Induction and Function of Antigen-specific Sialylated IgG Antibodies

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Das Leben ist wert, gelebt zu werden, sagt die Kunst, [...];

das Leben ist wert, erkannt zu werden, sagt die Wissenschaft.

Friedrich Nietzsche

Für Mama und Papa

Abstract

IgG antibodies are important regulators of the immune system and mediate pro- and anti-inflammatory effector functions through selective binding to activating and inhibitory Fcγ-receptors on immune cells, respectively. Recently, it has been shown that these distinct effects additionally result from differential Fc-associated *N*-linked IgG glycosylation. Terminal galactosylation and sialylation of IgG antibodies have been associated with the maintenance of tolerance in healthy individuals, whereas de-galactosylation and de-sialylation of IgG autoantibodies correlate with inflammatory immune responses and disease activity in patients with *rheumatoid arthritis*. Further, the antigen-unspecific immunosuppressive effect of intravenous immunoglobulin (IVIg), used for the treatment of several autoimmune diseases, is shown to be mediated by its minor fraction of sialylated IgG antibodies. However, neither regulation of IgG antibody sialylation during humoral immune responses nor effector functions of antigen-specific sialylated IgG antibodies have been investigated so far. This work evaluated the impact of T cell independent (TI) and T cell dependent (TD) B cell activation under pro- and non-inflammatory conditions on the development of pathogenic immune responses and additionally investigated the immune regulatory functions of generating IgG antibodies. I could show that immunisation with TD and TI antigens in the absence of inflammatory co-stimuli induces peripheral tolerance and protects from subsequent pathogenic immune responses. This tolerogenic immunisation is accompanied by the generation of antigen-specific sialylated serum IgG antibodies and the development of antigen-specific plasma cells expressing high levels of α-2,6-sialyltransferase. By contrast, TI and TD antigens provided with additional co-stimulatory molecules elicit pathogenic immune responses and promote inflammation, leading to the induction of antigen-specific non-sialylated IgG antibodies and plasma cells expressing reduced levels of α-2,6-sialyltransferase. These findings indicate that Fc-sialylation might be crucial for mediating anti-inflammatory IgG effector functions during tolerance induction. Indeed, transfer of *in vivo* generated antigen-specific sialylated IgG

antibodies as well as transfer of sialylated monoclonal IgG antibodies mediate anti-inflammatory effects and protect from the induction of pathogenic immune responses, such as inflammation and allergy, in an antigen-specific manner. Compared to systemic immunosuppressive effects of high dose IVIg therapy (2 g/kg of bodyweight), even low-dose administration (5 mg/kg of bodyweight) of sialylated monoclonal IgG antibodies is sufficient to efficiently induce antigen-specific protection independently of the inhibitory FcγRIIB. Thus, monoclonal sialylated IgG antibodies may provide a novel therapeutic approach to treat autoimmunity and allergy in an antigen-specific manner with the benefit of avoiding systemic immunosuppression.

Zusammenfassung

IgG Antikörper sind wichtige Regulatoren des Immunsystems. Sie vermitteln- je nach Bindungsaffinität zu aktivierenden oder inhibierenden Fcγ-Rezeptoren auf Immunzellen- inflammatorische oder anti-inflammatorische Effekte. Zuletzt konnte gezeigt werden, dass diese Effekte maßgeblich durch unterschiedliche Fc-assoziierte *N*-Glykosylierungen verursacht werden. Im gesunden Organismus sind Galaktosylierung und Sialylierung an IgG Antikörpern daran beteiligt, Toleranz aufrecht zu erhalten. Im Gegensatz dazu korreliert das Auftreten von de-galaktosylierten und de-sialylierten IgG Antikörpern mit inflammatorischen Immunantworten und dem Schweregrad der Krankheit in Patienten mit *Rheumatoider Arthritis*. Ferner wird durch einen geringen Anteil an sialylierten IgG Antikörpern auch der systemische immunsuppressive Effekt von intravenösem Immunglobulin (*engl. intravenous immunoglobulin*, IVIg), das zur Behandlung von verschiedenen Autoimmunerkrankungen verwendet wird, vermittelt. Wie genau der Vorgang der Sialylierung während einer humoralen Immunantwort reguliert wird und welche Aufgaben sialylierte antigen-spezifische IgG Antikörper ausüben, konnte bisher noch nicht gezeigt werden. Diese Arbeit untersuchte den Einfluss von T Zell abhängiger (*engl. T cell dependent*, TD) und T Zell unabhängiger (*engl. T cell independent*, TI) B Zellaktivierung unter „steady-state" und inflammatorischen Bedingungen auf die Entstehung von pathogenen Immunreaktionen. Zudem wurden die immunregulatorischen Funktionen der entstehenden IgG Antikörper analysiert. Ich konnte zeigen, dass eine Immunisierung mit TD und TI Antigenen ohne zusätzliche Stimuli zu peripherer Toleranz führt und vor nachfolgenden pathogenen Immunantworten schützt. Diese Immunisierung wird sowohl von der Entstehung antigenspezifischer sialylierter IgG Antikörper als auch von der Differenzierung antigenspezifischer Plasmazellen, die das Enzym α-2,6-Sialyltransferase in hohem Maße exprimieren, begleitet. Unter dem Einfluss von pro-inflammatorischen Signalen induzieren TD und TI Antigene jedoch pathogene Immunantworten und fördern die Entstehung von Entzündungsprozessen. Im Gegensatz zur tolerogenen Immunisierung entstehen hier antigenspezifische nicht-sialylierte IgG

Antikörper und Plasmazellen, die nur geringe Mengen α-2,6-Sialyltransferase exprimieren. Diese Ergebnisse deuten darauf hin, dass die Fc-assoziierte Sialylierung von IgG Antikörpern bei der Vermittlung von anti-inflammatorischen Effekten während der Entstehung von Toleranz beteiligt ist. So konnte der Transfer von *in vivo* induzierten antigenspezifischen sialylierten IgG Antikörpern sowie der Transfer von sialylierten monoklonalen IgG Antikörpern anschließende pathogene Immunatworten unterdrücken und somit vor der Entstehung von Entzündungen und Allergien schützen. Im Vergleich zu systemischen immunsuppressiven Effekten, die durch IVIg in hoher Dosierung (2 g/kg Körpergewicht) erzielt werden, sind schon geringe Mengen an sialylierten monoklonalen Antikörpern (5 mg/kg Körpergewicht) ausreichend, um antigen-spezifisch vor inflammatorischen Immunreaktionen zu schützen. Dieser Effekt wird dabei unabhängig von dem inhibitorischen Fcγ-Rezeptor IIB vermittelt. Folglich stellen monoklonale sialylierte IgG Antikörper einen neuwertigen Ansatz zur antigenspezifischen Therapie von Autoimmunerkrankungen und Allergien dar.

Diese Arbeit entstand im deutschen Rheumaforschungszentrum (DRFZ) in Berlin, einem Mitglied der Leibniz-Gesellschaft, im Labor für Toleranz und Autoimmunität unter der Leitung von Prof. Dr. rer. nat. Marc Ehlers. Die vorliegende Dissertation wurde in der Zeit von Oktober 2007 bis Dezember 2010 angefertigt.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbstständig und ohne Hilfe Dritter angefertigt wurde. Alle benutzten Hilfsmittel sind vollständig angegeben worden.

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Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotionseröffnungsverfahren beantragt habe.

Constanze Heß Berlin, den 15. November 2010

Table of Contents

List of Tables

List of Figures

Abbreviations

α-	anti-
ADCC	antibody dependent cell-mediated cytotoxicity
AgeI	restriction enzyme
AHR	airway hyper-responsiveness
A/I	activating-to-inhibitory ratio
APC	antigen presenting cell
APC	Allophycocyanine
APS	ammonium persulphate
Asn	asparagine
BALB/c	wildtype mouse strain
BCR	B cell receptor
BMDC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
C57BL/6	wildtype mouse strain
C	constant region
CCR	chemokine receptor
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CIDP	*chronic inflammatory demyelinating polyneuropathy*
CLP	common lymphoid progenitor cell
CMP	cytidine monophosphate
CpG	unmethylated sequences of DNA
CRP	C-reactive protein
CSR	class switch recombination
CTL	cytotoxic T lymphocyte
Cy5	carboxymethylindocyanine-5

C3a ..... complement subunit

C5a ..... complement subunit

DC ..... dendritic cell

DCIR ..... dendritic cell immunoreceptor

DC-SIGN ..... dendritic cell specific ICAM-3 grabbin non-integrin related

DHP ..... 2,5-dihydroxybenzoic acid

DMEM ..... Dulbecco/Vogt modified Eagle's minimal essential medium

DNA ..... desoxyribonucleic acid

dNTP ..... desoxyribonucleotide triphosphate

DTH ..... delayed type hypersensitivity

EDTA ..... ethylenediaminetetraacetic acid

ELISA ..... enzyme-linked immunosorbent assay

EndoS ..... endoglycosidase S

EtBr ..... ethidium bromide

Fab ..... antibody variable region; antigen binding fragment

FACS ..... fluorescence activated cell sorting

Fc ..... antibody constant region; fragment crystallizable

FcR ..... Fc-receptor

FcRn ..... neonatal Fc-receptor

FcγRIIB ..... Fcγ-receptor IIB

FCS ..... fetal calf serum

FDC ..... follicular dendritic cell

FITC ..... fluoresceinisithiocyanat

G0 ..... Fc-glycan with terminal N-acetylglucosamine

G1 ..... Fc-glycan with one terminal galactose

G2 ..... Fc-glycan with two terminal galactoses

GBM ..... glomerular basement membrane

GBS ..... *Guillain-Barre-Syndrom*

GC ..... germinal centre

GlcNac ..... N-acetylglucosamine

GM-CSF ..... granulocyte macrophage colony stimulating factor

Gr-1 ..... myleoid differentiation antigen on granulocytes

H ..... heavy

H+E ..... hematoxylin and eosin

HEK 293T ..... human embryonic kidney cells withT-antigen

HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HindIII ..... restriction enzyme

HRP ..... horseradish peroxidase

H37Ra ..... *Mycobacterium tuberculosis* strain

HRP ..... horse radish peroxidase

IC ..... immune complex

ICAM-3 ..... intercellular adhesion molecule

IFA ..... incomplete Freund`s adjuvant

IFN-γ ..... interferon γ

Ig ..... immunoglobulin

IgA ..... immunoglobulin A

IgE ..... immunoglobulin E

IgG ..... immunoglobulin G

IgH ..... Ig Heavy chain

IgL ..... Ig light chain

IgM ..... immunoglobulin M

IL ..... interleukin

IL-4Rα$^{-/-}$ ..... mouse model deficient in IL-4 receptor α

IMDM ..... Iscove's Modified Dulbecco's Medium

i.n. ..... intra-nasal

i.p. ..... intra-peritoneal

i.v. ..... intra-venous

ITAM ..... immunoreceptor tyrosine-based activation motif

ITIM ..... immunoreceptor tyrosine-based inhibitory motif

ITP ..... *idiopathic thrombocytopenic inflammatory*

IVIg ..... intravenous immunoglobulin G

kDa ..... kilo dalton

l ..... litre

L ..... light

La ..... protein binding to RNA

LN ..... lymph node

LPC ..... lymphoid progenitor cell

LPS ..... lipopolysaccharide

M ..... molar

Mac 1 ..... marker on macrophages

MALDI-TOF ..... Matrix-assisted laser desorption/ionisation

MBL ..... mannose-binding lectin

MES ..... 2-(*N*-morpholino)-ethansulfonic acid

MFI ..... mean fluorescent intensity

mg, µg, ng, pg ..... mili-, micro-, nano-, picogram

MHC ..... major histocompatibility complex

mM ..... millimolar

ml ..... mililitre

MOPS ..... 3-(N-morpholino)-propanesulfonic acid

MWCO ..... molecular weight cut off

MyD88 ..... myeloid differentiation factor 88

MZ ..... marginal zone

neg. ctrl ..... negative control

Neu5Ac ..... *N*-Acetylneuraminic acid, human sialic acid

Neu5Gc ..... N-glycolylneuraminic acid, non human sialic acid

NF ..... nuclear factor

NF-kB ..... nuclear factor kappa B

NheI ..... restriction enzyme

NK ..... natural killer

N-linked ..... chemical bound via nitrogen

NLR ..... nucleotide-binding oligomerisation domain

NTS ..... nephrotoxic serum

OD ..... optical density

OVA ..... chicken egg albumin, ovalbumin

PAMPs ..... pathogen-associated molecular patterns

PAGE ..... polyacrylamide gel electrophoresis

PBS ..... phosphate buffered saline

PC ..... plasma cell

PCR ..... polymerase chain reaction

Pe ..... phycoerythrin

PEI ..... polyethylene imide

PerCp ..... peridin-Clorophyll-protein

pH ..... potentiometric hydrogen ion concentration

PMA phorbol-12-myristate-13-acetate

PNGase ..... peptide N-Glycosidase

pos.ctrl. ..... positive control

PRR ..... pattern recognition receptor

PS ..... Penicillin/Streptomycin

RIIB$^{-/-}$ ..... mouse model deficient in FcγRIIB

RA ..... *rheumatoid arthritis*

RNA ..... ribonucleic acid

RPMI ..... *Roswell Park Memorial Institute Medium*

RT ..... room temperature

S1 ..... Fc-glycan with one terminal sialic acid molecule

S2 ..... Fc-glycan with two terminal sialic acid molecules

SDS ..... sodium dodecyl sulfate

SDS-PAGE ..... SDS - polyacrylamide gel electrophoresis

SEM ..... standard error of the mean

SiglecF ..... sialic acid

SIGN-R1 ..... specific ICAM-3 grabbin non-integrin related 1

SIT ..... specific immunotherapy

SLE ..... systemic lupus erythematosus

SPF ..... *specific pathogen free*

SRC ..... non-receptor thyrosine kinase

ST6GAL ..... β -1,6-sialyltransferase

SV40 ..... *Simian virus 40*, polyomavirus

Taq ..... DNA-polymerase from *Thermus aquaticus*

TAE ..... Tris, acetatic acid, EDTA buffer

TCR ..... T cell receptor

TCRβ-/- ..... mouse model deficient in TCRβ

TD ..... T cell dependent

TE ..... Tris/ EDTA

TEMED ..... tetramethylethylenediamine

TGF-β ..... transformal growth factor β

Th ..... T helper

Thy1.1 ..... thymocyte differentiation antigen 1, not expressed on C57BL/6 thymocytes

TI ..... T cell independent

TMB ..... tetramethylbenzidine

TLR ..... Toll-like receptor

TNF-β ..... tumor necrosis factor β

TNP ..... trinitrophenyl

TOF ..... time-of-flight

Treg ..... regulatory T cell

TRIF ..... (TIR)-domain-containing-apaptor inducing interferone β

UDP ..... uracil diphosphate

U ..... unit

V ..... variable region

1 Introduction

1.1 The immune system

The immune system of vertebrates is a highly complex system which defends the host against exogenic pathogens, like parasites, fungi, bacteria and viruses. Extensive research in the field of immunology has given insight into the complexity of this system but still, new sub-cell types, molecules and mechanisms contributing to this network are discovered regularly. In principle, the immune system can be subdivided into the innate or non-specific and the adaptive or specific immune system. To ensure that the immunological defense machinery is only directed against pathogens and abnormal self-structures, but not against endogenous self-structures and harmless foreign antigens the regulation of inflammatory immune responses and tolerance is an essential part of the immune system. Defects within this system lead to the false positive induction of an inflammatory adaptive immune response and are the cause of autoimmunity and allergies. One of the major goals of immunological research today is to understand such dysregulation and to develop strategies for the treatment of or the protection from harmful autoimmune diseases and allergies.

1.2 The innate immune system

Once a pathogen has overcome the first line of defense, such as mechanical barriers including the skin, the respiratory and epithelial tract, and successfully enters the organism, it is recognised by cellular or soluble components of the innate immune system of the host (Kimbrell and Beutler 2001). The complement system is a cascade involving blood-soluble proteins, which mediate the clearance of pathogens by direct lysis or engulfment by macrophages via opsonisation. The effector functions of complement can be activated through three different pathways. The MBLectin pathway is initiated by binding of mannose-binding lectin (MBL) to mannose containing carbohydrates or glycoproteins on bacteria or viruses (Turner and Hamvas 2000). The alternative pathway is triggered by

direct binding of complement components to the cell surface of pathogens, which in turn activates the complement cascade. The classical pathway is antibody-mediated and therefore considered as part of the adaptive immune system (Tomlinson 1993; Dunkelberger and Song 2010). Besides the complement system, many cell types are involved in the early defense mechanisms of the innate immune system, such as neutrophils, natural killer (NK) cells, monocytes, macrophages, and dendritic cells (DCs). Neutrophils are important for the fast defense against bacteria and fungi since they produce and release antimicrobial peptides and radical oxygen intermediates at the site of the infection (Godaly and Young 2005; Segal 2005) and recruit further immune cells. NK cells can directly bind to antibody-bound target cells and lyse infected cells by perforine and granzyme release (Biron, Nguyen et al. 1999; Boyington and Sun 2002). Monocytes are recruited from the blood stream and differentiate into macrophages, which can uptake antigens by phagocytosis (Aderem and Underhill 1999) and release inflammatory mediators, such as cytokines and chemokines, which are important for attracting other immune cells (Mogensen 1979; Luster 2002). Dendritic cells can also uptake and process antigens and therefore act as antigen-presenting cells. Once activated, they migrate to the lymph node where they interact with specific B and T cells to initiate and shape adaptive immune responses (Banchereau, Briere et al. 2000).

Recognition of pathogens by cells of the innate immune system is unspecific and relies on the recognition of pathogen-associated molecular patterns (PAMPs) like microbial and viral components, by pattern recognition receptors (PRRs) on the cells of the innate immune system. There are different families of PRRs including retinoid acid-inducible gene-1 receptors, nucleotide-binding oligomerisation domain-like receptors (NLRs) (Franchi, McDonald et al. 2006) and Toll-like receptors (TLRs) (Takeda and Akira 2005). TLRs are a well described group of PRRs and bind to microbial patterns, such as bacterial cell wall components (TLR 2, 4, 6), flagella (TLR 5), RNA (TLR 7, 8) or bacterial DNA (TLR 9) (Barton and Medzhitov 2002; Janeway and Medzhitov 2002; Takeda and Akira 2005). All TLRs except TLR 3, are coupled to the adapter molecule myeloid differentiation factor 88 (MyD88) and are able to activate nuclear factor (NF)-κB for induction of NF-κB dependent genes (Takeda and Akira 2005; Lanzavecchia and Sallusto 2007). Triggering of TLRs leads to the activation of macrophages and DCs inducing the up-regulation of co-stimulatory molecules and the secretion of inflammatory cytokines and chemokines (Banchereau, Briere et al. 2000). As the pathogen recognition process by the innate

immune system is limited to pathogens that own PAMPs and thus, cannot protect the organism from pathogens without PAMPs, a second line of defense is required. If the innate immune system fails to clear pathogens, highly specific adaptive B and T cell immune responses will be required (McHeyzer-Williams, McHeyzer-Williams et al. 2000).

1.3 Adaptive immune responses

Cells of the adaptive immune system bear receptors with random specificity on the cell surface and therefore provide the recognition of non-conserved structures on pathogens and induce specific defense strategies to protect the organism. Adaptive immune responses are mediated by B- and T-lymphocytes (B cells and T cells), which carry specific antigen-binding receptors on their cell surface, the B cell antigen receptor (BCR) and the T cell antigen receptor (TCR). The large repertoire of antigen-receptors on the surface of cells is due to rearrangment of germline encoded variable gene segments during developmental stages. Activation of B and T cells leads to the induction of specific humoral and cellular immune responses, respectively. The reactivity of antigen-specific B cells can further be enhanced by adaptive somatic hypermutations. Additionally, one major characteristic of the adaptive immune system is the ability to develop an immunological memory, which provides long-lasting protection against reinfection with the same microorganism (McHeyzer-Williams, McHeyzer-Williams et al. 2000). However, a false positive specific B and T cell response is responsible for the development of autoimmunity and allergy.

1.3.1 Antigen-presenting cells (APCs)

Antigen presenting cells (APCs) efficiently internalise antigens by phagocytosis or receptor-mediated endocytosis and present processed antigenic peptides to T cells. Professional APCs are macrophages, B cells and dendritic cells (DCs). In contrast to all nucleated cells, which express MHC class I molecules on their surface, APCs additionally express major histocompatibility complex (MHC) class II molecules to prime CD4 helper T cells. While macrophages and B cells up-regulate MHC class II expression subsequent to antigen encounter, DCs already express low levels of MHC class II molecules in an immature state (Unanue 1984; Forster, Schubel et al. 1999; Rodriguez-Pinto 2005).

DCs are considered to be crucial for the initiation and regulation of innate and adaptive immune responses (Banchereau, Briere et al. 2000); (Lee and Iwasaki 2007). Immature DCs are located in peripheral tissues, such as the skin and mucosal surfaces. Antigen binding to PRRs expressed on DCs leads to the activation and maturation of the DC, which in turn induces the up-regulation of MHC class II molecules and co-stimulatory molecules like B7.1 (CD80) and B7.2 (CD86) (Janeway and Medzhitov 2002). The expression of the chemokine receptor CCR7 on mature DCs promotes migration of DCs to secondary lymphoid organs, where they present the encountered antigen in complex with MHC class I and MHC class II to the TCRs of naïve T cells. Depending on the microenvironment, DCs can become potent stimulators of T cell-mediated immunity after undergoing differentiation and maturation in response to additional stimuli, such as CD40 ligation and TLR stimulation by microbial ligands (Forster, Schubel et al. 1999; Lee and Iwasaki 2007). By contrast, when encountering antigens without co-stimulation in the steady state, DCs cannot fully mature.

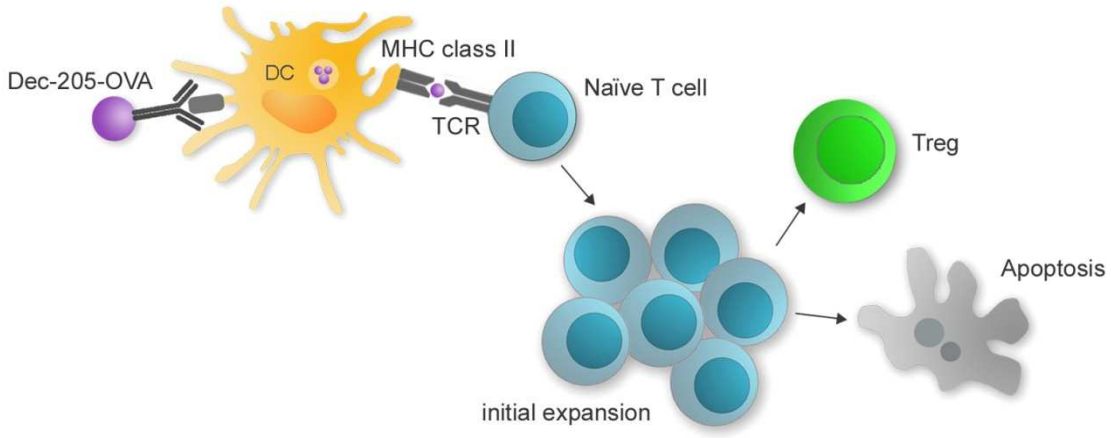


Figure 1.1: Dec-205-OVA mediated induction of peripheral tolerance. Anti-Dec-205 fused to Ovalbumin (OVA) is recognised by Dec-205 on DCs leading to antigen uptake, processing and presentation to naïve T cells. After initial T cell proliferation, peripheral tolerance is induced by the induction of regulatory T cells (Tregs) and apoptosis of specific T cells (adapted from (Tarner and Fathman 2006)).

Immature or semi-mature DCs induce tolerance to the encountered antigen by either deletion of antigen-specific T cells or the induction of regulatory T cells (Tregs) (Hawiger, Inaba et al. 2001; Steinman 2003). Recently, *in situ* studies revealed that the endocytotic

receptor Dec-205, which is expressed on DCs, is involved in the uptake of antigens by DCs. Targeting antigens to Dec-205 by anti-Dec-205 antibodies showed that this receptor induces a more efficient antigen processing and presentation of antibody associated peptides on MHC complexes. In the steady state, antigen uptake by Dec-205 is not associated with a phenotypical change of the DC, but with the induction of peripheral tolerance (*Figure 1.1*). By contrast, Dec-205-mediated antigen uptake in the presence of co-stimulatory molecules leads to strong immune responses, which are accompanied by the stimulation of T helper (Th) cells and the expression of pro-inflammatory cytokines, like IFN-γ. (Hawiger, Inaba et al. 2001; Steinman 2003; Steinman, Hawiger et al. 2003).

1.3.2 T lymphocytes

T lymphocytes (T cells) are primary effector cells of the adaptive immune system mediating cellular immune responses. T cells develop from lymphoid progenitor cells (LPCs), which migrate from the bone marrow to the thymus and differentiate to mature T cells (Carlyle and Zuniga-Pflucker 1998; Carpenter and Bosselut 2010). They are characterised by the possession of a specific antigen receptor, the TCR, which can recognise antigenic peptides if presented by MHC class I and MHC class II molecules and mediate T cell activation via cytosolic immunoreceptor tyrosine-based activation motifs (ITAMs). T cells undergo rearrangement of genes for the TCR α, β, γ and δ chains which contribute to the huge repertoire of TCRs (Kisielow and von Boehmer 1995). There are two major subspecies of T cells distinguished by the expression of the cell surface markers CD8 and CD4, the CD8 T cells and the CD4 T cells (Seder and Ahmed 2003). The proteins CD4 and CD8 recognise MHC class II and MHC class I, respectively, and thereby function as TCR co-receptors (Mosmann, Cherwinski et al. 1986; Barry and Bleackley 2002). Activation of CD8 T cells leads to the generation of cytotoxic T lymphocytes (CTLs) with the ability to eliminate altered self-cells, such as virus infected or tumor cells (Barry and Bleackley 2002). Dependent on the respective activating signals, naïve CD4 T cells differentiate into different T helper (Th) cell lineages (Mosmann, Cherwinski et al. 1986; Williams and Bevan 2007). T helper cells exhibit no direct cytotoxic or phagocytotic activity, but play an important role in activating and directing other immune cells, such as CTLs, phagocytes and B cells. The helping function is crucial for mediating cellular and humoral immune responses. Based on their cytokine expression, Th cells can be subdivided into Th1, Th2,

Th17 and Tregs which all fulfill different tasks (Mosmann, Cherwinski et al. 1986; Sakaguchi 2004; Dong 2006). Th1 cells are characterised by the production of IFN-γ, IL-2 and TNF-β, which activate macrophages and induce cell-mediated immunity. Th2 cells secrete IL-4, IL-5, IL-10 and IL-6, which activate eosinophils and induce antibody-production by activated B cells. While IL-17 producing Th17 cells are associated with autoimmunity (Dong 2006; Dong, Bachman et al. 2008), Tregs, which secrete TGF-β and IL-10 mediate immune suppression and tolerance. Memory T cells are antigen-specific and ensure a fast response to a second exposure of the specific antigen.

1.3.3 B lymphocytes

B lymphocytes (B cells) are the primary effector cells of the humoral immune response and maturate from LPCs in the bone marrow (Martynowicz, Walters et al.). Naïve B cells are characterised by the expression of antigen receptors on their surface, which are able to recognise soluble antigenic structures. The so-called BCR is composed of a membrane-bound immunoglobulin (Ig) and the associated heterodimer composed of the Igα- and the Igβ-chain. The membrane-boung Ig consists of two heavy (H) and two light (L) chains joined by disulfide bonds. Each chain is composed of variable (V) and constant (C) region. With its N-terminal variable regions, the BCR functions as an antigen-binding site. BCR binding leads to the phosphorylation of ITAMs in the cytosolic domains of the Igα and Igβ chains and thereby promotes downstream activating signalling (Ollila and Vihinen 2005). During B cell development, BCRs can reach a vast number of specificities due to unique gene segment rearrangements (somatic recombination) of the H and the L chain.

1.4 B cell activation

Naïve B cells circulate through the blood and lymph until they home to secondary lymphoid organs where they encounter their specific antigen. B cell activation is initiated by the recognition of soluble antigen by the BCR. Recognition of antigens by BCR binding is rarely sufficient to trigger various downstream activation pathways crucial for B cell proliferation and differentiation (Pasare and Medzhitov 2004). Besides antigen binding, additional stimulatory signals from other accessory cells, like macrophages, DCs and T cells are required for the activation of naïve B cells. Thus, the activation of B cells can be

classified in two different mechanisms, the T cell independent (TI) and the T cell dependent (TD) activation (*Figure 1.2*). Once activated, B cells proliferate and differentiate into plasma cells, which secrete large amounts of soluble Igs, the so called antibodies (Ollila and Vihinen 2005).

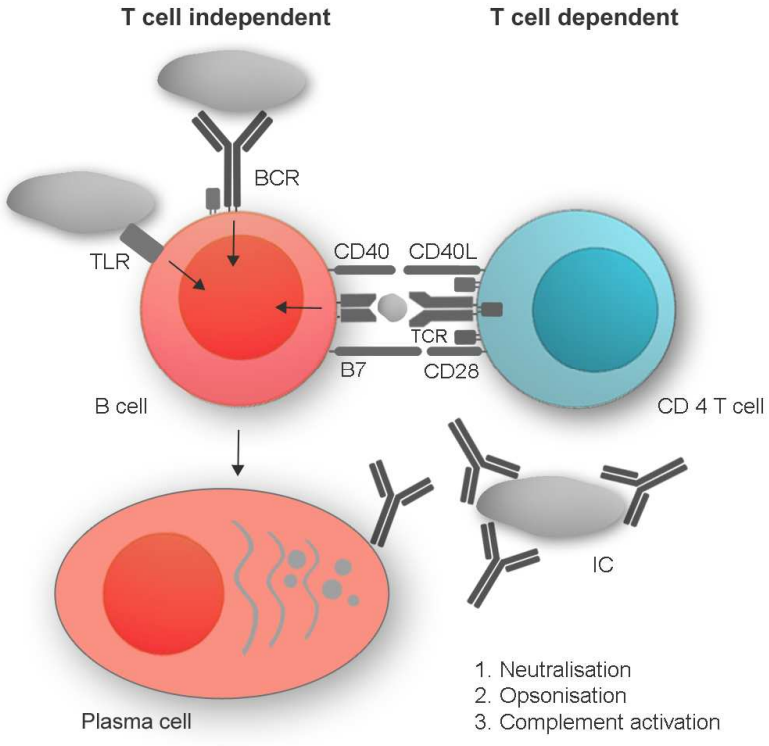


Figure 1.2: TI and TD B cell activation. B cells can be activated by BCR crosslinking (e.g. through bacterial polysaccharides) and TLR stimulation (e.g. through LPS). Most of all antigens are unable to crosslink BCRs and therefore require additional CD4 T cell help for B cell activation. CD4 T cells bind to antigenic peptides presented by MHC class II molecules and mediate B cell activation by additional binding to co-stimulatory receptors, like CD40. Activated B cells can differentiate into antibody secreting plasma cells. Antigen-specific antibodies can bind to the respective soluble antigen and form immune complexes, which mediate neutralisation and opsonisation or initiate antigen degradation by activation of the comlement cascade (adapted from (Ollila and Vihinen 2005)).

1.4.1 T cell dependent B cell activation

Most of all common antigens are protein-derived and thus, induce TD immune responses (McHeyzer-Williams, Malherbe et al. 2006). TD B cell activation is mediated by sequential integration of two signals, BCR ligation by an antigen and co-stimulation by the help of MHC class-II restricted CD4 T cells. After antigen binding to BCRs, BCR-antigen

complexes are internalised and processed. Processed antigenic peptides are presented on MHC class II molecules to respective CD4 T cells. Thereupon, the co-receptor CD40 on the B cell surface binds to CD40L (CD154) on activated T cells, which induces the production of B cell stimulatory cytokines in T cells (Jaiswal and Croft 1997) and the up-regulation of receptors for various cytokines and chemokines essential for proliferation, differentiation and migration of B cells (Burstein and Abbas 1991; Ruprecht and Lanzavecchia 2006). Activated B cells either migrate to extrafollicular sites and differentiate to short-lived plasma cells (Allen, Okada et al. 2007) or mature into germinal centre-precurser cells and move into primary follicles. In germinal centres, activated B cells displace naïve B cells to form the secondary follicle, which is also named the outer mantle zone (Hawiger, Inaba et al. 2001). In the dark zone of the germinal centre, activated B cells pass through a process of clonal expansion, which is often accompanied by class switch recombination of the H chain constant region (Klein and Dalla-Favera 2008) and several rounds of somatic hypermutation, the hallmark of germinal centre B cells, leading to BCRs with increased antigen affinities (Jacob, Kelsoe et al. 1991; Kelsoe 1991; Berek 1993). Germinal centre B cells migrate into the light zone, where follicular dendritic cells (FDCs) initiate positive selection of antigen-recognising B cells. Selected B cells mature and differentiate into long-lived plasma or memory B cells (Sze, Toellner et al. 2000; Allen, Okada et al. 2007; Klein and Dalla-Favera 2008). Memory B cells are generated from activated B cells after primary exposure to an antigen and persist long-term in the organism and hereby effectively protect against re-infection (McHeyzer-Williams and McHeyzer-Williams 2005; Ollila and Vihinen 2005).

