Aus der Klinik für Innere Medizin IV, Nieren- und Hochdruckkrankheiten
Direktor: Prof. Dr. Kunzendorf
im Universitätsklinikum Schleswig-Holstein, Campus Kiel
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INDIRECT EFFECTOR MECHANISMS OF ANTIBODIES IN HEMODIALYSIS PATIENTS

INDIREKTE EFFEKTOMECHANISMEN VON ANTIKÖRPERN BEI HÄMODIALYSEPATIENTEN

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Vorgelegt von
TILL JAKOB KOCH

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Referent: PD Dr. Michael Dechant,
Klinik für Innere Medizin IV, Nieren- und Hochdruckkrankheiten

Korreferent: Prof. Dr. A. Hauschild,
Klinik für Dermatologie, Venerologie und Allergologie

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Meinen Eltern

1 Glossary

A	Ampere
Ab	Antibody
Abs	Antibodies
ADCC	Antigen dependent cellular cytotoxicity
Ag	Antigen
APS	$(NH_4)_2S_2O_8$ = Ammoniumpersulfat
Basophil	Basophil granulocyte
BSA	Bovine serum albumine
CAPD	Continuous ambulatory peritoneal dialysis
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CKD	Chronic kidney disease
CRD	Chronic renal disease
CTNK	CTNK-cell, cytotoxic natural killer cell
DP	Dialysis patient
EC50	Half maximal effective concentration
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
et al.	et alia
Eosinophil	Eosinophil granulocyte
Erythrocyte	Red blood cell
ESRD	End stage renal disease
E:T ratio	effector : target ratio
e.g.	exempli gratia = for instance =
	z.B.
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FCS	Fetal calf serum
FcR	Fc receptor
FDA	Food and Drug Administration
GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O	Water
H_2O_{dest}	Distilled water
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
HD	Hemodialysis
HF	Hemofiltration
HI	Healthy individual
HRP	Horseraddish peroxidase
IF	Immunofluorescence
i.e.	id est = that is
IL	Interleukin
Incidence	Rate of new cases in a given period of time
Ig	Immunoglobulin
K^+	potassium
K/DOQI	Kidney Disease Outcomes Quality Initiative
Leukocyte	White blood cell
M	molar
mAb	Monoclonal antibody
MFI	Mean fluorescent intensity
min	minute
ml	milliliter

mm	millimeter
Neutrophil	Neutrophil granulocyte = PMN
ng	nanogram
Na^+	Sodium
NaOH	Sodium hydroxide
NaH_2PO_4	Natriumdihydrogenphosphate
Na_2HPO_4	Dinatriumhydrogenphosphate
n.d.	not described
NIPD	Nightly intermitting peritoneal dialysis
NK-cell	Natural killer cell
NKF	National kidney foundation
nl	nanoliter
nm	nanometer
PBMC	peripheral blood mononuclear cell
pH	Potentia hydrogenii
PD	Peritoneal dialysis
PMN	Polymorph nuclear cell = neutrophil
PO_4^{3-}	Phosphate
Prevalence	Number of affected patients in a population at a given time
RRT	Renal replacement therapy
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TDS	Technical data sheet
TEMED	N,N,N,N-Tetramethylethylendiamin
thrombo-cyte	Blood platelet
TNFα	Tumor necrosis factor α
V	Volt
VEGF	Vascular endothelial growth factor
W	Watt
µl	Microliter
µM	Micromolar
µm	Micrometer

2 Introduction

2.1 Chronic kidney disease

Chronic kidney disease (=CKD =chronic renal disease =CRD) is the progressive, irreversible and substantial loss of renal function over a period of time. Many people suffer from this disease, in the US the prevalence of CKD in adults was 13% between 1999 and 2004. There are no reliable nationwide statistics on the incidence of the first four stages of CKD in Germany. The reasons that lead to CKD are numerous, foremost are typical diseases of civilization like diabetes, atherosclerosis and hypertension. In 2006, 32% of the patients beginning renal replacement therapy (=RRT, necessary in the final stage of CKD) suffered diabetes mellitus type 2 and 24% nephrosclerosis due to hypertension[1].

The definition most commonly used to classify CKD in daily routine is the Kidney Disease Outcomes Quality Initiative (K/DOQI) of the American National Kidney Foundation (=NKF, Amerikanische Nierenstiftung). Depending on the glomerular filtration rate (GFR) and the existence of proteinuria, five stages of CKD can be distinguished:

1. Slightly diminished kidney function (GFR>89ml/min/1.73m², proteinuria existent)
2. Mild reduction in GFR (GFR 60-89ml/min/1.73m², proteinuria existent)
3. Moderate reduction in GFR (GFR 30-59ml/min/1.73m²)
4. Severe reduction in GFR (GFR 15-29ml/min/1.73m²)
5. Established renal failure (GFR <15ml/min/1.73m²), RRT necessary

According to the definitions of the NKF, CKD stage 5 is also called "established chronic kidney disease". Other terms for this stage include end-stage renal disease (ESRD), chronic kidney failure (CKF), chronic renal failure (CRF) or "terminal renal failure"[2].

In order to survive terminal kidney failure, patients have to undergo renal replacement therapy (RRT), a palliative treatment of the disease. In 2006, 91,718 patients were on RRT in Germany[1]. In principle, extracorporeal methods can be distinguished from intracorporeal ones. Major RRT methods are kidney transplantation (intracorporeal), peritoneal dialysis (intracorporeal), or hemodialysis (extracorporeal). CKD and RRT are, however, associated with a sharp increase of patient's mortality foremost due to cardiovascular and other complication[3]. The median 5-year survival rate is < 50%, even lower than that of

malignancies in general (55%). In Germany, 52% of the deaths among dialysis patients are due to cardiovascular diseases, followed by 18% infections and 10% malignancies[4, 5, 6].

The method offering the best long term outcome in regards of life quality and health of the RRT recipients is clearly kidney transplantation. It is, if available, much preferable over the dialysis methods, extending patients lives in average 15 years more than dialysis would[7]. When comparing the recipients of kidney transplantation to the patients receiving dialysis, McDonald et al. found an initial increase in mortality after the operation during the first three months. This risk fell below that of the dialysis group after 6 months and from 12 months onward after transplantation, the risk of mortality was reduced about 80% in transplant recipients over the dialysis group[8]. In Germany, 2,776 patients received a kidney transplant in 2006. However, not every patient fits the criteria required to undergo transplantation and even the ones that do fit often have to wait many years for a matching transplant organ, since there are much more potential recipients than there are donors. The mean waiting time required for a cadaveric kidney transplant in Germany was 40 months in 2006[1], so many patients depend on dialysis as a RRT, even if only temporarily.

2.2 Dialysis

Dialysis is a method to artificially remove unwanted solutes (=substances dissolved in a fluid) or free water from a patient's blood when the kidneys are unable to perform this task.

The principle of dialysis is an exchange of solutes by diffusion over a semipermeable membrane, a barrier that is penetrable for certain substances like Sodium (Na^+) or Potassium (K^+), while holding back others (protein, cells). This principle was discovered by Thomas Graham in the 1850s. There are different types of dialysis methods.

2.2.1 Hemodialysis

Hemodialysis (=HD, also haemodialysis) is an extracorporeal method of dialysis where patients typically undergo treatment three times a week for about four hours. A shunt, (an arteriovenous fistula, usually "cimino"-fistula between a. radialis and v. cephalica) is punctured and hooked to a dialysis machine[9]. To prevent clotting, the blood drained from the patient is mixed with an anticoagulant such as heparin, since the thrombocytes would quickly coagulate when confronted with the alien plastic surfaces of the dialysis filters. The

blood then flows through the dialysis machine, where within the dialysis filter, it encounters the dialysis solution on the other side of a semipermeable membrane. Counter current flow is used, meaning the blood and the solution flow in opposite directions, achieving higher concentration gradients and thus increasing the efficiency of the dialysis. Since the two compartments (blood and dialysis solution) have different concentrations of certain solutes which are able to penetrate the filter, an exchange of solutes happens. This cleanses the blood of unwanted solutes such as K^+, PO_4^{3-} (phosphate) or urea, while other solutes such as HCO_3^- (bicarbonate) can be substituted to the patient's blood. Free water can be removed from the blood by application of hydrostatic pressure, causing free water to move across the membrane following the pressure gradient. Hemodialysis was first described 1914 by John Abel (Baltimore) in animals[10] and 1924 by Georg Haas (Gießen) in humans[11]. In 1945, Wilhelm Kolff (Kampen) managed to save the first patients life with dialysis and proved that acute renal failure could be survived using hemodialysis.

2.2.2 Hemofiltration

Hemofiltration (=HF, also haemofiltration) is an extracorporeal method similar to hemodialysis and is mostly used at intensive care units for acute renal failure, since blood pressure and blood volume can be adjusted meticulously. It is a slower, continuous method, where no dialysis solution is used, but solutes are removed from the blood by convection rather than diffusion (as in HD). A hydrostatic pressure is applied to the blood flowing along a filter membrane, driving water and solutes out of the blood while retaining proteins and cells. Then, an isotonic replacement fluid is added to the blood to replace the lost fluid. HF is not a long-term RRT method.

2.2.3 Peritoneal dialysis

Peritoneal Dialysis (=PD) is an intracorporeal method that uses the patient's peritoneum (the serous membrane lining the abdominal cavity) as a natural semipermeable membrane. Dialysis fluid is inserted in the abdominal cavity through a permanently implanted catheter, where it remains at night while the patient sleeps (=nightly intermittent PD, NIPD), or it is cycled through the abdominal cavity several times during the day (=continuous ambulatory PD, CAPD).

Worldwide, HD is far more common than PD (in average 11% PD). Some countries like Germany have even lower PD percentages (only 4.8% PD in 2006), while others such as the UK or Mexico have up to 75% of their dialysis patients on PD[12].

PD offers some advantages over HD such as the ability to undertake treatment at home, being free to move during the day (NIPD) and better PO_4^{3-} clearing. The risks and expenses of the PD are comparable to HD, with the primary risk in PD being that of infection of the catheter followed by peritonitis. The advantages offered by HD are better control of blood pressure, less diet restrictions (severe protein loss in PD, i.e. albumin via the peritoneum) and better efficiency in removing wastes from the blood.

In Germany, 808 dialysis patients per million population (pmp) were recorded in 2006 (in total 66,508). By far the largest part was hemodialysis (87.5%) with ambulatory central hemodialysis (75.2%) as the major type. Peritoneal dialysis accounted for only 4.8% of all dialysis. The incidence of dialysis was 213pmp in 2006[1].

Since its introduction in the 1960s, dialysis has allowed many patients to survive with diseases which only 60 years ago meant certain death. But despite this enormous scientific progress it is important to remember that dialysis is no curative treatment (curing a disease) but merely a palliative means to elongate life. Because even if an almost normal life is possible with dialysis, the long-term consequences of end stage renal disease requiring dialysis are grave.

2.2.4 Consequences of ESRD and dialysis

CKD is a state of chronic, low-grade inflammation in which blood cells, most notably platelets and leukocytes, are constantly activated[13]. One explanation for this chronic state of inflammation is the increased exposure to lipopolysaccharide (LPS) from bacterial membranes in the blood due to uremia-related increase in intestinal permeability[14]. It has been shown that CKD patients have an increased risk to contract bacterial infections like pneumonia or die of it[15,16].

In dialysis patients, an additional factor comes into play. The contact of the blood to the surfaces of dialysis membranes seems to matter in the inflammation process[17]. The side-effects of the interaction between the blood cells and the foreign material of the dialysis

membrane have been described as "bioincompatibility", a form of inflammatory response[18] scaled both acute and long-term. The acute inflammatory response is regulated by numerous mediators, most notably the pro-inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor α (TNFα) and IL-6. The latter is mostly secreted from PBMC (peripheral blood mononuclear cells) in human blood and is the major cytokine to induce the production of acute phase proteins such as C- reactive protein (CRP)[19], which in daily routine is most commonly used to measure inflammatory status.

This permanent, long-term inflammation has been shown to massively influence the mortality in dialysis patients[20]. The increased mortality is mostly due to a dramatic rise in soft tissue calcification and atherosclerosis among dialysis patients[21]. Even young patients who undergo regular dialysis suffer diseases such as coronary artery disease (CAD) which are not usually seen in their age-group among non-CKD patients[22]. Patients receiving regular hemodialysis (hence called DP), in comparison to healthy individuals (hence called HI), show a significant cognitive decline[23] and have a 10 to 30 times higher cardiovascular mortality[24,] due to accelerated atherosclerosis and CAD, and age-related macula degeneration.

The risk to develop tumors is also increased in dialysis patients[25, 26]. In a big study by Maisonneuve et al., more than 830,000 patients who received dialysis for ESRD were observed for an average follow-up of 2.5 years. The risk of dialysis patients to develop cancer was increased by 80% (standard incidence rate i.e. the rate of observed to expected cancer, SIR 1.8) when dialysis patients of all age groups are observed. The risk was especially high in patients under the age of 35, with the relative risk going up more than 300% (SIR 3.7). In older patients, the risk was closer to that of the normal population (SIR 1.2), i.e. the relative risk gradually diminished with age. The tumor entities with the highest increase in risk were malignancies of the genitourinary tract such as bladder (1.5) and kidney (3.6) tumors. Thyroid and other endocrine cancers (2.3) and hematopoietic cancers such as multiple myeloma (3.5) were also more common than in the healthy population[27].

2.3 Antibodies

2.3.1 Structure of antibodies

Antibodies (=Ab), also called Immunoglobulins (=Ig), are proteins in the blood and other body fluids of humans which serve the immune system to defend against foreign particles that express certain antigens (=Ag) on their surface such as bacteria or tumor cells. They are produced in their soluble form by plasma cells, certain types of leukocytes as a reaction to the encounter of foreign antigens. The following picture (figure 1) demonstrates the design of an antibody:

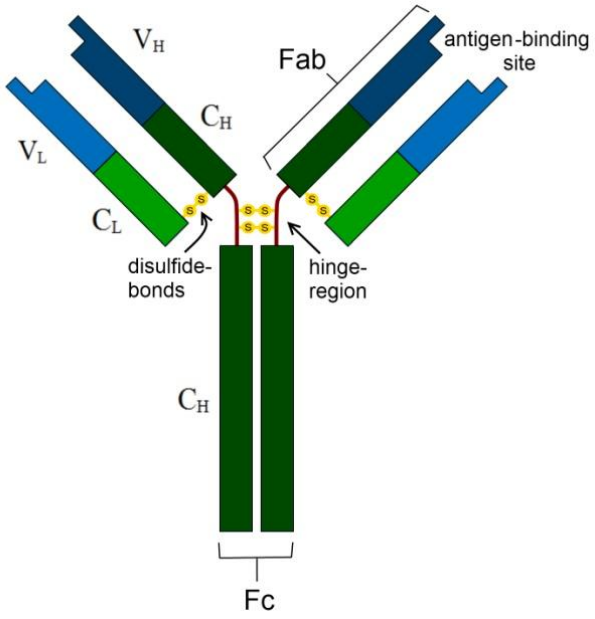


Figure 1: Schematic drawing of an antibody

Antibodies consist of two identical heavy chains (dark blue/green, V_H and C_H) and two identical light chains (bright blue/green, V_L and C_L) bound to each other by disulfide bonds. The antibody's subclass (IgA, D, E, G or M) is determined by its H-chain, while there are two subtypes of L-chain (κ or λ). Every type of H-chain can be combined with every type of L-chain. One Fc ("fragment crystallizable") and two Fab parts ("fragment antigen binding") of the antibody can be distinguished, the Fab fragments being bound to the Fc part via hinge-regions.
At the end of the Fab are the variable domains ("V", blue) while the rest of the Fab and the Fc make up the constant domains ("C", green). The end of the variable domain contains the antigen-binding site, which determines the epitope (region of the Ag recognized by the antibody) the antibody binds to[28, 29].

2.3.2 Antigens

Antigens (=Ag), which can trigger the production of antibodies and are bound by them, are foremost structures on the surface of pathogens like bacteria (such as LPS) or tumor cells. The antibody binds to a certain epitope of the antigen and triggers an immune reaction.

2.3.3 Antibody subtypes

There are five different subtypes of antibodies in humans (IgA, D, E, G and M), which differ in size, biological properties and location in the body. All of them can be found in the blood, while some also function as receptors in the membrane of cells. Table 1 gives an overview over antibodies found in humans.

Name	Subclasses	Molecular form	Serum concentration	Primary location
IgG	IgG1, 2, 3, 4	monomer	~ 12 mg/ml	serum
IgA	IgA1, 2	monomer, dimer	~ 3 mg/ml, 90%monomer, 10%dimer	epithelium
IgM	-	pentamer	~ 1.5mg/ml	serum
IgD	-	monomer	~ 30µg/ml	serum
IgE	-	monomer	~ 0.005µg/ml	serum

Table 1: Immunoglobulin subtypes in humans

IgG, a monomeric Ig with four subtypes (~70%IgG1, 20%IgG2, 5%IgG3 and 5%IgG4) is the antibody most often found in the blood and provides the main immunity against foreign pathogens. Since it is the smallest Ig, it is the only one which can pass through the placenta's blood/blood barrier to provide passive immunity to the fetus.

IgA, a dimeric Ig with two subclasses (IgA1 and 2) is secreted on all mucosa tissue such as the gastro-intestinal tract, the respiratory tract or the genitourinary tract as well as in various body fluids.

IgM, a pentameric Ig with only one subclass, fights pathogens in the early phase of immune reaction before enough IgG can be produced.

IgE, a monomeric Ig with only one subclass, plays an important role in allergic reactions, as it binds allergens and triggers histamine release from mast cells.

IgD, a monomeric Ig with only one subclass, activates basophilic granulocytes and mast cells.

2.3.4 Antibodies as drugs

Since the idea of antibodies was first established in the 1890s by Paul Ehrlich and others[30], much research has been conducted in this field. In the 1920s, Antibodies were shown to be a type of protein by Heidelberger and Avery[31]; in the 1940s, a lock-and key theory for antibodies was proven by Pauling. In the 1960s, the light chain, the Fab and Fc parts were described by Edelman and Porter[32]. A breakthrough was reached when the first monoclonal antibodies (mAbs: antibodies with exactly the same structure, i.e. identical antigen specificity) could be produced in 1975. This was achieved by Jerne, Köhler and Milstein through the fusing of immortal murine myeloma cells and antibody-producing B-cells, then selecting successfully fused cells by selection medium[33,34]. Monoclonal antibodies were first humanized in 1988. Before, the antibodies produced were murine (mouse-antibodies), or chimerical (variable region murine, constant region human). They were structurally similar but different enough to invoke severe immune reactions. The humanization (mostly human antibody except for antigen-determining region) was an important step to reduce allergic reaction to the foreign proteins experienced by early recipients of chimerical antibodies, thereby increasing antibody tolerance. By using transgenic mice, the production of fully human antibodies was eventually achieved in 1991, reducing allergic reactions further[35, 36].

Nowadays, many antibodies are approved as treatment of various diseases, foremost malignancies. In 2006, 160 different antibodies were in clinical trials or awaiting approval in the US. Antibody drugs are usually well tolerated by patients and offer potent therapeutic possibilities in addition to the usual approaches of surgery, radio therapy or chemotherapy.

2.4 EGFR

The transformation from normal cells to tumor cells (carcinogenesis) requires several alterations on the molecular level. One of these alterations can be an increased expression of growth receptors to increase stimuli by growth factors. The ErbB protein family is a group of such growth receptors that are found in high levels on the surface of many solid tumors[37]. The proteins are named after their genes, the proto-oncogenes c-erbB-1 to 4, whose abbreviation derives from the v-erbB, a viral homolog erythroblastic leukemia oncogene. The family of ErbB consists of four members, ErbB-1 (=EGFR, epidermal growth factor receptor =HER-1), ErbB-2 (=HER-2/neu), ErbB-3 (=HER-3) and ErbB-4 (=HER-4). ErbB-1, -2 and -4

possess tyrosine kinases in the intracellular compartment. ErbB-3 can only function via dimerization with one of the other ErbB subtypes.

ErbB are receptors for many ligands associated with cell growth. The most ligands have been described for EGFR, including EGF (=epidermal growth factor), TGFα (=transforming growth factor), HB-EGF (=heparin-binding EGF-like growth factor), AREG (=amphiregulin) and epiregulin, all members of the EGF-family. An over-expression of EGFR has been shown for numerous tumors[38], including colorectal carcinoma, anal cancer[39] or head and neck cancer[40].

When not bound by a ligand, EGFR has been shown to assume a "tethered" confirmation, in which the dimerization site is blocked by an amino acid side chain, disabling dimerization[41]. Only after binding of a ligand, the receptors turn towards an "untethered" confirmation, allowing the receptors to dimerize into homo- or heterodimers. They then autophosphorylize and start a downstream signaling cascade[42].

