The role of antibody affinity in mediating protection against autoimmune inflammatory diseases

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THE ROLE OF ANTIBODY AFFINITY IN MEDIATING PROTECTION AGAINST AUTOIMMUNE INFLAMMATORY DISEASES

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1 SUMMARY

Protective immunity relies on the interplay of a complex network of cells and molecules that make up the innate and the adaptive branch of the immune system. These immune cells depend on the ability to recognize and respond to invading pathogens or agents without inflicting damage on the host. While in most cases the immune system manages this challenging task of distinguishing self from non-self molecules, irregular immune responses do manifest themselves sometimes, entailing severe diseases, such as autoimmunity. In this particular case, tolerance mechanisms are broken or bypassed, disrupting the important balance of effector and regulatory immune functions, which in many cases are mediated by cytokines. It is thus apparent, that cytokines represent noteworthy targets for many therapeutic approaches. Monoclonal antibody (mAb) therapy emerged rather recently as a very promising way to block cytokines and inhibit their biological function and to date, more than 20 mAbs have been approved for therapy in humans. The general concept of affinity maturation predicts that germline antibodies expressed during early immune responses have low affinities, whereas late-response antibodies exhibit high affinities, accompanied by high neutralization and protective capacity, due to the accumulation of hypermutations in their variable regions (VRs). Based on this, the industry has made tremendous efforts to artificially increase mAb affinity, resulting in high costs of development. However, despite evidence from preclinical as well as clinical studies investigating mAbs indicating that affinity thresholds for clinical efficacy might exist, this theory has never been put to test.

The present study aimed to provide a better understanding of the role antibody affinity plays for protection against inflammatory autoimmune diseases. We therefore immunized mice using the established virus-like particle (VLP)-based vaccine Qβ-IL-17 and generated mAbs against interleukin-17 (IL-17), a specific cytokine involved in diseases such as rheumatoid arthritis (RA), psoriasis and multiple sclerosis (MS). Two particular mAbs were selected due to their high affinity for IL-17 and their variable heavy chain (VH) was mutated back towards germline sequence, with the prospect of generating antibodies recognizing the same epitope but binding with lower affinity.
In the first part the antibody aIL-17 2E1 was investigated, which had an affinity of 
<11 pM and showed protection in EAE, an animal model for MS. Surprisingly, the 
antibody engineered to exhibit no hypermutation and completely matching the 
germline sequence, exhibited only a reduced but still high affinity of 0.5 nM. Furthermore, 
not only the hypermutated but also the germline version of this mAb showed high in vivo 
neutralization capacity as well as protective effect in animal models for psoriasis and rheumatoid arthritis. These results suggest that increasing 
anti-cytokine mAb affinity above a certain threshold, presumably a limit <0.5 nM, does not necessarily translate into better clinical efficacy and that even germline 
mAbs generated with our VLP-based vaccines may be sufficient for protection, 
questioning the relevance of the industry’s efforts of engineering mAb affinity up to 
femtomolar levels.

The second part describes the mAb aIL-17 1B10, which also exhibits high affinity 
of <28 pM and of which the germline version did bind to IL-17 with a much lower 
affinity in the µM range. While the hypermutated 1B10 antibody was neutralizing in 
vitro and showed protective efficacy in EAE, the germline antibody failed to do so. After generating mAbs with intermediate numbers of hypermutations aiming at 
getting mAbs with intermediate affinities, we surprisingly found that all of them 
bound as strong to IL-17 as the fully hypermutated 1B10 mAb, suggesting that any 
hypermutation in the VH region is sufficient for proper folding of the antigen binding 
site. The successive backmutation of the VL and generation of additional mutants will 
hopefully result in mAbs binding with intermediate affinity and allow us to proceed to 
study in vivo efficacy and determine the exact affinity threshold required for 
protection against autoimmune diseases.
2 ZUSAMMENFASSUNG

Eine schützende Immunabwehr eines Organismus gegen Pathogene beruht auf dem Zusammenspiel eines komplexen Netzwerkes aus Zellen und Molekülen, welche die angeborene wie auch die adaptive Immunantwort vermitteln. Diese Immunzellen besitzen die Fähigkeit, eindringende Pathogene und Agenzien zu erkennen und daraufhin eine Immunantwort einzuleiten, ohne den Wirtsorganismus dabei zu schädigen. Während das Immunsystem der Herausforderung körpereigene von körperfremden Substanzen zu unterscheiden in den meisten Fällen gewachsen ist, können sich dennoch fehlgeleitete Immunantworten manifestieren, die Beschwerden wie Autoimmunerkrankungen mit sich ziehen. In diesem spezifischen Fall werden Immuntoleranz Mechanismen beeinträchtigt, was zu einer Störung des wichtigen Gleichgewichts von effektor und regulatorischen Funktionen führt, welche in vielen Fällen von Zytokinen ausgetragen werden. Folgegemäss repräsentieren Zytokine wichtige Zielgruppen für viele therapeutische Ansätze. Die Therapie mit monoklonalen Antikörpern (mAbs) hat sich als eine vielversprechende Methode Zytokine zu inhibieren hervorgetan und allein heute sind schon mehr als 20 mAbs für die Therapie im Menschen zugelassen. Das allgemeine Konzept der Affinitätsreifung besagt, dass Keimlinienantikörper der frühen Immunantwort eine niedrige Affinität für das jeweilige Antigen besitzen, währenddessen Antikörper der späten Immunantwort hohe Affinitäten wie auch hohes Neutralisations- und Schutzpotential aufzeigen, was wiederum auf der Akkumulation von Hypermutationen in ihren variablen Regionen (VRs) basiert. Als Folge hat die Industrie grösste Bestrebungen in die künstliche Erhöhung der mAb Affinität gesteckt, was zu immensen Entwicklungskosten führte. Trotz Indizien aus präklinischen wie auch klinischen Studien mit mAbs, welche darauf hinweisen, dass möglicherweise Affinitätsgrenzwerte für die klinische Wirksamkeit existieren, wurde diese Theorie nie geprüft.

Das Ziel der vorliegenden Studie war ein erweitertes Verständnis dafür zu erlangen, welche Rolle die Antikörperaffinität für den Schutz gegen entzündliche Autoimmunerkrankungen spielt. Für diesen Zweck wurden Mäuse mit dem etablierten, auf Virus-ähnlichen Partikeln (VLP)-basierenden Impfstoff Qß-IL-17
immunisiert, um Antikörper gegen Interleukin-17 (IL-17) zu erzeugen, einem Zytokin das in Krankheiten wie Rheumatoider Arthritis (RA), Psoriasis und Multipler Sklerose (MS) eine zentrale Rolle spielt. Aufgrund ihrer hohen Affinität für IL-17 wurden zwei mAbs ausgewählt und die Sequenz ihrer variablen Region der schweren Kette (VH) in Richtung Keimlinie zurückmutiert, mit der Erwartung Antikörper herzustellen, die an das gleiche Epitop mit niedrigerer Affinität binden.

Im ersten Teil dieser Studie wurde der Antikörper aIL-17 2E1 analysiert, welcher eine Affinität von <11 pM sowie einen schützenden Effekt in EAE aufwies, einem Tiermodell für MS. Überraschenderweise band der abgeleitete Keimlinienantikörper mit einer niedrigeren aber dennoch hohen Affinität von 0.5 nM. Des Weiteren zeigte nicht nur der hypermutierte sondern auch die Keimlinienversion dieses Antikörpers stark neutralisierende Eigenschaften in vivo, sowie einen schützenden Effekt in Tiermodellen für Psoriasis und RA. Diese Resultate deuten darauf hin, dass die Erhöhung der Affinität von anti-Zytokin Antikörpern über einen bestimmten Grenzwert, vermutlich <0.5 nM, nicht zwingendermassen mit einer besseren klinischen Wirksamkeit einhergehen muss. Ausserdem weisen sogar Keimlinienantikörper, die durch Impfung mit VLP-basierenden Impfstoffen entstehen, bereits schützende Eigenschaften auf, was die Relevanz der Bemühungen der Industrie, Antikörperaffinität bis zu femtomolaren Werten zu erhöhen, in Frage stellt. Der zweite Teil dieser Studie behandelt den mAb aIL-17 1B10, der ebenfalls eine hohe Affinität von <28 pM zeigte und dessen Keimlinienversion mit bedeutend niedrigerer Affinität im µM Bereich an IL-17 band. Der hypermutierte 1B10 mAb, aber nicht der entsprechende Keimlinienantikörper wies in vivo neutralisierende und in EAE schützende Eigenschaften auf. Als nächstes wurden mAbs mit unterschiedlicher Anzahl und Kombinationen von Hypermutationen generiert, mit dem Ziel mAbs unterschiedlicher Affinitäten zu erzeugen. Überraschenderweise zeigten jedoch alle so produzierten mAbs eine ähnlich hohe Affinität wie der ursprüngliche hypermutierte 1B10 mAb, was darauf hinweist, dass jede der Hypermutationen in der VH genügt, um zu einer Faltung der Antigen-bindenden Region zu führen. Die sukzessive Rückmutation der VL und das Generieren zusätzlicher Mutanten wird hoffentlich mAbs mit intermediärer Affinität erzeugen und uns erlauben, die in vivo Wirksamkeit dieser mAbs zu testen und den exakten Schwellenwert für den Schutz gegen entzündliche Autoimmunerkrankungen zu bestimmen.
3 **GENERAL INTRODUCTION**

3.1 **THE IMMUNE SYSTEM**

The immune system (IS) is defined as the collection of tissues, cells and molecules within an organism that protect against diseases mediated by infectious agents and cancer. Sophisticated defense mechanisms have evolved to specifically recognize, contain and eventually eradicate pathogens, while tight self-regulation avoids damage to the host by uncontrolled immune activation to self-molecules and host tissues. Protective immunity is mediated by a complex interplay of the two branches of the immune system. The innate IS provides an initial and rapid defense, whereas the adaptive IS develops later and provides a stronger, more specific response and is responsible for immunological memory. However, aberrant immune responses do occur and are the cause of severe diseases including chronic inflammation, autoimmunity, immunopathology and allergy.

3.2 **THE INNATE IMMUNE SYSTEM**

Immunity against invading pathogens is mediated by layered defense systems of increasing specificity. Mechanisms of the innate immune system represent the first line of defense and begin to act immediately upon encounter of the invading pathogen.

Epithelial cells lining the internal and external surfaces of the body, specifically the skin, gastrointestinal, respiratory and urogenital tracts, are held together by tight junctions and provide an initial, physical barrier that seals off the internal milieu to the pathogen-containing external environment. The cells of the internal mucosal epithelia secrete mucus, which coats the potentially harmful microorganisms and inhibits adhesion to the epithelial surface. Movement of epithelial cilia in the respiratory tract as well as peristaltic movement of the gut stimulate flow of mucus or
food, respectively, which leads to the expulsion of the microorganism or other irritants. In addition to mechanical defenses, epithelia provide a chemical barrier by secreting microbicidal substances. Gastric acid and digestive enzymes found in the gastrointestinal tract, antibacterial enzymes such as lysozyme and phospholipase A secreted in tears and saliva as well as antifungal or antimicrobial peptides such as β-defensins all serve as powerful chemical mechanisms to defeat the pathogen (Ryu, Kim et al. 2010; Maldonado-Contreras and McCormick 2011).

If a pathogen manages to breach an epithelial barrier it encounters phagocytes that recognize, engulf and destroy the invading microorganism. Two types of circulating phagocytes are initially recruited to the infection site, neutrophils (also known as polymorphonuclear leukocytes, PMNs) followed by monocytes. The less abundant monocytes enter extravascular tissues in response to infection, where they differentiate into tissue-resident macrophages or dendritic cells (DCs) and are able to survive long-term. Large numbers of these macrophages reside in connective tissues, beneath all epithelial surfaces and a number of organs such as the spleen, lymph nodes and liver. Neutrophils on the other hand are short-lived cells that circulate in the bloodstream in large numbers but do not reside in healthy tissues. Upon invasion of a pathogen, neutrophil production in the bone marrow is stimulated by cytokines known as colony-stimulating factors (CSFs), leading to rapid amplification of neutrophil proliferation and maturation. Like monocytes, neutrophils migrate to the site of infection where they ingest and destroy the microbes (Kantari, Pederzoli-Ribeil et al. 2008; Borregaard 2010; Geissmann, Manz et al. 2010). In contrast to macrophages, however, neutrophils do not persist in tissues.

All cell types participating in the innate immune response recognize certain classes of conserved microbial structures often unique to microbes termed pathogen associated molecular patterns (PAMPs), thus enabling to some extent the discrimination between self and non-self. Binding to these PAMPs is mediated via germline-encoded receptors, the so-called pathogen recognition receptors (PRRs), which are grouped into different classes including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), scavenger receptors and the RIG-like helicases (RLRs). In addition to PAMPs, PRRs have been reported to recognize damage-associated molecular patterns (DAMPs), which are endogenous molecules released from damaged cells. Receptor binding triggers the transcription of
proinflammatory cytokines, type I interferons (IFNs), chemokines, antimicrobial proteins and in many cases leads to phagocytosis (Takeuchi and Akira 2010).

In the process of phagocytosis, neutrophils and macrophages internalize the invading microbe into phagosomes. These membrane-enclosed vesicles become acidified and later fuse with another type of granules called lysosomes to form phagolysosomes. Within these phagolysosomes the pathogens come in contact with the lysosomal content. Enzymes, proteins as well as peptides act in concert with leukocyte-derived toxic products released in a process termed respiratory burst to eventually destroy the microbe (toxins include nitric oxide (NO), superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$)). While neutrophils die shortly after phagocytosis, macrophages live on to produce new lysosomes. In addition to phagocytosis, macrophage functions include the release of a wide array of cytokines, chemokines and complement proteins that stimulate local inflammation and neutrophil recruitment, as well as serving as antigen-presenting cells (APCs), able to activate the adaptive immune response (Murray and Wynn 2011; Amulic, Cazalet et al. 2012).

In addition to monocytes and neutrophils, other leukocytes such as phagocytic DCs, natural killer (NK) cells, mast cells, basophils and eosinophils contribute to inflammatory processes and the elimination of pathogens in the course of the innate immune response. While macrophage-derived cytokines and chemokines initiate the inflammatory response by stimulating leukocyte recruitment and extravasation, the later stages of inflammation are characterized by neutrophil and later monocyte attraction, differentiation of tissue-macrophages and DCs and finally eosinophil and lymphocyte migration, increase of vascular permeability and clotting in microvessels surrounding the site of infection. Similar to macrophages, immature DCs reside in tissues and display a high endocytic and phagocytic capacity, facilitating antigen capture. Upon antigen capture and processing, DCs become activated and migrate to secondary lymphoid organs where they serve as APCs expressing costimulatory molecules and present the antigen to CD4$^+$ helper T cells, thereby constituting an important link between the innate and the adaptive immune response (Banchereau, Briere et al. 2000). NK cells are lymphocytes that survey host tissue for infected, transformed and stress cells and respond by directly killing target cells infected by intracellular microbes such as viruses or by secreting interferon-$\gamma$ (IFN-$\gamma$), which in turn activates macrophages to kill phagocytosed microbes (Sun and Lanier 2011).
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Mast cells are tissue-resident cells of the connective tissue and mucosa and besides being the major effector cells of immediate hypersensitivity reactions (allergy) they are also implicated in host responses to pathogens. When activated by stimuli such as direct physical or chemical injury, complement proteins or cross-linking of their surface receptors by IgE (FcεRI receptor), mast cells undergo degranulation. In this process, the contents of their characteristic cytoplasmic granules, particularly histamine, hormonal mediators and cytokines, get released into the interstitium, where they cause vasodilation, increase blood vessel permeability and recruit macrophages and neutrophils. Eosinophils are granulocytes that along with mast cells regulate mechanisms associated with allergic reactions and asthma and play an important role in defense against extracellular parasites, such as helminths. Effector functions are carried out by proinflammatory mediators including granule-stored proteins, cytokines as well as newly synthesized eicosanoids, which get secreted by the eosinophils upon activation. Basophils represent the least common type of granulocytes and share certain characteristics with mast cells. If activated, basophils degranulate to release histamine, proteoglycans, proteolytic enzymes and cytokines/chemokines, thereby contributing to inflammation as well as allergy (Stone, Prussin et al. 2010).

In addition to the abundant cellular components of the immune system, a collection of circulating and membrane-associated proteins called the complement system plays a central role in the innate defense. Besides eliminating pathogens and clearing the host of apoptotic cells and immune complexes, the complement system orchestrates immune responses, is involved in tissue development and repair and contributes to homeostasis. The complement system can be activated through three different pathways, the initiating stimuli being either components of the innate branch of the IS, such as microbes or mannose-binding lectin (MBL), or part of the adaptive IS, specifically antibody-antigen complexes. Either way, all three initiating factors trigger a biochemical cascade where activated complement proteins act as proteolytic enzymes ultimately providing different effector proteins with functions in host defense. In the process of opsonization, invading microbes are coated by a complement protein called C3b, which promotes phagocyte interaction and phagocytosis. Proteolytic fragments of complement proteins promote inflammation by serving as chemoattractants for phagocytes and leukocytes. In addition, activation of
complement can lead to the formation of the so-called membrane attack complex, which causes holes in the pathogen membrane, thereby inducing osmotic lysis, or apoptosis of pathogens. Finally, the complement system can stimulate the adaptive immune response by marking antigen-antibody complexes for the trapping by follicular dendritic cells in germinal centers, leading to antigen-display and further development of humoral immunity (Ricklin, Hajishengallis et al. 2010).

### 3.3 The Adaptive Immune System

The adaptive immune system is composed of specialized lymphocytes and their products evolved to mediate antigen-specific immunity. While the innate immune system initially protects against infections mediated by pathogens displaying conserved molecular patterns, the adaptive immune system is able to express receptors that specifically recognize and adapt to a great variety of pathogenic as well as noninfectious molecules and thereby complements innate processes by specifically combating different types of microbes that have evaded primary immune barriers. The highly diverse lymphocyte repertoire is the result of clonal selection processes where the rearrangement of a limited number of germline-encoded variable gene segments generates a vast variety of unique genes encoding distinct antigen-specific receptors. Somatic hypermutations further enhance binding capacity and clonal expansion processes then increase the number of lymphocytes in order to keep pace with the proliferation rate of microbes. In the course of the primary adaptive immune response, in which full protective capacity is reached after five to seven days, memory lymphocytes are generated, which enable the body to mount a more rapid secondary immune response in the case of subsequent encounters with the same pathogen.

Similar to the innate immune system, one can discriminate between two different branches of the adaptive immune system, each of which provides protection against different pathogens and uses distinct effector cells. Cell-mediated immunity is designed to combat intracellular microbes and is mediated mainly by T lymphocytes whereas humoral immunity defends the body against extracellular microbes via antibodies derived from B lymphocytes.
3.3.1 **T lymphocytes**

**Maturation and selection of T lymphocytes**

Multipotent hematopoietic stem cells (HSCs) in the bone marrow (BM) are the main source of T lymphocytes and undergo a gradual differentiation process during which they become restricted to the lymphoid lineage (Metcalf 1998). They then leave the BM as double negative (CD4−CD8−) T progenitor cells to migrate towards the thymus, where the entire maturation process takes place. These earliest progenitors contain genes encoding the T cell receptor (TCR) in their germline configuration, which usually exists of an α and β chain or in certain cases a γ and δ chain (Brenner, McLean et al. 1986). Of these cells, a small population expresses not only α:β T cell receptors but also the NK1.1 receptor and are therefore known as NKT cells. Whereas the major α:β lineages recognize MHC molecules (which will be illustrated in later sections), these NKT cells rather recognize CD1 molecules and will not be discussed further (Hu, Gimferrer et al. 2011). In the α:β and γ:δ progenitors however, each receptor chain loci harbors multiple variable region (V) genes and constant region (C) genes. Between these genes lie small stretches of joining (J) nucleotides and in the case of the β and δ chains also diversity (D) gene segments, which enable a sequence of somatic DNA recombinations (VDJ recombinations) followed by RNA splicing events, leading to the production of a diverse repertoire of TCRs (Bassing, Swat et al. 2002). While cells that fail to successfully rearrange the β- respectively the δ-chain locus or do not express the complete TCR will die, the surviving α:β cells express CD4 and CD8 co-receptors, thus called double positive (CD4+CD8+) thymocytes, and begin to proliferate (Ciofani, Knowles et al. 2006; Bhandoola, von Boehmer et al. 2007). In contrast, γ:δ T cells do not express these co-receptors, even when mature, and are exported to the periphery, primarily epithelia, as double negative cells (Raulet 1989; Xiong and Raulet 2007). After TCR rearrangement, survival of the short-lived α:β double positive thymocytes depends on signaling via the TCR by self-ligands in the thymic cortex, which subsequently induce positive or negative TCR-specific thymic selection. Binding of high affinity ligands stimulates negative selection and clonal deletion of double positive cells, inhibiting
further differentiation (Pircher, Rohrer et al. 1991; Pobezinsky, Angelov et al. 2012). Low affinity ligands on the other hand trigger positive selection to preserve self-MHC restricted T cells by eliminating cells that do not recognize an MHC molecule expressed on cells of the thymic cortex. Cells recognizing MHC class I-peptide complexes will further express CD8 while loosing CD4 expression, whereas cells recognizing MHC class II-peptide complexes will maintain only CD4 expression. These single-positive T cells are then subjected to negative selection, where cells with too strong receptor-MHC-peptide complex binding undergo apoptosis, hence ensuring the elimination of potentially self-reactive T lymphocytes. Consequently, the resulting mature T-cell repertoire is both MHC-restricted and self-tolerant (Starr, Jameson et al. 2003; Koch and Radtke 2011). Once the maturation process is completed, naïve T lymphocytes expressing the chemokine receptor CCR7 enter the bloodstream and are guided to T-cell rich zones of peripheral lymphoid tissues (spleen and lymph nodes), where they interact with other cell types (Forster, Schubel et al. 1999).

**Antigen presentation and T cell activation**

As opposed to B lymphocytes, the majority of T lymphocytes requires a process called antigen presentation in order to recognize any antigen other than the ones belonging to the group of superantigens, which are capable of activating T cells directly. Furthermore, antigen recognition by T lymphocytes is largely restricted to protein antigens, whereas B cells are able to detect not only proteins but also polysaccharides, lipids and other nonprotein antigens. Adaptive immune responses are triggered immediately after an antigen passes through the epithelial barrier. Professional antigen presenting cells (APCs), such as DCs and to a lesser extent macrophages take up the antigen, process and present it to naïve T cells via a molecule called major histocompatibility complex (MHC) on their cell surface. Subsequently, clonal expansion and differentiation into effector T cells is induced, followed by the activation of their effector functions.

