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MODULATION OF EFFECTOR FUNCTIONS OF NK CELLS BY HUMAN PURIFIED IMMUNOGLOBULIN G AND RECOMBINANT PROTEIN ANALOGS

MASTER THESIS

VOLUME 1

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Abbreviations

7-AAD	7-aminoactinomycin D
Ab	Antibody
ADCC	Antibody Dependent-Cell Cytotoxicity
Ag	Antigen
ATCC	American Type Culture Collection
BATDA	bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate
BCR	B cells receptor
BSA	Bovine Serum Albumin
BSI	British Society for Immunology
BV421	Brilliant Violet 421
BV605	Brilliant Violet 605
c	Control tube
CBA	Cytometric Bead Array
CD	Cluster of differentiation
CDC	Complement-dependent Cytotoxicity
DPBS	Dulbecco's Phosphate-buffered Saline
E:T	Effector to target
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic
Fab	Fragment Antigen-binding
FACS	Fluorescence-activated Cell Sorting
FasL	Fas Ligand
FBS	Fetal Bovine Serum
Fc	Fragment Crystallizable

FcαR	Fc-alpha Receptor
FcδR	Fc-delta Receptor
FcεR	Fc-epsilon Receptor
FcγR	Fc-gamma Receptor
FcμR	Fc-mu Receptor
FcR	Fc Receptor
FCS	Fetal Calf Serum
FcγRIIIA	Fc-gamma receptor III A
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-macrophage Colony Stimulating Factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HEX	Hexamer
$HEX_{low}R2$	Hexamer with low binding to FcγR2
$HEX_{low}R3$	Hexamer with low binding to FcγR3
IFN	Interferon
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
ITP	Immune Thrombocytopenia
IVIG	Intravenous Immunoglobulin G
MCP-1	Monocyte Chemoattractant Protein-1
MHC	Major Histocompatibility Complex
MONO	Monomer
NK	Natural Killer
ON	Overnight
PAMP	Pathogen-associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PE	Phycoerythrin
Pen/Strep	Penicillin Streptomycin
PRR	Pattern Recognition Receptor
RBC	Red Blood Cell
rec	Recombinant
RNase	Ribonuclease
RT	Room Temperature
SD	Standard Deviation
Signif	Significance

Th	Tyrosine Hydroxylase
TLR	Toll-like receptors
TNFalpha	Tumor Necrosis Factor alpha
TRAIL	TNF-related Apoptosis-inducing Ligand

Resumo

As células natural killer (NK) têm um papel fundamental na resposta inata do sistema imunitário através da eliminação de células infetadas com vírus, células em situações de stress, células cancerígenas e células estranhas ao corpo sem necessidade de reconhecimento de antigénios. As células NK eliminam os seus alvos através de mecanismos de citotoxicidade direta, citotoxicidade celular dependente de anticorpos (ADCC) e através de vias como FasL/TRAIL, bem como através da produção de citocinas, incluindo interferon-gama (IFN-γ) e o fator de necrose tumoral (TNFalpha).

A perda da capacidade do sistema imunitário de diferenciar entre antigénios "próprios" e "não-próprios" é a principal causa relacionada com autoimunidade. Para além de corticoides e fármacos imunossupressores, uma segunda linha de tratamento para patologias autoimunes são as imunoglobulinas intravenosas (IVIG).

A grande necessidade das IVIG juntamente com a disponibilidade limitada de plasma humano, a matéria-prima para a purificação das IVIG, resultou numa escassez a nível mundial, que se agravou com a pandemia da Covid-19. Uma alternativa é a produção de imunoglobulinas G recombinantes (IgG), como por exemplo um hexâmero (HEX) composto pela porção Fc da subclasse IgG1 fundido com a parte final (μ-tailpiece) de uma imunoglobulina M (IgM). No entanto, antes de se realizarem estudos clínicos com esta molécula, será necessário realizar estudos mecanísticos adicionais.

Esta tese teve como objetivo principal:

(i) Estabelecer ensaios de citotoxicidade direta e citotoxicidade dependente de anticorpos (ADCC) para explorar o efeito das IVIG ou de moléculas humanas IgG recombinantes e seus análogos na atividade citotóxica das células NK.

(ii) Caracterizar o perfil de citocinas libertadas pelas células NK na presença das IVIG e de moléculas humanas IgG recombinantes.

Inicialmente foram estabelecidas as condições experimentais com o intuito de ter ensaios de citotoxicidade direta e de ADCC confiáveis, utilizando linhas celulares K562 e Daudi como alvos, respetivamente. De seguida, foi avaliado o impacto das IVIG e das moléculas IgG recombinantes e os seus análogos na atividade citotóxica das células NK. Por fim, através da utilização de ensaios de cytometric bead array (CBA), o perfil de citocinas libertadas pelas

células NK após incubação noturna com IVIG e das moléculas IgG recombinantes com diferentes afinidades aos recetores das NK ($HEX_{low}R3$, $HEX_{low}R2$ and MONO) foi caracterizado. Os resultados desta tese demonstram que o HEX é o inibidor mais potente dos ensaios de ADCC utilizando um anticorpo monoclonal anti-CD20 e células Daudi como alvo. No caso de ensaios de citotoxicidade direta, as IVIG induziram maior inibição. Também se observou que o HEX, na concentração mais alta testada (0.25 mg/ml), aumentou os níveis de citocinas libertados pelas células NK; em particular, a secreção de TNFalpha foi maior em comparação com IFN-γ, apesar de terem sido detetadas diferenças entre os diferentes dadores.

Em suma, os resultados apresentados nesta tese contribuíram para uma melhor compreensão dos mecanismos de ação das IVIG e das moléculas $_{rec}$IgG e os seus análogos. As isoformas do HEX poderão ter bastante potencial clínico em termos de tratamento de doenças desencadeadas por autoanticorpos.

Palavras-chave: Anticorpos, NK cells, ensaios funcionais, modulação

Abstract

Natural killer (NK) cells play a fundamental role in innate immune responses by eliminating virally infected, stressed, cancer and foreign cells in the absence of antigen presentation. NK cells eliminate their targets by direct cytotoxicity, antibody dependent-cell cytotoxicity (ADCC) and death ligand pathways (FasL/TRAIL) mechanisms in addition to the production of cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNFalpha).

The loss of the immune system's capacity to differentiate between “self” and “nonself” antigens is the primary cause of autoimmunity. Beside corticosteroids and classical immunosuppressive drugs, a second line of treatment for autoimmune pathologies are intravenous immunoglobulins (IVIG).

The high clinical demand for IVIG along with a limited supply of human plasma, the raw material for the purification of IVIG, has resulted in a worldwide shortage, which has been aggravated by the Covid-19 pandemics. An alternative is to manufacture recombinant immunoglobulin G (IgG), for example a hexamer (HEX) composed of IgG1 subclass Fc portions fused to an IgM µ-tailpiece. However, before clinic studies can be conducted with this product, additional mechanistic studies are needed.

The overall goal of this thesis was:

(i) To establish reliable direct cytotoxicity and ADCC assays to further explore the impact of IVIG or recombinant human IgGs molecules and analogs on the cytotoxic activity of NK cells.

(ii) To characterize the cytokine profile released by NK cells in the presence of IVIG and recombinant human IgG molecules.

First, it was established the detailed experimental conditions for reliable direct cytotoxicity and ADCC assays using the K562 and Daudi cell lines as target cells, respectively. Further, the impact of IVIG or recombinant human IgGs molecules and analogs on the cytotoxic activity of NK cells was assessed. Finally, using cytometric bead array (CBA) assays it was characterized the cytokine profile released by NK cells after overnight incubation with IVIG or $_{rec}$IgGs molecules and analogs of $_{rec}$IgG with different affinities to NK cells’ receptors ($HEX_{low}R3$, $HEX_{low}R2$ and MONO).

This thesis findings show that HEX is the most potent inhibitor in ADCC assays using a monoclonal anti-CD20 antibody and Daudi cells as targets. In the case of direct cytotoxicity assays, IVIG induced the greatest inhibition. It was also observed that HEX, at the highest concentrations tested (0.25 mg/ml), increased the amounts of cytokines released by NK cells; in particular, TNFalpha secretion was higher in comparison with IFN-γ, although interindividual differences were detected among the donors.

Overall, the results of this thesis contribute to a better understanding of the mechanisms of action of IVIG and $_{rec}$IgG molecules and analogs. HEX isoforms may have a potential in the clinic as treatment for autoantibody-mediated diseases.

Key words: Antibodies, NK cells, functional assay, modulation

Introduction

The immune system has an important role in protecting the human body from potentially harmful pathogens, substances, and cell mutations, and it has various lines of defense to serve that purpose. However, when the immune system fails, it can result in diseases such as recurrent infections, autoimmunity, allergy, and cancer. [4]

The skin, mucosal surfaces, sebaceous glands, tears, and saliva are the first physical defenses of the human body to protect it against infection. The first contact of the pathogen with the body is the skin, which is comprised of keratinized cells that form a thick layer, named epithelium, forming the outer surface of the body. In case of wounds, burns, surgical procedures, or other physical or chemical damage, this layer is disrupted, exposing the soft tissues, and providing an easy way for pathogens to enter the human body. At this point, the immune system enters into action. In terms of the immune response, there are innate and adaptive immunity and the immune response differs depending on which is activated. [5] Within innate and adaptive response, several cell types of leukocytes are involved, including granulocytes, monocytes, and lymphocytes.

Leukocytes, also known as white blood cells, are one of the key components of the immune system and are derived from a common progenitor, the pluripotent hematopoietic stem cell.[6] Pluripotent hematopoietic stem cell can further divide, mature, and differentiate into one of three lineages: erythroid, myeloid, and lymphoid, as shown in **Figure 1.** The common lymphoid progenitor can differentiate into B cells, T cells, and Natural Killer (NK) cells. In case of infection, B cells can further mature into plasma cells and secrete specific antibodies. Finally, the common myeloid progenitor differentiates into granulocytes (neutrophils, eosinophils, and basophils), monocytes, and dendritic cells. [7]

All these cells of the immune system serve different and specific functions. These cells can be divided according to their innate or adaptive function.

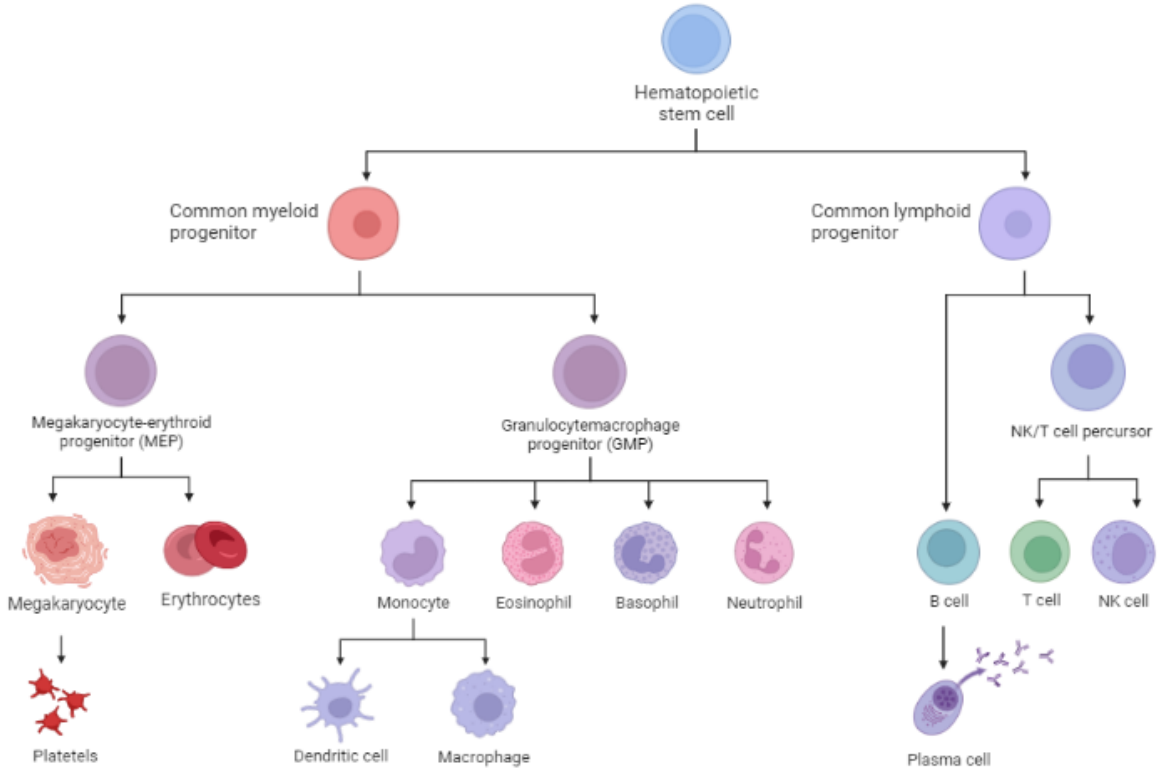


Figure 1. Blood cells derived from a common hematopoietic stem cell. The pluripotent stem cell differentiates to myeloid, erythroid, and lymphoid lineages. The myeloid progenitor differentiates into three types of granulocytes (eosinophil, basophil, and neutrophil), and monocytes that will further give rise to dendritic cells and macrophages. The lymphoid progenitor differentiates in B cells, T cells and NK cells. B cells give rise to plasma cells that secrete specific antibodies in case of infection. (Created in BioRender).

Innate and adaptive immunity

The first line of defense is innate immunity, comprising physical barriers and chemical defenses, as described above; and cellular defenses, like phagocytes, NK, dendritic cells, and mast cells. Innate immunity entails a rapid response within minutes of exposure to a threat, by the secretion of an extensive nonspecific array of signaling molecules. This response has no immunologic memory, can last from two to fourteen days, and if it lasts too long, it can cause tissue damage in the host. The pattern recognition receptors (PRR), pathogen-associated molecular patterns (PAMPs) and danger signals or alarmins (DAMPs) are signaling pathways used by the innate immune cells as a defensive response. The innate immunity is not very specific, since it depends on the recognition of conserved molecular structures common to many pathogens but absent in the host, stimulating an inflammatory response.[8]

After the first contact with the pathogen, the innate immune system usually induces the second line of defense or adaptive immunity.

The adaptive immune response provides long-lasting protection and is highly specific to the pathogen that induced the response.[9] However, this response is not immediate. The pathogens' surface antigens are the eliciting substances that will induce an immune response.[5] Leukocytes, especially lymphocytes, are the main cells involved in this immune response. Lymphocytes B or T cells can enhance antibody responses as well as cell-mediated immune responses. When the antibody response is activated, B cells secrete antibodies, called immunoglobulins (Ig), which circulate in the bloodstream and permeate other body fluids, where they bind specifically to the foreign antigen that stimulated their production. When the cell-mediated immune response is activated, T cells will react directly against the antigen presented to them via major histocompatibility complex (MHC) class-I or -II by professional antigen presenting cells.[10] Finally, the adaptive immune system generates memory cells, allowing the host to recognize and fight the pathogen faster upon a second infection.

The major differences between these two types of immunity are highlighted in **Table 1**.

Table 1. Main characteristics of Innate and Adaptive immunity.

