Immunogenicity of therapeutic antibodies

Immunological mechanisms & clinical consequences

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Monoclonal antibody therapy has revolutionized the treatment of many diseases, including chronic inflammatory diseases and cancer. Antibody therapy can unfortunately also elicit an unwanted immune response, leading to anti-drug antibodies (ADA). It is well known that ADA can lower the level of free drug, which may lead to a reduced clinical response. Additionally, for some antibody therapeutics, more adverse events are reported in ADA positive patients. A rather neglected result of immunogenicity is the formation of immune complexes between drug and ADA that likely occurs in all ADA positive patients. Although the high dosages drug and ADA could possibly lead to a very high amount of immune complexes, the biological and clinical effects of these complexes are largely unknown. The aim of this thesis is to provide a detailed characterization of the ADA response and its associated immune complex formation, to better understand the causes and mechanisms behind the adverse clinical observations.

How antibodies are formed

The immune system is a highly complex and sophisticated network of cells and proteins, evolved to protect us against harmful pathogens. Antibodies, or immunoglobulins, form a crucial part of this system. They rapidly neutralize invading pathogens and subsequently mark them to be removed from the host. The formation of an antibody response involves many processes, of which the main steps are described below.

Upon infection, invading pathogens are rapidly captured via cell surface molecules on the membrane of macrophages and immature dendritic cells (DCs). Whereas macrophages mainly serve to engulf and destruct the pathogen and cause local inflammation, DCs mature upon activation of pattern recognition receptors (PRRs) expressed by the DC, which recognize pathogen-associated molecular patterns (PAMPs), and migrate via the lymphatic system to lymph nodes. Here, the DCs present degraded parts of the pathogen, in the form of peptides in complex with major histocompatibility complex (MHC) class II molecules to naïve CD4+ T-cells, which leads to activation of the latter. Additional costimulatory signals and cytokines delivered by the mature DCs induce proliferation of the activated T-cells and their subsequent differentiation into T-helper cells. In the process, the T-helper cells migrate to the border of the B/T-cell zones of the lymph node.

Smaller antigenic particles such as toxins and immune complexes do not require DC-mediated transportation, but can diffuse directly via the lymphatic system to the lymph nodes. Here, specific B-cells may recognize specific antigens of the intact pathogen via their B-cell receptor (BCR). This ensures activation of the B-cell, which also induces their migration to the border of the B/T-cell zones. In this process, B-cells will also internalize antigens, or even whole pathogens, and present these as peptides on
their membrane in the context of MHC class II to cognate T-helper cells. B-cells that recognize the same cognate antigen as the T-cells will receive T-cell cytokines and costimulatory signals via CD40 on the B-cell and CD40L on the cognate T-cell, leading to survival, proliferation and differentiation of the activated B-cells.\textsuperscript{3,4}

In order to provide a first antibody-mediated defense against the pathogen, B-cells with the highest affinities will migrate to the medullary chords in the lymph node, where they rapidly proliferate and differentiate into antibody secreting cells.\textsuperscript{3,5} The majority of the secreted antibodies is of the pentameric IgM type, and whereas their affinity is still rather low, this is compensated by their high valency. These antibodies aid the adaptive immune system by facilitating phagocytosis and antigen presentation through complex formation with the pathogen.

To eventually obtain high-affinity antibodies, activated B-cells with lower affinities return to the B-cell follicle where their vigorous proliferation initiates formation of the dark zone of a new germinal center (GC).\textsuperscript{3,5} During proliferation, the process of somatic hypermutation (SHM) gives rise to daughter cells with mutated variable domain sequences, which can affect the affinity of the BCR (either positively or negatively). After a number of days, the B-cells exit the dark zone and migrate to the light zone of the GCs, where they compete for intact antigen by follicular dendritic cells and additionally have to compete for sufficient help of follicular T-helper cells. This way, only the B-cells with the highest affinities will survive.\textsuperscript{6,7} In addition, follicular T-helper cells secrete cytokines that induce class switch recombination in B-cells, leading to the production of other isotypes such as IgG, IgA and IgE.\textsuperscript{8} B-cells can undergo multiple rounds through the dark and the light zone to increase their affinity for the antigen.\textsuperscript{4} After several rounds, they can leave the germinal center to become a plasma cell or memory B-cell. Plasma cells travel to the bone marrow where they occupy a niche and continuously produce high-affinity antibodies for constant protection. Memory B-cells circulate through the lymphoid system in a dormant state, but upon contact with their antigen reactivate, and initiate a rapid secondary antibody response to protect the host from infection.\textsuperscript{5,9}