By the overexpression of EGFR on the cell surface, activating dimers like the strongly signaling EGFR/HER2neu heterodimer become more numerous on the cells surface. Downstream signaling cascades become stronger activated, leading to heightened cell proliferation and angiogenesis[43], an inhibition of apoptosis[44] and metastasis of tumor cells[45].

In a therapeutic approach, monoclonal antibodies (=mAbs) are directed against the extracellular domain of the EGFR. Binding of the EGFR by monoclonal antibodies suppresses ligand binding and prevents dimerization, thus inhibiting intracellular signaling, resulting in halted proliferation and induction of apoptosis[46]. Apart from these direct effector mechanisms, there are also indirect ones (see 2.6.2 below). Antibodies directed against EGFR currently approved for clinical application are cetuximab (in Europe marketed by Merck as Erbitux®) and panitumumab (marketed by Amgen as Vectibix®). They are both approved for the treatment of metastatic colorectal cancer; cetuximab is also approved for the treatment of squamous cell carcinoma of the head and neck. Several other monoclonal antibodies directed against EGFR are in clinical testing and awaiting FDA approval, e.g. nimotuzumab (YM Biosciences, Habana), zalutumumab (Genmab, Utrecht) or necitumumab[47].

2.5 Fc receptors

Fc receptors (=FcR) are proteins on cell surfaces that recognize the Fc part of an antibody. They play an important role in immune response and can be found on the surface of almost all cell types of the immune system[48] except possibly on T-cells. There are several types of FcR in humans, classified by the types of antibodies they recognize. FcαR, FcγR, FcδR, FcεR and FcμR bind IgA, IgG, IgD, IgE and IgM, respectively.

2.5.1 FcγR

FcγR, the most important group of the FcR for inducing phagocytosis[49], can be divided further into subclasses. They differ in their molecular structures and their IgG affinity. The following table gives an overview over the FcγR subclass:

Name	CD	Affinity	Signaling motif	Cellular distribution
FcγRI	64	high	γ-chain ITAM	Macrophages, monocytes, immature neutrophils
FcγRIIa	32	low	α-chain ITAM	Macrophages, monocytes, neutrophils, platelets, Langerhans cells
FcγRIIb	32	low	α-chain ITIM	Macrophages, monocytes, B-cells
FcγRIIc	32	low	α-chain ITAM	Macrophages, monocytes, neutrophils, B-cells
FcγRIIIa	16a	medium	γ-chain ITAM	Macrophages, monocytes, NK-cells, γδ T-cells
FcγRIIIb	16b	low	GPI-linked receptor	Neutrophils (exclusively)

Table 2: FcγR subclasses

FcγR are expressed on almost all leukocytic cells such as NK-cells, monocytes, macrophages, dendritic cells, neutrophils, basophils or mast cells. They bind the Fc part of IgG and are important in determining the leukocyte's response to antibodies in the serum. They belong to the large immunoglobulin superfamily (IgSF), a group of proteins involved in cell binding and adhesion that is based on shared structural similarity with Igs (=antibodies). All FcγR have an extracellular ligand-binding α-chain, a transmembrane region and an intracellular domain; only FcγRIIIb is composed slightly different with a GPI-linked receptor. FcγRI and FcγRIIIa also have an intracellular γ-chain dimer carrying ITAMs (immunoreceptor tyrosine based activating motifs), while the other activating FcγR have the ITAM on their α-chain. FcγRIIb, the only inhibitory FcγR, expresses an ITIM (immunoreceptor tyrosine based inhibitory motif) on its α-chain. FcγRIIIb, which is exclusively expressed on neutrophils, is a

glycosyl-phosphatidylinositol (GPI)-linked receptor. FcγRI is the only high affinity receptor with an affinity up to 1000 fold that of the other receptors, leading to a constant saturation of FcγRI with ligands. However, activation of this receptor only occurs after the receptors have been crosslinked by antigen. The lower affinity of the other FcγRs prevents the binding of Ig which are always present in the serum and thus avoids uncontrolled activation of the receptors[50]. FcγRIIb, with its unique inhibitory abilities, plays a critical role in restricting B-cell activation. Auto-reactive B-cells constantly arise due to random recombination of immunoglobulin loci, which generates B-cell diversity. By restricting their activation via FcγRIIb, B-cell tolerance can be maintained and autoinflammation prevented[51].

2.6 Antibodies' mechanism of action

When regarding the mode of operation of antibodies, direct and indirect effector mechanisms can be distinguished, both of which contribute to the abatement of tumor cells in vivo.

2.6.1 Direct effector mechanisms

The direct effector mechanisms, mediated by the Fab part of the antibody, include the inhibition of ligand binding to the receptor (e.g. EGF or TGFα for EGFR), inhibition of receptor dimerization or phosphorylation, downregulation of the receptor or induction of apoptosis in target cells. The induction of direct effector mechanisms by antibodies is followed by regressing in tumor growth[52]. Since only the antibody is required to induce these effects, they depend solely on the properties of the target cell and the antibody.

2.6.2 Indirect effector mechanisms

The indirect effector mechanisms, mediated by the Fc part of the antibody, include ADCC, CDC and phagocytosis. The antibody binds its antigen with the Fab part, while its Fc part interacts with effector cells (leukocytes) or complement factors.

2.6.2.1 ADCC

In ADCC (antibody dependent cellular cytotoxicity), antibodies mediate the lysis of tumor cells (=targets) through the interaction with effector cells[53] (e.g. NK cells, neutrophils (=PMN) or monocytes) (see figure 2).

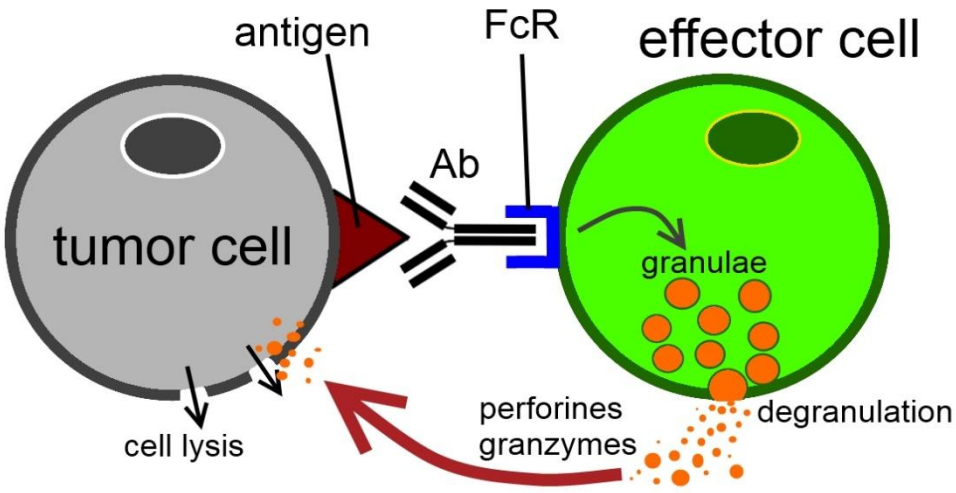


Figure 2: Schematic principle of ADCC:

A target cell (tumor cell) expresses an antigen such as EGFR on its surface, which is bound by the Fab part of an antibody such as cetuximab. The antibodies Fc part is bound by the FcR of an effector cell such as NK-cells, monocytes or neutrophils (PMN). The binding of the antibody to the FcR triggers degranulation of granulae in the effector cells, liberating perforines, granzymes and other substances which lead to cell lysis of the target cell.

2.6.2.2 CDC

CDC (complement dependent cytotoxicity) mediates the lysis of tumor cells through the activation of complement factors, soluble proteins synthesized mostly in liver hepatocytes, but also in leukocytes or epithelial cells. Since CDC is not influenced by effector cells, it was not tested in these experiments.

2.6.3 Phagocytosis

Phagocytosis (greek phago="eating", kytos="cell", osis="process"), a special form of endocytosis, is the ingestion of solid particles of subcellular size by scavenger cells (phagocytes) such as neutrophils or macrophages. The phagocytes recognize the Fc part of the antibody that binds the antigen on the particles surface with its Fab, engulf the particle in spurs of the membrane and internalize them in phagosomes. These phagosomes fuse with lysosomes, intracellular vesicles with low pH, to form phagolysosomes and digest the particle.

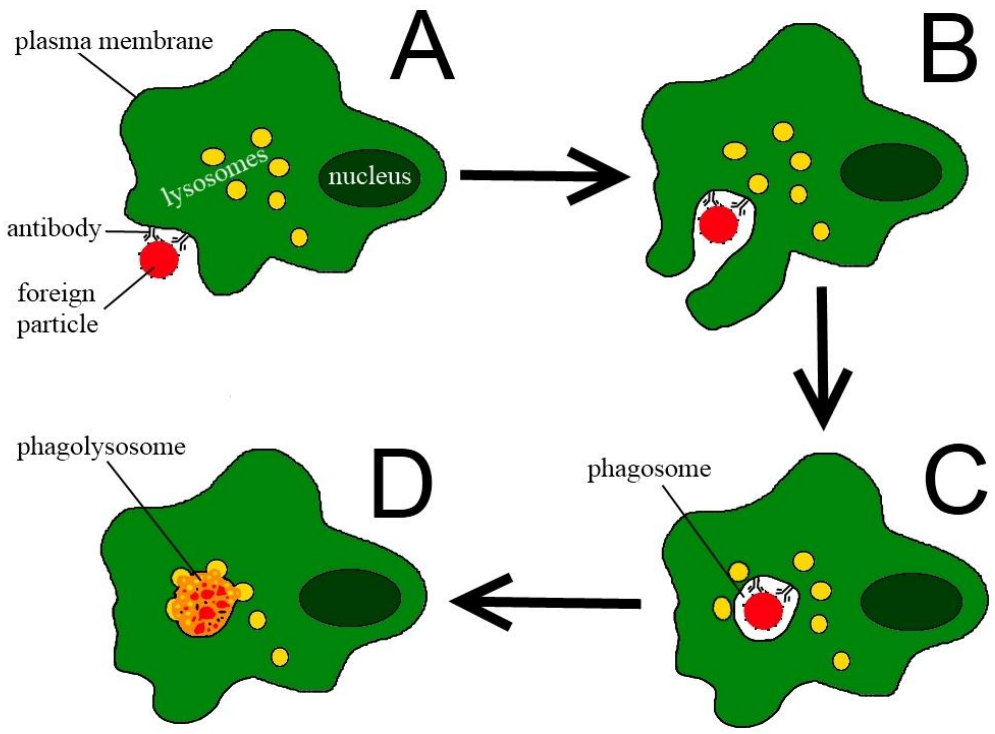


Figure 3: Schematic principle of Phagocytosis:

A phagocyte (green), such as a neutrophil, monocyte or macrophage, binds a foreign particle antibody-mediated (A). It engulfs the particle in membrane spurs (B), internalizing it into a phagosome (C). It then fuses the lysosomes (yellow) with the phagosome into a phagolysosome (D), digesting the particle.

2.6.4 Leukocyte subpopulations

The indirect effector mechanisms are influenced not only by the properties of the target cell and the antibody, but in case of ADCC and phagocytosis, also of the effector cells (leukocytes). The leukocytes are influenced constantly by a number of cytokines and surface molecules they interact with. The leukocytes are not one homogenous group but consist of numerous subgroups (see figure 4), which react differently on these impulses. In hemodialysis patients, the leukocytes are regularly in close contact to the plastic membrane of the dialysis filters, surfaces with entirely foreign molecules. This can be assumed to lead to changes in leukocyte composition and activation status.

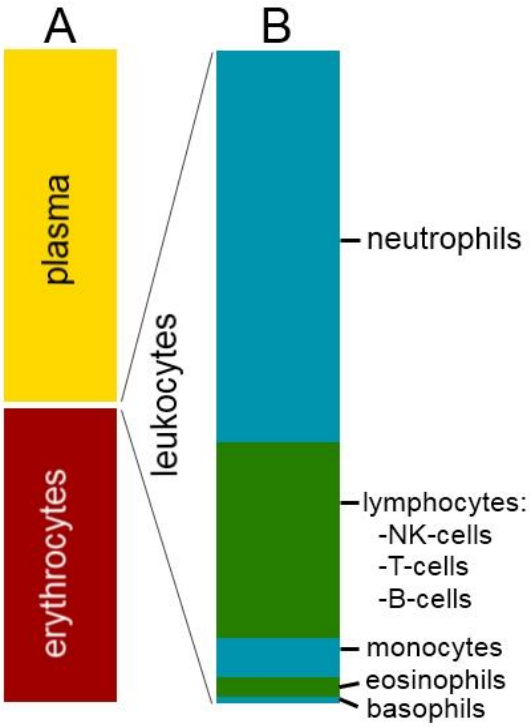


Figure 4: Composition of human blood.
Human blood consists of about 45% erythrocytes ("red blood cells", red in A), 55% plasma (yellow in A) and less than 1% of leukocytes ("white blood cells", white in A). The leukocytes (shown magnified in B) can be subdivided into 60% neutrophilic granulocytes ("neutrophils"), 30% lymphocytes (consisting mostly of NK-, T- and B-cells), 6% monocytes, 3% eosinophilic granulocytes ("eosinophils") and 1% basophilic granulocytes ("basophils").

2.7 Purpose of the study:

ESRD patients undergoing regular hemodialysis (=DP) have a permanently activated immune system and are in a constant state on low-grade inflammation. The uremic solutes in the blood and the constant encounter of foreign surfaces influence the blood's leukocytes and change their state of activation. While the body immediately answers these pro-inflammatory impulses with an activation of the immune system, the impulses seem to reduce the immune status when given continually over a long period of time, as in patients

receiving regular hemodialysis. This is indicated by their increase in bacterial infection and tumor incidence.

There is yet little data on how uremia and hemodialysis influence the leukocyte's abilities to fend off tumor cells via ADCC or phagocytosis. Treatment with monoclonal antibodies is a relatively recent therapeutic option that has seen a dramatic increase in usage in the last decade as more and more antibody-drugs are approved, and will certainly become even more important in the future. Therefore, knowing whether or not dialysis patients profit from the usually cost-intensive antibody-drugs in the same way that healthy individuals (=HI, individuals without ESRD, not on dialysis) do is immediately important for clinical decisions.

Interestingly, two contrary hypotheses can be formulated. While one could argue for decreased abilities of leukocytes to fend off tumor cells in vivo via ADCC due to the increased tumor incidence found in DP, one could also argue for an increased ability due to the permanent state of low-grade inflammation and the constant impulse of pro-inflammatory cytokines.

The data available on whether the ADCC capacity is different in DP from HI is very limited, old and somewhat contradicting. In 1981, Badger at al. did a series of experiments on ADCC with blood from 23 DP. They found equal or increased lysis rates in ADCC in most of the DP[54]. Also in 1981, Lang et al. showed reduced lysis rates in ADCC among 15 DP[55]. Both groups investigated solely mononuclear cells as effector cell population. To our knowledge, no data on neutrophil's ADCC capacity were reported. These two contradicting studies have insufficiently explained how ESRD and/or hemodialysis alters ADCC capacity. Further efforts are necessary to clarify these questions.

If a difference in the abilities of DP's leukocytes to fight against tumor cells using antibodies can be proposed, be it increased or decreased, then differences in FcγR expression are a plausible reason for it. FcγR are the receptors for IgG antibodies and play a vital role in the intracellular downstream signaling after encountering foreign antigens. A change in FcγR expression among certain leukocytes subpopulations could therefore be proposed. One paper from 2002 by Kawanaka et al. showed increased CD16 (FcγRIII) on monocytes from DP[56]. No data has yet been published on the other types of FcγR, or on expression on different leukocyte subpopulations from DP such as monocytes or PMN. In a study from

2004, Bouts et al. investigated FcR expression of lymphocytes, monocytes and neutrophils, and showed significantly reduced levels of FcγRII on all three populations[57]. Unfortunately, this was only investigated in uremic children. Another deficiency in this study was the presentation of the results. For FcR quantification, only the measured mean fluorescent intensity (MFI) was shown, in oppose to presenting the actual number of molecules on the cells surface. No reference particles with defined antigen density (as in Dako's Qifikit) were entrained, only the cells labeled with fluorescent antibody were measured using flow cytometry. Since the MFI varies from assay to assay, this method is less reliable than the direct quantification where an actual number is calculated each day from reference particles with defined antigen density.

In a study from 1996 by Carcamo et al reduced expression of FcγRI and FcγRIII could be shown[58], but only on peritoneal macrophages and only in patients undergoing peritoneal dialysis. Once again, further investigation is necessary to clarify the question of FcR expression alteration in DP.

Apart from ADCC, phagocytosis is the second major form of indirect effector mechanism of antibodies. It plays an important role in the body's fight to fend off alien particles like bacteria or tumor cells. In order to contribute data to the question whether dialysis influences the leukocyte's ability for phagocytosis, a methodical problem had to be solved. There is no easily conductible experimental assay that allows measurement of antibody-dependent phagocytosis of particles coated with EGFR as an antigen.

The questions and goals of this study are therefore:

- Are antibody-drugs as effectively mediating ADCC and phagocytosis in DP as in HI?
- If there are differences between DP and HI, what are possible causes?
- Does the expression of FcR differ in DP from HI?
- Establishing an assay for antibody-dependent phagocytosis of EGFR coated microspheres

3 Methods

3.1 Study population and consent

Written informed consent was obtained from fifteen dialysis patients undergoing regular hemodialysis (DP) in the department of internal medicine at the University Hospital Schleswig-Holstein in Kiel, Germany. Written informed consent was also obtained from fifteen healthy individuals (HI). The study protocol was approved by the hospital ethics committee before the start of the study.

3.1.1 Taking of blood samples

After written informed consent was obtained, blood was taken from DP immediately prior to the connection of the dialysis machine.

Blood was taken from HI by disinfection of the skin (Kodan Hautantiseptikum, Schülke), puncturing a venous blood vessel at the arm. The blood was collected into a 10ml tube (Monovette-Kanüle, Sarstedt) which included citrate as an anticoagulant and was inverted several times.

Up to 30ml of blood was taken from dialysis patients, up to 100ml from healthy individuals.

3.2 Cell biological methods

All cell biological experiments using cell cultures were performed under sterile conditions under a sterile lamina airflow workbench (HeraSafe). Only sterile materials, buffers and media were used.

3.2.1 Cell Culture

Adherent cells (A1207, A431) were prepared two times a week. Medium in the cell culture flask was decanted, cells were washed twice with 10ml PBS, then incubated with 1 ml pre-warmed Trypsin-EDTA solution (0.25%) at 37°C for several minutes, detaching them of the flasks plastic wall. Detached cells were decanted into 20ml pre-warmed medium, stopping the Trypsin's enzymatic reaction. 1ml of this solution was diluted 1:20 by adding 19ml of new medium and returned to a new flask. Cell culture flasks were stored at 37°C and 5% CO_2. Contamination with Mycoplasma bacteria was controlled monthly by Lonza Kit.

3.2.2 Vitality testing, cell count

Cells were dyed with trypan blue and diluted as required. For a 1:10 dilution, 10µl cell suspension was added to 90µl of trypan blue, dispersed, added to a counting chamber (Neubauer-Zählkammer, VWR) and viewed through a microscope. Avital cells were dyed blue, vital cells remained white.

Cells in the 4 x 4 Squares of the counting chamber were counted, the number of cells was calculated using the following formula: cells/ml = counted cells in 4 squares / (4 x dilution factor x 10.000).

3.2.3 Isolation of Leukocyte -Subpopulations

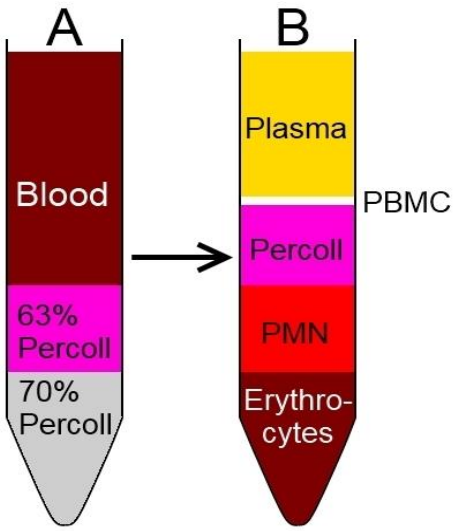


Figure 5: Isolation of PBMC, PMN.
Percoll gradients of two different densities (63% and 70% percoll) are carefully layered atop one another. Blood is layered on top (A) and the whole tube is centrifuged at 2500min^{-1} for 20min. This results in a separation of the blood's components according to their density (B): beneath the low-density plasma is the “buffy coat” containing the PBMC. The PMN are mainly in the layer between the percoll and the erythrocytes.

3.2.3.1 PBMC, PMN by density gradient

A density gradient consisting of 3ml of two different percoll layers (70%, then 63%) was layered briefly with 10ml citrate-anticoagulated blood in a 15ml tube. After centrifugation (2500min^{-1}, 20min, no break) the blood's components were separated according to their density (see Figure 5).