Immature DCs residing in epithelia and subepithelial tissues as well as in peripheral lymphoid organs are the major players in antigen presentation to T cells. Upon antigen encounter, DCs often bind the microbe through membrane receptors
such as the terminal-mannose receptor, and ingest the protein via different mechanisms depending on the nature of the antigen, particularly phago- and receptor-independent macropinocytosis (Inaba, Inaba et al. 1993; Sallusto, Cella et al. 1995). Simultaneously, the pathogen induces innate immune reactions by stimulating the PRRs (e.g. TLRs) not only on DCs but epithelial cells and tissue-resident macrophages, which consequently leads to the secretion of cytokines such as TNF-α, IL-1β and IL-6 (Reis e Sousa 2001). Once DCs have taken up the antigen and are stimulated by the proinflammatory microenvironment, they mature into active DCs. This process is accompanied by a reduced adhesiveness for epithelia, downregulation of endocytotic and phagocytotic receptors as well as upregulation of costimulatory molecules, for instance CD40, CD80 and CD86 (Banchereau, Briere et al. 2000). Moreover, mature DCs express CCR7, a surface receptor for the chemokines CCL19 and CCL21, which guides their subsequent migration to the paracortical zones of draining lymph nodes or the spleen, where the antigen is concentrated and encounters T lymphocytes (Dieu, Vanbervliet et al. 1998). During this migration, the maturation process of DCs continues in a CCL19-dependent fashion, marked by an increased and stable expression of the MHC glycoproteins (Marsland, Battig et al. 2005). In contrast to large antigens, soluble or smaller antigens such as virus like particles (VLPs) do not depend on this cellular transport by DCs, but rather drain freely to secondary lymphoid organs where they are picked up and processed by tissue-resident DCs (Manolova, Flace et al. 2008).

Exogenous protein antigens such as microbes or toxins are transported to the late endosomal MHC class II compartment (MIIC) within the DC, where they encounter a heterotrimeric complex composed of the MHC class II molecule and an invariant chain, which originally assembled in the ER (Peters, Neefjes et al. 1991; Busch, Doebele et al. 2000). The proteins as well as the invariant chain are then processed by endosomal proteases and the resulting antigenic peptide is integrated into the peptide-binding groove of the complex, replacing the degraded invariant chain. Once the peptide is bound, the generated peptide-MHC II complex becomes stable and is transported to the plasma membrane, where the antigen is then displayed to CD4+ T lymphocytes (Wubbolts, Fernandez-Borja et al. 1996; Cella, Engering et al. 1997). In contrast to the MHC class I molecule, the MHC class II protein is solely expressed by professional APCs (Bryant and Ploegh 2004). While the peptide within the complex is
recognized and bound by the T cell receptor (TCR) of CD4\(^+\) T helper cells (T\(_H\)), the MHC II molecule is bound by the CD4 co-receptor molecule, providing the initiating signal for T cell activation, given that multiple receptors and co-receptors are engaged simultaneously. Adhesion molecules on T cells, such as the integrin leukocyte function-associated antigen-1 (LFA-1), further stabilize this connection and enhance T cell responses by binding to ligands on APCs, such as the intercellular adhesion molecule-1 (ICAM-1) (Dustin, Rothlein et al. 1986; Dustin and Springer 1989). Ultimately, the costimulators expressed on APCs are bound by the receptors such as CD28 and CD40L expressed on all T lymphocytes, enhancing T cell activation and cytokine expression by APCs. In general, the sum of these stimuli orchestrates a set of responses including the release of cytokines and expression of cytokine receptors, the clonal expansion of the antigen-specific lymphocyte clones and the differentiation of naïve T cells into effector and memory cells. Naïve CD4\(^+\) T lymphocytes for instance differentiate into various T\(_H\) cell subsets that differ in cellular phenotype and function and will be described in more detail in the following sections (Vyas, Van der Veen et al. 2008; Neefjes, Jongsma et al. 2011).

In contrast to exogenous proteins, intracellular protein antigens derived from viruses or cytoplasmic bacteria are processed into protein fragments by cytosolic or nuclear proteasomes within the DC. The resulting peptides are transported into the ER, where the MHC class I molecule is being assembled, via the transporter associated with antigen presentation (TAP) (Mester, Hoffmann et al. 2011). Upon binding of the antigenic peptide, the generated peptide-MHC I complex leaves the ER for presentation at the cell surface of essentially all nucleated cells. The peptide is recognized and bound by the TCR of CD8\(^-\) cytotoxic T lymphocytes (CTLs), whereas the ubiquitously expressed MHC I molecule is bound by the CD8 co-receptor molecule. Full activation of these T lymphocytes also requires costimulators and adhesion molecules in addition to antigen and co-receptor binding. However, the presentation of a cytoplasmic antigen from another cell by the DC, a process known as cross-presentation, represents an additional and unique feature of CD8\(^-\) T cell activation (Rock, Gamble et al. 1990). Once activated, CD8\(^-\) cytotoxic T cells serve to directly kill infected target cells and tumor cells through a process involving the release of membrane pore-forming proteins and proteolytic enzymes from cytoplasmic granules (Vyas, Van der Veen et al. 2008; Neefjes, Jongsma et al. 2011).
3.3.2 **CD4\(^+\) T cells and the plasticity of the TH lineage differentiation**

CD4\(^+\) T helper (TH) lymphocytes are important mediators in the generation of both humoral and cell mediated immune responses and in many instances act through recruitment of other cellular components of the immune system. Upon interaction with antigen presented by antigen-presenting cells (APCs) such as dendritic cells (DCs), naïve CD4\(^+\) T cells differentiate into distinct effector TH subsets with specific immunological functions suited to act in host defense against the invading pathogen at the site of infection. At the same time a population of central memory cells develops, which instead of entering the tissue, resides in the lymph nodes and spleen and gives rise to further effector cells upon subsequent antigenic stimulation (Zhu, Yamane et al. 2010).

**TH cell lineages and their signature cytokines**

The T helper 1 (TH1) cell and TH2 cell paradigm proposed by Mosmann and Coffman in 1986 first described two distinct TH cell subsets, classified based on their unique cytokine secretion profiles that defined their function (Mosmann, Cherwinski et al. 1986). In the past 25 years, numerous studies have helped to further characterize TH lineages by additional factors including their expression of surface molecules and especially the transcription factors that define their signature gene-expression profile (Glimcher and Murphy 2000; Zhou, Chong et al. 2009).

In regard to cytokine expression, the classical TH1 cells secrete interferon-\(\gamma\) (IFN\(\gamma\)) as their signature cytokine, which is important for macrophage activation and cellular immunity against intracellular microorganisms including viruses. TH2 cells produce IL-4, IL-5 and IL-13, later being critical for IgE production, eosinophil recruitment and clearance of extracellular parasites such as helminths (Szabo, Sullivan et al. 2003; Ansel, Djuretic et al. 2006). More recently discovered TH17 cells constitute a third major effector population derived from naïve CD4\(^+\) T cells and are characterized by the secretion of IL-17A, IL-17F, IL-22 and, although not exclusively, IL-21.
General Introduction

(Harrington, Hatton et al. 2005; Zhou, Ivanov et al. 2007). This T cell subset has been shown to be a crucial mediator of both host protection against extracellular bacteria and fungi and autoimmune inflammatory processes (McGeachy and Cua 2008; Ishigame, Kakuta et al. 2009). Natural regulatory T cells (nTReg) are recognized as a bona fide CD4+ T cell lineage, as they are not derived in the periphery from the naïve precursor Th1, Th2 and Th17 cells have in common, but rather develop in the thymus (Sakaguchi, Sakaguchi et al. 1995). Induced regulatory T cells (iTReg) however constitute a fourth effector lineage, as they were shown to differentiate from naïve CD4+ T cells in the periphery and share many if not all of the important immunesuppressive functions of nTReg cells, such as secretion of TGF-β (Chen, Jin et al. 2003; Littman and Rudensky 2010). The CD4+ T cells which enter the germinal center and mediate B cell help for antibody production as well as the regulation of immunoglobulin class switching and B cell affinity maturation are termed follicular helper T cells (T FH) cells (Fazilleau, Mark et al. 2009; Crotty 2011). Until recently it was highly disagreed upon whether this cell type in fact constituted an independent lineage, comparable to TH1, TH2, TH17 and iTRegs, or rather a phenotypic state of each of the effector lineages (Zhu, Yamane et al. 2010). Recent findings concerning T FH and the identification of a master regulator of T FH differentiation however, have now established these cells as a distinct lineage (Crotty 2011). Other potential TH lineages have been proposed, including IL-9 producing TH9 (Locksley 2009), IL-22-producing TH22 cells (Locksley 2009; Trifari, Kaplan et al. 2009), TH3 cells (TGFβ-producing CD4+ T cells) and Tr1 cells (IL-10 secreting CD4+ cells) (Groux, O'Garra et al. 1997; Weiner 2001).

The cytokine environment is crucial for fate determination

Both, T cell receptor (TCR)– as well as cytokine-mediated signaling is crucial for promoting TH cell fate determination and selective expansion of committed TH cells. Through activation of downstream signaling molecules, early cytokine production is induced and the master transcription factor is upregulated, which induces secondary transcription factors that in turn act together with the master transcription factor not only to enhance the expression of lineage-specific cytokines and cytokine receptors
but to also regulate genes associated with selective growth of the favored lineage or with repression of alternative lineage fates (Zhu, Yamane et al. 2010).

The differentiation of naïve CD4\(^+\) T cells in response to pathogenic microbes is regulated predominantly by the distinct cytokine milieu resulting from the infection by a specific pathogen present during the TCR-mediated activation process (Glimcher and Murphy 2000). Differentiation into T\(_{H1}\) cells is driven by IL-12 produced by innate immune cells such as DCs and macrophages as well as IFN-\(\gamma\) produced by both NK cells and T cells (Hsieh, Macatonia et al. 1993; Zhu, Yamane et al. 2010).

T\(_{H2}\) cell differentiation requires the presence of IL-4, of which the source is less clearly defined as for the T\(_{H1}\)-promoting cytokines. It appears that if the infectious microbe does not induce IL-12 production by APCs, which may be the case for helminths, it is the T cells themselves, which produce IL-4. In addition, helminths may also induce cells of the mast cell lineage to secrete IL-4.

In contrast to T\(_{H1}\) and T\(_{H2}\) cell differentiation, which both require their respective signature cytokines, T\(_{H17}\) cells do not depend on IL-17. Murine T\(_{H17}\) cells were shown to differentiate from naïve CD4\(^+\) T cells in response to TGF-\(\beta\) and IL-6, while further progression of T\(_{H17}\) development depends on the autocrine secreted factor IL-21 as well as DC derived IL-23 (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006). In humans, induction of differentiation relies on TGF-\(\beta\) in combination with IL-1\(\beta\), IL-6 or IL-21 (Manel, Unutmaz et al. 2008; Yang, Anderson et al. 2008). The number of specific cytokine receptors that exist at any one time may determine the relative efficacy of each of the different cytokines involved. IL-23 receptors for example, are not expressed until after the naïve cell has partially completed its differentiation, and thus IL-23 has little effect in the initial T\(_{H17}\) differentiation induction but rather in expansion and stabilization of the cells to produce their signature cytokines (Bettelli, Korn et al. 2008).

While the findings for cytokines initiating T\(_{FH}\) development are still controversial, it has been shown that the induction of iTregs from naïve CD4\(^+\) T cells depends on T cell activation in the presence of TGF-\(\beta\) and IL-2, as well as retinoic acid, and thereby also supports the principle, that a major product of the differentiated cell contributes to induction, fostering the development of T cell lineages by positive feed-back loop (Vogelzang, McGuire et al. 2008; Zhu, Yamane et al. 2010).
The differentiation-inducing cytokines bind to receptors of the Type I/II receptor superfamily and initiate signaling. Signal transducer and activator of transcription (STAT) factors have crucial roles in sensing these extrinsic cues and transmitting cytokine-mediated signals generally via the JAK/STAT pathway to regulate the expression of lineage-defining master regulators which control selective gene expression, defining a specific phenotype. In collaboration with these master regulators, STAT proteins additionally control cytokine production (Hirahara, Vahedi et al. 2011; O’Shea, Lahesmaa et al. 2011).

Of the seven defined STAT family members it is the STAT1 and STAT4 proteins, which are involved in Th1 differentiation. As opposed to other STATs, which are expressed by a wide range of cell types, STAT4 is mainly expressed by immune cells. It is predominantly activated by IL-12 and thereby promotes Th1 development and expression of the key regulator T-box 21 (Tbx21, also called T-bet) (Kaplan, Sun et al. 1996; Thierfelder, van Deursen et al. 1996; Szabo, Kim et al. 2000). STAT4 also plays an important role in Ifng expression in innate immune cells such as natural killer (NK) cells (Miyagi, Gil et al. 2007). STAT1, also essential for Th1 differentiation, is activated through IFN-γ, which acts through T-bet to induce more INF-γ, indicating a positive feedback loop for the amplification of Th1 responses (Lighvani, Frucht et al. 2001; Afkarian, Sedy et al. 2002). For INF-γ induction, T-bet functions partly by remodeling the Ifng gene and through upregulating IL-12Rβ2 expression, consequently stimulating IFN-γ expression as well as selective Th1 cell expansion in response to IL-12 (Mullen, High et al. 2001).

Th2 development is initiated by the activation of the major signal transducer STAT6 through IL-4, which induces the key transcription factor GATA-binding protein 3 (Gata3) (Kaplan, Schindler et al. 1996; Takeda, Tanaka et al. 1996). IL-4 induced regulation of gene transcription has been shown to be highly dynamic. While the initiation of Th2 differentiation depends largely on STAT6, which acts as a major switch signal soon after IL-4 exposure, the maintenance of the acquired phenotype requires additional factors at later stages (Elo, Jarvenpaa et al. 2010). Among the direct targets of STAT6 are genes of other transcription factors, including RUNX1, EPAS1 (endothelial PAS domain protein 1) and BATF (basic leucine zipper transcriptional factor ATF-like) that are crucial for programming Th2 cells (O’Shea, Lahesmaa et al. 2011).
The T\textsubscript{H}17 cell differentiation promoting cytokines IL-6, IL-21 and IL-23 all signal through STAT3, and in combination with TGFβ induce the master regulators ROR\textgamma t and ROR\textalpha. STAT3 binds directly to *Il17* and *Il21* and induces the expression of ROR\textgamma t and the IL-23R (Mangan, Harrington et al. 2006; Korn, Bettelli et al. 2007; Nurieva, Yang et al. 2007; Wei, Laurence et al. 2007; Zhou, Ivanov et al. 2007). ROR\textgamma t and STAT3 both bind to the *Il17a/Ii17f* locus and collaborate in driving T\textsubscript{H}17 differentiation. While it has been shown, that TGF-β-independent activation of STAT3 together with IL-1 is sufficient to promote expression of IL-21 and IL-23, this effect is amplified in the presence of TGF-β (Nurieva, Yang et al. 2007; Zhou, Ivanov et al. 2007; Ghoreschi, Laurence et al. 2010).

Development, stabilization and maintenance of the T\textsubscript{Reg} cell phenotype as well as T\textsubscript{Reg} survival depend on Foxp3, the master transcription factor of nT\textsubscript{Reg} (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003). A variety of distinct T cell populations exhibiting a regulatory phenotype can be induced in peripheral naïve CD4\textsuperscript{+} T cells but are not yet well described. Foxp3 expression can be stimulated through TGF-β and IL-2, which signals through STAT5, as well as retinoic acid and continuous expression of Foxp3 is required to maintain the suppressive activity of these iT\textsubscript{Reg} cells (Davidson, DiPaolo et al. 2007; Williams and Rudensky 2007; Zheng and Rudensky 2007; Elias, Laurence et al. 2008).

Generally, transcription factors that drive lineage commitment positively control the expression of lineage-defining genes, but are also capable of suppressing the expression of genes associated with alternative fates. Such inhibition is achieved either by repression of transcription factors important for lineage determination or by preventing the production of cytokines of other lineages. By this means, STAT6 target genes are actively repressed by STAT4 in T\textsubscript{H}1 cells and T-bet suppresses GATA3-expression, while GATA3 on the other hand suppresses T\textsubscript{H}1 differentiation by downregulating STAT4 (Usui, Preiss et al. 2006; Zhu, Yamane et al. 2006; O'Shea, Lahesmaa et al. 2011). In addition, the IL-6/STAT3 pathway suppresses the TGF-β mediated expression of Foxp3, a negative regulator of ROR\textgamma t function. *Stat3* deletion studies in T cells indicate that intrinsic activation of STAT3 in T\textsubscript{Reg} cells enables these cells to specifically suppress T\textsubscript{H}17 cell responses (Chaudhry, Rudra et al. 2009). Furthermore, a recent report indicates that the positive effects of STAT3 on T\textsubscript{H}17 development are balanced by competitive binding of STAT5 at the same locus, which
explains the competitive and dose-dependent regulation of IL-17 production by IL-6 and IL-2 (Gaffen 2011; Yang, Ghoreschi et al. 2011).

In addition to priming cytokines, the CD4^+ T cell differentiation is controlled by the antigen recognition by the T cell antigen receptor (TCR) and the signaling events triggered by this interaction. The overall TCR-mediated signal strength, antigen dosage as well as costimulation provided by APCs influence signaling cascades critical for early cytokine production and the upregulation of the lineage-defining master transcription factor. The TCR signaling strength, composed of the TCR affinity, ligand density and the MHC genotype, may be reflected by the level of activation of major signal transduction pathways, including the ERK/MAPK, calcium/calcineurin, and PKC/NF-κB pathways and has been described to regulate T_H1/T_H1 polarization during *in vitro* differentiation. Early studies have shown, that in general strong signaling favors T_H1 differentiation while weaker signaling leads to T_H2 differentiation (Constant and Bottomly 1997; Murray 1998; Boyton and Altmann 2002; Nakayama and Yamashita 2010). Although there appears to be contradictory evidence suggesting that very low but also very high doses of antigen lead to T_H2 differentiation, while mid-ranged concentrations lead to T_H1 differentiation (Burstein and Abbas 1993; Hosken, Shibuya et al. 1995), more recent insights into the mechanisms governing the differentiation decision support the initial hypothesis. The interaction of the accessory protein CD40, constitutively expressed on all APCs, and CD40 ligand (CD40L), induced in activated CD4^+ T cells, has been stated to promote the activation of APCs and T cells (van Essen, Kikutani et al. 1995). T_H1 lineage commitment is favored by high dose antigen, which, through up regulation of CD40L, induces CD40-mediated IL-12 production by APCs. Low antigen doses on the other hand do not reach the threshold required for CD40L induction and instead induce *de novo* GATA3-dependent IL-4 production from naïve CD4^+ T cells, which promotes autocrine T_H2 differentiation (Ruedl, Bachmann et al. 2000; Yamane, Zhu et al. 2005). It should be noted, however, that presence of TLR-ligands likely is much more important than antigen-dose in driving T_H1 versus T_H2 responses.

Thus, TCR- as well as cytokine-mediated signaling acts in concert to activate distinct downstream pathways which eventually lead to the induction of selective lineage-promoting transcription factors, central for promoting T_H cell fate
determination. However, in order for the transcription factors to activate their target genes, chromatin accessibility is required, which in turn is highly regulated, also in the long-term, through epigenetic modifications.

**Epigenetic modifications influence cellular phenotype and CD4$^+$ T cell plasticity**

Among the epigenetic changes that alter chromatin structure is the selective positioning of nucleosomes, the structural units of chromatin composed of four core histones (H3, H4, H2A, H2B) around which 147 bp of DNA are wrapped. This process is mediated by ATP-dependent chromatin remodeling complexes, which influences protein binding to cis-regulatory regions such as promoters, enhancers or silencers (Eberharter and Becker 2004; Saha, Wittmeyer et al. 2006). Alterations in the histone-content of nucleosomes as well as changes in modification status of histones and DNA represent additional epigenetic mechanisms, which regulate the chromatin state (Ansel, Lee et al. 2003). The N-terminal tails as well as some internal sites of histones can undergo covalent modifications by specific enzymes, including phosphorylation, acetylation, methylation and ubiquitination, which condense or relax the chromatin structure to negatively or actively regulate transcription. The majority of these modifications are considered to be highly dynamic, as enzymes that remove the modifications have been found (Kouzarides 2007). Permissive histone changes including trimethylation of H3K4 (H3K4me3) and H3K79 (H3K79me3) as well as monomethylation of H3K27, H3K9 and H3K20, are found at promoters and enhancers of active genes, whereas di- and trimethylation of H3K27 (H3K27me2, H3K27me3) represent repressive marks correlating with inactive genes (Bernstein, Mikkelsen et al. 2006; Roh, Cuddapah et al. 2006). Each pattern of histone modifications recruits a unique set of regulatory proteins, which then define the overall gene activity. The most relevant modification of DNA is the cytosine methylation of CpG dinucleotides, which leads to recruitment of methyl-CpG binding proteins that are involved with the formation of inaccessible chromatin and thus strongly correlate with gene silencing in mammals (Ansel, Lee et al. 2003).

The standard lineage commitment model of CD4$^+$ T helper cell differentiation implies that the T cell subsets, such as T$_{H1}$, T$_{H2}$ and T$_{H17}$, behave like terminally
differentiated cells with stable and heritable phenotypes (Hirahara, Vahedi et al. 2011). This was initially thought to involve stable programs of gene expression, which correlate with epigenetic changes at loci encoding cytokine genes, thus maintaining their unique cytokine profile, even in conditions that promoted other effector lineages (Murphy, Shibuya et al. 1996; Bird, Brown et al. 1998; Zhou, Chong et al. 2009). While to some extent this might be true, this view has undergone considerable re-evaluation. Recent findings, based on new insights into T\textsubscript{H}17 and T\textsubscript{Reg} differentiation, indicate that T helper cells also have the capacity for more plasticity and, as a response to exogenous signals, can alter their cellular phenotype.