1.4.2 T cell independent B cell activation

Certain non-protein derived antigens can also activate B cells independently of T cell help. These antigens are classified as T cell-independent (TI) antigens. TI antigens can be further subdivided into TI type 1 and 2 antigens (Mond, Vos et al. 1995). TI type 1 (TI-1) antigens are typically coupled to TLR ligands, like lipopolysaccharide (LPS), which interact with TLRs. Thus, the TI-1 antigen binds to the BCR, whereas the TLR ligand LPS activates TLR 4 to co-stimulate the B cell. Classical TI-1 antigens in research are nitrophenylated LPS (NP-LPS) and trinitrophenylated LPS (TNP-LPS) inducing NP- and TNP-specific antibodies, respectively (Fidler 1975; Humbert, Motta et al. 1979). Recently, it has been

demonstrated that RNA or DNA containing TI antigens are sufficient to induce IgG autoantibodies via TLR 7 and TLR 9 co-stimulation, respectively. However, their role in the induction of autoimmunity is still unknown (Lanzavecchia and Sallusto 2007; Herlands, Christensen et al. 2008; Tsao, Jiao et al. 2008). TI type 2 (TI-2) antigens, such as polysaccharides, are large molecules with repeating epitopes that are able to simultaneously engage and extensively cross-link multiple BCRs and thereby activate B cells in the absence of T cells (Mond, Lees et al. 1995). TI-2 antigens lack intrinsic mitogenicity and therefore, do not activate TLRs. B cell activation by TI-2 antigens leads to IgM and IgG antibody responses (Vos, Lees et al. 2000). Experiments with TI-2 antigens have suggested an immune regulatory function of resulting IgG antibodies, including the ability to suppress a second challenge with the same TI-2 antigen (Brodeur and Wortis 1980; Obukhanych and Nussenzweig 2006). Thus, B cell activation with TI-2 antigens has always been related with tolerisation of B cells.

1.5 Humoral immune responses- antibody functions

Humoral immune responses are mediated by antibodies, which are secreted in plasma cells that are generated upon B cell activation. Serum levels vary considerably in the different antibody subclasses (Clynes and Ravetch 1995; Waldmann 2003). Roughly 5% of serum antibody levels are comprised of IgM, which is the first Ig expressed at elevated levels upon B cell activation. After Ig class-switch recombination during germinal centre reactions, other isotypes, such as IgG and IgA and IgE, are produced. IgG is the most abundantly expressed isotype in vertebrates, like humans and mice, and makes up about 85% of the immunoglobulin level. (Manz, Hauser et al. 2005). IgG antibodies are the major mediators of the humoral immunity and contribute to the clearance of pathogens and the neutralisation of toxins. Antibodies have various protective effects which promote the elimination of foreign antigens.

Secreted antibodies can bind to soluble antigens and form immune complexes (ICs) and therby modulate immune responses in different ways. Antibodies protect the host from pathogens by neutralisation, opsonisation, complement cascade activation and antibody-dependent cell-mediated cytotoxicity (ADCC) (Chan and Carter 2010). They can easily neutralise antigens (e.g. toxins and bacteria) by binding and thereby inhibiting their interactions with cellular components. Antibodies bound to soluble antigens have the

ability to activate the complement system which in turn leads to the eradication of pathogens (e.g. bacteria) (Brown, Hussell et al. 2002). Another function of antibodies is the opsonisation of pathogens. Opsonised pathogens will be taken-up by effector cells via Fc-receptor binding (Metzger and Kinet 1988), which internalise and process pathogenic molecules (phagocytosis). Furthermore, antibodies that bind to abnormal self cells activate NK cells by binding to their Fc-receptors thus mediating the ADCC pathway which leads to the eradication of antibody-loaded cells (Chan and Carter 2010).

1.6 Fcγ-receptors and their functions

Fcγ-receptors (FcγRs) are Fc-receptors which are expressed on the surface of adaptive and innate immune cells. FcγRs bind to the Fc-part of IgG molecules with high affinity when complexed with an antigen. Binding of IgG immune complexes to FcγRs leads to either inflammatory or anti-inflammatory immune responses depending on the signal. When stimulatory signals dominate, cell-activation and inflammatory immune responses are induced which in turn leads to the elimination of pathogenic antigens or abnormal self-cells. In the absence of positive signals, cell activation is blocked and anti-inflammatory responses can occur (Nimmerjahn and Ravetch 2008). Loss of the balance between these binary systems results in aberrant immune responses which induces the loss of tolerance and the development of autoimmunity or immuno-deficiency. Until now, there are four classes of FcγRs in mice: FcγRI, FcγRIIB, FcγRIII and FcγRIV. Besides FcγRs, one IgA and IgM binding Fcα/μ-receptor and one Fcε-receptor was identified on murine immune cells. The human FcR systems are more complex as it can be distinguished between the FcγRI gene family (*Fcgr1a, Fcgr1b* and *Fcgr1c*), the FcγRII gene family (*Fcgr2a* and *Fcgr2c*) and the existence of several allelic FcγR variants. Based on their functionality, FcγRs can be divided into activating and inhibitory receptors. In mice, there are three activating (FcγRI, FcγRIII and FcγRIV) and one inhibitory receptor (FcγRIIB). Innate immune cells such as monocytes, macrophages and DCs express both activating and inhibitory FcγRs whereas B cells only express the inhibitory FcγRIIB and NK cells the activating FcγRIII (Nimmerjahn and Ravetch 2008). Binding of complexed IgG to activating or inhibitory FcγRs on immune cells leads to the modulation of the effector functions of the innate and the adaptive immune system (Figure 3). FcγRs have a key function whether an immunogenic or

a tolerogenic response is initiated. Thus, they are involved in regulating IgG responses. Activating FcγRs cannot transmit activating signals in the absence of the common γ-chain which carries the ITAM in the receptor associated adaptor molecules. Significant defects in antibody-mediated effector cell responses were observed in mice carrying a γ-chain knockout (Nimmerjahn and Ravetch 2006). Cross-linking of activating FcγRs by immune complexes initiates signalling pathways starting with the tyrosine phosphorylation of the ITAMs by kinases of the Src family. This process is followed by various downstream signalling targets, which lead to the activation of effector immune cells (Brownlie, Lawlor et al. 2008; Nimmerjahn and Ravetch 2008).

There is only one inhibitory FcγR in humans and mice, FcγRIIB, which is expressed on all leucocytes except on NK cells and T cells. FcγRIIB is a single chain receptor which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic region (Nimmerjahn and Ravetch 2006). Complexed IgG triggers ITIMs and BCRs simultaneously which results in the activation of phosphatases of the SHIP-family. Thereby, ITIM mediated signals interfere with downstream pathways necessary for activating signals. Thus, FcγRIIB functions as an important regulator of the activating signals that are transmitted by the BCR (Nimmerjahn and Ravetch 2008). FcγRIIB plays a unique role in the regulation of innate and adaptive immune responses and is actively involved in the maintenance of tolerance. FcγRIIB regulates the activation of DCs and therefore prevents from spontaneous maturation of DCs under steady-state conditions and the induction of self-destructive responses. As a regulator of innate immune cells, FcγRIIB controls the initiation of pro-inflammatory immune responses. Furthermore, negative feedback regulation by FcγRIIB in B cells has emerged as a late checkpoint regulating plasma cell survival and apoptosis in B cells. Complexed IgG binds to FcγRIIB on B cells and induces pro-apoptotic signals. This regulatory mechanism is important for the deletion of autoreactive plasma cells and overreaching antibody production. Impaired FcγRIIB expression leads to enhanced cell activation and predisposition for generating autoimmune diseases (Brownlie, Lawlor et al. 2008; Nimmerjahn and Ravetch 2008).

1.7 Autoimmunity and allergy

Autoimmunity is the failure of an organism to recognise its own constituent parts as *self*, which allows an immune response against its own cells and tissues. Any disease that results from such an aberrant immune response is termed an autoimmune disease. It is essential that all defence mechanisms are only directed against pathogenic structures. Therefore, it is important that the complex system of the immune system can distinguish between self and non-self (Goodnow 1996). Central and peripheral tolerance mechanism prevent the development of self-reactive antigen-receptors by clonal deletion or anergy and apoptosis (Goodnow, Adelstein et al. 1990); (Nemazee and Buerki 1989; Nemazee and Burki 1989). Genetic dispositions or unfavourable environmental factors provoke inaccurate regulation of tolerance mechanism. Resultant development of self-reactive antibodies or self-reactive T effector cells subsequently lead to the development of autoimmunity (Kotzin 1996).

By contrast, allergy is an exaberated immune response directed against normally harmless environmental substances, known as allergens. Allergy is a hypersensitive disorder of the immune system and belongs to the hypersensitivity type I. These reactions are aquired and proceed rapidly. A hallmark of allergy is the detectability of increased serum IgE antibodies and eosinophils in the lung (Venarske and deShazo 2003). In the healty environment, IgE and eosinophils are involved in the protective immunity against parasitic infections. Eosinophils belong to the group of granulocytes and secrete a range of highly toxic proteins and free radicals that are effective in killing parasites. An allergen encountering the human organism for the first time causes a Th-2 cell-mediated immune response. Allergen-specific Th-2 cells are activated by allergen-presenting cells (Venarske and deShazo 2003), like DCs, and start to secrete the cytokines IL-4 and IL-13, which induce class-switching in B cells and the promote the production of IgE antibodies. Additionally, the expression of CD40L on the surface of Th2 cells is up-regulated. The interaction of CD40L with CD40 on B cells, as well as further stimulation by IL-4, induces the secretion of IgE by allergen-specific B cells. IgE can bind to the high-affinity Fcε-receptor, which is mainly expressed on mast cells, basophils and eosinophils (Broide 2001). The IgE coated immune cells are sensitised at this stage. When activated during a second exposure to the same allergen, the allergen binds to the IgE molecules on the surface of mastcells or basophils. When sensitised mast cells are activated by allergen cross-linking IgE molecules, they undergo a process called degranulation and rapidly release histamine and other pro-inflammatory

mediators, such as heparin, prostaglandin D2 and IL-4 and IL-13 cytokines (Williams and Galli 2000). Pro-inflammatory mediators dilate blood vessels and cause the characteristic signs of inflammation, such as mucosal edema, itching, smooth muscle contraction and mucus secretion. Thus, type I allergy is characterised by an increased number of IL-4 producing Th2 cells and eosinophils and further, by the production of allergen-specific IgE antibodies.

1.8 Immunoglobulin G

IgG antibodies are glycoproteins of a Y-shaped structure and consist of two identical heavy (H) and two identical light (L) chains which are linked by disulfide bonds. While the light chains consist of a variable and a constant region, V_L and C_L, the heavy chains are composed of a variable, V_H and three constant regions, C_H1, C_H2 and C_H3. The variable domains of the heavy and the light chain make up the antigen-binding site of the antibody whereas the C-domains determine the Ig isotype and the corresponding functional properties. Cleavage of antibodies with the proteolytic enzyme called papain leads to antibody peptide fragments. Papain cleaves antibodies in the hinge region between the C_H1 and the C_H2 domain releasing 2 antigen binding fragments (Fab) and the C_H effector dimer (Fc, fragment crystallizable).

IgG antibodies are the primary mediators of protective humoral immunity and inflammation. Due to their different binding properties to Fcγ-receptors, the four IgG subclasses (IgG1-4 in humans, IgG1, IgG2b and IgG2c, IgG3 in mice) mediate diverse effector functions (Ravetch and Bolland 2001; Nimmerjahn and Ravetch 2005). The affinity of IgG isotypes for functional distinct Fcγ-receptors is described as a ratio, referred to as the activating-to-inhibitory (A/I) ratio (*Table 1.1*) (Nimmerjahn and Ravetch 2005). Due to the A/I ratio, IgG1 is associated with anti-inflammatory properties whereas IgG2b and IgG2c are considered to have a high pathogenic potential to trigger inflammation and dominate autoimmune diseases (Nimmerjahn and Ravetch 2006).

	IgG1 (FcγRIII/IIB)	IgG2b (FcγRIV/IIB)	IgG2c (FcγRIV/IIB)
A/I ratio	0.1	7	69

Table 1.1: Activating-to-inhibitory (A/I) ratio describes the affinity of IgG isotypes to Fcγ-receptors. A/I ratio IgG2c>>IgG2b>IgG1 reveals that the IgG2c isotype has the highest activating potential contrary to IgG1.

1.8.1 Glycosylation of the Fc-domain

The biological activity of IgG antibodies is modulated by their glycosylation pattern. The Fc-parts of IgG antibodies contain a highly conserved *N*-glycosylation site at asparagine 297 (Asn297) in both heavy chains. IgG Fc *N*-glycans are biantennary structures and consist of a core heptapolysaccharide containing N-acetylglucosamine (GlcNac) and mannose. Further glycosylation modifications of the core glycan structures are observed in serum IgG antibodies (*Figure 1.3*). Fucose, bisecting GlcNac, galactose and terminal sialic acid moieties are present in the glycan structures (Kaneko, Nimmerjahn et al. 2006; Huhn, Selman et al. 2009). As conformation of the IgG Fc-part is influenced by their glycan pattern, glycosylation of IgGs is essential for binding to Fcγ-receptor. Specific cleavage of Fc-linked glycans by the bacterial enzyme endoglycosidase (EndoS) completely abrogates the functional properties of IgGs (Collin and Olsen 2001; Collin, Shannon et al. 2008).

1.8.2 Fc-glycosylation in autoimmune diseases

Parekh *et al.* have reported in the 1980's that patients with rheumatoid arthritis show serum IgG antibodies which differ from the glycosylation pattern of healthy individuals. In healthy individuals glycosylation of IgG antibodis varies considerably between individuals of the same sex and age. While galactosylation decreases during the lifespan, pregnancy is associated with increased galactosylation. Most of all IgG antibodies from healthy donors are of the G1 and G2 isoform, which means that the core heptapolysaccharide carries one or two terminal galactose residues, respectively. Interestingly, patients with chronic *rheumatoid arthritis* (RA) generate IgG antibodies with shorter Fc-glycan structures. More than 60% of all IgG antibodies are of the G0 glycan type, which only consists of the core

glycan structure without galactose. Moreover, in healthy individuals, up to 15% of the serum IgG antibodies have oligosacharides displaying terminal sialic acid, by contrast, IgG glycan structures in patients with RA carry no terminal sialic acid. These findings were the first to associate the influence of Fc-glycosylation of IgG antibodies to their function in chronic inflammatory diseases (Parekh, Dwek et al. 1985; Parekh, Dwek et al. 1988). Sialylation of IgG antibodies reduced their cytotoxicity due to reduced affinities of IgGs to corresponding Fcγ-receptors. Sialylation of steady state IgG ensures the maintenance of the anti-inflammatory state. When challenged upon pathogenic stimulation, antigen-specific IgGs switch to less sialylated IgG isoforms and provide inflammatory effector functions (Nimmerjahn and Ravetch 2005; Kaneko, Nimmerjahn et al. 2006).

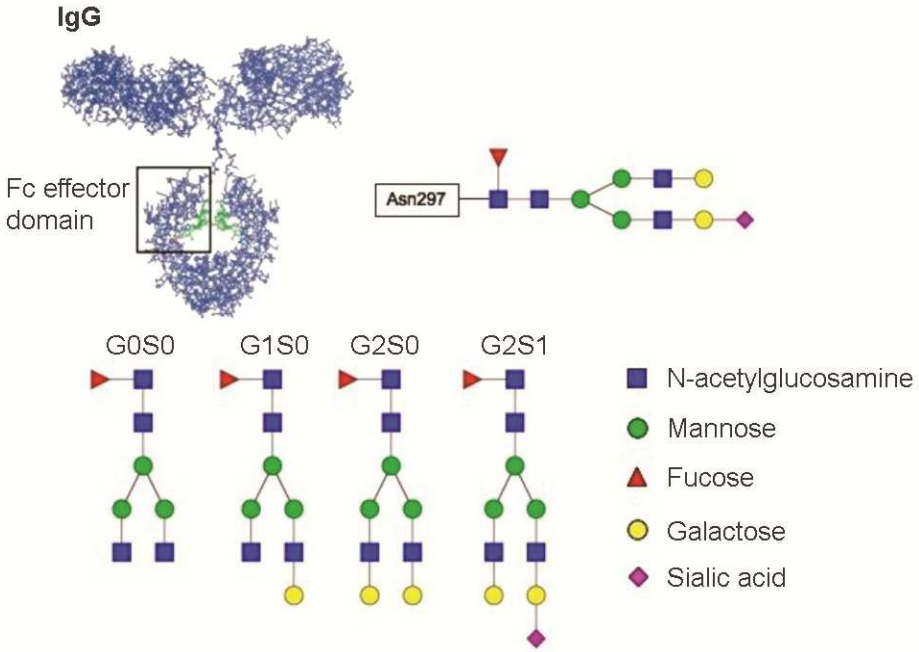


Figure 1.3: Fc-glycan structures. Primary *N*-acetylglucosamine is attached to the Asn297 of each heavy chain in the C_H2 domain. Depending on the terminal sugar this Fc-core oligosaccharide is associated with different effector functions. G0, G1 and G2 isoforms have no, 1 or 2 terminal galactoses, respectively. S1 and S2 isoforms display 1 or 2 terminal sialic acids. About 10% - 15% of serum IgG antibodies from healthy donors carry Fc-glycans with terminal sialic acid, which provide antibodies with anti-inflammatory properties.

1.8.3 Intravenous immunoglobulin (IVIg)

High dose intravenous immunoglobulin (IVIg) preparations are used currently for the treatment of many inflammatory and autoimmune diseases, such as thrombocytopenia, RA and systemic lupus erythematosus. Therapeutic Ig preparations are comprised of polyclonal and polyspecific Ig, mainly IgG, derived from pooled serum of thousands of healthy donors. Surprisingly, IVIg can have both pro- and anti-inflammatory effects depending on its concentration. Low-dose IVIg activates calcium-dependent signalling pathways and finally leads to pro-inflammatory cell activation (Durandy, Kaveri et al. 2009). By contrast, due to reconstitution of the normal IgG glycosylation pattern in autoimmune patients, high concentrations (1 to 2 g/kg body weight) (Anthony, Nimmerjahn et al. 2008) of IVIg mediate anti-inflammatory properties. Until now, studies have proven therapeutic effects of high dose IVIg in autoimmune diseases, but the mechanism of action remains poorly understood.

1.8.4 Mechanism of IVIg

Previous studies have revealed that IVIg acts on different components of the innate and the adaptive immune system, such as DCs, macrophages, NK cells, T cells, B cells and various complement factors (Figure 7). High dose IVIg inhibits the up-regulation of CD80 and CD86 on DCs and abrogates their ability to process and present antigens (Bayry, Lacroix-Desmazes et al. 2003). Furthermore, IVIg increases the expression of inhibitory FcγRIIB on the surface of effector macrophages in mice. Increase of FcγRIIB expression might result in an enhanced binding of IgG antibodies to inhibitory FcγRIIB, which leads to enhanced inhibitory signalling which results in diminished secretion of pro-inflammatory cytokines (Dhodapkar, Kaufman et al. 2005). As the ratio of activating to inhibitory receptors decreases, the threshold triggering pro-inflammatory immune responses rises. In addition, FcγRIIB-mediated negative signalling on B cells suppresses the expansion of autoreactive B cells and down-modulates the production of autoantibodies (Lemieux, Bazin et al. 2005). How IVIg mediates its suppressive effects on T cells and whether IVIg induces Tregs is still poorly understood (Durandy, Kaveri et al. 2009). There are only few publications suggesting any functional pathway how IVIg works in detail. Several studies revealed that the well known anti-inflammatory activities of IVIg are attributed to the sugar

moiety on Asn297 in the C_H2 domain of the IgG Fc-part. Treatment of IVIg with peptide N-glycosidase (PNGase) F, which removes *N*-linked carbohydrates structures, leads to the inability to mediate anti-inflammatory effects in the K/N serum model of RA (Kaneko, Nimmerjahn et al. 2006). Furthermore, Kaneko *et al.* demonstrated the sialic acid to be essential for IgG antibodies to mediate the anti-inflammatory effects of IVIg (Kaneko, Nimmerjahn et al. 2006). In addition, Samuelsson *et al.* could show a clear requirement of the inhibitory FcγRIIB for the protective effect of IVIg (Samuelsson, Towers et al. 2001). Sialylated IgGs have reduced affinities to Fcγ-receptors in general, but rather bind to the inhibitory FcγRIIB as IVIg induces an enhanced expression of FcγRIIB on immune cells. In 2008, Anthony *et al.* recapitulated the anti-inflammatory activity of IVIg with sialylated Fc portions. 19 IgG Fc dimers without Fab fragments could provide the same benefit as full-length IVIg antibodies and this effect was only due to terminal sialic acid on the Fc-glycan. Fc-fragments without terminal sialic acid showed no beneficial efficacy in autoimmune disease models (Kaneko, Nimmerjahn et al. 2006; Anthony, Nimmerjahn et al. 2008). It has been demonstrated that the anti-inflammatory effect of IVIG can be translated by splenic marginal zone macrophages via a C-type lectin receptor called SIGN-R1 (specific ICAM-3 grabbing non-integrin related 1) in mice (Anthony, Wermeling et al. 2008).This receptor is only expressed on splenic marginal zone macrophages and is crucial for the anti-inflammatory function of IVIg because upon binding of sialylated Fc-parts, marginal zone macrophages induce up-regulation of the inhibitory FcγRIIB on effector macrophages, so the ratio of inhibitory to activating Fcγ-receptors increases. The human homologue DC-SIGN is not expressed on macrophages but on dendritic cells. Therefore results concerning the activity of this receptor must be translated into the human system carefully (Bayry, Bansal et al. 2009). Recent findings have described the interference of IVIg in terms of antigen presentation and herby influencing the activation of T-cells indirectly (Nandakumar, Backlund et al. 2004; MacMillan, Lee et al. 2009). IVIg-Fc binding reduced the ability of antigen-presenting cells to present antigens via MHC class II to $CD4^+$ T cells, which did not become activated. This effect of IVIG was shown to be independent of the inhibitory FcγRIIB (Aubin, Lemieux et al. 2010). Binding of IVIg-Fc to antigen-presenting cells occurred via activating Fcγ-receptors.

1.8.5 Contra-indications of IVIg

Although high dose IVIg therapy is successfully used to treat several autoimmune diseases, the administration of IVIg to patients has some disadvantages. IVIg has to be administered in very high doses of 1 to 2 g/kg body weight per month to eclipse anti-inflammatory IgG effects. Due to the production procedure, treatment with IVIg is a very expensive form of therapy, which is not accessible for everyone. Besides cost intensity, side effects like headache and fatigue are reported frequently. Furthermore, as IVIg mediates its effect systemically, the transfer of allergies from donors to recipients, have been reported (Bayry, Lacroix-Desmazes et al. 2007; Nimmerjahn and Ravetch 2007; Anthony, Nimmerjahn et al. 2008; Anthony, Wermeling et al. 2008; Durandy, Kaveri et al. 2009). All things considered, exploiting the beneficial effects of IVIg is of great value to strive for more specific and efficient approaches treating autoimmune disease patients.

1.9 Therapeutic IgGs

Since the 1990's therapeutic antigen-specific monoclonal IgG antibodies have become the fastest growing class of human therapeutics and the second largest class of drugs after vaccines. Moboclonal IgG antibodies were approved for passive immunisation to clear pathogens as well as for the treatment of chronic inflammatory diseases by inhibiting inflammatory mediators such as TNF-α (Williams, Ghrayeb et al. 1995). Recently, IgG Fc-glycans were suggested to have strong modulating effects. Removing core fucose from the glycan structure, antibodies display increased cytotoxic effector functions (Satoh, Iida et al. 2006). Sialylation of recombinant proteins has been carried out by industry to increase the half-life of biologics, but recent findings propose that sialylation of IgG Fc-glycans reduces their ability to engage Fcγ-receptors. Furthermore, sialylated IgG antibodies contained in IVIg have been demonstrated to mediate unspecific immunosuppressive effects (Nimmerjahn and Ravetch 2007). However, the potential of sialylated monoclonal IgG antibodies to inhibit antigen-specific inflammatory immune responses has not been investigated yet.

1.10 Inflammatory mouse models

1.10.1 Nephrotoxic nephritis

Nephritis is an inflammation of the kidney due to the deposition of immune complexes and the recruitment of inflammatory immune cells in the kidney. The damage of the kidney which is directly attached to the inflammation leads to proteinuria. Proteinuria describes the pathogenic state of nephritis, where proteins occur in the urine since they cannot be held back by glomeruli and tubuli in the kidney. Pre-sensitisation of C57BL/6 (B6) mice with sheep IgG and CFA followed by the application of sheep anti-mouse glomerular basement membrane (GBM) containing serum (nephrotoxic serum, NTS) four days later induces acute glomerulonephritis with renal injury. Renal pathology is dependent on the presence of sheep IgG-specific for GBM and mouse anti-sheep IgG2b and IgG2c antibodies, which co-localise with the glomeruli and inflammatory immune cells such as macrophages expressing FcγRIV (*Figure 1.4*). The introduction of nephritis results in acute renal injury, which is associated with proteinuria and organ failure followed by mortality. Nephritis can be inhibited by the administration of IVIg. (Madaio and Harrington 2001; Kaneko, Nimmerjahn et al. 2006).

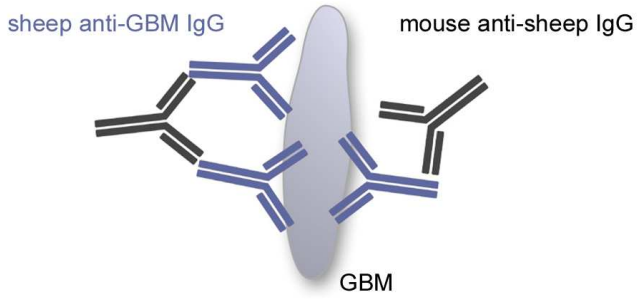


Figure 1.4: Nephrotoxic serum (NTS) induced immune complex deposition in the kidney. *In vivo* induced murine anti-sheep IgG antibodies bind to transferred sheep anti-GBM IgG antibodies contained in NTS and form immune complexes, which then bind to GBM protein in the kidney. Deposition of immune complexes in the kidney leads to the recruitment of inflammatory macrophages and finally results in the induction of nephritis.

1.10.2 Delayed typer hypersensitivity (DTH)

Delayed-type I hypersensitivity (DTH) reactions are well characterised inflammatory responses dependent on T cells. Classical DTH elicited by immunisation with antigen together with co-stimuli followed by local antigen challenge in the ear or the footpad is accompanied by a strong cellular infiltrate, including neutrophils, macrophages and T lymphocytes. The infiltrates are initiated by local release of pro-inflammatory cytokines and chemokines as well as by up-regulation of adhesion molecules on endothelial cells. Naïve CD4 T helper cells initially bind to antigenic peptides via MHC class II molecules on APCs followed by differentiation to pro-inflammatory Th cells. Subesequent to local antigenic challenge, antigen-specific Th cells induce a granulomatous inflammation at the site of the antigen injection, which further causes erythema (Cher and Mosmann 1987; Kobayashi, Kaneda et al. 2001; Doebis, Siegmund et al. 2008). Until now, the effect of IVIg in DTH mouse models has not been investigated.

1.10.3 Allergic airway inflammation model

Murine models of allergic airway inflammation have many similar traits to allergy and asthma in humans and can be used to investigate developmental stages of disease development. After systemic immunisation with an antigen and Th2 cell-stimulating adjuvant, such as aluminum hydroxide ($Al(OH)_3$) and subsequent pulmonary antigen challenge, various strains of mice develop airway hyper-responsiveness (AHR) which has been regarded as an outcome indicator of asthma (Wanner 1990; Zhu and Gilmour 2009). In this model of allergic airway inflammation, mice were sensitised twice by intraperitoneal injection of ovalbumin (OVA) together with Alum (aluminum potassium sulphate; $AlK(SO_4)_2$) followed by two airway allergen challenges, which result in the development of OVA-specific antibodies, lung injury and eosionphils in the lung.

1.11 Aim of the thesis

Immune complexes consisting of IgG antibodies exhibit strong immune regulatory functions, including enhancement and suppression of inflammatory immune responses (Heyman 2000; Nimmerjahn and Ravetch 2006). These distinct activities of IgG antibodies can be explained by binding to activating and inhibitory Fcγ-receptors on immune cells (Nimmerjahn and Ravetch 2005), but it has also been reported that the glycosylation pattern is crucial for mediating IgG effector functions (Parekh, Dwek et al. 1985; Albert, Collin et al. 2008; Huhn, Selman et al. 2009). In particular, IgG Fc-sialylation has been suggested to be involved in anti-inflammatory activities and thus, to be associated with the maintenance of tolerance.

The first aim of my work was to investigate the impact of T cell independent (TI) and T cell dependent (TD) immunisation under pro- or non-inflammatory conditions on IgG antibody responses and their enhancing or inhibiting influence on pathogenic immune responses in various mouse models described above. Furthermore, I aimed to evaluate whether TI and TD IgG antibodies generated under pro- and non-inflammatory conditions display different sialylation patterns and thus, contribute to the establishment of inflammation or tolerance. Analyses in this part of the work will provide new insight into the process of IgG antibody sialylation.

In the second part of my work I aimed to substitute the systemic immunosuppressive effect of high dose IVIg therapy (2g/kg of body weight) by a low dose application of antigen-specific sialylated monoclonal IgG antibodies (5 mg/ kg of body weight). Antigen-specific induction of tolerance might provide a novel therapeutic approach to treat autoimmune diseases and allergy in an antigen-specific manner to avoid unspecific immunosuppression. Furthermore, I addressed the question how sialylated IgG antibodies mediate anti-inflammatory effects.

2 Material and Methods

2.1 Mice

Mice used in this dissertation are listed in *table 2.1*. TCRβ$^{-/-}$, FcγRIIB$^{-/-}$, IL4Rα$^{-/-}$ mice were backcrossed for at least eight generations to the C57BL/6 background. Exclusively 8 to 12 weeks old mice were analysed. Unless stated otherwise mice were bred in the animal breeding facility of the Deutsches Rheumaforschungszentrum and the Max-Planck-Institute for infection biology in Berlin-Marienfelde under SPF conditions. The feeding took place *ad libitum*.

Table 2.1: Mice.

Abbreviation	General remarks & references
C57BL/6	purchased from Charles River Laboratories
BALB/c	Purchased from Charles River Laboratories
TCRβ$^{-/-}$	purchased from Jackson Laboratories
FcγRIIB$^{-/-}$	(Takai et al.1996, Bolland and Ravetch 2000)
IL4Rα$^{-/-}$	(Barner et al. 1998)

2.2 Material

2.2.1 Cells

In *table 2.2* you find an overview of all the used cell lines.

2.2.2 Primer

All primers used for cloning of monoclonal anti-TNP IgG1 antibodies are listed in *table 2.3*.

Table 2.2: Cells.

Name	Supplier
H5 hybridoma cell line	House facility
OX-7 hybridoma cell line	House facility
4C9 SK19 hybridoma cell line	House facility
Human embryonic kidney cell line stably expressing the large T-antigen of SV40 (HEK 293T)	House facility

Table 2.3: Primer sequences for cloning of monoclonal anti-TNP IgG1 antibodies.

Name	Sequence 5` → 3`
Murine IgE HC variable region fw	tct acc ggt gta cat tcc gag gtg cag ctt cag gag tca
Murine IgE HC variable region rev	gca ggg cta gct gca gag aca gtg acc aga gtc cc
Murine IgG1 HC constant region fw	cct cgc gct agc acg aca ccc cca tct gtc tat cca c
Murine IgG1 HC constant region rev	tta ttc ggc gta cgc gtc att tac cag gag agt ggg ag
Murine kappa LC variable and constant region fw	gtc acc ggt gta cat tca gac att gtg atg tca cag tct
Murine kappa LC variable and constant region rev	tta ttc gga agc ttt caa cac tca ttc ctg ttg aag

2.2.3 Plasmids

The plasmids used for cloning of monoclonal anti-TNP IgG1 antibodies are the expression vectors mgO53 heavy and light chain (Wardemann, Yurasov et al. 2003).

2.2.4 Media

Media used for cell culture are listed in *table 2.4* and were purchased from Gibco/ Invitrogen.

Table 2.4: Media

Name	Composition
RPMI GlutaMAX-I (complete)	RPMI 1640 Glutamax; 10% FCS; 20 µM 2-mercaptoethanol (both Sigma-Aldrich); 100 U/l Penicillin; 100 U/l Streptomycin (both Seromed)
DMEM GlutaMAX-I (complete)	DMEM Glutamax; 10% FCS; 20 µM 2-mercaptoethanol (both Sigma-Aldrich); 100 U/l Penicillin; 100 U/l Streptomycin (both Seromed)
IMDM (complete)	IMDM; 2mM L-Guanine; 10% FCS; 20 µM 2-mercaptoethanol (both Sigma-Aldrich); 25 mM HEPES; non essential amino acids; 100 U/l Penicillin; 100 U/l Streptomycin (both Seromed)

2.2.5 Antibodies

In *table 2.5* and *2.6* you find all antibodies used for detection in ELISA and flow cytometry analysis or used for injections.

2.2.6 Enzymes and substrates

Substrates and enzymes used for glycosylation modifications of IgG antibodies are listed in *table 2.7*.

2.2.7 Antigens, adjuvants and other biological derivatives

Antigens and adjuvants used for immunisation are combined in *table 2.8*.

Table 2.5: Conjugated antibodies.