**Antibody structure and function**

An antibody is a Y-shaped protein consisting of two linked heavy chains that each pair a light chain (Figure 1A). An antibody can furthermore be divided into two antigen binding fragments (Fab) and a crystallizable fragment (Fc, Figure 1B). Whereas the Fab specifically binds the antigen, the Fc exerts the effector functions of the antibody.
Antibodies can be divided in several ways.

**Fab**

The antibody repertoire needs to recognize an immense variety of pathogens. To enable this, several mechanisms have evolved to diversify the antibody repertoire. The variety of an antibody lies in the variable domains of the Fab (Figure 1C), in the antigen binding site. The set of determinants (i.e. idiotopes) that is unique for each antibody clone is called the idiotype (Figure 1D).

The highest diversity in the variable domains is found in the complementarity determining regions (CDRs), which make up the loops that are involved in antigen binding. This diversity partly arises from the many V-, D- and J-genes that can encode for the variable domain within each developing B-cell. A single V-gene and J-gene segment (and in case of the heavy chain also a D-gene segment) is randomly selected during B-cell development and combined together to form one unique variable domain in each B-cell. Introduction of random nucleotides at the V/D/J joining sites during this recombination process further diversifies the variable part, and the matching of a unique heavy chain with a unique light chain within each B-cell results in an even higher repertoire of BCRs, and hence antibodies. Finally, B-cells that undergo SHM during the germinal center reaction introduce point mutations specifically in the CDRs to further increase the affinity for their antigen.

Through an ingenious way of selection, auto-reactive B-cells are removed from the B-cell pool, while a highly diverse B-cell repertoire is maintained that can even further diversify and increase affinity upon recognition of their antigen.

**Fc**

B-cells that undergo class switch recombination in the germinal center alter the expression of the IgM constant heavy chain into another isotype, and thereby change the Fc part of the antibody. There are five immunoglobulin isotypes: IgA, IgD, IgE, IgG and IgM. Of IgA, two subclasses are expressed, IgA1 and IgA2, and IgG is expressed in four different subclasses, IgG1, IgG2, IgG3 and IgG4. Each isotype or subclass exerts distinct effector functions through their unique Fc part, shaping the antibody response to most efficiently attack and clear different types of pathogens. For instance, IgE is
produced to combat helminths, whereas IgA mainly protects the mucosa from invading pathogens.\textsuperscript{10}

All classes of antibodies can neutralize pathogens by inhibiting their functions. Additionally, binding of antibodies to a single pathogen also brings antibodies close together. This opsonization further leads to clearance, in which phagocytic cells (e.g. liver and spleen macrophages) engulf and destruct the pathogens.\textsuperscript{11} This may occur via two mechanisms: Fc receptor binding or complement activation; both mechanisms are described below.

First, opsonization of pathogens facilitates their internalization by phagocytic cells such as macrophages and dendritic cells. Antibody-mediated phagocytosis occurs via Fc\(\alpha\) and Fc\(\gamma\) receptors that bind IgA and IgG, respectively.\textsuperscript{12,13} In case of IgG, most efficient phagocytosis is observed for IgG1 and IgG3, due to their higher affinity for Fc\(\gamma\) receptors compared to IgG2 and IgG4.\textsuperscript{10} Phagocytosed pathogens are killed in the phagolysosome, and subsequently presented again via MHC class II to further stimulate the pathogen-specific immune response. Second, opsonization of pathogens leads to antibody-dependent cell-mediated cytotoxicity (ADCC), a mechanism in which primarily NK-cells are activated by Fc receptor crosslinking, thereby triggering the secretion of cytotoxic compounds to kill the target cell.