While proximal promoters of cytokine genes generally exhibit permissive histone methylation marks for lineage-defining cytokines in the respective lineages and repressive marks for the cytokine genes of opposing subsets, the molecular mechanism underlying this plasticity may be related to poised bivalent epigenetic states at the transcriptional regulator gene loci (Wei, Wei et al. 2009).

Generation of genome-wide maps for histone modifications in various CD4\textsuperscript{+} T cell subsets revealed that Th1 cells exhibit H3K4 trimethylation at the \textit{Ifng} gene locus and H3K27me3 marks at \textit{Il4}, \textit{Il17} and \textit{Il21} gene loci, correlating precisely with the key cytokine expressed. Similarly, Th2 cells show H3K4me3 marks at \textit{Il4} locus but repressive H3K27me3 at \textit{Ifng}, \textit{Il17} and \textit{Il21} loci, also supporting the dualistic view of fate determination with Th1 and Th2 cells being two mutually exclusive lineages (Ansel, Djuretic et al. 2006; Hatton, Harrington et al. 2006; Schoenborn, Dorschner et al. 2007; Wei, Wei et al. 2009). While in Th17 cells the \textit{Il17} and \textit{Il17f} loci are marked by permissive H3 acetylation and H3K4 trimethylation (Akimzhanov, Yang et al. 2007) and the \textit{Ifng} locus exhibits H3K27 trimethylation, intriguingly the \textit{Il4} locus shows only low levels of H3K27 trimethylation which suggests that these cells harbor the potential for IL-4 production. Comparably, iTregs show extensive H3K27 trimethylation in \textit{Il17} and \textit{Il21} loci, while the \textit{Il4} as well as \textit{Ifng} loci show only little or no repressive marks, and thereby may have potential to be expressed (Wei, Wei et al. 2009).

In addition to cytokine loci, genes encoding transcription factors are also subject to epigenetic modifications, contributing to regulation of T helper cell differentiation.
As mentioned above, STAT family transcription factors are central for T\textsubscript{H} differentiation, however, their activation alone is not sufficient to drive lineage commitment. Consistently, the lineage-promoting STATs are typically associated with unopposed H3K4me3 marks in their promoter region, which correlates with their ubiquitous expression patterns in each of the T cell subtypes (Wei, Wei et al. 2009).

However, STAT transcription factors themselves contribute to distinctive epigenetic modifications in helper T cell subsets. Using genome-wide ChIP-Seq technology and comparative epigenetic analysis of wildtype versus STAT-deficient T\textsubscript{H} cell subsets, the extent of genes having epigenetic modifications depending on a certain STAT protein became evident. In T\textsubscript{H}1 cells, many of the expected target genes such as \textit{Ifng}, \textit{Tbx21} and \textit{IL18r} showed STAT4-dependent epigenetic modifications, while in T\textsubscript{H}2 cells among others the \textit{IL4} and \textit{Gata3} loci displayed STAT6-dependent modifications (Wei, Vahedi et al. 2010). Similarly, STAT3 targets many genes implicated in T\textsubscript{H}17 differentiation for epigenetic changes, including \textit{IL17a}, \textit{IL17f} and \textit{IL21} (Durant, Watford et al. 2010). Nevertheless, not all STATs affect the epigenetic landscape in the same way. STAT4, for example, enhances permissive H3K4me3 marks, while STAT6 regulates suppressive H3K27me3 modifications. This finding is especially interesting regarding a subset of genes bound by STAT4 in T\textsubscript{H}1 but by STAT6 in T\textsubscript{H}2 cells, including the IL18r1-IL18rap locus, on which these two transcription factors have divergent effects (Wei, Vahedi et al. 2010; Hirahara, Vahedi et al. 2011).

As opposed to STATs, master transcription factors such as T-bet, GATA3, ROR\textgamma and Foxp3 are capable of promoting and maintaining at least some properties of the lineage phenotypes. While strong H3K4me3 marks are found on the gene coding for the lineage-specific key regulator, distinct epigenetic states exist for the same gene in different nonexpressing lineages of opposed subtypes. Several of these genomic regions display colocalized permissive H3K4me3 and repressive H3K27me3 marks. Such bivalent epigenetic states may poise these genes driving lineage specification to remain ready for either activation under different polarizing conditions, hence reprogramming these cells into other lineages (Zhou, Chong et al. 2009).

Of the four T helper cell subsets, T\textsubscript{Reg} and T\textsubscript{H}17 cells contain the highest disposition for plasticity (Fig. 1). In nT\textsubscript{Reg}s, the \textit{Foxp3} locus is demethylated and displays H3K4me3 marks, while the \textit{Tbx21}, \textit{Gata3} and \textit{Rorc} genes are all bivalently modified (Floess, Freyer et al. 2007; Janson, Winerdal et al. 2008). Together with
the fact that the *Ifng* as well as the *Il4* locus is not at all or only minimally repressed suggests it may be possible for nT\(_{\text{Reg}}\)s to switch to IFN-\(\gamma\)- or IL-4-producing phenotypes, which indeed has been shown to happen (Wan and Flavell 2007). However, iT\(_{\text{Reg}}\)s seem to differ from nT\(_{\text{Reg}}\)s in their propensity for reprogramming, as iT\(_{\text{Reg}}\)s exhibit permissive histone marks in their *Rorc* locus and repressive ones on the *Il17* region. This is consistent with the finding that it is possible for TGF-\(\beta\) to induce ROR\(\gamma\)t expression in these cells, while ROR\(\gamma\)t-induced IL-17 expression is inhibited by Foxp3 (Zhou, Lopes et al. 2008; Wei, Wei et al. 2009). In addition, iT\(_{\text{Reg}}\)s differ from nT\(_{\text{Reg}}\)s in the stability of Foxp3 expression. The *Foxp3* locus is only partially demethylated in iT\(_{\text{Reg}}\)s, which, despite Foxp3 expression and suppressive function, can lose their Foxp3 expression once restimulated in absence of TGF-\(\beta\) (Floess, Freyer et al. 2007; Huehn, Polansky et al. 2009).

In T\(_{\text{H17}}\) cells, a bivalent epigenetic state is found on the *Tbx21* and *Gata3* loci, while the *Foxp3* locus is neither repressed nor does it show activating histone marks. This suggests that these cells may be redirected toward T\(_{\text{H1}}\) or T\(_{\text{H2}}\) cell fates and is consistent with the finding that Foxp3 can be expressed in T\(_{\text{H17}}\) cells (Zhou, Lopes et al. 2008).

In T\(_{\text{H1}}\) and T\(_{\text{H2}}\) cells, the *Rorc* and *Foxp3* loci are repressed while the *Gata3* and the *Tbx21* genes, respectively, display bivalent modifications, which correlates to a report revealing that interconversion between these two T\(_{\text{H}}\) cell subsets can occur under the appropriate polarizing conditions (Krawczyk, Shen et al. 2007).

Although some T helper cell subsets appear to be terminally differentiated on the basis of epigenetic marks on cytokine loci, the finding that signature genes are not always repressed by H3K27me3 modifications in subsets where they are not expressed, as well as the bivalent modification pattern on key transcription factor loci supports a more dynamic regulation, which may reverse the suppression of signature cytokines in favor of plasticity of helper T cells.
Figure 1 | Epigenetic states of gene loci encoding master regulator transcription factors in CD4⁺ T cells. Key transcription factors of a certain TH subtype show permissive H3K4me3 marks, while others may display bivalent epigenetic states (permissive H3K4me3 modifications together with suppressive H3K27me3 marks), allowing plasticity of these cells in regard to their cellular phenotype. Picture was taken from (Zhou, Chong et al. 2009).

3.3.3 THE INTERLEUKIN-17 FAMILY

Structure

The IL-17 family of cytokines consists of six structurally related members designated IL-17A-F. IL-17A was first described and cloned in 1993 by Rouvier et al. from a murine cytotoxic T lymphocyte (CTL) hybridoma cDNA library and originally termed CTLA-8 (Rouvier, Luciani et al. 1993). Database searches and degenerative PCR strategies have later revealed the five additional homologous cytokines IL-17B, IL-17C, IL-17E (also known as IL-25) and IL-17F, completing the IL-17 family (Li, Chen et al. 2000; Fort, Cheung et al. 2001; Lee, Ho et al. 2001; Starnes, Robertson et al. 2001; Starnes, Broxmeyer et al. 2002). Murine IL-17A is a secreted glycoprotein consisting of 147 amino acid residues, which shares 63% amino acid homology with
the human IL-17A, which in turn consists of 155 amino acids and has a molecular mass of around 35kDa (Yao, Fanslow et al. 1995; Kolls and Linden 2004). Among the IL-17 family members, IL-17F shares the highest amino acid sequence identity with IL-17A, as they are to 50% homologous, whereas IL-17E is the least related, with only 16% homology. While all of the family members share a similar amino acid sequence in their C-termini, the sequences of IL-17B, IL-17C and IL-17E differ greatly from those of IL-17A and IL-17F in the N-terminus, since extensions are longer for the former three cytokines. While the \textit{Ili17a} gene is closely linked to the \textit{Ili17f} gene in all species examined to date (for instance on mouse chromosome 1 or human chromosome 6), the genes for the remaining family members map to distinct chromosomes (Weaver, Hatton et al. 2007; Iwakura, Ishigame et al. 2011). Regarding protein conformation, the crystal structure of IL-17F was the first one to be solved and revealed that IL-17 family members exert their actions as a disulfide-linked homodimeric glycoprotein and adopt a cysteine knot fold involving four of the five spatially conserved C-terminal cysteines, similar to the canonical cysteine knot found in TGF-β, bone morphogenetic protein (BMP) and nerve growth factor (NGF) superfamilies (Fig. 2). However, IL-17F lacks the disulfide responsible for forming the classical canonical knot motif and instead has two conserved serine residues replacing these cysteines (Hymowitz, Filvaroff et al. 2001).

Figure 2 | The structure of IL-17F. A | Ribbon trace of the IL-17F monomer with labeled strands and sulfur atoms involved in disulfide bonds highlighted in yellow. Inset: Canonical cysteine knot fold with cysteine residues in yellow and missing cysteines in black. B | Ribbon trace of the IL-17F dimer, color labeling shown as in A. Picture was taken from (Weaver, Hatton et al. 2007), however original figure can be found in (Hymowitz, Filvaroff et al. 2001).
In addition it has been shown that IL-17A and IL-17F are able to form not only functional homo- but also heterodimers in human T cells, mainly due to their sequence homology and overlapping expression pattern (Wright, Guo et al. 2007). Later, a study analyzing IL-17A in complex with a human neutralizing antibody confirmed the conserved structure found for IL-17F and revealed that the homodimeric cytokine was indeed sandwiched between two antibody-derived Fab fragments, with each fragment contacting both monomers in the dimer (Gerhardt, Abbott et al. 2009).

**Cellular sources**

IL-17A is the most prominent member of the IL-17 family and was initially designated product of activated CD4$^+$ memory T lymphocytes before gaining especial attention with the discovery of T$_{H}^{17}$ cells as a CD4$^+$ T cell subset distinct from the classical T$_{H}^{1}$ and T$_{H}^{2}$ lineages, which produce IL-17A as their main signature cytokine (Yao, Fanslow et al. 1995; Fossiez, Djossou et al. 1996; Harrington, Hatton et al. 2005). Due to the tightly clustered genomic organization and coordinate regulation during effector T cell development, the expression of IL-17A and IL-17F from immune cells seems to be coherent (Weaver, Hatton et al. 2007). However, IL-17A and IL-17F expression does not appear to be limited to CD4$^+$ effector cells. A wide variety of T cells represent additional sources of these two cytokines, including cytotoxic CD8$^+$ T cells (Tc17), distinct populations of $\gamma\delta$T (γδ-17) cells and NKT (NKT-17) cells (Lockhart, Green et al. 2006; Liu, Tsai et al. 2007; Cua and Tato 2010). Among them, γδ-17 and NKT-17 cells belong to the cell populations, which act rapidly during infection, before the onset of adaptive T cell responses and therefore represent two innate sources of IL-17. More recently, studies have also demonstrated rapid IL-17 expression in innate populations of neutrophils, monocytes, T$_{FH}$ and lymphoid tissue inducer (LTi)-like cells among others (Cua and Tato 2010; Iwakura, Ishigame et al. 2011). These sources of IL-17 play substantial roles in regulating IL-17 dependent immune responses, especially at mucosal surfaces (Gaffen 2011).

IL-17B is expressed as a noncovalent dimer in the pancreas, small intestine, stomach and in the spinal cord with especially high expression levels in chondrocytes.
and neurons (Li, Chen et al. 2000; Shi, Ullrich et al. 2000). IL-17C however can be detected in human testes, thymus, spleen and prostate and is expressed in CD4$^+$ T cells and macrophages at sites of inflammation (Li, Chen et al. 2000; Yamaguchi, Fujio et al. 2007). Like IL-17B, IL-17D expression is found in several tissues including skeletal muscle and neuronal cells, whereas, in immune cells, expression may be induced only in resting CD4$^+$ T and B cells. Unlike IL-17A and IL-17F, IL-17D is not expressed in activated B- or T-lymphocytes (Starnes, Broxmeyer et al. 2002). Low levels of IL-17E, the most divergent IL-17 family member, have been detected in the lung, brain, prostate and testis in human tissues as well as in murine \textit{in vitro}-derived T$_H$2 lineages (Fort, Cheung et al. 2001; Lee, Ho et al. 2001).

### 3.3.4 The IL-17 Receptor Complex and IL-17 Mediated Signaling

The IL-17 receptor (IL-17R) family contains five receptor subunits IL-17RA – IL-17RE, among which IL-17RA is by far the most important member and represents a common signalling subunit used by at least four of the IL-17 cytokines (Aggarwal and Gurney 2002). All of these receptors are not homologous to any known receptors and therefore constitute a unique family of cytokine receptors, with the individual family members exhibiting considerable sequence divergence. Similar to the IL-17 family of cytokines, the five members of the IL-17R family share unique structural features. All receptor subunits are Type I single-pass transmembrane proteins and appear to contain several conserved structural motifs, comprising a unusual large cytoplasmic tail with a SEF/IL-17R (SEFIR) domain and an extracellular amino-terminus with a fibronectin III-like domain, later being an essential component of the IL-17A-binding domain (Kramer, Yi et al. 2006; Kramer, Hanel et al. 2007). With exception of IL-17RA, the additional five receptors IL-17RB - IL-17RE display significant alternative splicing, resulting in some forms being secreted as soluble proteins whilst retaining their ligand binding capacities (Haudenschild, Moseley et al. 2002).
Receptor complex formation and composition

Initial studies recognized IL-17RA as the receptor required not only for IL-17A signalling, but also for signal transduction mediated by IL-17F and the IL-17A/IL-17F heterodimer (Yao, Fanslow et al. 1995; Hymowitz, Filvaroff et al. 2001). However it was found that the affinity of this receptor for IL-17A was lower than the minimal concentration necessary for signal transduction, indicating that an additional subunit must be involved. Indeed, in order to induce responses to these cytokines, IL-17RA pairs with IL-17RC to form a heteromeric complex. Furthermore, it has been shown that Il17ra−/− and Il17rc−/− mice fail to respond to either cytokine, IL-17A and IL-17F, revealing that both receptor subunits are involved in signal transduction (Toy, Kugler et al. 2006; Kuestner, Taft et al. 2007; Wright, Bennett et al. 2008). However, the two receptors have different affinities for IL-17A and IL-17F; in humans, IL-17RA has higher affinity for IL-17A than for IL-17F, while IL-17RC binds to both cytokines with comparable affinity. Murine IL-17RC on the other hand binds predominantly to IL-17F (Hymowitz, Filvaroff et al. 2001; Kuestner, Taft et al. 2007). There is some evidence that IL-17RA associates with other subunits as well. The heterodimer composed of IL-17RA and IL-17RB for instance serves as a receptor for IL-17E whereas the proposed existence of a complex formed by IL-17RA with IL-17RD has yet to be validated. To date, the exact mechanism of the receptor complex formation is not well defined. The classical view of cytokine receptor signalling suggests that individual receptor subunit monomers reside in the membrane and oligomerize upon ligand binding thereby juxtaposing receptor-associated signalling intermediates. In contrast, recent studies could show that IL-17RA forms ligand-independent complexes, with the pre-assembly of receptor subunits allowing for a more rapid and ligand-specific response (Kramer, Yi et al. 2006; Kramer and Gaffen 2007; Kramer, Hanel et al. 2007). While IL-17RA may exist as a preformed homodimer on the cell surface, the formation of the heteromeric complex with IL-17RC is ligand-dependent, as IL-17RA undergoes a conformational change upon IL-17 binding, which enables the association with IL-7RC resulting in a heterodimeric signalling complex (Kramer, Yi et al. 2006). More recent studies using surface plasmon resonance (SPR) strategies demonstrated a stepwise association of receptor/ligand binding. After engagement of either IL-17RA or IL-17RC by IL-17A or IL-17F, the affinity of the second binding-
site for the binding of the alternative receptor subunit is increased, encouraging the formation of a heterodimeric complex (Ely, Fischer et al. 2009). While the exact stoichiometry of the biologically relevant form of the receptor complex remains to be fully determined, native gel analyses of IL-17RC point to the existence of a complex composed one IL-17RC subunit and two IL-17RA subunits (You, Shi et al. 2006).

**Receptor expression**

Although IL-17A and IL-17F both bind and signal through the same receptors, the two cytokines exhibit distinct biological effects, which may be explained with the cell type- and tissue-specific receptor distribution. Recent studies have revealed that IL-17RA for instance is distributed primarily in immune cells such as murine macrophages and T cells, human peripheral blood T lymphocytes and vascular endothelial cells, where expression of the receptor is high. A slightly lower expression is observed on fibroblasts, osteoblasts, endothelial and epithelial cells, while macrophages and DCs are only marginally responsive (Moseley, Haudenschild et al. 2003; Ishigame, Kakuta et al. 2009). In contrast, IL-17RC has been shown to be preferentially expressed in nonimmune cells such as murine colonic epithelial cells as well as human cells of the prostate, liver, kidney, thyroid and joints (Haudenschild, Moseley et al. 2002; Kuestner, Taft et al. 2007; Gaffen 2009; Ishigame, Kakuta et al. 2009). As mentioned above, IL-17RA and IL-17RC exhibit different binding preferences and affinities for IL-17A and IL-17F, which additionally supports the possibility, that the two cytokines make differential use of the receptor subunits. Moreover it could be shown, that the cell-surface expression level of IL-17RA, which is dynamically regulated by factors such as IL-15, IL-21 and PI3K, correlates with ligand-induced signalling strength and that signalling strength in terms of downstream gene activation induced by IL-17F is decreased up to 30 fold compared with IL-17A, with the IL-17A/IL-17F heterodimer acting at an intermediate level (Gaffen 2009).
Signalling pathways induced via the IL-17RA/RC complex

Receptors of the IL-17R family mediate signalling events that differ remarkably from those triggered by other cytokine receptors usually involved in adaptive immunity. The signature cytokines of the classical T\textsubscript{H}1 and T\textsubscript{H}2 lineages induce signalling cascades via the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins. In particular, the T\textsubscript{H}1-type cytokine INF-\gamma acts via the JAK1- and JAK2-STAT1-dependent pathway, while the T\textsubscript{H}2-type cytokine IL-4 activates a JAK1- and JAK3-STAT6 dependent pathway. T\textsubscript{H}17-derived IL-17A and IL-17F on the other hand, trigger a signalling pathway depending on Act1 (also known as CIKS) and TNFR-associated factor 6 (TRAF6), eventually leading to the induction of pro-inflammatory mediators such as nuclear factor-\kappaB (NF-\kappaB), an event usually related to receptors associated with innate immunity, for instance IL-1 family receptors (Fig. 3). However, another minor T\textsubscript{H}17 signature cytokine, IL-22, has the ability to activate a second signal transduction pathway which involves JAK1- and TYK2-STAT3 signalling, which targets downstream genes similar to the ones induced by IL-17. By this means, the rather unusual signalling properties of T\textsubscript{H}17 cells promote signals common to early inflammatory events and thereby serve to bridge innate and adaptive immunity (Yu and Gaffen 2008; Gaffen 2009).

The IL-17R family members IL-17RA and IL-17RC, which together form the IL-17 receptor complex, both encode conserved SEF/IL-17R (SEFIR) domains. However, only the SEFIR motif of the IL-17RA subunit has remarkable sequence homology to the Toll/IL-1R (TIR) BB-loop and is therefore termed TIR-like loop (TILL) (Novatchkova, Leibbrandt et al. 2003; Maitra, Shen et al. 2007). While the TIR domain found in Toll and IL-1 receptors provides docking site for intracellular adaptors such as MyD88, IL-17RA rather engages the adaptor Act1, which contains a SEFIR as well as a TRAF-binding domain and mediates diverse downstream events (Chang, Park et al. 2006). How exactly IL-17RC participates in signalling and whether it binds to the distinct downstream molecules observed for IL-17RA remains to be elucidated. Upon IL-17 binding, Act1 is rapidly phosphorylated by yet unknown kinases and recruits central upstream activators of the canonical NF-\kappaB pathway, explicitly the NF-\kappaB-activating TNFR-associated factor 6 (TRAF6) and TAK1 as
well as the inhibitory TRAF3 (Schwandner, Yamaguchi et al. 2000; Qian, Liu et al. 2007; Zhu, Pan et al. 2010). Furthermore, IL-17 activates several NF-κB downstream targets including inhibitor of NF-κB-ζ (IκBζ) and the transcription factors CCAAT/enhancer-binding proteins (mainly C/EBPβ and C/EBPδ) as well as mitogen-activated protein kinase (MAPK) and possibly PI3K (Ruddy, Wong et al. 2004; Gaffen 2009).