	Innate immunity	***Adaptive immunity***
Response time	Immediate reaction	Delayed reaction
	General defense	Specific defense
Specificity	Non-specific	Specific
	Always present	Requires activation
Memory	Absent	Present
Cells involved	Macrophages	Antigen presenting cells (dendritic cells)
	Dendritic cells	
	Phagocytes	B cells
	Neutrophils	T cells
	NK cells	

Natural Killer cells

NK cells belong to the lymphoid lineage and represent 5-10% of all peripheral blood

lymphocytes and play a fundamental role in innate immune responses.[11] NK cells are characterized by the lack of CD3 and the expression of CD56 and CD16. Two subsets have been characterized, based on the expression of these last two surface markers, $CD56^{bright}$ $CD16^{dim/-}$ and $CD56^{dim}$ $CD16^{+}$.[12] The Fc-gamma receptor III (FcγRIIIA/CD16), which is an activating receptor, plays an important role in killing infected antibody-coated target cells through antibody-dependent cellular cytotoxicity (ADCC).[13] The repertoire of cytotoxic components of NK cells allows them to directly lyse infected cells, first by releasing cytoplasmic granules containing perforin and granzymes, and second through a non-secretory via based on FasL/TRAIL contact. [14]

NK cells can kill fast and effectively virally infected, stressed and cancer cells in the absence of antigen presentation, which distinguishes them from cytotoxic of CD8 T cells.[15] Contrary to monocytes, neutrophils, or macrophages that phagocyte infectious particles, NK cells produce granules containing lytic molecules, such as perforin and granzymes that induce cell lysis. When activated, NK cells also release cytokines and chemokines, which influence the inflammatory environment.[16] Tumor necrosis factor alpha (TNFalpha) and interferon-gamma (IFN-γ) are the most important cytokines produced by NK cells. These two cytokines have a major role in the clearance of virally infected and tumor cells since they can induce apoptosis. Therefore, NK cells' cytotoxic properties make them very relevant in the clinical context, especially for viral infections, transplantation and cancer immunotherapy.[17,18]

The NK cell's potential to kill target cells is determined by a balance of signals from activating and inhibitory receptors that can monitor the ligands expressed by cells throughout the body. The recognition of self MHC class I molecules on potential target cells is the major mechanisms to induce a strong inhibitory signal to NK cells, suppressing their lytic activity. Allogenic, virus-infected or tumor cells, on the other hand, increase the activating signal or have their MHC class I expression downregulated and can no longer deliver a strong inhibitory signal, leading to the killing of the target cell by direct NK cytotoxicity, as shown in **Figure 2**.[2,19]

Another feature of NK cells is their ability to induce immunoregulatory signals through the secretion of specific cytokines, such as IL-5, IL-10, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF). [20]

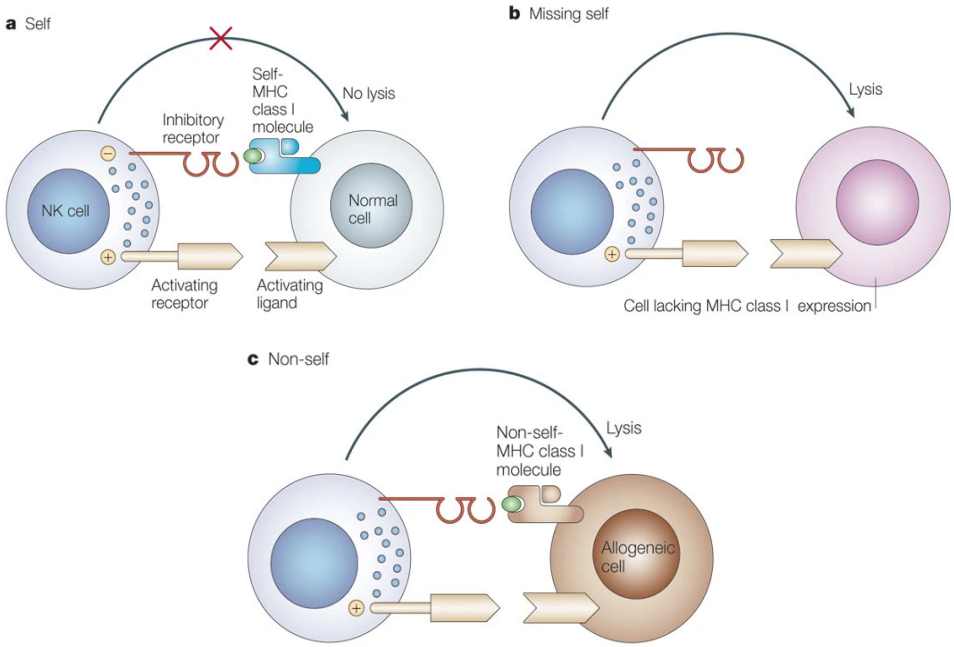


Figure 2. How NK cells recognize "self" from "non-self". NK cell's potential to kill target cells is determined by a balance of signals from activating and inhibitory receptors **(A)** when interacting with a normal target cell and despite receiving activating signals, NK cell does not lyse the cell due to inhibitory signals from the NK cell's surface ligated MHC-binding receptor, which recognizes the self MHC-class I molecule. **(B)** In case the target cell does not have MHC-class I molecule as a result of viral infection or transformation, the MHC-binding inhibitory receptor is not activated, and thus the NK cell does not receive inhibitory signals, resulting in cell lysis. **(C)** For instance, an allogenic target cell expressing foreign non-self MHC-class I molecule does not engage all the inhibitory receptors leading to lysis by NK cells. (Adapted from [2])

Immunoglobulins

Immunoglobulins (Ig), also known as antibodies (Ab), are glycoproteins having a Y shape, produced by memory B and plasma cells, which can be found in human serum. Igs are a heterogeneous mixture with different Ag recognition specificities. Igs are homodimers, composed of a fragment antigen-binding (Fab) region and a fragment crystallizable (Fc) region. This region identifies the different isotypes: IgG, IgA, IgM, IgD, and IgE. Igs are present in different concentrations in human serum (IgG > IgA > IgM > IgD > IgE) and each one has distinct functions briefly described in **Figure 3**. The major function of Igs are antigen binding via the Fab region (humoral activity) and Fc-mediated effector functions (blocking, complement-dependent cytotoxicity - CDC - and ADCC). [21]

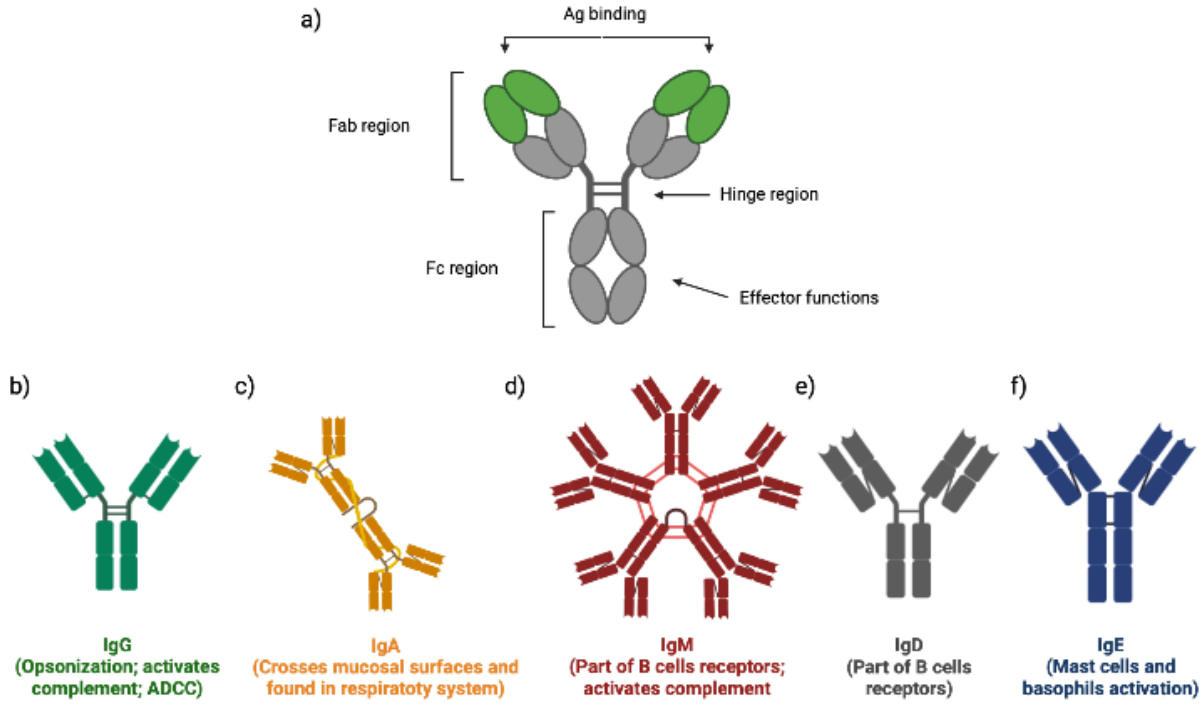


Figure 3: Immunoglobulin structure and different functions of each isotype. On top is represented **(A)** the structure of an immunoglobulin, with both Fab fragments, where the antigens bind the Fc part, responsible for the effector functions and the hinge region. Below it is represented the different isotypes of immunoglobulins that can be found in the human serum ordered based on its availability percentage. **(B)** IgG opsonizes infected target cells, activates complement and antibody-dependent cellular cytotoxicity (ADCC) by NK cells; **(C)** IgA crosses the mucosal surfaces and are found in the respiratory system; **(D)** IgM is part of the B cells receptors (BCR) and activates complement; **(E)** IgD is part of BCR and **(F)** IgE provokes mast cells and basophils activation. (Created in BioRender)

These Igs can modulate the immune response through the interaction of their Fc part with Fc

receptors (FcR). FcRs are expressed widely throughout the immune system by cells of hematopoietic origin and play an important role in protecting the human body against pathogens. Each Ig isotype corresponds to a different type of FcR with different effector functions. IgA corresponds to FcαR, to IgE corresponds FcεR, to IgG corresponds FcγR, to IgD corresponds FcδR and to IgM corresponds FcμR.[22]A well-balanced immune response is triggered by positive and negative signals that affect both innate and adaptive immune cells. [23]Homeostasis is very important, and the FcRs can either activate or inhibit immune response.

The primary cause of autoantibody (autoAb) development is a loss of the immune system's capacity to differentiate between “self” and “non-self” Ags. AutoAbs are Abs produced by the immune system that specifically react with “self” Ags. As a result, autoAb are at the root of many autoimmune diseases. AutoAbs can bind to the FcγR expressed on NK cells (*i.e.*, CD16/FcγRIIIa) and trigger ADCC. In some cases, NK cells secrete cytokines that recruit and activate other immune cells, indirectly increasing inflammation and tissue damage. [24]

Direct cytotoxicity

NK cells recognize the target cell with the receptors expressed on the surface, triggering signaling pathways in the effector cell leading to the death of the target cell. The self-missing MHC-I trigger this type of cytotoxicity. The pathways that include the killing of the target cells include the release of cytotoxic granules such as perforin and granzyme, TNFalpha family receptors signaling and pro-inflammatory cytokines release such as IFN-γ. The cytotoxic granules and the TNFalpha family receptors induce target cell apoptosis and IFN-γ upregulates FasL/TRAIL expression on the surface of NK cells, as shown in **Figure 4**. [25]

Antibody-dependent cellular cytotoxicity

For antibody-dependent cellular cytotoxicity (ADCC), it is necessary that Ab bound to a target cell and interact with FcγR of the effector cell. CD16/FcγRIIIa is the major FcγR found on human NK cells, it is an activating receptor with a potent cytotoxic and cytokine production capacity. [26] Ab binding creates a crosslinking between the target cell and the effector cell, which induces a signaling cascade, similar to direct cytotoxicity, and eventually target cell death, as shown in **Figure 4.** [25]

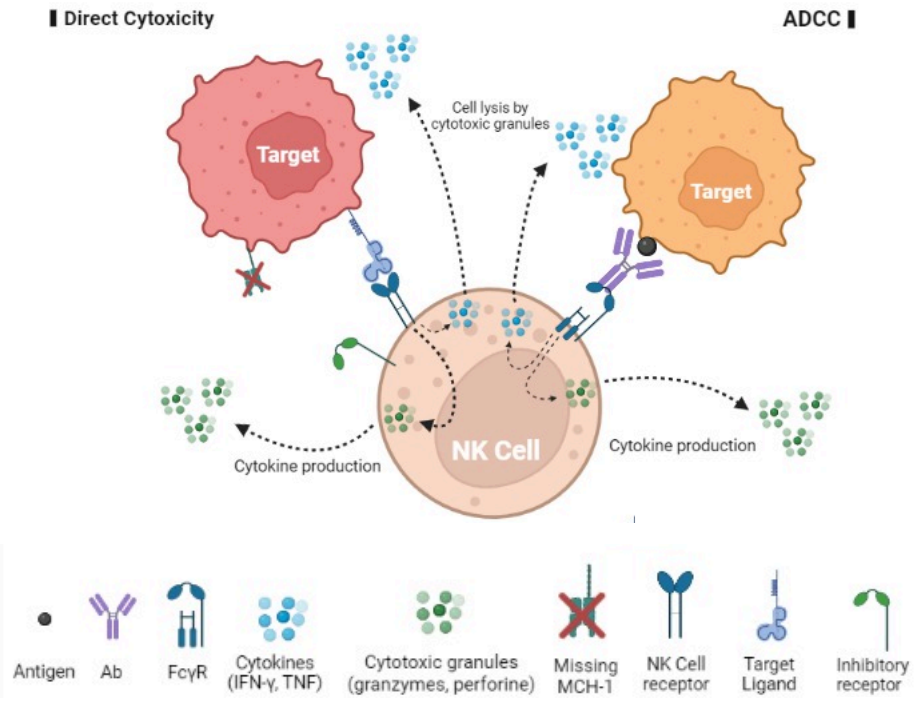


Figure 4. NK cells mediate direct cytotoxicity and antibody-dependent cellular cytotoxicity. NK cells express numerous inhibitory and activating receptors and their balance define the fate of target cells. In case of **direct cytotoxicity**, if a target cell does not express MHC class I molecule activating receptors will send a strong signal to NK cells leading to the secretion of cytokines and cell lysis by cytotoxic granules. In case of **ADCC**, the binding of an Ab to the antigen in the target cell and FcγR in the effector cell leads to the killing of the target by cytotoxic granules and cytokines secretions. (Created in BioRender)

Intravenous immunoglobulin

Therapeutic effects of intravenous immunoglobulin (IVIG) were first studied at the end of the 19th century when it was shown that immunized blood serum from a rabbit protected a naïve rabbit against tetanus disease, and since then, this therapy has come a long way. [27]

Nowadays, IVIG is a well-established treatment for autoimmune diseases, consisting of an isolated and pooled preparation of normal human IgG obtained from thousands (from 1,000 to 15,000) of healthy donors. [28] In addition to IgG monomers (>96%), IVIG preparations contain a small percentage of IgG dimers, IgM, and IgA. IVIG is given at a low dose (400mg/kg body weight) at regular intervals, usually 4 week due to a half-life of approximately 20 days, replacement treatment in primary or acquired immunodeficiencies where IgG plasma concentrations are very low. IVIG is also used to substitute decreased IgG levels secondary due to other medications. Inversely, at larger doses (1–2 g/kg), IVIG is used as an

immunomodulatory therapy in a variety of autoimmune or auto-inflammatory disorders, such as Kawasaki disease and immune thrombocytopenia. [29,30] IVIG has multiple and complex mechanisms of action and a single mechanism may not account for its therapeutic benefit, implying that these mechanisms may work synergistically. [31] These potential mechanisms can be classified into two groups based on whether they are dependent on the Fc or Fab parts of the IgG molecule, as shown in **Figure 5**. [32]