Second, multimerization of IgG1, IgG3 and to a certain extent also IgG2, induces activation of the classical pathway of the complement system.\textsuperscript{14} IgM is a potent complement activator as well, but does not require multimerization due to its pre-existing pentameric form. Fluid phase complement activation via IgM is prevented by a conformational change that is required before IgM can bind C1q, the first component of the classical pathway. Only IgM that has bound its target undergoes this conformational change and exposes its C1q binding sites.\textsuperscript{15} After C1q binding to IgM or multimerized IgG, a cascade of complement proteins is cleaved. This eventually results in the assembly of the membrane attack complex, which forms pores in the membrane of pathogens to inflict lysis. Furthermore, several cleaved complement proteins have anaphylatoxin activity and attract immune cells, whereas other cleavage products mark the debris to be phagocytosed.

**Therapeutic monoclonal antibodies**

The high specificity and affinity of antibodies make them ideal to use as therapeutics. This was already appreciated more than a hundred years ago, when the first serum therapy was developed. Initially, serum of immunized horses, containing antibodies against a certain toxin, was used to treat patients.\textsuperscript{16} However, since this serum also
contained many other foreign molecules, strong side effects could occur, making serum therapy a dangerous treatment at that time.\textsuperscript{16}

Nowadays, polyclonal antibodies are still used as therapy, but in a highly purified and generally safe form, for instance to provide passive immunity against infectious agents, or as intravenous immunoglobulin (IVIg) to treat many health conditions.\textsuperscript{17,18} Furthermore, the last few decades considerable improvements have been made in the development of monoclonal antibody therapy. The high specificity of antibodies is used to very specifically target cytokines, receptors or cells. The effector functions of the different antibody classes are furthermore exploited to optimize the therapeutic effect. The three main working mechanisms of therapeutic antibodies currently used in the clinic are neutralization, blocking of receptors and cell depletion.\textsuperscript{19}

\textit{Neutralization}
Neutralization of pro-inflammatory cytokines such as IL-6 or TNF blocks pro-inflammatory pathways and ameliorates chronic inflammation. Since these targets are small and soluble, the isotype of the therapeutic antibodies is less relevant. Most of these blocking antibodies, such as the anti-TNF therapeutics infliximab, adalimumab and golimumab, and the anti-IL-6 antibody tocilizumab are of the IgG1 class. An exception is eculizumab (anti-C5 of the complement system) which is a combination between IgG2 and IgG4 to remove all effector functions; IgG2 and IgG4 intrinsically have a low Fc receptor binding capacity and IgG4 is a poor complement activator.\textsuperscript{20} Another way of eliminating effector functions is by using only the Fab, as is done with the anti-TNF therapeutic certolizumab.

\textit{Blocking of receptors}
Inflammatory diseases can also be treated with antibodies that block cellular receptors. Blocking antibodies only inhibit the inflammatory functions of the cell; the cells are not depleted.

Different mechanisms are used to obtain anti-inflammatory effects. First, instead of targeting the cytokine itself, the cytokine receptor is antagonized, for example tocilizumab that targets the IL-6 receptor. Second, activation of immune cells can be prevented. For instance, the fusion protein abatacept (CTLA-4 fused with an IgG1-Fc) prevents activation of T-cells by antigen presenting cells and thereby reduces inflammation.\textsuperscript{21} Third, receptors that facilitate migration of immune cells can be blocked. This is especially useful in case of local inflammation, as for instance with multiple sclerosis (MS). MS can be treated with natalizumab that blocks the interaction between $\alpha_4\beta_1$ integrin expressed on lymphocytes with VCAM-1 on epithelial cells, thereby preventing
the influx of lymphocytes into the brain. The IgG4 subclass of natalizumab, which has poor FcγR binding and complement activation, ensures that the lymphocytes are not depleted and are thus still able to combat infections in the periphery.

Cell depletion

In a similar way as pathogens are opsonized and removed from the host, therapeutic antibodies can be used to remove pro-inflammatory cells or tumor cells. The Fc of the therapeutic plays a crucial role in this process. Antibody-mediated phagocytosis, ADCC and complement dependent cytotoxicity (CDC) occurs best with IgG1 or IgG3 antibodies. However, since IgG3 has a shorter half-life, a longer hinge region and a higher allotypic variation than IgG1, IgG3 is less suitable as therapeutic and has therefore not been used for therapeutic antibody development.