Among the various MAPK stimulated by IL-17, the extracellular signal-regulated kinase 1 (ERK1) and ERK2, are the ones most strongly and rapidly phosphorylated and in turn mediate phosphorylation of C/EBPβ on threonine 188 within 15 minutes of IL-17 stimulation. This modification serves as a first event to regulate the transcription factor. The second event is the phosphorylation of threonine 179 in the regulatory site within C/EBPβ by glycogen synthase 3β (GSK3β), which occurs after one hour and requires prior phosphorylation by ERK. While regulation of ERK is mediated by the SEFIR and TILL domains of IL-17RA, the regulation of GSK3β is mediated by a different domain in the distal tail of the receptor, known as C/EBPβ-activation domain (CBAD) (Ramji and Foka 2002; Maitra, Shen et al. 2007). Together with the SEFIR and TILL domains, this CBAD domain is also involved regulating the IL-17-dependent expression of the tree existing C/EBPβ isoforms generated by alternative translation, particularly the liver-enriched activator protein LAP* (the other two being the shorter and transcriptionally more active LAP and the liver-enriched inhibitory protein LIP). Promoters of IL-17A target genes are enriched for C/EBP-binding elements and binding of these transcription factors is required for the activation of certain promoters such as the promoter for the Il6 gene. Surprisingly, the two IL-17-induced phosphorylation events of C/EBPβ eventually lead to the downregulation of its transcriptional capacity. However, since this negative regulation as well as the alternative translation of C/EBPβ occurs in a time frame in which IL-17A also upregulates the C/EBPδ expression via the canonical NF-κB pathway, the transcriptional control of the C/EBP-dependent IL-17 target genes seems dynamically regulated. Hence, C/EBP transcription factors serve as important mediators of IL-17-induced signalling but are also targets thereof, as their expression is regulated by IL-17 (Ruddy, Wong et al. 2004; Shen, Li et al. 2009).
**Figure 3 | The IL-17-mediated signalling pathways.** The IL-17 receptor complex consists of the subunits IL-17RA and IL-17RC. It contains different functional receptor motifs (SEFIR, TILL, CBAD), through which it mediates several downstream events. The Receptor engages the adaptor Act1 that upon activation recruits upstream activators of the canonical NF-κB pathway such as TRAF6, TRAF3 and TAK1, which leads to the activation of the downstream targets IκBζ and c/EBPδ. In addition, IL-17 induces the activation of the transcription factor C/EBPβ, which exists in three splice variants, of which two are depicted here (LAP and LAP*). This activation process is mediated via the kinases ERK and GSK3β. Moreover, IL-17 leads to the activation of different MAPKs and possibly PI3K and induces the stabilization of various mRNAs encoding target cytokines and chemokines.

CBAD, C/EBPβ-activation domain; C/EBP, CCAAT/enhancer-binding protein; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; IκBζ, inhibitor of NF-κB-ζ; LAP, liver-enriched activator protein; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; SEFIR, SEF/IL-17R domain; TAK1, TGF-β-activated kinase 1; TILL, Toll/IL-1 R-like loop; TRAF, TNFR-associated factor. Picture taken from (Gaffen 2009).

The most important role of the IL-17 induced MAPK pathway is regulating the stability of several target mRNAs, particularly those encoding chemokines and cytokines. TRAF6 and TAK1 have been shown to activate MAPKs including p38, which have well-established roles in promoting mRNA stabilization. Nevertheless, recent findings propose a second, TRAF-6-independent mechanism (Hartupee, Liu et al. 2009). Surprisingly IL-17A, IL-17F but also IL-17A/IL-17F display rather weak signal activity, even at high concentrations. However, these cytokines are able to enter effective synergy with factors such as TNFα, IFN-γ as well as IL-1β (Gaffen 2011). It is in cooperation with these factors, that IL-17 potentially inhibits mRNA...
destabilizing proteins, leading to increased mRNA transcript half-life. Expression of IL-6 and CXCL1 for instance is only modestly activated by IL-17, but together with TNFα IL-17 enhances transcript half-life considerably, a process which has been shown to depend on the adaptor protein Act1, but not TRAF6 (Hartupee, Liu et al. 2009).

3.3.5 IL-17 EFFECTOR FUNCTIONS

As mentioned above, IL-17A and IL-17F possess high sequence identity and are both secreted as homo- or heterodimers, in which form they bind to the same receptor, letting suggest that these two cytokines may have similar biological functions. Indeed, IL-17A and IL-17F have been shown to be potent stimulators of host protection and inflammatory processes and activate immune cells such as T- and B-cells as well as macrophages, to promote T cell priming, antibody production and the expression of certain proinflammatory cytokines (in the case of macrophages, these are IL-1, IL-6, TNF and IL-12). IL-17A and IL-17F are also capable of inducing the expression of genes encoding specific proinflammatory mediators including cytokines (TNF, IL-1, IL-6, G-CSF and GM-CSF), chemokines (CXCL1, CXCL5, IL-8, CCL2 and CCL7), antimicrobial peptides (β-defensins, AMPs, cathelicidin, RegIII, lipocalin 2, salivary histatins), and matrix metalloproteinases (MMP1, MMP3 and MMP13), which are expressed by nonimmune cells such as epithelial cells, endothelial cells and fibroblasts. Subsequently IL-17A contributes to the generation and accumulation of neutrophils to sites of inflammation via the neutrophil-activating factors G-CSF and CXC chemokines. In addition, IL-17A induces SCF- and G-CSF-mediated granulopoiesis and stimulates the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 in chondrocytes as well as intercellular cell adhesion molecule 1 (ICAM-1) in keratinocytes. Together these mediators not only coordinate inflammatory processes and protect against infection of fungi and bacteria, they also promote local tissue destruction, enhance osteoclastogenesis and induce neovascularization in tumors, resulting in development of various diseases. However, the fact that IL-17F is a less potent inducer of the expression of proinflammatory cytokines as well as the previously mentioned distinct tissue distribution of IL-17RA and IL-17RC account for some functional specialization of these two cytokines.
While IL-17A plays important roles in autoimmune and allergic responses, host defence against bacterial and fungal infections and tumor development, IL-17F is mainly involved in the host resistance against bacteria and inflammation in epithelial tissues (Gaffen 2011; Iwakura, Ishigame et al. 2011). The origin, effector functions and targets of all the IL-17 family members are summarized in Table 1.

**Table 1 | Origin, effector functions and targets of the IL-17 family members.**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
<th>Percent Homology with IL-17A</th>
<th>Percent Homology with Human</th>
<th>Producer Cells</th>
<th>Target Cells</th>
<th>Proposed Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17A</td>
<td>IL-17RA and IL-17RC</td>
<td>100</td>
<td>63</td>
<td>CD4⁺ T cell, CD8⁺ T cell, γδ T cell, NKT cell, LTi-like cell, neutrophil and Paneth cell</td>
<td>epithelial cell, fibroblast, keratinocyte, synovioocyte, endothelial cell, T cell, B cell and macrophage</td>
<td>proinflammatory cytokine and chemokine induction; neutrophil recruitment; antimicrobial peptide induction; osteoclastogenesis; angiogenesis; promotion of T-cell priming and Ab production</td>
</tr>
<tr>
<td>IL-17B</td>
<td>IL-17RB</td>
<td>24</td>
<td>88</td>
<td>chondrocyte and neuron</td>
<td>monocyte and endothelial cell</td>
<td>proinflammatory cytokine induction; neutrophil recruitment</td>
</tr>
<tr>
<td>IL-17C</td>
<td>IL-17RE</td>
<td>26</td>
<td>83</td>
<td>CD4⁺ T cell, DC, macrophage and keratinocyte</td>
<td>monocyte</td>
<td>proinflammatory cytokine induction; neutrophil recruitment</td>
</tr>
<tr>
<td>IL-17D</td>
<td>unknown</td>
<td>30</td>
<td>78</td>
<td>CD4⁺ T cell and B cell</td>
<td>endothelial cell and myeloid progenitor</td>
<td>proinflammatory cytokine induction</td>
</tr>
<tr>
<td>IL-17E</td>
<td>IL-17RA and IL-17RB</td>
<td>16</td>
<td>81</td>
<td>CD4⁺ cell, CD8⁺ T cell, mast cell, eosinophil, epithelial cell and endothelial cell</td>
<td>T cell, macrophage, epithelial cell, NK, MΦLin⁺ cell, nucocyte, and Th2 cell</td>
<td>eosinophil recruitment; promotion of Th2 and Th9 cell responses; suppression of Th1 and Th17 cell responses</td>
</tr>
<tr>
<td>IL-17F</td>
<td>IL-17RA and IL-17RC</td>
<td>50</td>
<td>77</td>
<td>CD4⁺ cell, CD8⁺ T cell, γδ T cell, NKT cell, LTi-like cell, and epithelial cell</td>
<td>epithelial cell, fibroblast, keratinocyte, synovioocyte and endothelial cell</td>
<td>proinflammatory cytokine and chemokine induction; neutrophil recruitment; antimicrobial peptide induction</td>
</tr>
</tbody>
</table>

Taken from (Iwakura, Ishigame et al. 2011).

**IL-17 in autoimmunity**

Autoimmunity is defined as an uncontrolled immune response against autologous antigens and may precede the clinical detectable onset of disease up to years. There is mounting evidence that disease development is influenced not only by genetic predisposition but also environmental factors. Susceptibility genes may interfere with the maintenance of self-tolerance and result in the appearance of autoreactive T- and B cells. Infections and other inflammatory stimuli have been suggested to promote the influx of cellular components into tissues and lead to the activation of these self-
reactive lymphocytes, which contribute to pathology. Key to understanding disease-underlying mechanisms and to ultimately develop effective therapies are pre-clinical animal models, as patient material is usually hard to retrieve or in certain cases not even accessible (Pollinger 2012).

Two of the most common autoimmune disorders to date are multiple sclerosis (MS) and rheumatoid arthritis (RA). Although they differ in their specific clinical symptoms, both diseases are initiated by inflammatory events, which result in the destruction of distinct target tissues.

Multiple Sclerosis

Multiple sclerosis is a heterogeneous autoimmune disease that is caused by an inappropriate immune response to antigens of the central nervous system (CNS) and targets the myelin sheet surrounding axons of the brain and spinal cord, which eventually leads to inflammation, demyelination and axonal injury. Clinical symptoms usually manifest themselves in young adulthood, with females being 2-3 times more affected than males, and consist of intermittent phases of neurological dysfunctions including impairment of motor and sensory function, ataxia and in defects in the autonomic, visual and cognitive systems (Chen, Wang et al. 2012). While in a majority of MS patients the disease presents itself in a relapse-remission manner at early stages, the recovery of these patients is impaired as the disease advances and ultimately results in irreversible progression, so called secondary progressive MS (SPMS). Around 10% of patients diagnosed with MS do not experience any remission phases and suffer from a continuous form of the disease termed primary progressive MS (PPMS) (Glass, Saijo et al. 2010). While the specific biological mechanisms underlying the disease are not completely understood, viral and bacterial infections potentially act as triggers for the initiation of MS. As an example of molecular mimicry, certain regions of pathogen-related antigenic proteins resemble components of the myelin sheet, such as the myelin basic protein (MBP). Pathogenic antigens recognized to date include a peptide from hepatitis B virus (HBV) and possibly peptides of the Epstein-Barr virus (EBV) as well as some enterobacteria (Fujinami and Oldstone 1985; Glass, Saijo et al. 2010). As proposed for many autoimmune disorders, environmental factors such as infections are thought to act in concert with certain genetic predispositions to initiate disease. For MS there
are several polymorphisms suggested to underlie disease susceptibility, the most prominent ones being MHC class II molecules including the two HLA-DRB* genes of the HLA-DR15 haplotype DRB1*1501 and DRB5*0101 as well as genes expressing proteins regulating differentiation, activation and migration of effector T and B lymphocytes (Oksenberg and Hauser 2005; McFarland and Martin 2007). Furthermore it is believed that while both the innate and the adaptive immune system are involved in MS pathology, it is the innate immune cells outside of the CNS, such as DCs, that play a crucial role for initiation of the disease (Bailey, Schreiner et al. 2007; Glass, Saijo et al. 2010). Immature DCs are known to generally maintain peripheral tolerance by stimulating suppressive and anti-inflammatory functions of T_{Reg} and T_{H2} cells, respectively. In MS however, abnormally activated mature DCs present the autoantigen to T lymphocytes, provide costimulation and secrete proinflammatory cytokines. In addition to providing the stimulus for differentiation of effector T lymphocytes, DCs are also involved in generating other autoreactive adaptive immune effectors, in particular B cells and CTL/CD8^+ T cells, which play roles as amplifiers and effectors in MS (Glass, Saijo et al. 2010). These myelin-reactive cells have been shown to exhibit an increased expression of surface molecules such as the adhesion molecule α4β5-integrin (VLA4) that allow them to penetrate the blood brain barrier (BBB) and the blood cerebrospinal fluid barrier (BCSFB) by binding to endothelial cell adhesion molecules (ECAMs), such as VCAM1, E-selectin, ICAM-1 and PECAM-1 (Elves, Osborn et al. 1990; Dore-Duffy, Washington et al. 1993; Elovaara, Ukkonen et al. 2000). Proinflammatory cytokines and chemokines released from autoreactive T lymphocytes, primarily TNF-α, IL-1β, IL-17, IFN-γ, IL-12 and IL-6, initiate and sustain inflammatory responses in MS. They not only induce the expression of the ECAMs and thereby increase adhesion of the leukocytes to the endothelium, but also disrupt the BBB by disorganizing cell-cell junctions and promote migration of the autoreactive effector cells into the CNS (Woodroffe and Cuzner 1993; Minagar and Alexander 2003). Consequently, acute inflammatory lesions (plaques) in the perivascular region of the brain and spinal cord white matter are generated, which are characterized by infiltration of self-reactive lymphocytes and antibody-producing plasma cells as well as the presence of autoreactive CNS components such as astrocytes and proliferating oligodendrocytes, also activated by the proinflammatory cytokine milieu (Lopez-Diego and Weiner 2008). As the disease progresses, APCs such as DCs migrate into the CNS, where
they reactivate lymphocytes and initiate the inflammatory response within the CNS compartment. Subsequently, inflammatory and innate immune cells such as macrophages, granulocytes and natural killer cells are attracted into the CNS compartment while more CNS resident microglia and astrocytes accumulate during this later development of lesions. The aberrant activation of these cells leads to an upregulated release of proinflammatory cytokines and chemokines, MMPs, toxic molecules such as reactive nitrogen species (RNS), reactive oxygen species (ROS), glutamate-mediated exitotoxicity and autoantibody production, which together maintain inflammation and mediate demyelination and neurodegeneration (Glass, Saijo et al. 2010; Chen, Wang et al. 2012). In addition, CTL/CD8+ T cells mediate direct cytotoxicity on MHC class I-expressing cells including oligodendrocytes and neurons and deposition of antibodies and complement around demyelinated lesions promotes further axonal degeneration (Skulina, Schmidt et al. 2004; Zang, Li et al. 2004; O'Connor, Appel et al. 2005). Large amounts of chronically activated B cells appear to correlate with the rate of disease progression and activated astrocytes have been reported to overexpress BAFF, a survival factor for autoreactive B cells, further stabilizing this effector cell line (Krumbholz, Theil et al. 2005). While remyelination of neurons might occur as part of normal repair it is severely impaired in the presence of chronic inflammation as observed for MS and can therefore not counteract the degenerative effects of this autoimmune disease (Glass, Saijo et al. 2010).

The widely accepted animal model for MS is experimental autoimmune encephalomyelitis (EAE), which in addition to rodents can elicit demyelinating paralysis in a variety of animals such as monkey, sheep and dogs (Freund, Stern et al. 1947; Kabat, Wolf et al. 1947; Lipton and Freund 1952). However, the clinical course of the disease as well as histopathology differ tremendously depending on the genetic background of the animals, the source and application mode of the antigenic material (Mix, Meyer-Rienecker et al. 2010). In general, EAE is induced actively (A-EAE) or passively (P-EAE) and more recently, several spontaneous EAE models have been analyzed. A-EAE is induced actively by the subcutaneous immunization with an antigen emulsified in CFA containing Mycobacterium tuberculosis followed by an intraperitoneal injection of pertussis toxin (P.tx.). In this setting, the CFA serves as a stimulator for the innate immune to promote an inflammatory response which results in effective antigen-presentation that in turn leads to the generation of CD4+ T
lymphocytes and activated macrophages. The P.tx. serves as an adjuvant for T cells, induces activation of Toll-like receptor 4 (TLR4) on innate immune cells and manages to break self-tolerance in EAE-resistant mouse strains (Hofstetter, Shive et al. 2002; Waldner, Collins et al. 2004; Pollinger 2012). P-EAE on the other hand is induced by the transfer of in vitro generated and activated antigenic T cell lines into recipient animals, nevertheless the cellular phenotype may never reach complete identity with in vivo generated T cell clones (Mokhtarian, McFarlin et al. 1984). Since the initial studies on EAE models, the complexity of the antigenic material used to trigger the disease has been stepwise reduced from simple brain tissue and central myelin and non-myelin proteins to small encephalitogenic peptides, with which the various models now have become more reproducible. The most common peptides are derived from myelin proteins including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) (Einstein, Robertson et al. 1962; Lennon, Wilks et al. 1970; Lebar, Lubetzki et al. 1986; Tuohy, Lu et al. 1988; Mix, Meyer-Rienecker et al. 2010). Despite certain limitations, the MOG35-55-EAE induced in C57BL/6 mice is the most frequently used murine model for MS to date and has been valuable for studying the contributions of various cell types to pathogenesis of this disease (Egen and Ouyang 2010). In this model, first clinical symptoms manifest 10-20 days after disease induction and present as chronic progressive paralysis starting with a loss of tail tonus to partial and eventually entire paralysis of tail, hind limbs and ultimately, tetraplegia (Pollinger 2012). A disadvantage of this model, as in any actively induced EAE model, is the large and permanent applied CFA deposits, which are a strain for the animals complete immune system. In addition it is not yet fully understood, whether the integrity of the BBB in human MS is as disrupted as in EAE, a circumstance particularly relevant if therapies targeting cytokines are developed, since this may limit the ability of the therapeutic molecule to reach a target inside the CNS (Mix, Meyer-Rienecker et al. 2010).

**Rheumatoid Arthritis**

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects around 1% of the population and primarily targets the bone, cartilage and the synovial membrane. Early autoimmunity manifests itself in the production of autoantibodies against IgG (known as rheumatoid factors) and cyclic citrullinated peptides, however
little is known about the mechanisms involved in breaching B- and T-cell tolerance and the triggers crucial for the onset of inflammatory synovitis (McInnes and Schett 2007). Several genetic loci are implicated with the susceptibility to RA, including the well-established HLA-DR4 alleles. Among the environmental risk factors associated with RA, smoking has been shown to critically promote disease-development in HLA-DR4 positive individuals, which is particularly interesting since it is known that smoking stimulates the citrullination of self-proteins (Klareskog, Padyukov et al. 2007). Disease onset is characterized by the inflammation and hyperplasia of the synovial membrane, in which a superficial layer of synovial fibroblast and macrophages covers an interstitial zone of cellular infiltrate comprising synovial fibroblasts, macrophages, B and plasma cells, CD4$^+$ and CD8$^+$ T cells, NK and NKT cells. This cellular infiltrate then invades the adjacent cartilage and eventually also the bone, resulting in progressive synovial fibroblast- and osteoclast-mediated joint destruction. This degradation process possibly leads to the generation of new antigens thereby further promoting autoimmunity. In addition, the arthritic joint is severely hypoxic, which promotes angiogenesis, a characteristic feature of RA. The joint-resident osteoclasts and synoviocytes as well as immune cells are believed to communicate via signaling molecules such as cytokines. Moreover, the specific cytokine milieu observed in the synovium, consisting of IL-1$\beta$, IL-6, IL-12, IL-15, IL-8, IL-23 and TGF-$\beta$, is not only responsible for the maintenance of the chronic inflammation of the synovium but also drives the tissue destruction of cartilage and bone as well as promotes the differentiation of synovial effector and regulatory T cells and regulates their phenotype. While several cytokines including TNF-$\alpha$, IL-1, IL-10 and IL-18 have been implicated with the disease and the current standard therapy is targeting TNF-$\alpha$, dissecting the role of IL-17 in RA has been of particular interest in the past years (Lubberts, Koenders et al. 2005; McInnes and Schett 2007).

Most of the knowledge about the underlying patho-mechanisms has been gained from the use of the collagen-induced arthritis (CIA) model, however other models such as the antigen-induced arthritis (AIA) model have proven beneficial (Pollinger, Junt et al. 2011). In general, induction of arthritis in the CIA model is achieved by immunizing with type II collagen in combination with complete Freund’s adjuvants (CFA), generating antibodies against this particular collagen, a component found in the mature adult cartilage. Within 23 days after disease induction, arthritic mice
develop symptoms resembling human RA, in particular following initial inflammation the peripheral joints develop edemas, which eventually results in pannus formation and finally erosion of bone and cartilage. In contrast to most of the EAE models, which are known to have a strong T lymphocyte contribution, the components initiating models for RA are more diverse and besides T cells include also B cells, antibodies and innate immune cells (Pollinger 2012).