The PBMC ("buffy coat") were collected from the plasma/percoll interface, PMN (neutrophils) were collected from beneath the percoll layer. The PBMC were placed in a new 50ml tube, washed with PBS and poured through a strainer with pores of 30µm size to remove clotted cells. The remaining erythrocytes were then removed by hypotonic lysis. For this, PBMC were filled up to 50ml with PBS, centrifuged (1800min^{-1}, 5min), the pellet consequently resuspended in 45ml of 4°C cold distilled water and inverted several times.

After 30 seconds, 5ml of 10xPBS were applied to restore the concentration of the 1xPBS phosphate buffer and again the tube was inverted several times. Since the erythrocytes are less stable to the osmotic stress than the leukocytes, the erythrocytes were lysed while the leukocytes survived.

The PMN were also placed in a new 50ml tube, washed with PBS (1800min^{-1}, 5min, no break) and exposed to hypotonic lysis twice, since many more erythrocytes are mixed in the PMN layer than in the PBMC layer.

PBMC and PMN were counted in a counting chamber and tested for vitality via trypan blue exclusion. Their purity was tested by FSC/SSC in flow cytometry. Both were higher than 95%. Subsequently, PBMC and PMN were counted and their concentration adjusted to the desired level in R10+ medium.

3.2.3.2 NK-Cells untouched by NK Cell Isolation Kit (Miltenyi Biotec)

PBMCs were isolated, counted and adjusted to the desired cell number. After centrifugation (10min, 2000min^{-1}), the pellet was resuspended in 40µl MACS-Buffer/10^7 cells.

10µl „NK Cell Biotin-Antibody Cocktail“/10^7 cells was added, then incubated for 10min at 4°C. Subsequently, 30µl MACS-Buffer/10^7 cells were added, then 20µl „NK Cell Microbead Cocktail“/10^7 cells and incubated anew for 15min at 4°C. Thereafter, 1-2ml MACS-Buffer/10^7 cells was added, centrifuged (10min, 2000min^{-1}) and the pellet resuspended in 500µl MACS-Buffer.

An LS-Separation Column was placed in a strong magnetic field of a suitable MACS Separator and rinsed with 3ml MACS-Buffer. The cell suspension was added and washed three times with 3 ml MACS-Buffer. The collected effluent represented the enriched NK-cell fraction, which was subsequently counted and adjusted to the desired concentration.

3.2.3.3 Monocytes touched by CD14 Microbeads (Miltenyi Biotec)

PBMCs were isolated, counted and adjusted to the desired cell number. After centrifugation (10min, 2000min^{-1}), the pellet was resuspended in 40µl MACS-Buffer/10^7 cells.

20µl „CD14 MicroBeads“/10^7 cells was added, then incubated for 15min at 4°C. Subsequently, 1-2ml MACS-Buffer/10^7 cells was added, centrifuged (10min, 2000min^{-1}) and the pellet resuspended in 500µl MACS-Buffer.

An LS-Separation Column was placed in a strong magnetic field of a suitable MACS Separator and rinsed with 3ml MACS-Buffer. The cell suspension was added and washed three times

with 3 ml MACS-Buffer. The collected effluent represented the CD14 negative cell fraction. The column was removed from the magnetic field and placed atop a new tube. 5ml of MACS-Buffer were added and the plunger was pushed firmly into the column, washing out the labeled CD14+ cells, which were subsequently counted and adjusted to the desired concentration.

3.2.3.4 Monocytes untouched by Monocyte Isolation Kit II (Miltenyi Biotec)

PBMCs were isolated, counted and adjusted to the desired cell number. After centrifugation (10min, 2000min^{-1}), the pellet was resuspended in 40µl MACS-Buffer/10^7 cells.
10µl „Fcγ-Blocking Reagent“/10^7 cells and 10µl „Biotin-Antibody-Cocktail“/10^7 cells were added, respectively, then incubated for 10 min at 4°C. Subsequently, 30µl MACS-Buffer/10^7 cells were added, then 20µl „Anti-Biotin Microbeads“/10^7 cells and incubated anew for 15min at 4°C. Thereafter, 1-2ml MACS-Buffer/10^7 cells was added, centrifuged (10min, 2000min^{-1}) and the pellet resuspended in 500µl MACS-Buffer.
An LS-Separation Column was placed in a strong magnetic field of a suitable MACS Separator and rinsed with 3ml MACS-Buffer. The cell suspension was added and washed three times with 3 ml MACS-Buffer. The collected effluent represented the enriched monocyte cell fraction, which was subsequently counted and adjusted to the desired concentration.

3.2.4 Fluorescence microscopy

In order to visualize the phagocytosis of microspheres by PMN, the beads were incubated at 37°C with different concentrations of PMN over different periods of time. Subsequently, cells were washed twice by 100µl of FACS-Buffer, then applied to a glass slide and viewed under a fluorescent microscope (Zeiss Imager Z1). If the samples were irradiated by laser light (Zeiss ApoTome), the beads fluoresced.

3.2.5 Flow Cytometry

3.2.5.1 Immunofluorescence theory

Direct (primary) and indirect (secondary) immunofluorescence (IF) can be distinguished; both can be measured using a flow cytometer.

Particles are sped through a capillary at high velocity, passing through a beam of laser light of a certain wavelength. Each particle passing through this beam scatters the light, and fluorescent parts of the particle (fluorophores) may be exited into emitting light of different wavelengths. The light is measured by several detectors, the FSC (forward scatter) which changes according to the particle's volume and the SSC (sidewards scatter) which changes according to the particle's granularity.

For the direct immunofluorescence (see figure 6), a fluorescently marked antibody, directed against an antigen at the particles cell surface, is applied, e.g. a PC-7 labeled antibody against human CD56 on the surface of NK-cells. The antibody binds the antigen and can then be detected through flow cytometry.

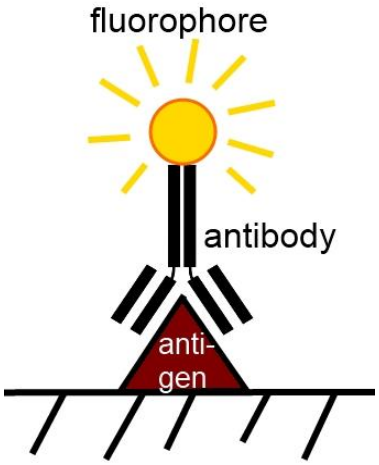


Figure 6: Direct immunofluorescence.
An antibody binds to an antigen with its Fab part. Its Fc part is marked by a fluorophore, allowing its detection in flow cytometry.

For the indirect immunofluorescence (see figure 7), an unmarked primary antibody is added, binding to the Ag with its Fab part. Subsequently, a fluorescently marked secondary antibody, directed against the Fc-part of the primary antibody is added and binds its target, the primary antibody, allowing the particle to be detected through flow cytometry.

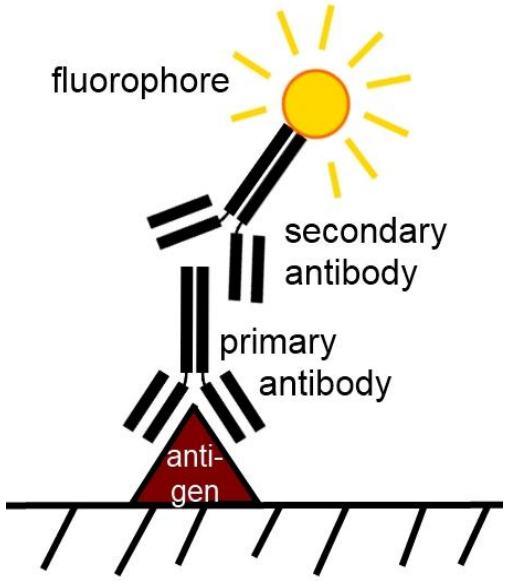


Figure 7: Indirect immunofluorescence.
An antibody binds to an antigen with its Fab part. Its Fc part is consequently bound by a secondary antibody, which is marked at its Fc part with a fluorophore, allowing its detection in flow cytometry.

Direct IF is faster and has less background-signals, while indirect IF can be cheaper, as many primary antibodies can be marked by a single secondary antibody, avoiding the necessity to fluorescently mark each primary antibody individually.
There are many different fluorophores, the ones used here are the following:

name	**emission wavelength (nm)**
FITC	519
PE	578
ECD	615
PC-5	670
PC-7	767

Table 3: Fluorophores used in immunofluorescence

3.2.5.2 Assay procedures

The Qifikit assay should serve as an example for similar assays. 0.5 x 10^6 cells/well were applied to a 96-well V-Bottom microplate and centrifuged (2000min^{-1}, 2min). Fc receptors (FcR) were blocked by adding 5µl/well Polyglobin (10mg/ml) per well, saturating the binding capacity of the FcR by unspecific human IgGs. 5µl of the primary antibodies (200µg/ml) were added /well, which could now only bind to the FcR with their Fab- parts. After incubation for 15min at 4°C, cells were washed two times by 100µl Facs-Buffer/well. At the indirect IF, the secondary antibody was now applied (5µl of the 200µg/ml concentration), incubated for 30min at 4°C and washed twice again by 100µl Facs-Buffer/well. The cell pellet was then resuspended in 100µl PBS, placed in a tube containing 400µl of PBS and measured in a flow cytometer.

The following other flow cytometry assays were performed:

Assay	Antigen (Target)	Primary antibody	Secondary antibody	Incuba-tion time
Without immunofluorescence				
Control of the PBMC/PMN-purification	-	-	-	-
Direct immunofluorescence				
Control of NK-cell and monocyte purification	human CD 14, 16, 56	Isotype controls: IgG -FITC, -PE, -PC5 measuring antibodies: CD14-FITC, CD16-PC5, CD56-PE	-	15min at 4°C
Composition of PBMC: NK-cells, monocytes, T- and B-cells, CTNK-cell	human CD 3, 16, 19, 32, 56, 64	Isotype controls: IgG -FITC, -PE, -ECD, -PC5, -PC7 measuring antibodies: CD45-FITC, CD16-PC5, CD56-PE	-	15min at 4°C
Indirect immunofluorescence				
Qifikit	FcR	mTib92, mTH69, mW6/32, m22, mIV.3, m3G8, mA77	gαm IgG FITC	15min at 4°C
Binding of EGFR-antibodies to EGFR-coupled beads	EGFR on coupled beads	IgG1, Rituximab, Herceptin, 2F8T, 2F8T-Fab, IgG2, cetuximab, hR3, panitumumab, 003, 005, 018, h425, IgA2	gαh IgG-PerCP (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:20	45min at 4°C

Table 4: Flow cytometry assays

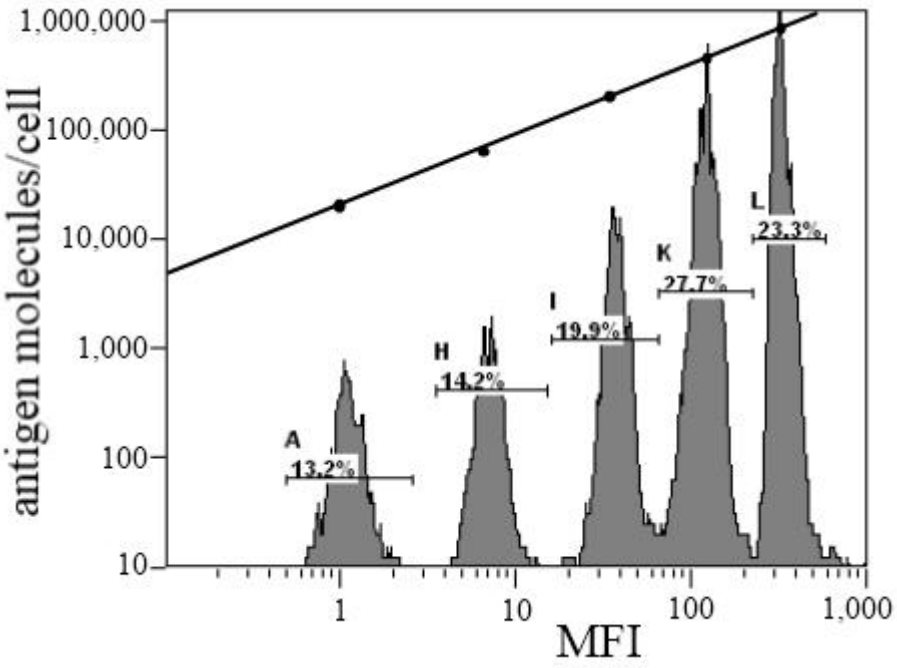


Figure 8: Qifikit calibration beads used to set up a calibration curve.
The measured MFI (mean fluorescent intensities) for the 5 peaks of the 5 different beads populations (X axis) are shown as points against the antibodies per bead population (as specified by the producer). A calibration curve is laid to best fit the 5 points. Calibration curve graph is overlaid with flow cytometry data of the calibration beads to visualize the process.

3.2.5.3 Qifikit

The Qifikit sold by Dako allows a reliable quantitative determination of surface antigen on cells by flow cytometry using indirect immunofluorescence (IF). Cells are labeled with a murine primary antibody against the Ag of interest (here: FcR), while other cells are labeled with an unspecific mouse antibody as control. Subsequently, cells, setup beads and calibration beads from the kit are labeled with the same secondary FITC-labeled anti-mouse antibody (gαm-FITC). All antibodies are used at saturating concentration. The calibration beads provided by the kit are then used to construct a calibration curve. There are five different populations of calibration beads, each with a different amount of antigen on its surface. More antigen results in more secondary antibody binding to the beads, yielding a higher MFI (mean fluorescent intensity, a measure for the level of fluorescence) in the flow cytometry. Therefore, a calibration curve can be constructed by assigning the MFI to the X-axis and the number of antigens on the surface to the Y-axis.
By measuring the MFI of the cells analyzed, one can then calculate the antigen density on the cell surface according to the manufacturer's formula.

3.3 Biochemical methods

3.3.1 ADCC

The desired subpopulations of leukocytes, called effectors (PBMC, PMN, monocytes) were first purified (see above) and applied in triplets (to reduce variability) to the wells of a 96-well roundbottom plate. 0.4 x 10^6 (PMN/PBMC) or 0.2 x 10^6 (monocytes) effectors were applied per well, the antibodies had a concentration of 2µg/ml in the final volume of 200µl (less if dilution was used to measure different concentrations).

Of the targets ("target cells" = A1207, A431), which expressed EGFR on their surface, 0.6 x 10^6 per plate were transferred to a 15ml tube and incubated with 100µl ^{51}Cr per 1.2 x 10^6 cells for 2h at 37°C, 5% CO_2.

Subsequently, the targets were washed three times with medium and adjusted to a concentration of 10^5 /ml. 5000 targets were added per well, yielding a final E:T-ratio (ratio of effectors vs. targets) of 80:1 (PMN, PBMC) or 40:1 (monocytes) and a final volume of 200µl/well. After further incubation for 3h at 37°C, 5% CO_2, cells were centrifuged (2000min^{-1}, 5min) and 25µl of the supernatant were carefully transferred to 150µl of a scintillation fluid ("Szintillationsflüssigkeit"). This solution was shaken 15min by an orbital shaker and the ^{51}Cr released by the lysis of the target cells was then measured in a scintillation analyzer (MicroTriLux, PerkinElmer).

The lysis rate of each triplet of wells was calculated using the following formula:

$Lysis = (well_{sci}-BR_{sci})*100/(MR_{sci}-BR_{sci})$

where $well_{sci}$ was the mean of the scintillation measured in a triplet of wells, BR_{sci} was the scintillation of the basal release triplet (no cells, no antibodies) and MR_{sci} was the scintillation measured in the maximal release triplet (targets incubated in 2% TritonX, yielding a complete lysis).

The specific lysis rate was then calculated by dividing the lysis rate for each triplet where antibody and effectors were combined, by the lysis rate of the triplets in which the effectors were incubated without antibody.

3.3.2 Westernblot

The Westernblot technique was used to analyze proteins by their molecular weight, for example to verify the content of ordered protein solutions like EGFR before coupling it on microspheres. It uses gel electrophoresis, in which a voltage is applied to a polyacrylamide

gel containing the protein soluted in buffers. After application of a strong denaturing reagent (sodium dodecyl sulfate, SDS), the tertiary structure of the protein is lost and is becomes covered in the negatively charges SDS. When voltage is applied, the negatively charged proteins migrate towards the positively charged electrode through the mesh of the acrylamid gel. Smaller proteins migrate faster, thus sorting the proteins by size. Size can be determined by comparing the analyzed protein to standards with known molecular weight. 10ml of a Polyacrylamid gel was prepared by combining 4ml H_2O_{dest}, 3.3ml Acrylamid, 2.5ml Tris-buffer, 100µl SDS, 100µl APS and 4µl TEMED, the latter serving as catalyst for the polymerization. Protein samples were loaded into the prepared wells in the gel with Laemmli-buffer. Separation was achieved by applying 135V, 3A, 300W for about 90min. Proteins were then transferred to a nitrocellulose membrane. For this, the membrane was placed atop the gel, covered in turn by a stack of filter paper. Buffer solution is applied to the entire stack, moving up the papers by capillary force and transferring the protein from the gel to the membrane. Subsequently, proteins were detected by a primary antibody and the latter marked by a HRP-marked secondary antibody. The enzyme produced luminescence in proportion to the amount of protein existent, once a chemiluminescent agent (SuperSignal®) was applied. The membrane was viewed under UV light and photographs were taken for documantation.

3.3.3 ELISA

ELISA (enzyme linked immunosorbent assay) was used on several occasions. For example, the binding of antibodies to EGFR coupled beads was controlled by classical sandwich-ELISA. 8-well strips (Nunc Immunosorp) were incubated with an anti-EGFR capture-antibody (100 µl cetuximab, 5µg/ml) at 4°C overnight, chemically binding the capture antibody to the bottom of the wells. All following steps were performed at room temperature. The wells were washed three times by 200µl ELISA-washing-Buffer each and then incubated for 1h with Facs-Buffer. Since Facs-Buffer contains BSA, this step served to block the remaining protein binding sites on the wells' bottom with unspecific protein.

Now the samples (EGFR coupled beads) were applied together with several standards (proteins with known weight) in 7 decreasing concentration steps (dilution 1:2). These samples/standards were bound by the capture antibody. After incubation for 1.5h, the strips were washed three times again with 200µl ELISA-washing-Buffer each, then incubated for 1.5h with a murine detection-antibody (m425), which also recognized EGFR. Subsequently,

the strips were once more washed three times by 200µl ELISA-washing-Buffer each, then incubated for 0.5h by a goat anti-mouse antibody coupled with HRP (horseraddish peroxidase), allowing this antibody (gαm-IgG-HRP) to bind to the secondary antibody's Fc part. 50µl of o-Phenylenediamine dihydrochloride, a substrate of the HRP, was applied, triggering a visible reaction in which the HRP transposed its substrate within a few seconds. After 20sec, the reaction was stopped by 3M HCl and the Adsorption of the samples was measured in an absorption-reader (Tecan Sunrise) at 492nm wavelength. Since the concentration of the proteins working as standards was known, we could derive a calibration curve, allowing us to calculate the approximate concentration of EGFR bound to the beads' surface.

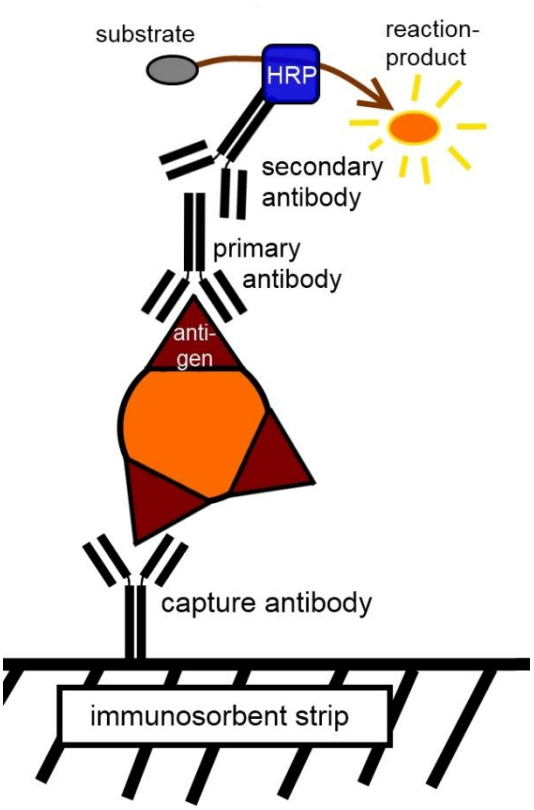


Figure 9: Principle of ELISA
A capture antibody is bound to an immunosorbent strip by its Fc part, allowing it to bind a antigen on the surface of a particle with its Fab part. The antigen is then bound by a primary antibody, which in turn is bound at its Fc part by a secondary antibody. Coupled to the Fc part of the secondary antibody is an enzyme (here HRP= horseraddish peroxidase), which can converse an uncolored substrate to a visible reaction product

3.4 Phagocytosis-assay

We wanted to establish a model for the phagocytosis of tumor cells or parts of tumor cells expressing EGFR by granulocytes (PMN) in order to test whether dialysis patients' ability to phagocyte EGFR-coated particles would differ from healthy donors. We chose fluorescent polysterene microspheres for this ("beads": Fluoresbrite Carboxylate YG 1.0 Micron Microspheres Cat#15702), as they are easy to handle and detect and were available in the desired size of 1µm, so the granulocytes could ingest several beads at once. Since we would couple EGFR on the surface of the beads, our hypothesis was that the degree of phagocytosis observed (number of beads ingested) would depend on the amount of anti-EGFR antibody applied in the experiment. The antibody would bind the EGFR on the beads surface with their Fab part, leaving the Fc part to be detected and consequently bound by the FcγR of the granulocytes, thus inducing phagocytosis of the beads.