Other mechanisms

The very specific target binding of monoclonal antibody therapeutics does not necessarily mean that their effects are equally restricted. Because of the high complexity of the immune system, the inhibition of one cytokine or depletion of a single cell type may have many downstream effects. On the other hand, these multiple effects (including unanticipated ones) may supplement each other for higher efficacy. For instance in cancer treatment, antibodies against tumor antigens may not only induce ADCC or CDC of tumor cells, but can also increase tumor antigen presentation, leading to a stronger adaptive anti-tumor immune response.

Anti-TNF therapeutics for the treatment of Crohn’s disease possibly also work via multiple mechanisms. Whereas infliximab and adalimumab are found effective, certolizumab shows varying clinical results and etanercept did not show effectivity. This suggests that blocking TNF alone may not be sufficient. Several proposed additional mechanisms include reduction of the neutrophil count, apoptosis of immune cells via membrane bound TNF, and Fc-mediated immune regulation.

Complete knowledge of all significant effects of a new therapeutic is thus a challenging goal. Animal studies may solve this in part, but the efficacy and working mechanisms of a new therapeutic likely only become apparent during a clinical trial, or even much later during patient studies.

Next generation therapeutic antibodies

Since antibodies are well-studied proteins, the effector mechanisms of the Fc domain can now be pinpointed to specific amino acids and glycans. This enables antibody
engineering, in which the existing antibody structure is altered in order to gain or remove certain antibody functions.

Antibody engineering is done on various levels and ranges from removing a single monosaccharide up to the addition of entire variable domains. Nonetheless, we have to keep in mind that even small modifications to the antibody framework can cause considerable changes in effector functions.

Glycosylation of the Fc domain for example, greatly influences the affinity of the antibody for Fc receptors. Depletion of cells via antibody-dependent cellular cytotoxicity (ADCC) depends on this affinity, and can be significantly enhanced when the Fc-glycan is de-fucosylated. It was found that equal or even improved efficacy was reached with much lower antibody concentrations. De-fucosylation is especially a valuable tool for the development of anti-cancer therapeutics, but clinical trials with de-fucosylated antibodies to treat other diseases are currently ongoing as well.

More drastic modifications are found in dual-variable-domain immunoglobulins (DVD-Ig). The four variable domains of two antibodies are then fused into one antibody molecule, creating four antigen binding sites. In this way, each modified Fab can bind two different targets, for instance IL-1α and IL-1β for treatment of osteoarthritis. Apart from the increased binding capacity, DVD-Ig therapeutics do not have additional benefits over ‘general’ therapeutic antibodies, and their use in the clinic is therefore limited.

A more ‘natural’ form of antibodies that bind two different targets are the bispecifics. This type of next generation therapeutics is copied from IgG4, which has the peculiar ability to exchange half-molecules (i.e. one heavy and one light chain). During this process, two IgG4 antibody clones with different specificities exchange half-molecules, thereby forming molecules that each can bind two distinct targets, thus being bispecific. Because of this ability, bispecific IgG4 antibodies are naturally found in the (healthy) population. Therapeutic bispecifics can be made in all isotypes, and various techniques have been developed to make the exchange as efficient as possible. Bispecifics are generally used to bring two or even three cell types together. Catumaxomab is such a bispecific, and targets EpCAM on tumor cells and CD3 on T-cells, and with its Fc domain it furthermore recruits NK-cells for ADCC as well as phagocytes to increase antigen presentation of tumor antigens.

Next to the modified intact antibodies, a plethora of antibody fragments and fusions of fragments are under development. One such a fusion construct already used in the
Clinic is blinatumomab, in which the variable domains of an anti-CD3 are linked to the variable domains of an anti-CD19 antibody. This construct links T-cells to (leukemic) B-cells and thus has an immunomodulatory working mechanism. The absence of an Fc-domain and part of the variable domains reduces the distance between the two cells and prevents effector mechanisms, but also greatly reduces its half-life to 2-3 hours instead of 3 weeks for IgG1. Still, the very low levels required for therapeutic efficacy and the high response rates likely outweigh these disadvantages.