Specificity	Clone	Conjugate	Supplier
mouse CD4	YTS191	FITC	House facility
mouse CD4	RM4-5	PerCp	BD Biosciences
mouse CD40	1C10	none	House facility
mouse CD11c	N418	Cy5, biotin	House facility
mouse CD138	218-2	Pe	BD Biosciences
mouse CD86	GL1	FITC	eBiosciences
mouse CD154	MR1	APC	eBiosciences
mouse Gr-1	RB68C5	FITC	House facility
mouse IL-17	TC11-18H10	Pe	BD Biosciences
mouse IL-4	11B11	Pe	BD Biosciences
mouse IFN-γ	AN18.17.24	Pe	BD Biosciences
mouse MacI	M1/70.15.11	Cy5	House facility
mouse MHCII	M5/114	Pe	House facility
mouse SiglecF	E50-2440	Pe	BD Biosciences
Mouse α-2,6-sialyl-transferase	M2	none	IBL International
mouse IgG1	polyclonal goat	HRP, FITC	Bethyl
mouse IgG2a	polyclonal goat	HRP	Bethyl
mouse IgG2b	polyclonal goat	HRP, FITC	Bethyl
mouse IgG2c	polyclonal goat	HRP, FITC	Bethyl
mouse IgM	polyclonal goat	HRP	Bethyl
rabbit IgG	polyclonal goat	Cy5	Invitrogen

2.2.8 Conjugated biological derivatives

For flow cytometry analysis, streptavidin-coupled APC (BD Biosciences) was used to detect anti-mouse CD11c-biotin. For antigen-specific plasma cell stainings, streptavidin-coupled PerCp was used to detect biotinylated OVA and TNP-BSA.

Table 2.6: Unconjugated antibodies.

Specificity	Clone	Supplier
mouse CD154	MR-1	House Facility
mouse CD4	GK 1.5	House facility
mouse IgG1	polyclonal goat	Bethyl
TNP	H5	House facility
OVA	4C9 SK19	House facility
Thy1.1	OX-7	House facility

Table 2.7: Enzymes and substrates.

Name	Supplier
β-1,4-galactosyltransferase	Calbiochem
UDP-β-D-Galactose, disodium salt	Calbiochem
Human α-2,6-(N)-sialyltransferase	Calbiochem
CMP-Sialic acid, disodium salt	Calbiochem
Sialidase A	Prozyme
Taq	Genexpress
T4 DNA-Ligase	NEB
BsiW I	NEB
Hind III	NEB
AgeI	NEB
Nhe I	NEB

2.2.9 Buffers and solutions

Buffers and solution used in this work are listed in *table 2.9*.

Table 2.8: Antigens, adjuvants and other biological derivatives.

Name	Supplier
Chicken egg albumin (OVA)	Calbiochem
Sheep IgG	Bethyl
TNP(0.4)-LPS	Biosearch Technology
TNP(9)-BSA	Biosearch Technology
TNP(12)-Ficoll	Biosearch Technology
TNP(3)-OVA	House facility
TNP(13)-sheep IgG	House facility
Montanide Iso 50V	Seppic
Freund's adjuvant, complete (CFA)	Sigma-Aldrich
Freund's adjuvant, incomplete (IFA)	Sigma-Aldrich
Mycobacterium tuberkulosis H37 RA	DIFCO laboratories
Beriglobin (IVIg)	Behringer Ingelheim

Table 2.9: Buffers and solutions.

Name	Composition
Block/ wash buffer	PBS; 3% BSA (Sigma-Aldrich); 1mM EDTA (Sigma-Aldrich); 0.1% gelatine Sigma-Aldrich)
Coating buffer	0.05 M Carbonate-bicarbonate (Fluka); pH 9.6
Coomassie destaining solution	5% methanol (Sigma-Aldrich)/ 5% acetic acid (Sigma-Aldrich)
Coomassie staining solution	1% Coomassie brilliant blue R250 (Frova, Guarino et al.); 50% methanol (Sigma-Aldrich)/ 10% acetic acid (Sigma-Aldrich)
Coupling buffer	0.1 M $NaHCO_3$ (Sigma-Aldrich); 0.5 M NaCl (Sigma-Aldrich)
Elution buffer	0.1 M glycine

PBS	137 mM NaCl (Sigma-Aldrich); 2.7 mM KCl (Sigma-Aldrich); 8 mM Na_2HPO_4 (Sigma-Aldrich); 1.5 mM NaH_2PO_4 (Sigma-Aldrich); pH 7.2
PBS/ BSA	PBS; 0.5% BSA (Sigma-Aldrich)
Protein sample buffer (4x)	240 mM Tris/HCl (Sigma-Aldrich) pH 6.8; 8% SDS (Merck); 5% 2-mercaptoethanol (Sigma-Aldrich); 0.04% bromphenol blue; 40% Glycerol
Lysis buffer	0.15 M NH_4Cl (Sigma-Aldrich); 1 mM $KHCO_3$ (Roth); 0.1 mM EDTA (Sigma-Aldrich); pH 7.2
MES buffer	50 mM 2-(N-morpholino)ethanesulfonic acid (Sigma); pH 6.0
MOPS buffer	50 mM 3-morpholinopropanesulfonic acid (Roth); pH 7.2
Neutral washing buffer	0.1 M Tris/HCl (Sigma-Aldrich); 0.5 M NaCl (Sigma-Aldrich); pH 8.0
Phosphate buffer	20 mM Na_2HPO_4(Sigma-Aldrich); 20 mM NaH_2PO_4 (Sigma-Aldrich); pH 7.0
SDS-PAGE buffer (10x)	250 mM Tris (Sigma-Aldrich); 1.92 M glycine; 1% SDS (Merck); pH 8.3
SDS resolving gel buffer	1.5 M Tris/HCl (Sigma-Aldrich) pH 8.8; 0.4% SDS (Merck)
SDS stacking gel buffer	1 M Tris/HCl (Sigma-Aldrich) pH 6.8; 0.8% SDS (Merck)

2.2.10 Kits

The kits listed in *table 2.10* were applied according to the manufacturer's instructions.

Table 2.10: Kits.

Name	Supplier
Bio-plex pro cytokines assay (mouse IL-6)	BioRad
Sialidase kit (GK80040)	Prozyme
NucleoBond Xtra Maxi kit	Macherey-Nagel
QIAprep Spin Miniprep kit	Quiagen
Nucleospin Extract II kit	Macherey-Nagel

2.2.11 Software

The software programs used in this work are registered in *table 2.11*.

Table 2.11: Software.

Name	Supplier
FlowJo	Tree Star
GraphPad Prism 4.0	GraphPad Software
ND1000	Thermo Fisher Scientific
Softmax PRO v3.0	Molecular Devices
EndNote X3	

2.3 Molecular and genetic methods

2.3.1 Transformation of *E.coli*

50 µl of competent *E.coli* Top10 cells (in house preparation) thawed on ice were mixed with 1µl of plasmid DNA or 5 µl ligation products. The mixture was incubated for 20 minutes on ice followed by a heat shock through 2 minute incubation at 42°C. Subsequently, 500 µl of LB-medium without any antiobiotics were added and the culture was gently rotated at 37°C for 45 minutes. Afterwards the bacteria were spun down and incubated either in 3ml fluid LB medium containing 50 µg/ml ampicillin (Sigma-Aldrich)

or on LB agar plates containing 50 µg/ml ampicillin over night at 37°C. Selected bacterial clones were picked and transferred into 3 ml LB medium containing 50 µg/ml ampicillin and incubated over night at 37°C.

2.3.2 Plasmid isolation

Overnight cultures of bacteria in LB medium containing ampillicin were inoculated with a single colony. Preperation of Plasmid DNA was achieved by the principle of alkaline lysis. Small (3 ml, mini) scale preperations were purified with the QIAprep Spin Miniprep-Kit (Qiagen) and large scale (200- 500 ml, maxi) preperations were performed with the NucleoBond Xtra Maxi kit (Macherey-Nagel) according to the manufacturer`s instructions. DNA was finally resolved in TE buffer.

2.3.3 Quantification of DNA

By photometric measurement of the optical densitiy at 260 and 280 nm wavelength the concentration and the purity of an aqueous nucleic acid solution can be determined. With help of a Nanodrop (Peqlab) the extinction at 260 nm (OD_{260nm}) and 280 nm (OD_{280nm}) wave length was measured. The TE buffer used for elution was used as reference. The quotient OD_{260nm}/OD_{280nm} is an indication for the purity of the nucleic acid solution. At high purity a value between 1.8 and 2.0 is reached. Lower values suggest a contamination with proteins.

2.3.4 Agarose gel electrophoresis

Seperation of DNA fragments according to their size was achieved by agarose gel electrophoresis. DNA is negatively charged and due to this runs in a stress field towards the positive pole. 1-2% gels were prepared in 1x TAE buffer, containing 1 µg/ml Ethidiumbromide (EtBr) (Carl-Roth, Karlsruhe, Germany), which intercalates into the DNA and by this stains the DNA fragments. DNA samples were mixed with 6x DNA loading buffer (Orange G) and loaded on the gel, which was run with 5-10V/cm in 1x TAE buffer. To determine the length of the separated DNA fragments, a molecular weight marker (100 bp DNA - Ladder Plus marker; Fermentas) was run simultaneously on the gel. Separated DNA was documented by UV radiation at 312 nm wavelength and a video

supported system. If necessary, DNA fragments were seized from the agarose gel and purified with the Nucleospin Extract II kit (Machery Nagel) according to the manufacturer`s recommendations.

2.3.5 Cloning of monoclonal anti-TNP IgG1

The variable VDJ heavy chain region (NCBI X65772) and complete kappa light chain gene (NCBI X65774) of the murine anti-TNP IgE hybridoma IgELa2 (ATCC-TIB142) (Kofler, Schnegg et al. 1992) were amplified by PCR from cDNA (anti-TNP variable VDJ heavy chain part, forward primer and reverse primer (table 2.3); anti-TNP complete VJ kappa light chain, forward primer and reverse primer (table 2.3)). To synthesise a murine anti-TNP IgG1 antibody the variable heavy chain region (*AgeI-NheI*) in combination with an amplified murine C57BL/6 IgG1 heavy constant region (*NheI-BsiWI*) (forward and reverse primer (table 2.3)) and the complete kappa light chain gene (*AgeI-HindIII*) were cloned into recently described expression vectors (Wardemann, Yurasov et al. 2003). The leader sequences of the described expression vectors were used (cloning was performed by Alexandra Lorenz, AG Ehlers, DRFZ).

2.4 Expression and purification of IgG antibodies

2.4.1 Cell culture

2.4.1.1 Cultivation of human embryonic kidney cells (HEK 293T)

For the expression of anti-TNP IgG1 and anti-OVA IgG1 antibodies we used human embryonic kidney 293T cells (HEK293T), which stably express the large T antigen from SV40 virus. Cells were cultured in DMDM high glucose (Gibco/Invitrogen) supplemented with 10% FCS (Sigma-Aldrich) and 1% PS (Seromed) at 37°C and 5% CO_2 in 145 mm tissue plates. Optimal growth was achieved by passaging the cells twice a week at a confluency of 80%.

2.4.1.2 Cultivation of hybridoma cell lines

For the production of murine anti-TNP IgG1, anti-OVA IgG1 and anti-Thy1.1 IgG1 we used the hybridoma cell lines H5 (Wernersson, Karlsson et al. 1999), 4C9 (unpublished) and MRC OX-7. All hybridoma cell lines were cultured in RPMI high clucose (Gibco/Invitrogen) supplemented with 2% FCS* (IgG-free FCS, house preparation), 1% PS (Seromed), 1% Primatone RL/UF (MP Biomedicals) and 50 μM 2-mercaptoethanol at 37°C and 5% CO_2 in 145 mm tissue plates. Optimal growth was achieved by passaging the cells once or twice a week.

2.4.2 Production of IgG antibodies

2.4.2.1 Expression of anti-TNP IgG1 antibodies

Recombinant monoclonal anti-TNP IgG1 antibodies were produced by polyethylenimine (PEI)-mediated co-transfection of HEK 293T cells with the respective IgH and IgL chain encoding plasmid DNA (2.2.3). For transfection, cells were grown to 80% confluency, washed with PBS and cultured in 25ml DMEM high glucose containing 1% Primatone RL (MP Biomedicals) and 1% PS (Seromed). For anti-TNP IgG1, 10 μg of IgH and 10 μg IgL chain encoding plasmid DNA were diluted in 3 ml PBS and mixed thoroughly with 100 μl (0.6 mg/ml H_2O) polyethylenimine (PEI) (Sigma-Aldrich). After an incubation of 10 minutes at room temperature, the DNA-PEI mix was added to the culture plates. Supernatants of transfected cells were collected after five to seven days of culture. Then, anti-TNP IgG1 antibodies were purified with Protein-G-Sepharose and dialysed against PBS.

2.4.2.2 Expression of Dec-205-OVA

Dec-205-OVA (Dec-OVA) antibodies were produced by polyethylenimine-mediated co-transfection of HEK293T cells with 20 μg of each IgH and IgL chain encoding plasmid DNA (Boscardin, Hafalla et al. 2006) as described in 2.5.2.1. Supernatants were collected after five to seven days of culture and Dec-OVA IgG1 antibodies were purified with Protein-G-Sepharose and dialysed against PBS.

2.4.2.3 Production of hybridoma-derived IgG1 antibodies

For antibody production, murine anti-TNP IgG1 (clone H5) (Wernersson, Karlsson et al. 1999), anti-OVA IgG1 (clones 4C9 SK15) and anti-Thy1.1 IgG1 (clone MRC OX-7) hybridomas were grown in RPMI high glucose (Gibco/Invitrogen) supplemented with 1% Primatone RL/UF (Sheffield BioScience) and 1% PS (Seromed). 14 days later, IgG1 antibodies were purified from cell culture supernatants with Protein-G-Sepharose and dialysed against PBS.

2.4.3 Purification of IgG antibodies

2.4.3.1 Protein-G Sepharose column for purification of IgG

To analyse immunoglobulins from serum samples and from cell culture, the IgG antibodies were purified via affinity chromatography using Protein G sepharose. Protein G is a protein that is located in the bacterial cell wall and shows a high binding affinity to the Fc part of IgG molecules. This protein is coupled to sepharose and therefore enables purification via affinity chromatography. The binding capacity of Protein G accounts 20 mg IgG pro ml gel.

In brief, supernatants from hybridoma cultures or serum samples, diluted with PBS, were applicated to the Protein G sepharose column. After extensively washing off unspecific proteins or serum IgM with PBS, IgG molecules were eluted with 0.1 M glycine pH 2.7 and immediately neutralised in 1/10 volume of 1 M Tris-HCl pH 9.0. Then, collected IgG fractions were dialysed against PBS over night at 4°C. The determination of the IgG concentration was done photometrically at 280 nm using Nanodrop spectrophotometer. If necessary, the IgG molecules were concentrated by a centrifugation device (BioRad) with a MWCO of 50 kDa. The IgG molecules were stored at -20°C.

2.4.3.2 Preparation of an antigen-specific Purification Column

For purification of OVA-specific and TNP-specific IgG antibodies OVA and TNP-BSA, respectively, was coupled to CnBr-activated Sepharose 4B (GE-Healthcare). In brief, the CnBr-activated Sepharose was swelled in 1 mM HCl for 15 minutes. After washing with 200ml 1 mM HCl, the CnBr-activated Sepharose was incubated with 1-2 mg OVA

(Calbiochem) or TNP-BSA (Biosearch Technologies) per ml Coupling Solution over night at 4°C. To block remaining unreacted groups, the CNBr-activated Sepharose, was washed with 0.1 M Tris-HCl pH8 and rotated at 4°C for 2 hours. After blocking was completed, the CnBr-activated Sepharose was washed three times with alternating acidic and neutral washing buffer. The Sepharose Column was stored at 4°C in 20% Ethanol in PBS.

2.4.3.3 Purification of antigen-specific IgG antibodies

Analysing anti-TNP and anti-OVA IgG molecules from serum samples, IgG molecules were initially purified with Protein G sepharose as described above. Subsequent, TNP or OVA-reactive serum IgG antibodies were purified using TNP-BSA or OVA coupled CNBr-activated sepharose 4B columns. In detail, serum samples, diluted with PBS, were applied to the column and after washing off unspecific proteins with PBS, specific IgG molecules were eluted with 0.1 M glycine pH 2.7. Collected fractions were neutralised in 1/10 volume of 1 M Tris-HCl pH 9.0 and dialysed against PBS over night at 4°C. Determination of the antibody concentration was done photometrically at 280 nm using Nanodrop spectrophotometer. If necessary, the IgG molecules were concentrated by a centrifugation device with a MWCO of 50 kDa. The IgG molecules were stored at -20°C.

2.4.4 Glycosylation modification of IgG antibodies

2.4.4.1 Enzymatic glycosylation

Monoclonal murine anti-TNP H5 IgG1 and anti-Thy1.1 OX-7 IgG1 hybridoma antibodies have Fc-glycans lacking terminal sialic acid. About two-thirds of the bisecting Fc-glycans are also lacking galactose. In order to check the impact of terminal sialic acid on the antibodies, we performed an enzymatic glycosylation of the IgG molecules *in vitro*. The glycosylation process consists of two steps, galactosylation and sialylation, and has been described previously (Anthony et al., 2008).

Galactosylation

Antibodies were first dialysed against 50 mM MOPS (Sigma-Aldrich) pH 7.2 overnight at 4°C and then concentrated to 5 mg/ml. To 5 mg/ml antibodies 10 μM UDP-Galactose (Calbiochem), 75 mU β-1,4-galactosyltransferase (Calbiochem) and 20 mM $MnCl_2$ were

added and incubated for 48 hours at 37°C while constantly shaking at 350 rpm. A buffer exchange to 50 mM MES (Sigma-Aldrich) pH 6.0 overnight at 4°C was followed by the sialylation step.

Sialylation

25 mU human α-2,6-(N)-sialyltransferase (Calbiochem) and 0.5 mM CMP-sialic acid (CMP-Neu5Ac, Calbiochem) was added to the galctosylated antibodies and incubated for 48 h at 37°C while constantly shaking at 350 rpm. Finally a two-step buffer exchange to 100 mM NaCl (Sigma-Aldrich) and afterwards to PBS was done using a centrifugation device with a MWCO of 50 kDa. Concentrations of differentially glycosylated IgG1 antibodies were determined by Nanodrop spectrophotometer. Antibody integrity was analysed by SDS-PAGE, anti-TNP reactivity was tested by ELISA and N-glycosylation of IgG Fc fragments was characterized by MALDI-TOF mass spectrometry.

2.4.4.2 Enzymatic de-sialylation

To remove sialic acid from the antibodies, IgG molecules were treated with the enzyme sialidase A (Prozyme), which has the ability to cleave all non-reducing terminal sialic acids.

De-sialylation of either polyclonal or monoclonal anti-TNP and anti-OVA IgG antibodies was performed using the Sialidase kit (Prozyme) on the basis of the manufacturer´s instruction. In detail, appropriate volume of five-fold reaction buffer (250 mM sodium phospahate, pH 6.0) was added to the antibodies to achieve a 50 mM phosphate buffered solution, pH 6.0. Sialidase A was added at 60 mU per milligram antibody. Following incubation at 37°C over night, the antibodies were separated from sialidase A by dialysing against PBS using a centrifugation device with a MWCO of 100 kDa. N-glycosylation of IgG Fc-fragments was characterised by MALDI-TOF mass spectrometry.

2.5 Biochemical methods

2.5.1 MALDI-TOF Mass spectrometry

Glycan analysis of IgG antibodies was performed using MALDI-TOF mass spectrometry. Matrix-assisted laser desorption/ ionization (MALDI) is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (such as proteins, peptides and

sugars) and large organic molecules, which tend to be fragile and fragment when ionized by more conventional ionization methods. The most commonly used type of mass spectrometer is the time-of-flight (TOF) spectometer.

The MALDI-TOF analyses for this work were done by Andre Winkler in co-operation with the group of Dr. Markus Berger (Charite, Universitaetsmedizin Berlin, Glykodesign und Glykoanalytik). In brief, IgG samples were digested with endoglycosidase S (EndoS) purified from *Streptococcus pyogenes* (Collin and Olsen 2001). EndoS specifically cleaves the N-linked glycan at the Fc fragment exclusively from IgGs between the first and second GlcNAc (Collin and Olsen 2001). Resulting N-glycans were purified by solid phase extraction using reversed-phase C18 and graphitized carbon columns (Alltech, Deerfield, IL). Samples were permethylated according to standard protocols and further investigated by MALDI-TOF mass spectrometry. Spectra were recorded on an Ultraflex III mass spectrometer (Bruker Daltonics) equipped with a Smartbeam laser. Calibration was performed on a glucose ladder and DHB was used as matrix. Spectra were recorded in reflector positive ionization mode and mass spectra from 3000 laser shots were accumulated.

2.5.2 Enzyme-linked immunosorbent Assay (ELISA)

ELISA is a biochemical technique used to detect the presence of an antibody or antigen in a liquid sample and is based on an enzymatic colour change. Here, ELISA was applied to detect antigen-specific antibodies in serum samples or antibodies that were expressed in cell culture. To detect antibodies directed against a particular antigen, the plastic surface of a microtiter plate was coated with this particular antigen. After that a non-reacting protein, such as bovine serum albumin (BSA), is added to block the uncoated parts of the microtiter plate surface. Then the samples containing antibodies can be added. During an incubtion period specific antibodies can bind to the coated antigen. Afterwards, a secondary antibody, which in turn can bind to the specific primary antibody, is applied. This secondary antibody is coupled to an enzyme, here the horse radish peroxidase (HRP) that is able to convert the chromogen tetramethylbenzidin (TMB) from a colourless substrate to a blue end product. The intensity of the colourimetric turnover is proportional to the quantity of the bound secondary antibody. This reaction is stopped by the application of 1 N H_2SO_4 and the absorption of the sample is measured at 450 nm.

In this work, monoclonal and serum antibodies specific for OVA, TNP or sheep IgG were detected in ELISA. To determine OVA-specific antibodies, 96-well plates (flat-bottom, Costar) were coated with 100 µl of 50 µg/ml OVA (Calbiochem) in coating buffer for 2 hours at room temperatur or overnight at 4°C. For the detection of TNP- and sheep IgG-specific antibodies, ELISA microtiter plates were incubated with 10 µg/ml TNP-BSA (Biosearch Technologies) or 10 µg/ml sheep IgG (Bethyl) as described above. Subsequent to the coating procedure, the wells were washed triply with PBS and then incubated with 100 µl Block/wash buffer for 1 hour at room temperature. Serum samples were diluted 1:100 in Block/wash buffer and incubated for 1 hour at room temperature in the 96-well microtiter plate. Monoclonal antibodies were incubated in a 1:3 dilution series, starting with a concentration of 5 µg/ml. After washing off the samples, the detection of the specific antibodies was performed using 100 µl HRP-coupled polyclonal goat anti-mouse IgG1, IgG2a, IgG2b, IgG2b, IgG3 and IgM in a 1:5000 dilution. After washing off the HRP-coupled antibodies intensively, the colourimetric turnover was induced by application of 100 µl TMB (BD Bioscience) and the reaction was stopped with 100 µl 1N H_2SO_4 (Roth). Absorption was measured at 450 nm using a photometric ELISA plate reader (Tekan SpektraFluor).

2.5.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The integrity of the expressed antibodies was verified by denaturating polyacrylamid gel electrophoresis. In SDS-PAGE, proteins can be separated according to their molecular weight and size. In brief, proteins were first heated in a reducing SDS (Sigma-Aldrich) and 2-mercaptoethanol (Sigma-Aldrich) containing buffer for 5 minutes at 95°C. The high temperature and the 2-mercaptoethanol denature the proteins by reducing disulfide linkages and breaking up hydrogen bonds, thus cracking quaternary protein structures. In addition, SDS is an anionic detergent which denatures secondary and non-disulfide linked tertiary structures, and applies a negative charge to each protein in proportion to its mass.

To generate the resolving gel, a solution with 15% acrylamide (Sigma-Aldrich) (stock 30% acryl-bisacrylamide) concentration was induced to polymerase with 0.1% TEMED (Invitrogen) and 0.1% ammonium persulphate (APS, Carl-Roth) and immediately poured into the gel chamber (BioRad). The stacking gel, which leads to the concentration of the protein samples at the border between the stacking gel and the resolving gel, contained a

5% acrylamide solution. After loading of the heated protein samples, gel electrophoresis was performed in 1x SDS PAGE buffer with 25 mA/ gel. To estimate the size of the proteins, a marker (Kaleidoscope, BioRad) with proteins of defined size was loaded next to the samples. Proteins were then detected by staining of the gel with Coomassie staining solution.

2.6 Mouse experimental methods

2.6.1 Blood sampling

Many analyses require serum from mice. To obtain serum the mice were warmed-up under a red light and taped at the tail vein. The serum was collected in a serum collecting tube (Greiner Bio-One) which contains a special gel cushion that allows blood cells to go through and therefore seperates blood cells from the serum. The serum was then transfered into a fresh 1.5 ml reaction tube (Sarstedt) and freezed at -20°C.

2.6.2 Preparation of organs and cells

For the removal of organs, mice were sacrificed by cervical dislocation. Organs and cells were processed as described in the preparation protocols below.

2.6.2.1 Preparation of lymphocytes from spleen and lymphnode

For the generation of lymphocytes, draining lymphnodes or spleens were homogenised in PBS/BSA. Afterwards, cells were treated with 600 µl Lysis buffer in order to remove all erythrocytes from the suspension. Subsequent to 5 minutes of incubation, lysis was stopped by adding 600 µl PBS/BSA, cells were filtrated by using a 30 µm pre-separation filter (Miltenyi Biotec) and centrifugated. Then, cells were washed with 1 ml PBS/BSA and finally resuspended in the appropriate volume of PBS/BSA or medium.

2.6.2.2 Preparation of BM-DCs from bone marrow

For the generation of BM-derived DCs, cells were first washed out from the femur and tibia of B6 or FcγRIIB$^{-/-}$ mice. Then, BM cells were treated with 600 µl Lysis buffer in

order to remove all erythrocytes from the suspension. Subsequent to 5 minutes of incubation, lysis was stopped by adding 600 µl PBS/BSA, cells were filtrated by using a 30 µm preseparation filter (Miltenyi Biotec). After washing the cells with 1 ml PBS/BSA, cells were finally resuspended in 1 ml IMDM (Invitrogen) supplemented with nonessential amino acids (Gibco/Invitrogen), 10% FCS* (Gibco/Invitrogen), 1% PS (Gibco/Invitrogen), and 50 µM 2-mercaptoethanol. Subsequent, cell numbers were determined using the Neubauer counting-chamber.

2.6.2.3 Preparation of lymphocytes and eosinophiles from lung

For the generation of lung lymphocytes and eosinophils, lungs were collected and transferred to C-tubes (Miltenyi Biotec) containing 3 ml 1640 RPMI (Gibco/Invitrogen) with 0.5% BSA (Sigma-Aldrich), 1.5 mg collagenase D (Roche), and 60 µg DNAse I (Roche). Lungs were sheared using the GentleMACS Dissociator (Miltenyi Biotec). Following, erythrocytes were removed by treating the suspension with Lysis buffer.

2.6.3 Depletion of CD4 T cells

To deplete CD4 T cells, mice were treated with 250 µg anti-mouse CD4 antibody (clone GK1.5). The anti-CD4 antibody was injected i.p. into the mice on day -1 and 4. Efficiency of CD4 T cell depletion was verified by flow cytometry analysis using FITC-coupled anti-CD4 antibody (clone YTS191).

2.6.4 Inhibition of CD40/CD40L interaction

To block CD40/CD40L interaction between B and T cells, mice were treated with 250 µg anti-mouse CD154 antibody (clone MR-1). The anti-CD154 antibody was injected i.p. on day -1, 3 and 7 (Foy et al. 1994).

2.6.5 Immunisation protocols

2.6.5.1 Immunisation with CFA and CFA^{high}

Co-administration of antigen with CFA is commonly used to induce antigen-specific inflammatory immune reactions. CFA (Sigma-Aldrich) contains 1 mg/ml heat-killed *mycobacteria tuberkulosis*. To induce even stronger inflammatory responses, enriched CFA (CFA^{high}) (Marty, Peclat et al. 2001) was prepared adding 5 mg/ml *Mycobacteria tuberkulosis* H37 RA (DIFCO laboratories) to IFA (Sigma-Aldrich).

In detail, CFA or CFA^{high} was added to the antigen solution in equal parts. Antigen solution was prepared diluting certain concentrations of TNP(4)-LPS, TNP(13)-BSA (both Biosearch Technologies), TNP(3)-OVA, TNP(13)-sheep IgG (both house preparations), OVA (Calbiochem) or sheep IgG (Bethyl) in PBS. Emulsion was vortexed thoroughly and homogenised using a sonicator. 100 µl of stiff, homogenised emulsion was injected s.c. into the mice.

2.6.5.2 Immunisation with alum

Alum is commonly used to enhance immune respones to antigens. In detail, OVA (Calbiochem), TNP(3)-OVA and TNP(13)-sheep IgG (both house preparations) in Alum was prepared by combining 1 ml of antigen in PBS (5 mg/ml) with 1.25 ml of $KAl(SO_4)_2$ (Sigma-Aldrich). The pH was neutralised with 10 M NaOH (Roth) and the precipitate was washed and resuspended in 1 ml PBS (5 mg/ml). Requested antigen concentrations were injected i.p. into the mice.

2.6.5.3 Immunisation with anti-CD40

Anti-CD40 is a co-stimulatory molecule used for induction of immune responses in mice. Here, 5 µg Dec-205-OVA or 100 µg TNP(3)-OVA (Biosearch Technologies) and 50 µg anti-CD40 (clone 1C10) were diluted in 100 µl PBS and injected i.p. into the mice.

2.6.6 Delayed type hypersensitivity mouse model

Delayed type hypersensitivity (DTH) reactions are elicited by local antigen challenge of already immunised mice, which is accompanied by a strong cellular infiltrate including neutrophils, macrophages and T lymphocytes at the site of the antigen injection.

To induce a DTH response, B6 and FcγRIIB$^{-/-}$ mice were first immunised with the antigen on day 0, followed by local application of the respective antigen on day 12 to 27, depending on the experimental setup. For immunisation, antigens were used in combination with different adjuvants or co-stimuli (*table 2.12*) and injected i.p. in a volume of 100 µl. Local challenge was performed by applicating 5 µl of antigen together with montanide ISA 50V (Seppic) into the right footpad of pre-immunised mice. PBS co-administered with montanide ISA 50V (Seppic) was injected into the left footpad as a negative control for unspecific footpad swelling. Inflammatory reaction intensity correlates with footpad thickness determined using an Oditest micrometer gauge in blind tests (Kroeplin Laengenmesstechnik, Germany).

immunisation		challenge
100 µg TNP(3)-OVA	/ CFA	37,5 µg OVA
100 µg TNP(3)-OVA	/ Alum	37,5 µg OVA
100 µg TNP(3)-OVA	/ 50 µg anti-CD40	37,5 µg OVA
5 µg Dec-205-OVA	/ 50 µg anti-CD40	37,5 µg OVA
100 µg TNP(12)-sheep IgG	/ CFA	12,5 µg sheep IgG
100 µg sheep IgG	/ CFA	12,5 µg sheep IgG

Table 2.12: Immunisation and challenge in different DTH reactions.

2.6.7 Inducible nephritis mouse model

Nephritis is an inflammation of the kidney due to the deposition of immune complexes in the kidney. Inflammation of the kidney is directly attached to an irreversible damage of the kidney followed by nephritis-associated mortality. In this work, the antibody-mediated nephrotoxic nephritis model was used, which has been described previously (Madaio, Salant et al. 1984).

In detail, nephritis was induced in FcγRIIB$^{-/-}$ mice injecting i.p. either 100 µg TNP-sheepIgG or 100 µg sheep IgG (Seppic) in CFA on day 0 followed by i.v. injecting 100 µl anti-mouse glomerular basment membrane (GBM) nephrotoxic serum (NTS) on day 4/5.

2.6.8 Allergic airway inflammation mouse model

BALB/c and FcγRIIB$^{-/-}$ mice were injected i.p. with 50 µg OVA in alum on day 0 and 14. On day 28 and 29, mice were anesthetised with ketamine/xylazine (94 mg/kg and 6.25 mg/kg, respectively) (Pharmacia GmbH) and 50 µg OVA (Calbiochem) in PBS was applied intranasal (i.n.) to the mice. Mice were sacrificed on day 35 and organs were collected for further analysis.

2.6.9 Lung histology

For histology, lungs were first flushed with PBS and then filled with 50% OCT medium (Tissue-Tek) compound in PBS. Prepared lungs were transferred into histocassettes (Tissue-Tek), embedded in OCT medium and quick frozen on dry ice. Fine sections (5 µm) were produced using the microtom (Kryostat Microm-HM 500 OM, Microm). Lung sections were gently transferred on glass frosted slide (Menzel-Glaeser) and fixed with cold acetone (Roth) for 10 min at -20°C. After fixation, fine lung sections were either stored at -80°C or immediately stained with hematoxylin and eosin, in co-operation with Prof. Dr. Loddenkemper (Institute for Pathology, Benjamin-Franklin-Campus, Berlin).