Firstly, we tried the coupling of proteins to the surface of microspheres by cheap, easily available protein. Polyglobin (Intratect, Biotest Pharma GmbH), i.e. unspecific polyclonal human IgGs, was used as a positive control, since the coupling would leave some of the antibodies facing outward with their Fc parts, thus encouraging phagocytosis. Albumin (BSA= Albumin FraktionV, Carl Roth GmbH) served as a negative control, since it bears no resemblance to antibody structure. Later, we coupled EGFR (Genmab, Utrecht).

Protein was coupled to the beads surface according to the "Carbodiimid-method" of the TDS 238C from Polysciences Inc.

0.5ml of Beads-solution (~$2.275*10^{10}$ Beads, concentration $4.55*10^{7}$/µl) were washed twice with Carbonate-buffer (pH=9.6), then thrice with MES-buffer (pH=6.0). The -COOH groups ("carboxy goups") on the surface of the beads were then activated by carbodiimid. For this, we resuspended the beads in MES-buffer, then added a freshly set up 2% carbodiimid-solution in a 1:1 dilution and incubated for 3h on a rolling mixer. Since Carbodiimid is known to be unstable in hydrous solution and degrade to urea, which in turn denatures protein, the Carbodiimid solution had to be freshly set up (younger than 15min) and the beads had to be washed twice again with MES-buffer after the incubation. Subsequently, beads were resuspended in 0.2M Borate-buffer (pH=8.5).

Now protein was added. The amount of protein needed to achieve a monolayer of protein on the beads surface was calculated according to the manufacturer's formula:

$S = (6 / \rho * d))*(C)$

Where S = amount of protein needed for a monolayer

ρ = density of microspheres (= 1.05g/cm³)

d = diameter of microspheres (= 1µm)

C = microsphere capacity for a certain protein (Albumin, MW 65kD → 3mg/m²; IgG, MW 150kD → 2.5 mg/m²)

For albumin, we calculated an amount of 18mg albumin / g microspheres. For polyglobin, we calculated an amount of 18mg polyglobin / g microspheres. For EGFR, we calculated 21mg / g microspheres

The beads solution purchased by Polysciences Inc consisted of 2.66% beads with a density of 1.05g/cm³. Thus, there were 1ml*0.0266*1.05 = 0.0273g/ml (= 27.3mg/ml) beads in the manufacturers solution.

The appropriate amount of protein needed was added and incubated on a rolling mixer in the dark over night. On the next morning, Ethanolamine was added to saturate -COOH groups still unbound by protein. To block unspecific protein binding sites, the beads were incubated in Albumin-Solution (10mg BSA in 1ml Borate-buffer) for 30min.

Lastly, beads were resuspended in 0.5 ml Storage-buffer (0.01M Phosphate-buffer, 1% BSA, 0.1% Sodium-Acid, 5% Glycerol), delivering a concentration of about $4.55*10^7$ beads/µl.

To examine the phagocytosis, we incubated 5µl of the beads solution (diluted 1:10, ~$2.275*10^7$ Beads) with 5µl of the antibody (200µg/ml) in a 96 well V-bottom shaped plate for 30 min to allow the binding of the antibodies to EGFR. We then set the purified PMN obtained from healthy donors to the desired concentration of 10^7/ml, then added 50µl ($0.5x10^6$ cells) per well and filled up the wells with medium to 100µl volume. After incubation for 120min at 37°C we washed twice with 100µl Facs-buffer and measured the cells with flow cytometry.

When beads coated only with albumin or polyglobin were used, no antibodies were applied, only beads and the PMN were incubated for 120min at 37°C.

4 Results

4.1 Comparison of DP and HI ADCC capacity

We wanted to examine whether differences could be found in the ADCC capacity of ESRD-patients undergoing regular hemodialysis (DP) from healthy individuals (HI).
Blood samples were taken from DP before the start of hemodialysis procedure. HI served as a control group.
Since EGFR is expressed on many solid tumors such as colorectal carcinoma or head and neck cancer, two FDA-approved antibodies against EGFR which are currently in clinical use were used. As an IgG1 antibody, we used cetuximab (marketed by Merck as Erbitux®), a chimerical monoclonal antibody approved for the treatment of EGFR positive colorectal cancer (about 75% of all colorectal cancer) and head and neck cancer. As an IgG2 antibody, we used panitumumab (marketed by Amgen as Vectibix®), a human monoclonal antibody approved for the treatment of EGFR positive colorectal cancer. When examining PMN, we also used 225 IgA2, since PMN show lower lysis rates with cetuximab than PBMC, but show nearly 100% lysis rates with 225 IgA2 when used at saturating concentration. 225 IgA2 was therefore used as a positive control for PMN.

4.1.1 ADCC of neutrophils against A1207 using cetuximab, panitumumab, 225 IgA2

We firstly examined whether DP would profit from anti-EGFR antibodies to the same extent as HI by conducting ^{51}Cr release assays with dilution rows of cetuximab and panitumumab. PMN (neutrophils) served as effectors (400,000/well) while A1207 served as targets (5000/well) in an E:T-Ratio of 80:1. The specific lysis rate (X-axis) was plotted against the antibody concentration (Y-axis). An unspecific antibody was always entrained as a negative control. Since 225 IgA2 has been shown to mediate strong ADCC with PMN, 225 IgA2 was entrained as a positive control.

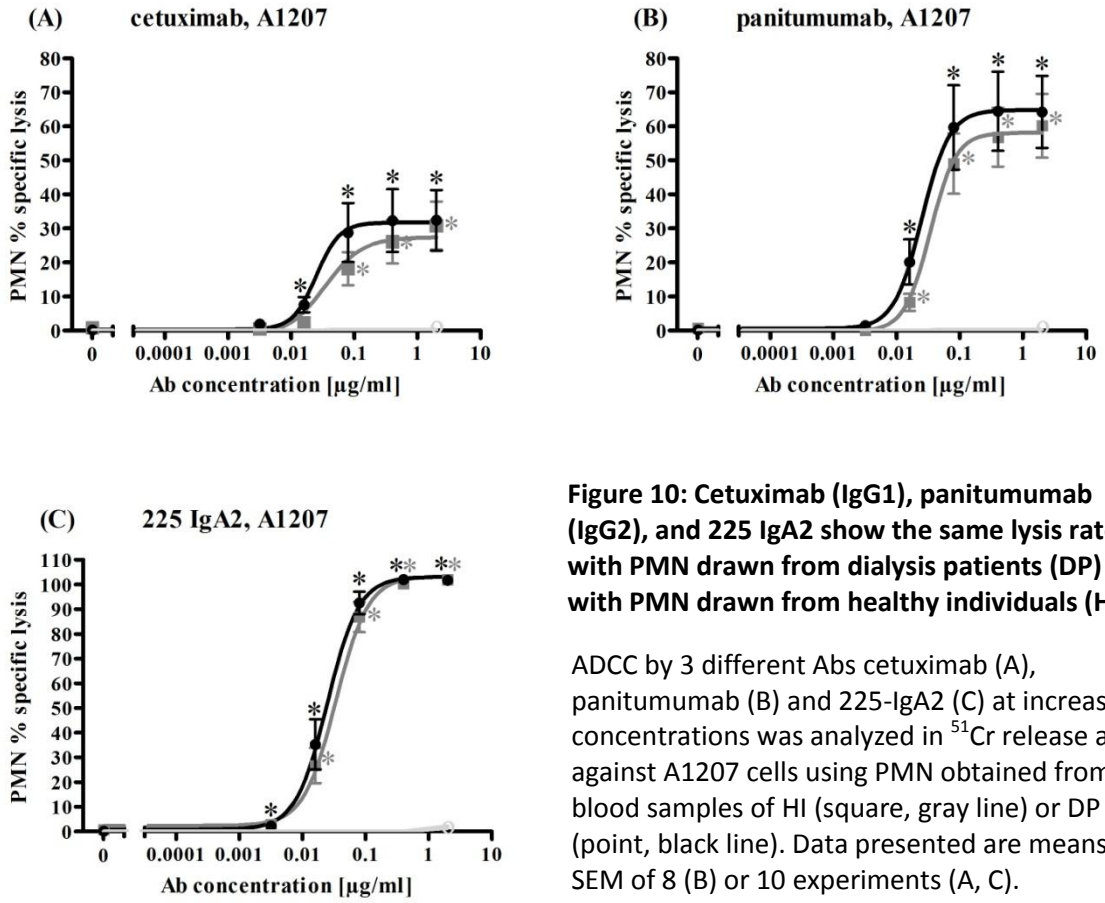


Figure 10: Cetuximab (IgG1), panitumumab (IgG2), and 225 IgA2 show the same lysis rates with PMN drawn from dialysis patients (DP) as with PMN drawn from healthy individuals (HI)

ADCC by 3 different Abs cetuximab (A), panitumumab (B) and 225-IgA2 (C) at increasing concentrations was analyzed in ^{51}Cr release assay against A1207 cells using PMN obtained from blood samples of HI (square, gray line) or DP (point, black line). Data presented are means ± SEM of 8 (B) or 10 experiments (A, C). Significance was accepted when $p < 0.05$. An asterisk (*) indicates significant specific lysis compared to control antibody (open circle, light gray line).

While the specific lysis rates of DP with PMN was slightly higher than that of HI and also the EC50 was slightly lower for cetuximab, panitumumab and 225 IgA2, the difference was far from significant.

Antibody	HI-mean (95%CI)	DP-mean (95%CI)
cetuximab • plateau (95%CI) • EC50 (95%CI)	 29.5 (21.3-37.7) 0.064 (0.024-0.168)	 32.5 (22.8-42.1) 0.030 (0.008-0.112)
panitumumab • plateau (95%CI) • EC50 (95%CI)	 58.8 (49.2-68.3) 0.038 (0.02-0.08)	 64.5 (51.7-77.2) 0.024 (0.010-0.054)
225 IgA2 • plateau (95%CI) • EC50 (95%CI)	 101.8 (95.6-107.9) 0.029 (0.023-0.038)	 102.0 (94.9-109.2) 0.023 (0.017-0.030)

Table 5: Corresponding data for figure 10

4.1.2 ADCC of mononuclear cells against A1207 using cetuximab, panitumumab

The lysis rates of DP with PBMC and cetuximab bore no significant difference, similar to the PMN with cetuximab. For panitumumab, however, a significant difference was observed at the three highest concentrations (0.08, 0.4 and 2µg/ml).

The data indicated a normal distribution of the measurements according to D'Agostino & Pearson omnibus K2 normality test. Main effect in 2-way-ANOVA: no significance between cases: P=0.133, F=2.30; significant difference between healthy/dialysis: P<0.0001, F=24.99. Then paired T-Test, two-tailed: P=0.005, 0.005, 0.006 and R^2=0.71, 0.70 and 0.68 for antibody concentrations of 2µg/ml, 0.4µg/ml and 0.08µg/ml, respectively.

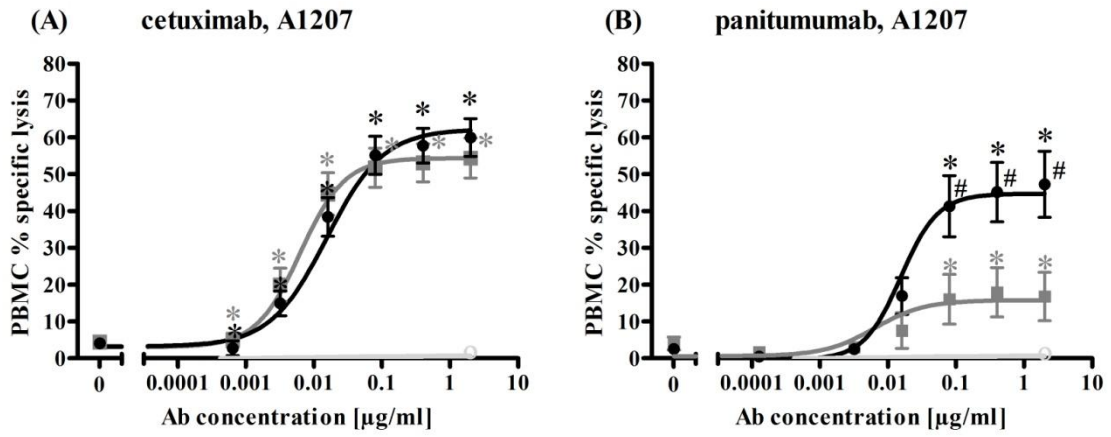


Figure 11: Cetuximab shows the same lysis rate with PBMC drawn from DP as with PBMC drawn from HI. Panitumumab shows significantly higher lysis rates with PBMC from DP than from HI

ADCC by 2 different antibodies cetuximab (A) and panitumumab (B) at increasing concentrations was analyzed in ^{51}Cr release assay against A1207 cells using PBMC obtained from blood samples of HI (square, gray line) or DP (point, black line). Data presented are means ± SEM of 8 (B) or 10 experiments (A). Significance was accepted when $p < 0.05$. An asterisk (*) indicates significant specific lysis compared to control antibody (open circle, light gray line); pound (#) indicates significant difference between HI and DP.

Antibody	HI-mean (95%CI)	DP-mean (95%CI)
cetuximab • Plateau (95%CI) • EC50 (95%CI)	 53.5 (48.2-58.9) 0.005 (0.003-0.008)	 59.4 (53.7-65.0) 0.010 (0.006-0.016)
panitumumab • Plateau (95%CI) • EC50 (95%CI)	 17.4 (10.8-24.1) 0.022 (0.004-0.112)	 46.5 (38.3-54.7) 0.023 (0.011-0.047)

Table 6: Corresponding data for figure 11

4.1.3 ADCC of mononuclear cells against A431 using cetuximab, panitumumab

Similar experiments were now performed on another target cell line expressing EGFR, A431. PBMC of HI and DP were compared by ^{51}Cr release assays with a single antibody concentration of cetuximab and panitumumab, reliably in the saturating region (2µg/ml). Once again, we found slightly elevated rates of specific lysis for DP, but the differences were not significant (n=8, HI-mean=34.7, 95%CI 17.7-51.6. DP-mean=48.5, 95%CI 33.0-64.0; R^2=0.278; P=0.145). For panitumumab, we once again observed significantly elevated rate of specific lysis of DP over HI (n=8, HI-mean=9.0, 95%CI 1.7-16.4; DP-mean=15.7, 95%CI 7.0-24.4; P=0.040; R^2=0.474. paired T-Test, two-tailed).

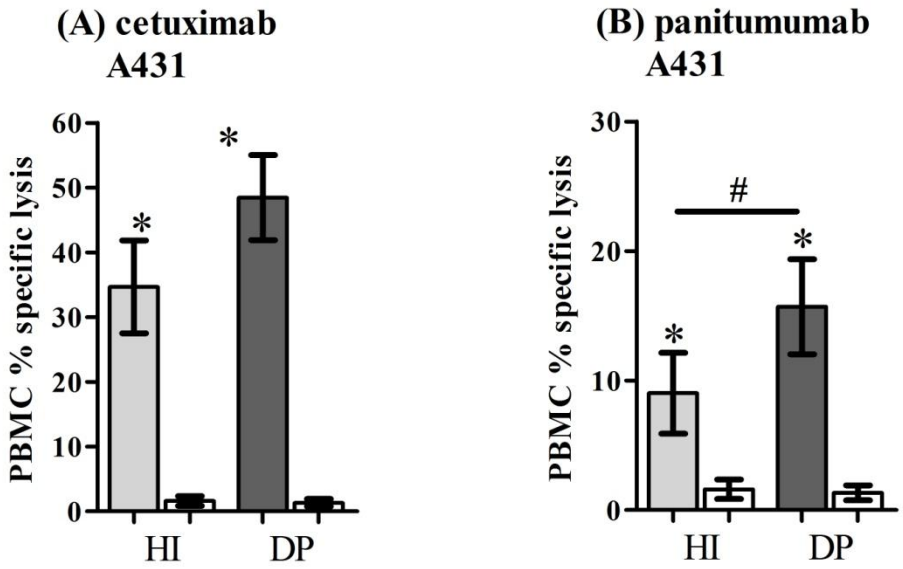


Figure 12: Cetuximab shows slightly higher lysis rates on PBMC drawn from DP as on PBMC drawn from HI. Panitumumab shows significantly higher lysis rates with PBMC from DP than from HI

ADCC by 2 different antibodies cetuximab (A) and panitumumab (B) at 2µg/ml concentration was analyzed in ^{51}Cr release assay against A431 cells using PBMC obtained from blood samples of HI (light gray bar) or DP (dark gray bar). White bars indicate background lysis by respective effectors via control Ab. Data presented are means ± SEM of 8 experiments. Significance was accepted when $p < 0.05$. An asterisk (*) indicates significant specific lysis compared to control antibody; pound (#) indicates significant difference between HI and DP.

4.2 Phagocytosis assay

We had observed an effect of DP's PBMC showing an increased capacity in IgG2-mediated ADCC. We now wanted to see whether this meant that other antibody-dependent methods of defense against tumor cells expressing EGFR like phagocytosis were altered in DP as well. Therefore, we had to establish an assay for antibody-dependent phagocytosis. As a model, we chose EGFR-coated microbeads (=polysterene microspheres), which can easily be detected by flow cytometry.

4.2.1 General practicability, setup of the assay

We first had to successfully couple protein to the bead's surface, then in a next step set out to examine which parameters influenced the phagocytosis of the beads by neutrophils (PMN). In order to show general practicability of the assay, we first coupled the readily available polyglobin (unspecific polyclonal human IgGs, used as a positive control), and albumin (BSA, Albumin FraktionV, used as a negative control). We wanted to determine optimal experimental conditions and find out factors the phagocytosis depended on so that we could improve the assay.

For the phagocytosis assay, we incubated granulocytes obtained from HI with beads, washed twice with Facs-buffer, then measured the cells using flow cytometry (data shown here was measured in Epics XL-MCL, Beckman Coulter). Since the beads are fluorescent at 520nm wavelength (channel F1 in Epics XL-MCL), we could identify the cells that had absorbed fluorescent beads.

We could show that phagocytosis was influenced by the following parameters:

- Amount of protein coupled to the beads surface: more protein meant higher rate of phagocytosis (see figure 14)
- Incubation time: longer incubation time meant higher rate of phagocytosis (30, 75, 120, 240min.) (see figure 15)
- Stimulation of PMN: adding GM-CSF unspecifically activated the granulocytes to a higher rate of phagocytosis for albumin as well as polyglobin (see figure 16)
- Temperature during incubation: while phagocytosis was observed when incubating at 37°C, practically no phagocytosis occurred when incubating at 0°C, even if GM-CSF was added (see figure 17)

The following pictures show 3 graphs per measured sample: FSC_{lin}/SSC_{lin} on the left, $F1_{LOG}/SSC_{lin}$ in the middle and $F1_{LOG}$ on the right. The gate "PMN" in FSC_{lin}/SSC_{lin} is located to fit the solitary PMN and the PMN that have ingested beads, and exclude the solitary beads. The next window, $F1_{LOG}/SSC_{lin}$, only shows events from "PMN", and F1 (X-axis) is plotted against SSC (Y-axis). As PMN ingest more beads, they become more granular, thus receiving higher measurement in SSC, and the beads within the PMN make the cell more fluorescent, thus measuring peaks in the F1 channel. The last window, $F1_{LOG}$, shows only the F1 channel of the "PMN" gate. As more beads get ingested by PMN, peaks appear on the right side of the window, indicating fluorescent cells. Besides the first big peak (only PMN), the first couple of peaks can be identified, interpreted as PMN having ingested one, two and more beads.