**Discovery of T\textsubscript{H}17 cells as drivers of autoimmunity**

Many organ-specific autoimmune diseases have long been believed to be the outcome of dysregulated autoantigen-specific T\textsubscript{H}1 cell responses mediated by this T\textsubscript{H} subset effector cytokines, as a pathogenic effect has been shown in various animal models of human diseases. However, more recent lines of evidence have challenged this concept by proving that the IL-23-IL-17 rather than the IL-12-INF-\textgamma signaling axis promotes autoimmunity, particularly in the CIA and EAE, as well as inflammatory bowel disease (IBD) (Cua, Sherlock et al. 2003; Murphy, Langrish et al. 2003; Lubberts, Koenders et al. 2004). Genetic or antibody-mediated depletion of the T\textsubscript{H}1 effector cytokine IFN-\gamma leads to enhanced inflammatory symptoms in EAE and CIA and results from studies in mice deficient for the T\textsubscript{H}1-pathway molecules IFN-\gammaR, IL-12(p35) and IL-12R\textbeta2 additionally contradict the importance of T\textsubscript{H}1 cells in these diseases (Billiau, Heremans et al. 1988; Ferber, Brocke et al. 1996; Zhang, Yu et al. 2003; McGechay and Cua 2008). Studies of the relative contribution of IL-12 and IL-23 revealed that while deficiency in the IL-12p35 subunit has no influence on progression of EAE, the lack of either the p40 or the p35 subunit, which together make up IL-23, results in protection from EAE as well as CIA (Cua, Sherlock et al. 2003; Murphy, Langrish et al. 2003). Around the same time, IL-23 was recognized as the main cytokine driving the expansion of an IL-17-secreting CD4\textsuperscript{+} T cell population, subsequently termed T\textsubscript{H}17 (Aggarwal, Ghilardi et al. 2003). Similar to IL23a\textsuperscript{−/−} mice, which exhibit a reduced number of T\textsubscript{H}17 cells and are resistant to EAE and CIA, development of these diseases is attenuated in IL17\textsuperscript{−/−} mice (Murphy, Langrish et al. 2003; Langrish, Chen et al. 2005). In addition it was shown that the passive transfer of IL-17 producing memory activated CD4\textsuperscript{+} T cells, which were expanded *in vitro* with IL-23, resulted in severe EAE, finally substantiating the concept of T\textsubscript{H}17 cells being critical drivers of autoimmunity (Langrish, Chen et al. 2005).
Nevertheless, there are contradicting studies showing that IL17<sup>−/−</sup> mice are at least in part still susceptible to inflammation following MOG immunization, suggesting the contribution of other IL-23-induced factors in disease progression, such as the T<sub>H</sub>17-derived IL-17F or IL-22 (Haak, Croxford et al. 2009). However, in 2009 Ishigame et al. presented extensive experiments using IL17<sub>a</sub><sup>−/−</sup>, IL17<sub>f</sub><sup>−/−</sup> and IL17<sub>a</sub><sup>−/−</sup>IL17<sub>f</sub><sup>−/−</sup> mice to analyze the distinct functions of IL-17A and IL-17F in immune disorders. They convincingly demonstrated that IL-17A contributes critically to the development of EAE, CIA, among other autoimmune disorders, whereas IL-17F is dispensable for the induction of these diseases. Furthermore, disease severity was unchanged in IL-17<sub>a</sub><sup>−/−</sup>IL17<sub>f</sub><sup>−/−</sup> mice compared to IL-17<sub>a</sub><sup>−/−</sup> mice, suggesting that IL-17F does not have any additive, compensatory or synergistic properties to those of IL-17A. Also, IL17-F is less capable of inducing cytokines from macrophages or T-cells compared to IL-17A, which may explain the inefficiency of this cytokine in immune responses even though it is produced simultaneously as IL-17A from T<sub>H</sub>17 cells (Ishigame, Kakuta et al. 2009). As opposed to IL-17F, IL-22 has been shown to play an essential role in regulating inflammatory processes associated with psoriasis, an autoimmune inflammatory skin disease characterized by leukocyte-infiltration into the epidermis and dermis and hyperplasia of the epidermis, whereas its contribution to other autoimmune disorders such as inflammatory bowel disease and arthritis remains to be established (Wolk, Kunz et al. 2004; Zheng, Danilenko et al. 2007). In a murine model of psoriasis, IL22<sup>−/−</sup> mice lacking the cytokine or blockage of the cytokine with an IL-22-specific antibody leads to a reduction of IL-23-mediated dermal inflammation (Zheng, Danilenko et al. 2007; Ma, Liang et al. 2008). In addition, IL22<sup>−/−</sup> mice show considerably attenuated CIA, whereas IL-22 is dispensable for EAE development, even though it has been shown to be capable of disrupting tight junctions joining endothelial cells of the blood-brain barrier (Kebir, Kreyborg et al. 2007; Kreyborg, Etzensperger et al. 2007; Geboes, Dumoutier et al. 2009).

**T<sub>H</sub>17 cells: Effect in Rheumatoid Arthritis and Multiple Sclerosis**

In summary, T<sub>H</sub>17 cells orchestrate diverse immune functions in the development of RA, mainly through the release of cytokines such as IL-17. Not only do these effector T cells stimulate the production of osteopontin, an extracellular matrix protein acting as paracrine and autocrine amplification factor for cytokine release in
GENERAL INTRODUCTION

joints (Yumoto, Ishijima et al. 2002; Xu, Nie et al. 2005), but also induce matrix metalloproteinase (MMP) synthesis, release GM-CSF and stimulate the expression of receptor activator for NF-κB ligand (RANKL), a factor which has been shown to be critical stimulating osteoclastogenesis (Fossiez, Djossou et al. 1996; Kotake, Udagawa et al. 1999; Pollinger, Jun et al. 2011). The secreted cytokines act in the downstream-activation of leukocytes and mesenchymal cells, whereas joint-resident macrophages, synovial fibroblasts and epithelial cells are activated in a cell-contact mediated manner, which in turn elicits them to release cytokines, chemokines and other proinflammatory mediators. Furthermore T_{H}17 have been shown to provide help to B cells and drive neutrophil differentiation, maturation and activation (McInnes and Schett 2007).

Also in pathogenesis of MS T_{H}17 cells have been attributed essential roles. Human MS brain biopsies showed that these IL-17 producing CD4^{+} T cells are enriched in acute and chronic compared to inactive lesions (Tzartos, Friese et al. 2008). Genomic profiling studies of brain-infiltrating T-cells confirmed these results by revealing the upregulation of the expression of several genes encoding cytokines regulating T_{H}17 cell expansion as well as of the T_{H}17-promoting transcription factor RORC (Montes, Zhang et al. 2009). In addition, magnetic resonance imaging (MRI) of patients suffering from relapsing-remitting MS (RR-MS) demonstrated that the presence of active lesions might be associated with the IL-17 release from myelin-reactive peripheral CD4^{+} T lymphocytes (Hedegaard, Krakauer et al. 2008). Accordingly it is believed that in MS, the abnormally activated peripheral DCs polarize naïve CD4^{+} T cells not only into T_{H}1, but also into T_{H}17 cells. T_{H}17 cell are known to require IL-23 exposure to become encephalitogenic and a recent report demonstrated that this cytokine induces GM-CSF production, which contributes to the phenotypic maturation into T_{H}17 effector cells (Cua, Sherlock et al. 2003; El-Behi, Ciric et al. 2011). The T_{H}17 effector cytokines IL-17 and IL-22 target corresponding receptors expressed on endothelial cells of the BBB and by modulating the expression of molecules comprising BBB tight junctions and remodeling the set barrier, contribute to alter the BBB integrity and increase its permeability. The T_{H}17-derived cytokines also induce the expression of IL-6 and CXCL8 from BBB endothelial cells, which allows the autoreactive T helper cells to migrate from the systemic compartment into the CNS (Kebir, Kreyemborg et al. 2007; Linker, Luhder et al. 2008). Epithelial cells of the choroid plexus have been shown to express CCL20, a
ligand for the chemokine receptor CCR6 expressed on autoreactive T_{H}17 cells that guides the initial migration wave, whereas the expression of the same ligand by activated astrocytes at later stages of the disease is thought to recruit T_{H}17 cells into the brain parenchyma (Reboldi, Coisne et al. 2009). As opposed to T_{H}1 cells, which depend on the integrin $\alpha 4\beta 1$ (VLA-4) to enter the CNS, T_{H}17 cells rather infiltrate the brain parenchyma via the expression of $\alpha L\beta 2$ (LFA-1) (Rothhammer, Heink et al. 2011). Within the CNS T_{H}17 cells are reactivated by DCs, recruit further T helper cells and initiate CNS inflammation. T_{H}17-derived GM-CSF has been shown to activate CNS microglia, which are producers of IL-23 and in turn may promote local activation as well as expansion of the T_{H}17 cell population (Li, Gran et al. 2003; Ponomarev, Shriver et al. 2007). T_{H}17-originated IL-17 also synergizes with IL-6 to enhance the IL-6 signaling cascade in astrocytes, which potentially acts as a positive feedback stimulus on T_{H}17 differentiation (Ma, Reynolds et al. 2010). In addition to secreting proinflammatory cytokines, T_{H}17 cells have been suggested to release granzyme B, with its cytolytic activity contributing directly to neurodegeneration observed in MS (Kebir, Kreymborg et al. 2007). Therefore, both the interactions of T_{H}17 cells with CNS-resident DCs such as astrocytes and microglia cells as well as with peripheral DCs play an essential role in the immunopathogenesis of MS (Chen, Wang et al. 2012).

In conclusion, the IL-17 pathway contributes significantly to the development of many inflammatory autoimmune diseases and its importance is highlighted by the current success of several clinical trials with antibodies directly blocking the cytokine or inhibiting T_{H}17 cell development with positive effects in RA, psoriasis and uveitis (Genovese, Van den Bosch et al. 2010; Hueber, Patel et al. 2010).
3.3.6 **BLymphocytes**

The humoral immune response represents, in addition to the cell-mediated immune response mediated by T lymphocytes, the second arm of the adaptive immune system and is primarily governed by B lymphocytes and their products.

**Antibodies**

The B lymphocyte antigen-receptors (BCRs) are membrane bound or secreted immunoglobulins (Igs), also called antibodies, which are composed of four polypeptide chains. Two of these chains are identical disulfide-linked heavy (H) chains, each made up of one antigen-specific variable (V) region as well as three to four conserved constant (C) regions. Antibodies may belong to five distinct isotypes differing in their C region, particularly IgM, IgD, IgG, IgE or IgA, depending on the type of heavy chain they incorporate, either µ, δ, γ, ε or α. The other two chains are the light (L) chains of either κ or λ subtype, which are attached to the heavy chains via disulfide bonds and consist of one V and one C region (Schroeder and Cavacini 2010). Among the various antibody classes, IgG prevails in the human serum, comprising around 75% of the total volume of serum antibodies (Altshuler, Serebryanaya et al. 2010). Within the V regions of the heavy (V_H) as well as the light chain (V_L) lay short stretches known as hypervariable complementarity-determining regions (CDRs) that mediate antigen binding, the highest variability being in the CDR3 (Padlan, Abergel et al. 1995; Altshuler, Serebryanaya et al. 2010). The antibody molecule can be divided into two subregions based on its proteolytic fragments, the first being the amino-terminal fragment antigen-binding (Fab) region comprised of the light chain as well as the V_H and C_H1. The second region is linked to the Fab fragments via a flexible hinge region and is known as the fragment crystalline (Fc) region, which consists of the carboxy-terminal C_H domains and mediates the antibody effector functions (Schroeder and Cavacini 2010) (Fig. 4A).
Figure 4 | Antibody formation. A | The immunoglobulin structure. The antibody molecule consists of two identical heavy chains and two light chains, connected by disulfide bonds. The variable regions $V_H$ and $V_L$ (yellow) recognize and bind the antigen whereas the heavy chain constant region (red) determines effector functions. B | Primary diversification of the antibody repertoire through immunoglobulin rearrangement of the light- (L, top) and heavy-chain (H, bottom) genes during early B lymphocyte development. In the L- and H-chain loci, a functional V region is assembled through a process called V(D)J recombination. In the L-chain, the Variable (V) and Joining (J) genes randomly selected from a pool of germline segments are combined and juxtaposed to a Constant (C) gene, while the intervening DNA is deleted. For the formation of the H-chain V region, one Diversity (D) gene is joined with a J gene, followed by the assembly of the V genes with this DJ-complex. Taken from (Nossal 2003).

Development of B lymphocytes

Like all cells of the lymphoid lineage, B lymphocytes originate from pluripotent HSCs in the bone marrow, where they undergo a sequential maturation process. Connective tissue stromal cells provide signals such as IL-7, CXCL12 and stem cell factor (SCF) that generate a microenvironment which stimulates lymphocyte progenitor development, differentiation and proliferation (Nagasawa 2006). Within the earliest B lymphocyte progenitor called the pre-B cell, the Ig genes are rearranged in an antigen-independent manner, resulting in an immature B cell carrying a cell-surface antigen receptor, IgM, now allowing the interaction with antigens. Similar to the TCR, the Ig heavy chain and light chain loci contain several V and C regions with intermediate stretches of J genes, while only the Ig heavy chain loci additionally carry D gene segments. Expression of the Ig heavy chain involves two gene recombination events mediated by VDJ recombinases, the first joining the D and J
segments in both alleles, the second linking the V region to the DJ complex, which in contrast to the previous rearrangement occurs only on one chromosome. If this initial VDJ joining fails, rearrangement on the other chromosome is initiated (Bassing, Swat et al. 2002). Following successful recombination, the primary RNA transcript is processed and the VDJ complex is spliced onto the first C segment, giving rise to the \( \mu \) mRNA which results in the production of the \( \mu \) heavy chain protein. While the major fraction of this protein remains in the cytoplasm, some is expressed on the cell surface where it associates with the so called invariant surrogate light chain (SLC) as well as Ig\( \alpha \) and Ig\( \beta \) signaling molecules to generate the pre-B cell receptor (pre-BCR) complex (Martensson, Keenan et al. 2007). This receptor complex provides survival signals and stimulates allelic exclusion, meaning the inhibition of Ig heavy chain recombination events occurring on the second chromosome (Vettermann and Schlissel 2010). Furthermore, recombination of the \( \kappa \) and \( \lambda \) Ig light chain loci is triggered, with the sequence of recombination and splicing processes being essentially identical to the heavy chain recombination, except that the V gene is directly joined to a J gene segment (Fig. 4B). The membrane-bound \( \mu \) heavy chain together with either a \( \kappa \) or \( \lambda \) light chain constitutes the complete membrane-bound BCR on the immature B cell and only cells successfully expressing the complete BCR survive (Kurosaki, Shinohara et al. 2010). Combinatorial diversity, meaning the joining of different V, D and J gene combinations, as well as junctional diversity, which is the introduction of alterations in nucleotide sequences at gene loci junctions, both ensure the generation of maximal variability in the BCR repertoire. At this point a series of central tolerance mechanisms come into play, ensuring the elimination of self-reactive immature B cells. Negative selection mechanisms in the bone marrow are strongly antigen-dependent. Cells recognizing abundant or multivalent cell-surface antigens, which lead to strong BCR cross-linking, will either undergo clonal deletion, resulting in apoptosis, or receptor editing, a process of secondary light chain recombination to reduce the receptors reactivity to autologous antigens. Cells binding to soluble self-antigens causing low-level BCR cross-linking on the other hand will induce anergy, the downregulation of IgM receptor expression, rendering them unresponsive to self-antigens (Hartley, Crosbie et al. 1991; Goodnow 1996; Casellas, Shih et al. 2001; Goodnow, Sprent et al. 2005; Link and Bachmann 2010; Gonzalez, Degn et al. 2011). Surviving immature B cells then migrate to the peripheral lymphoid organs, where they mature to express IgD in addition to IgM, which is the result of another
alternative splicing event targeting the VDJ heavy chain RNA that comprises both Cµ and Cδ exons (Chen and Cerutti 2010). The major population of these naïve B cells consists of the so-called follicular (FO) B lymphocytes, which either reside in the follicles of secondary lymphoid organs and respond to protein antigens or recirculate through the bone marrow, where they respond to blood borne pathogens (Cariappa, Mazo et al. 2005; Pillai and Cariappa 2009). The minor B cell populations include the marginal zone (MZ) B lymphocytes in the marginal zones of splenic white pulp, which respond to blood borne polysaccharide and lipid antigens and enter circulation (Lopes-Carvalho and Kearney 2004; Casola 2007). Additionally, B-1 B cells residing in the peritoneum and mucosal tissues elicit responses to nonprotein antigens (Hardy 2006). These naïve B cells are activated following encounter of foreign antigens concentrated in peripheral lymphoid tissues.

**Activation of B lymphocytes and humoral immune responses**

Within the B cell-rich zones of follicles and marginal zones of the spleen, lymph nodes and mucosal lymphoid tissues, cells such as macrophages, DCs and follicular dendritic cells (FDCs) capture the antigens and present them to the B lymphocytes (Huang, Han et al. 2005; Batista and Harwood 2009). In contrast to T lymphocytes, B lymphocytes are able to recognize antigens in their native conformation via the membrane-bound BCRs. Binding of antigens forming aggregates or exhibiting multiple epitopes induces clustering and cross-linking of membrane Ig receptors and triggers signaling events initially transduced by the Igα and Igβ molecules of the BCR complex (Harwood and Batista 2010). Moreover, B lymphocytes express TLRs as well as a receptor for fragments of complement proteins generated during innate immune responses to coat the invading microbe (Chaturvedi, Dorward et al. 2008). Hence, B lymphocytes receive activating signals not only from antigen binding but also through the recognition of complement protein and microbial PAMPs.

Upon antigen-mediated activation, B lymphocyte responses can be classified into T-cell dependent or T-cell independent responses, depending on the nature of the antigen.
T-dependent antigens are mainly protein antigens ranging from soluble proteins to whole cells, which lack repetitive structures and require T cell help to induce a strong antibody response. As mentioned in previous sections, invading microbes or protein antigens are internalized, processed and displayed as peptides via MHC class II molecules by APCs, mainly DCs, in the T cell zones of peripheral lymphoid tissues. Upon peptide recognition, naïve CD4\(^+\) T cells are activated and stimulated to proliferate and differentiate into distinct T\(_{H}\) effector cells, which then migrate into the periphery. While some of these effector cells enter the circulation, others increase expression of the CXCR5 receptor and migrate toward the borders of lymphoid follicles where they are prone to encounter antigen-stimulated follicular B cells. In B lymphocytes, protein antigens that are bound by the BCR mediate receptor cross-linking, resulting in antigen endocytosis, processing and display as a MHC class II-peptide complex for the recognition by cognate T\(_{H}\) cells (Lanzavecchia 1990; Kurosaki, Shinohara et al. 2010; Vinuesa, Linterman et al. 2010). In addition, antigen binding leads to an increased expression of B7 costimulators, and a reduced expression of the follicle homing chemokine CXCR5 whereas the CCR7 expression increases, leading the migration out of the follicle (Han, Hathcock et al. 1995; Reif, Ekland et al. 2002). At the edges of the T cell zone of the secondary lymphoid organs, the effector T\(_{H}\) cells recognize and bind the antigen peptide-MHC II complex presented on B lymphocytes as well as the costimulatory molecules. Consequently, the T\(_{H}\) cells are activated, resulting in expression of CD40L and secretion of cytokines. Thus, CD40L-CD40 interactions are promoted, providing the final signal for the complete activation of the B lymphocyte and subsequent proliferation (clonal expansion) to form extrafollicular foci and differentiate into short-lived antibody-secreting plasma cells providing immediate protection (Han, Hathcock et al. 1995; McHeyzer-Williams and McHeyzer-Williams 2005; Kurosaki, Shinohara et al. 2010). Some of the proliferating yet still undifferentiated B lymphocytes however migrate into a primary lymphoid follicle where the formation of a germinal center (GC) is induced. Germinal centers are specific microenvironments within the B cell follicles of secondary lymphoid organs that contain B lymphocytes gathered around a network of FDCs. Within these sites, mature B lymphocytes undergo extensive proliferation, somatic hypermutation (SHM), class switch recombination (CSR) and finally differentiation into high affinity antibody-secreting plasma cells and long-lived memory cells (Gonzalez, Degn et al. 2011; Victora and Nussenzweig 2012). While
the initial generation of a diverse antibody repertoire occurs early in the B cell development, the GC reaction induces a second wave of antibody diversification through the processes of CSR, which broadens the functional capabilities, and SHM, which increases antibody affinity for the antigen (Li, Woo et al. 2004; MacLennan 2005). CD40L-mediated signals initiate the heavy chain isotype class switching in antigen-stimulated B lymphocytes by inducing the expression of activation-induced deaminase (AID) (Muramatsu, Kinoshita et al. 2000). AID promotes intrachromosomal gene rearrangements that cause the replacement of the Cµ segment by downstream Cγ, Cε or Cα stretches, thus placing each antigen-binding (VDJ) region next to distinct C regions. Now the humoral immune response is capable of generating IgG, IgE and IgA antibodies in addition to IgM and IgD, allowing each V region to mediate different effector functions (Li, Woo et al. 2004; Maizels 2005). Th1-derived effector cytokines control the type of heavy chain C region participating in the recombination process, thereby promoting the production of the isotype best suited to complement their specific Th1 cell effector functions. The Th1-derived IFN-γ for instance drives the isotype switch to IgG2a and IgG2b, two opsonizing antibodies capable of binding to phagocyte Fc receptors and promoting phagocytosis of pathogens such as bacteria and viruses. The Th2 signature cytokine IL-4 on the other hand mediates the switch to IgE, an antibody effective in eliminating helminths (Coffman, Lebman et al. 1993). Besides triggering CSR, the above-mentioned enzyme AID also initiates the introduction of various point mutations in the antibody heavy and light chain V genes during B cell proliferation in the GCs, a process known as SHM. These mutations translate into amino acid exchanges resulting in affinity maturation, optimization of binding specificity and consequently the generation of a variety of high affinity neutralizing antibodies. While these point mutations do not extend into the C-region exons, the highest mutation frequency can be found in the V(D)J coding regions as well as the J introns with a mutation rate of around $10^{-5}$–$10^{-3}$/base pair/generation, which is around $10^3$–fold greater than the basal genomic mutation level and in general proportional to the transcription rate (Fukita, Jacobs et al. 1998; Bachl, Carlson et al. 2001; Longerich, Tanaka et al. 2005; Peled, Kuang et al. 2008). Following clonal expansion and BCR diversification, B lymphocytes expressing high-affinity BCRs undergo positive selection. GC-resident FDCs present the antigen by binding immune complexes via receptors for antibody-Fc regions or complement. Continuous scanning of B lymphocytes over these FDC networks
eventually leads to antigen binding of high affinity mutants. While these high affinity clones receive survival signals through their BCRs, the B lymphocytes expressing a receptor with lower affinity will die by apoptosis (Allen, Okada et al. 2007; Schwickert, Lindquist et al. 2007). Positive selection stimulates B lymphocytes to compete for the interaction with GC-resident cognate T<sub>H</sub> cells, which if successful may result in re-entry into the GC-cycle (Allen, Okada et al. 2007). In addition to positive selection, high-affinity self-reactive GC B lymphocytes may be eliminated during negative selection within or after the GC reaction, although the precise nature of this checkpoint yet remains to be elucidated (Victora and Nussenzweig 2012). Receptor specificity is continually redefined through several rounds of BCR diversification and selection, a process referred to as affinity maturation. Alternatively, affinity-matured B lymphocytes may leave GC cycle and differentiate into either non-secreting memory cells, which provide long-term protection, or antibody-secreting plasma cells (McHeyzer-Williams, Okitsu et al. 2012). While plasma cells from the GCs in the spleen and lymph nodes home to the bone marrow, where they continue to produce antibody and thus provide serological protection, some plasma cells of GCs in mucosal tissues that are primarily switched to IgA remain in the mucosal system.