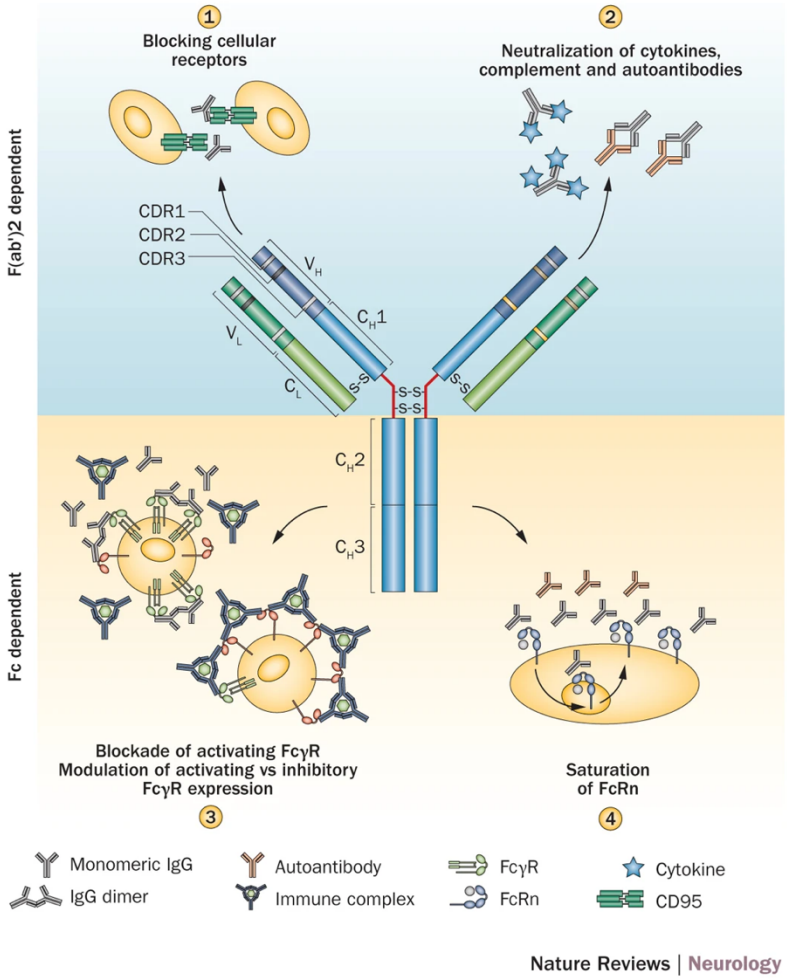


Figure 5. Figure 5. Mechanisms of action of IVIG. IVIG's mechanisms of action can be either Fab or Fc dependent. In the Fab fragment of the immunoglobulin, which determines the specificity of the antibody molecule, two different mechanisms take place: (1) blockade of cell-cell interactions mediated by surface receptors, namely Fas-Fas ligand, that interfere with autoimmune pathology, and (2) neutralization of cytokines, complement and autoantibodies onto the cell surface, hence avoiding complement-mediated tissue damage. Other two mechanisms operate in the Fc fragment, which is critical for the initiation of effector responses, including: (3) blockade of activating FcγRs, modulation of activating and inhibitory FcγRs expression and (4) saturation of FcRn. (Adapted from [3])

The high clinical demand for IVIG for many medical conditions, such as acquired-hypogammaglobulinemia, primary immunodeficiencies, immune thrombocytopenic or solid organ transplantation, along with a limited supply of human plasma, has resulted in procurement issues and worldwide shortages, which have been aggravated by the Covid-19 pandemics that caused a lack of donors of blood and plasma.[33,34] These shortages can severely influence the clinical state of patients due to a significant number of therapy adjustments, which include IVIG course delay, dose decrease, and even treatment termination. The high demand for IVIG products lead to health care institutions acquiring it from commercial manufacturers, where blood donors are paid for giving blood, contributing to an increase in the cost of manufacturing of an already expensive therapy. [35] Depending on the patient's weight, the number of infusions required and hospitalization requirement, IVIG can cost up to 100,000€ per year, making it unaffordable for the average patient. [36] This, combined with limited supply, makes it critical to find new approaches considered a much more cost-effective use of health care resources.

Hexamer

Activation of FcγRs and complement by immune complexes is a common important pathogenic trigger in many autoimmune diseases. Therefore, blockade of these innate immune pathways may be an attractive target for the treatment of immune complex-mediated mechanisms. Many autoantibody-mediated diseases are currently treated with intravenous immunoglobulins.[37]

It is hypothesized that recombinant high-avidity multivalent Fc molecules have the potential for the treatment of autoimmune diseases in replacement of IVIG. Considering their structure, these molecules should achieve effective receptor blockade at lower doses than IVIG. [1,38-40] To this end, a recombinant Fc hexamer (termed Fc-μTP-L30C) was designed to fuse the IgM μ-tailpiece to the C terminus of human IgG1 Fc, as shown in **Figure 6**.[1] It has already been reported to bind with high avidity to FcγR and thereby to inhibit Fc receptor-mediated effector activities, demonstrating its potential therapeutic usefulness.[1,38-40]

The potential benefits of substituting IVIG with recombinant IgG molecules warrant further investigation. Primarily, healthcare systems would gain greatly from this substitution since IVIG has a limited supply and high related expenses, whereas recombinant IgG molecules allow producing large volumes of recombinant products with uniform quality regardless of plasma availability. More importantly, this might significantly improve the quality of life for patients who require frequent IVIG infusions, considering its higher efficacy is achieved with significantly lower doses that may be administered subcutaneously from the comfort of the patient's home. [41]

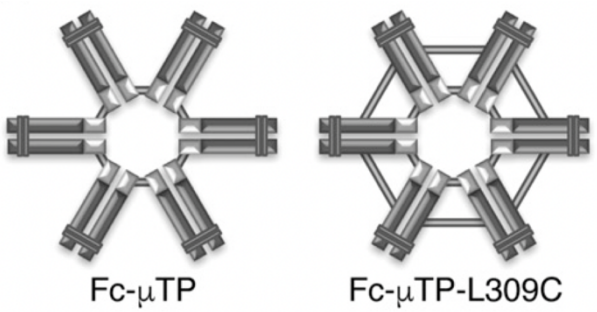


Figure 6. Recombinant IgG molecule's structure. Schematic representation of Fc-μTP and Fc-μTP-L309C structure. Light grey represents the IgM tailpiece, with the Fc fragment of human IgG1 represented in dark grey. The disulfide bounds are represented forming a hexamer around the IgG1 Fc fragment of Fc-μTP-L309C. (Adapted from [1])

Goal

The overall goal of this thesis was to better understand the molecular mechanisms involved in the antibody target cell destruction mediated by natural killer (NK) cells in the presence of external recombinant immunoglobulins (IgGs). In this context, the impact of intravenous immunoglobulins (IVIG) or recombinant human IgGs ($_{rec}$IgGs) and analogues on the cytotoxic activity of NK cells was assessed.

In order to achieve this goal, the following specific aims (SA) were defined:

SA1. To compare the effect of IVIG and $_{rec}$IgGs on NK cytotoxicity

For such, the standardization of two main techniques used to test ADCC and direct cytotoxicity by NK cells with IVIG and $_{rec}$IgG was necessary:

SA1.1. To establish a reliable ADCC assay to elucidate the interactions between the Fc region of $_{rec}$IgG and IVIG with the Fc-gamma receptor IIIa (CD16/FcγRIIIa) expressed on NK cells in the presence of anti-CD20 monoclonal antibody obinutuzumab, and the Daudi target cell line.

SA1.2. To establish a reliable direct cytotoxicity assay using the K562 target cell line in the presence of IVIG and $_{rec}$IgG .

SA2. To characterize the cytokine profile released by NK cells in the presence of IVIG and $_{rec}$IgG molecules.

To elucidate the cytokines released by NK cells after overnight incubation with IVIG or $_{rec}$IgGs molecules and analogs.

Materials and methods

Reagents

Antibodies. Details for the antibodies used to assess NK cell purity by flow cytometry are provided in **Table 2**. Before use, the optimal working dilution was established.

Biologicals. The intravenous immunoglobulins (IVIG), Monomer (MONO), Hexamer (HEX), Hexamer with low binding to FcγR3 ($HEX_{low}R3$), and Hexamer with low binding to FcγR2 ($HEX_{low}R2$) were purchased from CSL Behring AG (Bern, Switzerland). [1] The humanized anti-CD20 antibody was provided by Roche Innovation Center, Zurich (Zurich, Switzerland).

Chemicals. Ethylenediaminetetraacetic (EDTA) was acquired from Sigma-Aldrich® (St. Louis, MO, US). 7-aminoactinomycin D (7-AAD) was purchased from BD Pharmingen (San Diego, CA, USA).

Culture media and buffers. RPMI-1640, AIM-V®, Dulbecco's phosphate-buffered saline (DPBS), Penicillin streptomycin (Pen/Strep), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchase from Gibco (Grand Island, NY, USA). L-Alanyl-L-Glutamine was acquired from Bioswisstec (Schaffhausen, Switzerland); and Ficoll-Paque Plus from GE Healthcare (Uppsala, Sweden). Fetal bovine serum (FCS), Bovine Serum albumin (BSA) and probenecid were from Sigma-Aldrich®. MACS® buffer consisted of 0.5% BSA and 2mM of EDTA in DPBS, followed by 30min de-bubble using the vacuum pump, aliquoted and kept at 4°C for future experiments. FACS buffer was prepared by adding 0.1% of BSA and 0.05% of NaN_3 in DPBS.

Kits. Human NK cell isolation kit was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), DELFIA® kit from PerkinElmer® (Waltham, Massachusetts, United States), and QIAmp DNA Blood Mini Kit was purchased from Qiagen (Qiagen, Hilden, Germany). Cytometric Beads Array (CBA) Human Th1/Th2 Cytokine Kit II from R&D Systems (Minneapolis, Minnesota, United States) was kindly provided by the flow cytometry core facility of the University of Geneva.

Ethical issues. This project was approved by the Regional Research Ethics Committee (CCER)

(CCER # 2018-00552).

Cell lines and culture conditions

The cell lines Daudi (ATCC CCL-213) and K562 (ATCC CCL-243) were purchased from American Type Culture Collection (ATCC®). Daudi cells are B lymphoblasts derived from a 16-year-old male with EBV-related Burkitt's lymphoma. Daudi cells express CD20, an extracellular membrane channel involved in the regulation of cellular calcium influx necessary for the development, differentiation, and activation of B-lymphocytes.[42] [43] [44] On the other side, K562 derives from a pleural effusion of a 53-year-old female with chronic myelogenous leukemia, having the particularity of lacking MHC class-I, making them susceptible to the attack of NK cells.[45]

Both cell lines grow in suspension in a culture media consisting of RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 100μg/ml streptomycin, 100U/ml penicillin and 2mM of L-Alanyl-L-Glutamine. Cells were kept in culture at 37°C and 5% CO_2 humidified incubator. Daudi and K562 were seeded at 0.15 × 10^6 cells/ml and split every four and three days, respectively.

Peripheral blood mononuclear cells (PBMCs) isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Paque gradient centrifugation from buffy coats, a white blood cell-rich concentrate prepared by centrifuging 500ml of blood token from a healthy donor, as shown in **Figure 7**. Each Buffy coat was diluted 1:4 ratio 0.2% EDTA in DPBS, layered over the Ficoll-Paque gradient and centrifugated at 900 × *g* for 20min at room temperature (RT) and no brake. Plasma (upper layer) was aspirated leaving the thin white layer where PBMCs are located. Next, PBMCs were collected, resuspended with DPBS containing 0.2% EDTA and spun at 200 × *g* for 10min. Two extra washes were performed at 300 × *g* for 10min to remove remaining platelets. Finally, the cell pellet was resuspended, counted and when necessary, adjusted cell concentration of 250x10^6 PBMCs for the NK isolation.

In those cases where red blood cells (RBC) were present, lysis was performed after PBMC collection. For that, 18ml of sterile H_2O and 2ml of 10 × DPBS were added to resuspend the cell pellet, for a maximum of 30sec. The rest of the protocol was maintained as described

before.

NK cells isolation

Following PBMCs isolation, NK cell purification was performed using the Miltenyi NK cell negative isolation kit, according to the manufacturer's instructions.

On the day of the isolation, the PBMC cell number was determined, spun at 300 × *g* for 10min, and kept at 4°C during the entire protocol, as well as all the medium used. NK cell Biotin-Antibody Cocktail followed by NK cell MicroBead Cocktail were incubated at 5min and 10min, respectively, to label the antigens not expressed by NK cells (negative isolation). Next, the cell suspension was applied to the column, and the flow-through containing enriched unlabeled NK cells, was collected.

Determination of the purity of NK cells by flow cytometry

Both, the samples and FACS buffer, were kept on ice and light protected.

PBMCs and NK cells were seeded in a V-bottom 96-well plate and spun at 350 × *g* for 7min. Each well was resuspended in 50µL of previously prepared antibody mix as represented in **Table 3** and the plate was incubated for 25min at 4°C in the dark. Next, cells were washed twice at 350 × *g*, for 7min. Finally, 7-AAD was added at final concentration of 0.12µg/ml in 300µL of FACS buffer and incubated for 10 min in the dark at RT. Sample was acquired using flow cytometer Attune NxT (ThermoFisher, USA).

DNA extraction

DNA extraction was performed using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany), using a protocol adapted from the manufacturer as shown in **Figure 8.** Briefly, for DNA extraction, RNase was added to an Eppendorf, followed by 100mg/ml of sample and proteinase K. After adding AL buffer, the sample was incubated for 30min at 56°C. Next, 200µL 100% ethanol was added. The sample was applied to the column and spun three times at 24,100 × *g* for 1min, adding the respective volume of AW1 buffer. After, AW2 buffer was added twice and spun for 5min at 24,100 × *g*.

The preparation was transferred into a new collection tube, spun at 24,100 × *g* for 2min and changed to an Eppendorf tube, which was spun for another 2min at 24,100 × *g* following a

2min incubation with pre-warmed water . The samples were then read using a NanoDrop™ spectrophotometer to determinate concentration and kept at -20°C.

ADCC and Direct cytotoxicity assays using fresh NK cells

Two different approaches were investigated during ADCC and direct cytotoxicity, *(1)* the effect of serial dilutions of NK cell numbers to a fixed number of target cells, *i.e.*, different effector to target ratio (E:T); and *(2)* the response of NK cells to different doses of biologicals at a fixed E:T ratio. In both cases, fresh NK cells isolated from buffy coats were incubated overnight (ON, 16h) with IVIG, HEX, $HEX_{low}R3$, $HEX_{low}R2$ or just with culture media.

Different E:T ratios

All the NK cells were plated at 2-fold serial dilutions in AIM-V supplemented with 2% HEPES and incubated ON, to later use at different E:T ratios (from 20:1 to 2.5:1). 2.5mg/ml of IVIGs were added to the ON culture having a control in which IVIG was absent.

Next day, 6×10^6 Daudi or K562 cells, for ADCC or direct cytotoxicity assays, respectively, were labelled with 1.25µL/million cells of BATDA for 20min at 37°C in the water bath and protected from light. After labelling, cells were washed three times, for 4min at 250 × *g* using DPBS with 250mM probenecid (washing buffer). Next, the pellet was resuspended in 1ml of RPMI-1640 with 250mM probenecid and the cells counted.