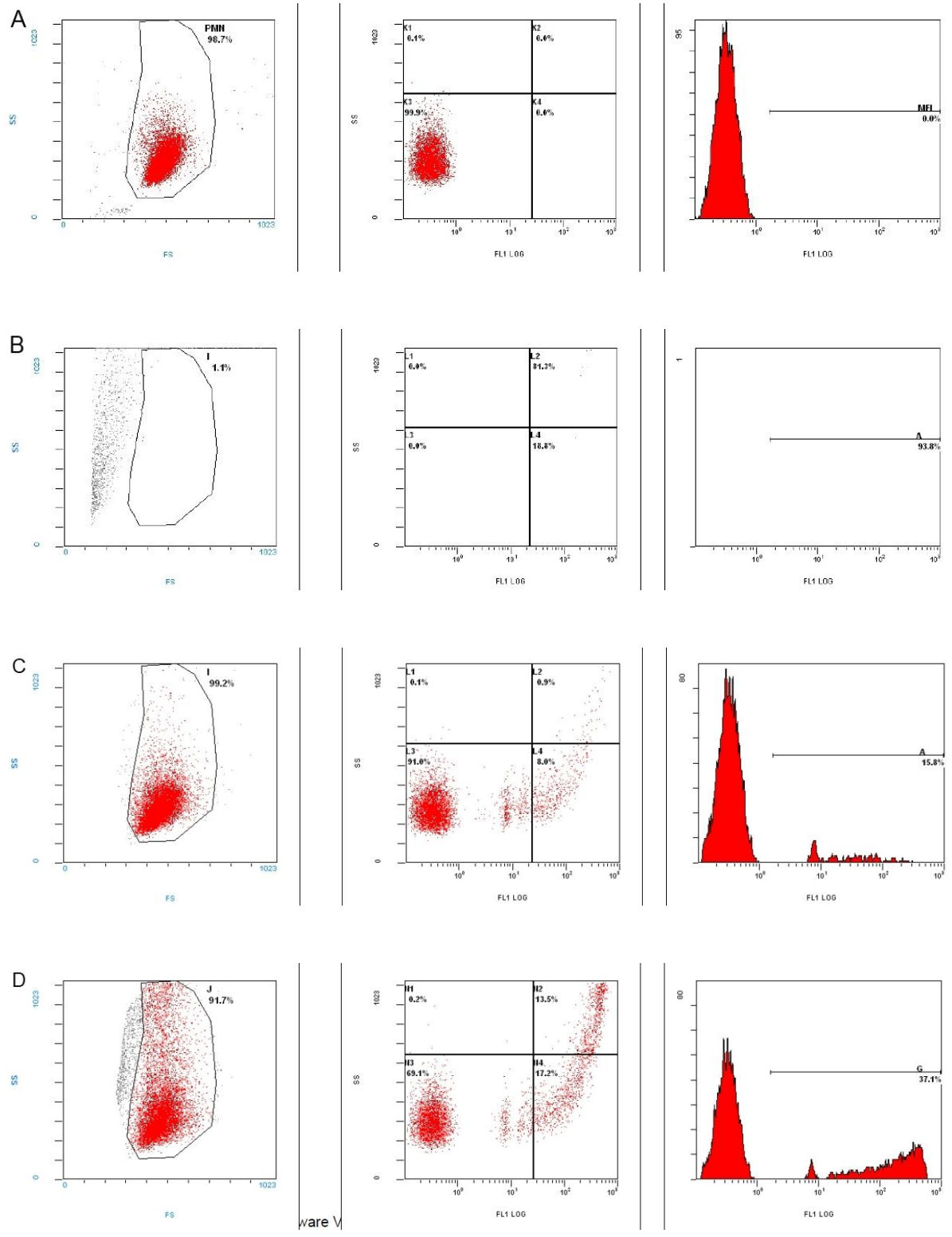
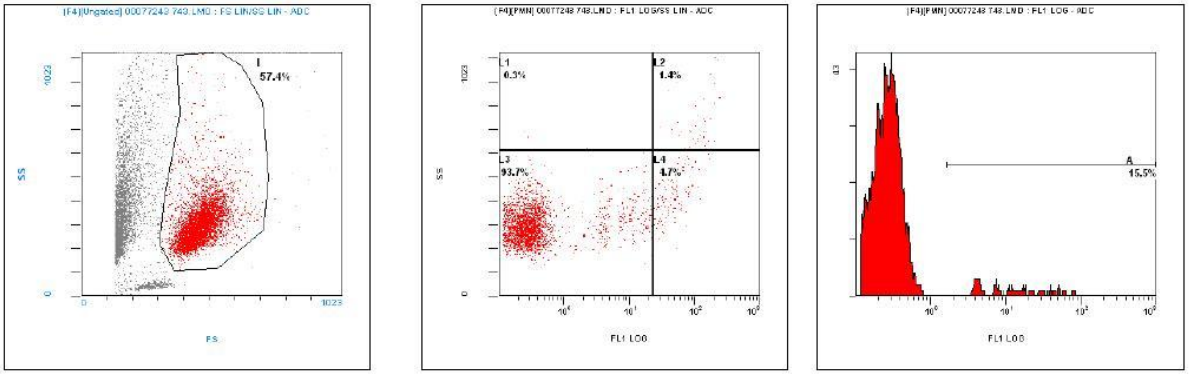
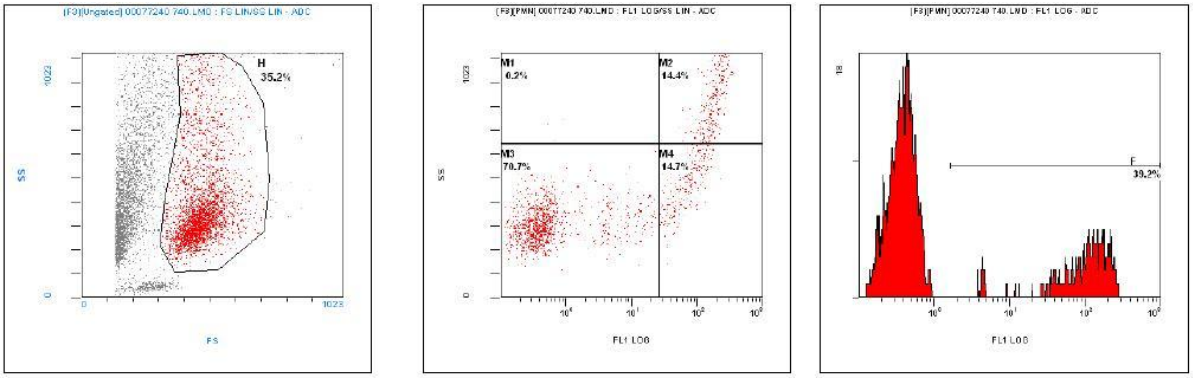


Figure 13: Typical flow cytometry data from phagocytosis assay

PMN alone (A), beads alone (B), PMN + beads coupled with albumin (C), PMN + beads coupled with polyglobin (D), all after incubation at 37°C for 120min. Pictures show 3 graphs per measured sample: FSC_{lin}/SSC_{lin}, (left) $F1_{LOG}/SSC_{lin}$ (middle) and $F1_{LOG}$ (right). The gate seen on the right in the $F1_{LOG}$ window contains the PMN that ingested fluorescent beads.



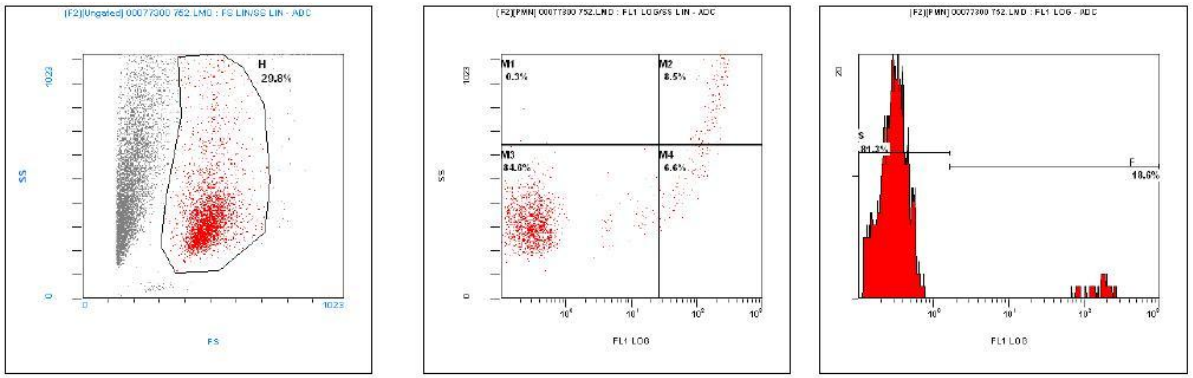
A: small amount of protein



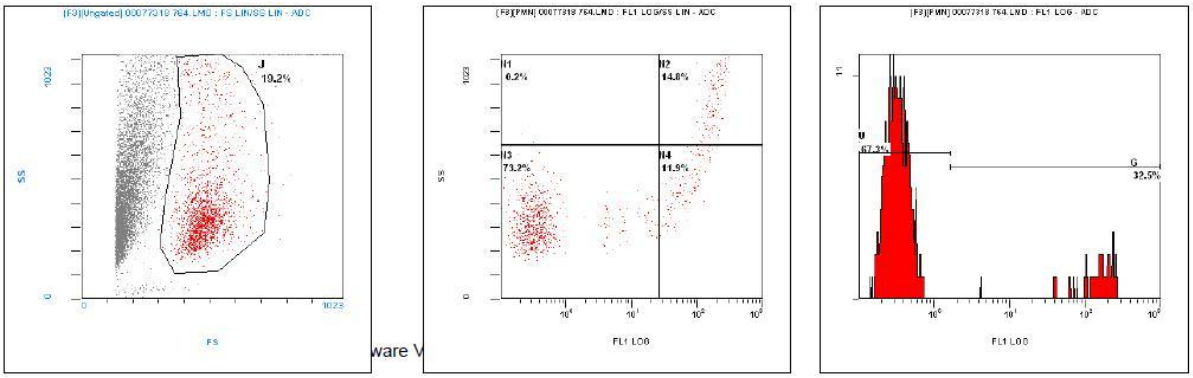
B: large amount of protein

Figure 14: Rate of phagocytosis is influenced by the amount of protein coupled to the beads surface

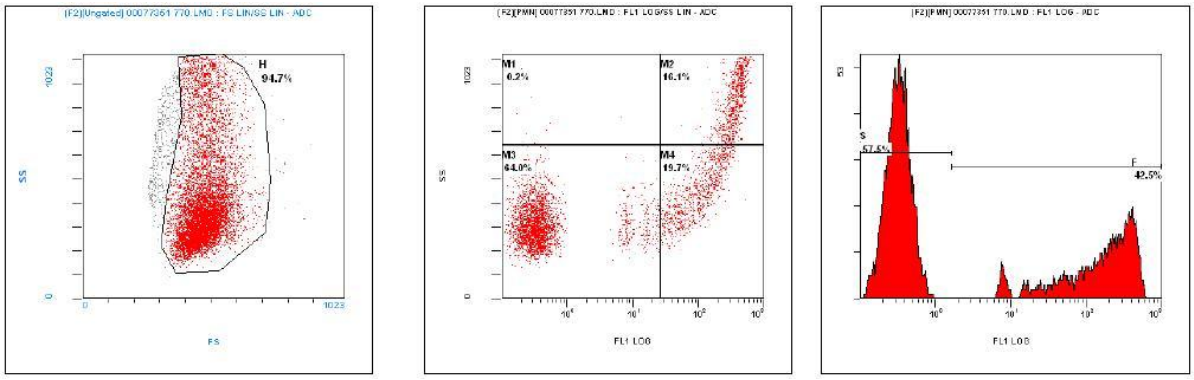
Incubation of PMN and beads coated with small (A) or large (B) amounts of polyglobin for 120min at 37°C.



A: 30min



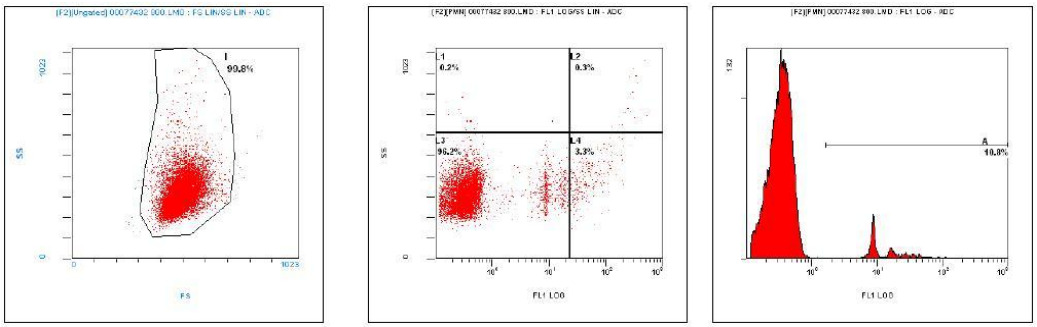
B: 75min.



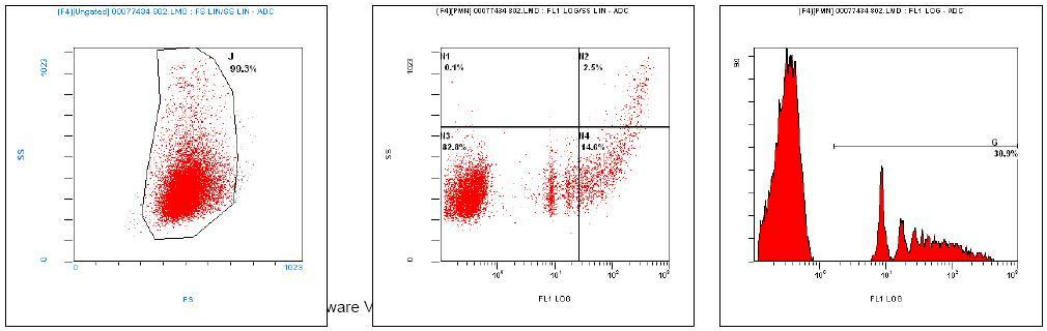
C: 120min.

Figure 15: Rate of phagocytosis is influenced by the incubation time

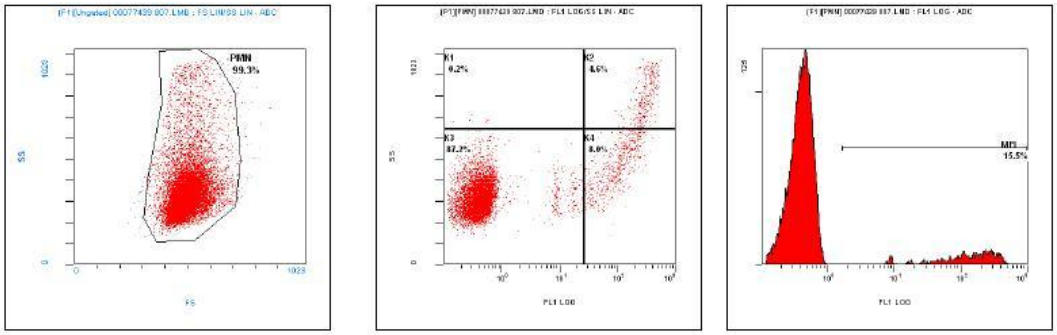
Incubation of PMN with beads coated with polyglobin for 30 (A), 75 (B) and 120min (C) at 37°C.



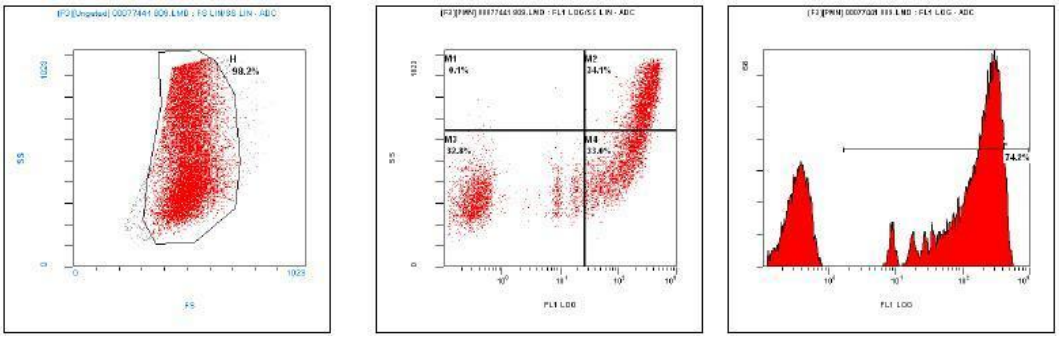
A: Albumin beads



B: Albumin beads with GM-CSF



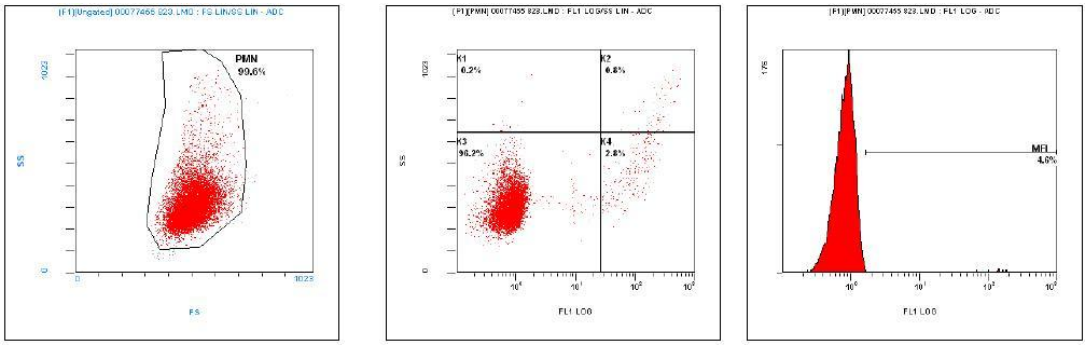
C: Polyglobin beads



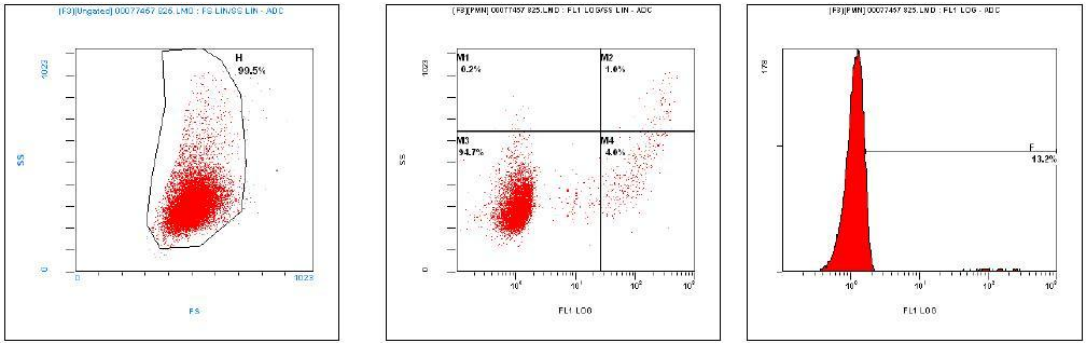
D: Polyglobin beads with GM-CSF

Figure 16: Stimulation of PMN by adding GM-CSF unspecifically activates the granulocytes

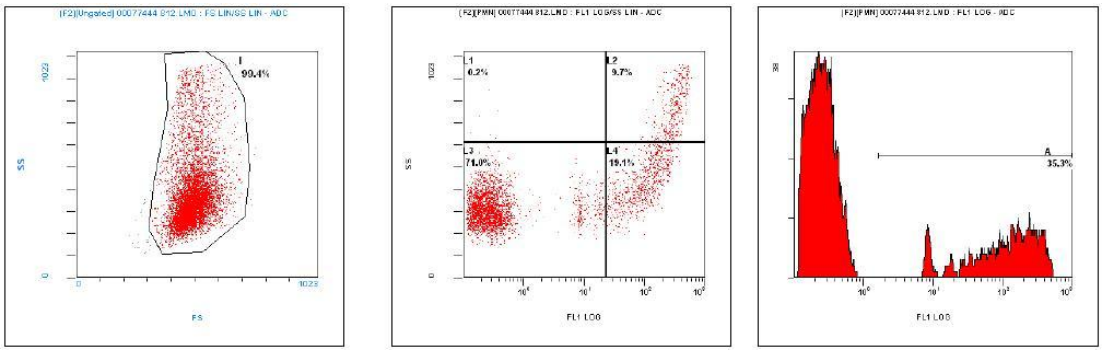
A higher rate of phagocytosis can be observed when incubating PMN with GM-CSF (B and D) and beads coupled with albumin (A and B) as well as polyglobin (C and D), although the effect is remarkably higher on polyglobin-coupled beads. All incubated at 37°C for 120min.