2.7 Immunological Methods

2.7.1 Lymphocyte stimulation

2.7.1.1 Unspecific stimulation with PMA/ ionomycin

Determination of intracellular proteins, like e.g. cytokines, requires the stimulation of the cells, as cytokines are usually only present in low detecting amounts. Stimulation promotes the secretion and enrichment of the cytokines in the cell. In detail, prepared lymphocytes were resuspended in 200 µl RPMI supplemented with 10 ng/ml PMA (Sigma-Aldrich) and

1 µg/ml ionomycin (Sigma-Aldrich) and incubated for 1 hour at 37°C. PMA acts as a stimulant on the activation status of the T cells independent of their antigen-specificity. Ionomycin is an ionophore and enables PMA to get into the cell. To inhibit the excretion of secreted cytokines, the intracellular transport of proteins is blocked by 5 µg/ml brefeldin A (Sigma-Aldrich). Brefeldin A has the ability to inhibit the vesicular transport from the endoplasmatic reticulum to the Golgi apparatus and therfore promotes the accumulation of cytokines in the cell. After an incubation period of 3 hours at 37°C, the cells can be washed and resuspended in PBS/BSA. According to intracellular staining, cytokine production can now be examined by flow cytometry.

2.7.1.2 Antigen-specific re-stimulation

For antigen-specific re-stimulation of lymph node T cells after DTH induction, single cell suspensions of popliteal lymph nodes were re-stimulated with 2 mg/ml ovalbumin in RPMI (Gibco/Invitrogen) containing 1% Penicillin/Streptomycin (PS) (Seromed), 2-mercaptoethanol and 10% FCS (Sigma-Aldrich) for 6 hours at 37°C. Brefeldin A (5 µg/ml) (Sigma-Aldrich) was added after 2 hours of stimulation. OVA-specific CD4+ T cells were CD154 positive after re-stimulation (Kirchhoff, Frentsch et al. 2007).

2.7.2 Flow cytometry analysis

With flow cytometry analysis (or fluorescence activated cell sorting, FACS), cells can be detected on a single cell level, while passing a laser beam within a liquid flow. Certain parameters of the cells, as e.g. the size, the granularity and the fluorescence can be quantified. As only very few cells *per se* emit flourescent light, cells are stained with antibodies coupled to a fluorophore to detect fluorescence. Those antibodies are mostly directed against cell surface or intracellular proteins. Every cell passing the laser beam disperses the light of the laser and every fluorescent molecule is excitated. The dispersed light gives information about the size and the granularity of the cell and fluorescence gives information about the expression of a certain protein.

2.7.2.1 Staining of cell surface proteins

Cell surface protein stainings for FACS were performed in cold PBS/BSA and in 96-well plates (V-bottom, non-treated) (Costar). For the staining, cells were incubated with the appropriate primary antibody in a total volume of 50 µl, for 15 minutes on ice (see table). The proper dilution of the antibody was determined by preliminary test stainings. Usually, for flow cytometry analysis, the concentration of all antibodies was 1-5 µg/ml. Subsequent to inbubation, cells were washed with 200 µl PBS/BSA and after that either prepared for a following intracellular staining or directly resuspended in 200 µl PBS/BSA for flow cytometry analysis. Biotinylated anti-mouse CD154 antibody (see table) requires additional staining with SA-coupled APC (see table) for detection in flow cytometry analysis.

2.7.2.2 Staining of intracellular proteins

For intracellular staining of proteins, cells were fixed with 100 µl Cytofix/Cytoperm (BD Biosciences) for 20 minutes on ice. This step enables fixation and permeabilisation of the cells, which is necessary for intracellular staining of proteins, e.g. cytokines and IgG antibodies. After washing the cells with 200 µl Perm/wash buffer (BD Biosciences) and then stained with a primary antibody (see table) in a total volume of 50 µl Perm/wash buffer for 30 minutes on ice.

2.8 *In vitro* assay with bone marrow-derived DCs (BMDCs)

2.8.1 *C*ultivation of BMDCs *ex vivo*

Concentration of BM-derived cells was adjusted to $1x10^6$/ml and cell suspension was supplemented with additional 10 ng/ml rekombinant IL-4 (R&D Systems) and GM-CSF (supernatant, DRFZ, Dr. Simon Fillatreau), diluted 1:25. The dilution was elucidated in a preliminary experiment. $1x10^6$ cells were plated per well and ml in a six well plate (Greiner). BM-derived DCs were generated over 9 days in complete IMDM (Gibco/Invitrogen) (see table) containing IL-4 and GM-CSF. Every third day, the medium was changed by removing half of the volume and replacing it with IMDM containing 10ng/ml IL-4 and GM-CSF, diluted 1:50.

2.8.2 Stimulation of BMDCs

On day 9, BMDCs were stimulated with the indicated immune complexes (ICs) over night, usually for 16 hours, in each well with 1 ml IMDM, containing non-assential amino acids, (Gibco/Invitrogen), 10% FCS* (IgG-free FCS, house preparation), 1% PS (Seromed) and 50 μM 2-mercaptoethanol (Sigma-Aldrich). IC-formation was performed incubating antigen and antibody in a ratio of 1:4 at 37°C for 1 hour. In detail, TNP-OVA ICs were prepared incubating 10 μg TNP(3)-OVA (House preparation) with 40 μg of the indicated anti-TNP IgG1 H5 antibodies. IVIg was added to the BMDCs in a concentration of 10 mg/ml. IL-6 concentrations in the culture supernatants were detected by Bio-Plex (BioRad) analysis as described by the manufacturer's instructions.

2.9 Statistical analysis

Statistical analyses were performed with Graph Pad Prism 4 using Student's *t* test or Logrank test for survival curves: n.s. $P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

3 Results

3.1 T cell independent (TI) and T cell dependent (TD) B cell activation induce sialylated IgG antibodies and tolerance under non-inflammatory conditions

3.1.1 T cell independent antigens induce peripheral tolerance and protect from inflammatory immune responses

It has been shown that there are several distinct pathways leading to the activation of B cells, the T cell independent (TI) and the T cell dependent (TD) pathway (Mond, Vos et al. 1995; McHeyzer-Williams, McHeyzer-Williams et al. 2000; McHeyzer-Williams, Malherbe et al. 2006). While TD antigens together with TLR co-stimulation induce full-blown inflammatory T and B cell responses, the quality of TI B cell activation has not been understood yet. On the one hand, TI B cell activation via TLR-MyD88 signalling has been demonstrated to play a major role in the development of IgG autoantibodies with unknown function (Berland, Fernandez et al. 2006; Christensen, Shupe et al. 2006; Ehlers, Fukuyama et al. 2006; Lanzavecchia and Sallusto 2007; Herlands, Christensen et al. 2008; Tsao, Jiao et al. 2008), on the other hand TI type 2 antigens induce IgG antibodies, which suppress a secondary B cell response when challenged with the same antigen independently of FcγRIIB (Brodeur and Wortis 1980; Heyman 2003).

To address the question whether immunisation with TI antigens exhibit distinct immune inhibitory functions, the impact of TI-1 TNP-LPS and TI-2 TNP-Ficoll was studied on the generation of antigen-specific IgM and IgG antibodies as well as on the development of inflammatory immune responses in the nephrotoxic nephritis mouse model (*Figure 3.1A*). To exclude any role of FcγRIIB, the experiments were performed in C57BL/6 mice (B6) lacking the inhibitory FcγRIIB (RIIB$^{-/-}$). Determination of antibody levels in the serum of RIIB$^{-/-}$ mice showed that immunisation with both TI antigens induced high titers of anti-TNP IgM antibodies (*Figure 3.1B*).

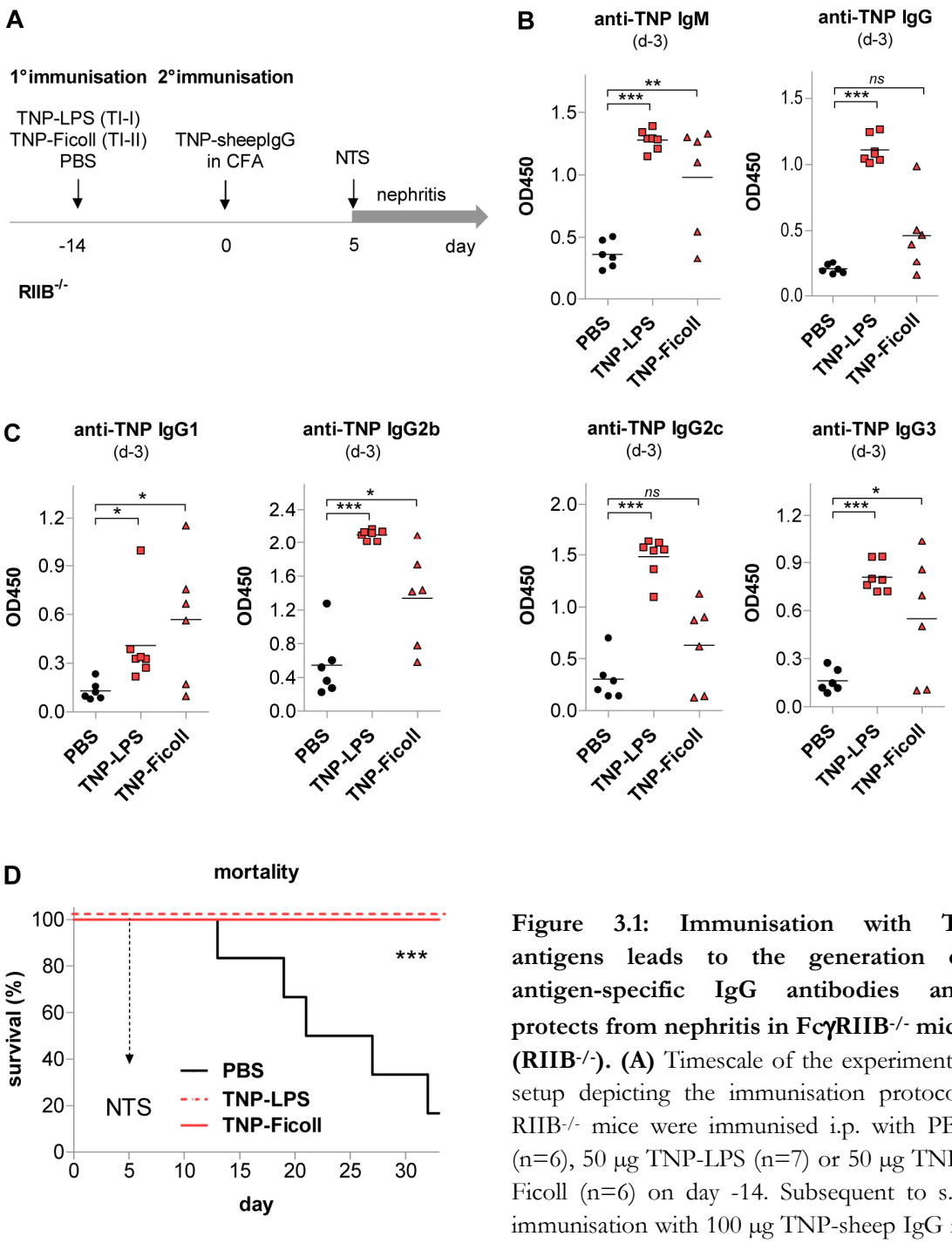


Figure 3.1: Immunisation with TI antigens leads to the generation of antigen-specific IgG antibodies and protects from nephritis in FcγRIIB$^{-/-}$ mice (RIIB$^{-/-}$). (A) Timescale of the experimental setup depicting the immunisation protocol: RIIB$^{-/-}$ mice were immunised i.p. with PBS (n=6), 50 µg TNP-LPS (n=7) or 50 µg TNP-Ficoll (n=6) on day -14. Subsequent to s.c. immunisation with 100 µg TNP-sheep IgG in CFA on day 0, nephritis was induced by i.v. injection of 100 µl NTS on day 5. **(B)** Anti-TNP IgM and IgG and **(C)** anti-TNP IgG1, IgG2b, IgG2c and IgG3 serum antibody levels of immunised mice as analysed in ELISA on day -3. Symbols represent data from individual mice. The mean value with standard error of the mean (SEM) is shown for each group **(D)** Kaplan-Meier survival curve for mice treated with NTS. One representative out of two independent experiments is shown. (***$P < 0.001$).

However, immunisation with TNP-LPS led to a more efficient induction of anti-TNP IgG antibodies than immunisation with TNP-Ficoll, while anti-TNP IgM production was comparable in both immunised groups (*Figure 3.1B*). TNP-LPS induced the production of predominantly anti-TNP IgG2b (*Figure 3.1C, second panel*), IgG2c (*Figure 3.1C, third panel*) and IgG3 (*Figure 3.1C, fourth panel*) antibodies, whereas TNP-Ficoll generated predominantly anti-TNP IgG1 antibodies (*Figure 3.1C, first panel*). However, pre-immunisation with TNP-LPS and TNP-Ficoll protected $RIIB^{-/-}$ mice from nephritis associated mortality after disease induction by the transfer of sheep anti-mouse GBM antibodies (nephrotoxic serum, NTS) (*Figure 3.1D*) (Madaio and Harrington 2001).

To confirm that immunisation with TI antigens has the potential to protect from inflammatory immune responses, the effect of TNP-LPS and TNP-Ficoll was accessorily examined in a T cell-mediated DTH response, where footpad swelling was induced by local antigen challenge in immunised B6 mice (*Figure 3.2A*) (Marty, Peclat et al. 2001). It could be clearly demonstrated that antigen-specific footpad swelling of TI pre-immunised mice was significantly reduced compared to untreated control mice, which displayed pronounced antigen-specific footpad swelling (*Figure 3.2B*).

These results indicate that immunisation with TNP-LPS and TNP-Ficoll lead to the generation of TNP-specific IgM and IgG antibodies and furthermore prevent the development of inflammatory disease symptoms as shown by attenuation of a DTH response and the protection from nephritis-mediated mortality.

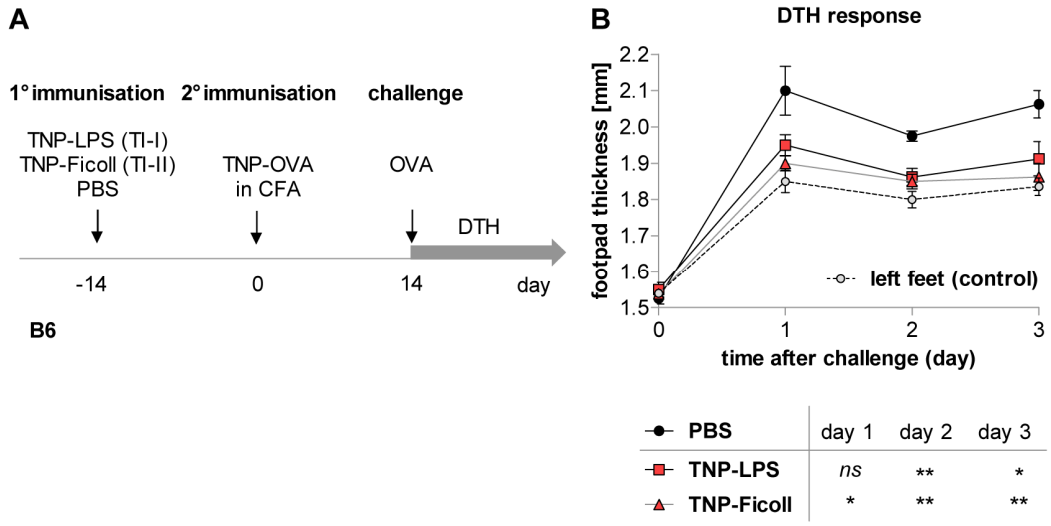


Figure 3.2: Immunisation with TI antigens attenuates a delayed type hypersensitivity (DTH) response in B6 mice. (A) Timescale of the experimental setup depicting the immunisation protocol: B6 mice were immunised i.p. with PBS (n=6), 50 μg TNP-LPS (n=6) or 50 μg TNP-Ficoll (n=6) on day -14. Subsequent to s.c. immunisation with 100 μg TNP-OVA in CFA on day 0, DTH was initiated by local injection of 37,5 μg OVA in montanide into the right footpad on day 14. As a control for unspecific swelling, PBS in montanide was injected into the left footpad. **(B)** OVA-specific DTH response depicted in a diagram as measured by the absolute footpad thickness (mm) of mice at indicated time points. Symbols represent data from one group. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. **(C)** Table depicting significances in footpad swelling comparing immunised and untreated mice. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$).

To investigate the influence of pro-inflammatory stimuli on TI immune responses, the impact of CFA was evaluated on TD and TI immunisation in a DTH response (*Figure 3.3A*). While pre-immunisation with TI TNP-LPS prevented a DTH response in immunised mice, immunisation with TD TNP-BSA in CFA exhibited no inhibitory effect on the antigen-specific footpad swelling (*Figure 3.3B*). Furthermore, compared to immunisation with TD antigens, TI TNP-LPS led to significantly reduced anti-OVA IgG2b serum antibody titers, which are induced upon TD immunisation (*Figure 3.3C*) concluding that the OVA-specific B cell response was suppressed. When TI TNP-LPS was co-administered with the co-stimulus CFA, the protective effect of TI immunisation was still sustained as could be demonstrated by reduced antigen-specific footpad swelling (*Figure 3.3B*) and diminished OVA-specific IgG2b serum antibody titers (*Figure 3.3C*). Only in the presence of the *Mycobacterium tuberculosis* enriched adjuvant CFA (CFA^{high}), the protective

effect of TI immunisation was abrogated, indicated by pronounced antigen-specific footpad swelling (*Figure 3.3B*) and high antigen-specific IgG2b serum antibody titers (*Figure 3.3C*).

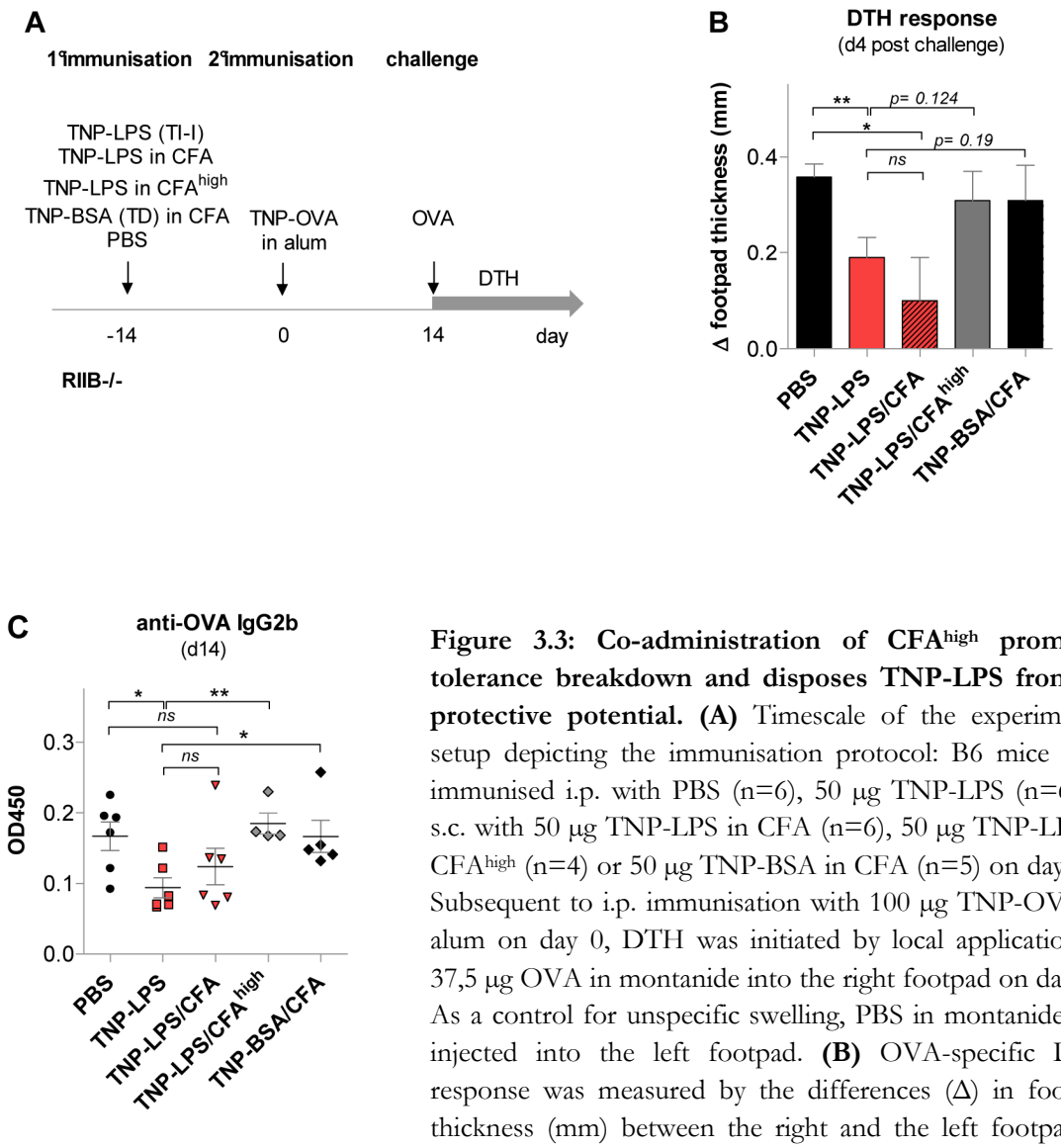


Figure 3.3: Co-administration of CFA^{high} promotes tolerance breakdown and disposes TNP-LPS from its protective potential. (A) Timescale of the experimental setup depicting the immunisation protocol: B6 mice were immunised i.p. with PBS (n=6), 50 µg TNP-LPS (n=6) or s.c. with 50 µg TNP-LPS in CFA (n=6), 50 µg TNP-LPS in CFA^{high} (n=4) or 50 µg TNP-BSA in CFA (n=5) on day -14. Subsequent to i.p. immunisation with 100 µg TNP-OVA in alum on day 0, DTH was initiated by local application of 37,5 µg OVA in montanide into the right footpad on day 14. As a control for unspecific swelling, PBS in montanide was injected into the left footpad. **(B)** OVA-specific DTH response was measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice. Day 4 after challenge was shown as representative data for the DTH response over time. Bar graphs show the mean value with standard error of the mean (SEM) for each group. **(C)** Serum anti-OVA IgG2b antibody levels as analysed by ELISA on day 14. Symbols represent data from individual mice. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$ and $***P < 0.001$).

Taken together, immunisation with TI antigens induces TNP-specific IgG antibodies and protects B6 mice from TD induced inflammatory immune responses independently of inhibitory FcγRIIB. The anti-inflammatory effect of TI immunisation in a DTH reaction

can be overcome only in a strong, CFA^{high}-mediated inflammatory milieu. CFA alone is not able to break tolerance when co-administered with TNP-LPS, but promotes the establishment of a DTH response when administered together with the TD antigen TNP-BSA

3.1.2 TI antigens induce antigen-specific sialylated IgG antibodies

The previous experiments revealed that TI immunisation protects from inflammatory immune responses and leads to the generation of antigen-specific IgG antibodies. *N*-linked glycosylation of IgG Fc-regions, particularly α-2,6-sialyltransferase-mediated sialylation, has been associated with immune regulatory functions (Kaneko, Nimmerjahn et al. 2006; Anthony, Nimmerjahn et al. 2008; Anthony, Wermeling et al. 2008; Nimmerjahn and Ravetch 2008).

I examined whether the Fc-sialylation of IgG antibodies generated after tolerogenic immunisation with TI antigens differs from IgG antibodies that were induced after pro-inflammatory TD immunisation. The Fc-linked sialic acid content of purified TI and TD induced antigen-specific serum IgG antibodies was analysed 14 days after immunisation. The sialic acid content of TNP-specific IgG antibodies induced with TNP-LPS or TNP-Ficoll was comparable to sialic acid levels measured in total serum IgG antibodies purified from untreated control mice (*Figure 3.4A*).

Around 13-15% of the TI induced anti-TNP IgG antibodies carried glycan structures with at least one terminal sialic acid molecule (S1). Furthermore, approximately 1.5% of the induced IgG antibodies displayed two molecules of terminal sialic acid attached to their Fc-mediated glycan structure (S2). Immunisation with comparable amounts of TD antigen without inflammatory co-stimuli was not able to induce detectable titers of antigen-specific IgG antibodies. Contrary, TNP- and OVA-specific IgG antibodies induced by co-administration of TNP-OVA with CFA or immunisation with OVA in combination with CFA or CFA^{high} showed significantly lower sialic acid contents than TI induced TNP-specific IgG antibodies (*Figure 3.4A*). Only 5% of TD IgG antibodies were sialylated and display at most one terminal sialic acid molecule (S1). Partial de-sialylation of anti-TNP IgG antibodies induced with TNP-LPS was observed when administered together with CFA. Around 10% of these TNP-specific IgG antibodies still carried terminal sialic acid molecules attached to their Fc-glycan structure though. Low sialylation levels comparable

to antigen-specific IgG antibodies in response to TD immunisation were merely observed when TNP-LPS was immunised in the presence of CFA^{high} (*Figure 3.4A*).

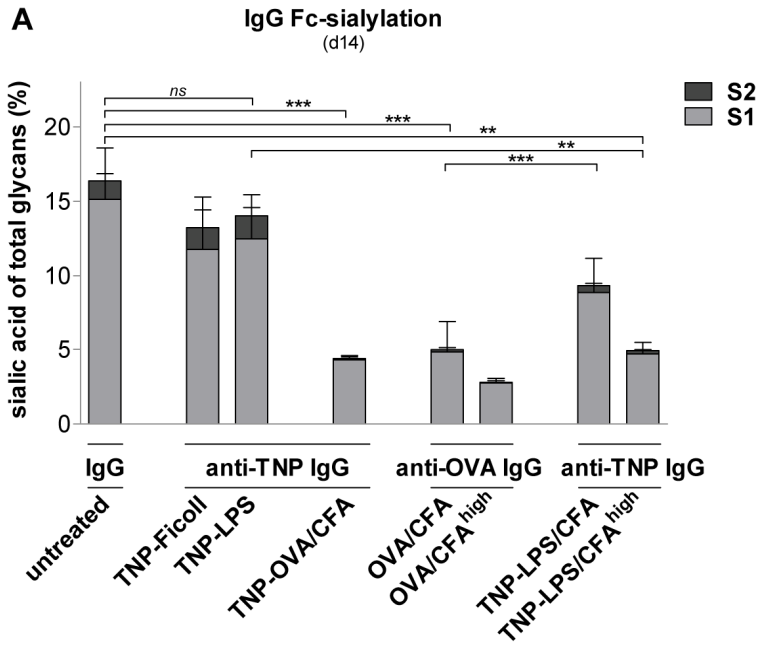


Figure 3.4: Immunisation with TI antigens induces the generation of antigen-specific sialylated IgG antibodies. Antigen-specific IgG antibodies were purified from pooled sera of treated B6 mice 14 days after immunisation **(A)** Fc sialic acid content of total IgG antibodies purified from sera of untreated B6 mice (n=11) or TNP- or OVA-specific IgG antibodies purified from sera of mice immunised with 50 μg TNP-Ficoll (n=10), 50 μg TNP-LPS (n=5), 100 μg TNP-OVA in CFA (n=3), 100 μg OVA in CFA (n=25), 100 μg OVA in CFA^{high} (n=3), 50 μg TNP-LPS in CFA (n=6) and 50 μg TNP-LPS in CFA^{high} (n=3) as analysed by MALDI-TOF mass spectrometry. Bar graphs indicate the frequency of glycan structures with 1 and 2 murine Neu5Gc sialic acid (S1, S2) residues. The mean value with standard error of the mean (SEM) is shown for each group. ($P > 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

In summary, TI immunisation leads to the generation of sialylated antigen-specific serum IgG antibodies contrary to TD antigens together with pro-infalmmatory co-stimuli, which induce the generation of IgG antibodies with low sialylation content. De-sialylation of IgG antibodies induced upon TI immunisation requires stronger CFA-mediated stimulation than TD immunisation.

3.1.3 Sialylation levels of TI IgG antibodies correlate with α-2,6-sialyltransferase expression in splenic plasma cells

Sialyltransferases are enzymes that add sialic acid molecules to certain glycan structures of nascent oligosaccharides. To examine whether the observed differences in the IgG Fc-sialylation correlate with a differential expression of α-2,6-sialyltransferase in IgG producing $CD138^+$ plasma cells, the protein level of α-2,6-sialyltransferase was measured in antigen-specific splenic PCs isolated from TI and TD immunised mice (*Figure 3.5*). Determination of α-2,6-sialyltransferase expression levels was performed eight days after immunisation. Independently of the IgG isotype, OVA-specific PCs induced by TD immunisation with OVA in CFA showed significantly lower α-2,6-sialyltransferase expression levels than unspecific PCs isolated from untreated control mice (*Figure 3.5A-D*). The expression of α-2,6-sialyltransferase in PCs was further reduced when OVA was administered with CFA^{high}. By contrast, immunisation with TNP-LPS induced TNP-specific PCs, which expressed α-2,6-sialyltransferase levels comparable to unspecific PCs generated under steady state conditions in untreated control mice (*Figure 3.5A*). However, when TNP-LPS was applied in combination with CFA or CFA^{high}, the expression of α-2,6-sialyltransferase in TNP-specific PCs decreased to levels measured in OVA-specific PCs induced after TD immunisation (*Figure 3.5A*). Observations concerning α-2,6-sialyltransferase expression levels in all PCs, were conferrable to IgG1 (*Figure 3.5B*), IgG2b (*Figure 3.5C*) as well as IgG2c (*Figure 3.5D*) producing PCs.

Thus, TI immunisation with TNP-LPS leads to the generation of TNP-specific sialylated IgG antibodies correlating with the induction of TNP-specific PCs expressing high levels of α-2,6-sialyltransferase. Co-administration of CFA and CFA^{high} gradually reduces α-2,6-sialyltransferase expression levels in antigen-specific splenic PCs of TI and TD immunised mice.

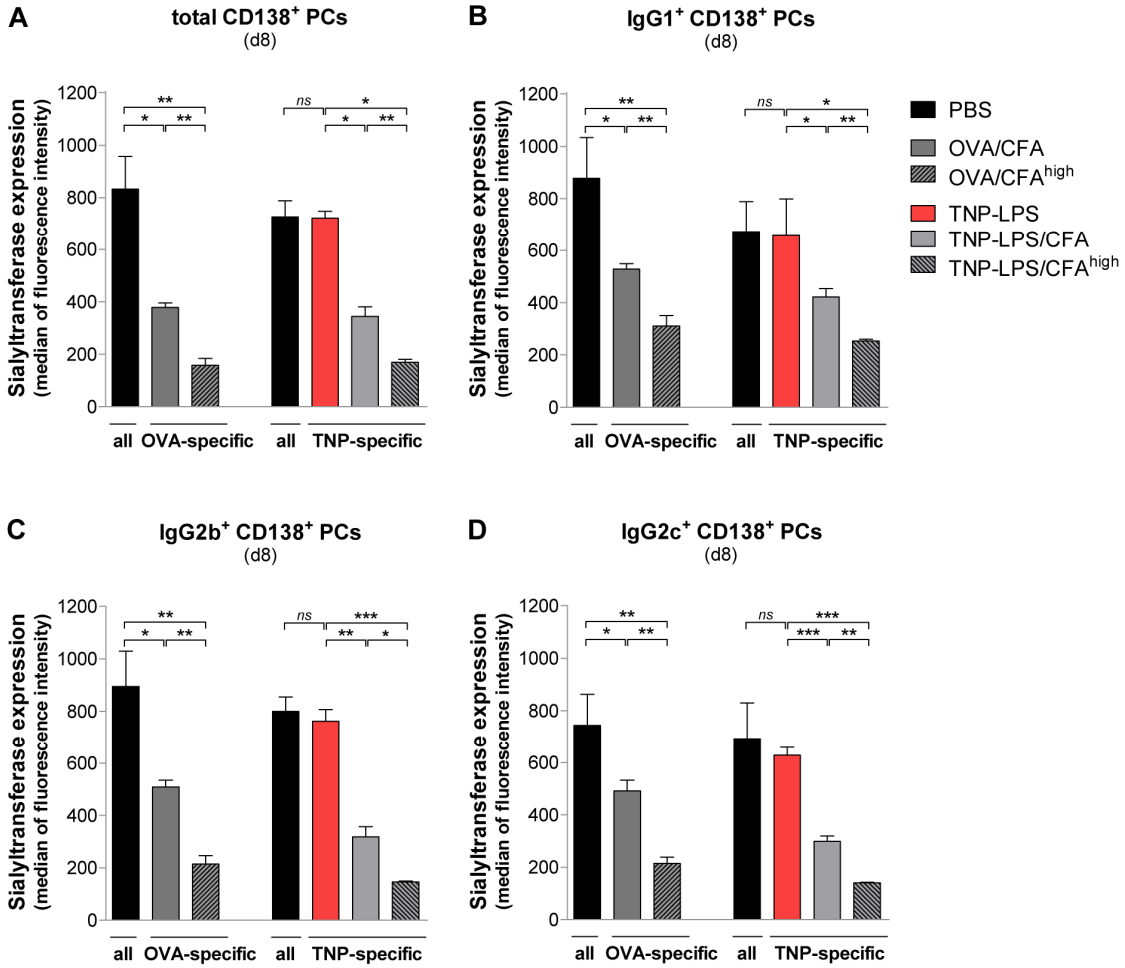


Figure 3.5: Immunisation with TI antigens induces antigen-specific plasma cells expressing high levels of α-2,6-sialyltransferase. (A-D) B6 mice were immunised with 100 µg OVA in CFA (n=3), 100 µg OVA in CFA^{high} (n=3), 50 µg TNP-LPS (n=3), 50 µg TNP-LPS in CFA (n=3), or 100 µg TNP-LPS in CFA^{high} (n=3). On day 8 post immunisation, splenic lymphocytes were stained for CD138 expression and OVA and TNP reactivity. Antigen-specific CD138$^+$ PCs from immunised and total CD138$^+$ PCs from age-matched untreated control mice (n=3) were analysed for intracellular α-2,6-sialyltransferase and IgG isotype expression by flow cytometry analysis. **(A)** Median values of α-2,6-sialyltransferase expression levels shown for all CD138+ PCs and TNP- or OVA-specific CD138$^+$ PCs of untreated mice and immunised mice, respectively. Median values of α-2,6-sialyltransferase expression levels shown for IgG1 **(B)**, IgG2b **(C)** and IgG2c **(D)** expressing CD138$^+$ PCs or TNP- or OVA-specific CD138$^+$ PCs. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$).