T-independent (TI) antigens comprise multivalent molecules exhibiting repetitive structures, as found in many bacterial antigens consisting of polysaccharides and lipids. Type I TI-antigens such as lipopolysaccharides contain an intrinsic B-cell stimulating activity and are able to induce polyclonal B cell activation at high concentrations. Type II TI-antigens on the other hand lack this intrinsic activity and in contrast to type I antigens, only act on mature and not immature B lymphocytes. They consist of multiple identical epitopes are capable of cross-linking a great number of BCRs on the B cell surface, while macrophages and DCs provide co-stimulatory signals, resulting in strong proliferation and differentiation of the B lymphocyte into IgM secreting plasma cells. As opposed to responses to T-dependent antigens, responses to these antigens neither induce memory B cells, nor are they accompanied by extensive CSR (Mond, Lees et al. 1995; Jeurissen, Ceuppens et al. 2004).

Interestingly, antiviral B cell responses have been shown to exhibit TD as well as TI features. Similar to soluble protein antigens, viral protein components are able to
induce CSR, GC formation, SHM as well as memory B cell formation in a T cell dependent manner (Bachmann and Zinkernagel 1997). The high immunogenicity of viruses rests on the highly repetitive organization of surface B cell epitopes that has been shown to render viruses capable of inducing T-independent B cell responses (Bachmann and Zinkernagel 1996). In addition, similar as shown for bacteria, certain viruses have been proposed capable of stimulating B lymphocyte TLRs, thus directly activating B cells in complete absence of T cell help, however more recent studies link the expression of TLR ligands particularly to the determination of isotype patterns (Yamamoto, Yamamoto et al. 1992; Krieg, Yi et al. 1995; Hemmi, Takeuchi et al. 2000). In murine antiviral responses, this CSR has been shown to be largely restricted to IgG2a (Coutelier, van der Logt et al. 1987). While in addition to T H cell dependent CD40-CD40L interactions initiating CSR, T H effector cytokines such as IFN-γ are believed to determine the particular isotype, other studies have reported IFN-γ-independent CSR to IgG2a in antiviral responses (Coffman, Seymour et al. 1988; Finkelman, Katona et al. 1988; Kawabe, Naka et al. 1994; Markine-Goriaynoff, van der Logt et al. 2000). A recent study using virus-like particles (VLPs) loaded with distinct TLR ligands revealed that rather than the T H effector cytokine IFN-γ, it is the direct stimulation of B cell TLR9 through ligands such as CpG, which is the driver of CSR to IgG2a in vivo (Jegerlehner, Maurer et al. 2007). As opposed to responses against soluble proteins, where the FO B cells generate the initial wave of antibodies, and antibacterial responses, where this function is attributed to MZ B cells, studies with the VLP QB could show, that in antiviral responses both, FO as well as MZ B cells respond to immunization and undergo isotype switching, resulting in TD IgG and TI IgM production (Gatto, Ruedl et al. 2004).
4 RESULTS

4.1 MANUSCRIPT I

PROTECTIVE EFFECT OF A GERMLINE ANTIBODY IN AUTOIMMUNE INFLAMMATORY DISEASES

Kiran Dallenbach, Patrick Maurer, Till Röhn, Manfred Kopf, Martin F. Bachmann

Monoclonal antibodies (mAbs) have recently emerged as new drug modalities for the treatment of chronic inflammation. Indeed, inhibiting cytokines using mAbs is an established disease-modifying therapy for inflammatory diseases. Interleukin-17 (IL-17) is a T cell derived central mediator of host protection as well as autoimmune diseases such as rheumatoid arthritis and psoriasis. The importance of IL-17 in mediating host protection is underscored by the observation that autoantibodies against IL-17 have recently been implicated in Candida albicans infection in Aire-deficient patients. Immunization with Qβ-IL-17, a virus-like particle (VLP)-based vaccine, has been shown to produce autoantibodies in mice and was effective in ameliorating disease symptoms in animal models of inflammatory autoimmune disorders. To characterize autoantibodies induced by vaccination at the molecular level, we generated mouse mAbs specific for IL-17 and compared them to germline Ig sequences. The variable regions of a selected hypermutated high affinity anti-IL-17 antibody differed in only three amino acid residues compared to the likely progenitor. An antibody, which was back-mutated to germline sequence, maintained a surprisingly high affinity for IL-17 in the nM range. The ability of the hypermutated and germline antibody, which recognize the same epitope, to block chronic inflammation was subsequently tested in murine models of autoimmunity. The parental lead antibody as well as the derived germline antibody were both able to delay disease onset and significantly reduced disease severity. These results indicate that the affinity of germline cytokine-specific antibodies may be sufficient for protection against autoimmune inflammatory diseases and questions the clinical
relevance of the industry’s effort to artificially enhance mAb affinity up to femtomolar levels.
4.1.1 INTRODUCTION

Cytokines are a large family of proteins and peptides that hold key immunoregulatory functions in a vast number of biological processes comprising, amongst others, cell proliferation and migration, angiogenesis and fibrosis (Feldmann 2008). Most notably, disruption of the subtle balance of pro-and anti-inflammatory cytokines contributes fundamentally to the development and pathogenesis of many inflammatory diseases. Cytokines thus represent promising targets for a variety of therapeutic strategies (Kopf, Bachmann et al. 2010).

Monoclonal antibodies (mAbs) recently emerged as new drug modalities to block small molecules such as cytokines. Specific features such as high specificity and target selectivity offer tremendous advantages over conventional therapies with immunosuppressant and anti-inflammatory drugs previously used for the treatment of autoimmunity, which had severe side effects such as opportunistic infections and high toxicity (Bach 1993). Major advances in antibody engineering over the past thirty years have allowed the design of safe and moderately immunogenic antibodies with high affinities. To date more than 20 mAbs have been approved by the FDA for therapy in humans and among them, nine alone are targeting cytokines (Presta 2008; Hansel, Kropshofer et al. 2010).

Interleukin (IL)-17A is one particular cytokine that has raised substantial interest in the recent years, as it has been implicated in several autoimmune diseases such as rheumatoid arthritis (RA), psoriasis and multiple sclerosis (MS) (Teunissen, Koomen et al. 1998; Matussevicius, Kivisakk et al. 1999). This primarily T cell derived cytokine promotes inflammation through induction of other proinflammatory cytokines and chemokines, recruitment of neutrophils, enhancement of antibody production and promotion of T cell priming (Iwakura, Ishigame et al. 2011). Preclinical experiments in animal models as well as the current success of anti IL-17 mAbs in clinical trials have validated the relevance of IL-17 as a target for the treatment of autoimmunity (Nakae, Nambu et al. 2003; Komiyama, Nakae et al. 2006; Rohn, Jennings et al. 2006; Uyttenhove and Van Snick 2006; Hueber, Patel et al. 2010). In addition to its proinflammatory effect, IL-17 exhibits anti-inflammatory properties and, through the induction of antimicrobial peptides, serves a crucial mediator of host protection, as exemplified by increased susceptibility to Candida...
albicans in IL-17R-deficient mice (Huang, Na et al. 2004). A recent study investigating patients suffering from autoimmune polyendocrine syndrome type I (APS-I) demonstrated that the majority of them also displayed an increased sensitivity to chronic mucocutaneous candidiasis (CMC), which was then found to correlate with increased levels of autoantibodies against IL-17A, IL-17F and/or IL-22 (Puel, Doffinger et al. 2010). This is particularly interesting, since the APS-I -underlying cause is a deficiency in the transcriptional regulator Aire, which is known to contribute to T cell tolerance through clonal deletion in the thymus but not B cell tolerance or host defense (Nagamine, Peterson et al. 1997; Mathis and Benoist 2009).

It is thus of interest to gain a better insight into the nature of antibodies induced by our established VLP-based vaccine Qβ-IL-17, which in previous studies has shown protective potential in animal models for rheumatoid arthritis, encephalomyelitis and myocarditis (Rohn, Jennings et al. 2006; Uyttenhove and Van Snick 2006). We here describe an anti-IL-17 monoclonal antibody generated by vaccination with Qβ-IL-17, which exhibits high affinity and protective in vivo capacity even in germline configuration. The existence of such an antibody has implications for B cell tolerance as well as the regulation of cytokine-networks.
4.1.2 MATERIALS & METHODS

Mice

Female C57BL/6 mice were purchased from Charles River, male DBA/1 mice from Harlan Netherlands B.V. Female and male IL-17 deficient (knockout (K.O.)) mice were kindly provided by Prof. M. Kopf (ETH Zurich, Switzerland). All mice were maintained under specific pathogen-free conditions and used for experimentation according to protocols approved by the Swiss Federal Veterinary Office.

Cloning, expression, purification and refolding of recombinant IL-17A

As shown previously (Rohn, Jennings et al. 2006), murine IL-17 (mIL-17) was expressed in *Echerichia coli* BL21 cells using the plasmid pMOD_His_mIL-17_C, which encodes a protein comprised of a N-terminal hexahistidine tag followed by mIL-17 (aa 26-158) and a C-terminal linker sequence consisting of five glycine residues and one cysteine residue. The protein was solubilized from inclusion bodies and purified over a Ni²⁺-NTA agarose column (Qiagen), followed by purification of the eluted fractions over a HiLoad Superdex 75 prep grade gel-filtration column (Amersham). Next, the mIL-17 protein was refolded in a two-step dialysis procedure and authentic refolding of the protein was confirmed using a mIL-17 sandwich ELISA (BD PharMingen).

Production of Qβ-IL-17 conjugate vaccine

Virus-like particles (VLPs) derived from the bacteriophage Qβ were expressed in *E. coli* JM109 and purified as shown previously (Cielens, Ose et al. 2000). In a two-step procedure, the mIL-17 was covalently conjugated to Qβ, as described in (Rohn, Jennings et al. 2006). First, Qβ-VLP were reacted at room temperature with a 20-fold molar excess of the heterobifunctional chemical cross-inker, succinimidyl-6-(β-maleimidopropionamido) hexanoate, followed by dialysis to remove unreacted cross-linker and incubation with an 10-fold excess of tri(2-carboxyethyl)phosphine hydrochloride to reduce any cysteine residues in the linker. Next, mIL-17 was
covalently linked to the derivatized Qβ by reacting equimolar amounts of mIL-17 and Qβ at room temperature. The vaccine was analyzed by SDS-PAGE and Immunoblotting with anti-Qβ and anti-His antibodies.

**Generation of mIL-17-specific Hybridoma IgG antibodies**

For the generation of monoclonal neutralizing IgG antibodies against mIL-17, mice were immunized twice subcutaneously with 50µg Qb-IL-17 on day 0 and day 14. Four days after the booster immunization, their splenocytes were fused to murine myeloma cells (Sp2/0Ag14, ATCC) using standard methods (Collaboration with EMBL, European Molecular Biology Laboratory, Italy). Hybridomas were screened by ELISA for binding of IgG to purified mIL-17. In brief, IL-17 was coated to ELISA plates (Nunc) at a concentration of 10 µg/mL and incubated with supernatant of hybridoma cells serially diluted in PBS / 0.05% Tween-20 / 2% BSA starting at an antibody concentration of 0.1 µg/mL. Bound IgG was detected with HRP-conjugated goat anti mouse IgG Fcγ fragment specific secondary antibody (Jackson) at a dilution of 1:1000 in PBS / 0.05% Tween-20 / 2% BSA. Fusions gave rise to eight IgG-secreting hybridomas, out of which two were only able to bind to recombinant mIL-17 but not mIL-17 produced in eukaryotic cells. Selected hybridoma clones were cultured in Dulbecco’s Modified Eagle Medium (DMEM GlutaMAX, Gibco) supplemented with 20% FBS (Gibco), 50 mg/mL Gentamicin (Gibco) and Hybridoma cloning factor (HCF, BioVeris). Antibodies were purified from culture supernatants by affinity chromatography over a protein G-Sepharose column (GE Healthcare). Purified antibodies were characterized for binding to refolded mIL-17, commercial available IL-17 (R&D), IL-17 without linker and IL-17F by ELISA. The IL-17 proteins were coated at a density of 0.5 µg/mL to ELISA plates (Nunc) and aIL-17 2E1 serially diluted 1:3 in PBS / 0.05% Tween-20 / 2% BSA was added, starting at a concentration of 1 µg/mL. Bound antibody was detected with HRP-conjugated goat anti mouse IgG (Fc gamma specific) (Jackson) diluted 1:1000 in PBS / 0.05% Tween-20 / 2% BSA. Affinity of aIL-17 2E1 for mIL-17 was determined by limiting coating ELISA.
RESULTS

Isolation of V_H and V_L Genes and generation of scFv antibody fragments

Total RNA was isolated from IL-17-specific hybridoma cells using TRI reagent (Molecular Research, Inc.) and cDNA was generated using Super Script II RT (Invitrogen). HCVRs and LCVRs were amplified and assembled to scFv coding regions by PCR using primers and protocols described previously (Beerli, Bauer et al. 2008). The resulting PCR products encoded scFv comprised of a 5’ LCVR (κ) and a 3’ HCVR joined by an 18aa-flexible linker, flanked by two SfiI restriction sites. To create fusion proteins consisting of N-terminal murine scFv linked to a C-terminal Fc-γ1 domain, the scFv coding regions were digested with the restriction endonuclease SfiI and ligated into the SfiI-digested expression vector pCEP-SP-Fc. Being a derivative of the episomal mammalian expression vector pCEP4 (Invitrogen), this vector contains the EBV replication origin and encodes the EBV nuclear antigen (EBNA-1) allowing for extra chromosomal replication, and carries a puromycin selection marker instead of the original hygromycin B resistance gene. The resulting constructs drive expression of secreted scFv-Fc domain fusion proteins under the regulation of a CMV promoter.

For expression of recombinant scFv fusion proteins, 293T HEK cells cultured in DMEM (10%FBS) were transformed with various scFv-Fc expression constructs using Lipofectamine 2000 (Invitrogen) and supernatants were collected after 48h. The concentration and IL-17 binding of scFv-Fc in the supernatants was determined by ELISA. For determining concentration, F(ab)_2 goat anti hu IgG Fc specific (Jackson) was coated to ELISA plates at a concentration of 2.5 µg/mL and incubated with 1:10 prediluted cell supernatants serially diluted 1:3 in PBS / 0.05% Tween-20 / 2% BSA. Bound scFv-Fc was detected with HRP-conjugated goat anti human IgG secondary antibody (Jackson) at a dilution of 1:1000 in PBS / 0.05% Tween-20 / 2% BSA. For determining IL-17 binding, ELISA plates were coated with 5 µg/mL mIL-17 and incubated with 1:10 prediluted supernatants serially diluted 1:3 in PBS / 0.05% Tween-20 / 2% BSA. Bound scFv-Fc was detected with HRP-conjugated goat anti human IgG (Jackson) at a dilution of 1:1000 in PBS / 0.05% Tween-20 / 2% BSA.

After sequence verification and comparison of VRs to antibody germline sequence (IMGT database), hypermutated V_H segments of selected IL-17 binding scFv were reverted to germline (Geneart) and mutagenized scFv were cloned into the expression vector pCEP-SP-Fc.
RESULTS

Cloning, expression and purification of recombinant monoclonal antibodies

For the generation of full-length murine IgG2aκ antibodies, the coding segments of selected IL-17-specific scFv antibodies were amplified by PCR using VR-specific transfer primers. Then κLC γ2HC expression constructs were assembled in pCMV-Script (Stratagene) and finally HC and LC coding regions were combined into the episomal expression vector pCB15, as described by (Beerli, Bauer et al. 2008).

Antibody plasmids were transfected into the HEK 293T cell line for expression using Lipofectamin Plus (Invitrogen). For large-scale production, stable transfectants were expanded and selected under puromycin (Sigma) pressure. Resistant protein-expressing cells were cultured in serum-free medium in Poly-L-Lysine-coated roller bottles for up to two months and antibodies were purified from supernatants by affinity chromatography over a protein G-Sepharose column (GE Healthcare).

Affinity measurement

Limiting coating ELISA:

To analyze the affinity of hybridoma-derived and recombinant anti IL-17 antibodies by ELISA, limiting coating condition were exploited to minimize avidity effects. Murine IL-17 was coated onto ELISA plates (Nunc) in a 3-fold serial dilution, starting at 10 µg/mL. Purified 2E1 antibody was added starting at a concentration of 0.5 µg/mL and serially diluted 1:3 in PBS / 0.05% Tween-20 / 2% BSA in the direction opposite to the coating dilution series. Bound antibody was detected with an HRP-conjugated goat anti mouse IgG (Fc gamma specific) (Jackson) diluted 1:1000 in PBS / 0.05% Tween-20 / 2% BSA. $K_D$ values were calculated from ELISA titers measured by determining half-maximal optical density (OD) at the limiting coating concentration of 370.37 ng/mL. Values are shown as OD at 450 nm.

Binding kinetics by Biacore:

The interaction of soluble mIL-17 with immobilized anti IL-17 antibodies 2E1 hypermut and 2E1 germline was assessed by surface plasmon resonance (SPR) detection using a Biacore T100 instrument (GE Healthcare) and data were analyzed with the Biacore T100 control software package version 2.0.3(GE Healthcare). For all runs, amine-free running buffer consisting of 10mM NaH$_2$PO$_4$ and 150mM NaCl was used. To prepare the Biacore surfaces, a standard amine-coupling protocol was applied. Flow cells of a carboxyl methylxylaran (CM5) sensor chip (GE Healthcare)
RESULTS

were activated individually by injecting a freshly prepared mixture of 0.4 M EDC and 0.1 M NHS for 7 min at a flow rate of 5 µL/min. 100 µg/mL anti IL-17 antibody 2E1 hypermut or 2E1 germline diluted in 10mM NaAc buffer pH 5.5 were injected for 7-14 min over the sample cells, until the desired level of immobilization was reached, typically at 5000-7500 resonance units (RU). To provide a reference surface, 100 µg/mL BSA (GE Healthcare, 2D Quant Kit) diluted in 10 mM NaAc buffer pH 4 was injected for 7 min over the reference cell, reaching coating densities of around 2000 RU. Excess reactive esters were quenched by injecting 1 M Ethanolamine-HCl for 7 min. To measure the kinetics of mIL-17 over the antibodies on the chip, a twofold serial dilution of mIL-17 (for 2E1 hypermut: 0.0625 – 8 nM mIL-17, for 2E1 germline: 0.125 – 32 nM mIL-17, with all concentrations in duplicates) was injected for 3 min at a flow rate of 30 µL/min, allowing a dissociation phase of 10 min. Surfaces were regenerated with two pulses of 2M Guanidine-HCl with stabilization periods of 5 min.

**In vivo neutralization assays**

To find the optimal dose of recombinant mIL-17 and the optimal time point for induction of murine KC (CXCL1) chemokine, a time and dose ranging experiment was performed. Female C57/BL6 mice (n = 3 per group, age = 8 weeks) were injected s.c either with 1, 10 or 100 µg recombinant mIL-17. Individual sera taken before, 2h and 4h after immunization and CXCL1 levels were determined by ELISA (KC Quantikine kit, R&D Systems).

To determine the neutralization capacity of the antibodies, female C57/BL6 mice (n = 5 per group, age = 8 weeks) were injected i.p. with 100 µg 2E1 hypermut IgG, or 2E1 germline IgG or an isotype matched anti-Feld1 antibody as control, 30 min prior to a s.c injection of 10 µg mIL-17. Two hours after mIL-17 administration, CXCL1 levels were determined by ELISA (R&D Systems). To further analyse neutralization, the experiment was repeated using female C57/BL6 mice (n = 10, age = 8 weeks) and serum IL-6 levels were measured by ELISA (IL-6 Quantikine kit, R&D Systems).
RESULTS

**Determination of *in vivo* protective capacity of IL-17-neutralizing IgGs**

**CIA model:**

Groups of male DBA/1 mice (n = 10, age = 8 weeks) were immunized s.c. with 50 µg Qβ-IL-17 on days -43, -29 and -10. Alternatively mice were injected i.p. twice per week starting on day -1 with 100 µg of either Anti-Feld1 IgG2α, 2E1 hypermut IgG2ακ or 2E1 germline IgG2ακ. On day 0, all mice were injected subcutaneously in the tail with 200µg bovine type II collagen (Chondrex, 4 mg/mL in 10 mM acetic acid) emulsified in an equal volume of Complete Freund’s adjuvant (CFA, Difco). On day 21, all mice received a booster injection intradermally into the base of the tail with the same amount of collagen emulsified in Incomplete Freund’s adjuvant (IFA, Difco). After disease induction, mice were monitored daily over three consecutive weeks for the development of arthritis by assigning visual clinical cores to each limb and measuring ankle thickness of hind limbs. As done previously (Rohn, Jennings et al. 2006; Spohn, Guler et al. 2007), clinical scores from 0 to 3 were assigned according to following definitions: 0 = normal, no swelling or reddening, 1 = mild reddening and/or swelling of digits/paw, 2 = reddening and swelling extending over whole paw/joint, 3= strong swelling, deformation of paw/joint, with ankylosis. Cumulative clinical scores of individual mice were calculated as the sum of clinical scores of all four limbs. Animals that displayed a clinical score of 3 on one or more limbs for 3 consecutive days or lost >15% body weight were euthanized.

**EAE model:**

Groups of female C57BL/6 mice (n = 5, age = 8 weeks) were immunized i.p. twice per week with 100 µg of either mIgG2ακ control mAb (BD Pharmingen, #553454) or hybridoma-derived anti-IL-17 2E1, starting one day before disease induction. For the induction of EAE, each mouse was injected s.c. into the right or left flank with 100 µg / 200 µL MOG (35-55) peptide (Biotrend) diluted in PBS-CFA (0.5 mg/mL) followed by the injection of 400 ng of pertussis toxin i.p. Starting eight days after disease induction, mice were monitored daily for three consecutive weeks for the development of EAE by visual scoring of clinical signs of disease. As done previously (Rohn, Jennings et al. 2006), clinical scores from 0 to 3 were assigned according to the following scoring scheme: 0 = normal, 0.5 = distal tail paralysis, 1 = complete tail paralysis, 1.5 = complete tail paralysis and unilateral hind limb weakness, 2 =
RESULTS

bilateral hind limb weakness, 2.5 = unilateral hind limb paralysis, 3 = bilateral hind limb paralysis. As an indicator of disease, body weight was also measured.