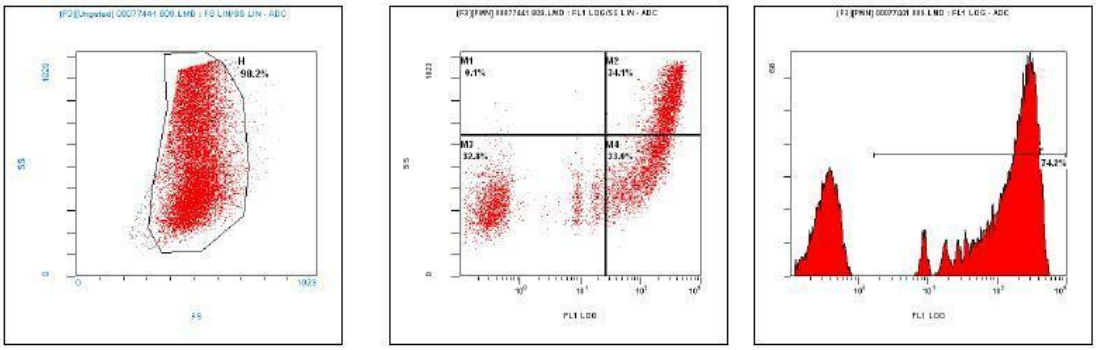
A: incubated at 0°C



B: incubated at 0°C with GM-CSF



C: incubated at 37°C



D: incubated at 37°C with GM-CSF

Figure 17: Rate of phagocytosis is influenced by the temperature during incubation

PMN are incubated with beads coupled with polyglobin for 120min at 0°C (A and B) and at 37°C (C and D) without (A and C) and with GM-CSF (B and D).

We also experimented with the shape of wells in the 96 well plates used (V-bottom vs. Flat bottom, no difference observed, no pictures shown) and the total volume in which to incubate the PMN and the beads for the given time (100, 150, 200µl; no significant difference observed but defined 100µl as standard procedure for easier handling, no pictures shown). We then determined it was best to incubate the beads and the Abs without the PMN first for 30min (allowing the Abs to bind to the EGFR on the beads surface), then adding the PMN and incubating for 120min.

As the optimum of experimental conditions, we identified:

1. Coupling an amount of protein to the beads surface to suffice for at least one monolayer
2. First add the beads and antibodies, incubate for 30min, then add PMN, fill to 100µl/well, then incubate.
3. 120min of incubation time at 37°C
4. No GM-CSF added, as this activated the granulocytes unspecifically, inducing phagocytosis of practically everything (even uncoated or albumin-coated beads)
5. Incubation in a V-bottom shaped 96 well plate (easier handling than flat bottom)
6. 100µl of incubation volume (easier handling than higher volumes)

4.2.2 Coupling of EGFR to microspheres

In a next step, we coupled EGFR on the beads' surface. Since only a few mg of this rather expensive protein were available, we down-scaled the coupling assay by 1/10th, starting with 50µl of beads-solution instead of 0.5ml. The calculated amount of EGFR for one monolayer when starting with 50µl of beads-solution was about 22µg.

Since the process of coupling protein to the beads surface involved chemicals with potentially denaturing effect (most notably ethanolamine and carbodiimid), we tried three different coupling procedures.

1. Covalent coupling according to the manufacturer's instructions ("carbodiimid-method", see methods, from Polyscience TDS 238C). Included ethanolamine and carbodiimid.
2. Covalent coupling ("carbodiimid-method"). Without ethanolamine.
3. Coupling by adsorption to non-carboxylated beads (Fluoresbrite Plain YG 1.0 Micron Microspheres Cat#17154) according to manufacturer's instructions (Polyscience TDS 238E). Without ethanolamine or carbodiimid.

For the phagocytosis assays of the EGFR coated beads, we incubated PMN with different Abs: Cetuximab (IgG1) and panitumumab (IgG2) as anti-EGFR antibodies, unspecific IgG1 or IgG2, or antibodies directed against other antigens such as rituximab (CD20) or trastuzumab (HER2/neu, both IgG1) as control antibodies.

No difference was observed between 1. and 2. The method of coupling protein to the surface of beads by adsorption (3.) was abandoned, since differences between albumin-coated and EGFR-coated beads were smaller than with the covalently coupled proteins (no pictures shown).

4.2.3 Assay for IgG antibodies

In our first ADCC experiments, we had observed a heightened ADCC capacity of DP's PBMC with IgG2. We wanted to examine whether this effect indicated a higher rate of IgG2 mediated phagocytosis of EGFR-coated beads, as well. We observed a higher rate of EGFR-coated beads ingested by PMN when they were incubated with panitumumab (37%) than without antibodies (3%), or with unspecific IgG2 (17%).

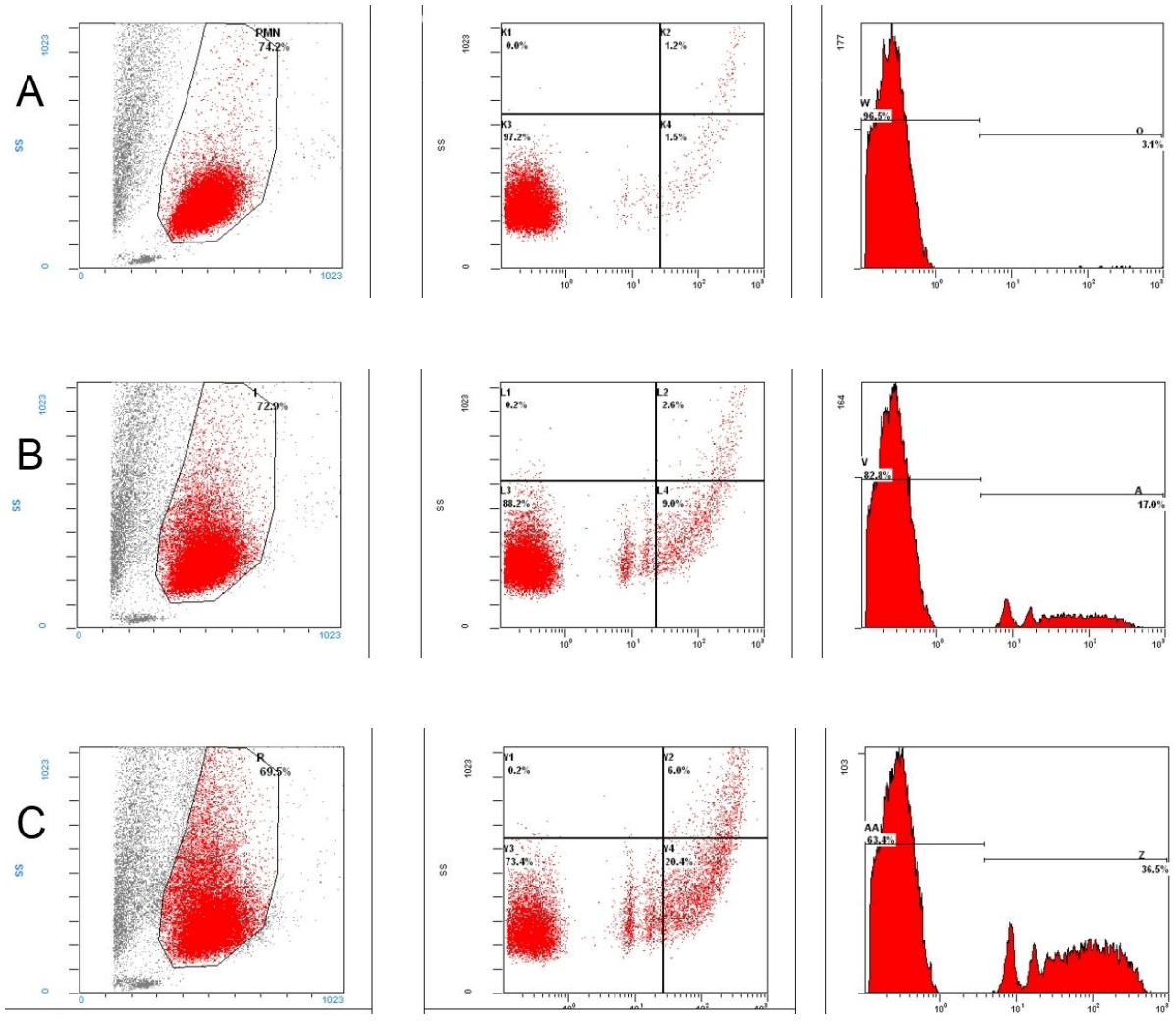


Figure 18: Phagocytosis of EGFR-coated polysterene microspheres (beads) influenced by specificity of IgG2 antibody

Of PMN incubated with EGFR-coated beads and no antibody (A), about 3% ingest beads. Of PMN incubated with EGFR-coated beads and unspecific IgG2 (B), about 17% ingest beads. Of PMN incubated with EGFR-coated beads and panitumumab (C), about 37% ingest beads.

In an immunofluorescence flow cytometry testing, we examined whether or not panitumumab bound to the EGFR-coated beads by first incubating beads and panitumumab, then adding a secondary antibody against human IgG, marked by another fluorophore (gαh PerCP anti-human IgG, diluted 1:20). 47% of the beads were bound by panitumumab, while only 1% of the beads were bound by the corresponding control antibody.

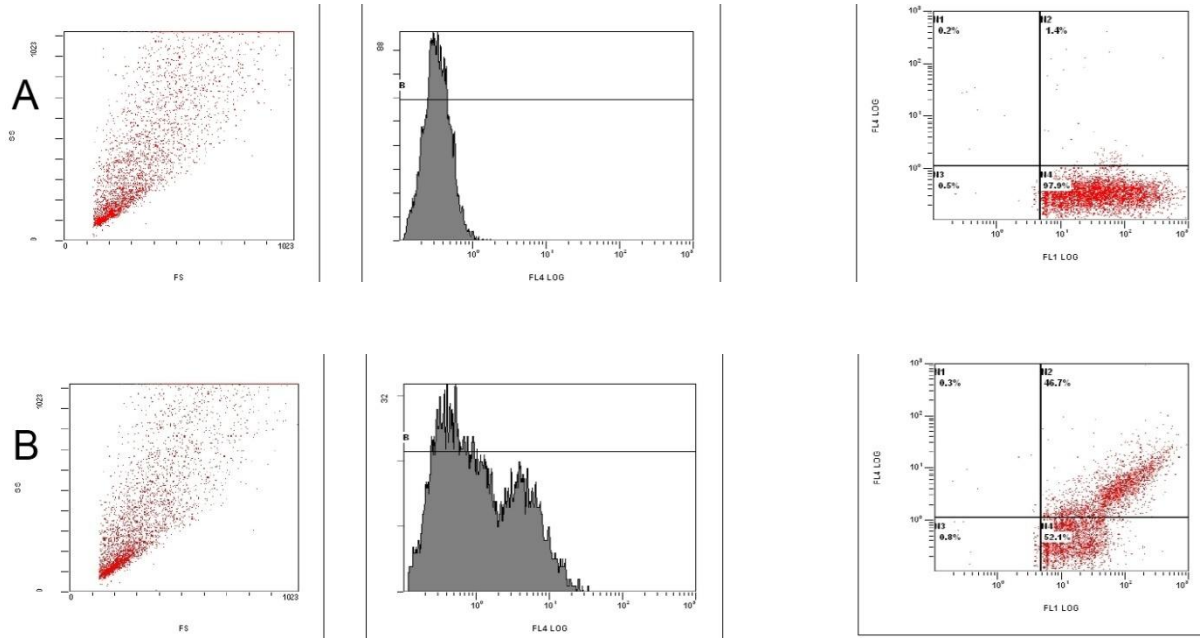


Figure 19: IgG2 antibody panitumumab binds EGFR coupled to the surface of polysterene microspheres (beads), its control antibody (unspecific IgG2) does not

Beads coupled with EGFR were incubated for 30min at 37°C with unspecific IgG2 (A) or panitumumab (B), then subsequently incubated with a secondary antibody (gαh PerCP anti-human IgG) for another 30min at 37°C, then measured using flow cytometry. Beads fluoresced in F1, while the fluorophore of the secondary antibody fluoresced in channel F4. Pictures show 3 graphs per measured sample: FSC_{lin}/SSC_{lin}, (left) $F1_{LOG}$ (middle) and $F1_{LOG}$ /$F4_{LOG}$ (right). The upper right gate in $F1_{LOG}$ /$F4_{LOG}$ shows the beads bound by the IgG2 Ab. About 1.4% of the EGFR-coated beads were bound by unspecific IgG2 (A), while about 46.7% of the EGFR-coated beads were bound by panitumumab (B, lower right).

However, we were unable to reproduce this effect for the IgG1 antibodies and when we examined the circumstances more closely, problems started to occur. At first we tested cetuximab and its control antibody, unspecific human IgG1 in the phagocytosis assay, finding an even higher phagocytosis rate in unspecific IgG1 than in cetuximab.

When we tested the binding ability of cetuximab to the EGFR-coated beads in an immunofluorescence flow cytometry experiment, we found that both cetuximab and its control antibody did not bind to the EGFR-coated beads.

We then tested several other IgG1 antibodies, including 003, 005, 018, 2F8T, hR3 and h425 directed against EGFR, as well as unspecific IgG1, IgG2 and Herceptin (also IgG1) directed against antigens other than EGFR. We tested both for induction of phagocytosis and for the antibodies' binding capacity of the EGFR-coated beads via immunofluorescence and flow cytometry. The antibodies showed a big diversity in the phagocytosis rates they induced. Consistent rates of phagocytosis were found for each antibody in repeated measurements. Confusingly, the phagocytosis rates did not correspond with the binding capacity to the bead of that particular antibody. The following table gives an overview of phagocytosis / binding capacity interactions.

Antibody	**Phagocytosis [%]**	**binding capacity of beads [%]**
anti-EGFR Abs		
cetuximab	25.0	4.4
panitumumab	29.1	46.7
OO3	45.2	7.1
OO5	37.3	70.9
O18	24.8	57.0
2F8T	33	28.1
hR3	37.2	4.0
h425	23.3	49.5
unspecific Abs		
IgG1	41.6	1.4
IgG2	12.1	1.4
Herceptin	17.5	2.5

Table 7: Phagocytosis and bead-binding capacity of several antibodies

We found Panitumumab, 005, 018 and h425 binding well to the EGFR-coated beads, while cetuximab, 003 and hR3 did not bind at all.
Some of the Abs which did not bind to the beads induced phagocytosis by neutrophils (e.g. 003: 45% phagocytosis, 7.1% binding) and several of the Abs that did bind the beads induced less phagocytosis (e.g. 005: 37% phagocytosis, 70.1% binding).
With a large number of tested Abs and the inconsistency in phagocytosis-binding capacity, the data indicated that the antibody-dependent phagocytosis of EGFR coated beads did not work with the construct of EGFR used here. This may be caused by an alteration of the EGFR protein during the coupling process, resulting in a loss of recognition by the Abs for its specific EGFR epitope. More experiments have to be conducted in order to solve these issues, a task that was beyond the scope of this doctoral thesis.

4.3 Differences in mononuclear cells' ADCC capacity further examined

4.3.1 ADCC of monocytes against A431 using cetuximab, panitumumab

We wanted to examine the effect of PBMC's increased ADCC capacity in DP further. Within the heterogeneous population of PBMC, several subpopulations came to mind which could have been responsible for the observed effect, foremost monocytes and NK-cells, as both have been shown to mediate ADCC.

We first conducted a series of experiments of ^{51}Cr release assays with purified monocytes ("untouched") against A431 at a single antibody concentration of cetuximab and panitumumab, reliably in the saturating region (2µg/ml). Monocytes of DP showed a higher rate of specific lysis with cetuximab over monocytes of HI, but not significantly elevated (n=5, HI-mean=11.9, 95%CI 3.00-20.81. DP-mean=20.74, 95%CI 6.19-35.29; R^2=0.4510; P=0.1441 Paired T-Test, two-tailed). With panitumumab, we found a significantly higher specific lysis rate of DP's monocytes over HI's monocytes (n=5, HI-mean=8.90, 95%CI 0.35-17.45; DP-mean=20.48, 95%CI 6.52-34.44; R^2=0.7641 P=0.0228. Paired T-Test, two-tailed).

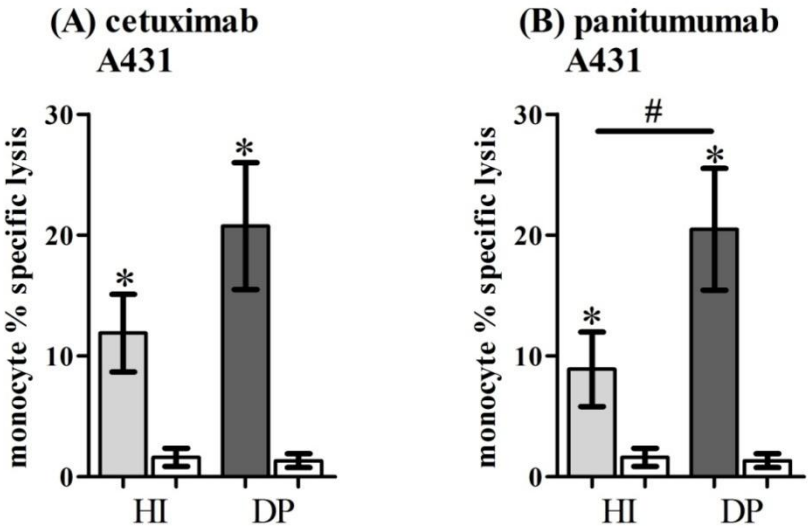


Figure 20: Cetuximab shows slightly higher lysis rates with monocytes drawn from DP than with monocytes drawn from HI. Panitumumab shows significantly higher lysis rates with DP's monocytes than HI's monocytes

ADCC by 2 different antibody cetuximab (A) and panitumumab (B) at 2µg/ml concentration was analyzed in ^{51}Cr release assay against A431 cells using monocytes obtained from blood samples of HI (light gray bar) or DP (dark gray bar). White bars indicate background lysis by respective effectors via control Ab. Data presented are means ± SEM of 8 experiments. Significance was accepted when $p < 0.05$. An asterisk (*) indicates significant specific lysis compared to control antibody; pound (#) indicates significant difference between HI and DP.

Since these experiments were performed at the same E:T ratio for HI and DP, the data indicates an enhanced ADCC capacity of DP's monocytes.

4.3.2 FcR quantification on monocytes and neutrophils

One hypothesis for the explanation of the heightened state of activation of DP monocytes over HI monocytes was the different expression of FcR, most notably CD32 (FcγRII).
It has been shown that the IgG2-antibody panitumumab, for which a significant difference was observed in ADCC lysis rate of the PBMC and monocytes between HI and DP, is most strongly bound with its Fc part by CD32[59]. The expression of CD32 was therefore especially interesting.
In order to quantify the FcR, we used the Qifikit (Dako) on purified monocytes ("untouched") and PMN. However, monocytes as well as PMN showed no significant difference in the expression of FcR.

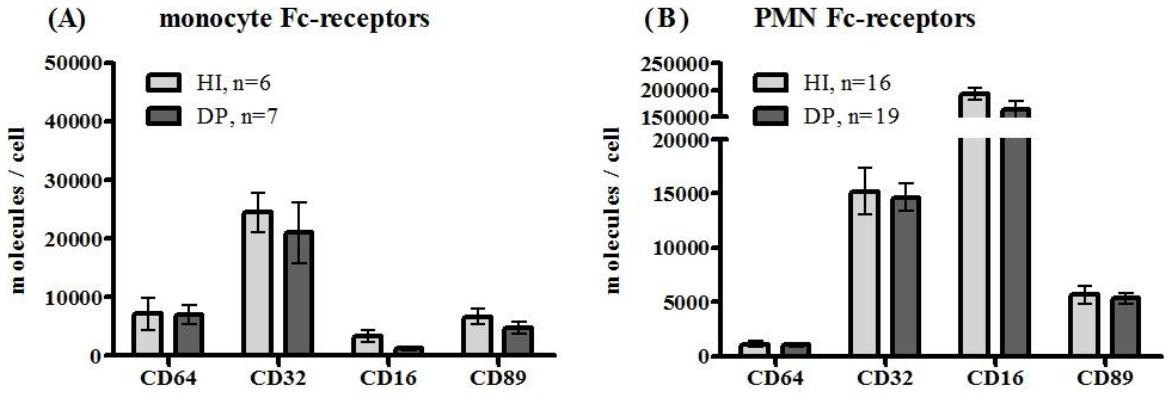


Figure 21: Quantification of Fc-receptors on monocytes and PMN from both HI and DP reveals roughly the same levels

Monocytes (purified "untouched") and PMN were obtained from blood samples of HI (light gray bars) or DP (dark gray bars). Data presented are means ± SEM of 6 to 19 experiments.

4.3.3 NK-cells examined for CD32 expression

Having found no difference in FcR expression of DP's monocytes, we wanted to examine whether or not NK-cells of DP expressed CD32 on their surface. In HI, there is no expression of CD32 on NK-cells.

For this, we purified NK-cells from pairs of HI and DP and examined them by direct immunofluorescence (CD45-FITC, 7AAD-PC-5, IgG-PE or CD32-PE, CD56-PC-7) and flow cytometry. NK-cells were defined as lying in the lymphocytes gate in FSC/SSC, CD45+, 7AAD-neg. (=vital), CD56+.