3.1.4 TI induced IgG antibodies mediate anti-inflammatory effector functions

As shown before, immunisation with TNP-LPS prevents inflammatory immune responses and leads to the generation of antigen-specific sialylated IgG antibodies. This correlates with the induction of antigen-specific PCs expressing high levels of α-2,6-sialyltransferase.

To determine whether TI induced sialylated IgG antibodies directly mediate anti-inflammatory effects dependent on their Fc sialylation content, I investigated the impact of transferred sialylated (+sial) (*Figure 3.6A*) and de-sialylated (sialidase treated, -sial) (*Figure 3.6A*) anti-TNP IgG antibodies on a TD immunisation and subsequent DTH reaction in RIIB-/- mice (*Figure 3.6B*). Transfer of TI induced IgG antibodies revealed that sialylated anti-TNP IgG antibodies (10 mg/kg body weight) were able to attenuate an inflammatory DTH response in $RIIB^{-/-}$ mice (*Figure 3.6C*). However, the application of de-sialylated IgG antibodies (10 mg/kg body weight) did not reduce a DTH response (*Figure 3.6C*). Furthermore, sialylated anti-TNP IgG antibodies were able to suppress the generation of anti-OVA IgG2b serum antibodies, which are induced upon an inflammatory TD immune reaction (*Figure 3.6D*). Moreover, sialylated IgG antibodies prevented the infiltration of $CD4^+$ T cells into the popliteal lymph node of antigen-challenged footpads, which was observed in untreated mice (*Figure 3.6E*). By contrast, de-sialylated TNP-specific IgG antibodies were not able to reduce neither anti-OVA IgG2b antibody titers (*Figure 3.6D*) nor $CD4^+$ T cell infiltration (*Figure 3.6E*). This indicates that Fc-sialic acid content of TI induced IgG antibodies is crucial for mediating the anti-inflammatory effector functions observed in TI immunised mice.

Thus, TI immunisation leads to the generation of TNP-specific sialylated IgG antibodies, which are *per se* sufficient to reduce an antigen-specific DTH response independently of FcγRIIB.

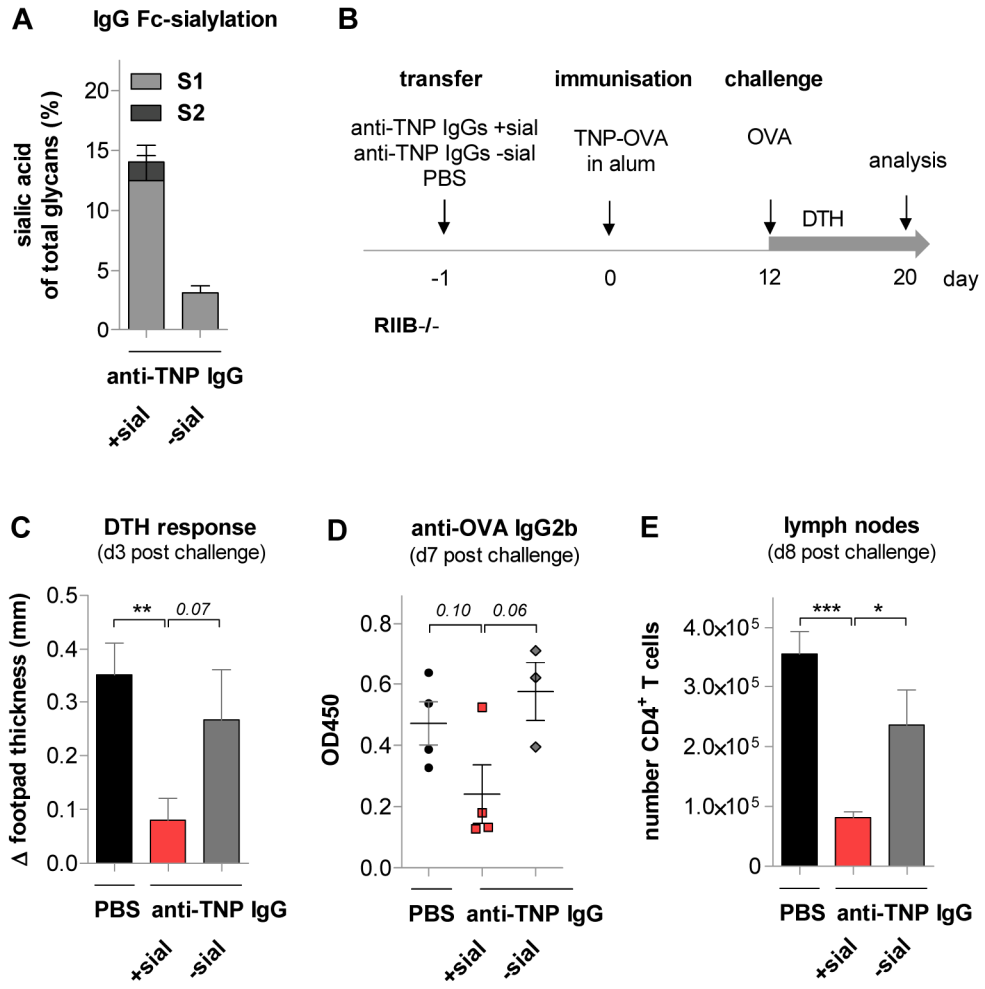


Figure 3.6: Transfer of purified TI induced TNP-specific sialylated IgG antibodies protect from a DTH response. TNP-specific sialylated IgG antibodies used in **B-E** were purified from pooled sera of TNP-LPS immunised mice 14 days post immunisation. Purified TNP-specific IgG antibodies were additionally treated with sialidase A. **(A)** Frequencies of Fc sialic acid moieties (one or two murine Neu5Gc; S1 and S2) of sialylated (+sial) and sialidase-treated (-sial) TNP-specific IgG antibodies as determined by MALDI-TOF mass spectrometry. **(B)** Schematic plan of the experimental approach followed in C-E. One day before i.p. immunisation with 100 µg TNP-OVA in alum, 200 µg of sialylated (n=4) or de-sialylated (n=3) anti-TNP IgG antibodies were tranfserred i.v. into RIIB$^{-/-}$ mice. On day 12, DTH was induced by local application of 37,5 µg OVA in montanide into the right footpad. As a control for unspecific swelling, PBS in montanide was injected into the left footpad. **(C)** OVA-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice. Day 3 after challenge was shown as representative data for the DTH response over time. Bar graphs show the mean value with standard error of the mean (SEM) for each group. **(D)** Serum anti-OVA IgG2b antibody levels as analysed by ELISA on day 7 post challenge. Symbols represent data from individual mice. The mean value with standard error of the mean (SEM) is shown for each group. **(E)** Total CD4^{+} T cell numbers isolated from popliteal lymph nodes of mice as analysed by flow cytometry analysis on day 8 post challenge. Bar graphs indicate the mean values with standard error

of the mean (SEM). One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$).

3.1.5 T cell dependent antigens under non-inflammatory conditions induce peripheral tolerance and protect from inflammatory immune responses

While TD immunisation with co-stimulation leads to inflammatory immune responses, as demonstrated by pronounced footpad swelling and mortality from nephritis, TD immunisation under non-inflammatory conditions has been shown to induce regulatory T cells and thereby leads to peripheral tolerance (Kretschmer et al., 2005; Steinman et al. 2003; Yamazaki et al., 2008).

Hence, the impact of TD immunisation under non-inflammatory conditions was evaluated on the generation of antigen-specific sialylated IgG antibodies and the protection from inflammatory immune reactions in the DTH mouse model (*Figure 3.7A*). Immunisation was performed using the fusion protein Dec-205-OVA (Dec-OVA), which delivers TD OVA to DCs by binding to their cell surface marker CD205 more efficiently than achieved with pure OVA immunisation (Steinman, Hawiger et al. 2003; Kretschmer, Apostolou et al. 2005; Boscardin, Hafalla et al. 2006; Yamazaki, Dudziak et al. 2008). Determination of antibody levels in the serum showed that immunisation with Dec-OVA induced high titers of OVA-specific IgG1 antibodies (*Figure 3.7C, first panel*). However, immunisation with Dec-OVA induced tolerance to OVA and protected from a subsequent inflammatory DTH response as assessed by a reduced OVA-specific footpad swelling compared to untreated control mice (*Figure 3.7B*). OVA-specific tolerance blocked the generation of inflammatory anti-OVA IgG2b (*Figure 3.7C, second panel*) and IgG2c (*Figure 3.7C, third panel*) serum antibodies as well as the accumulation of OVA-specific activated $CD4^+$ T cells (*Figure 3.7D*) and IFN-γ producing $CD4^+$ T helper (Th) 1 cells (*Figure 3.7E*).

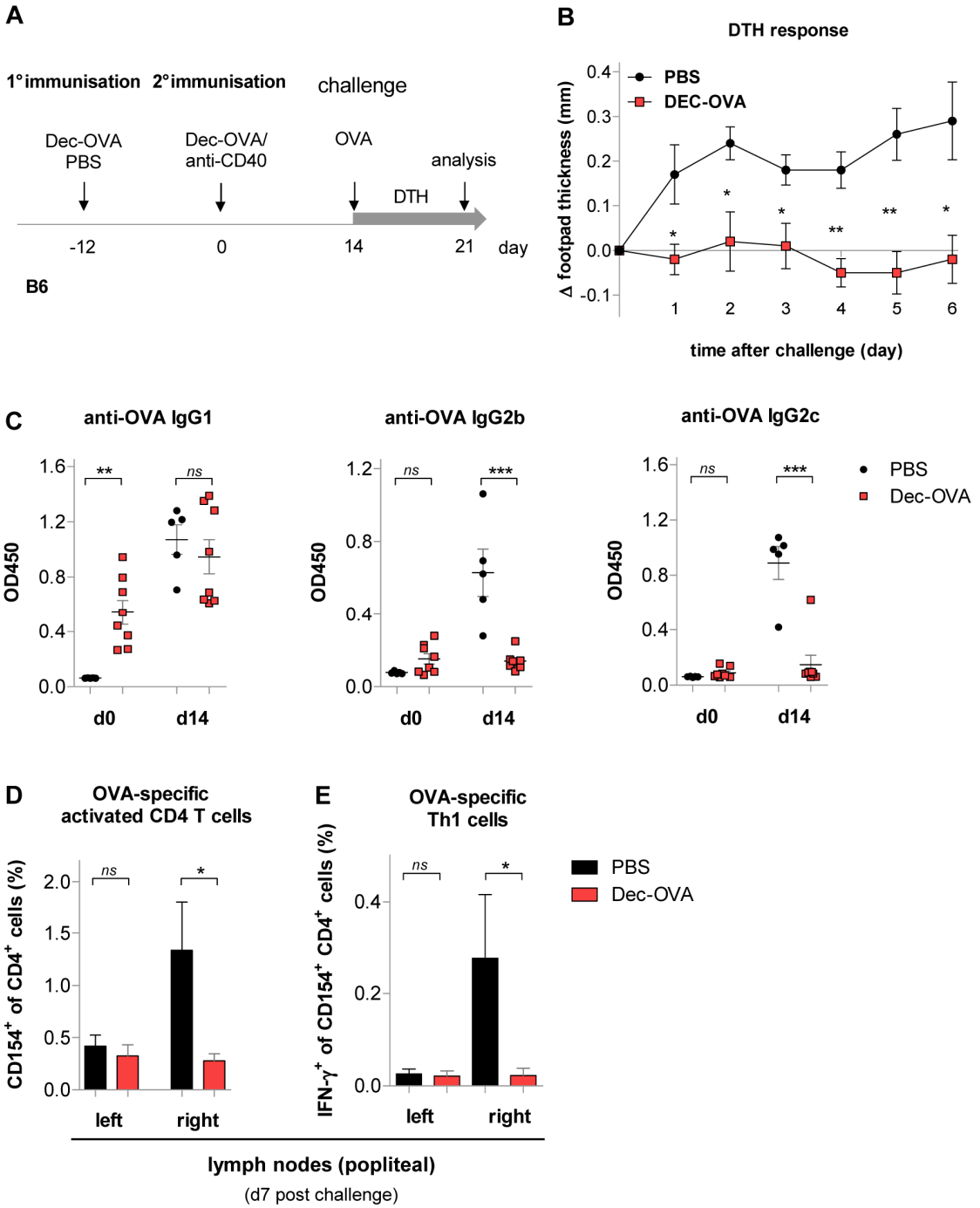


Figure 3.7: Immunisation with TD antigen Dec-OVA induces predominantly OVA-specific IgG1 antibodies and protects from a DTH response. (A) Timescale of the experimental setup depicting the immunisation protocol: B6 mice were injected i.p. with PBS (n=5) or 5 µg Dec-OVA (n=8) on day -12. Subsequent to i.p. immunisation with 5 µg Dec-OVA/50 µg anti-CD40 (clone 1C10) on day 0, DTH was initiated by local injection of 37,5 µg OVA in montanide into the right footpad on day 14. As a control for unspecific swelling, PBS in montanide was injected into the left footpad. **(B)** OVA-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of mice over time. Symbols represent data from one group. The mean value with standard error of the mean (SEM) is shown for each group. **(C)** Serum

anti-OVA IgG1, IgG2b and IgG2c antibody levels as determined by ELISA before (day 0) and after (day 14) stimulation. Symbols represent data from individual mice. The mean value with standard error of the mean (SEM) is shown for each group. (D-E) Lymphocytes isolated from popliteal lymph nodes of mice were re-stimulated with OVA. OVA-specific CD4+ T cells were further analysed for CD154 and IFN-γ expression in flow cytometry analysis. **(D)** Frequencies of $CD154^+$ CD4 T cells and **(E)** $IFN\text{-}\gamma^+$ $CD154^+$ CD4 T cells. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of four independent experiments is shown. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$).

Accordingly, immunisation with high doses of OVA (*Figure 3.8A*) also induced the generation of predominantly OVA-specific IgG1 serum antibodies (*Figure 3.8B, first panel*) and protected from a DTH response and the development of inflammation associated anti-OVA IgG2b (*Figure 3.8B, second panel*) and IgG2c (*Figure 3.8B, third panel*) antibodies.

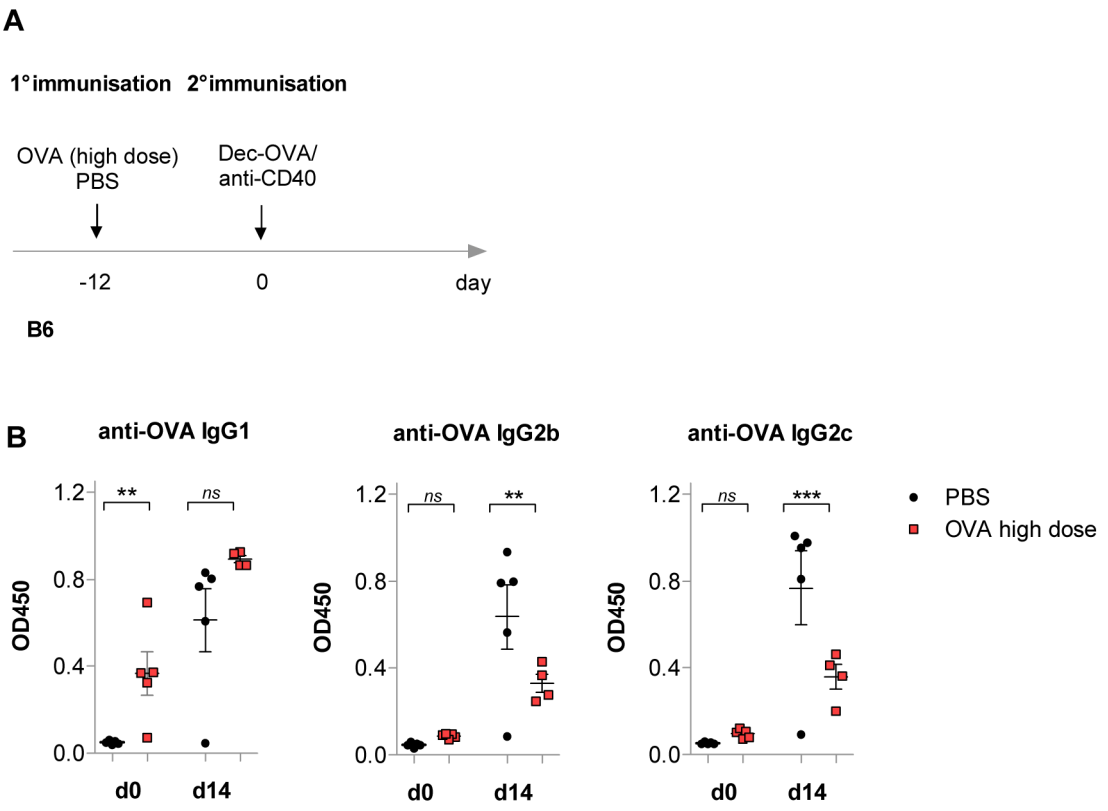


Figure 3.8: Immunisation with high doses of OVA induces OVA-specific IgG1 antibodies and protects from the generation of OVA-specific IgG2b and IgG2c antibodies after 2° immunisation. (A) Schematic plan of the experimental approach: B6 mice were pre-immunised i.p. with PBS (n=5) or 2 mg OVA (n=4) on day -12. On day 0, mice were immunised i.p. with 5 µg Dec-OVA/ 50 µg anti-CD40 (clone 1C10). **(B)** Serum anti-OVA IgG1, IgG2b and IgG2c antibody levels as determined by ELISA before (day 0) and after (day 14) second immunisation. Symbols represent data from individual mice. The mean value with standard error of the mean (SEM) is

shown for each group. One representative out of two independent experiments is shown. ($P > 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

In summary, immunisation with TD antigen under non-inflammatory conditions reduces the generation of anti-OVA IgG2b and IgG2c antibodies subsequent to inflammatory TD immunisation and thereby protects from DTH responses.

3.1.6 TD antigens induce the production of antigen-specific sialylated IgG antibody under non-inflammatory conditions

So far, the previous results revealed, that immunisation with Dec-OVA predominantly induces the generation of anti-OVA IgG1 antibodies and further protects from inflammatory immune reactions. As not only regulatory T cells but also antibody producing B cells have been described to exhibit immune regulatory functions (DiLillo, Matsushita et al. 2010) and Fc-sialylation of IgG antibodies are associated with anti-inflammatory effects (*see chapter 3.1.4.*), the sialylation pattern of TD generated IgG antibodies was analysed 14 days after immunisation (*Figure 3.9A*).

OVA-specific IgG antibodies induced by immunisation with Dec-OVA or high doses of OVA under non-inflammatory-conditions was similar to the sialic acid levels measured in purified serum IgG antibodies from untreated control mice (*Figure 3.9A*). While 12-14% of these TD induced anti-OVA IgG antibodies carried glycan structures with at least one terminal sialic acid molecule (S1), OVA-specific IgG antibodies generated upon immunisation with Dec-OVA and CD40 co-stimulation showed significantly lower sialylation levels (*Figure 3.9A*). Here, only 5-6% of the OVA-specific IgG antibodies were sialylated and display merely one terminal sialic acid attached to their Fc-part. Comparable low Fc-sialylation levels of OVA-specific IgG antibodies were observed when OVA was immunised together with CFA (*Figure 3.9A*).

In summary, similar to immunisation with TI antigens under non-inflammatory conditions (*Figure 3.4*), immunisation with TD antigens induces the generation of antigen-specific sialylated IgG antibodies. Contrary to this, pro-inflammatory immunisation with TD antigens leads to the induction of non-sialylated IgG antibodies.

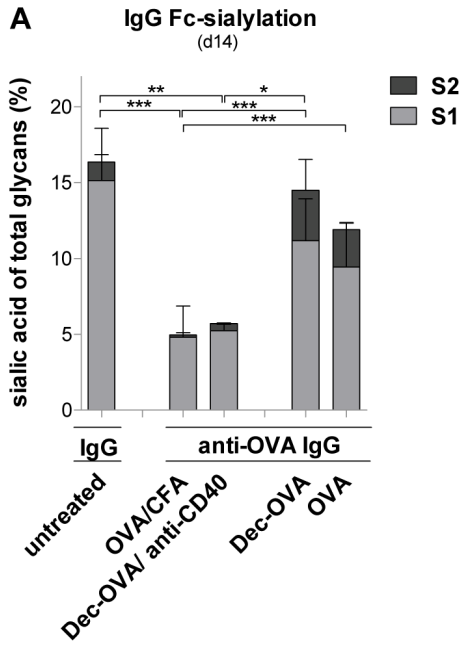


Figure 3.9: TD immunisation induces the generation of antigen-specific sialylated IgG antibodies under non-inflammatory conditions. OVA-specific IgG antibodies were purified from pooled sera of immunised B6 mice 14 days after immunisation. Total IgG antibodies were purified from pooled sera of untreated age-matched B6 mice **(A)** Fc sialic acid content of total IgG antibodies purified from sera of untreated B6 mice (n=11) or OVA-specific IgG antibodies purified from sera of mice immunised with 100 µg OVA in CFA (n=25), 5 µg Dec-OVA/50 µg anti-CD40 (n=3), 5 µg Dec-OVA (n=5) and 2 mg OVA (n=3) as analysed by MALDI-TOF mass spectrometry. Bar graphs indicate the frequency of glycan structures with 1 and 2 murine Neu5Gc sialic acid (S1, S2) residues. The mean value with standard error of the mean (SEM) is shown for each group. (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

3.1.7 Sialylation levels of TD IgG antibodies correlate with α-2,6-sialyltransferase expression in splenic plasma cells

To examine whether differences in the sialylation content of TD generated antigen-specific serum IgG antibodies are associated with differential expression levels of α-2,6-sialyltransferase, the protein levels were measured in antigen-specific splenic CD138$^+$ PCs isolated from mice 8 days after TD immunisation (*Figure 3.10*). OVA-specific splenic PCs induced upon immunisation with Dec-OVA/anti-CD40 or OVA together with CFA showed significantly lower levels of α-2,6-sialyltransferase than PCs from untreated control mice (*Figure 3.10A*). By contrast, PCs generated upon immunisation with high doses of OVA expressed high levels of α-2,6-sialyltransferase comparable to levels measured in PCs

isolated from untreated mice (*Figure 3.10A*). Due to insufficient induction of OVA-specific PCs, α-2,6-sialyltransferase expression levels could not be assessed upon immunisation with Dec-OVA.

However, TD immunisation under non-inflammatory conditions leads to the induction of antigen-specific PCs, which express high levels of α-2,6-sialyltransferase correlating with the secretion of sialylated IgG antibodies, contrary to TD immunisation under inflammatory conditions.

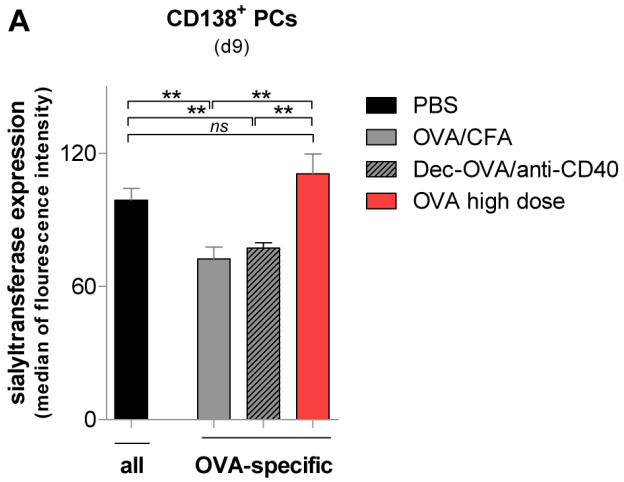


Figure 3.10: Non-inflammatory TD immunisation induces antigen-specific plasma cells expressing high levels of α-2,6-sialyl-transferase. B6 mice were immunised i.p. with 5 μg Dec-OVA/ 50 μg anti-CD40 (n=6), 2 mg OVA (n=4) or s.c. with 100 μg OVA in CFA (n=6). On day 9 post immunisation, splenic lymphocytes were stained for CD138 expression and OVA reactivity. Antigen-specific CD138+ PCs from immunised and total CD138+ PCs from age-matched untreated control mice (n=3) were analysed for intracellular α-2,6-sialyltransferase expression by flow cytometry analysis. **(A)** Median values of α-2,6-sialyltransferase expression levels shown for all CD138+ PCs and OVA-specific CD138+ PCs of untreated and immunised mice, respectively. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of two independent experiments is shown. ($P > 0.05$ and $^{**}P < 0.01$).

3.1.8 Generation of TD antigen-specific sialylated IgG1 antibodies is dependent on CD40-CD40 ligand interaction and IL-4 receptor alpha signalling

To determine signals crucial for the induction of sialylated anti-OVA IgG antibodies, the generation of OVA-specific serum IgG1 antibodies was analysed after immunisation with Dec-OVA under the influence of different depleting (anti-CD4) and blocking (anti-CD154) antibodies or in B6 mice lacking the receptor α for IL-4 binding (IL-4Rα$^{-/-}$) (*Figure 3.11*).

Depletion of $CD4^+$ T cells (*Figure 3.11B*) or blocking of the CD40-CD40L (*Figure 3.11C*) interaction as well as the lack of IL-4Rα signalling (*Figure 3.11D*) significantly abolished the induction of OVA-specific serum IgG1 antibodies. This indicates that the generation of antigen-specific sialylated IgG1 antibodies is dependent on CD4, CD40L and IL-4Rα signalling.

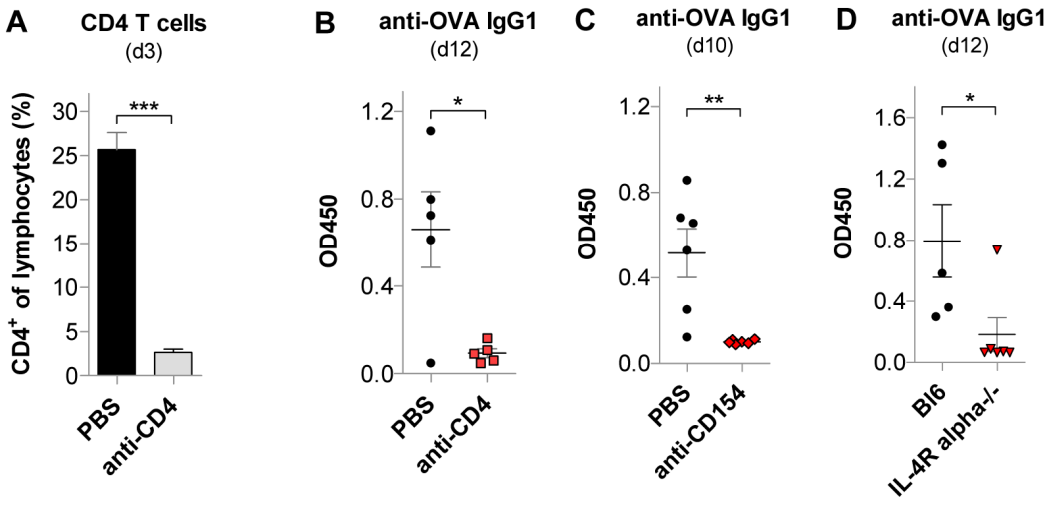


Figure 3.11: Development of OVA-specific sialylated IgG1 antibodies is dependent on CD4 T cell help, CD40-CD40L interaction and IL-4Rα signalling. For **A-B**, B6 mice were immunised i.p. with 5 µg Dec-OVA and additionally treated with PBS (n=5) or sequential i.p. injections of 250 µg anti-CD4 antibody (n=5). **(A)** Frequencies of CD4+ T cells isolated from peripheral blood of anti-CD4 antibody treated and untreated mice depicting the efficiency of CD4+ T cell depletion as analysed by flow cytometry analysis on day 3 post immunisation. Bar graphs show the mean value with standard error of the mean (SEM) for each group. **(B)** Serum anti-OVA IgG1 antibody levels of anti-CD4 antibody treated mice as determined by ELISA on day 12 post immunisation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. For C, B6 mice were immunised i.p. with 5 µg Dec-OVA and additionally treated with PBS (n=5) or sequential i.p. injections of 250 µg anti-CD154 antibody (n=5). **(C)** Serum anti-OVA IgG1 antibody levels from B6 mice treated with anti-CD154 antibody as determined by ELISA on day 10 post immunisation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. For D, B6 or IL-4R α-/- mice were immunised i.p. with 5 µg Dec-OVA. **(D)** Serum anti-OVA IgG1 antibody levels from B6 and IL-4Rα-/- mice as determined by ELISA on day 12 post immunisation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$).

3.1.9 TD induced sialylated IgG antibodies mediate anti-inflammatory effects

It was shown that TI generated antigen-specific sialylated IgG antibodies mediate anti-inflammatory effects and are able to protect from subsequent inflammatory immune reactions (*Figure 3.6*). To determine whether IgG antibodies generated upon immunisation with TD antigen under non-inflammatory conditions are sufficient to suppress inflammatory immune responses, the protective potential of OVA-specific IgG antibodies purified from pooled sera of Dec-OVA immunised mice was evaluated in a DTH response (*Figure 3.12A*).

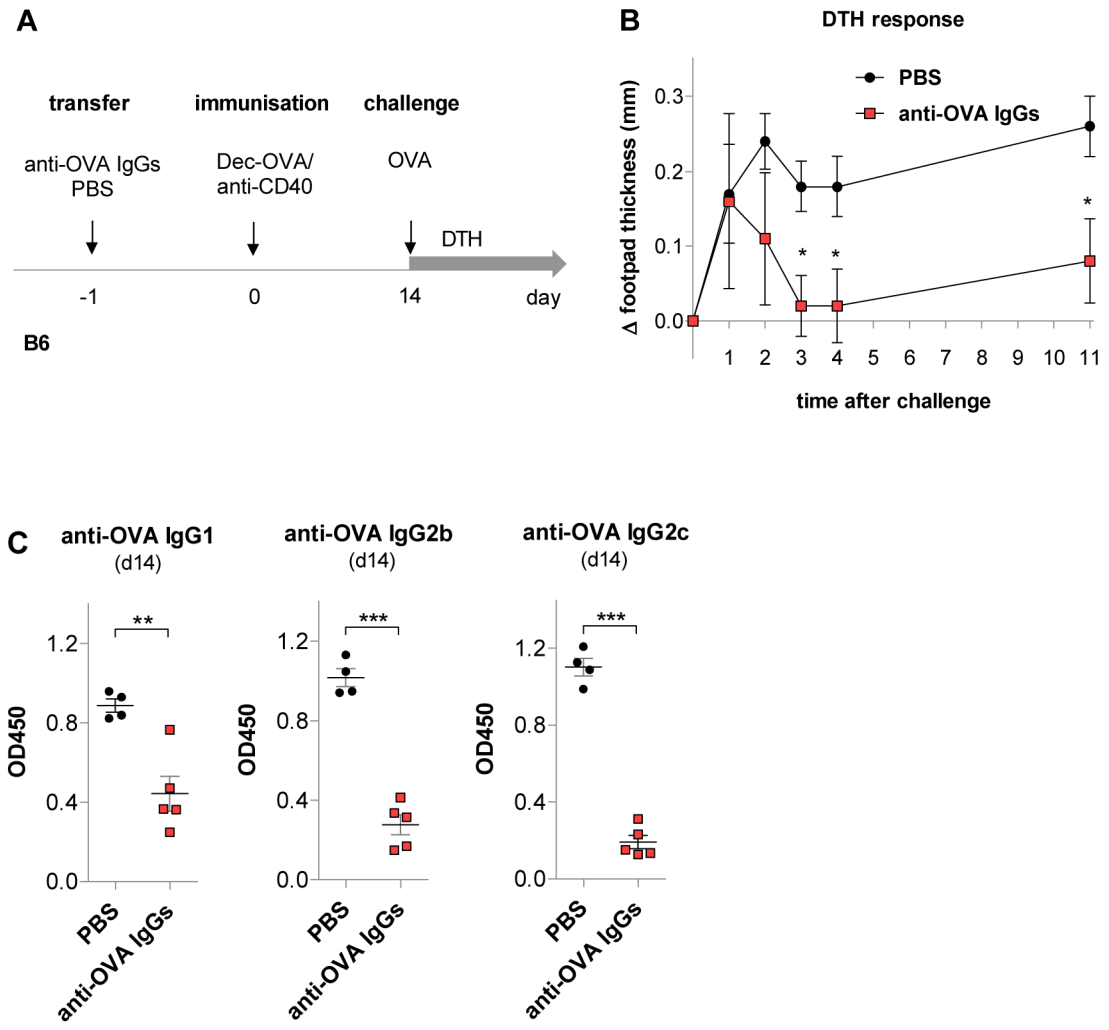


Figure 3.12: Transfer of purified TD induced OVA-specific sialylated IgG antibodies protect from a DTH response. OVA-specific sialylated IgG antibodies used in **A-C** were purified from pooled sera of Dec-OVA immunised mice 14 days post immunisation. **(A)** Timescale of the experimental setup depicting the immunisation protocol: One day before immunisation with 5 µg

Dec-205-OVA/50 μg anti-CD40 (clone 1C10), PBS (n=4) or 100 μg anti-OVA IgG antibodies (n=5) were transferred i.v. into B6 mice. On day 14, DTH was induced by local application of 37,5 μg OVA in montanide into the right footpad of mice. As a control for unspecific swelling, PBS in montanide was injected into the left footpad. **(B)** OVA-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice over time. Symbols represent data from one group. The mean value with standard error of the mean (SEM) is shown for each group. **(C)** Serum anti-OVA IgG1, IgG2b and IgG2c antibody levels as determined by ELISA on day 14 post stimulation. Symbols represent data from individual animals. One representative out of two independent experiments is shown. (*P < 0.05, **P < 0.01 and ***P < 0.001).