Immunogenicity of therapeutic antibodies

The first monoclonal antibodies used in the clinic were of murine origin, but the abundance of non-human determinants in these antibodies resulted in a broad and strong immune response towards these therapeutics, making them unsuitable for long-term treatment. Replacement of the murine heavy chain for a human one, thus making the antibody chimeric, has greatly reduced their side effects. Nevertheless, chimeric antibodies were still (strongly) immunogenic in a subset of patients, which lead to further humanization of the therapeutics. Additional replacement of the murine variable framework regions for human ones has resulted in humanized antibodies, after which new techniques such as phage display made it possible to create fully human antibodies. Interestingly, this initial drive to humanize antibodies in order to reduce immunogenicity has now somewhat shifted with the introduction of the mutated next generation therapeutics. Currently, chimeric, humanized and fully human antibodies are frequently used in the clinic, but despite the efforts of humanization even fully human antibodies can be immunogenic.

This is not completely surprising, since many human protein therapeutics, either plasma derived or recombinantly produced, are found to be immunogenic and can elicit an immune response. Known causes for immunogenicity are non-human or altered glycosylation, and protein aggregation, the latter likely due to the increased amount of identical epitopes on the aggregate, which may facilitate B-cell activation and could enhance phagocytosis and antigen peptide presentation to T-cells.

Furthermore, even autologous human antibodies contain foreign determinants. Due to the highly diversified antibody repertoire, antibodies inherently contain determinants in the idiotype that are unique to the host. This makes the idiotype potentially immunogenic, and may result in an anti-idiotype antibody response, as was already proposed in the 70’s by Jerne in the network theory. This theory suggests that autologous antibodies and B-cells expressing surface bound immunoglobulins may be targeted by autologous anti-idiotype antibodies. Therefore, a very small part of the antibody response is thought
to target itself, but at the same time it immediately neutralizes itself, leading to regulation
and suppression of the anti-idiotype response.\textsuperscript{41,42}

Even fully human therapeutic antibodies thus still contain ‘non-human’ idiotopes. Therefore, all therapeutic antibodies are potentially immunogenic and may elicit an antibody response known as anti-drug antibodies (ADA). It seems likely that ADA towards fully human antibodies predominantly target the idiotype, since this is the only foreign part of the drug. Furthermore, T-cell epitopes are found in the CDRs of several therapeutics.\textsuperscript{43} Previous work by our group (van Schouwenburg et al.\textsuperscript{44,45}) confirmed this anti-idiotype response for ADA towards the fully human therapeutic adalimumab. The polyclonal anti-adalimumab response was found to bind distinct but overlapping epitopes on the idiotype of adalimumab. However, it is unknown whether this restricted antibody response only occurs in case of adalimumab, or that fully human antibodies are invariably targeted on their idiotype.

In contrast to fully human antibodies, humanized and chimeric antibodies contain much more non-human determinants and could thus elicit a much broader response. Possibly, the polyclonal ADA of such a broad response may bind to more than two sites of one drug molecule, resulting in aggregation or complex formation. The study of Kosmac and colleagues however shows that the response against the chimeric anti-TNF antibody infliximab at least in part is neutralizing, meaning that ADA compete with TNF for infliximab and thus bind (close to) the antigen binding site.\textsuperscript{46} Furthermore, a study using linear oligopeptides of infliximab indicated that predominantly the TNF-binding sites are targeted by the ADA response.\textsuperscript{47}

These studies indicate that at least part of the antibody response against the chimeric infliximab binds (close to) the idiotype. Nevertheless, the question remains to what extent these antibodies are neutralizing and whether part of the antibody response might still bind other non-human sites of the drug. Furthermore, very little is known about the broadness of the antibody response towards other chimeric and humanized antibodies. This is therefore further investigated in Chapter 4 and Chapter 5 of this thesis. Together, these chapters provide new insight into the anti-idiotype concept.

\textbf{The effect of ADA in the clinic}

The clinical consequence of ADA to therapeutic antibodies is associated with their amount. This can be explained by the binding of ADA to the drug, thus reducing the free drug level. However, the mechanisms behind this effect are not fully known, and might either occur through neutralization (i.e. ADA bind the antigen binding site and thus compete with the drug’s target), through increased clearance due to complex
formation of drug and ADA, or through a combination of both. It could furthermore be that the proportion of neutralization or increased clearance by ADA differ for each therapeutic antibody. Nevertheless, answering these basic immunological questions could be valuable for the development of new therapeutic antibodies. The neutralizing capacity of ADA and the formation and clearance of ADA-drug complexes is therefore further investigated in Chapter 4, 5 and 7.

