These receptors regulate the immune response by a balanced expression of activating and inhibitory FcγRs and an altered balance may induce autoimmune disorders [104, 205].

In this thesis we have studied the role of FcγRs in systemic autoimmunity induced by mercury. The use of mice deficient for the different FcγRs have revealed that activating FcγRs are important for development and for the titre level of IC in tissues. Lack of activating FcγRs also increased development CIC in serum. Activating FcγRs were important for development and the level of autoantibodies and serum IgG1. Activating FcγRs were as well important for the induction of Th1 cytokines and inflammatory markers and IL-21.

Inhibitory FcγRIIB were important for the regulation of serum IgG1 and IgE but only showed minor effects on the autoantibody production.

We therefore conclude that both activating and inhibitory FcγRs are important for full-blown induction of HgIA and that an altered balance of these receptors can affect the induction.

The knowledge reached in this thesis has given us another piece to the big puzzle of systemic autoimmunity on the way to explore the mechanisms of this disorder.

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