To set up the ADCC assay, first 0.05µg/ml final concentration of anti-CD20 was added on top of NK cells resting in the 96-well plate from the night before. Followed by the addition of Daudi cells. The 96-well plate was spun at 150 × *g* for 1min before resting for 10min at RT and then placed for 1h in a humidified incubator at 37°C and 5% CO_2. At the end point, the plate was centrifuged for 5min at 500 × *g*, and 20µL of the supernatant were transferred to a flat bottom plate (supplied by the DELFIA® kit). Following addition of 200µL Europium Solution the 96-well plates were incubated at RT for 15min, light protected, on a plate shaker at 100 rpm, and read using EnVision multimode plate reader, by PerkinElmer®.

For direct cytotoxicity, anti-CD20 was omitted, and the target cells replaced by K562 instead of Daudi cells. The rest of the assay conditions remained the same.

Dose response at fix E:T ratio

Fresh NK cells were incubated ON with different concentrations of IVIG, HEX, $HEX_{low}R3$,

HEX_{low}R2 or just media; starting from 0.25 up to 0.00025mg/ml using 10-fold serial dilutions.

The following day, Daudi and K562 cells were labelled as describe before. The E:T ratios for ADCC and direct cytotoxicity were 5:1 and 10:1, respectively.

The assay controls were set up as follows. The positive control (maximum release) consisted of Daudi or K562 which were cultured with AIM-V + 2% HEPES, and 250mM probenecid in RPMI before the addition of 10µl of lysis buffer. The negative control (spontaneous release) consisted of target cells cultured with AIM-V + 2% HEPES and 250mM probenecid in RPMI.

Specific lysis calculi

Both ADCC and direct cytotoxicity assays were expressed as the percentage (%) of specific lysis and calculated by the subtraction of the readout of each assay sample and the spontaneous release, divided by the subtraction of the maximum release and the spontaneous release, respectively.

$$Specific\ lysis = \frac{sample - spontaneous\ release}{maximum\ release - spontaneous\ release} \times 100$$

Percentage of inhibition calculi

In order to calculate the percentage of inhibition, the maximum specific lysis was assumed to represent 100% of killing. Maximum killing was defined as the specific lysis given by the co-culture of NK cells with Daudi and anti-CD20, for ADCC experiments, or NK cells plus K562 in the case of direct cytotoxicity.

$$Inhibition\ (\%) = 100 - \frac{100\ \times\ sample}{maximum\ killing}$$

Cytokine measurement by Cytometric Bead Array (CBA)

NK cells were incubated ON with in the presence of different concentrations of IVIG and IgG recombinant molecules. The following day, 50µL of supernatant was collected and kept at -20°C until analysis by CBA.

This assay was conducted using a V-bottom 96-well plate. Before start, all the reagents reached RT. Standards were reconstituted using assay diluent and serial dilutions were

performed. Capture beads were prepared, vortexed vigorously and added to the sample's wells. Standards and supernatant of the biological samples were added to the plate. Finally, PE detection reagent was added to all the wells. The plate was covered and shacked for 5min at 500 rpm and incubated for 3h at RT and light protected. Next, the plate was centrifuged at 200 × *g* for 5min, and the beads were resuspended in 200µL of wash buffer. The limit of detection of each cytokine was 2.6 pg/ml for IL-2 and IL-4, 3 pg/ml for IL-6, 2.8 pg/ml for IL-10 and TNFalpha and 7.1 pg/ml for IFN-γ.

Statistical analysis

The analysis was performed using GraphPad (version 9.4.0). Comparisons were done by two-way ANOVA with Geisser-Greenhouse correction followed by Tukey's multiple comparisons test when comparisons among different recombinant IgG molecules of interest were performed. Significance is indicated by ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ and ns (not significant), $P \geq 0.05$.

Results

Two functional assays were performed to understand the mechanisms of inhibition of IVIG and other recombinant IgG molecules of interest: ADCC and direct cytotoxicity. For ADCC experiments, the CD20 expressing Daudi cell line and non-glycoengineered obinutuzumab (anti-CD20) were used. For direct cytotoxicity experiments, the MHC class-I lacking K562 cell line was used as target for NK cells.

The purity and viability of NK cells after isolation from buffy coats were optimal for the assays

The effector cells used in all the assays were obtained by negative selection from buffy coats provided by the Geneva Blood Transfusion Center (CTS). Thus, to ensure the quality of the starting material, the purity and viability were checked of the isolated cells, using as a reference the PBMC from the correspondent donor. **Figure 9B** shows a representative plot for the purity of NK cells reaching 97.42% purity. The overall purity of NK cells from the 11 donors belonging to this study was 94.7% and the viability of 97.4% as shown in **Table 4**. The most common contaminant cells after negative selection were monocytes (mean of 4.6%, range 1.55 to 9.2%); whereas contaminant B and T cells were found in very low numbers (< 0.6% combined) (**Table 5**). **Figure 9** shows the gating strategy used for the analysis.

Thus, the NK cells used in the current work were pure enough to discard other cells' contributions to the assays, and the viability was high to trust the data.

Best conditions to determine ADCC and direct cytotoxicity mediated by NK cells

To start, standardization steps were needed. For ADCC and direct cytotoxicity, it was tested *(i)* different E:T ratios (from 20:1 to 2.5:1, in 2-fold dilutions), *(ii)* different periods for the duration of the assay, and *(iii)* different concentrations of IVIG. In addition, for ADCC it was checked *(iv)* anti-CD20 concentrations, and *(v)* the presence or not of IL-2 during the ON incubation.

The optimal effector to target (E:T) ratio for ADCC assays is 5:1

At first, to determine the best E:T ratio, it was chosen a fix concentration of anti-CD20 antibody and IVIG of 0.5μg/ml and 2.5mg/ml, respectively, in a 2h assay using Daudi cells as

targets.

Compared to direct cytotoxicity (no antibody, blue rhomboid), as expected an enhancement of NK cell-mediated killing of Daudi cells in the presence of anti-CD20 (ADCC, pink circles) was observed. Whereas, the overnight treatment of NK cells with IVIG and during ADCC, reduced the killing of Daudi cells (half-pink square), but not the direct killing of the targets (orange squares), **Figure 10**. For all assayed conditions, there was an effect of the ratio between NK cells and Daudi cells (E:T ratio), being more pronounced in the ADCC assays.

In the representative plot shown in **Figure 10**, specific lysis values reached levels above the expected 100%. The sample release was higher than the maximum release control (see Material and Methods).This phenomenon may be explained due to target cells' stress that would spontaneously die, releasing BATDA to the supernatant and showing higher reading.

To choose an optimal E:T ratio for the following optimization steps, it is necessary that an assay readout was high enough to individually see differences among the conditions. Having this information into consideration, 5:1 and 2.5:1 ratio fulfill the criteria, and thus, were chosen for further experiments.

ADCC of Daudi targets by NK cells is optimal at 0.05 μg/ml of the anti-CD20 antibody in one-hour assay

For ADCC experiments, the three key components were the effectors (NK cells), the targets (Daudi cells), and the antibody binding the targets (anti-CD20). Thus, the next step was finding the optimal anti-CD20 concentration for the assay. Additionally, in the BATDA non-radioactive cytotoxicity assay the optimal time was needed to obtain the most reproducible maximum and minimum release. Once again, the concentration of IVIG was fixed to 2.5mg/ml.

Thus, 10-fold serial dilutions of anti-CD20 antibody were tested, from 0.5 to 0.0005μg/ml. The co-culture of effectors with targets was for 1h and 2h, using 5:1 and 2.5:1 E:T ratios.

Figure 11A and **Figure 11B** show dose-response curves tested at two different E:T ratios and assay times. At both, 5:1 and 2.5:1 E:T ratios, there was notable ADCC, but very reduced when IVIG was added ON to the NK cells. ADCC was depending on the anti-CD20 concentration (filled symbols), whereas 2.5mg/ml IVIG strongly inhibited the killing (open symbols). The ADCC rendered higher specific lysis at 2h assay, but which values were far beyond the 100%

specific lysis, that could be related with the external addition of IL-2.

ADCC was measurable starting from 0.05μg/ml of anti-CD20 antibody, reaching a plateau at 0.5μg/ml. Therefore, this was the concentration chosen for future experiments. The decision to use 1h assays and not 2h assays was derived from the specific lysis values closer to 100%.

In summary, the best E:T ratio was 5:1 since it showed better fold change between the concentrations of anti-CD20 and IVIG conditions for both co-culture times. The higher killing observed could be related to the experimental release that in some cases was higher than the maximum release control, not allowing the formula to be correctly applied.

The supply of Interleukin-2 (IL-2) overnight to NK cells before ADCC enhances their cytotoxicity

The enhanced activity of NK cells in the previous assays (specific lysis above 100%) could be related to the presence of IL-2 during the ON culture. To test this hypothesis, the ADCC assay was repeated with NK cells cultured or not with 50U/ml of IL-2 at two different concentrations of anti-CD20 antibody (0.05 and 0.005 μg/ml).

The anti-CD20 dose-response effect on ADCC was observed with and without IL-2 supply to NK cells. However, the specific lysis observed was higher with IL-2, **Figure 12A**. On the other hand, in the presence of IVIG, the anti-CD20-mediated killing is diminished for both conditions, **Figure 12B**. Furthermore, the addition of IL-2 to ON culture enhances the NK cells' direct cytotoxicity (dashed lines) as well.

Considering all parameters, IL-2 was excluded from further assays, and the chosen concentration of anti-CD20 was 0.05 μg/ml due to the better difference between anti-CD20 and IVIG conditions.

Reduction of IVIG in one order of magnitude keeps inhibiting ADCC by NK cells in a dose-response manner

Until now, 2.5mg/ml of IVIG was used to inhibit ADCC during the standardization process. However, it was aimed to reduce at least one order of magnitude the amount needed to inhibit ADCC in future experiments. Thus, for the next experiments, parameters of 5:1 E:T ratio, 0.05μg/ml of anti-CD20, two assay times: 1h and 2h, and no IL-2 during the ON culture of NK cells were kept as fixed conditions; and concentrations of IVIG, from 0.25 to

0.00025mg/ml, in 10-fold serial dilutions NK cells were tested.

Dose-inhibition curves were observed in both co-culture times, where no inhibition of ADCC occurred in the absence of IVIG (**Figure 13A-B**). In case the of IVIG, the higher inhibition was observed at 0.25mg/ml for 1h and 2h co-culture. Whereas, no ADCC inhibition by IVIG was detected from 0.0025mg/ml and lower.

In summary, after all the standardization steps, the best conditions for future ADCC assays using purified human NK cells as effectors and Daudi cells as targets were *(i)* an E:T ratio of 5:1, *(ii)* 0.05μg/ml of anti-CD20 antibody, *(iii)* 1h ADCC assay, *(iv)* no IL-2, and *(v)* IVIG concentration from 0.25mg/ml.

Direct cytotoxicity requires an E:T ratio of 10:1, 2-hour assay, and IVIG concentrations below 2.5 mg/ml

In direct cytotoxicity assays, the targets consisted of the K562 cell line susceptible to NK-killing due to the lack of MHC class-I. Therefore, no monoclonal antibody optimization was necessary. The testing of IL-2 was omitted because, as shown in **Figure 12**, IL-2 increased the direct cytotoxicity of Daudi cells and the option was to not treat NK cells with the cytokine.

Figure 14A-B shows the effect of the ratio between effectors (NK cells) and targets (K562 cells) and the assay time. The more NK cells, the higher the specific lysis. In parallel, the time that the assay last also affected the percentage of K562 cells lysis (specific lysis). From these data, an E:T ratio of 10:1 and 2h assays provide the best results in terms of specific lysis.

As to IVIG, it was tested whether different concentrations could inhibit direct cytotoxicity. Thus, a dose-response of the effect of IVIG was noticed for direct NK cytotoxicity (**Figure 15**). IVIG inhibited NK function from 0.0025mg/ml when added ON before the cytotoxicity assay.

In summary, after these standardization steps, the best conditions for future direct cytotoxicity assays using purified human NK cells as effectors and K562 cells as targets were

(i) an E:T ratio of 10:1, *(ii)* 2h direct cytotoxicity assay, and *(iii)* IVIG concentration from 0.25mg/ml.

Recombinant multimer analogs of IgG alter the effector functions of Natural killer cells *in vitro*

It is known that IVIG can affect NK cell functions including cytokine production and cytotoxicity.[46] To assess whether recombinant analogs of IgG (HEX, HEX variants, and MONO) have the same effect as IVIG on NK cells, human NK cells were isolated and incubated ON with 10-fold serial dilutions of IVIG, and recombinant molecules, previous to ADCC and direct cytotoxicity assays. Statistical analysis was performed on this data using two-way ANOVA and Tukey's multiple comparison test and analyzed as described in the Materials and Methods.

Hexamer (HEX) is more potent than IVIG in inhibiting NK ADCC

The aim of this study was to determine whether recombinant multimer and monomer IVIG analogs have a similar effect on ADCC mediated by human NK cells.

Eight different human healthy volunteers' NK cells were tested in ADCC assays. A representative dose-response plot from one donor shows the effect of IVIG and analogs on the target cells' specific lysis (**Figure 16A**). HEX strongly inhibited NK cell-mediated ADCC, compared with IVIG which was effective starting from 0.025mg/ml. MONO only showed a slight inhibition at the highest dose (0.25 mg/ml).

To be able to pool all donors, data were expressed as a percentage of ADCC inhibition. **Figure 16B** shows the dose-inhibition curves. HEX was the most potent inhibitor of ADCC mediated by NK cells, even higher than IVIG; whereas the recombinant version in the monomeric form (MONO) displayed a slight inhibition at the highest tested dose (0.25 mg/ml). Comparisons between the three groups (HEX *versus* MONO, IVIG *versus* MONO, and IVIG *versus* HEX) showed significant differences. **Table S1** summarizes the *p*-values' respective significance among the IVIG and recombinant IgG molecules of interest.

Molecular variants of the HEX with lower binding to Fc-gamma receptors 2 and 3 displayed lower inhibition of antibody-mediated function *in vitro*

Thanks to molecular engineering, it is possible to further modify the Fc part of IgG that binds to its receptor and to test these molecular variants in ADCC assays. Thus, hexamers with low

binding to FcγR2 and FcγR3 (HEX_{low}R2 and HEX_{low}R3, respectively) were provided by CSL and tested in the current study. NK cells from three different human healthy volunteers were cultured in parallel with the different HEXs before ADCC assays.

Figure 17A shows a representative dose-response plot for the specific lysis of Daudi cells coated with anti-CD20 antibody in the presence or not of the recombinant IgG. HEX was the most potent inhibitor of ADCC. The recombinant multimer analog HEX_{low}R3 showed higher inhibition than IVIG, whereas HEX_{low}R2 only started inhibiting at 0.025mg/ml.

Once again, donors were pooled by calculating the percentage of ADCC inhibition and the dose-inhibition curve of pooled data expressed as the percentage of ADCC inhibitions was observed. In these three donors, HEX had the strongest percentage of inhibition, followed by HEX_{low}R3 and to a lesser extent by HEX_{low}R2; whereas the controls using IVIG and MONO provided the same results as described in the eight first donors. **Table S2** gives the *p*-values and respective significance among the IVIG and recombinant IgG molecules of interest.

The NK cell direct cytotoxicity is inhibited by IVIG and to a lesser extent by Hexamer (HEX) and variants

Another important function of NK cells is direct cytotoxicity, where the target cell elimination process depends on the fine-tune balance between activating and inhibitory receptors, independently of antibodies. Nonetheless, previous observations of the lab showed that IVIG, when added ON to human NK cells, inhibited the lysis of pig endothelial cell by human NK cells. [47] Thus, the second interest was to confirm the previous results but using the prototypic NK cell target cell, K562. Furthermore, it was of interest to determine whether the recombinant analogs of IVIG show similar inhibition of direct cytotoxicity mediated by purified human NK cells.