Either through neutralization of via complex formation, the amount of ADA produced by the patient thus likely determines to what extent the drug free level is reduced, and consequently what level of free drug remains. Generally, a certain amount of free drug is required to obtain a clinical effect. For instance, adalimumab has an optimal clinical efficacy between 5-8 µg/ml in serum; higher concentrations do not give an additional effect.48 Nevertheless, the ‘one-fits-all’ dosing regimen of adalimumab results in a wide variation in drug concentrations between patients. Especially patients with an initial low drug level may be more prone to lose clinical efficacy due to ADA formation. However, even in patients with initial adequate drug levels, persistent high ADA production may reduce the free drug concentration to suboptimal levels, causing reduced clinical efficacy or complete non-response.

For certain therapeutic antibodies (e.g. infliximab, natalizumab49,50), ADA positivity increases the chance of adverse events. For most other therapeutics however, the chance of adverse events is similar in ADA positive and ADA negative patients,51,52 suggesting that ADA can have differential clinical effects. The exact cause for ADA-mediated adverse events is not clear, but immune complex formation seems to play a role.53 Moreover, some debate exists on the role of IgE anti-drug antibodies that induce hypersensitivity reactions.54–57 Both possible causes for adverse events are investigated in this thesis, and are described in Chapter 6 (IgE-ADA) and Chapter 7 (complex formation).

Reducing immunogenicity

Due to the negative consequences of ADA on the clinical efficacy of antibody therapeutics, several developments have been made to reduce immunogenicity to the minimum. Immunogenicity of existing therapeutics can only be suppressed on a patient level. It is now widely accepted that in RA and IBD patients, ADA formation towards anti-TNF therapeutics is reduced with concomitant immunosuppressant therapy such as methotrexate or azathioprine.58 For example, patients treated with a combination of adalimumab and methotrexate show significantly lower ADA levels and higher drug levels than patients treated with adalimumab as monotherapy.59–61 In contrast, immunosuppressant therapy has shown no beneficial effect in ankylosing spondylitis.
patients treated with infliximab. The question therefore remains via which mechanism immunosuppressants reduce immunogenicity.\textsuperscript{62–64}

Next to concomitant therapy, possibly the dosing regimen also influences ADA formation. Tolerance for the antibody therapeutic may be induced by initiating therapy with one or several high dosages. Studies on high dose tolerance induction indeed showed lower ADA responses in mice treated with an anti-IL-6 receptor antibody and in cynomolgus monkeys treated with adalimumab.\textsuperscript{65,66} It should be mentioned however, that both studies used drug-intolerant assays to measure ADA, and the high amounts of drug could have masked ADA formation. While higher dosing possibly induces tolerance for antibody therapeutics, the opposite is found for the therapeutic protein Factor VIII for hemophilia A treatment. Formation of inhibitory antibodies to Factor VIII is increased after more frequent exposure and peak treatments.\textsuperscript{67} Dosing induced tolerance may thus work via multiple mechanisms, and should be investigated for each individual therapeutic.

In the clinic, dosing of several antibody therapeutics (e.g. infliximab, adalimumab) starts with an induction phase in which the drug concentration rapidly increases, followed by a maintenance phase with a lower dosing or longer dosing interval. The rationale behind this dosing regimen may be to quickly obtain effective drug levels, to reduce immunogenicity or a combination of both.

Great effort is furthermore put in reducing the immunogenicity of newly developed antibody therapeutics. Where the humanization of therapeutics was the first successful accomplishment, the next challenge lies in de-immunizing the idiotype without disrupting antigen binding. Characterization of ADA towards existing therapeutics showed that ADA are mainly of the IgG isotype, indicating that the response is T-cell mediated.\textsuperscript{46,68,69} Furthermore, for natalizumab and infliximab associations are made between ADA formation and certain HLA-types.\textsuperscript{70,71} De-immunization of new therapeutics is therefore largely focused on the deletion of T-cell epitopes. There are multiple approaches to achieve this, for instance by \textit{in silico} predicting MHC class II binding to CDR peptide stretches, or by co-culturing T-cells with therapeutic-loaded APCs to determine T-cell activation.\textsuperscript{72} Newly approved therapeutics indeed seem less immunogenic, although this could also be the result of higher selection pressure during clinical trials and a better understanding of how to measure ADA responses.