Psoriasis model:

Female C57/BL6 mice (n=4 per group, age = 8 weeks) received a daily topical dose of 30 mg of commercially available Imiquimod (IMQ) cream (5%) (Aldara, MEDA Pharm) on both ears for 8 consecutive days. Mice of each group were immunized on day 0, 3 and 5, relative to the start of IMQ application, with 100 µg anti IL-17 antibody 2E1 hypermut, 2E1 germline or isotype matched anti-Feld1 as control. As a control, IL-17 K.O. mice (n = 4, two male and two female) received essentially the same IMQ treatment and injections of the anti-Feld1 control antibody. To score the severity of skin inflammation, ear thickening was monitored daily using a micrometer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Two-way ANOVA with Bonferroni’s post test was used to determine the significance of group differences in EAE, IMQ-induced psoriasis and CIA experiments. Student’s t-test was used to determine the significance of group differences in serum CXCL1 and serum IL-6 concentrations measured in the in vivo neutralization assays. A p value less than 0.05 was considered significant.
4.1.3 RESULTS

IL-17-Specific monoclonal antibody 2E1 binds with high affinity and shows protective effect \textit{in vivo}

In previous experiments we have demonstrated that immunization of mice with IL-17 displayed on virus-like particles (VLPs) induces potent IL-17-neutralizing antibodies, which protect mice against EAE as well as CIA (Rohn, Jennings et al. 2006). In order to test the hypothesis that mAb affinity above a certain threshold has a major influence on clinical efficacy in different murine models of diseases, mice were immunized twice with Qβ-IL-17 followed by the generation of IL-17-specific IgG-secreting hybridomas. Out of the hybridomas screened, the monoclonal antibody (mAb) secreted by the clone 2E1 (aIL-17 2E1 hybridoma) was identified to bind specifically to mIL-17A (Fig. 1A). Due to its high affinity (K_D in the range of $4.5 \times 10^{-11}$ M), as determined by limiting coating ELISA (Fig. 1B), this antibody was selected for further analysis. While the mAb bound to recombinant refolded IL-17 as well as commercially available IL-17 lacking the His-Tag and linker incorporated in the IL-17 used in the vaccine, it did not recognize IL-17F, although this member of the IL-17 family shares 50% sequence homology with IL-17A (Weaver, Hatton et al. 2007) (Fig. 1A). To test the therapeutic potential of the mAb raised by vaccination, we examined the effect of aIL-17 2E1 immunization in an acute model of MS, MOG$_{35-55}$- induced EAE. As shown in Fig. 1C, female C57BL/6 mice were injected with aIL-17 2E1 hybridoma or an isotype control antibody (aKLH mIgG2aκ) twice per week and MOG$_{35-55}$ formulated in CFA was used together with pertussis toxin to induce EAE. While mice receiving the control mAb developed severe signs of EAE, peaking with an average score of 2.9 at day 19 after disease induction, mice treated with aIL-17 2E1 hybridoma displayed significantly reduced symptoms, with a maximum score of 2 on day 12 ($p<0.0001$ aIL-17 2E1 vs. aKLH). Interestingly, these mice showed milder symptoms towards the end of the disease, with a maximal average of 1.1 after day 24. In addition, disease incidence in the aIL-17 2E1-treated mice was reduced. While three out of five mice of the control group reached the maximal score of 3 during the course of the disease, only one mouse of the aIL-17 2E1 hybridoma group reached this level.
RESULTS

Figure 1 | Functional characterization of the hybridoma-derived aIL-17 monoclonal antibody 2E1. A | aIL-17 2E1 hybridoma antibody was analyzed for its ability to recognize recombinant refolded mIL-17A, IL-17F, linker-free IL-17A and commercially available mIL-17 (R&D Systems) by ELISA. Values are shown as OD at 450 nm. B | Analysis of the affinity of aIL-17 2E1 hybridoma for mIL-17 under limiting coating conditions by ELISA. Serially diluted antibody was applied to mIL-17-coated ELISA plates and $K_D$ was determined at coating concentration 370.37 ng/mL. Values are shown as OD at 450 nm. C | 2E1 antibody shows disease-modifying effect in EAE. Female C57BL/6 mice (n = 5) were immunized i.p. with 100 µg 2E1 anti IL-17 or mouse isotype control antibody aKLH IgG2aκ (BD Pharmingen) twice per week, starting one day before disease induction. On day 0, disease was induced by injecting 100 µg MOG35-55 peptide in CFA s.c. and 400 ng pertussis toxin i.p.. Mice were examined daily and clinical scores were assigned according to the degree of paralysis. Shown are mean values with SEM (p<0.0001 vs. aKLH, two-way ANOVA).

Sequence comparison of the variable regions (VRs) of the 2E1 scFv fragment to germline sequence

To analyze somatic mutations in the variable regions (VRs) of the 2E1 antibody, RNA was isolated from hybridoma cells and the genes of the immunoglobulin heavy ($V_H$) and light ($V_L$) chains were amplified and assembled to scFvs. Binding to mIL-17 was confirmed by ELISA upon fusion of the scFv to the C-terminal Fc-$\gamma$1 domains and expression in HEK 293T cells (data not shown). To determine specific hypermutations, the variable regions were sequenced and compared to germline Ig
RESULTS

Sequence alignment revealed that the HCVR comprised gene segments belonging to the IgHV1 family, while the LCVR used gene segments of the IgKV4 family. The V\textsubscript{H} region differed at four positions compared to germline, resulting in three amino acid substitutions of which one was located in V\textsubscript{H}-CDR1 (Ile-to-Val) and one in VH-CDR2 region (Asn-to-Tyr), while the other two point mutations encoded the same amino acid replacement (Val-to-Lys) and were located adjoining the V\textsubscript{H}-CDR2 in the V\textsubscript{H}-FR3 segment (Fig. 2). The V\textsubscript{L} sequence of the 2E1 scFv on the other hand was devoid of somatic mutations. In a next step, the hypermutated V\textsubscript{H} segment of the scFv was reverted to germline. To generate full-length antibodies, the VL region as well as the hypermutated and germline VH segments were amplified, assembled and transferred into an expression constructs as described in materials and methods, resulting in a hypermutated (aIL-17 2E1 hypermut) and a germline IgG2aκ antibody (aIL-17 2E1 germline), sharing the same isotype as the mAb originally isolated from hybridoma cells.

**Figure 2** | Sequence alignment of V\textsubscript{L} and V\textsubscript{H} of the scFv fragment derived from the aIL-17 2E1 antibody and germline VRs. Total RNA was isolated from hybridoma cells and V\textsubscript{H} and V\textsubscript{L} regions were amplified from cDNA and assembled to antigen-specific single-chain Fv (scFvs). Sequences were aligned to germline sequences available on the international ImMunoGene Tics Information system (IMGT) database. Shown are the 2E1 aIL-17 scFv nucleotide sequence (black) and deduced amino acids by the single-letter code (green) with linker (☐), framework (FR) and CDR regions (☐). Nucleotides differing from germline sequence and resulting in amino acid replacement mutations are highlighted (■) and the corresponding germline amino acid is indicated (↵).
RESULTS

2E1 IgG antibody backmutation to germline maintains high affinity for mIL-17

The affinity of the interaction of the purified recombinant antibodies with mIL-17 was first studied by ELISA (Fig. 3A). IL-17 is an obligate homodimer and competition assays indicated that the aIL-17 2E1 mAb bound to a single epitope. Thus limiting coating conditions were used to minimize avidity effects, as previously shown for the affinity assessment of the interaction of tanezumab with the nerve growth factor (NGF), which shares a similar tertiary structure with IL-17, as both cytokines adopt a cysteine knot fold (Abdiche, Malashock et al. 2008; Zhang, Angkasekwinai et al. 2011). $K_D$ values were calculated from ELISA titers measured by determining half-maximal optical density (OD). AIL-17 2E1 hypermut bound as strong to mIL-17 as the hybridoma-derived antibody, with a $K_D$ value of $2 \times 10^{-11}$ M. Although backmutation of the hypermutated $V_H$ to germline reduced affinity around 100-fold, binding of the aIL-17 2E1 germline still was remarkably strong with a $K_D$ of $2 \times 10^{-9}$ M, considering that germline antibodies are believed not to be neutralizing or protective in vivo, in particular for a self-molecule. For exact $K_D$ determination, a global kinetic analysis using the Biacore T100 system was conducted. Ideal analysis of kinetic rate constants would require a monomeric analyte, allowing modelling using a simple bimolecular reaction mechanism (Myszka 1999; Abdiche, Malashock et al. 2008). To avoid avidity effects in our measurements, one experimental setup comprised flowing antibody over mIL-17 coated at low density of 400RU on a CM5 Biacore surface (data not shown). It soon became evident, that measuring the $k_d$ of the aIL-17 2E1 hypermut would require long dissociation periods. To extract reliable affinity data, several measurement cycles with different analyte-concentrations need to be run, resulting in our case in overall measurement periods of at least 7 hours. In addition, the strong interaction of the antibody with the ligand required regeneration with at least two 30s pulses of 2M Guanidine-HCl, conditions which lead to the continuous degeneration mIL-17 on the surface. Hence, the mAb/m-IL-17 interaction was studied in reverse orientation by flowing mIL-17 over aIL-17 2E1 hypermut or aIL-17 2E1 germline immobilized on a CM5 chip (Fig. 3B).
Figure 3 | Determination of affinity of the aIL-17 2E1 hypermut and 2E1 germline IgGs for mIL-17. A | Analysis of the affinity of aIL-17 2E1 hypermut and 2E1 germline for mIL-17 under limiting coating conditions by ELISA with hybridoma-derived aIL-17 2E1 as control. Serially diluted antibody was applied to mIL-17-coated ELISA plates and $K_D$ was determined at coating concentration 370.37 ng/mL. $K_D$ 2E1 hypermut was $2 \times 10^{-11}$ M and $K_D$ 2E1 germline $2 \times 10^{-9}$ M. Values are shown as OD at 450 nm. B | Global kinetic analysis of mIL-17 binding aIL-17 2E1 hypermut (top panel) and aIL-17 2E1 germline (bottom panel) immobilized on CM5 chip surface using Biacore T100. The mIL-17 concentrations analyzed spanned 0.0625 – 8 nM and 0.125 – 32 nM, respectively. For the 2E1 hypermut antibody, the association rate constant ($k_a$) was determined to be $9.362 \times 10^5$ M$^{-1}$s$^{-1}$. Since no decay in binding signal was detected, the dissociation constant ($k_d$) was defined to be <10$^{-5}$ s$^{-1}$ and the $K_D$ was <11 pM. For the 2E1 germline antibody, the globally fit $k_a$ was $4.961 \times 10^5$ M$^{-1}$s$^{-1}$ and the $k_d$ $2.632 \times 10^{-4}$ s$^{-1}$, resulting in a $K_D$ of 0.5 nM. Results are shown as time (s) plotted against resonance units (RU). In the overlay plots, sensorgrams are presented as colored lines and simulated curve fits as black lines.
The mAbs were captured at low coating concentrations to maximize univalent binding to mIL-17 and global kinetic analysis was performed as described. Dissociation of the aIL-17 2E1 hypermut antibody was too slow to be measured, forcing us to place an upper limit on the $k_d$ value. According to the “5% rule” (Katsamba, Navratilova et al. 2006), an exact $k_d$ value can only be measured, if at least 5% of the bound material dissociates, otherwise the $k_d$ is given as $< -\ln (0.95) / t_d$, with $t_d$ being the time allowed for dissociation (s) (Abdiche, Malashock et al. 2008). The this way calculated $k_d$ of $8.5 \times 10^{-5}$ s$^{-1}$ matched the instrument-given limit of $<10^{-5}$ s$^{-1}$, which was then used to determine the $K_D$ of $<11$ pM. The aIL-17 2E1 germline on the other hand showed faster and therefore measurable dissociation, resulting in a $K_D$ of 0.5 nM, a value that like the $K_D$ of the hypermutated antibody was slightly lower than the results obtained with limiting coating ELISA.

**Hypermuted and germline antibody neutralize the biological activity of IL-17 in vivo**

In order to test the ability of the hypermutated and germline 2E1 antibodies to neutralize the proinflammatory effector functions of IL-17 in vivo, C57BL/6 mice were immunized either with 100 µg aIL-17 2E1 hypermut, aIL-17 2E1 germline or an aFeld1 antibody as isotype-matched control and then challenged with 10 µg recombinant mIL-17. Since various cell types such as fibroblasts secrete distinct chemokines and cytokines in response to IL-17, we measured serum levels of CXCL-1 and IL-6 2h after injection as readout of the inflammatory response. As shown in Fig. 4, both antibodies, hypermutated and germline, significantly blocked the cytokine- and chemokine-inducing activities of IL-17, suggesting that the aIL-17 2E1 hypermut as well as the aIL-17 2E1 germline are both neutralizing.
RESULTS

Figure 4 | Inhibition of IL-17 biological activity in mice immunized with aIL-17 2E1 hypermut or aIL-17 2E1 germline. Female C57/BL6 mice were injected i.p. with 100 µg of either aIL-17 2E1 hypermut, aIL-17 2E1 germline IgG or aFeld1 IgG2a, followed by a s.c. injection of 10 µg mIL-17 after 30 min. Serum CXCL-1 or IL-6 levels were measured 2h after injection by ELISA (R&D Systems). Background levels of mIL-17 were in the range of <10 pg/mL and thus not shown. Depicted are mean values with SEM (*p<0.05 for aIL-17 2E1 hypermut vs. aFeld1 and aIL-17 2E1 germline vs. aFeld1).

Hypermutated and germline aIL-17 antibody show protective capacity in animal models of autoimmune inflammatory diseases

IL-17 has been shown to play a crucial role as proinflammatory mediator in various autoimmune diseases, including rheumatoid arthritis (RA) and psoriasis. Blocking IL-17 by the VLP-based vaccine Qβ-IL-17 or monoclonal antibodies such as the AIN457 by Novartis, have shown promising results in animal models as well as phase II studies in humans for the treatment of psoriasis and RA (Rohn, Jennings et al. 2006; Hueber, Patel et al. 2010). To investigate the therapeutic potential of our aIL-17 2E1 hypermut and aIL-17 2E1 germline antibodies, we assessed the effect of mAb treatment using two established murine models of autoimmunity, Imiquimod (IMQ)-induced skin inflammation and CIA (van der Fits, Mourits et al. 2009; Pantelyushin, Haak et al. 2012). In the first experiment, groups of female C57BL/6 and IL-17-deficient control mice were immunized three times with 100 µg aIL-17 2E1 hypermut, aIL-17 2E1 germline or isotype-matched aFeld1 control antibody as described. To induce psoriasis-like skin inflammation, IMQ cream was applied daily on both ears for seven consecutive days, resulting in signs of erythema, scaling and ear thickening after day 2. Control mAb-treated WT mice developed severe skin inflammation from day 2 onward with continually increasing scores until the end of
the experiment (Fig. 5A). In contrast, groups of WT mice treated with aIL-17 2E1 hypermut or aIL-17 2E1 germline showed substantially lower scores (p<0.0001 vs. WT (aFeld1) for both groups). Disease scores were similar as those observed in IL-17-deficient mice, indicating that neutralization of IL-17 was indeed rather complete.

In a next step, we tested the protective capacity of our antibodies in CIA, also in comparison to Qβ-IL-17, which has previously been shown to successfully suppress disease development. Prior to disease induction, groups of male DBA/1 mice were either immunized three times with Qβ-IL-17 or injected twice per week with 100 µg aIL-17 2E1 hypermut, aIL-17 2E1 germline or isotype-matched aFeld1 as control. CIA was induced by injection of collagen in CFA, followed by a booster injection of collagen in IFA. As shown in Fig. 5B, mice treated with the control antibody developed severe symptoms, peaking with an average score of 7.2 on day 36. In contrast, mice injected with the hypermutated anti IL-17 antibody, as well as the ones injected with the antibody mutated back to germline, showed substantially reduced disease severity (p<0.0001 vs. aFeld1 for both groups), with a maximum score of 2.8 and 3.5, respectively. Furthermore, disease incidence was reduced in both groups treated with anti IL-17 mAbs. While all mice of the group injected with aFeld1 developed high scores on one or more limbs during the course of the experiment, three out of ten mice treated with aIL-17 2E1 germline and even four out of 10 mice of the aIL-17 2E1 hypermut group developed none or only background-level scores. The protective capacity of the two monoclonal antibodies was comparable to the effect of the polyclonal antibody response induced by Qβ-IL-17 (p<0.0001 vs. aFeld1), with mice reaching a maximum score of 3.9 at the end of the experiment. This experiment suggests that an anti IL-17 germline antibody raised by vaccination with a VLP-vaccine has the same neutralizing and protective potential, as its hypermutated affinity-matured form.
RESULTS

Figure 5 | Protective capacity of hypermutated aIL-17 2E1 hypermut and germline aIL-17 2E1 germline in mice. A | Immunization with aIL-17 2E1 hypermut or aIL-17 2E1 germline suppresses development of IMQ-induced skin inflammation. Female C57BL/6 mice (WT) and IL-17 K.O. mice (n = 4) were treated daily with 30 mg IMQ cream on both ears to induce inflammation. WT Mice were immunized on day 0, 3 and 5 with 100 µg anti aIL-17 2E1 hypermut, aIL-17 2E1 germline or aFeld1 as control. IL-17 K.O. mice received aFeld1 injections. Ear thickening was measured daily. Shown are mean values with SEM (p<0.0001 vs. WT (aFeld1) for all groups, two-way ANOVA). B | Immunization with aIL-17 2E1 hypermut or aIL-17 2E1 germline reduces clinical signs of inflammation in CIA. Male DBA/1 mice (n = 10) were immunized either with 50 µg Qβ-IL-17 s.c. on days -43, -29 and -10 or twice per week starting on day -1 with 100 µg of either aIL-17 2E1 hypermut, aIL-17 2E1 germline or aFeld1 i.p. Arthritis was induced by injection of collagen in CFA s.c. into the tail (day 0) and a booster injection intradermally into the base of the tail with collagen in IFA (day 21). Progression of disease was monitored daily and visual clinical scores ranging from 0 to 3 were assigned to each limb according to the degree of joint swelling and reddening. Shown are mean values of the cumulative scores of all limbs per mouse with SEM (p<0.0001 vs. aFeld1 for all groups, two-way ANOVA).
4.1.4 Discussion

Therapeutic vaccines against potentially pathogenic self-molecules are a new class of drugs that offer several advantages over monoclonal antibodies in such as their production is far less cost-intensive, fewer administrations of lower dosages are required and the induced polyclonal immune responses may result in more rapid clearance of immune complexes (Bachmann and Dyer 2004; Kopf, Bachmann et al. 2010). However, in order to induce an immune response against autologous antigens, the immune system’s tolerance mechanisms have to be overcome to stimulate the production of autoantibodies with affinities high enough to reach clinical efficacy (Link and Bachmann 2010). Immunodrugs are therapeutic vaccines composed of self-molecules conjugated to Qβ, a bacteriophage-derived VLP, which due to its repetitive surfaces is able to activate antigen-specific B cells and exhibits foreign T\textsubscript{H} cell epitopes, which prime CD\textsuperscript{4} T lymphocytes that then provide B cell help and stimulate autoantibody production (Chackerian 2007; Jennings and Bachmann 2009). Preclinical proof of concept studies have been successfully conducted for numerous antigens, among them the proinflammatory cytokine IL-17A. Effective induction of autoantibodies and subsequent protection through immunization with Qβ-IL-17 has been confirmed for animal models of rheumatoid arthritis, multiple sclerosis and myocarditis. Of note, induction of self-specific B cell responses has always been possible without breaking T\textsubscript{H} cell tolerance, leading to reversible antibody responses without boosting by endogenous cytokines (Rohn, Jennings et al. 2006; Uyttenhove and Van Snick 2006).

In order to further investigate the nature of these immune responses, we have immunized mice with Qβ-IL-17 and generated a panel of monoclonal antibodies specifically binding mIL-17A, of which aIL-17 2E1 was selected for further analysis due to its high affinity.

EAE has been shown to be initiated by myelin-reactive cells, among others effector T lymphocytes, that penetrate the blood brain barrier (BBB) and release proinflammatory cytokines, such as IL-17, which in turn initiate and maintain inflammation in the central nervous system (CNS) and promote the infiltration of further autoreactive immune cells, resulting in lesion and demyelination (Woodroffe and Cuzner 1993; Minagar and Alexander 2003; Glass, Saijo et al. 2010). While there
have been reports that blockage of mIL-17 with mAbs is not very effective in preventing the development of EAE (Haak, Croxford et al. 2009), our study demonstrated that immunization with aIL-17 2E1 was able to ameliorate disease severity, which corroborates our previous results employing active vaccination with Qβ-IL-17 (Rohn, Jennings et al. 2006).

To shed light onto the precise role of hypermutation and affinity maturation, which the antibodies induced by the Qβ-IL-17 vaccine undergo, we mutated the hypermutated antibody back to germline sequence, generating a set of two antibodies with V_{H} regions differing in three amino acids but still recognizing the same epitope. As basic immunization studies with haptens demonstrated that during the course of an immune response, antibodies undergo a substantial increase in affinity as consequence of accumulation of point mutations in the variable regions, accompanied by faster on-rates, we assumed this backmutation would lead to a reduction in affinity (Eisen and Siskind 1964; Weigert, Cesari et al. 1970; Wysocki, Manser et al. 1986; Foote and Milstein 1991; Kalinke, Bucher et al. 1996). Surprisingly, affinity assessment revealed that the aIL-17 2E1 germline antibody still exhibited a high affinity with a \(K_D\) of 0.5 nM compared to the \(K_D\) of <11 pM resolved for the aIL-17 2E1 hypermut. Furthermore, \textit{in vivo} neutralization assays demonstrated that both, the hypermutated as well as the germline antibody significantly neutralized mIL-17 and blocked its biological activity. We next assessed the protective capacity of the aIL-17 2E1 hypermut and aIL-17 2E1 germline in animal models of autoimmunity. IL-17 has been shown to contribute to the synovial inflammation, bone and cartilage destruction of arthritic joints in collagen-induced arthritis as well as the pathogenesis of IMQ-induced psoriatic skin inflammation (van der Fits, Mourits et al. 2009; Pantelyushin, Haak et al. 2012; Pollinger 2012). In both models, the hypermutated and the germline antibody achieved significant reduction of disease symptoms, indicating effective neutralization of mIL-17 \textit{in vivo} and in the case of the psoriasis model, even an effect comparable to the complete deletion of mIL-17 in IL-17 knockout mice. However, the observation that an anti-cytokine germline antibody has the same neutralizing and protective capacity as the corresponding hypermutated mAb stands in harsh contrast to the general concept of affinity maturation (Foote and Eisen 1995). Our findings suggests that while anti-cytokine antibodies induced by VLP-based vaccines such as Qβ-IL17 undergo affinity maturation, increasing binding strength above a certain threshold does not translate into better efficacy. Interestingly there are several
findings in support of our hypothesis, among them studies investigating the immune response against vesicular stomatitis virus (VSV) (Newman, Mainhart et al. 1992; Foote and Eisen 1995). These data demonstrate that anti-VSV germline antibodies isolated during early immune response already possess high affinities and are protective in vivo, suggesting the existence of an affinity threshold, above which further affinity maturation does not increase the protective capacity, given a minimum serum concentration (Roost, Bachmann et al. 1995; Bachmann, Kalinke et al. 1997; Kalinke, Oxenius et al. 2000).