Three different human healthy volunteers' NK cells were tested. As shown for a representative donor, **Figure 18A**, overall IVIG was the most potent inhibitor of direct cytotoxicity starting already at 0.00025mg/ml. The recombinant multimer analogs HEX_{low}R3 and HEX_{low}R2 showed similar levels of inhibition and the HEX recombinant version was almost

as strong as IVIG. In contrast, the monomeric form (MONO) showed the lowest level of inhibition but had the highest fold-change difference among the two highest concentrations.

Pooled data expressed as a percentage of direct cytotoxicity inhibitions in **Figure 18B** show that IVIG had a higher percentage of inhibition than all other IgG molecules at higher concentrations, reaching a maximum of 50% inhibition at 0.025mg/ml. On the other hand, the MONO had the lowest percentage of inhibition in a dose-dependent manner. $HEX_{low}R2$ and $HEX_{low}R3$ showed similar levels of inhibition. Intriguingly, HEX showed a decreasing percentage of inhibition at higher concentrations of IgG molecules. **Table S3** presents the *p*-values' respective significance among the IVIG and recombinant IgG molecules of interest.

In summary, it was established a robust and reliable ADCC and direct cytotoxicity assays. HEX and HEX variants showed similar behavior to IVIG by inhibiting ADCC mediated by NK cells. HEX was the most potent inhibitor and MONO the less potent inhibitor. For direct cytotoxicity, HEX and its variants showed a similar inhibition behavior but to a lesser extent than in ADCC assays. IVIG was the most potent inhibitor of direct cytotoxicity mediated by NK cells.

IVIG and recombinant analogs trigger interferon-gamma and tumor necrosis factor secretion by NK cells during overnight *in vitro* cultures

Another important function of NK cells is the secretion of cytokines like IFN-γ and TNFalpha upon stimulation.[20] Therefore, it was collected a fraction of the ON supernatants of the culture of NK cells with the IgG molecules and determined the secreted cytokines using the CBA detection system.

From the panel of cytokines, IL-2, IL-4, IL-6, and IL-10 were under the detection limit; while IFN-γ and TNFalpha were measurable in the culture supernatants after adding IVIG and analogs to the NK cells. It is important to notice that despite the secretion of cytokines of IgG molecules were under the detection limit for IFN-γ, there were still dose-response curves for HEX, but not MONO (donor HD.CMU.131, **Figure 19A-B**). Whereas when data were pooled for 0.25mg/ml concentration of the biologicals, HEX was the stronger inducer of IFN-γ, followed by $HEX_{low}R3$, IVIG, and to a lesser extent by $HEX_{low}R2$; whereas MONO had an almost no effect. The results for TNFalpha showed a similar pattern with higher concentrations of the cytokine detected in the supernatants **(Figure 19C-D)**. In summary, there was a large

dispersion of the data based on the donors where it was seen a trend, but significance could not be found.

Discussion

The ability of NK cells to kill targets is regulated by a balance of signals from activating and inhibitory receptors. In the case of autoimmune diseases, autoAbs are produced and react with self-Ags. Consequently, NK cells may bind to these autoAbs *via* CD16a leading to antibody-dependent cellular-mediated cytotoxicity (ADCC).[24] On the other hand, IVIG represent a well-established second line treatment for autoimmune diseases, having the capability of reducing autoAb levels among other mechanisms of action including FcRn antagonism leading to a shorter half-life of Igs.[48] However, the shortage of plasma donors, and the high costs, stimulate the exploration of new approaches.[33] Currently, $_{rec}$IgG molecules are under investigation to assess their effects in comparison to IVIG. These molecules have not reached clinical studies yet, but preliminary *in vitro* and *in vivo* animal studies have shown them to be stronger inhibitors of ADCC, complement and phagocytosis than IVIG. [1,39,49]

Thus, the goal of this thesis was to assess the impact of IVIG or human recombinant IgG molecules and analogs on the cytotoxic activity of NK cells and cytokine production. For such, a reliable functional assays had to be standardized, in particular, ADCC and direct cytotoxicity, in order to evaluate the effect of IVIG and $_{rec}$IgG molecules.

Both ADCC and direct cytotoxicity assays were previously used by other members of the laboratory.[47,50] However, for the data shown in this thesis, additional standardization steps were needed, such as adjusting the co-culture time, the presence of IL-2, and the E:T ratios used for each assay. It is known that the presence of IL-2 influences the activation status of NK cells.[51] [52,53] The standardization step where NK cells were cultured or not overnight in the presence of IL-2 is in accordance with this data, showing higher ADCC when NK cells were cultured ON with IL-2. Thus, taking into consideration the high readouts of cytotoxicity (specific lysis) in the presence of IL-2, IL-2 was removed from the final assay.

In addition, the standardization procedures allowed to decrease the concentration of IVIG previously used from 2.5mg/ml to 0.25mg/ml with observable effects even as low as 0.0025mg/ml for direct cytotoxicity (Fig 14B). Using the same concentrations for IVIG and for the $_{rec}$IgG molecules, while maintaining a reliable readout, allowed direct comparisons of the effect. For the assays where different E:T ratios were tested, a higher amount of effector cells led to a higher specific lysis; the same principle was applied to the duration of the cytotoxicity assays. With that in mind, it was necessary to find a correct balance between maximum and

minimum readouts to ensure the proportion between them was good enough to best observe changes induced by the Igs compounds. A very low amount of IVIG was sufficient to achieve inhibition; however, this amount tested *in vitro* is far from the levels detected in blood after IVIG therapy. [54]

Previous studies showed that IVIG and $_{rec}$IgG molecules could abrogate ADCC and direct cytotoxicity *in vitro*.[48,47,51,55] The data presented here align with those results demonstrating the capacity of IVIG and $_{rec}$IgG molecules (HEX, HEX_{low}R3, HEX_{low}R2, and MONO) to inhibit ADCC mediated by NK cells after the challenge with specific targets *in vitro*. In particular, HEX was the most potent inhibitor for ADCC assay, and IVIG was the most potent inhibitor of direct cytotoxicity function. A dose-response effect for IgG molecules on NK cytotoxicity was observed in both functional assays. This data is consistent with previous work from our lab in which NK cells were exposed to IVIG for ON incubation.[47]

A previous study by Spirig *et al.* showed the effect of HEX compared to IVIG *in vitro*. The authors proved the ability of HEX not only to bind to the different FcγRs, but also to inhibit the classical complement pathway, and effector functions.[1] Additionally, HEX showed effectiveness in mitigating inflammation *in vivo* using collagen-Ab induced arthritis animal models and immune thrombocytopenia (ITP).[1] In the same study, HEX consistently outperformed IVIG, with higher inhibition *in vitro* and *in vivo*. Other reports inform about the inhibitory potential of IVIG *in vitro* and *in vivo*. [1,38] Independently, Jacobi *et al*. used whole blood cultures exposed to 20mg/ml of IVIG to test the cytotoxic function of NK cells, showing that IVIG treatment reduced the function of the NK cells compared to the control without IVIG. [51] My results are consistent with the observations of Jacobi *et al*.

This thesis demonstrates that not only HEX was a more potent inhibitor of ADCC than IVIG, but also that other forms of HEX (HEX_{low}R3 and HEX_{low}R2) inhibit in a dose-response dependent manner on NK cells after ON incubation, although to a lesser extent. However, MONO only showed a small decrease at the highest dose when tested in ADCC and direct cytotoxicity assays. In contrast, other studies showed that the inhibition mediated by the MONO remained stable across all the concentrations. [49,56] This difference in the inhibition mediated by the MONO can be explained due to the different magnitude of the

concentrations (μg/ml) relatively to the results presented in this thesis (mg/ml).

NK cells incubated ON with IgG induced TNFalpha and IFN-γ secretion, although IFN-γ is in lesser extent.[20,57] The data presented here showed the same trend of secretion of cytokines as described by Fauriat *et al*.[20] NK cells were exposed to different concentrations of IVIG, HEX, $HEX_{low}R3$, $HEX_{low}R2$, or MONO, and for all concentrations and IgG molecules tested, the levels of secretion of TNFalpha were higher than IFN-γ. Fauriat *et al.* showed previously that NK cells coated with IgG release several chemokines and cytokines. [20] For that reason, BD™ Cytometric Bead Array (CBA) Human Th1/Th2 cytokine kit II was used to measure IL-2, IL-4, IL-6, IL-10, TNFalpha, and IFN-γ in this thesis. Interestingly, NK cells' maximum release was observed using the highest concentration of IgG molecules (0.25mg/ml) after ON incubation; with HEX leading the secretion of TNFalpha and IFN-γ.

The first researcher to describe the concept of engineered IgG in hexameric form with the carboxyl-terminal tailpiece from IgM (μtp) was Smith *et al.*, using the four subclasses of IgG (IgG1, IgG2, IgG3, and IgG4). This study showed that recombinant IgG hexameric molecules had stronger effector functions than wild-type Abs.[49] Later studies showed IgG1 Fc with a fraction of the IgM tail including mutations, namely, hIgG1-Fc-LH309/310CL-TP would provide better binding to the FcγR of the effector cells.[58] Engineered IgG1 and IgG4 hexameric forms by fusion of an IgM tailpiece were described by Rowley *et al*.. The study assessed different domain residues to maximize the potency of these molecules. IgG1 presented better outcomes than IgG4 independently of the domain residues in the study. [38] The HEX used in this study was generated by fusion of 18 amino acids (aa) of an IgM tailpiece with a human IgG1 Fc fragment. The purpose of their study was to assess if recombinant IgG molecule was able to inhibit FcgR-mediated functions of pathological autoantibodies and to compare its efficacy with IVIG treatment.[1] They showed Fc-μTP-L309C to be more potent than IVIG. This thesis showed that HEX had statistically higher inhibition compared with IVIG using *in vitro* studies.

Conclusion and future perspectives

This thesis showed that HEX is a potent inhibitor in ADCC assays using anti-CD20 monoclonal antibody and Daudi cells as targets. In contrast, as to direct NK cytotoxicity assays, IVIG demonstrated higher inhibition. It was also observed that at maximum concentrations tested of HEX, there was an increase of cytokines released by NK cells; TNFalpha was produced more in comparison to IFN-γ in terms of concentration, although heterogeneity was seen among the donors. The doses of IVIG and $_{rec}$IgG analogs used to fulfill the aims of this study were based on clinically relevant doses. HEX isoforms may have potential in the clinic as recombinant IVIG mimics for the treatment of autoantibodies-mediated diseases.

In the future, it would be interesting to *(i)* analyze the cytokine release mediated by NK cells after ON incubation with IVIG and analogs by using an enhanced kit to be able to have a better readout; *(ii)* perform ADCC and direct cytotoxicity assays adding directly IVIG and IgG molecules in the assays instead of ON treatment of the NK cells.

Overall, the results of this thesis contribute to a better understanding of the mechanisms of action of IVIG and recombinant IgG molecules and analogs. HEX isoforms may have a therapeutic potential in the clinic as recombinant IVIG alternatives for the treatment of autoantibodies-mediated diseases with better inhibition of effector functions including ADCC.

Tables

Table 2. Characteristics of the antibodies used for the assessment of NK cells purity.

Antibody	Fluorochrome	Dilution	Clone	Host species, Isotype	Company	Catalogue number
CD45	**FITC**	1:200	HI30	m, IgG1	Biolegend	304006
CD45	**PE**	1:200	HI30	m, IgG1	Biolegend	304008
CD45	**VioGreen**	1:50	5BI	m, IgG1	Miltenyi	130113-124
CD45	**BV605**	1:100	HI30	m, IgG1	Biolegend	304042
CD45	**BV421**	1:600	HI30	m, IgG1	Biolegend	304032
isotype control	**FITC**	1:200	MOPC-21	m, IgG1	BioLegend	400108
isotype control	**PE**	1:400	MOPC-21	m, IgG1	BioLegend	400112
isotype control	**BV421**	1:400	MOPC-21	m, IgG1	Biolegend	400158
isotype control	**BV605**	1:200	MOPC-21	m, IgG1	Biolegend	400162
CD3	**FITC**	1:200	UCHT1	m, IgG1	Biolegend	300406
CD3	**PE**	1 :250	OKT3	m, IgG1	Biolegend	317308
CD14	**FITC**	1:200	HCD14	m, IgG1	Biolegend	325604
CD16	**BV605**	1:200	3G8	m, IgG1	Biolegend	302040
CD19	**PE**	1:40	HIB19	m, IgG1	Biolegend	302208
CD33	**FITC**	1:200	FMY9S5	m, IgG1	Biolegend	366620
CD56	**BV421**	1:200	HCD56	m, IgG1	Biolegend	318328

Abbreviations: BV421, brilliant violet 421; BV605, brilliant violet 605; FITC, fluorescein isothiocyanate; m, mouse; PE, phycoerythrin.

Table 3. Template for the determination of NK cells' purity using PBMCs as compensation.

Tube	Cells	BL-1 FITC	BL-3 7AAD	Vl-1 BV421	VL-2 VioGreen	VL-3 BV605	YL-1 PE
c1	PBMC	-	-	-	-	-	-
c2	PBMC	CD45	-	-	-	-	-
c3	PBMC	-	7-AAD	-	-	-	-
c4	PBMC	-	-	CD45	-	-	-
c5	PBMC	-	-	-	CD45	-	-
c6	PBMC	-	-	-	-	CD45	-
c7	PBMC	-	-	-	-	-	CD45
1	PBMC	mIgG1	7AAD	mIgG1	CD45	mIgG1	mIgG1
2	PBMC	CD3+CD33+CD14	7AAD	CD56	CD45	CD16	CD3
3	PBMC	CD3+CD33+CD14	7AAD	CD56	CD45	CD16	CD19
4	NK cells	mIgG1	7AAD	mIgG1	CD45	mIgG1	mIgG1
5	NK cells	CD3+CD33+CD14	7AAD	CD56	CD45	CD16	CD3
6	NK cells	CD3+CD33+CD14	7AAD	CD56	CD45	CD16	CD19

Abbreviations: 7-AAD, 7-aminoactinomycin D; c, control tube; CD, Cluster differentiation; m, mouse; NK, natural killer; PBMC, peripheral blood mononuclear cells.

Table 4. Summary of NK cells percentage and viability for PBMCs and isolated NK cells. Mean calculated from 11 donors.

	NK cells (%) **Mean ± SD**	**Viability (%)** **Mean ± SD**
PBMC	14.41 ± 4.48	95.70 ± 2.35
Isolated NK cells	94.66 ± 2.79	97.42 ± 1.90

Abbreviations: NK, natural killer; PBMC, peripheral blood mononuclear cell; SD, standard deviation.

Table 5. Summary for the content of T cells, B cells and monocytes contained in the purified NK cells. Mean calculated from 11 donors.

	Mean ± SD (%)	**Range (Min Max)**
T cells ($CD3^+$)	0.31 ± 0.39	0.05 – 1.31
B cells ($CD19^+$)	0.26 ± 0.21	0.04 – 0.63
Monocytes ($CD14^+CD33^+$)	4.60 ± 2.52	1.55 – 9.20

Abbreviations: NK, natural killer; SD, standard deviation.