\textit{Measuring the ADA response}

The immunogenic properties of a therapeutic antibody are usually investigated by determining the level of ADA formation. Several assays have been developed to
quantify the ADA response, with drug-tolerance as their main difference. Drug-intolerant assays, such as the bridging ELISA and the ABT, only measure free ADA and cannot detect ADA in complex with drug. Therefore, low ADA responses are generally missed in these assays. To determine the total immunogenicity of a therapeutic antibody, drug tolerant assays were developed.\textsuperscript{73,74} Often an acid step is introduced to dissociate the ADA-drug complexes, after which the sample is neutralized in presence of an excess of labeled drug, enabling detection of all ADA in the sample.

For clinical use, however, a drug level test will provide more information on the clinical efficacy, whereas drug-tolerant or –intolerant assays may give the reason for lack of response.\textsuperscript{59,75} Furthermore, drug tolerant assays are of major importance for drug development and for fundamental insight into the immune response, since these give the most accurate representation of the total immunogenicity.\textsuperscript{74} During assay development however, one should be aware that many (unexpected) factors may interfere with the assay, sometimes causing false positive results. These signals are obviously undesirable, and may for instance hamper drug development by masking the expected feasibility of a new therapeutic.\textsuperscript{74} The pitfalls that can hinder the interpretation of the results, as well as several assays to measure ADA are described in detail in Chapter 2.

**Therapeutic antibodies to treat inflammatory diseases**

Therapeutic antibodies cannot cure chronic inflammatory diseases (yet), but they are very effective in ameliorating their symptoms. Since inflammatory diseases are often driven by similar pro-inflammatory cytokines such as TNF, IL-6, IL-17 and IL-23,\textsuperscript{76,77} multiple therapeutic antibodies have been developed to inhibit these cytokines or their receptors. Interestingly, not all therapeutics are equally effective for all inflammatory diseases, which emphasizes that although these cytokines are overexpressed in multiple diseases, the underlying mechanisms of disease differ to a significant extent.\textsuperscript{31}

In this thesis, the anti-TNF agents infliximab, adalimumab, golimumab and certolizumab and the anti-α4-integrin natalizumab are used to investigate different aspects of the ADA response. Although these therapeutics are used to treat many inflammatory conditions, the focus in this thesis lies on three main (groups of) diseases: rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis; their characteristics are briefly described below.

**Rheumatoid arthritis**

Rheumatoid arthritis is a chronic auto-immune disease involving multiple inflammatory pathways and immune cells that cause inflammation in the joints. Without adequate treatment, irreversible bone destruction of the joints will occur.\textsuperscript{76,78} A crucial pro-
inflammatory cytokine is TNF, and inhibition of TNF with anti-TNF therapeutics reduces the symptoms of RA. Currently, five TNF-inhibitors (infliximab, adalimumab, certolizumab, golimumab and etanercept) are used in the clinic, and they are proven equally effective in reducing the symptoms. Nevertheless, some patients do not respond at all to anti-TNF therapy, suggesting that in these cases the disease is not TNF-mediated. However, other therapeutic antibodies, such as tocilizumab (anti-IL6) or rituximab (anti-CD20, B-cell depletion) may still prove effective for these patients.

**Inflammatory bowel disease**

Due to the important role TNF plays in inflammation, several TNF inhibitors are also successfully used in the treatment of other inflammatory disorders, such as ulcerative colitis or Crohn’s disease, together called inflammatory bowel disease or IBD. In these diseases, different parts of the colon and small intestine are inflamed.

Most patients respond well to TNF inhibitors, but as described above not all TNF inhibitors work equally well suggesting that blocking of TNF alone is not sufficient to reduce inflammation. This makes immunogenicity of TNF inhibitors particularly troublesome in these patients, since the options to switch between biologicals due to ADA formation are very limited. However, recently vedolizumab (anti-αβ integrin, blocking leukocyte migration) has been approved to treat IBD, and the therapeutic etrolizumab (anti-β integrin) is currently under investigation.