At n=4 pairs of HI/DP, we found no CD32 on NK-cell surface.

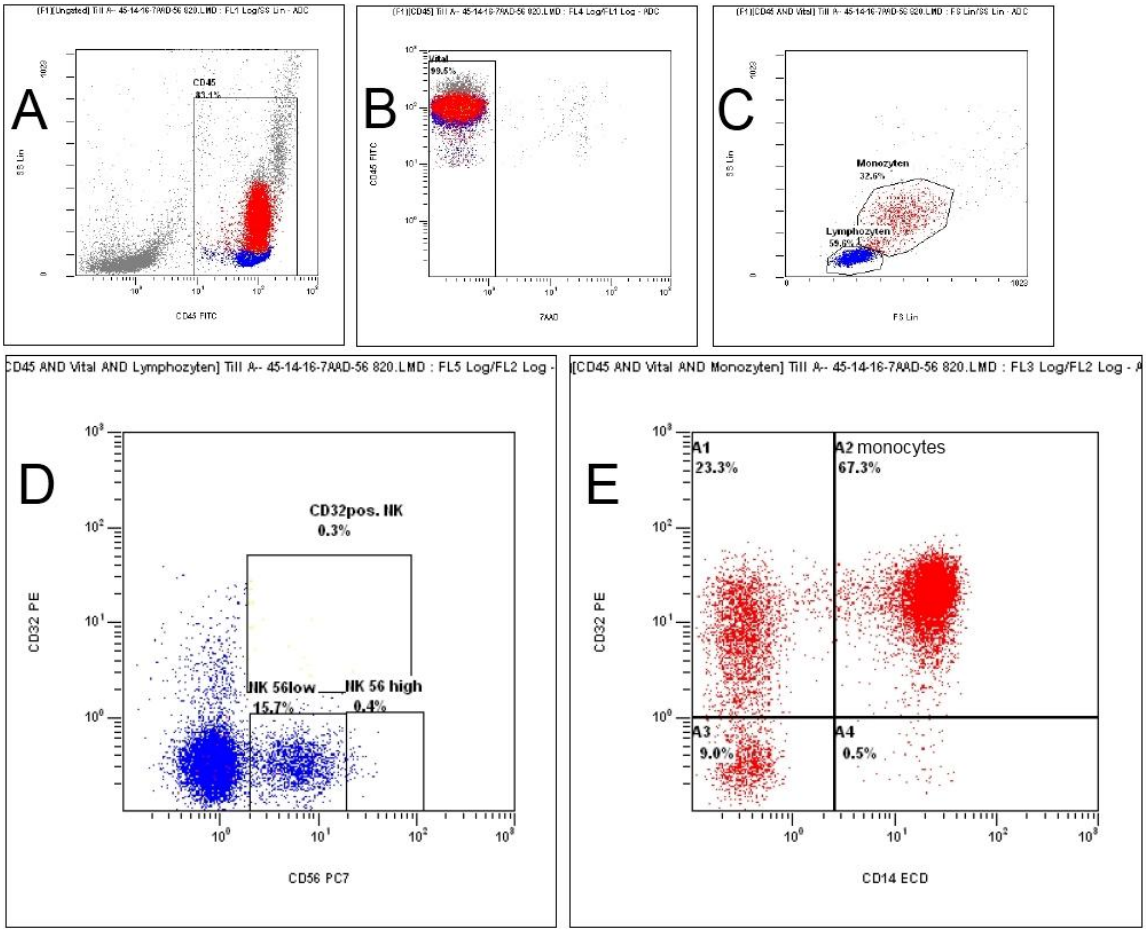


Figure 22: NK-cells of DP do not express CD32

Flow cytometry analysis of direct immunofluorescence. Cells were dyed with CD45-FITC, 7AAD-PC-5, IgG-PE or CD32-PE, CD56-PC-7. Successively, we gated on CD45+ cells (A) and 7AAD-negative =vital cells (B). FSC/SSC (C) was used to gate on lymphocytes (D, blue) according to size (FSC) and granularity (SSC). NK-cells were then defined as lying in the lymphocytes gate, CD56+. Of these, close to 0% showed to be CD32+ when compared to the negative control (unspecific IgG-PE, not shown). Red=monocyte gate. The shown pictures are representative for n=4 pairs of HI/DP.

4.3.4 Quantification of PBMC-subpopulations

Another explanation of the observed effect of DP's PBMC showing increased ADCC activity with panitumumab was found when analyzing the composition of the heterogeneous PBMC population. For n=4 pairs of HI/DP, PBMC were purified and dyed with several Antibodies, allowing us to quantify the individual subpopulations that make up the PBMC population. The following table shows the antibodies used:

Tube Nr.	HI or DP	FITC	PE	ECD	PC-5	PC-7
1	HI	CD45	IgG	IgG	7AAD	IgG
2	HI	CD45	CD32	CD16	7AAD	CD56
3	HI	CD45	CD32	CD14	7AAD	CD56
4	HI	CD45	CD19	CD3	7AAD	CD56
5	DP	CD45	IgG	IgG	7AAD	IgG
6	DP	CD45	CD32	CD16	7AAD	CD56
7	DP	CD45	CD32	CD14	7AAD	CD56
8	DP	CD45	CD19	CD3	7AAD	CD56

Table 8: antibodies used for quantification of PBMC subpopulations

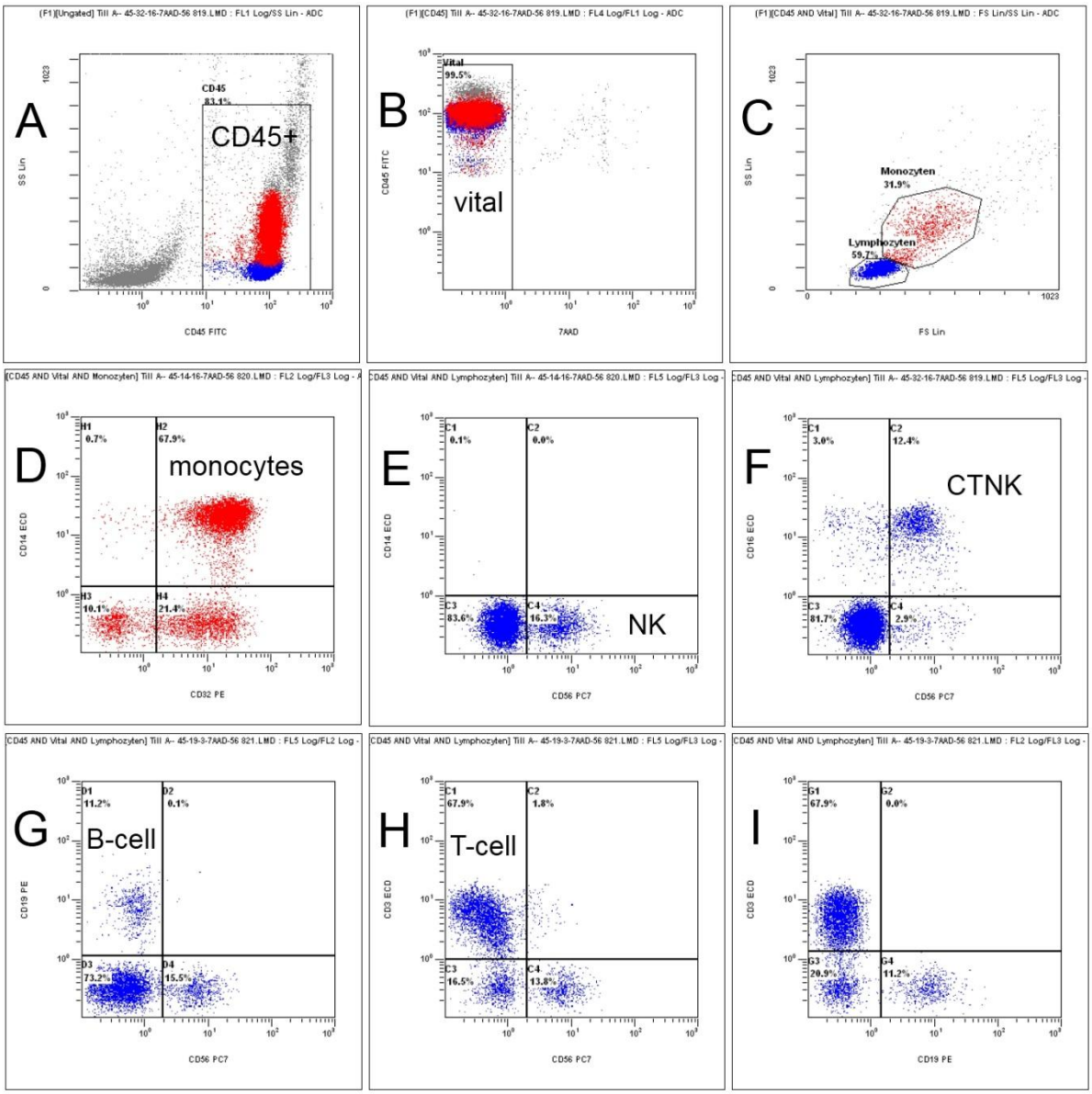


Figure 23: Typical Flow cytometry data from quantification of the PBMCs subpopulations

Flow cytometry analysis of direct immunofluorescence of several samples from one dialysis patient. Cells were dyed with CD45-FITC, 7AAD-PC-5, IgG-PE or CD32-PE or CD19-PE, IgG-ECD or CD16-ECD or CD3-ECD, IgG-PC-7 or CD56-PC-7. Successively, we gated on CD45+ cells (A) and 7AAD-negative =vital cells (B). FSC/SSC (C) was used to gate on monocytes (D, red) or lymphocytes (E-I, blue) according to size (FSC) and granularity (SSC). The shown pictures are representative for n=4 pairs of H/D.

The following table shows the definition of the PBMC subpopulations used for the quantification:

PBMC-Subpopulations	gate
Monocytes	CD14+ in monocyte gate in FSC/SSC of CD45+, vital cells
Lymphocytes	Lymphocyte gate in FSC/SSC of CD45+, vital cells
T-cells	CD3+ in lymphocyte gate in FSC/SSC of CD45+, vital cells
B-cells	CD19+ in lymphocyte gate in FSC/SSC of CD45+, vital cells
NK-cells("NK")	CD56+ in lymphocyte gate in FSC/SSC of CD45+, vital cells
CTNK-cells ("CTNK")	CD16+ and CD56+ in lymphocyte gate in FSC/SSC of CD45+, vital cells

Table 9: Gates used for quantification of PBMC subpopulations

Significantly elevated levels of monocytes in DP were observed compared to HI. In lymphocytes, DP showed significantly lower percentages than HI, while the decrease in lymphocytes was mostly due to the reduction of T-cells in DP over HI. The other populations analyzed, namely B-cells, NK-cells and CTNK-cells (= cytotoxic NK-cells) showed no significant difference between HI and DP. (n=4. Mean of HI's monocytes=11.4, 95%CI 3.2-19.5; mean of DP's monocytes=30.5, 95%CI 17.4-43.5; P=0.037; R^2=0.81.
Mean of HI's lymphocytes=80.7, 95%CI 69.4-91.9; mean of DP's lymphocytes=53.1, 95%CI 32.2-74.1; P=0.045; R^2=0.79.
Mean of HI's NK=15.0, 95%CI 5.2-24.8; mean of DP's NK=16.3, 95%CI 2.6-30.2.
Mean of HI's CTNK=10.3, 95%CI 0.4-30.2; mean of DP's CTNK=10.5, 95%CI 3.6-17.5. Mean of HI's T-cells=56.3, 95%CI 41.3-71.4; mean of DP's T-cells=32.7, 95%CI 0.0-67.5; P=0.114; R^2=0.79.
Mean of HI's B-cell=5.1, 95%CI 0.0-12.7; mean of DP's B-cell=4.4, 95%CI 0.0-12.5. Paired T-test, two-tailed.)

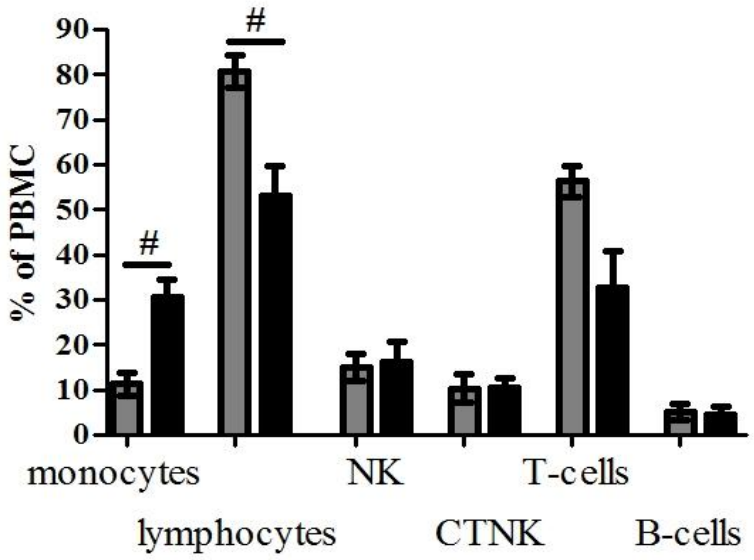


Figure 24: Differences in PBMC composition in HI and DP

PBMC from both groups (HI, gray bars; DP, black bars) were obtained from blood samples, then subsequently analyzed using direct immunofluorescence and flow cytometry. Monocytes were defined as CD14+ in FSC/SSC monocyte-gate of CD45+, vital cells. Lymphocytes were defined as FS/SS-lymphocyte-gate in CD45+, vital cells. Natural killer cells („NK“) were defined as CD56+ in FS/SS-lymphocyte-gate of CD45+, vital cells. Cytotoxic natural killer cells („CTNK“) were defined as both CD16+ and CD56+ in FS/SS-lymphocyte-gate of CD45+, vital cells. T-cells were defined as CD3+ in FS/SS-lymphocyte-gate of CD45+, vital cells. B-cells were defined as CD19+ in FS/SS-lymphocyte-gate of CD45+, vital cells. Data presented are means ± SEM of 4 experiments. Significance was accepted when $p < 0.05$. Pound (#) indicates significantly different percentages between HI and DP.

This data indicates that the increased ability of the PMBC in DP to mediate ADCC with IgG2 Abs can be attributed to two different causes. The first is the heightened state of monocyte activation in DP, while the second is the heightened proportion of monocytes among the subpopulations of the PBMC in DP.

5 Discussion

The concept of personalized tumor therapy aims to select the right drug for the right patient, among others by determination of a variety of biomarkers[60]. As examples for specific tumor characteristics, EGFR kinase mutations were positively correlated with response to EGFR tyrosine kinase inhibitors erlotinib and gefitinib in lung cancer, whereas the presence of KRAS mutations was highly predictive of resistance to EGFR-antibodies cetuximab and panitumumab in colorectal cancer. As example for predictive patient characteristics, FcγR polymorphisms were demonstrated to influence the outcome of treatment with cetuximab in colorectal cancer[61].

In this context, the aim of this study was to compare leukocytes of patients with ESRD undergoing regular hemodialysis (DP) to those of healthy individuals (HI) in their capacity to kill tumor cells with the mediation of antibodies. To our knowledge, only three studies are published addressing ADCC in DP at all. These studies are rather incomplete and results remain unclear. In one study, isolated PBMC of DP were tested in ADCC against chicken red blood cells (CRBC). Results were heterogeneous: PBMC from some patients performed better, some identical and some worse than PBMC from HI[54]. The other two studies can hardly be interpreted as they only used phagocyte depleted lymphocytes as effectors[55, 62].

We used the EGFR as target antigen, as it is overexpressed on many solid tumors and has been shown to play an important role in development and growth of tumors. To date, two EGFR-specific antibodies are FDA approved: cetuximab, which is of human IgG1 isotype, and panitumumab, which is of human IgG2 isotype. Comparison of both antibodies seemed especially interesting since due to their divergent isotypes both differ in their binding characteristics to FcR and thus in their recruitment of effector cell populations.

We planned to examine the two main indirect effector mechanisms, ADCC and phagocytosis. The classical ^{51}Cr release assay has already been established in the lab group as an in vitro model for ADCC, whereas a method to test antibody-mediated phagocytosis had to be established.

5.1 ADCC of PMN and PBMC

With isolated neutrophils (PMN) as effector cells, both EGFR-antibodies significantly triggered ADCC against EGFR-expressing A1207 tumor cell line, and the human IgG2 antibody panitumumab proved to be significantly more effective than the IgG1 antibody cetuximab, as described before[59]. However, we did not observe any difference between PMN isolated from DP and HI.

We then tested PBMC effector cells consisting mainly of lymphocytes, NK-cells and monocytes. With these effectors, ADCC levels were generally higher when mediated by antibodies of the IgG1 isotype than IgG2, an effect also described before[59]. With IgG1 antibody cetuximab, PBMC-ADCC was similar between DP and HI.

Interestingly, however, we observed a strong and significant difference in IgG2-mediated ADCC comparing DP and HI: PBMC from DP proved to be significantly more cytotoxic than those from HI. To exclude a cell line specific effect, we performed corresponding ADCC experiments with a second EGFR-positive tumor cell line, A431, confirming these results.

5.2 Antibody-dependent phagocytosis

Next, we aimed to investigate if this effect was restricted to ADCC or if it occurred in phagocytosis as well. Therefore, we had to establish an assay for antibody-dependent phagocytosis. As a model, we chose EGFR-coated microbeads, which can easily be detected by flow cytometry.

Using polyglobin- and albumin-coated beads as positive or negative control, we determined optimal experimental conditions to maximize the observed phagocytosis effect.

Phagocytosis rate proved to depend on a number of conditions such as incubation time, temperature, amount of protein coated on the beads surface and activation status of the effector cells. Importantly, principal conductibility and practicability of the chosen method could be demonstrated.

To test for antigen specific phagocytosis and influence of the antibodies isotype on the phagocytosis process, we coupled recombinantly produced EGFR to the beads surface. However, during the coupling process antigen characteristics of EGFR were obviously altered, resulting in EGFR-antibodies being unable to specifically bind to the beads. Most likely, the tertiary structure of EGFR was changed by the coupling conditions. We tried

various coupling conditions and beads, but failed to set up a stable and reliable test. Further experiments need to be conducted to optimize the assay, thus allowing investigation of antigen specific antibody-dependent phagocytosis. Amongst others, additional beads and coupling procedures have to be tried, as well as different recombinantly produced EGFR constructs. This task, however, was beyond the scope of this doctoral thesis.

5.3 Mechanisms of increased IgG2-mediated ADCC in DP further examined

Next, we searched for the underlying mechanism of the observed improved IgG2-mediated ADCC capacity of PBMC from DP. Basically, three mechanisms were to be considered:
Firstly, cytotoxic cells of DP could be preactivated.
Secondly, certain cytotoxic cell subpopulations may be altered in DP, e.g. in terms of different expression patterns of FcγR.
Thirdly, DP may have more cytotoxic cells susceptible to triggering ADCC by IgG2 antibodies.

5.3.1 Increased activation of DP's monocytes

Human IgG2 antibodies have been demonstrated not to bind to FcγRI (CD64) and to bind to FcγRIII (CD16) only at concentrations high above the physiological level. However, human IgG2 antibodies have been shown to bind strongly to FcγRII (CD32), in particular to the activatory FcγRIIa[59]. Within PBMC, the only cytotoxic cells naturally expressing FcγRIIa on their surface are monocytes. Therefore, we conducted a series of experiments examining ADCC capacity of monocytes of DP and HI, respectively. With isolated monocytes as effectors, we observed the same effects as with the unseparated PBMC: ADCC levels with effectors from DP were higher compared to HI with both the IgG1 and the IgG2 antibody, but the difference in IgG2-mediated ADCC was stronger and statistically significant. As we employed the monocytes at a fixed effector-to-target-ratio, this result indicated an activated state of monocytes in DP.
We also observed an increased ratio of CD14+ CD16+ monocytes in DP, an effect described before[63].

5.3.2 Differences in FcR expression of monocytes and PMN

As differences in FcR expression, in particular FcγRIIa, could explain the improved ADCC capacity of monocytes in DP, we measured expression levels of cytotoxic trigger molecules FcγRI, FcγRII, FcγRIII and FcαR on monocytes and on PMN. However, we could not observe any significant difference between DP and HI. Thus, the different state of activation observed in DP monocytes is likely to be due to a mechanism downstream the signaling cascade of FcR, allowing a stronger response to FcR-binding while maintaining the same level of FcR surface expression.

In our flow cytometry assays, we formally did not distinguish between the activatory FcγRIIa and the inhibitory FcγRIIb isoform. But as normal monocytes hardly express relevant numbers of FcγRIIb on their surface[64], an altered ratio of FcγRIIa to FcγRIIb (referred to as activating-to-inhibitiory or A/I-ratio) in DP could only result in weaker triggering of monocytes by binding of IgG2 in DP. One would thus expect lower lysis rates in DP and not higher ones as observed. Lastly, there are no recombinant murine antibodies available that exclusively target FcγRIIa or FcγRIIb, respectively and thus would allow one to determine the expression of FcγRII-subtypes.