Figures

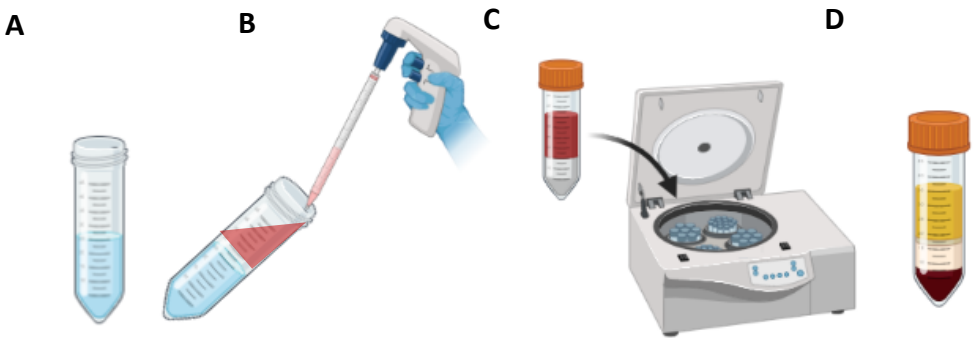


Figure 7. Main steps of PBMC isolation from a buffy coat. (A) Addition of Ficoll-Paque to a 50ml Falcon. **(B)** Slowly addition of diluted blood to the previous Falcon positioned at 45° angle to avoid that the two layers form by mixing. **(C)** Centrifugation with brake off to do not disturb the phases. **(D)** Buffy coat is separated into four layers: the red blood cells in the bottom, followed by the density gradient, a thin layer with PBMCs and finally plasma as the upper layer. (Created in BioRender)

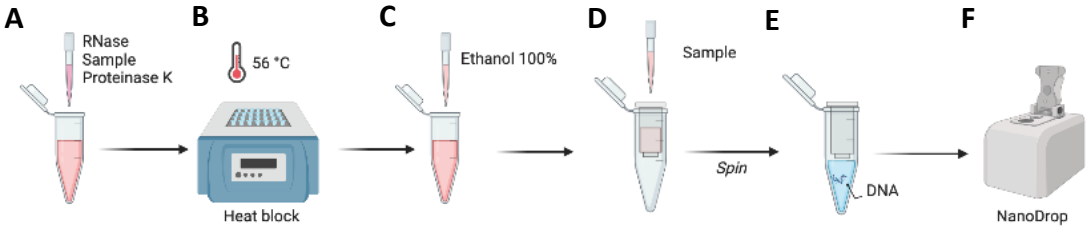
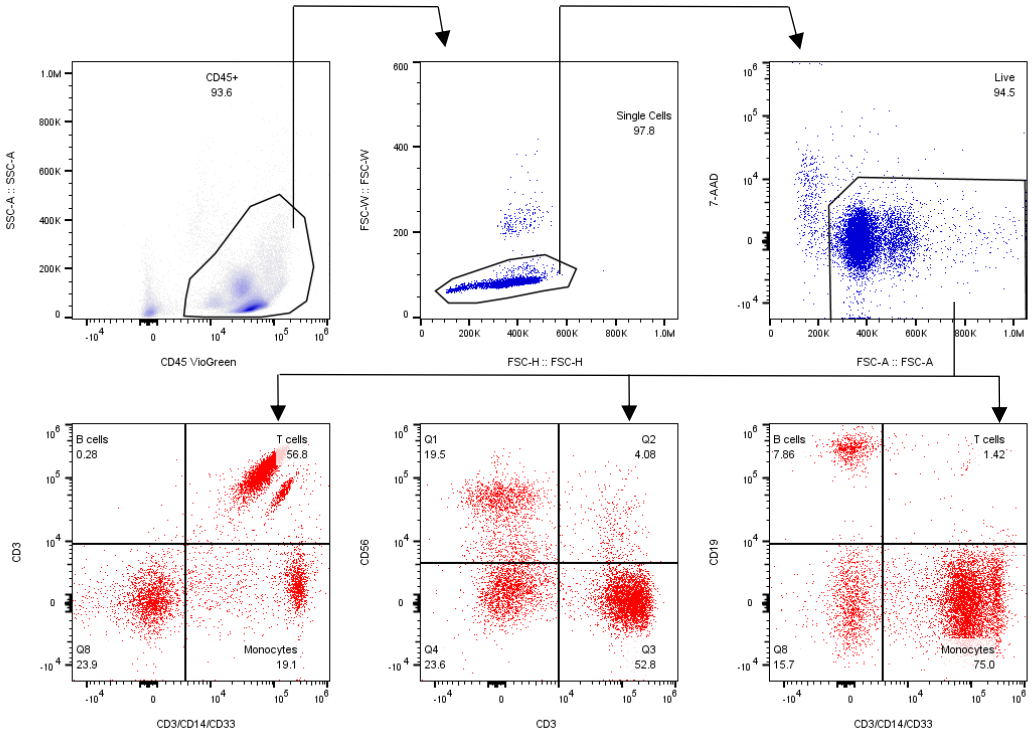


Figure 8. DNA extraction workflow. (A) RNase, sample, and proteinase K are added to an Eppendorf and **(B)** incubated at 56°C for 30 minutes. **(C)** Ethanol 100% is added to the sample and **(D)** transferred to a column, followed by three spins. **(E)** DNA is collected in an Eppendorf and **(F)** measured the concentration using NanoDrop™. (Created in BioRender)

A



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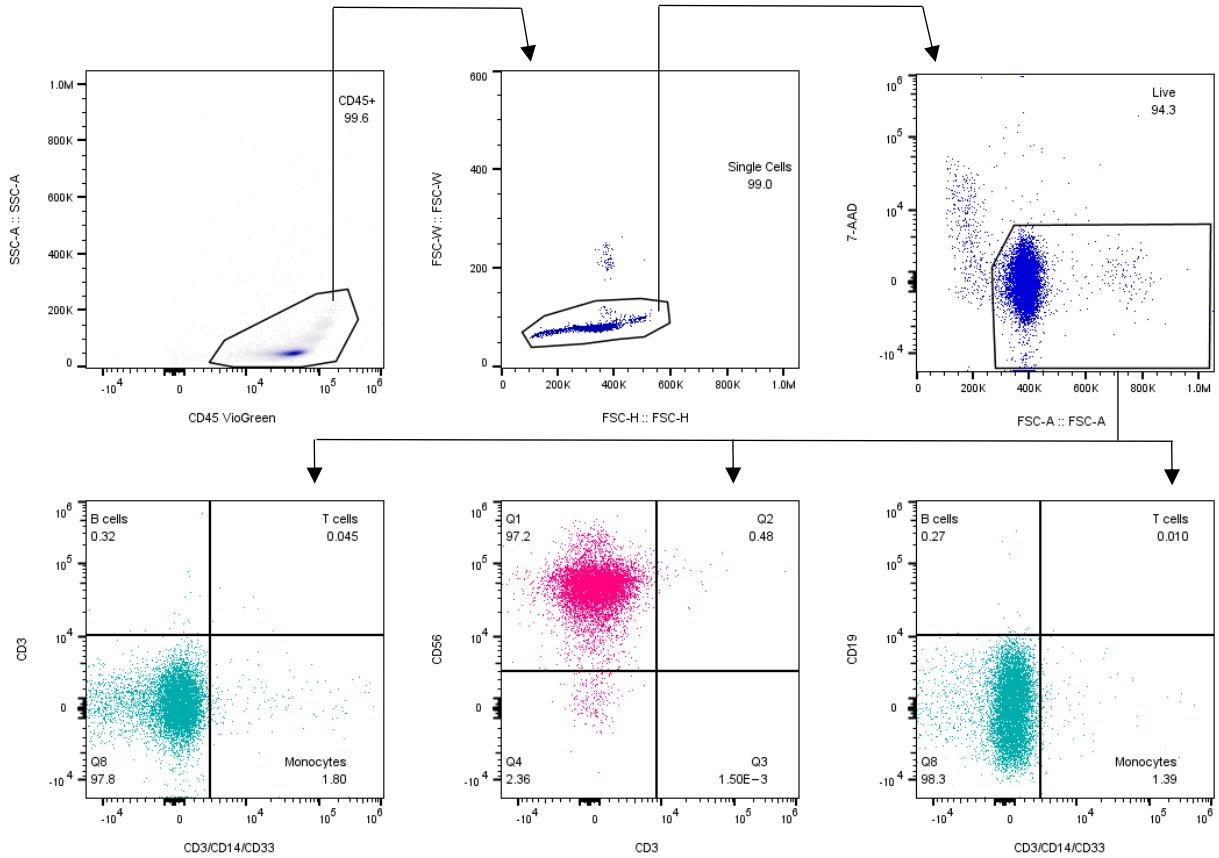

Figure 9. Gating strategy to assess NK cells' percentage in PBMCs and their purity after isolation. NK cells were isolated by negative magnetic isolation from PBMCs obtained from healthy donors. PBMCs and NK cells were identified by sequential gating on $CD45^+$ cells, single cells using FSC height and FSC width followed by live cells ($7AAD^-$) gating. **(A)** From live cells, to identify the different populations present in PBMCs, CD3/CD14/CD33 *versus* CD3 (far left) or *versus* CD19 (far right) were gated; where T, B cells are identified as only $CD3^+$ and $CD19^+$, respectively. Monocytes are represented by the $CD3^-CD14^+CD33^+$ population; whereas NK are the $CD56^+$ $CD3^-$ cells (middle). **(B)** A similar gating strategy was used to assess the purity of NK cells and its contaminants.

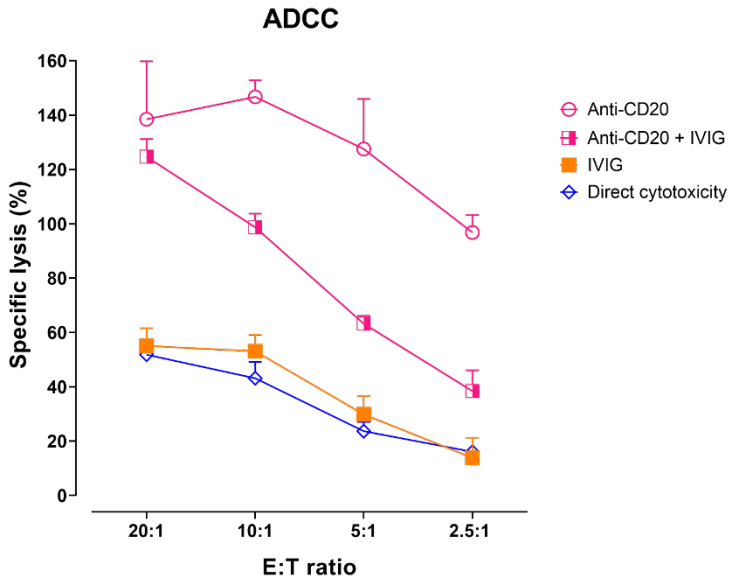

Figure 10. ADCC of anti-CD20 coated Daudi cells is dependent on the number of NK effector cells present during the assay. A representative cytotoxicity plot shows the ADCC mediated by NK cells that were incubated overnight in the presence of 50U/ml of IL-2 and 2.5mg/ml of IVIG (square conditions only). The next day, 0.5µg/ml of anti-CD20 (pink symbols) was added, followed by co-culture for 2h with Daudi. Assay control includes direct cytotoxicity, i.e., Daudi cells in the absence of anti-CD20 and NK cells incubated overnight with IL-2. *Abbreviations:* ADCC, antibody-dependent cellular-mediated cytotoxicity; E:T, effector to target; IL-2, interleukin-2; IVIG, intravenous immunoglobulin; NK, natural killer.

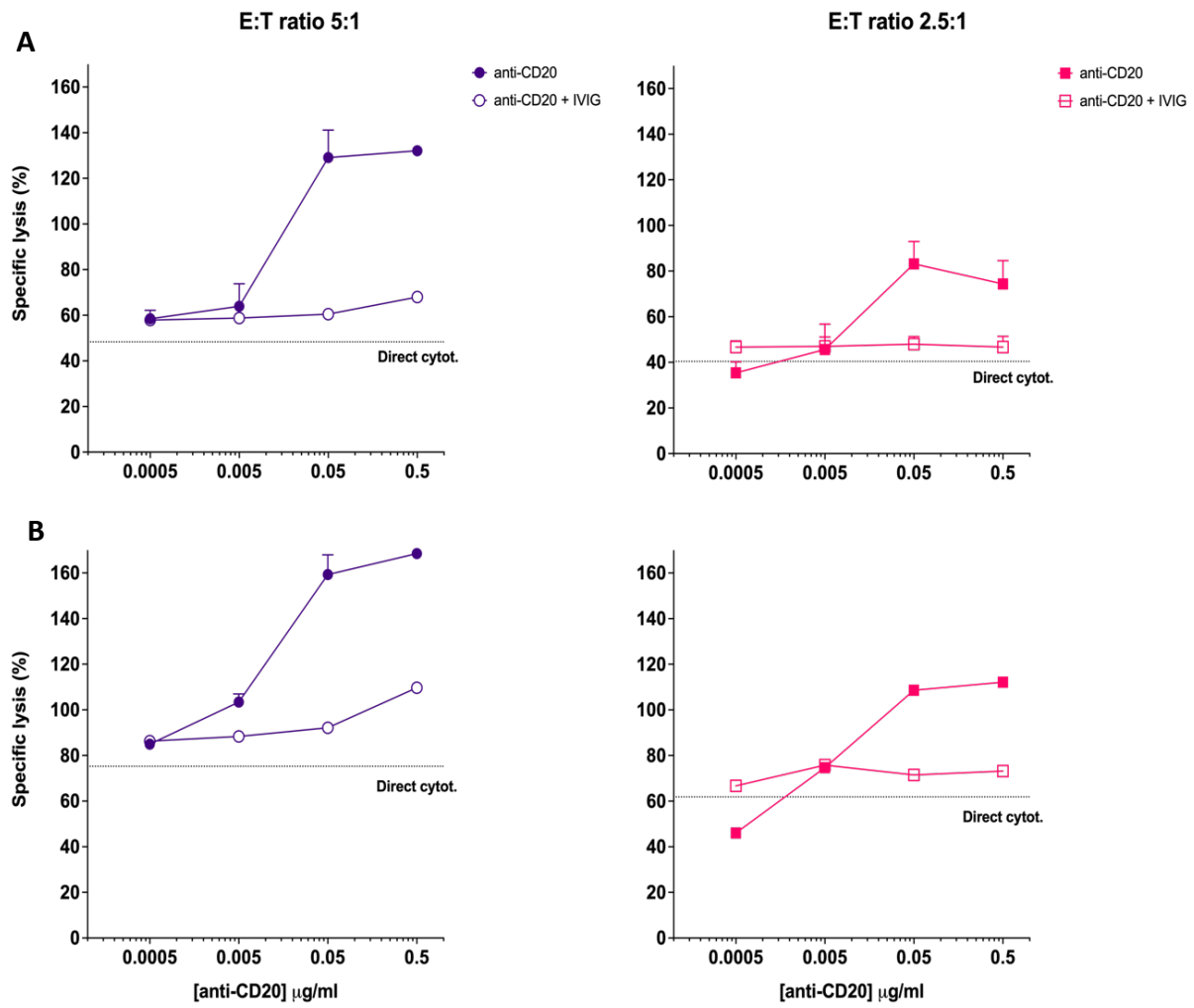

Figure 11. The effect of time of the ADCC assay at two different effector-to-target ratios. Representative anti-CD20 dose-response plots where NK cells and Daudi cells were assayed at 5:1 and 2.5 E:T ratios representing target cell elimination as a percentage of lysis (%). NK cells were cultured overnight in the presence of 50U/mL of IL-2 only (close symbols) or IL-2 plus a fixed concentration of 2.5mg/ml IVIG (open symbols). The following morning, target Daudi cells and 10-fold decreasing concentrations of anti-CD20, from 0.5 to 0.0005μg/ml alone (filled symbols) were added to the assay plate and co-cultured for **(A)** 1h and **(B)** 2h. The direct cytotoxicity control is represented as a black dotted line. (n = 1). *Abbreviations:* E:T, effector to target; IVIG, intravenous immunoglobulin; NK, natural killer.