Transfer of purified OVA-specific IgG antibodies revealed that TD induced antigen-specific sialylated antibodies (10 mg/kg body weight) inhibited a DTH response as could be determined by a reduced OVA-specific footpad swelling (*Figure 3.12B*). Additionally, the anti-inflammatory effect was accompanied by the reduction of anti-OVA IgG1 (*Figure 3.12C, first panel*), IgG2b (*Figure 3.12C, second panel*) and IgG2c (*Figure 3.12C, third panel*) serum antibody titers, which were induced after TD immunisation with Dec-OVA and CD40 co-stimulation in untreated control mice.

Thus, these observations indicate that immunisation with TD antigen under non-inflammatory conditions induces antigen-specific sialylated IgG antibodies that are able to suppress an antigen-specific DTH response.

3.2 Sialylated monoclonal IgG antibodies mediate anti-inflammatory effector functions

3.2.1 Anti-inflammatory property of monoclonal IgG antibodies is dependent on Fc-sialylation

To investigate whether monoclonal sialylated antibodies exhibit the potential to mediate anti-inflammatory effects, hybridoma-derived antigen-specific IgG antibodies were enzymatically sialylated (*Figure 3.13A*) and compared to their non-sialylated analogue during inflammatory immune responses (*Figure 3.13C*). Neither enzymatic sialylation nor de-sialylation of anti-OVA IgG1 antibodies influenced the OVA binding (*Figure 3.13B*). While the transfer of *in vitro* sialylated (80% sialylation) monoclonal anti-OVA IgG1 antibodies (clone 4C9) (10 mg/kg of body weight) protected B6 mice from an OVA-specific DTH

responses (*Figure 3.13D*), non-sialylated (< 2% sialylation) anti-OVA IgG1 antibodies (10 mg/kg of body weight) were not able to suppress an OVA-specific immune reaction.

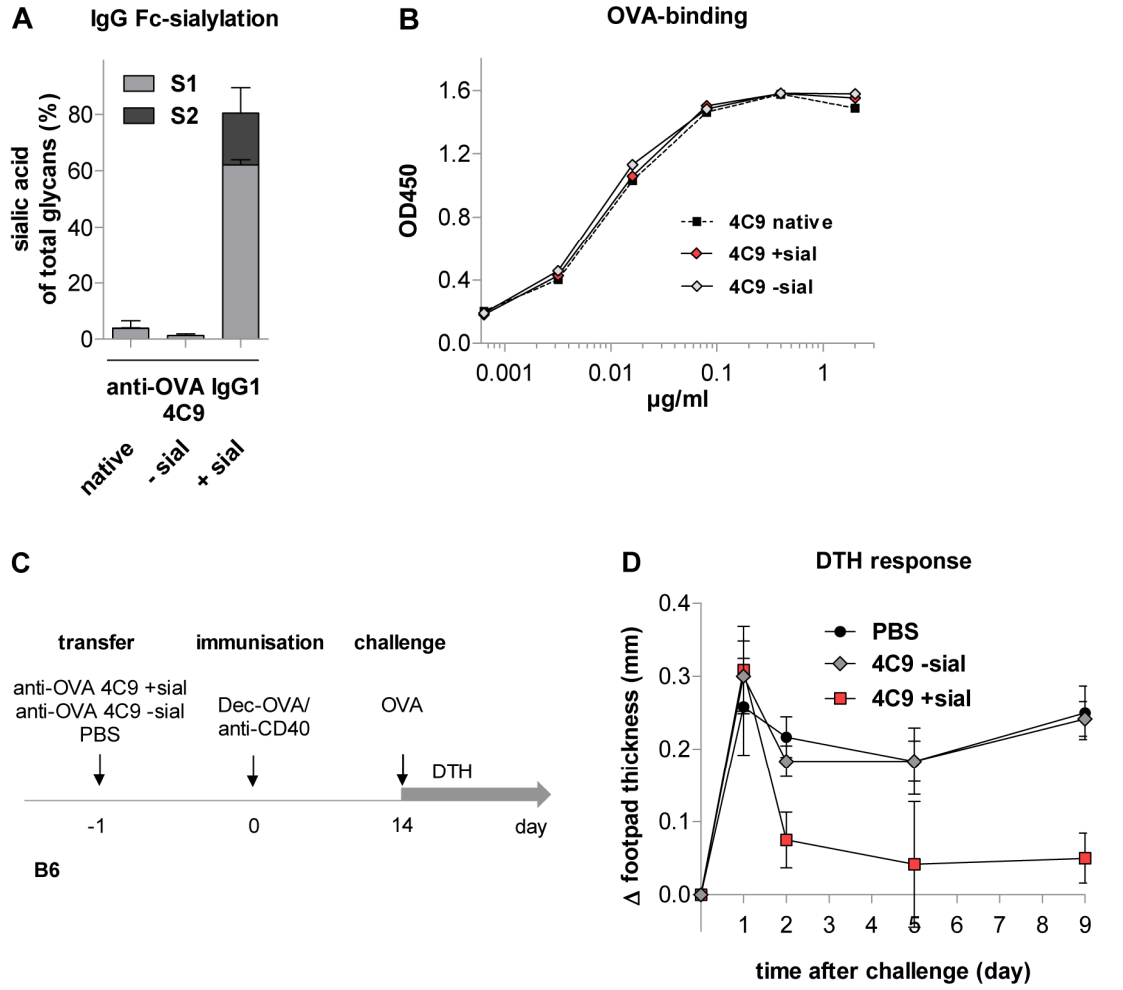


Figure 3.13: Transfer of sialylated monoclonal anti-OVA IgG1 antibodies protect from DTH response. Anti-OVA IgG1 antibodies (clone 4C9) were sialylated enzymatically. De-sialylation of anti-OVA IgG1 4C9 was performed using sialidase A. **(A)** Fc sialic acid content (one and two human sialic acid molecules (Neu5Ac); S1 and S2) of native (n=2), *in vitro* sialylated (+sial) (n=2) or de-sialylated (-sial) (n=2) anti-OVA IgG1 4C9 antibodies as analysed by MALDI-TOF mass spectrometry. Bar graphs indicate the frequency of glycan structures with 1 and 2 murine Neu5Gc sialic acid (S1, S2) residues. The mean value with standard error of the mean (SEM) is shown for each group. **(B)** Binding analysis of the monoclonal sialylated IgG1 antibodies to OVA in comparison to native or de-sialylated monoclonal IgG1 antibodies as determined by ELISA. One representative out of at least two independent ELISA is shown for each plot. **(C)** Timescale of the experimental setup depicting the immunisation protocol: One day prior to immunisation with 5 µg Dec-OVA/50 µg anti-CD40, PBS (n=6) or 200 µg of de-sialylated (-sial) (n=6) or *in vitro* sialylated (+sial) (n=6) OVA-specific IgG1 4C9 antibodies were transferred i.v. into B6 mice. On day 14, the

DTH response was induced by local application of 37,5 µg OVA in montanide into the right footpad of mice. As a control for unspecific swelling, PBS in montanide was injected into the left footpad. **(D)** OVA-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice over time. Symbols represent data from one group. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$).

This finding could be verified when *in vitro* sialylated (70% sialylation) (*Figure 3.14A*) and non-sialylated (0% sialylation) (*Figure 3.14A*) anti-TNP IgG1 antibodies (clone H5) were transferred into B6 mice (*Figure 3.14C*). *In vitro* sialylation of anti-TNP IgG1 H5 antibodies did not influence TNP-binding (*Figure 3.14B*). However, only *in vitro* sialylated anti-TNP IgG1 antibodies (5 mg/kg of body weight) were able to reduce antigen-specific footpad swelling in a DTH response, whereas non-sialylated anti-TNP IgG1 antibodies (5 mg/kg of body weight) were not sufficient to ameliorate antigen-specific footpad swelling (*Figure 3.14D*). Furthermore, the observed anti-inflammatory effect of sialylated anti-TNP IgG1 antibodies was accompanied by significantly reduced antigen-specific IgG2b antibody titers, which generate subsequent to TD induced immune reactions in untreated mice (*Figure 3.14E*).

Taken together, these findings clearly demonstrate that the anti-inflammatory effector functions of monoclonal IgG1 antibodies is directly associated with their Fc-sialylation levels.

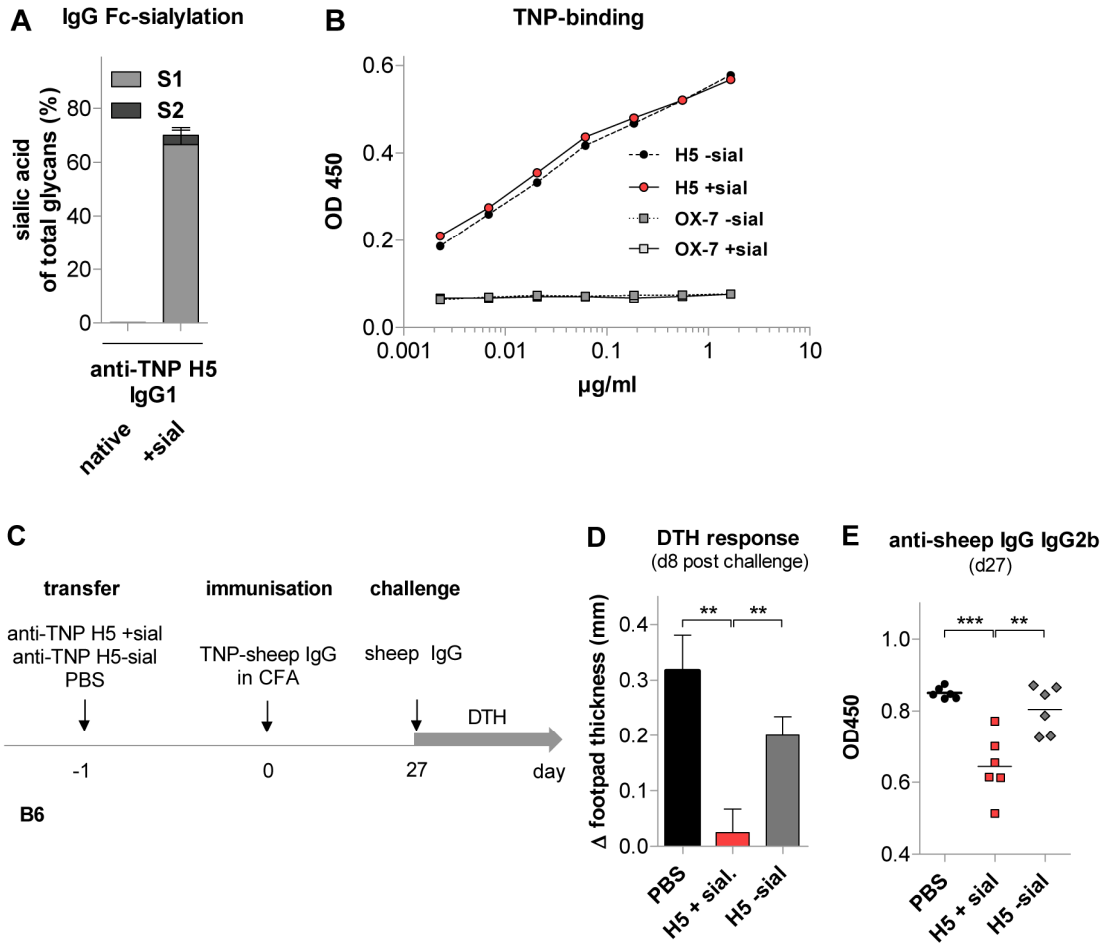


Figure 3.14: Transfer of sialylated monoclonal anti-TNP IgG1 antibodies protect from DTH response. Anti-TNP IgG1 antibodies (clone H5) were sialylated enzymatically. **(A)** Fc sialic acid content (one and two human sialic acid molecules (Neu5Ac); S1 and S2) of native (n=3) or *in vitro* sialylated (+sial) (n=3) anti-TNP IgG1 H5 antibodies as analysed by MALDI-TOF mass spectrometry. Bar graphs indicate the frequency of glycan structures with 1 and 2 murine Neu5Gc sialic acid (S1, S2) residues. The mean value with standard error of the mean (SEM) is shown for each group. **(B)** Binding analysis of the monoclonal sialylated IgG1 antibodies to TNP in comparison to native monoclonal IgG1 antibodies as determined by ELISA. One representative out of at least two independent ELISA is shown for each plot. **(C)** Timescale of the experimental approach followed in B-C. One day prior to s.c. immunisation with 100 µg TNP-sheep IgG in CFA, B6 mice were injected i.v. with PBS (n=6) or 100 µg of native (n=6) *in vitro* sialylated (n=6) TNP-specific H5 IgG1 antibodies. On day 27, the DTH response was induced by local application of 12,5 µg sheep IgG into the right footpad. As a control for unspecific swelling, PBS was injected into the left footpad. **(D)** Sheep IgG-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice. Day 8 post challenge was shown as representative data for the DTH response over time. Bar graphs show the mean value with standard error of the mean (SEM) for each group. **(E)** Serum anti-sheep IgG IgG2b antibody levels as determined by ELISA on day 27 post immunisation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for

each group. One representative out of three independent experiments is shown. (**$P < 0.01$ and ***$P < 0.001$).

3.2.2 Monoclonal sialylated IgG antibodies protect in an antigen-specific manner

To analyse whether the observed protective effects of sialylated antibodies is dependent on the antigen-specificity, the impact of antigen-unspecific and antigen-specific sialylated monoclonal IgG antibodies was compared and investigated in a DTH response (*Figure 3.15B*). Therefore, hybridoma-derived IgG1 antibody specific for Thy-1.1 (clone OX-7) was enzymatically sialylated (60% sialylation) (Figure 15A) and transferred into RIIB$^{-/-}$ mice (*Figure 3.15B*).

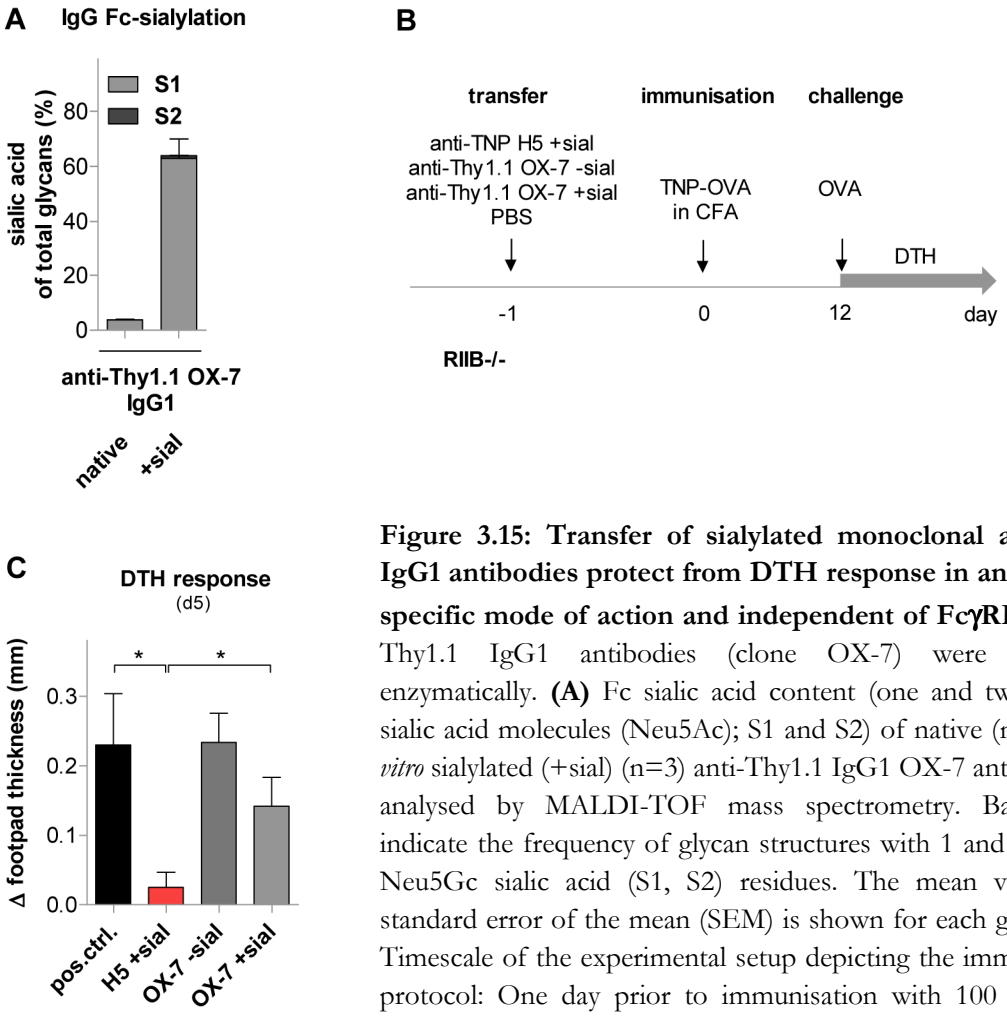


Figure 3.15: Transfer of sialylated monoclonal anti-TNP IgG1 antibodies protect from DTH response in an antigen-specific mode of action and independent of FcγRIIB. Anti-Thy1.1 IgG1 antibodies (clone OX-7) were sialylated enzymatically. **(A)** Fc sialic acid content (one and two human sialic acid molecules (Neu5Ac); S1 and S2) of native (n=3) or *in vitro* sialylated (+sial) (n=3) anti-Thy1.1 IgG1 OX-7 antibodies as analysed by MALDI-TOF mass spectrometry. Bar graphs indicate the frequency of glycan structures with 1 and 2 murine Neu5Gc sialic acid (S1, S2) residues. The mean value with standard error of the mean (SEM) is shown for each group. **(B)** Timescale of the experimental setup depicting the immunisation protocol: One day prior to immunisation with 100 µg TNP-OVA in CFA, RIIB$^{-/-}$ mice were injected i.v. with PBS (n=6), 500 µg of *in vitro* sialylated (n=6) anti-TNP H5 IgG1 or 500 µg of native (n=6) or in vitro sialylated (n=6) Thy1.1-specific OX-7 IgG1 antibodies. On day 12, the DTH reaction was induced by local application of 37,5 µg OVA into the right footpad. As a control for unspecific swelling, PBS was

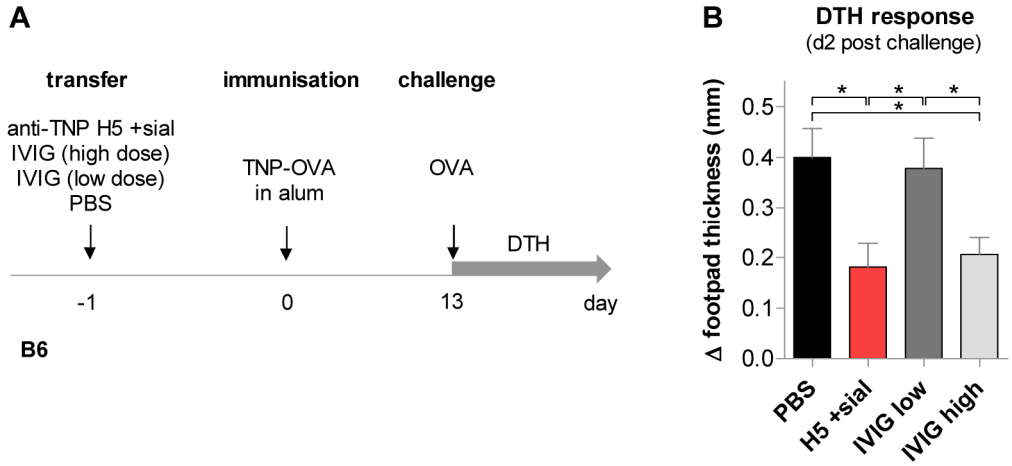
injected into the left footpad. **(C)** OVA-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice. Day 5 post challenge was shown as representative data for the DTH response over time. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of two independent experiments is shown. (*$P < 0.05$).

Anti-Thy-1.1 IgG1 antibodies did not bind TNP, which was confirmed by ELISA (*Figure 3.14B*). While antigen-specific sialylated anti-TNP IgG1 (70% sialylation) (*Figure 3.14A*) was sufficient to attenuate a DTH response, sialylated Thy1.1-specific IgG1 antibodies (both 25 mg/kg bogy weight) failed to inhibit a TNP-specific immune reaction and thus could not suppress an antigen-specific DTH response (*Figure 3.15C*).

In summary, these results indicate that sialylated monoclonal IgG1 antibodies have the ability to protect from inflammatory DTH responses in an antigen-specific manner independently of FcγRIIB.

To compare the inhibitory potential of antigen-specific sialylated antibodies to the immunosuppressive effect of IVIg in a DTH response, B6 mice were treated with sialylated anti-TNP IgG1 (70% sialylation) (*Figure 3.14A*) and different amounts of IVIg (*Figure 3.16A*). It has been shown previously, that IVIg exhibits immunosuppressive effects when administered at high doses (Nimmerjahn and Ravetch 2008).

Sialylated anti-TNP IgG1 antibodies (25 mg/kg of body weight) and high doses of IVIg ($IVIG^{high}$, 2 g/kg of body weight) significantly attenuated an antigen-specific inflammatory DTH response, as measured by reduced antigen-specific footpad swelling (*Figure 3.16B*). Furthermore, antigen-specific and IVIg-mediated unspecific inhibitory effects were accompanied by reduced levels of antigen-specific IgG2c serum antibodies, which were generated upon TD immunisation in untreated control mice (*Figure 3.16C*). In contrast, mice treated with low amounts of IVIg ($IVIg^{low}$, 112 mg/kg of body weight) were not protected from a DTH response (Figure 16B) and the generation of antigen-specific serum IgG2c antibody levels (*Figure 3.16C*). While antigen-specific sialylated IgG antibodies mediate their anti-inflammatory effects already at low doses (5-25 mg/kg of body weight), IVIg exhibits its immunosuppressive potential only at 80-400-fold higher doses (2 g/kg of body weight), indicating that antigen-specific inhibitory effects are induced more efficiently.



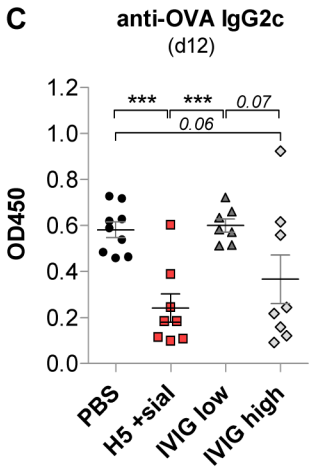


Figure 3.16: Low-dose administration of antigen-specific sialylated monoclonal IgG1 antibodies suppresses a DTH response comparable to high-dose IVIg treatment. (A) Timescale of the experimental approach depicting the immunisation protocol: One day prior to immunisation with 100 µg TNP-OVA in alum, PBS (n=8), 500 µg *in vitro* sialylated TNP-specific H5 IgG1 antibodies (H5 +sial) (n=8), 2.25 mg IVIG (IVIG low) (n=7) or 40 mg IVIG (IVIG high) (n=8) was transferred i.v. into B6 mice. IVIG high treatment was repeated on day 4 and 11. On day 13, the DTH reaction was induced by local application of 37.5 µg OVA into the right footpad. As a control for unspecific swelling, PBS was injected into the left footpad. **(B)** OVA-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice. Day 2 post challenge was shown as representative data for the DTH response over time. Bar graphs show the mean value with standard error of the mean (SEM) for each group. One representative out of two independent experiments is shown. **(C)** Serum anti-OVA IgG2c antibody levels as determined by ELISA on day 12. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$)

3.2.3 Sialylated monoclonal IgG antibodies induce peripheral tolerance

To further investigate whether sialylated monoclonal IgG antibodies have the ability to induce tolerance to TD antigens, the applicatipon of immune complexes (ICs) containing the respective antigen was evaluated on subsequent inflammatory immune responses in B6 mice (*Figure 3.17A*).

Pre-immunisation of mice with ICs containing sialylated TNP-specific IgG1 antibodies (70% sialylation) (*Figure 3.14A*) (4 mg/kg bodyweight) and the respective antigen 14 days before TD immunisation attenuated antigen-specific footpad swelling in a DTH response (*Figure 3.17B*) and furthermore reduced the development of anti-TNP IgG2b antibodies (*Figure 3.17C*). B6 mice pre-immunised with non-sialylated anti-TNP IgG1 antibodies containing ICs showed neither a reduction of antigen-specific footpad swelling in a DTH response (*Figure 3.17B*) nor diminished TNP-specific IgG2b serum antibody levels (*Figure 3.17C*).

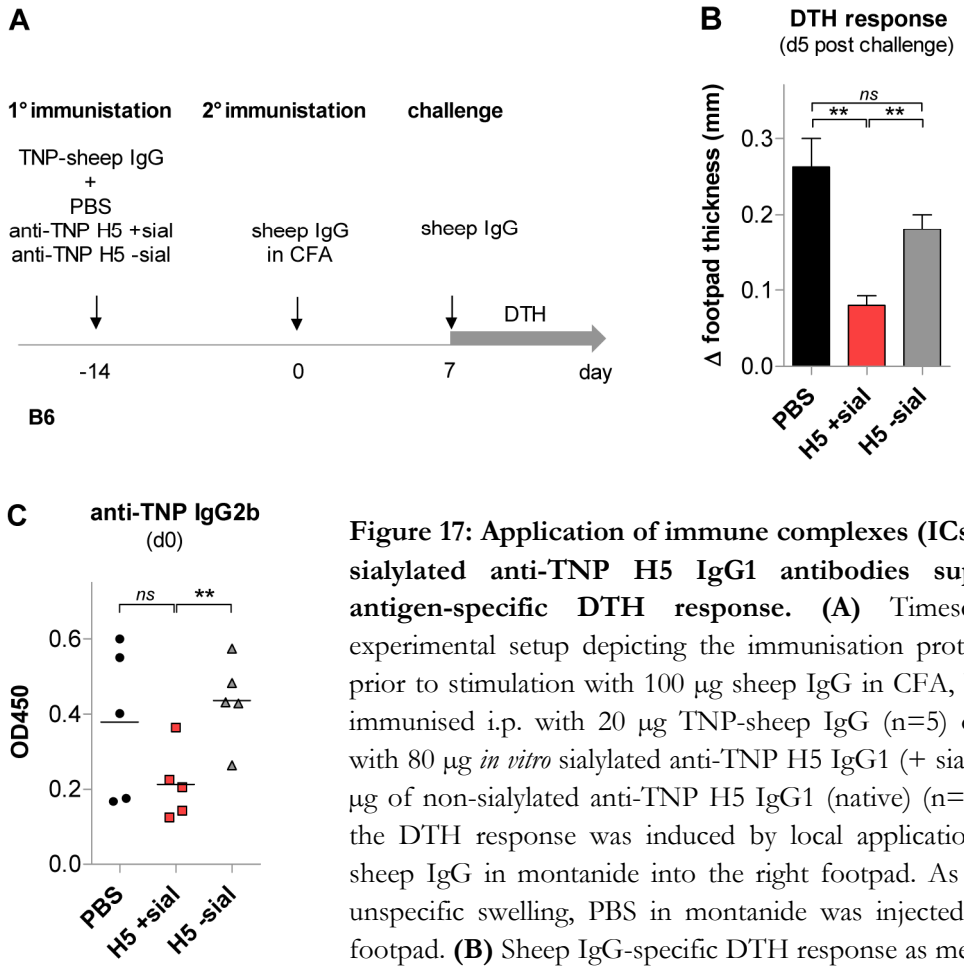


Figure 17: Application of immune complexes (ICs) containing sialylated anti-TNP H5 IgG1 antibodies suppresses an antigen-specific DTH response. (A) Timescale of the experimental setup depicting the immunisation protocol: 14 days prior to stimulation with 100 µg sheep IgG in CFA, B6 mice were immunised i.p. with 20 µg TNP-sheep IgG (n=5) or complexed with 80 µg *in vitro* sialylated anti-TNP H5 IgG1 (+ sial) (n=5) or 80 µg of non-sialylated anti-TNP H5 IgG1 (native) (n=5). On day 7, the DTH response was induced by local application of 12.5 µg sheep IgG in montanide into the right footpad. As a control for unspecific swelling, PBS in montanide was injected into the left footpad. **(B)** Sheep IgG-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice. Day 5 post challenge was shown as representative data for the DTH response over time. Bar graphs show the mean value with standard error of the mean (SEM) for each group. **(C)** Serum anti-TNP IgG2b antibody levels as determined by ELISA on day 0 before second immunisation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. ($P > 0.05$ and $^{**}P < 0.01$).

The tolerogenic potential of sialylated ICs could be confirmed in the nephrotoxic nephritis model (*Figure 3.18A*). Pre-immunisation with ICs containing sialylated anti-TNP IgG1 antibodies (11% sialylation) (*Figure 3.18A*) protected RIIB$^{-/-}$ mice from nephritis-mediated mortality (*Figure 3.18C*). By contrast, ICs containing non-sialylated anti-TNP IgG1 antibodies (< 1% sialylation) (*Figure 3.18A*) were not sufficient to prevent mice from mortality. Here, nephritis-associated mortality was comparable to untreated positive control mice (*Figure 3.18C*).

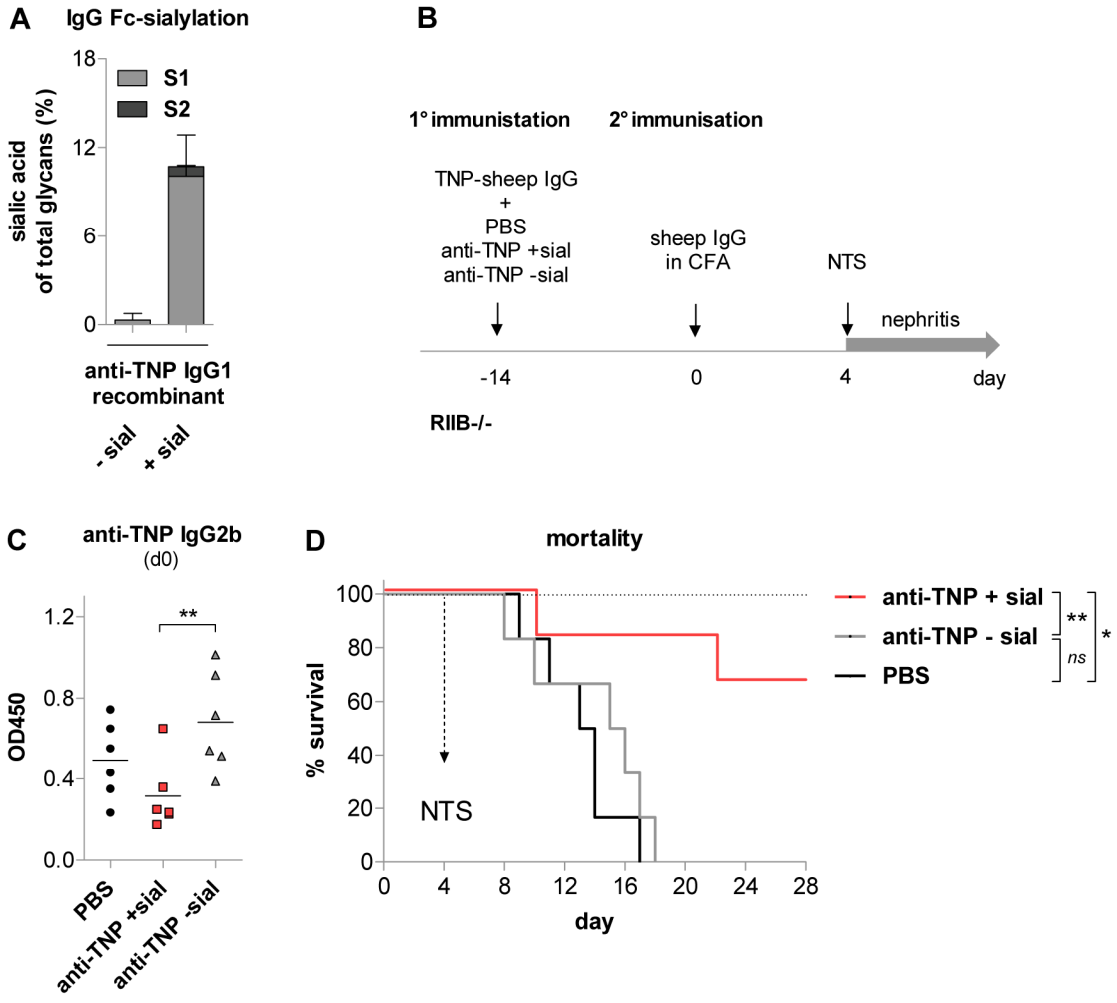


Figure 3.18: Application of immune complexes (ICs) containing sialylated recombinant anti-TNP IgG1 antibodies protect from nephritis independently of FcγRIIB. Recombinant anti-TNP IgG1 antibodies produced in 293 T HEK cells were sialylated enzymatically. **(A)** Frequencies of Fc sialic acid moieties (one or two murine Neu5Gc; S1 and S2) of recombinant sialylated anti-TNP IgG1 (+ sial) and non-sialyated anti-TNP IgG1 (- sial) antibodies as determined by MALDI-TOF mass spectrometry. Bar graphs indicate the frequency of glycan structures with 1 and 2 murine Neu5Gc sialic acid (S1, S2) residues. The mean value with standard error of the mean (SEM) is shown for each group. **(B)** Timescale of the experimental setup depicting the

immunisation protocol: RIIB$^{-/-}$ mice were immunised i.p. with 20 μg TNP-sheep IgG in PBS (n=6) or complexed with either 80 μg of sialylated recombinant anti-TNP IgG1 (+ sial) (n=5) or 80 μg of non-sialylated anti-TNP IgG1 (- sial) (n=4) on day -14. Subsequent to immunisation with 100 μg sheep IgG in CFA on day 0, nephritis was induced by i.v. injection of 100 μl NTS on day 4. **(C)** Serum anti-TNP IgG2b antibody levels as determined by ELISA on day 0 before second immunisation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. **(D)** Kaplan-Meier survival curve for mice treated with NTS. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$ and $**P < 0.01$).