**Multiple sclerosis**

In multiple sclerosis, the myelin sheets of the neurons in the brain are attacked by the immune system, leading to brain lesions. The symptoms are very diverse and include muscle weakness, cognitive impairment and fatigue. The inflammation in MS is restricted to the brain, and treatment is complicated by the blood-brain-barrier. Antibody therapy therefore focusses on the migration of lymphocytes from the periphery to the brain in order to reduce cell-mediated inflammation. The humanized antibody natalizumab targets the lymphocytes via the αβ subunit of the αβ integrin. For extravasation, interaction of αβ of the leukocytes with VCAM-1 on epithelial cells is required, and blocking this interaction thus blocks their influx into the brain. Many patients respond to natalizumab and have a significant reduction in the amount of brain lesions. Notably, a considerable part of the patients are transiently positive for ADA to natalizumab without significantly affecting treatment response.

**Scope of this thesis**

Immunogenicity of antibody therapeutics is an accepted phenomenon, and the clinical effects regarding lower therapeutic efficacy are well-studied. The mechanisms that are
involved in inflicting these clinical adverse events however, are largely unknown. The focus of this thesis therefore lies on elucidating the immunological mechanisms behind the observed unwanted clinical effects. We therefore investigated the way in which ADA reduce the free drug level, how ADA induce adverse events through complex formation, and also why complex formation induces no adverse events at all in many ADA positive patients.

To assess immunogenicity, robust assays are required to measure ADA responses. Many test formats are available, but the right assay can only be chosen if the research question is clearly formulated. One should also be aware of interference by antibodies that are not elicited upon treatment, the so called pre-existing and cross-reactive antibodies. These antibodies, together with how to avoid their inference in immunogenicity assays, are reviewed in Chapter 2.

TNF is a key mediator in inflammatory diseases such as RA and IBD, and the inhibition of TNF thus reduces inflammation. Currently, five anti-TNF therapeutic antibodies are in clinical use. In Chapter 3, TNF itself is investigated. TNF is an unstable homotrimer that rapidly loses its activity under physiological conditions. In this chapter we investigated how TNF becomes inactive, and the role that TNF-inhibitors play in this process.

Anti-drug antibodies towards anti-TNF therapeutics are known to reduce the level of free drug. The mechanism behind this is unclear, and could be either neutralization or faster clearance due to complex formation. Previous work of our group has shown that the ADA response towards the fully human antibody adalimumab is largely neutralizing; the response towards the other anti-TNF therapeutics is unknown. However, the non-human determinants in these therapeutics could potentially induce a broader immune response, facilitating formation of large immune complexes and faster clearance. In Chapter 4 we therefore determined to what extent ADA towards all four anti-TNF therapeutic antibodies are neutralizing.

The results of Chapter 4 have raised new questions regarding the immunogenicity of the idiotype. In Chapter 5 we have therefore investigated the neutralizing capacity of ADA towards natalizumab, which is in many aspects different than the anti-TNF therapeutics, most importantly because it targets a different molecule. The results of Chapter 4 and Chapter 5 combined give more insight into the general antibody response towards therapeutic antibodies.

Anti-TNF therapy is effective in most patients, but a small subset develops adverse events called infusion reactions. The symptoms of these reactions resemble that of an
IgE-mediated Type I hypersensitivity. However, the role of IgE anti-infliximab is under debate since the assays used to measure IgE anti-infliximab are not optimal, largely because of the lack of a positive control. In Chapter 6, a recombinant human monoclonal IgE anti-infliximab antibody was constructed and a robust IgE anti-infliximab assay was developed. With this assay, the incidence of IgE anti-infliximab was determined in infliximab-treated patients and furthermore the association between IgE anti-infliximab and infusion reactions was evaluated.

Binding of ADA to therapeutic antibodies results in the formation of a specific type of immune complexes, and this will likely occur in all ADA positive patients that are treated with a therapeutic. In Chapter 7, the factors that influence formation of these complexes are investigated, as well as their clearance by macrophages. Finally, it is investigated which effector functions these complexes can provoke, and to which degree these could also play a role in a clinical setting.
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