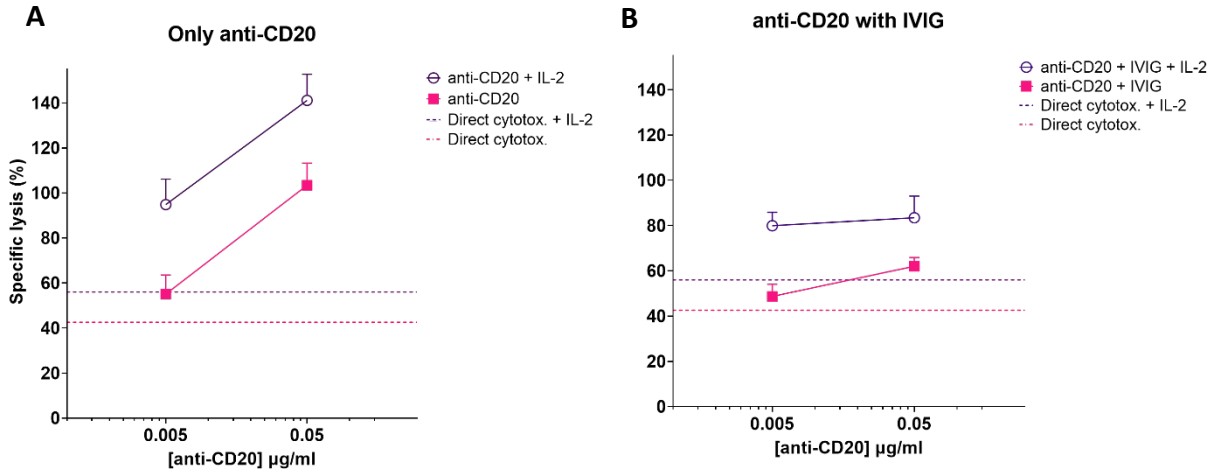

Figure 12. NK cells have enhanced cytotoxicity after overnight culture with IL-2. After isolation, NK cells cultured overnight in the presence of 50U/mL IL-2 (open symbols) or not (close symbols). The ADCC assay considered two different concentrations of anti-CD20, 0.05 and 0.005µg/ml given to **(A)** NK cells or **(B)** NK cells cultured overnight with 2.5mg/ml IVIG. The other assay conditions consisted of a 1h assay and an E:T ratio of 5:1 ratios and plots representing target cell elimination as a percentage of lysis (%). The direct cytotoxicity control is represented as purple and pink dashed lines for the absence or presence of IL-2, respectively. (n = 1) *Abbreviations:* E:T, effector to target; IL-2, interleukin 2; IVIG, intravenous immunoglobulin; NK, natural killer.

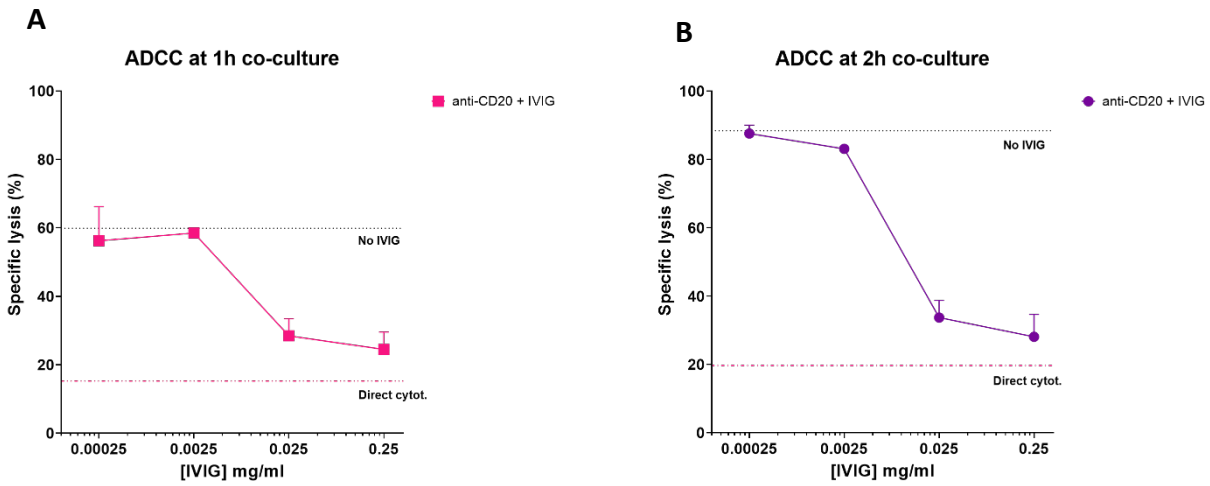

Figure 13. The inhibition of ADCC mediated by NK cells is IVIG dose-dependent at the two assayed times. NK cells were isolated and incubated overnight with decreasing concentrations of IVIG (from 0.25 to 0.00025mg/ml) (filled symbols). Next morning, Daudi cells and 0.05μg/mL of anti-CD20 were added to NK cells at a 5:1 E:T ratio, and the assay was run for **(A)** 1h and **(B)** 2h. Plots represent target cell elimination shown as a percentage of lysis (%). In both plots, controls included the conditions in the absence of IVIG (black dotted line) and direct cytotoxicity (pink dotted line). (n = 1) *Abbreviations:* E:T, effector to target; IVIG, intravenous immunoglobulin

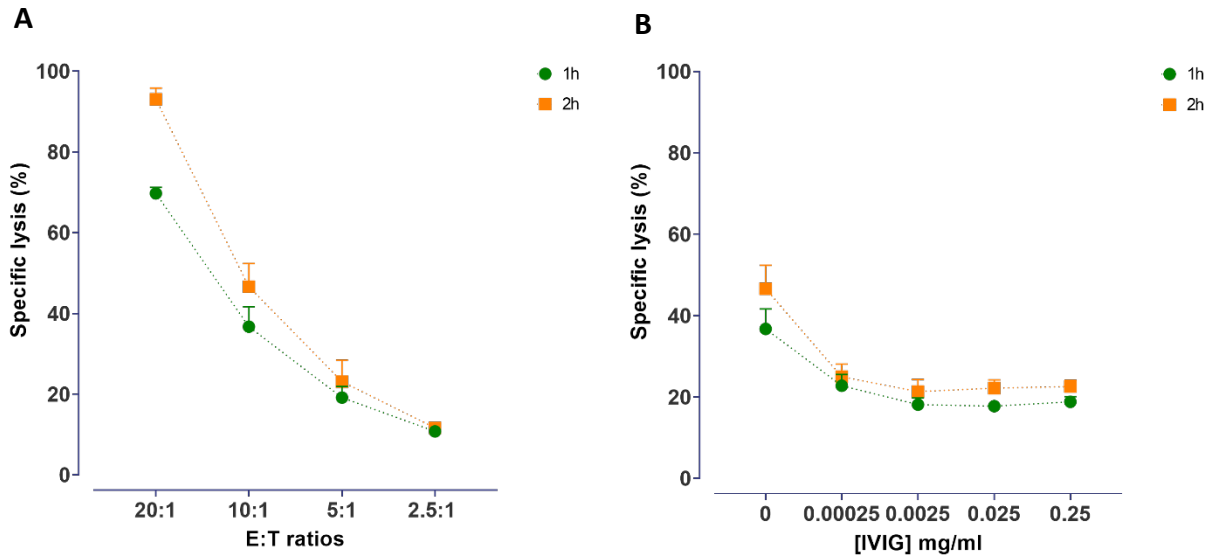

Figure 14. A two-hour assay increased cytotoxicity against K562 targets by NK cells for all E:T ratios and IVIG concentrations tested. Representative plots of K562 target cell elimination are shown as a percentage of lysis (%). NK cells were incubated overnight with decreasing concentrations of IVIG (from 0.25 to 0.00025 mg/ml). The next morning, **(A)** NK cells were challenged with K562 cells at 2-fold decreasing E:T ratios (from 20:1 to 2.5:1). **(B)** NK cells were pre-treated overnight with 10-fold decreasing concentrations of IVIG, followed by co-culture during 1h (green) and 2h (yellow) with K562. *Abbreviations:* E:T, effector to target; IVIG, intravenous immunoglobulin.

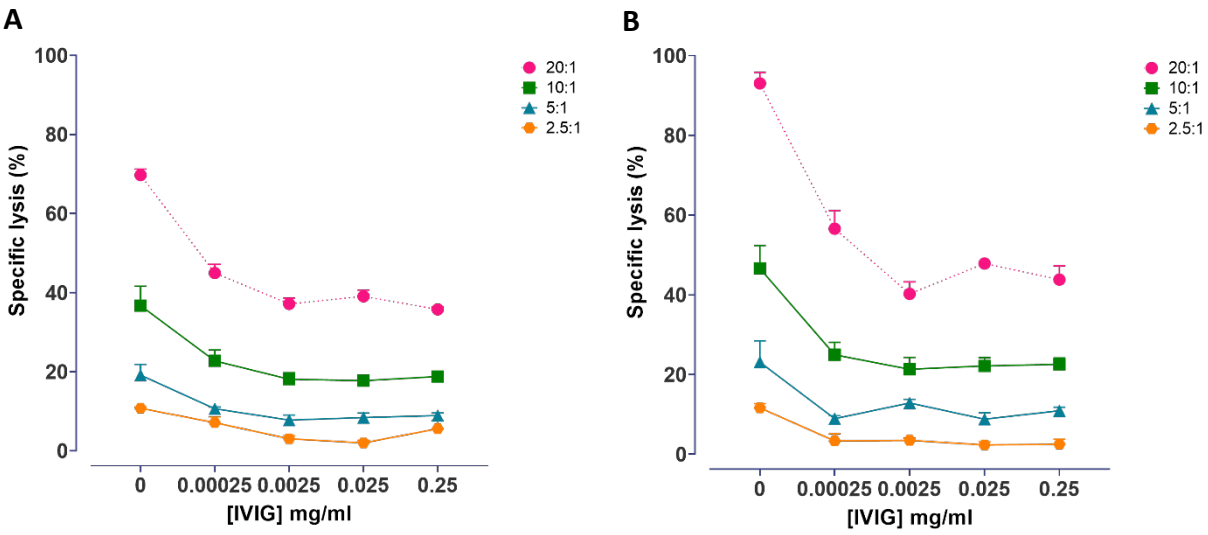

Figure 15. Direct cytotoxicity of K562 cells by NK cells is inhibited by IVIG in a dose- and time-dependent manner. Representative plots of K562 target cell elimination are shown as a percentage of lysis (%). NK cells were incubated overnight with decreasing concentrations of IVIG (from 0.25 to 0.00025 mg/ml). The next morning, NK cells were co-cultured with K562 cells at 2-fold decreasing E:T ratios (from 20:1 to 2.5:1) during **(A)** 1h and **(B)** 2h. *Abbreviations:* E:T, effector to target; IVIG, intravenous immunoglobulin.

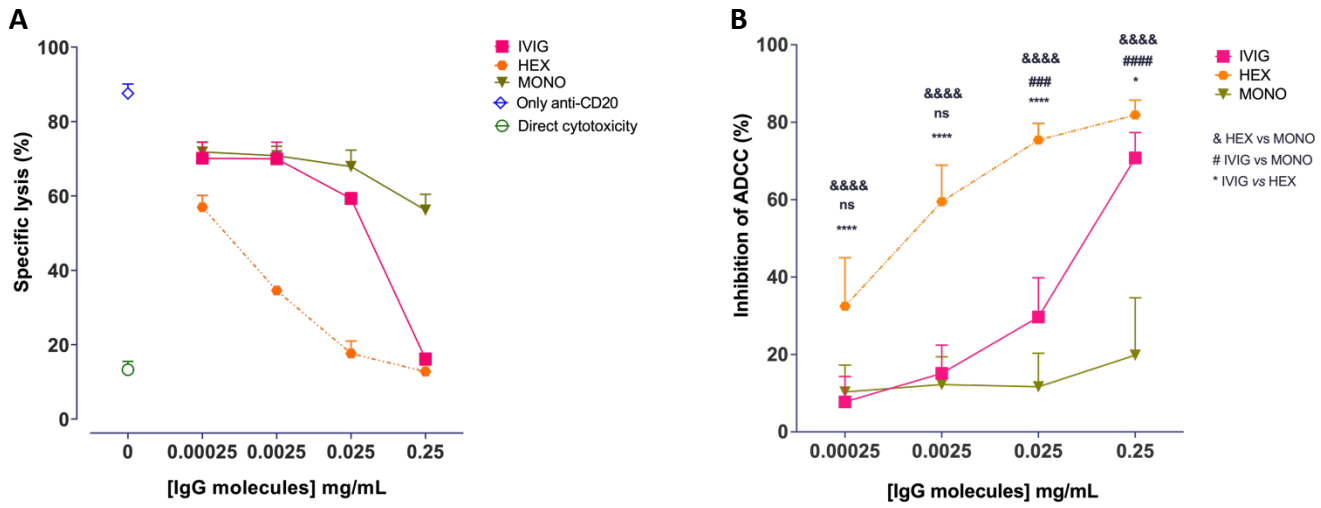

Figure 16. Hexamer has higher inhibition on ADCC than IVIG and Monomer. NK cells were incubated overnight in the presence of 10-fold decreasing concentrations of IVIG and IgG recombinant molecules (from 0.25 to 0.00025 mg/ml). The next morning for the ADCC assay, 0.05μg/ml of anti-CD20 antibody and the Daudi target cells were added, at an E:T ratio of 5:1, and the assay was run for 1h. **(A)** A representative donor is shown for the ADCC assay and expressed as the percentage of specific lysis (%) for the dose-response effect of IVIG (pink squares), HEX (yellow circles) and MONO (green triangles). Controls of direct cytotoxicity (open green circle) and only anti-CD20 on targets (open blue rhomboid) are indicated. **(B)** Pooled data of the mean of eight donors shown as the percentage of ADCC inhibition for the dose-response effect of the IVIG and IgG recombinant molecules. Comparisons between the three groups (HEX *versus* MONO, IVIG *versus* MONO and IVIG *versus* HEX) were done using Two-Way ANOVA and Tukey's multiple comparison test. Statistically significance in the figure used * (IVIG *vs* HEX), # (IVIG *vs* MONO) and & (HEX *vs* MONO) symbols for P * ≤ 0.05, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, respectively. The same principle was applied for the two other symbols, & and #. *Abbreviations*: ADCC, antibody-dependent cellular-mediated cytotoxicity; E:T, effector to target; HEX, hexamer, IVIG, intravenous immunoglobulins; MONO, monomer.