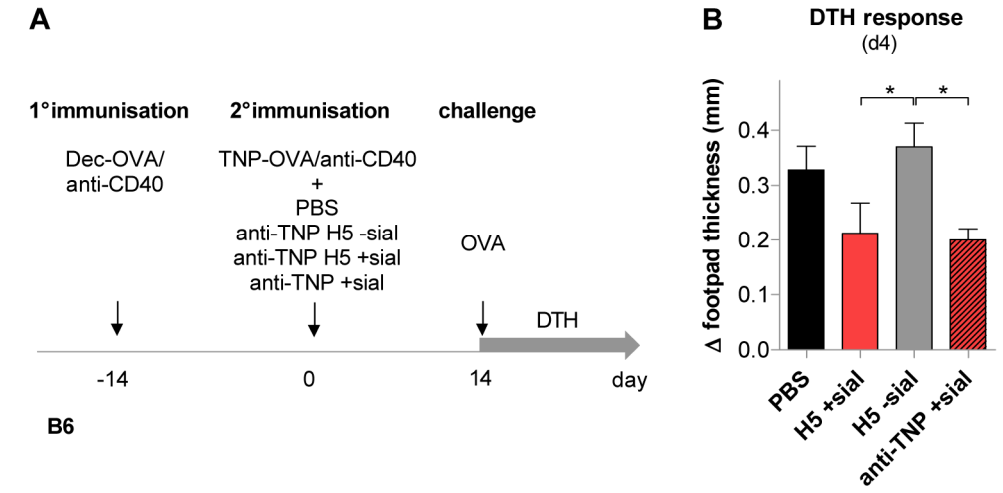
However, ICs containing antigen-specific sialylated IgG1 antibodies induce peripheral tolerance to TD antigens and protect from inflammatory immune responses independent of FcγRIIB.

3.2.4 Monoclonal sialylated IgG antibodies suppress ongoing inflammatory immune responses

To further examine whether sialylated antigen-specific IgG1 antibodies can also inhibit ongoing inflammatory immune reactions, already immunised B6 mice were treated with the antibodies two weeks after the onset of the inflammatory immunisation (*Figure 3.19A*). Here, the inhibitory potential of low sialylated recombinant anti-TNP-IgG1 antibodies (11% sialylation) (*Figure 3.18A*) and high sialylated hybridoma-derived anti-TNP IgG1 H5 antibodies (70% sialylation) (*Figure 3.14A*) was analysed in a DTH reaction.

The data clearly show, that the application of both, recombinantly expressed or hybridoma-derived, sialylated TNP-specific IgG1 antibodies (4 mg/kg of body weight) suppressed a DTH response determined by reduced antigen-specific footpad swelling (*Figure 3.19B*). By contrast, non-sialylated anti-TNP IgG1 H5 antibodies (4 mg/kg of body weight) did not exhibit any inhibitory effects on the DTH response. The antigen-specific footpad swelling was pronounced and comparable to the observed footpad swelling of untreated positive control mice (*Figure 3.19B*). Moreover, the administration of sialylated anti-TNP H5 IgG1 antibodies significantly reduced the generation of anti-OVA IgG2b antibodies in the serum (*Figure 3.19C*), whereas the application of non-sialylated anti-TNP IgG1 antibodies did not diminish OVA-specific IgG2b serum antibody titers (*Figure 3.19C*).

In summary, the data reveal that even low sialylated antigen-specific IgG1 antibodies have the potential to suppress ongoing inflammatory immune responses and protect from subsequent DTH reactions.



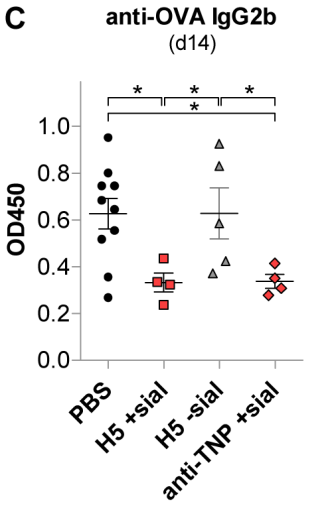


Figure 3.19: Therapeutic administration of sialylated antigen-specific IgG1 inhibits ongoing inflammatory immune response and suppresses a DTH reaction. (A) Timescale of the experimental setup depicting the immunisation protocol: TD immunity to OVA was induced in B6 mice by i.p. injection of 5 µg Dec-OVA/50 µg anti-CD40 on day -14. On day 0, pre-sensitised mice were treated i.p. with anti-CD40 and 20 µg TNP-OVA alone (n=9) or complexed with 80 µg of native non-sialylated anti-TNP H5 IgG1 (n=5) or *in vitro* sialylated anti-TNP H5 IgG1 (n=4) or sialylated recombinant anti-TNP IgG1 (+ sial). On day 14, the DTH response was induced by local application of 37.5 µg OVA in montanide into the right footpad. As a control for unspecific swelling, PBS was injected into the left footpad. **(B)** OVA-specific DTH response as measured by the differences (Δ) in footpad thickness (mm) between the right and the left footpad of indicated mice. Day 4 post challenge was shown as representative data for the DTH response over time. Bar graphs show the mean value with standard error of the mean (SEM) for each group. **(C)** Serum anti-OVA IgG2b antibody levels as determined by ELISA on day 14 post immunisation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. (*$P < 0.05$).

3.2.5 Monoclonal sialylated IgG1 antibodies inhibit IL-6 secretion by dendritic cells

Recently, it has been published that high amounts of IVIg influence the activation of immune cells such as regulatory macrophages in the marginal zone (Nimmerjahn and

Ravetch 2008); Anthony et al., 2008b). Additionally, dendritic cells (DCs) were shown to inhibit the activation of T cells independently of FcγRIIB (Aubin et al., 2010; Aubin, Lemieux et al. 2010).

To better understand the mechanism by which antigen-specific sialylated antibodies can influence T and B cell responses *in vivo*, the influence of sialylated IgG1 antibody immune complexes (ICs) on DCs was determined *in vitro* (*Figure 3.20*). Thus, the IL-6 secretion of RIIB$^{-/-}$ bone marrow-derived dendritic cells (BMDCs) was analysed subsequent to stimulation with ICs containing sialylated or non-sialylated anti-TNP IgG1 H5 antibodies (40 µg/ml) and TNP-OVA. 16 hours post incubation of BMDCs with ICs, IL-6 secretion was determined in the supernatants.

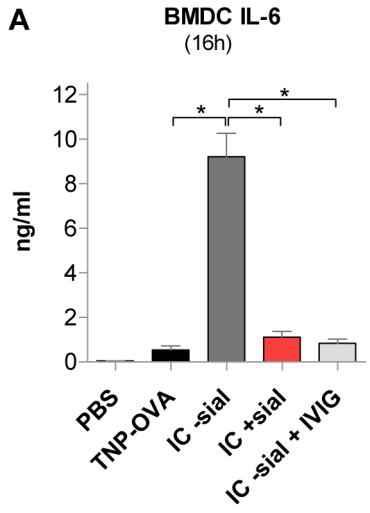


Figure 3.20: Immune complexes containing antigen-specific sialylated IgG1 antibodies inhibit IL-6 secretion of BMDCs independently of FcγRIIB. (A) IL-6 secretion as measured in the supernatants of BMDCs from RIIB-/- mice incubated for 16 hours with ICs containing sialylated (IC H5 +sial) or non-sialylated (IC H5 -sial) anti-TNP IgG1 H5 antibodies (20 µg/ml) and IVIg (10 mg/ml). Results shown are representative for three independent experiments. (*P < 0.05).

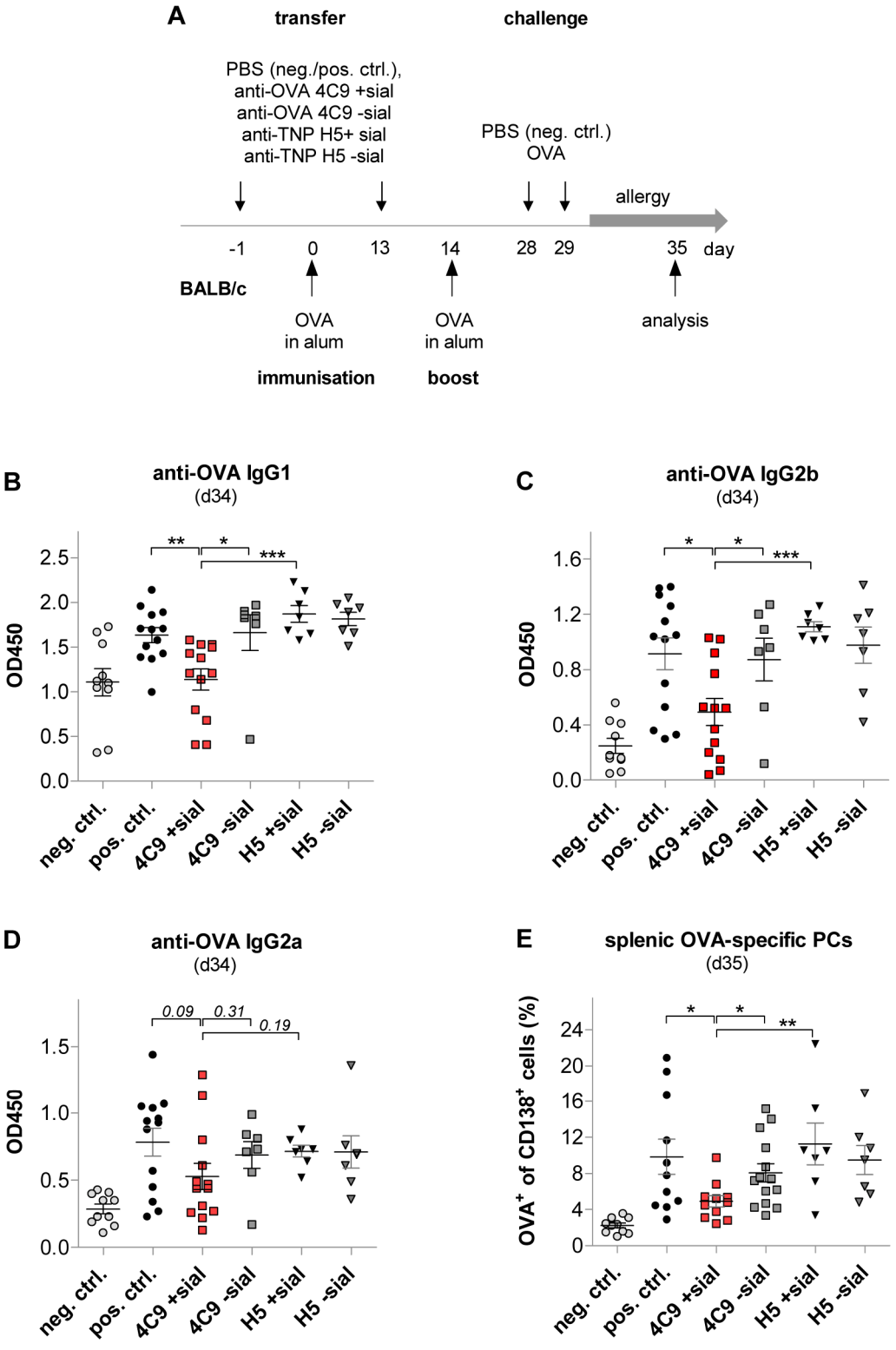
However, non-sialylated IgG1 ICs initiated the production of IL-6 in BMDCs, whereas sialylated IgG1 ICs were not able to induce the secretion of IL-6 (*Figure 3.20A*). Subsequent to incubation with non-sialylated IgG1 ICs, the addition of high dose IVIg (10 mg/ml) could significantly block IL-6 secretion in BMDCs (*Figure 3.20B*). These data indicate that ICs containing antigen-specific sialylated IgG1 antibodies prevent BMDCs from IL-6 secretion and therefore might contribute to inhibition of DC maturation.

3.3 Monoclonal siylalated IgG antibodies protect from pathology in the allergic airway inflammation mouse model

So far, I could show that monoclonal antigen-specific sialylated IgG1 antibodies suppress inflammatory immune responses in a DTH and a nephrotoxic nephritis mouse model.

To further strengthen the anti-inflammatory effects of antigen-specific sialylated IgG1 antibodies, we additionally investigated their impact in an OVA-induced allergic airway inflammation model in BALB/c mice (*Figure 3.21*). Allergic airway inflammation was induced by sensitisation with OVA in alum and subsequent intra-nasal (i.n.) challenge with OVA. To investigate the protective effect, mice were treated with antigen-specific *in vitro* sialylated or de-sialylated anti-OVA IgG1 antibodies (clone 4C9) (*Figure 3.13A*) or with *in vitro* sialylated or non-sialylated anti-TNP IgG1 antibodies (clone H5) (*Figure 3.14A*) before and after sensitisation (*Figure 3.21A*). The obtained data clearly demonstrate that only sialylated anti-OVA IgG1 antibodies (10 mg/kg of body weight) displayed the abilty to prevent from allergic airway inflammation, which is characterised by infiltration of eosinophils (*Figure 3.21F*) and CD4+ Th2 cells (*Figure 3.21G*) into the lung. Furthermore, the generation of OVA-specific serum IgG1 (*Figure 3.21B*), IgG2a (IgG2c, B6) (*Figure 3.21C*) and IgG2b antibody responses (*Figure 3.21D*) was diminished, correlating with reduced development of OVA-specific splenic PC (*Figure 3.21E*). De-sialylated anti-OVA as well as sialylated and non-sialylated anti-TNP IgG1 antibodies (10 mg/kg of body weight) were not capable of suppressing eosinophils (*Figure 3.21F*) and Th2 cell infiltration (*Figure 3.21G*) into the lung and reducing anti-OVA IgG1, IgG2b and IgG2a (IgG2c, B6) antibodies (*Figure 3.21B-D*). Haematoxylin and eosin (H+E) stained lung sections revealed that allergic mice (pos.ctrl.) display highly increased inflammatory infiltrates observed in peribronchial and perivascular areas compared to healthy mice (neg. ctrl.) (*Figure 3.21H*). Furthermore, hypertrophy of the smooth muscle cell layer was observed when allergy was induced. However, only the treatment of mice with sialylated anti-OVA IgG1 antibodies prevented the infiltration of inflammatory effector cells to bronchi and bronchioles as indicated by decreased number of infiltrates and the lack of hypertrophy of the smooth muscle cell layer (*Figure 3.21H*). In contrast, non-sialylated anti-OVA IgG1 did not protect from allergy-associated pathology (*Figure 3.21H*).

In summary, only antigen-specific sialylated IgG antibodies exhibit the ability to protect from allergy-associated pathology in the allergic airway inflammation model.


A
transfer
challenge
PBS (neg./pos. ctrl.),
anti-OVA 4C9 +sial
anti-OVA 4C9 -sial
anti-TNP H5+ sial
anti-TNP H5 -sial
PBS (neg. ctrl.)
OVA
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-1
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13
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28
29
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BALB/c
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anti-OVA IgG1
(d34)
OD450
C
anti-OVA IgG2b
(d34)
OD450
D
anti-OVA IgG2a
(d34)
OD450
0.09
0.31
0.19
E
splenic OVA-specific PCs
(d35)
OVA+ of CD138+ cells (%)
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pos. ctrl.
4C9 +sial
4C9 -sial
H5 +sial
H5 -sial

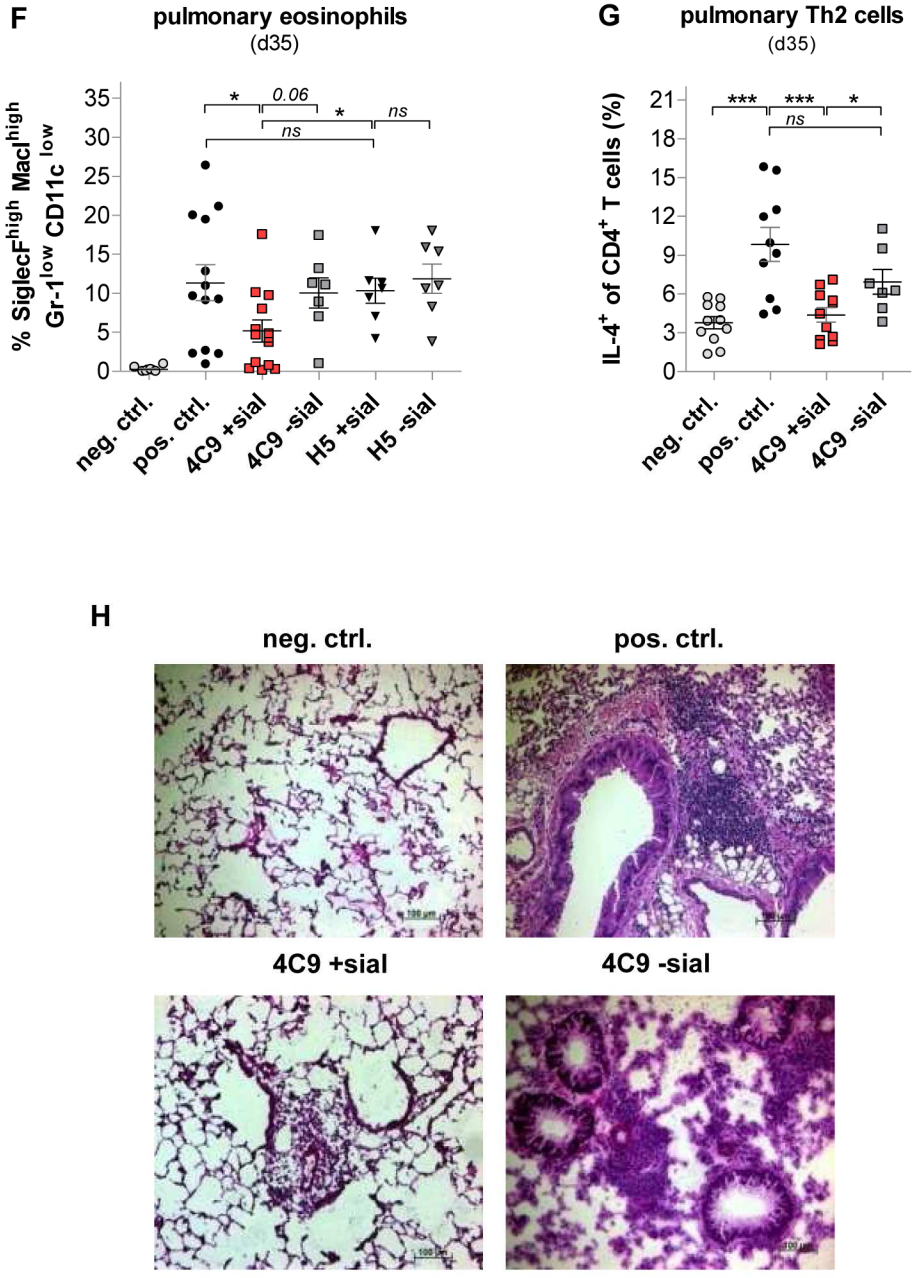

Figure 3.21: Antigen-specific sialylated, but not de-sialylated monoclonal IgG1 antibodies protect from allergic airway inflammation. (A) Schematic plan of the experimental approach followed in B-H. Allergic airway inflammation was induced by i.p. sensitisation of BALB/c mice with 50 µg of OVA in alum on day 0 and 14 followed by intra-nasal (i.n.) challenge with OVA on day 28 and 29. On day -1 and 13 the mice were injected i.v. with PBS (pos. ctrl.), 200 µg of *in vitro* sialylated anti-OVA IgG1 (anti-OVA 4C9 +sial), 200 µg of sialidase-treated anti-OVA IgG1 (anti-OVA 4C9 -sial), 200 µg of *in vitro* sialylated anti-TNP IgG1 (anti-TNP H5 +sial), or 200 µg of native non-sialylated anti-TNP IgG1 (anti-TNP H5 -sial). Negative control (neg. ctrl.) mice received OVA in alum and i.n. challenged with PBS. Serum anti-OVA IgG1 **(B)**, IgG2b **(C)** and IgG2a

(IgG2c, B6) **(D)** antibody levels as determined by ELISA on day 34 post stimulation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. **(E)** Frequencies of OVA-reactive cells from CD138+ splenic PCs as measured by flow cytometry analysis on day 35. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. Frequencies of lung **(F)** SiglecF^{high}MacI^{high}Gr-1^{low}CD11c^{low} eosinophils and **(G)** IL-4+ cells from CD4+ T cells as measured by flow cytometry analysis on day 35. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. **(H)** Histological analysis of hematoxylin and eosin (H+E) stained lung sections of indicated groups. Cell nuclei appear as purple dots, cytoplasm as violet structures. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$)

To verify that the observed anti-inflammatory effect of sialylated anti-OVA IgG1 antibodies in the OVA-induced allergic airway inflammation model is independent of the inhibitory FcγRIIB, the impact of sialylated anti-OVA IgG1 antibodies was further investigated in RIIB$^{-/-}$ mice (*Figure 3.22A*). To compare the inhibitory potential, OVA sensitised mice were treated with either non-sialylated or *in vitro* sialylated anti-OVA IgG1 antibodies (clone 4C9) (*Figure 3.13A*). Here, only sialylated, but not the non-sialylated anti-OVA IgG1 antibodies reduced the infiltration of eosinophils into the lung (*Figure 3.22C*) and suppressed the generation of anti-OVA IgG1 and IgG2c serum antibodies (*Figure 3.22B*).

Thus, the data show that sialylated, but not the non-sialylated antigen-specific IgG1 antibodies protect from pathology in the allergic airway inflammation mouse model independently of the inhibitory FcγRIIB.

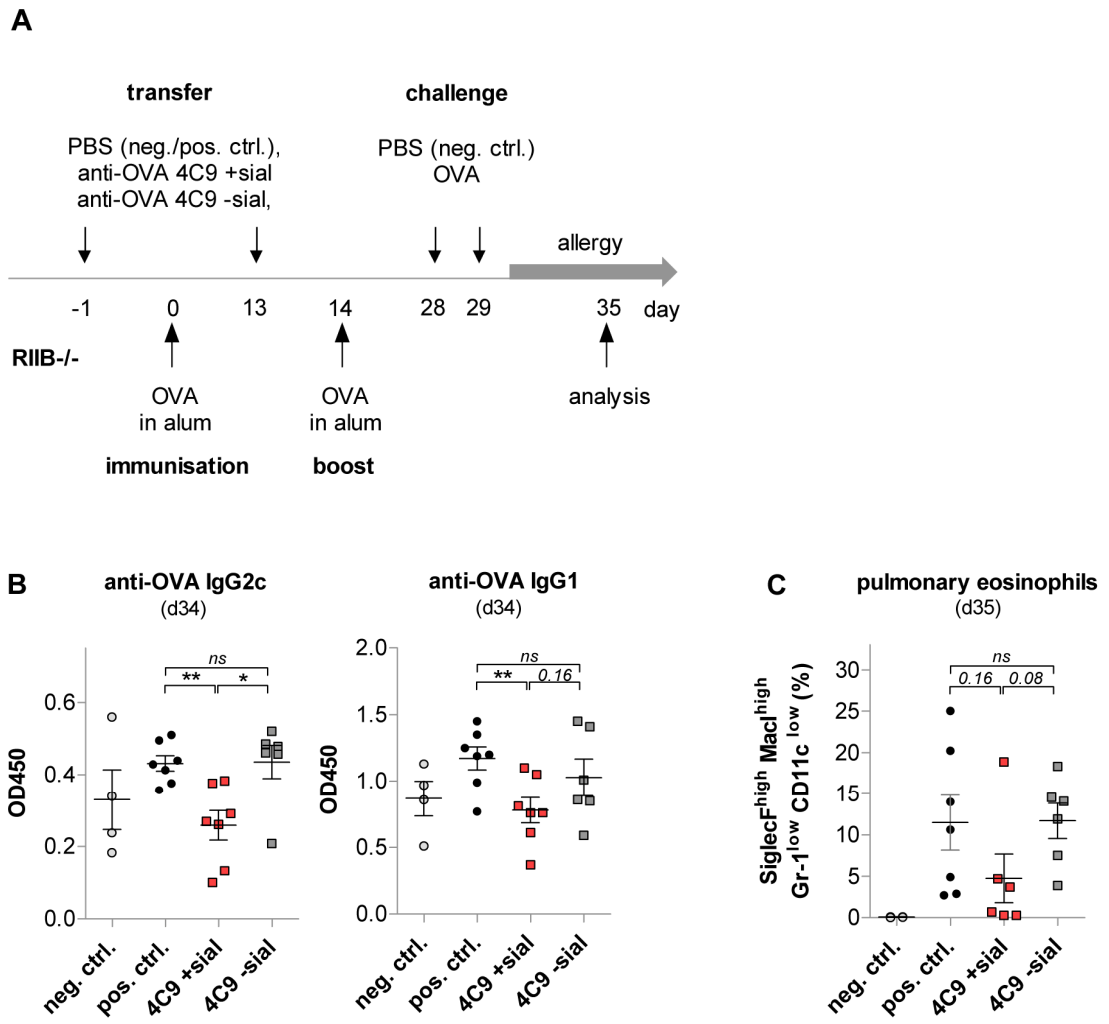

Figure 3.22: Antigen-specific sialylated, but not de-sialylated monoclonal IgG1 antibodies protect from allergic airway inflammation independently of FcγRIIB. (A) Schematic plan of the experimental approach depicting the immunisation protocol: Allergic airway inflammation was induced by i.p. sensitisation with 50 µg of OVA in alum on day 0 and 14 and i.n. application of OVA on day 28 and 29 in RIIB-/- mice. On day -1 and 13 the mice were injected i.v. with PBS (pos. ctrl.), 200 µg of in vitro sialylated anti-OVA IgG1 (anti-OVA 4C9 +sial), or 200 µg sialidase treated anti-OVA IgG1 (anti-OVA 4C9 -sial). Negative control (neg. ctrl.) mice received OVA in alum and mice were challenged i.n. with PBS. **(B)** Serum anti-OVA IgG1 and IgG2c antibody levels as determined by ELISA on day 34 post stimulation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. **(C)** Frequencies of lung SiglecFhighMacIhighGr-1lowCD11clow eosinophils as measured by flow cytometry analysis on day 35 post stimulation. Symbols represent data from individual animals. The mean value with standard error of the mean (SEM) is shown for each group. One representative out of two independent experiments is shown. ($P > 0.05$, $*P < 0.05$ and $**P < 0.01$).

4 Discussion

Immune complexes consisting of IgG antibodies exhibit different immune regulatory functions, including strong enhancement and suppression of inflammatory immune responses (Heyman 2000; Nimmerjahn and Ravetch 2006). These distinct activities of IgG antibodies can be explained by selective binding to activating and inhibitory Fcγ-receptors on immune cells (Nimmerjahn and Ravetch 2005), but it has also been reported that the glycosylation pattern is crucial for mediating IgG effector functions (Parekh, Dwek et al. 1985; Albert, Collin et al. 2008; Huhn, Selman et al. 2009). In particular, IgG Fc-sialylation has been suggested to be involved in anti-inflammatory activities and thus, is associated with the maintenance of tolerance. By contrast, de-sialylation of IgGs leads to a shift from anti-inflammatory to pro-inflammatory effector functions and thus, is associated with loss of tolerance in various autoimmune diseases. However, regulation and dynamics of IgG antibody sialylation during humoral immune responses had not been described before. This work provides new insight into the process of IgG antibody sialylation and further demonstrates that antigen-specific sialylated IgG antibodies mediate anti-inflammatory effects and can be used to inhibit inflammatory immune responses in an antigen-specific manner.

4.1 Induction of peripheral tolerance by TI and TD B cell activation

It has been shown that there are several distinct pathways leading to the activation of B cells, the T cell independent (TI) and the T cell dependent (TD) pathway (Mond, Vos et al. 1995; McHeyzer-Williams, McHeyzer-Williams et al. 2000; McHeyzer-Williams, Malherbe et al. 2006). Once activated, B cells proliferate and differentiate into plasma cells, which secrete large amounts of soluble IgM and IgG antibodies (Ollila and Vihinen 2005). Most of all notable antigens are derived from proteins and thus, activate B cells with the help of CD4 T cells. While high doses of T cell dependent (TD) antigens induce regulatory T cells (Tregs) and peripheral tolerance in the steady state (Steinman, Hawiger et al. 2003;

Yamazaki, Iyoda et al. 2003; Kretschmer, Apostolou et al. 2005), delivering TD antigens together with inflammatory co-stimuli, such as TLR and CD40 ligands, leads to the establishment of inflammatory B and T cell responses. By contrast, TI antigens activate B cells without MHC class II-restricted CD4 T cell help, either by TI type 2 (TI-2) antigens crosslinking BCRs or TI type 1 (TI-1) co-stimulating BCR and TLRs. During the last years, the role of TI B cell activation has been highly discussed as there are data revealing opposing inflammatory and inhibitory effects for TI antigens. TI B cell activation via TLR-MyD88 signalling has been demonstrated to play a major role in the development of IgG autoantibodies (Berland, Fernandez et al. 2006; Christensen, Shupe et al. 2006; Ehlers, Fukuyama et al. 2006; Lanzavecchia and Sallusto 2007; Herlands, Christensen et al. 2008; Tsao, Jiao et al. 2008). In contrast, TI type 2 (TI-2) antigens, such as polysaccharides, have been demonstrated to induce IgG antibodies, which suppress a second challenge with the same TI-2 antigen, including the inhibition of IgG antibody responses, independently of FcγRIIB (Brodeur and Wortis 1980; Sverremark and Fernandez 1998; Heyman 2003). Recent evidence suggests that not only Tregs but also B cells can exhibit immune regulatory functions. However, it has remained to be investigated whether TI and TD B cell activation under pro-and non-inflammatory conditions results in the generation of different IgG antibodies with rather tolerogenic or pathogenic properties. To address this issue, TD and TI immunisation under pro-and non-inflammatory conditions was evaluated on the generation of IgG antibodies and their impact on inflammatory immune responses. Consistent with previous observations, TD antigens together with TLR co-stimulation or other co-stimulatory molecules lead to B and T cell responses including the generation of IgG antibodies and the establishment of antigen-specific inflammatory immune reactions. Inflammatory TD immunisation initiates the induction of a T cell-mediated DTH response or the onset of nephritis in the antibody-mediated nephrotoxic nephritis mouse model. Pre-immunisation with TI-1 antigen TNP-LPS and TI-2 antigen TNP-Ficoll inhibits subsequent TD inflammatory immune responses and prevents mice from an antigen-specific DTH reaction as well as from the development of disease symptoms in the nephrotoxic nephritis model. However, TNP-LPS and TNP-Ficoll lead to the generation of IgG antibodies and the induction of peripheral tolerance. As TI antigens have been described to influence B cell responses, it is likely that the generated IgG antibodies participate in the process of mediating the observed immunosuppressive effects. While TD immunisation with co-stimulation leads to inflammatory immune responses, TD

immunisation under non-inflammatory conditions has been shown to induce peripheral tolerance including the development of regulatory T cells (Tregs) and the deletion of antigen-specific T cells (Verhasselt, Vosters et al. 2004); Kretschmer et al., 2005; Steinman et al. 2003; Yamazaki et al., 2008). However, immunisation with Dec-205-OVA (Dec-OVA), which delivers TD OVA to dendritic cells (DCs) more efficiently than immunisation with pure OVA (Steinman, Hawiger et al. 2003; Kretschmer, Apostolou et al. 2005; Boscardin, Hafalla et al. 2006; Yamazaki, Dudziak et al. 2008), protects mice from subsequent TD inflammatory immune responses in a DTH reaction. Although TD and TI antigens activate B cells differently, both lead to the induction of peripheral tolerance under non-inflammatory conditions. Since the generation of IgG antibodies is an obvious common feature of TD and TI immunisation, the functional properties of these IgG antibodies were further investigated. It has been described, that IgG antibodies fulfill important immune regulatory functions and can exhibit either inflammatory or anti-inflammatory effects, depending on their isotype and glycosylation pattern (Nimmerjahn, Bruhns et al. 2005; Kaneko, Nimmerjahn et al. 2006; Anthony, Nimmerjahn et al. 2008; Nimmerjahn and Ravetch 2008; Nimmerjahn and Ravetch 2008). Antibodies from the IgG2b and IgG2c (IgG2a in BALB/c) isotype are supposed to act pro-inflammatory. This could be shown for pathogenic autoantibodies, e.g. in lupus mouse models, which are primarily from the IgG2b and the IgG2c isotype and deposit in form of immune complexes in the kidney leading to inflammation (Ehlers, Fukuyama et al. 2006). IgG2b and IgG2c antibodies display high affinities for activating Fcγ-receptors (FcγRI, FcγRIII, FcγRIV), which activate pro-inflammatory signalling pathways and finally lead to pathology. With its high affinity to bind to the inhibitory FcγRIIB, IgG1 is the isotype, which is mainly associated with anti-inflammatory effects and protection (Nimmerjahn and Ravetch 2005). However, it remains to be investigated whether the presence of IgG autoantibodies generated in response to TI-1 B cell activation via TLR 7 or TLR 9 co-stimulation reflects inflammatory lupus disease and is associated with mortality in these models. In this work, it could be shown that immunisation with TD and TI antigens leads to the generation of distinct prevalent IgG isotypes. While inflammatory TD immunisation induces the generation of antigen-specific IgG1, IgG2b and IgG2c antibodies, tolerogenic immunisation with Dec-OVA leads to the development of predominantly IgG1 antibodies. However, TI-1 antigen (TNP-LPS) predominantly induces antigen-specific IgG2b and IgG2c antibodies, whereas TI-2 antigen (TNP-Ficoll) additionally leads to the development

of IgG1 antibodies. This observation demonstrating that tolerogenic and pathogenic immunisation with TD and TI antigens leads to the development of the same IgG antibody isotypes implies that the isotype-specific effector function of serum IgG antibodies is thus not the only issue to predict the quality of an immune response. This is in line with previous observations, which reveal that the glycosylation pattern of IgG antibodies plays an important role in mediating IgG dependent effector functions (Parekh, Dwek et al. 1985; Parekh, Dwek et al. 1988). In the 1980's, Parekh *et al.* reported that patients with rheumatoid arthritis show IgG serum antibodies which differ from the glycosylation pattern of healthy individuals. While most of all IgG antibodies in healthy donors display galactose, more than 60% of the IgG antibodies from patients with chronic rheumatoid arthritis carry de-galactosylated glycan structures. Moreover, terminal sialic acid is completely lacking in glycan structures of rheumatoid arthritis patients, whereas 15% of all IgG antibodies isolated from healthy individuals are sialylated. It has been observed that sialylation of Fc-parts reduces the affinity of IgG antibodies to bind to Fcγ-receptors and thereby heighten the threshold of activation by IgG2b and IgG2c antibodies (Parekh, Dwek et al. 1985). However, sialylation of steady state IgG antibodies ensures the maintenance of the anti-inflammatory state in healthy individuals. When challenged upon pathogenic stimulation, antigen-specific IgG antibodies switch to less sialylated IgG isoforms and provide pro-inflammatory effector functions (Nimmerjahn and Ravetch 2005; Kaneko, Nimmerjahn et al. 2006).