5.3.3 FcRII expression on NK-cells

In order to confirm that monocytes are the major subgroup of PBMC to generate the increased IgG2-mediated ADCC capacity in DP, we examined the role of additional PBMC-subpopulations, and whether they expressed FcγRII on their surface. NK cells are the main cytotoxic cells for IgG1-mediated ADCC and subsets of NK cells have been demonstrated to express FcγRII to some extent on their surface[65]. However, we could not detect FcγRII expression on the surface of NK cells either in DP or HI.

5.3.4 Biocompatibility and activation of leukocytes

A possible reason for the activated state of DP's leukocytes could be the blood's continuous contact with foreign surfaces while passing through the dialysis machine. Biocompatibility has been markedly improved in recent years by the production of more advanced membranes, but the foreign surfaces still lead to strong effects in the blood. Different types of dialysis membranes have been demonstrated to affect aggregation and activation of

leukocytes to a varying degree[66]. The hydroxyl groups of plastic seem to play an important role in the activation of blood components, as indicated by studies demonstrating membranes with less hydroxyl groups to reduce the relative risk of mortality in DP[67]. Certain Synthetic membranes lead to different expression patterns of genes contributing to cell adhesion[68].

The complement system (C3a, C5a) has also been proven to become activated by contact to hydroxyl groups[69,70]. Activated complement can induce chemotaxis and increased adherence of leukocytes to the endothelium[71]. Additionally, it has been demonstrated that gene expression patterns in PBMC vary according to the employed membrane. Mitochondrial dysfunction and apoptosis in PBMC was observed during hemodialysis sessions[72].

In summary, this data suggests an increased activation status of leukocytes in DP, consistent with the results of our study. However, further studies are required to explore why this difference seemed to be restricted to monocytes, and could not be observed in PMN in our study. Furthermore, it is unclear why monocyte activation results in significantly increased ADCC via the IgG2 but not via the IgG1 antibody.

5.3.5 Differences in PBMC composition

Next, we were interested if FcγRII-expressing cytotoxic cells were numerically expanded in DP. Therefore, we measured composition of PBMC subpopulations in DP and HI by 5-color flow cytometry, sorting cells according to size, granularity, vitality and expression of typical antigens. T-lymphocyte numbers were found to be decreased in DP. Since T-cells play an integral role in the defense against tumor cells, decreased cell count might contribute to the observed higher tumor incidence in DP[27]. Interestingly, monocyte numbers were significantly increased in DP compared to HI. This increased absolute number of monocytes combined with the activated status of monocytes could explain the observed superior IgG2-mediated ADCC capacity of DP's PBMC. An increased monocytic cell number in DP has been described before[73, 74]. The mechanisms that lead to increased monocyte counts in DP are still unclear, but systemic microinflammation and an altered cytokine environment, e.g. elevated macrophage colony-stimulating factor (M-CSF)[73, 75] are likely to be responsible.

5.4 Conclusion

In summary, our experiments demonstrate that cytotoxic effector cells (both PMN and PBMC) of patients with end-stage renal disease undergoing dialysis are fully capable of antibody mediated tumor cell killing. We did not find any evidence that DP's ability to perform ADCC is diminished compared to HI. Thus, we do not see any rational reason to withhold antibody-mediated tumor therapies from DP. Furthermore, our studies indicate that dialysis patients might profit particularly from certain therapeutic approaches which recruit monocytes as effector cells, such as IgG2 antibodies.

5.5 Publication

Parts of this thesis were published in the American Journal of Nephrology. The contribution entitled "Antibody dependent cellular cytotoxicity in patients on chronic hemodialysis" by Koch et al[76] appeared in October 2013. PMID: 24157422

6 Summary

Patients suffering from chronic kidney failure undergoing regular hemodialysis (DP) have been shown to suffer from increased incidence of solid tumors (e.g. colorectal carcinoma), which indicates a reduced immune status. At the same time, their immune system is constantly activated to a state of slight inflammation, likely due to the continuous interaction of leukocytes with the foreign surface of the dialysis filters. In the last decades, several novel antibody drugs have been approved for the treatment of tumors, most of them of the IgG isotype. Major effects of these drugs in defense against tumor cells are mediated through indirect effector mechanisms such as ADCC. These indirect effector mechanisms depend not only on the target cell (tumor) and the antibody but also on the effector cell (leukocyte). It is yet unclear in which way regular hemodialysis influences the leukocytes ability to defend against tumor cells using antibodies. In this study, we investigated this with the mediation of antibodies. We used in vitro methods like ^{51}Cr release assay or immunofluorescence to investigate the in vivo tumor defense and compared DP to healthy individuals (HI). We found an increased ability of DP's peripheral blood mononuclear cells (PBMC) to kill EGFR coated tumor cells when ADCC was mediated by anti-EGFR antibodies of the IgG2 isotype. When ADCC was mediated by antibodies of the IgG1 isotype, DP and HI showed no significant difference.

In order to examine whether the observed effect was restricted to ADCC or also occurred in antibody-dependent phagocytosis by cells such as PMN, we then strove to establish an assay using EGFR-coated microspheres. Principal conductibility and practicability of the chosen method could be demonstrated, and we determined optimal experimental conditions to maximize the observed phagocytosis effect. However, we were unable to show the antibody-dependency of phagocytosis, likely due to an alteration of the EGFR-molecule in the coupling procedure.

To investigate further the effect of increased IgG2-mediated ADCC capacity in DP, we then experimented with subpopulations of leukocytes such as monocytes and NK-cells. In the heterogeneous PBMC population, we identified monocytes as the major faction contributing to the observed effect. An expression of FcγRII on the surface of NK-cells of DP or HI could be ruled out.

The proportion of monocytes from all PBMC was numerically raised in DP and their monocytes were shown to be more potent in ADCC, suggesting a heightened activation status. The activation of monocytes seems not to be due to changes in the FcR expression, as both DP and HI showed the same level of FcR expression, but seems to be due to an effect downstream the signaling-cascade.

Our experiments indicate that cytotoxic effector cells of patients with end-stage renal disease undergoing dialysis are fully capable of antibody mediated tumor cell killing. We did not find any evidence that DP's ability to perform ADCC is diminished compared to HI. Furthermore, our studies indicate that dialysis patients might profit particularly from certain therapeutic approaches which recruit monocytes as effector cells, such as IgG2 antibodies.

Parts of this thesis were published in a paper called "Antibody dependent cellular cytotoxicity in patients on chronic hemodialysis" by Koch et al[76], which appeared in the American Journal of Nephrology in 2013.

7 Zusammenfassung

Patienten mit chronischer Niereninsuffizienz, welche sich regelmäßiger Hämodialyse unterziehen müssen (dialysis patients, DP), haben ein erhöhtes Risiko, solide Tumoren zu entwickeln, z.B. kolorektale Karzinome. Dies deutet auf einen reduzierten Immunschutz hin, während sich das Immunsystem von DP jedoch andererseits permanent im Zustand einer leichten Aktivierung befindet. Dies liegt u.a. an der Interaktion der Leukozyten mit den fremdartigen Oberflächen der Dialysefilter. In den letzten Jahren haben monoklonale Antikörper als Medikamente gegen Tumoren zunehmend an Bedeutung gewonnen, die meisten vom IgG-Isotyp. Antikörper wirken unter anderem über indirekte Effektormechanismen wie ADCC (antibody dependent cellular cytotoxicity, antikörpervermittelte zelluläre Zytotoxizität). Diese sind nicht nur von der Zielzelle (Tumorzelle) und dem Antikörper abhängig sondern auch vom Effektor (Leukozyt). Es ist noch unklar, auf welche Weise regelmäßige Hämodialyse die Fähigkeit von Leukozyten beeinflusst, mit Hilfe von Antikörpern Tumorzellen zu bekämpfen. Wir untersuchten dies indem wir DP mit gesunden Spendern (healthy individuals, HI) verglichen. In-vitro-Methoden wie ^{51}Cr-release-assay oder Immunfluoreszenz wurden benutzt um die Tumorabwehr in vivo zu simulieren. Wir fanden eine erhöhte ADCC-Kapazität gegen EGFR exprimierende Tumorzellen in den mononukleären Zellen (PBMC) von DP, wenn anti-EGFR Antikörper vom IgG2 Isotyp wie panitumumab eingesetzt wurden. Bei IgG1- Antikörpern unterschied sich die ADCC-Kapazität bei DP und HI dagegen nicht.

Um zu untersuchen, ob der beobachtete Effekt auf ADCC begrenzt war oder auch in Antikörper-vermittelter Phagozytose von Fresszellen wie z.B. Neutrophilen (PMN) eine Rolle spielt, begannen wir die Etablierung eines Assays auf Basis von Mikrosphären (beads) mit auf der Oberfläche fixierten EGFR-Molekülen. Die grundlegende Durchführbarkeit der Methode konnte gezeigt und optimale experimentelle Bedingungen definiert werden, doch es gelang uns nicht, die Abhängigkeit der Phagozytose von der Spezifität der IgG2 Antikörper zu zeigen.

Um den Effekt der erhöhten IgG2-vermittelten ADCC-Kapazität in Hämodialysepatienten (DP) weiter zu untersuchen, betrachteten wir als nächstes verschiedene Untergruppen von Leukozyten, insbesondere Monozyten und NK-Zellen. Besonders Monozyten schienen an

dem beobachteten Effekt beteiligt, eine Expression von FcγRII auf der Oberfläche von NK-Zellen in DP konnte als Ursache ausgeschlossen werden.
Der Anteil von Monozyten an PBMC war bei Hämodialysepatienten signifikant erhöht, und die vorhandenen Monozyten zeigten eine erhöhte Lyserate im ADCC, was auf einen erhöhten Aktivierungsstatus hinweist. Diese Aktivierung scheint jedoch nicht auf einer Veränderung der FcR-Expression zu beruhen, sondern auf einen Effekt in einem intrazellulären Abschnitt der Signalkaskade.
Unsere Experimente deuten darauf hin dass zytotoxische Effektorzellen von Dialysepatienten in der Lage sind Tumorzellen mit Hilfe von Antikörpern adäquat abzuwehren. Es ergaben sich keine Hinweise dass DP eine verminderte Fähigkeit zum ADCC gegenüber HI besitzen. Zudem deuten unsere Ergebnisse darauf hin, dass Dialysepatienten besonders von Therapieansätzen profitieren könnten, die mit Hilfe von IgG2- Antikörpern auf Monozyten als Effektorzellen beruhen.
Teile dieser Doktorarbeit wurden unter dem Titel “Antibody dependent cellular cytotoxicity in patients on chronic hemodialysis” von Koch et al[76] im „American Journal of Nephrology“ 2013 publiziert.

8 Appendix

8.1 Materials

8.1.1 Chemicals

Name	**Producer**
Ampicillin	Roth
APS (=$(NH_4)_2S_2O_8$ =Ammonium persulfate)	Sigma
Bovine Serum Albumin Fraktion V	Roth
Bromphenolblue	Sigma
β-Mercaptoethanol	Sigma
Chromium51	Hartmann Analytik
Destillied water	Invitrogen
Dimethylsulfoxid (DMSO)	Sigma-Aldrich
Ethanol	Roth
Fetal calve serum (fetales Kälberserum) = (FCS)	Invitrogen
Glycerin	Merck
Hanks	PAA
Natriumazid	Roth
Natriumchlorid (NaCl)	Roth
Natriumhydroxid (NaOH)	Roth
Natriumdihydrogenphosphate (NaH_2PO_4)	Roth
Dinatriumhydrogenphosphate (Na_2HPO_4)	Roth
Penicillin/Streptomycin (Pen/Strep)	PAA
Percoll	Biochrom, Berlin
HCl	Merck
Sodium dodecyl sulfate (SDS)	Sigma
Tris	Roth
TritonX-100	Merck
Trypanblau	Sigma-Aldrich
Trypsin-EDTA (0,25%)	Invitrogen
Tween20	Merck
1xPBS	PAA
10xPBS	Invitrogen

Szintillationsflüssigkeit	PerkinElmer
o-Phenylenediamine dihydrochloride	SIGMA-Aldrich, SIGMAFAST OPD P9187-50set
SuperSignal	Thermo Scientific
TEMED (N,N,N,N-Tetramethylethylendiamin)	Merck

8.1.2 Solutions

- Facs-buffer: PBS (PAA) +1% BSA +0.1% sodium azide (Roth)
- MACS-buffer: autoMACS rinsing solution, pH=7,2 (Miltenyi Biotec)
- ELISA-washbuffer: PBS (PAA) + 0.05% Tween20 (Merck)
- TAE running buffer:
- 63% Percoll: 18.5ml hanks (PAA) + 31.5ml percoll (Biochrome)
- 70% Percoll: 15ml PBS (PAA) + 35ml percoll (Biochrome)
- 0.1 M Carbonate-buffer: 0.1 M Na_2CO_3 titrated to pH=9.6 with 0.1M $NaHCO_3$
- 0.1 M MES-buffer: 19.2g 2-(N-Morpholino)ethansulfonsäure (MW 195.2) eluded in 900ml distilled water. Titrated to pH=5.5 with 1M NaOH, then filled to 1000ml with distilled water.
- 0.2 M Borate-buffer: 0.2 M H_3BO_3 titrated to pH= 8.5 with 1 M NaOH.
- BSA-Solution: 10mg/ml BSA (Roth) in 0.2M Borate-Buffer
- Storage-buffer: 19ml 0.,1 M NaH_2PO_4-Monohydrate + 81ml Na_2HPO_4-Heptahydrate + 1g BSA (Roth) + 5ml Glycerol (Merck) + 0.1g sodium azide (Roth) then filled up to 1000ml with destilled water
- FACS-buffer: PBS + 5g BSA (Roth) + 0.5ml sodium azide (Roth)
- 1.5M Tris-buffer: 181.7g Tris + 1 H_20_{dest}
- Laemmli-buffer: 3.2ml 1M Tris-buffer + 3.5ml Glycerin + 1.5g SDS + 100mg bromphenolblue + 2.5ml β-Mercaptoethanol
- R10+ medium: 500ml RPMI 1640 (PAA) +50ml (10%) FCS (Invitrogen), +5ml (1%) Penicillin/Streptomycin (PAA)
- D10+ medium: 500ml DMEM (PAA) +50ml (10%) FCS (Invitrogen), +5ml (1%) Penicillin/Streptomycin (PAA)

8.1.3 Cells

Name	**Medium**	**Producer**
A1207	D10+	DSMZ*
A431	R10+	DSMZ*

*Deutsche Sammlung von Mikroorganismen und Zellkulturen

8.1.4 Consumables

Name	Application	Producer
Serological Pipette	Fluid handling	Eppendorf
Cell culture Flask	Cell culture	Falcon, Heidelberg
Microtiter plates, 96 wells, V-shape	Flow cytometry	Sarstedt
Multiple Well Plate 96 wells, Round Bottom with Lid	ADCC	Sarstedt
8-Well Streifen Nunc Immunosorp™ Module	ELISA	Thermo Fisher Scientific, Roskilde, Dänemark
96-Well Sample Plate	Scintillation measurement	Wallac, Turku, Finnland
TopSeal-A	Scintillation measurement	PerkinElmer
MACS LS Separation column	Monocyte and NK-cell preparation	Milteny Biotec
Kodan Hautantiseptikum	Anti septic spray	Schülke
Monovette-Kanüle		Sarstedt
Soluble EGFR	EGFR construct used for coupling	Genmab, Utrecht
MACS Pre-Separation Filter	Cell strainer 30µm pores	Miltenyi Biotec
Fluoresbrite Carboxylate YG 1.0 Micron Microspheres Cat#15702	Beads	Polysciences Inc.
Fluoresbrite Plain YG 1.0 Micron Microspheres Cat#17154	Beads	Polysciences Inc.

8.1.5 Machines

Name	Application	Producer
HeraSafe	Lamina	Heraeus Instrument, Hanau
Coulter EPICS XL-MCL Durchflusszytometer	Flow cytometry	Beckman Coulter
Heraeus Multifuge 3S+	Centrifuge	Thermo Scientific
Heraeus Megafuge 1.0R	Centrifuge	Thermo Scientific
Orbitalschüttler Gyro Rocker SSL3	Mixing of supernatant with scintillation fluid	Stuart
1450 LSC MicroBeta TriLux,	Scintillation-measuring	PerkinElmer
Tecan Sunrise	Adsorbtion reader	Groeding, Austria
Pipetboy	Pipetting aid	Integra
Neubauer-Zählkammer	Counting chamber	VWR

8.1.6 Antibodies

Name	Antigen (Target)	Producer
Polyglobin (Intratect)	unspecific hIgG	Bayer
IgG-FITC	polyclonal	Beckman Coulter
IgG-PE	polyclonal	Beckman Coulter
IgG-ECD	polyclonal	Beckman Coulter
IgG-PC-5	polyclonal	Beckman Coulter
IgG-PC-7	polyclonal	Beckman Coulter
CD3-ECD	human CD3	Beckman Coulter
CD14-ECD	human CD14	Beckman Coulter
CD16-ECD	human CD16	Beckman Coulter
CD19-PE	human CD19	Beckman Coulter
CD32-PE	human CD32	Beckman Coulter
CD45-FITC	human CD45	Beckman Coulter
CD56-PC-7	human CD56	Beckman Coulter
CD89-PE	human CD89	Beckman Coulter
7AAD Viablity Dye	---	Beckman Coulter
cetuximab (Erbitux)	human EGFR	Merck
panitumumab (Vectibix)	human EGFR	Amgen
2F8T	human EGFR	Genmab
225 IgA2	human EGFR	Selfmade (Beyer JIM 2009)
m425	human EGFR	Merck
OO5	human EGFR	Genmab
OO8	human EGFR	Genmab
O13	human EGFR	Genmab
hR3	human EGFR	Nimotuzumab, TheraCim
Rituximab (Mabthera)	human CD20	Roche
Trastuzumab (Herceptin)	HER2/neu	Roche
KLH (Keyhole Limpet Hemocyanin)	---	Genmab
Unspezif. IgG1	---	Genmab
Unspezif. IgG2	---	Genmab
mTib92	---	ATCC
mTH69	---	Selfmade (Gramatzki)
mW6/32	human HLA-I	ATCC
m22	human CD64 (FcγRI)	Genmab

mIV.3	human CD32 (FcγRII)	Genmab
m3G8	human CD16 (FcγRIII)	Genmab
mA77	human CD89 (FcαR)	ATCC
gαm-IgG-HRP	murine Fc	Dako
gαm-FITC	murine Fc	Dako
gαh IgG-PerCP	human IgG	Jackson ImmunoResearch Laboratories, Inc

8.1.7 Kits

Name	**Application**	**Producer**
Qifikit	FcR-Quantification	Dako
CD14 MicroBeads	Monocyte purification (touched)	Miltenyi Biotec
Monocyte Isolation Kit II	Monocyte purification (untouched)	Miltenyi Biotec
NK Cell Isolation Kit	NK-cell purification (untouched)	Miltenyi Biotec
MycoAlert	Mycoplasma detection	Lonza AG

8.1.8 Sample material (blood)

- Written informed consent was obtained from all blood donors
- exclusion criteria: infection, low Hb, taking of immune modulating drugs

8.1.9 Software

Name	**Application**	**Producer**
GraphPad Prism 5	Analysis, statistics	GraphPad Software, Inc
OpenOffice, Word 2007	Writing	Openoffice.org, Microsoft
CXL	Analysis of flow cytometry	Beckman Coulter
Endnote X4	Citations	Thomson Reuters

8.2 Figure list

8.3 Table list

9 Declaration

I hereby declare that apart from the contribution of my advisers, this dissertation is completely my own work. No part of it has been submitted for previous dissertations. I have not undergone previous attempts of dissertation.

Hiermit erkläre ich, dass diese Dissertation, abgesehen von der Beratung durch meine akademischen Lehrer, nach Inhalt und Form meine eigene Arbeit ist. Sie hat weder im Ganzen noch zu Teilen an anderer Stelle im Rahmen eines Promotionsverfahrens vorgelegen. Ferner erkläre ich, dass ich noch keine früheren Promotionsversuche unternommen habe.

Kiel, _____

Till Koch

10 Curriculum vitae

Personal Information

Till Jakob Koch, born 16.03.1983 in Bielefeld, Germany

Education

1989-2002	Primary School and High School in Bielefeld and Münster
2003/2004	Studies of chemistry at the University of Rostock
2004-2006	Studies of dental medicine at the University of Kiel
2006-2012	Studies of medicine at the University of Kiel
2011-2013	Dissertation at Klinik für Innere Medizin IV - Nieren- und Hochdruckkrankheiten, UK-SH Kiel
Since 2012	Resident at Klinik für Innere Medizin I - Gastroenterologie, UK-SH Kiel

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