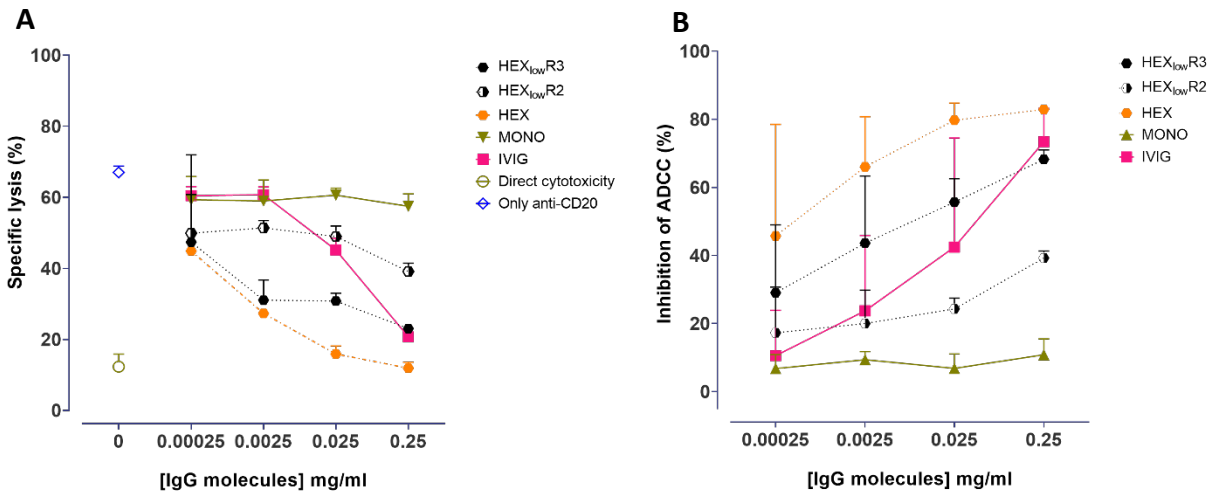

Figure 17. Hexamer is the most potent inhibitor of ADCC compared to other hexamer variants. NK cells were incubated overnight in the presence of 10-fold decreasing concentrations of IVIG and IgG recombinant molecules (from 0.25 to 0.00025 mg/ml). The next morning, 0.05μg/ml of anti-CD20 antibody and Daudi target cells were added, at an E:T ratio of 5:1, and incubated for 1h. **(A)** A representative donor is shown for the ADCC assay and expressed as the percentage of specific lysis (%) for the dose-response effect of IVIG (close pink squares), HEX (close yellow hexagon), $HEX_{low}R3$ (close black hexagon), $HEX_{low}R2$ (half black hexagon), and MONO (green triangles). Controls of direct cytotoxicity (open green circle) and only anti-CD20 on targets (open blue rhomboid) are indicated. **(B)** Pooled data of the mean of three donors is shown as the percentage of ADCC's inhibition for the dose-response effect of the IVIG and IgG recombinant molecules. *Abbreviations*: ADCC, antibody-dependent cellular-mediated cytotoxicity; E:T, effector to target; HEX, hexamer; $HEX_{low}R3$, hexamer with low binding to FcγR3; $HEX_{low}R2$, hexamer with low binding to FcγR3; IVIG, intravenous immunoglobulins; MONO, monomer.

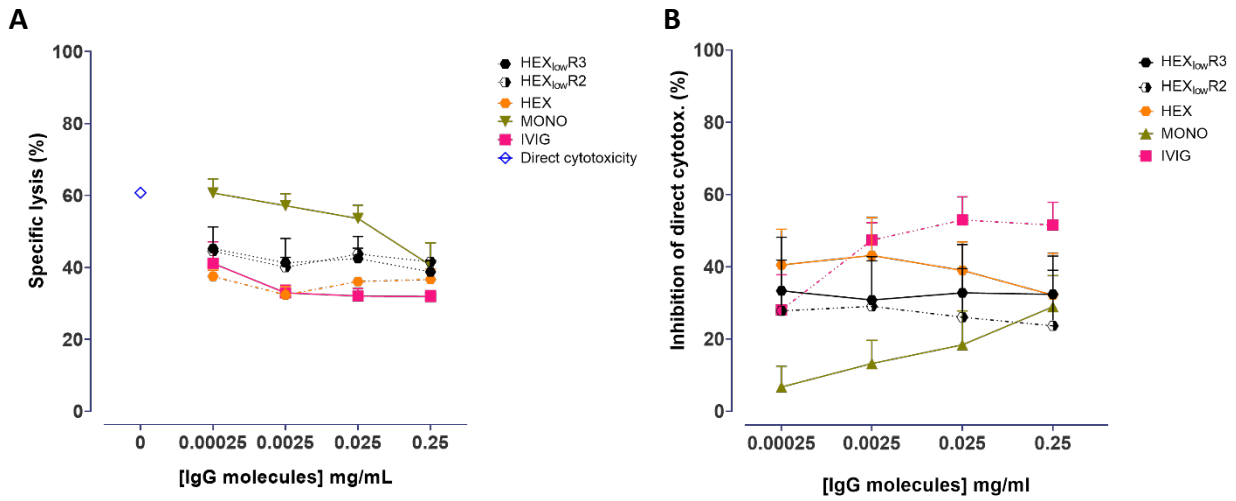

Figure 18. **IVIG is the stronger inhibitor of NK cell direct cytotoxicity.** NK cells were incubated overnight in the presence of 10-fold decreasing concentrations of IVIG and IgG recombinant molecules (from 0.25 to 0.00025 mg/ml). The next morning, for the direct cytotoxicity assay, K562 target cells were added, at an E:T ratio of 10:1, and the assay was run for 2h. **(A)** A representative donor is shown for the direct cytotoxicity assay and expressed as the percentage of specific lysis (%) for the dose-response effect of IVIG (close pink squares), HEX (close yellow hexagon), $HEX_{low}R3$ (close black hexagon), $HEX_{low}R2$ (half black hexagon), and MONO (green triangles). Control of direct cytotoxicity is represent as an open green circle. **(B)** Pooled direct cytotoxicity data of the mean three donors shown as the percentage of direct cytotoxicity inhibition for the dose-response effect of the IVIG and IgG recombinant molecules. *Abbreviations*: E:T, effector to target; HEX, hexamer; $HEX_{low}R3$, hexamer with low binding to FcγR3; $HEX_{low}R2$, hexamer with low binding to FcγR3; IVIG, intravenous immunoglobulins; MONO, monomer.

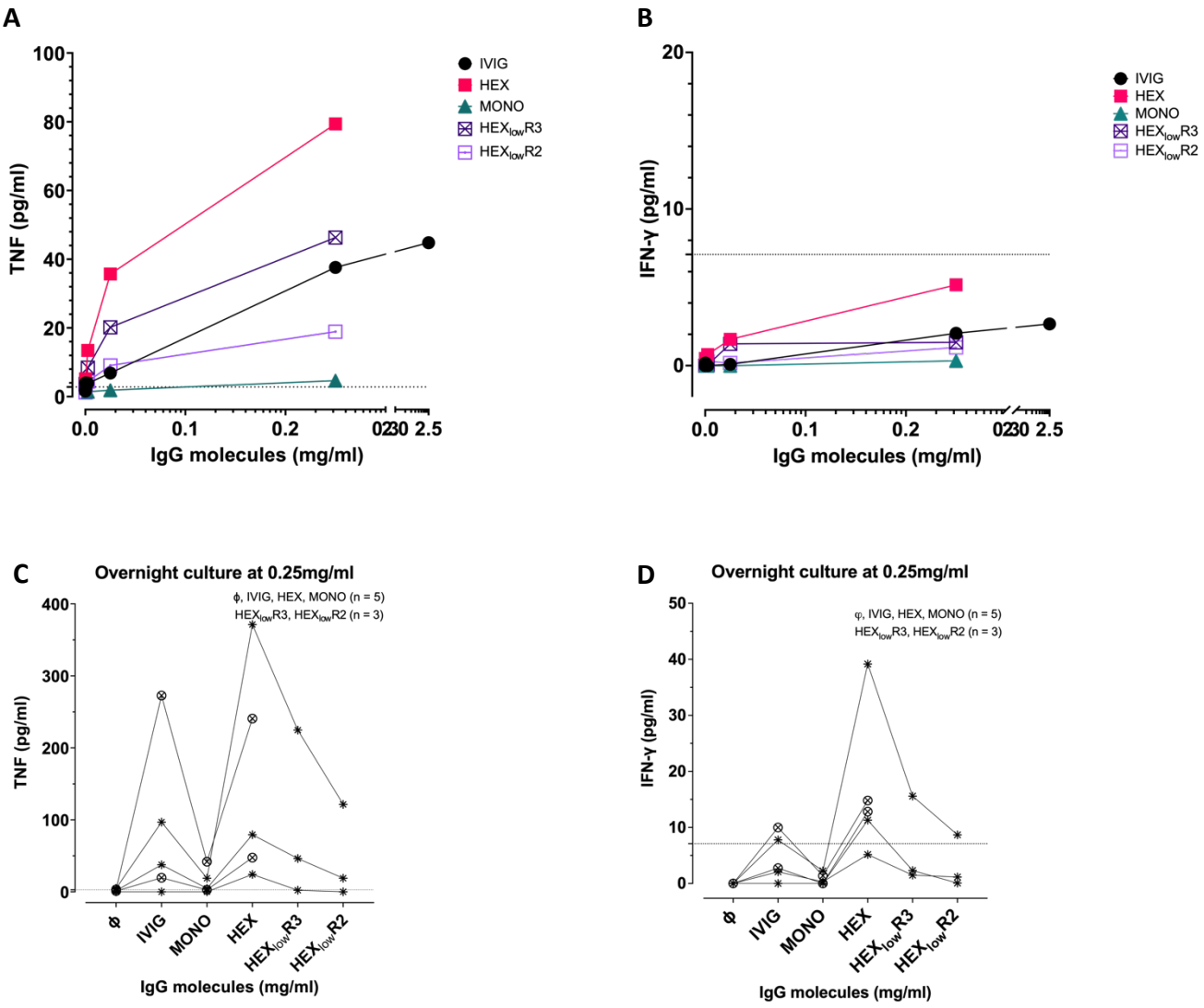

Figure 19. NK cells secrete higher cytokine levels after overnight incubation with HEX. NK cells were incubated overnight (16h) in the presence of 10-fold decreasing concentrations of IVIG and IgG recombinant molecules (from 2.5 to 0mg/ml). The following day, a fraction of supernatant was collected and analyzed using CBA assay. A representative donor (HD.CTS.131) is shown for **(A)** TNFalpha and **(B)** IFN-γ contained in the culture supernatant and expressed in pg/ml after treatment of NK cells with IVIG (close black circles), HEX (close pink square), MONO (close green triangle), $HEX_{low}R3$ (crossed purple square) and $HEX_{low}R2$ (open purple square). Pooled data of 0.25mg/ml of IVIG, HEX and MONO (n = 5); and $HEX_{low}R3$ and $HEX_{low}R2$ (n = 3) for **(C)** TNFalpha and **(D)** IFN-γ contained in the culture supernatants. *Abbreviations*: CBA, cytometric bead array; HEX, hexamer; $HEX_{low}R3$, hexamer with low binding to FcγR3; $HEX_{low}R2$, hexamer with low binding to FcγR3; IFN-γ, interferon-gamma; IVIG, intravenous immunoglobulins; MONO, monomer; NK, natural killer; TNFALPHA, tumor necrosis factor.

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Supplementary material

Table S1. Summary of the *p*-values for IVIG *vs* HEX, IVIG *vs* MONO and HEX *vs* MONO comparison post-test after 2-way ANOVA determinations in ADCC from eight different donors between the different IgG molecules.

	IVIG *vs* HEX		IVIG *vs* MONO		HEX *vs* MONO	
Concentration (mg/ml)	*P* value	Significance	*P* value	Significance	*P* value	Significance
0.00025	<0.0001	****	> 0.05	ns	<0.0001	&&&&
0.0025	<0.0001	****	> 0.05	ns	<0.0001	&&&&
0.025	<0.0001	****	0.0002	###	<0.0001	&&&&
0.25	0.0340	*	<0.0001	####	<0.0001	&&&&

Abbreviations: ADCC, antibody-dependent cellular-mediated cytotoxicity; HEX, hexamer; IVIG, intravenous immunoglobulin; MONO, monomer

Table S2. Summary of the *p*-values for IVIG *vs* MONO, IVIG *vs* HEX, IVIG *vs* $HEX_{low}R3$, IVIG *vs* $HEX_{low}R2$, HEX *vs* MONO, HEX *vs* $HEX_{low}R3$, HEX *vs* $HEX_{low}R2$, $HEX_{low}R3$ *vs* $HEX_{low}R2$, MONO *vs* $HEX_{low}R3$ and MONO *vs* $HEX_{low}R2$ comparison post-test after 2-way ANOVA determinations in ADCC from three different donors between the different IgG molecules

	IVIG *vs* MONO		IVIG *vs* HEX		IVIG *vs* $HEX_{low}3$		IVIG *vs* $HEX_{low}2$		HEX *vs* MONO		HEX *vs* $HEX_{low}3$		HEX *vs* $HEX_{low}2$	
Conc. (mg/ml)	*P* value	*P* value	*P* value	Signif.	*P* value	*P* value	*P* value	Signif.	*P* value	*P* value	*P* value	Signif.	*P* value	Signif.
0.00025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.0025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	0.0003	***	0.0432	*	0.0010	**
0.25	0.0099	**	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	0.0025	**	0.0172	*	0.0001	***

	$HEX_{low}3$ *vs* $HEX_{low}2$		MONO *vs* $HEX_{low}3$		MONO *vs* $HEX_{low}2$	
Conc. (mg/ml)	*P* value	Signif.	*P* value	Signif.	*P* value	Signif.
0.00025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.0025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.025	0.0258	*	0.0050	**	0.0251	*
0.25	0.0009	***	0.0010	***	0.0126	*

Abbreviations: ADCC, antibody-dependent cellular-mediated cytotoxicity; E:T, effector to target; HEX, hexamer; $HEX_{low}R3$, hexamer with low binding to FcγR3; $HEX_{low}R2$, hexamer with low binding to FcγR3; IVIG, intravenous immunoglobulins; MONO, monomer; Signif, significance.

Table S3. Summary of the *p*-values for IVIG *vs* MONO, IVIG *vs* HEX, IVIG *vs* $HEX_{low}R3$, IVIG *vs* $HEX_{low}R2$, HEX *vs* MONO, HEX *vs* $HEX_{low}R3$, HEX *vs* $HEX_{low}R2$, $HEX_{low}R3$ *vs* $HEX_{low}R2$, MONO *vs* $HEX_{low}R3$ and MONO *vs* $HEX_{low}R2$ comparison post-test after 2-way ANOVA determinations in ADCC from three different donors between the different IgG molecules

	IVIG *vs* MONO		IVIG *vs* HEX		IVIG *vs* $HEX_{low}3$		IVIG *vs* $HEX_{low}2$		HEX *vs* MONO		HEX *vs* $HEX_{low}3$		HEX *vs* $HEX_{low}2$	
Conc. (mg/ml)	*P* value	Signif.	*P* value	Signif.	*P* value	Signif.	*P* value	Signif.	*P* value	Signif.	*P* value	Signif.	*P* value	Signif.
0.00025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	0.0480	*	≥ 0.05	ns	≥ 0.05	ns
0.0025	0.0107	*	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.025	0.0367	*	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.25	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns

	$HEX_{low}3$ *vs* $HEX_{low}2$		MONO *vs* $HEX_{low}3$		MONO *vs* $HEX_{low}2$	
Conc. (mg/ml)	*P* value	Signif.	*P* value	Signif.	*P* value	Signif.
0.00025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.0025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.025	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns
0.25	≥ 0.05	ns	≥ 0.05	ns	≥ 0.05	ns

Abbreviations: ADCC, antibody-dependent cellular-mediated cytotoxicity; E:T, effector to target; HEX, hexamer; $HEX_{low}R3$, hexamer with low binding to FcγR3; $HEX_{low}R2$, hexamer with low binding to FcγR3; IVIG, intravenous immunoglobulins; MONO, monomer; Signif, significance.