4.2 Peripheral tolerance is associated with the generation of sialylated antigen-specific IgG antibodies

In general, it has been shown that sialylated IgG antibodies reflect the steady state situation in a healthy organism. Stimulation with an antigen leads to the development of non-sialylated antibodies in newly generated plasma cells and switches the immune system to an inflammatory state, which can trigger autoimmune diseases (Durandy, Kaveri et al. 2009). Considering the differential glycosylation pattern of IgG antibodies in healthy individuals and autoimmune disease patients, analysis of the Fc-sialylation from IgG antibodies revealed that IgG antibodies induced upon tolerance-associated immunisation display sialylated glycan structures including one or two terminal sialic acid residues. However, sialylation of antigen-specific IgG antibodies occurs independently of the type of antigen

(TI and TD) used for immunisation as well as independent of the predominant generated IgG isotypes. Thus, tolerogenic effects of TI and TD immunisation are associated with the development of antigen-specific sialylated IgG antibodies. By contrast, TD antigens when co-administered with CFA- or other co-stimuli induce inflammatory immune reactions, leading to the generation of non-sialylated IgG antibodies. Thus, my findings imply that non-sialylated IgG antibodies are associated with inflammation and immunity, whereas sialyation is crucial to mediate tolerogenic effects. However, regulation and dynamics of IgG antibody sialylation during humoral immune responses have not been described yet. In my work I could demonstrate, that tolerogenic immunisation with TD and TI antigen leads to the development of antigen-specific plasma cells expressing high levels of α-2,6-sialyltransferase, an enzyme that transfers sialic acid to *N*-glycans of nascent glycoproteins. Moreover, α-2,6-sialyltransferase expression levels correlate with the generation of antigen-specific sialylated IgG antibodies after tolerance induction. Levels of antigen-specific IgG sialylation and α-2,6-sialyltransferase expression are comparable to those observed for steady state IgG antibodies and PCs of untreated control mice. These findings support previous suggestions stating that steady-state autoreactive and polyreactive IgG antibodies contained in the serum might play a crucial role in the maintenance of self-tolerance (Egner 2000; Tiller, Tsuiji et al. 2007). By contrast, the generation of non-sialylated IgG antibodies during inflammatory TD immune responses is accompanied by the development of PCs expressing only low levels of α-2,6-sialyltransferase. The correlation of IgG sialylation and α-2,6-sialyltransferase expression implies that the process of IgG Fc-sialylation is regulated by different expression of the sialic acid transferring enzyme α-2,6-sialyltransferase. Investigating the influence of CFA on TI immunisation, I could show that sialylation of IgG antibodies was reduced compared to TI IgG antibodies generated in the absence of CFA, but still increased compared to antigen-specific IgG antibodies induced upon inflammatory TD immunisation. Only CFA with higher mycobacterial content (CFA^{high}) promotes efficient de-sialylation of TI antigen-specific serum IgG antibodies. This is in line with the findings demonstrating that only CFA^{high}, but not CFA, has the ability to suspend the tolerogenic potential of TI induced immune responses, as it abrogated the protective effect in a DTH response. Thus, stronger co-stimuli are needed to initiate the process, which leads to the generation of non-sialylated IgG antibodies during TI immunisation. This draws on previous publication reporting that high contents of *Mycobacterium tuberculosis* is required to induce the expression of pro-inflammatory cytokines and to trigger collagen-

induced arthritis in non-susceptible mouse strains (Kai, Shibuya et al. 2006). Surprisingly, both administration of both CFA and CFA^{high} was sufficient to reduce the expression levels of α-2,6-sialyltransferase in developing antigen-specific splenic PCs during immunisation with TI antigens. These findings imply that TI B cell activation might switch to TD B cell activation when provided with strong adjuvants, such as CFA^{high}. Since the activity of sialidases is associated with IFN-γ production in activated T cells (Nan, Carubelli et al. 2007) and the secretion of pro-inflammatory cytokines in DCs (Stamatos, Carubelli et al. 2010), cellular sialidases might also contribute to the dynamic changes in IgG sialylation in PCs.

So far, this work elucidates that inflammatory TD immunisation, which initiates antigen-specific immunity and promotes the induction of inflammatory immune responses are associated with the generation of non-sialylated IgG antibodies. Contrary, immunisation with both TI and TD antigens under non-inflammatory conditions lead to the induction of peripheral tolerance and the generation of antigen-specific sialylated antibodies. Thus, these findings indicate that IgG antibodies might directly influence the quality of an immune reaction regulated by their Fc-sialylation.

4.3 Protective effect of IgG antibodies depends on Fc-sialylation

Recent publications depict that IgG antibodies exhibit pro-inflammatory or anti-inflammatory effector functions according to their glycosylation pattern (Nimmerjahn, Bruhns et al. 2005; Kaneko, Nimmerjahn et al. 2006; Anthony, Nimmerjahn et al. 2008; Nimmerjahn and Ravetch 2008; Nimmerjahn and Ravetch 2008).Accordingly, I found a correlation between the effector function of antigen-specific TI and TD induced IgG antibodies and their Fc-sialylation pattern. It has not been demonstrated whether the generated IgG antibodies directly mediate the observed pro-or anti-inflammtatory effects. However, this work clearly shows that the transfer of *in vivo* generated antigen-specific sialylated IgG antibodies protects from subsequent inflammatory TD immune responses. De-sialylation of these *in vivo* induced antigen-specific sialylated IgG antibodies abrogated their protective potential. Thus, these findings demonstrate that IgG antibodies induced during tolerogenic immune responses directly mediate anti-inflammatory effector functions in dependency with their Fc-sialylation. Removal of their Fc-associated sialic acid abolishes

the protective potential of tolerogenic IgG antibodies on subsequent inflammatory TD immune responses.

4.4 Antigen-specific monoclonal sialylated IgG antibodies mediate anti-inflammatory effects

Until now, one of the few immunosuppressive drugs based on IgG antibodies is intravenious immunoglobulin (IVIg) (Nimmerjahn and Ravetch 2007; Durandy, Kaveri et al. 2009). High dose IVIg therapy is used in a variety of acute and chronic autoimmune diseases, such as *idiopathic thrombocytopenic purpura* (ITP), *Guillain-Barre-Syndrom* (GBS), *Kawasaki disease* and *chronic inflammatory demyelinating polyneuropathy* (CIDP) (Nimmerjahn and Ravetch 2008). IVIg is a primary blood product and is gained from pooled sera of thousands of donors, including the risk of increased susceptibility to pathogens and the transfer of inadvertent immune responses. However, transfer of IVIg exhibits a systemic immunosuppressive effect, when administered in high doses, but causes unspecific side effects like systemic immunosuppression, headache and fatigue. Despite beneficial effects of IVIg in various autoimmune diseases, there is still a wide range of inflammatory and autoimmune diseases that exclude the usage of IVIg therapy. Yet, the replacement of antigen-unspecific IVIg by antigen-specific IgG-based aproaches remains to be a promising future objective to treat autoimmune diseases. Based on my findings, which show that IgG antibodies exhibit anti-inflammatory properties dependent on their Fc-sialylation, hybridoma-derived monoclonal IgG antibodies were enzymatically sialylated and tested for their protective potential during inflammatory immune responses in various inflammatory mouse models. I could demonstrate that sialylated monoclonal IgG1 antibodies exhibit anti-inflammatory effects. The transfer of monoclonal sialylated IgG1 antibodies prevents from antigen-specific DTH responses and suppresses the development of allergy in the allergic airway inflammation model. I could point out that enzymatic sialylation of monoclonal IgG1 antibodies is crucial to switch their effector function to trigger anti-inflammatory effects and thereby protect from inflammatory immune responses. Furthermore, this work clearly depicts that the immunosuppressive antigen-unspecific effect of high dose IVIg therapy (2 g/kg of body weight) can be substituted by a singular administration of antigen-specific sialylated monoclonal IgG antibodies (5 mg/kg of body weight). By contrast, low dose treatment with IVIg (112 mg/kg of body weight) does not

ameliorate antigen-specific DTH immune responses. This finding supports previous observations showing that IVIg can only exert both anti-inflammatory activities, depending on its concentration (Durandy, Kaveri et al. 2009). The unspecific immunosuppressive effect of high dose IVIg therapy has been highly discussed and a number of hypotheses have been advanced to explain the paradoxical activity. Some investigators conducted its immunosuppressive effect to IgG Fab-fragments, suggesting that polyclonal IgG antibodies neutralize pro-inflammatory mediators, such as C3a-and C5a-anaphylatoxins (Prasad, Papoff et al. 1998; Basta, Van Goor et al. 2003). Others have focused on the Fc-fragment as the anti-inflammatory component and report IVIg-mediated saturation of the neonatal Fc-receptor (FcRn), which results in clearance of autoantibodies by shortening their half-life and thereby, in amelioration of autoimmune diseases (Liu, Garcia et al. 2007). Another possible Fc-dependent mechanism, which has been proposed to account for the activity of IVIg, is the blockade of activating Fcγ-receptors, which results in the abrogation of autoantibody activity in various autoimmune mouse models (Ghetie and Ward 2000; Song, Crow et al. 2003; Nimmerjahn and Ravetch 2006; Roopenian and Akilesh 2007). However, recent studies point out that only a minor fraction of IVIg, the sialylated IgG antibodies, is the active component to mediate the anti-inflammatory antigen-unspecific effects, which feasibly explains the requirement of high doses (Anthony, Wermeling et al. 2008; Nimmerjahn and Ravetch 2008; Bayry, Bansal et al. 2009; Durandy, Kaveri et al. 2009; MacMillan, Lee et al. 2009). The anti-inflammatory activity of IVIg has been demonstrated in a variety of animal models of autoimmunity, including autoantibody ITP (Samuelsson, Towers et al. 2001), serum-transfer arthritis (Bruhns, Samuelsson et al. 2003) and nephrotoxic nephritis (Kaneko, Nimmerjahn et al. 2006) and is a property of the Fc-fragment and its associated glycan structure (Samuelsson, Towers et al. 2001; Kaneko, Nimmerjahn et al. 2006; Nimmerjahn and Ravetch 2007). Removal of the terminal sialic acid from IVIg or its papain-derived Fc fragment abrogates the anti-inflammatory activity in these animal models. Conversely, enrichment of the sialylated fraction of IVIg enhances its activity. Recently, it has been shown that the immunosuppressive effect of IVIg can be reconstituted by a fully recombinant preparation of sialylated IgG Fc-fragments (Anthony, Wermeling et al. 2008; Nimmerjahn and Ravetch 2008; Bayry, Bansal et al. 2009; Durandy, Kaveri et al. 2009; MacMillan, Lee et al. 2009). The mechanism contributing to the systemic immunosuppressive effect of IVIg is widely discussed, but it has been demonstrated that sialylated IgG Fc-fragments require a specific C-type lectin, SIGN-R1 (specific ICAM-3

grabbing non-integrin-related 1), expressed on marginal zone macrophages (Anthony, Wermeling et al. 2008; Bayry, Bansal et al. 2009). The engagement of SIGN-RI induces a cellular program that results in the up-regulation of inhibitory FcγRIIB on effector macrophages at the site of the inflammation, thereby altering the threshold concentration of immune complexes necessary to trigger macrophage activation and inflammation (Samuelsson, Towers et al. 2001; Crow, Song et al. 2003; Kaneko, Nimmerjahn et al. 2006). These findings and others suggest that the expression of inhibitory FcγRIIB is indispensible for mediating the immunosuppressive effects of IVIg, when however indirectly. Contrary to this, my findings indicate that monoclonal sialylated IgG antibodies exhibit their early protective effects on adaptive immune responses and disease pathology in our mouse models independently of FcγRIIB. Transfer of antigen-specific sialylated IgG antibodies was sufficient to prevent a DTH response as well as allergic airway inflammation in wildtype B6 mice lacking FcγRIIB ($RIIB^{-/-}$ mice). This finding indicates that other receptors or molecules might contribute to the early protective potential of antigen-specific sialylated IgG1 antibodies.

Moreover, I proved that low dose administration of antigen-unspecific sialylated monoclonal IgG1 antibodies does not prevent inflammation in a DTH response confirming that the anti-inflammatory effect of sialylated monoclonal IgG1 antibodies is mediated in an antigen-specific manner. These observations indicate that the anti-inflammatory property of sialylated IgG Fc-regions is transferred by targeting the respective antigen with sialylated IgG antibodies and formation of sialylated immune complexes (ICs). This finding draws on previous observations, which demonstrate that ICs containing monoclonal IgG antibodies can recapitulate the therapeutic effect of IVIg in mouse ITP (Siragam, Crow et al. 2006). Here, the beneficial effect was explained by the interaction of IVIg with activating Fcγ-receptors on DCs, thus acting as primary functional targets. The pivotal role of DCs in orchestrating anti-inflammatory effects mediated by IVIg has been further demonstrated by others. Recently, it has been shown that IVIg inhibits effector CD4 T cell priming by antigen-presenting cells (APCs) such as bone marrow-derived DCs (BMDCs), independent of the inhibitory FcγRIIB (Aubin, Lemieux et al. 2010). In this work, I could show that non-sialylated, but not sialylated IgG antibodies induces the secretion of IL-6 and confirmed that high amounts of IVIg inhibit IL-6 production in BMDCs *in vitro*, independently of FcγRIIB. IL-6 is a pro-inflammatory

cytokine, which is produced in activated, mature DCs and promotes the activation of CD4 T cells and differentiation into inflammatory TH17 cells. Besides its effect on T cells, it is also crucial for the induction of B lymphocyte maturation into plasma cells and augmentation of antibody secretion (Bettelli, Carrier et al. 2006; Rabe, Chalaris et al. 2008). Furthermore, IL-6 has been described to promote the pathogenesis of autoimmune diseases as the deletion of IL-6 in lupus prone mice reduced renal macrophage infiltration and autoantibody production, thereby delaying the onset of lupus nephritis (Cash, Relle et al. 2010). Thus, my results indicate that sialylated IgG antibodies might prevent DCs from maturation and thereby inhibit T and B cell activation. I could show *in vivo*, that inflammatory immunisation is not only accompanied by the development of PCs producing antigen-specific non-sialylated pathogenic IgG antibodies, but also by the induction of antigen-specific activated CD4 T cells. As it has been reported previously, high dose application of IVIg has the ability to prevent DCs from CD4 T cell priming and hereby, inhibits CD4 T cell activation *in vitro* (Aubin, Lemieux et al. 2010). In line with these observations, I could verify *in vivo* that antigen-specific sialylated IgG antibodies are able to prevent infiltration of activated antigen-specific CD4 T cells and IFN-γ producing Th1 cells on the site of the inflammation and thereby might contribute to the observed anti-inflammatory effect. Furthermore, I could demonstrate that low dose administration of sialylated monoclonal IgG1 antibodies significantly reduced the generation of antigen-specific IgG2c and IgG2b antibodies, which have been described to exhibit pathogenic effector functions in autoimmune diseases (Ehlers, Fukuyama et al. 2006). The same effect could be observed for the treatment with high dose IVIg, which was also able to reduce serum titers of pathogenic IgG2b antibodies indicating that anti-inflammatory effects of low dose antigen-specific IgG antibodies are sufficient to recapitulate IVIg high dose therapy.

Taken together, these findings suggest that sialylated monoclonal IgG antibodies have the ability to prevent inflammatory immune reactions by inhibiting CD4 T cell activation and B cell-mediated antibody responses in an antigen-specific manner independently of the inhibitory FcγRIIB.

4.5 Antigen-specific treatment of allergy

It has been reported that specific immunotherapy (SIT) with TD antigen is associated with the development of antigen-specific human IgG4 antibodies (IgG1 in mice) and the protection from allergic reactions (Akdis 2006). However, acute allergic reactions are accompanied by the development of antigen-specific IgG4 and IgE antibodies and the infiltration of eosinophils into the lung (Berrens, Koers et al. 1977). The differences between IgG4 generated during acute allergy or after SIT are still unknown. Different IgG4 Fc-sialylation levels might be responsible for different effector functions of IgG4 after SIT or in acute allergic reactions and have to be investigated in future. Based on these observations and my previous findings, I investigated whether antigen-specific sialylated IgG1 antibodies are able to protect mice from allergy in the allergic airway inflammation model. My data clearly show that antigen-specific sialylated IgG1 antibodies are able to protect mice from allergy-mediated pathology in the allergic airway inflammation model. Anti-inflammatory effects of sialylated IgG1 antibodies are reflected in the reduction of allergy-associated IgG1, IgG2c (BALB/c IgG2a) and IgG2b isotypes as well as in decreased infiltration of eosinophils into the lung, a hallmark for allergic airway inflammation. Verifying my previous findings suggesting that sialylated IgG1 antibodies influence CD4 T cell responses, I could show that treatment of allergy with antigen-specific sialylated IgG1 antibodies leads to reduced infiltration of Th2 T cells into the lung. Th2 cells promote inflammatory immune responses in allergies by secretion of the pro-inflammatory cytokines IL-4, IL-9 and IL-13 (Louahed, Toda et al. 2000; Kuperman, Huang et al. 2002). However, the anti-inflammatory potential of sialylated IgG1 antibodies in the allergic airway inflammation model is carried out independently of the inhibitory FcγRIIB. This strengthens the suggestion that antigen-specific sialylated IgG mediate their anti-inflammatory effects by a yet unknown FcγRIIB-independent mechanism.

4.6 Monoclonal sialylated IgG immune complexes induce antigen-specific tolerance

Under inflammatory conditions, immune complexes (ICs) were formed by antigen-specific non-sialylated IgG antibodies and their respective immunogenic antigen. It has been shown that non-sialylated IgG ICs can bind to activating Fcγ-receptors expressed on innate

immune effector cells (Anthony, Wermeling et al. 2008; Nimmerjahn and Ravetch 2008). Cross-linking of activating Fcγ-receptors leads to the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in receptor-associated signalling adaptor proteins such as the common γ-chain by Src familiy kinases. Phosphorylation induces downstream pathways, resulting in calcium-dependent cell activation (Nimmerjahn and Ravetch 2008). Mediation of anti-inflammatory effects by blocking activating receptors is rather unlikely, as it has been shown that sialic acid-rich IgG antibodies have a decreased affinity to classical Fcγ-receptors in mice and humans (Kaneko, Nimmerjahn et al. 2006; Nimmerjahn and Ravetch 2008). These observations rather argue for a novel receptor on regulatory macrophages or other immune cells that specifically recognises sialylated IgG antibodies and mediate anti-inflammatory effects. This hypothesis is strengthened by my experiments, which show that the prophylactic application of low dose sialylated IgG antibodies in form of immune complexes induces antigen-specific tolerance and thereby efficiently suppresses subsequent inflammatory immune responses and pathology in the DTH and the nephrotoxic nephritis model. This approach indicates that the observed anti-inflammatory effect of sialylated IgG antibodies is not only mediated by diminished binding to activating Fcγ-receptors, but might have been carried out by a rather dominant mechanism of action. One possible explanation for the induction of tolerance by ICs could be that sialylated ICs bind to sialic acid binding receptors on antigen-presenting cells (APCs) and induce a cellular programm preventing APCs from fully maturation, thus, leading to impaired B and T cell responses. It has been shown, that the immune system can distinguish between sialylated and non-sialylated IgG antibodies, thereby protecting the organism against coincidential activation of inflammatory pathways in the absence of pathogenic challenge. C-type lectins, such as SIGN-R1 and the human homologue DC-SIGN, can bind to 2,6-sialylated IgG Fc-parts, thus, shifting the immune response towards an anti-inflammatory state resulting in the inhibition of effective immunity (Anthony, Wermeling et al. 2008). Binding to SIGN-R1 on marginal zone macrophages has been associated with the up-regulation of FcγRIIB on effector macrophages. Subsequently, enhanced binding to inhibitory FcγRIIB leads to inhibitory signalling pathways contributing to the unspecific effects underlying IVIg. By contrast, low dose monoclonal sialylated IgG antibodies mediate their anti-inflammatory effects independently of inhibitory signalling by FcγRIIB indicating that SIGN-R1 might be involved in other

regulatory pathways finally resulting in antigen-specific tolerance. Furthermore, it is likely that sialylated IgG ICs can bind to other lectin-receptors on the surface of immune cells. Possible candidates to be considered as receptors are e.g. other C-type lectin receptors (Bates, Fournier et al. 1999; Suzuki-Inoue, Fuller et al. 2006; Kumagai and Akira 2010), such as Dectin-1 and DCIR (dendritic cell immunoreceptor) (Bates, Fournier et al. 1999; Suzuki-Inoue, Fuller et al. 2006), which are expressed on different APC subsets and supposed to bind to certain carbohydrates. The intracellular domains of most C-type lectins contain either ITAMs or ITIMs. Possibly, only sialylated IgG ICs lead to the phosphorylation of ITIMs, initiating antigen-specific tolerogenic signalling pathways and inhibition of cellular activation. Sialylated IgG antibodies might directly bind to ITIM containing receptors or indirectly activate ITIMs by the interaction with another receptor. Immature or semi-mature DCs possibly induce tolerance to the respective antigen by either deletion or anergy of antigen-specific T cells or by the induction of regulatory T cells (Tregs) (Hawiger, Inaba et al. 2001; Steinman 2003). As I could show *in vitro,* sialylated IgG ICs cannot induce IL-6 production in DCs and thereby might contribute to the abrogation of inflammatory B and T cell responses and the induction of tolerance. This mutual mechanism would explain the observation that prophylactic administration of sialylated IgG ICs leads to reduced IgG antibody titers as well as to impaired CD4 T cell responses. The hypothesis supporting a dominant mechanism mediated by sialylated IgG antibodies is further confirmed by experiments demonstrating that the application of antigen-specific sialylated IgG antibodies is sufficient to suppress even ongoing inflammatory immune responses and prevent from subsequent DTH reactions. Here, APCs as crucial regulators of inflammatory and pro-inflammatory immune responses might be unlikely to exclusively orchestrate the observed inhibitory effects since inflammation and cell activation has already been ongoing, indicating that sialylated ICs might also directly influence effector immune cells, like macrophages, B and T cells, by a yet unknown receptor. CD22 is an inhibitory co-receptor of the BCR, and plays a critical role in modulating B cell signalling (Walker and Smith 2008). It is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family of adhesion molecules (Sgroi, Varki et al. 1993) and specifically binds to sialic acid attached in α-2,6-linkage to galactose residues on glycoproteins (Powell, Sgroi et al. 1993; Powell, Jain et al. 1995; Walker and Smith 2008). Besides its potential to provide positive signals to the B cell, CD22-deficient mice have been demonstrated that CD22 functions predominantly as an inhibitory receptor *in vivo* (O'Keefe, Williams et al. 1996;

Otipoby, Andersson et al. 1996; Nitschke, Carsetti et al. 1997). Furthermore, it has been shown that defects in CD22 might predispose to autoimmunity, likely in a similar fashion to FcγRIIB (Pritchard and Smith 2003). Although the role of CD22 is still being discussed, there are implications for CD22 playing a key part in establishing the BCR threshold and the generation of B cell hyper- and hypoactivity. Thus, investigating the role of CD22 in mediating anti-inflammatory effects by antigen-specific sialylated IgG antibodies might be a challenge in the future.

Taken together, my findings reveal that the administration of sialylated IgG ICs leads to the induction of antigen-specific tolerance, including impaired B and T cell responses and thereby leading to the protection from inflammatory immune responses independently of the inhibitory FcγRIIB. However, the mechanism behind the observed inhibitory effect has not been identified in this work and remains to be elucidated in the future (*Figure 4.1*).

4.7 Conclusion

In summary, I could show that TI as well as TD B cell activation under non-inflammatory conditions induces peripheral tolerance and prevents the establishment of a full-blown inflammatory immune response as demonstrated in a DTH and the nephrotoxic nephritis model. Furthermore, tolerogenic TI and TD immunisation is accompanied by the generation of antigen-specific sialylated IgG antibodies, which correlates with high expression levels of α-2,6-sialyltransferase in antigen-specific splenic PCs. These observations, which reveal a correlation between tolerance and IgG sialylation, support the idea of steady-state autoreactive and polyreactive IgG antibodies being sialylated and thus, maintaining self-tolerance. Investigating the anti-inflammatory potential of TI and TD induced tolerogenic IgG antibodies, I was the first to show that antigen-specific IgG antibodies per se are sufficient to mediate anti-inflammatory effects dependent on their Fc-sialyation and thereby, protect from inflammatory immune reactions in an antigen-specific manner. Sialylation of IgG antibodies seems to be crucial during the process of tolerance induction. In line with these findings, I could demonstrate that sialylation of monoclonal IgG antibodies induces anti-inflammatory properties. Compared to unspecific immunosuppressive effects of high-dose IVIg therapy, only low-dose administration of sialylated monoclonal IgG antibodies was sufficient to efficiently induce antigen-specific protection independently of the inhibitory FcγRIIB. The mechanism that mediates

inhibitory signalling pathways triggered by antigen-specific sialylated IgG antibodies still needs to be further explained.

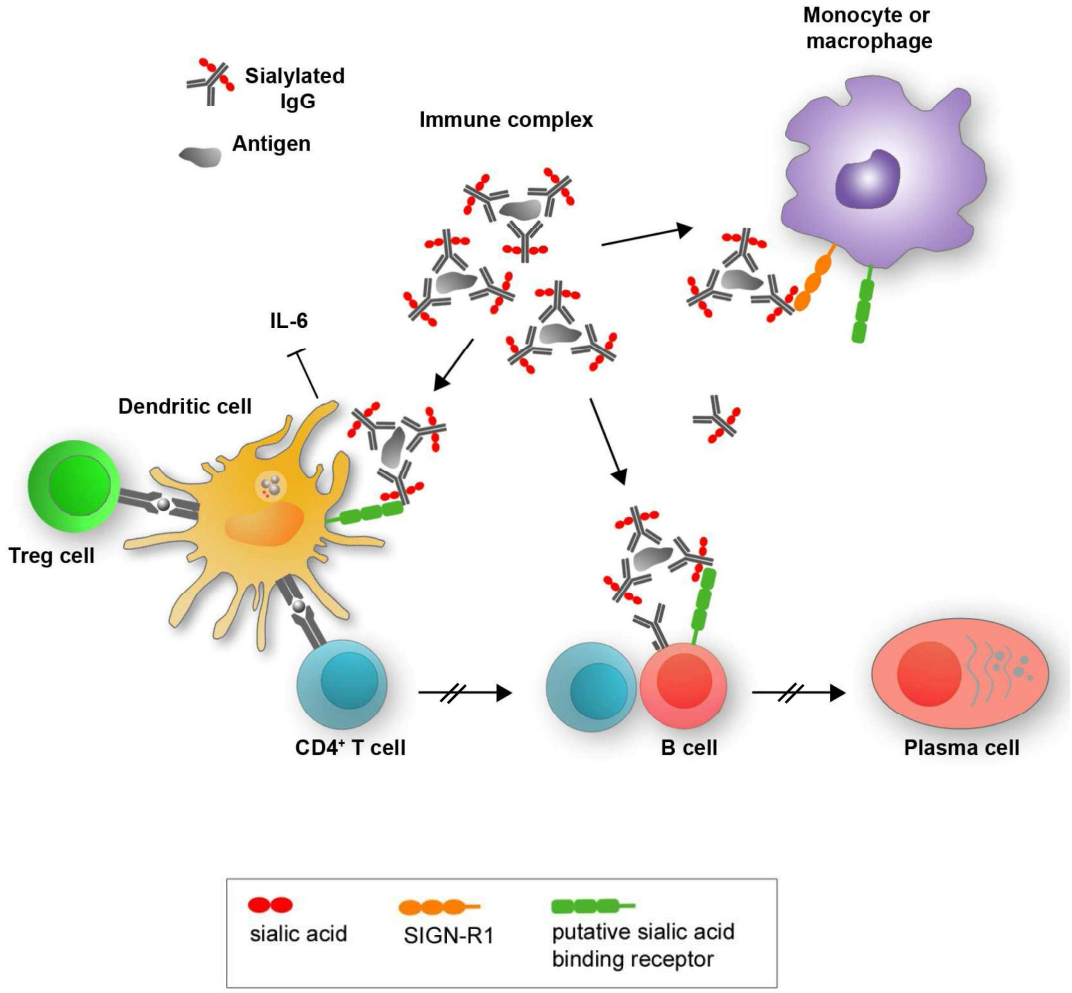

Figure 4.1: Antigen-specific sialylated IgG antibodies mediate anti-inflammatory effects by possible mechanisms. *In vivo* induced antigen-specific or transferred monoclonal sialylated IgG antibodies bind to their respective antigen and form immune complexes (ICs). Most likely, sialylated IgG ICs bind to DCs by a yet unknown sialic acid binding receptor (e.g. Siglec, Dectin). This interaction either induces the generation of antigen-specific regulatory T cells (Tregs) or the deletion or anergy of antigen-specific CD4 T cells, which consequently leads to insufficient B cell activation and IgG antibody production. Moreover, sialylated IgG ICs prevent DCs fromsecretion of the pro-inflammatory cytokine IL-6. Possibly, sialylated IgG ICs also bind to SIGN-RI on marginal zone macrophages, which lead to inhibitory signalling pathways independent of the up-regulation of inhibitory FcγRIIB on effector macrophages. Another feasible mechanism is that sialylated IgG ICs bind to silic acid binding receptors on B cells (e.g. CD22), which induce inhibitory signals. However, mechanisms contributing to the anti-inflammatory effects of sialylated IgG antibodies lead to reduced B and T cell responses and act in an antigen-specific manner independently of the expression of FcγRIIB.

To date, more than 20 recombinant IgG antibodies are licensed for therapeutic and diagnostic purposes. In chronic inflammatory autoimmune diseases therapeutic antibodies are used to block pro-inflammatory cytokines or eliminate effector immune cells. Antigen-specific sialylated IgG antibodies could now provide a novel tool for actively transfering tolerance to immunogenic structures or mediate antigen-specific anti-inflammatory properties in an already imbalanced system found under autoimmune and allergic conditions. Progress in the field of translational research will enable the identification of immunogenic structures crucial for autoimmune diseases and allergy and provide the basis for specific treatment with antigen-specific sialylated IgG antibodies. This basic principle would be transferrable to multiple autoimmune diseases and allergies, thus, creating a completely new approach to more individualised therapies with the possible profit of fewer side effects, like observed by the systemic immunosuppressive IVIg therapy.

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