Furthermore, the finding of an IL-17 specific germline antibody with early neutralizing capability as such might contribute to the understanding of another autoimmune disease recently linked to IL-17. Autoimmune polyendocrine syndrome type I (APS-I), characterized by symptoms including multiple autoimmune endocrinopathies and hypothyroidism, is caused by mutations in the AIRE gene coding for a transcriptional regulator of the expression of peripheral tissue antigens in the thymus, which contributes to central tolerance (Nagamine, Peterson et al. 1997; Mathis and Benoist 2009). These mutations result in self-reactive T lymphocytes evading clonal deletion, causing autoimmunity in the periphery (Liston, Lesage et al. 2003; Anderson, Venanzi et al. 2005). In addition to the hallmark autoantibodies against the type I interferons (IFN) IFN-α and IFN-ω, a recent study has now found high titers of autoantibodies against the T₇ signature cytokines IL-17A, IL-17F and IL-22 in APS-I patients additionally suffering from chronic mucocutaneous candidiasis (CMC) (Meager, Visvalingam et al. 2006; Puel, Doffinger et al. 2010). Interestingly, these autoantibodies seem to be produced even though there is little and contradictory evidence that B cell tolerance would be impaired in APS-I patients, while the lack of T cell tolerance towards tissue antigens is evident (Devoss, Shum et al. 2008; Gavanescu, Benoist et al. 2008). The presence of germline antibodies with IL-17 neutralizing capacities indicates that there is no immunological tolerance for IL-17 at the level of B cells. Hence, impaired T₇ cell tolerance is sufficient to drive IL-17 specific antibody responses.
4.2 Manuscript II

The role of antibody affinity in mediating protection against autoimmune inflammatory diseases

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Monoclonal antibodies (mAbs) represent a new class of therapeutic agents for the immunotherapy of chronic inflammatory diseases. Particularly the application of mAbs to block cytokines, which hold central roles in mediating immune responses, has emerged as a recognized medical intervention for many indications including inflammatory diseases such as rheumatoid arthritis. It is generally assumed that mAbs need to have a high affinity for the target cytokine in order to show efficacy and major efforts are being made by the industry to improve mAb affinity up to pico- and even femtomolar levels. However, so far no conclusive studies have been conducted, which directly address this issue. To elucidate this question, we generated a panel of mouse mAbs specific for Interleukin 17 (IL-17), which bind to the antigen with very high affinity. We hypothesized that mutating the antibody genes towards germline would affect their affinity for IL-17. Indeed, the deduced germline antibody exhibited extremely low affinities and in contrast to the hypermutated mAb, failed to protect in vivo against EAE, an animal model for multiple sclerosis. By introducing intermediate numbers of mutations in the heavy chain, we aimed to generate of a panel of antibodies binding the same epitope with intermediate affinities. Surprisingly, all 14 mutants exhibited high affinity for mIL-17. Even antibodies differing from germline with only a single mutation showed affinities in the $10^{-11}$ M range. We therefore will test antibodies, which also exhibit light-chains mutated towards germline.
4.2.1 INTRODUCTION

Monoclonal antibodies (mAbs) have become one of the most powerful tools for medical intervention. In addition to their high specificity and target selectivity, their ability to neutralize secreted proteins provides a major advantage over traditional therapeutic approaches. These properties have allowed generating a number of different mAbs neutralizing a set of target molecules that was previously poorly accessible for classical small molecules, particularly cytokines. As interaction of cytokines with their receptors involves protein-protein interactions with large surfaces, this makes blocking them with small molecules extremely difficult. Moreover, signal transduction molecules down-stream of these cytokine receptors typically comprise kinases. This class of molecules remains – despite recent breakthroughs – a challenging target, mostly due to limited specificity of small molecular compounds. This positions mAbs uniquely to block cytokines and other secreted proteins as well as their receptors.

Despite the high specificity of mAbs, relatively large amounts of protein are usually required to reach the therapeutic window (typically >100 mg/patient/dose), which renders these therapies expensive due to the high cost of goods (Kopf, Bachmann et al. 2010). From a theoretical point of view, it seems likely that lower doses of mAbs may be required if the antibody affinity is increased. Indeed, the law of mass action would predict this to be the case - provided concentrations of cytokines are low, which is expected to be the case in vivo (Link and Bachmann 2010). Hence, engineering mAbs to artificially increase affinities may be one way to reduce the protein amounts required and thus save expenses. Despite intensive efforts of industry as well as academia to improve the affinity of mAbs into the pico- or even femtomolar range (Altshuler, Serebryanaya et al. 2010), there is little in vivo data showing that this actually improves the efficacy of these antibodies. In fact, studies on vesicular stomatitis virus (VSV) immunity have revealed that even germline antibodies against VSV exhibit neutralizing and protective capacity, which does not significantly improve after affinity maturation during late immune response, thus suggesting the existence of an affinity threshold for protection (Roost, Bachmann et al. 1995; Bachmann, Kalinke et al. 1997; Kalinke, Oxenius et al. 2000). In addition, clinical experience from anti-cytokine-antibodies suggests that increasing antibody
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affinity not essentially correlates with enhanced efficacy and reduction of dosage, as exemplified by two antibodies targeting IL-1β, Novartis’ Ilaris and Xoma’s Xoma052. Even though affinities of these mAbs differ by a factor 100, the clinically administered doses are almost identical (Alten, Gram et al. 2008; Dhimolea 2010; Owyang, Issafras et al. 2011; Chakraborty, Tannenbaum et al. 2012). Nevertheless, the clinical relevance of improving affinity of anti-cytokine antibodies beyond a certain threshold has never been put to test.

We therefore established a system that allows investigating this question in murine disease models. We immunized mice with our virus-like particle (VLP)-based vaccine Qβ-IL-17 and generated a mAb with very high affinity for Interleukin-17 (IL-17) (Rohn, Jennings et al. 2006). By introducing specific mutations into the variable regions of this high affinity antibody, we stepwise mutated the antibody back to germline sequence in order to reduce affinity. Testing antibodies specific for the identical epitope but exhibiting different affinities for in vivo efficacy will eventually reveal, whether increasing the affinity of antibodies above a minimal threshold is a worthwhile step in the development of mAb therapies.
4.2.2 MATERIALS & METHODS

Mice

Female C57BL/6 mice were purchased from Charles River, male DBA/1 mice from Harlan Netherlands B.V. Female and male IL-17 deficient (knockout (K.O.)) mice were kindly provided by Prof. M. Kopf (ETH Zurich, Switzerland). All mice were maintained under specific pathogen-free conditions and used for experimentation according to protocols approved by the Swiss Federal Veterinary Office.

Cloning, expression, purification and refolding of recombinant IL-17A

As described previously (Rohn, Jennings et al. 2006), the plasmid MOD_His_mIL-17_C was expressed in Echerichia coli BL21 cells to produce a recombinant protein comprised of a N-terminal hexahistidine tag followed by mIL-17 (aa 26-158) and a C-terminal linker sequence consisting of five glycine residues and one cysteine residue. Following solubilization from inclusion bodies and purification over a Ni\(^{2+}\)-NTA agarose column (Qiagen), the protein was further purified over a HiLoad Superdex 75 prep grade gel-filtration column (Amersham). Refolding of the mIL-17 protein was performed in a two-step dialysis procedure and a mIL-17 sandwich ELISA (BD PharMingen) was used to confirm authentic refolding of the protein.

Production of Qβ-IL-17 conjugate vaccine

Virus-like particles (VLPs) derived from the bacteriophage Qβ were expressed in E. coli JM109 and purified as shown previously (Cielens, Ose et al. 2000). In a two-step procedure, the mIL-17 was covalently conjugated to Qβ, as described before (Rohn, Jennings et al. 2006). First, Qβ-VLP were reacted at room temperature with a 20-fold molar excess of the heterobifunctional chemical cross-inker, succinimidyl-6-(β-maleimidopropionamido) hexanoate, followed by dialysis to remove unreacted cross-linker and incubation with an 10-fold excess of tri(2-carboxyethyl)phosphine hydrochloride to reduce any cysteine residues in the linker. Next, mIL-17 was
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covalently linked to the derivatized Qβ by reacting equimolar amounts of mIL-17 and Qβ at room temperature. The vaccine was analyzed by SDS-PAGE and Immunoblotting with anti-Qβ and anti-His antibodies.

Generation of mIL-17-specific Hybridoma IgG antibodies

To induce neutralizing monoclonal IgG antibodies against mIL-17, mice were immunized twice subcutaneously with 50µg Qb-IL-17 on day 0 and day 14. Following the booster injection, the splenocytes were fused four days later to murine myeloma cells (Sp2/0Ag14, ATCC) using standard methods (Collaboration with EMBL, European Molecular Biology Laboratory, Italy). Hybridoma produced IgG was then screened for binding to purified mIL-17 by ELISA. Supernatants of hybridoma cells were serially diluted in PBS / 0.05% Tween-20 / 2% BSA starting at an antibody concentration of 0.1 µg/mL and applied to ELISA plates (Nunc) that had been coated with of 10 µg/mL IL-17. HRP-conjugated goat anti mouse IgG Fcγ fragment specific secondary antibody (Jackson) was used at a dilution of 1:1000 in PBS / 0.05% Tween-20 / 2% BSA to detect bound IgG. As a result, six out of eight IgG-secreting hybridomas were able to bind not only to recombinant mIL-17 but also mIL-17 produced in eukaryotic cells. Selected hybridoma clones were cultured in Dulbecco’s Modified Eagle Medium (DMEM GlutaMAX, Gibco) supplemented with 20% FBS (Gibco), 50 mg/mL Gentamicin (Gibco) and Hybridoma cloning factor (HCF, BioVeris). Affinity chromatography over a protein G-Sepharose column (GE Healthcare) was used to purify antibodies from culture supernatants. As one of the recovered antibodies, aIL-17 1B10 was then characterized by ELISA for binding to a variety of IL-17 proteins including refolded mIL-17, commercial available IL-17 (R&D), IL-17 without linker and IL-17F. In brief, ELISA plates (Nunc) were coated with IL-17 proteins at a density of 0.5 µg/mL before a 1:3 serial dilution of aIL-17 1B10 in PBS / 0.05% Tween-20 / 2% BSA was applied, starting at a concentration of 1 µg/mL. Antibody binding was detected using HRP-conjugated goat anti mouse IgG (Fc gamma specific) (Jackson) diluted 1:1000 in PBS / 0.05% Tween-20 / 2% BSA. Limiting coating ELISA was utilized to determine the affinity of aIL-17 1B10 for mIL-17.
Isolation of $V_H$ and $V_L$ Genes and generation of scFv antibody fragments

To isolate total RNA from IL-17-specific hybridoma cells TRI reagent (Molecular Research, Inc.) was utilized, followed by generation of cDNA using Super Script II RT (Invitrogen). After amplification of HCVRs and LCVRs, scFv coding regions were assembled by PCR applying primers and protocols described previously (Beerli, Bauer et al. 2008). As a result, PCR products encoded scFv consisting of a 5’ LCVR ($\kappa$) and a 3’ HCVR fused by an 18aa-flexible linker, and flanked by two SfiI restriction sites. For the generation of fusion proteins comprising a N-terminal murine scFv joined to a C-terminal Fc-\(\gamma\)1 domain, the restriction endonuclease SfiI was used for the digestion of the scFv coding regions, which were then ligated into the SfiI-digested expression vector pCEP-SP-Fc. As a derivative of the episomal mammalian expression vector pCEP4 (Invitrogen), this vector comprises the EBV replication origin and the EBV nuclear antigen (EBNA-1), which allows for extra chromosomal replication. Furthermore, this vector features a puromycin selection marker instead of the original hygromycin B resistance gene. In the resulting constructs a CMV promoter regulates the expression of secreted scFv-Fc domain fusion proteins.

For expression of recombinant scFv fusion proteins, Lipofectamine 2000 (Invitrogen) was used to transform 293T HEK cells grown in DMEM (10% FBS) with various scFv-Fc expression constructs, followed by collection of supernatants after 48h. Supernatants were then analyzed for concentration and IL-17 binding capacity of scFv-Fcs by ELISA. To determine the concentration of the produced molecules, ELISA plates (Nunc) were coated with F(ab)$_2$ goat anti hu IgG Fc specific (Jackson) at a concentration of 2.5 µg/mL followed by incubation with 1:10 prediluted cell supernatants serially diluted 1:3 in PBS / 0.05% Tween-20 / 2% BSA. HRP-conjugated goat anti human IgG secondary antibody (Jackson) was applied at a dilution of 1:1000 in PBS / 0.05% Tween-20 / 2% BSA to detect bound scFv-Fc. To screen for IL-17 binding, 5 µg/mL mIL-17 was coated onto ELISA plates (Nunc) and incubated with supernatants prediluted 1:10 and serially diluted 1:3 in PBS / 0.05% Tween-20 / 2% BSA. Bound scFv-Fc was detected with HRP-conjugated goat anti human IgG (Jackson).

Following sequencing, VRs of selected IL-17 binding scFv were compared to antibody germline sequence (IMGT database), hypermutated $V_H$ segments were
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reverted to germline as described in the results section (Geneart) and mutagenized scFv were cloned into the expression vector pCEP-SP-Fc.

Cloning, expression and purification of recombinant monoclonal antibodies and affinity mutants

Full-length murine IgG2α antibodies 1B10 hypermut and 1B10 germline were generated by amplifying the coding segments of selected IL-17-specific scFv antibodies by PCR, utilizing VR-specific transfer primers. After assembling κLC γ2HC expression constructs in pCMV-Script (Stratagene), HC and LC coding regions were joined in the episomal expression vector pCB15, as shown in (Beerli, Bauer et al. 2008). For the generation of aIL-17 1B10 mutants 1 – 14, the V\textsubscript{H} coding regions containing the desired point mutations (Geneart) were directly cloned into the pCMV-HC vectors and then assembled with the LC in the pCB15 vector.

For expression, antibody plasmids were transfected into the HEK 293T cell line using Lipofectamin Plus (Invitrogen). Stable transfectants were expanded and selected under puromycin (Sigma) pressure for production at larger scale. Resistant protein-expressing cells were cultured in serum-free medium in Poly-L-Lysine-coated roller bottles for up to two months and antibodies were purified from supernatants by affinity chromatography over a protein G-Sepharose column (GE Healthcare).

Affinity measurement

Limiting coating ELISA:

By limiting the coating concentrations in an ELISA binding assay, the affinity of hybridoma-derived and recombinant anti IL-17 antibodies could be measured with minimal influence of avidity effects. ELISA plates (Nunc) were coated with 1:3 serially diluted murine IL-17, starting at a concentration of 10 µg/mL (Fig 1B), 400ng/mL (3A) or 5 µg/mL (Table 2). Next, a 1:3 serial dilution of purified 1B10 antibody in PBS / 0.05% Tween-20 / 2% BSA was applied with a starting concentration of 0.5 µg/mL in the direction rotated by 90° to the coating dilution series. For the determination of affinity of 1B10 mutants 5-14, supernatants from transfected HEK 293T cells were applied instead of purified antibodies. This minimizes partial denaturation of antibodies due to purification procedures. HRP-conjugated goat anti mouse IgG (Fc gamma specific) (Jackson) diluted 1:1000 in PBS
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/ 0.05% Tween-20 / 2% BSA was used to detect bound antibody. $K_D$ values were calculated from ELISA titers measured by determining half-maximal optical density (OD) at the limiting coating concentration of 123.46 ng/mL (1B10 hybridoma, Fig. 1B), 133.33 ng/mL (1B10 hypermut and germline, Fig. 3A) or 61.73 ng/mL (1B10 mutants, Table 2). Values are given as OD at 450 nm.

Binding kinetics by Biacore:

The affinity of soluble mIL-17 for immobilized anti IL-17 antibodies 1B10 hypermut and 1B10 germline was also analyzed using surface plasmon resonance (SPR) detection measured with Biacore T100 instrument (GE Healthcare). Data were evaluated with the Biacore T100 control software package version 2.0.3(GE Healthcare). Running buffer was amine-free and consisted of 10mM NaH$_2$PO$_4$, 150mM NaCl and 0.05% Tween. Biacore surfaces were prepared using a standard amine-coupling protocol. To individually activate the flow cells of a carboxyl methylxamidexran (CM5) sensor chip (GE Healthcare), a freshly prepared mixture of 0.4 M EDC and 0.1 M NHS was injected for 7 min at a flow rate of 5 µL/min. To immobilize the antibody, anti IL-17 antibody 1B10 hypermut or 1B10 germline at a concentration of 100 µg/mL diluted in 10 mM NaAc buffer pH 5.5 were flowed over the sample cells for 11 - 18 min, until the desired level of immobilization was reached, usually at 5300 – 10’000 resonance units (RU). The reference surface was coated with 100 µg/mL BSA (GE Healthcare, 2D Quant Kit) diluted in 10 mM NaAc buffer pH 4, injected for 7 min, thus reaching coating densities of around 2000 RU. Excess reactive esters were quenched by injecting 1 M Ethanolamine-HCl for 7 min.

To measure the kinetics of mIL-17 over the antibodies on the chip, a three- or twofold serial dilution of mIL-17 (for 1B10 hypermut: 0.033 - 24.3 nM mIL-17, for 1B10 germline: 2.5 - 160 nM mIL-17, respectively, with all concentrations in duplicates) was injected for 3 min at a flow rate of 30 µL/min, permitting a dissociation phase of 10 min. Surface regeneration was achieved with two pulses of 2M Guanidine-HCl with stabilization periods of 5 min.

Measurement of neutralizing-activity: In vitro IL-6 reporter assay

To measure the inhibition of IL-17 induced IL-6 release from murine fibroblast cells, 20 µg/mL anti-IL-17 antibodies 1B10 hypermut, 1B10 germline or total mouse IgG (Jackson) were was serially diluted 1:5 in DMEM containing 20 ng/mL
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prokaryotically expressed, refolded mIL-17 and 2 ng/mL recombinant TNF-α. After incubating for 1h, the media containing antibody-bound and unbound IL-17 in equilibrium was added onto BALB/3T3 cells seeded into 96-well plates at a density of 2x10^4 cells / 100 µL. IL-6 concentrations in supernatants diluted 1:20 were measured 48h later by sandwich ELISA (BioLegend). Results are shown as OD at 450 nm.

**Determination of in vivo protective capacity of IL-17-neutralizing IgGs**

**EAE model:**

Groups of female C57BL/6 mice (n = 10, age = 8 weeks) were either immunized three times s.c with QB-IL-17 starting 36 days before disease induction or injected twice per week i.p. with 100 µg of either recombinant aIL-17 1B10 hypermut, aIL-17 1B10 germline or a control aKLH mIgG2aκ mAb (BD Pharmingen, #553454), starting one day before induction. EAE was induced on day 0 by injecting each mouse s.c. into the right or left flank with 100 µg / 200 µL MOG35-55 peptide (Biotrend) diluted in PBS-CFA (0.5 mg/mL) followed by the injection of 400 ng of pertussis toxin i.p. From day 7 on, mice were scored on a daily basis for three consecutive weeks for the development of clinical signs of disease as follows. As described previously (Rohn, Jennings et al. 2006), clinical scores from 0 to 3 were assigned according to the following scoring scheme: 0 = normal, 0.5 = distal tail paralysis, 1 = complete tail paralysis, 1.5 = complete tail paralysis and unilateral hind limb weakness, 2 = bilateral hind limb weakness, 2.5 = unilateral hind limb paralysis, 3 = bilateral hind limb paralysis. As and indicator of disease, body weight was also measured.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Two-way ANOVA with Bonferroni’s post test was used to determine the significance of group differences in the EAE experiment. A p value less than 0.05 was considered significant.
4.2.3 RESULTS

Monoclonal antibody 1B10 induced by Qb-IL-17 binds IL-17 with high affinity

In order to conduct a systematic comparison of the influence of antibody affinity on clinical efficacy we aimed to generate a very high affinity anti IL-17 antibody and mutants thereof to produce a panel of antibodies with a single specificity but varying affinities. To this end, we immunized mice twice with Qβ-IL-17 to obtain hybridoma cell lines secreting IgG antibodies specific for mIL-17. The monoclonal antibody (mAb) secreted by the hybridoma clone 1B10 (aIL-17 1B10 hybridoma) was able to specifically recognize mIL-17A (Fig. 1A). In order to distinguish the antibody from subsequently generated germline like versions, it was called mAb 1B10 hypermut. The antibody recognized commercially available mIL-17 with similar efficacy as the recombinant refolded IL-17, comprising a linker as well as a His-tag, which was utilized for production of the Qβ-vaccine. Despite the fact that IL-17A and its related IL-17 family member IL-17F are to 50% identical at the protein level, aIL-17 1B10 hypermut did not bind to IL-17F (Weaver, Hatton et al. 2007). As a first approach to investigate antibody affinity, binding of purified aIL-17 1B10 hypermut to mIL-17 was assessed under limiting coating conditions by ELISA and revealed a strong interaction with a $K_D$ of $4 \times 10^{-11}$ M (Fig. 1B).

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**Figure 1** Binding specificity and affinity determined for the hybridoma-derived aIL-17 monoclonal antibody 1B10. **A** Binding of aIL-17 1B10 hybridoma antibody to recombinant refolded mIL-17A, IL-17F, linker-free IL-17A and commercially available mIL-17 (R&D Systems) was assessed by ELISA. Values are given as OD at 450 nm. **B** Limiting coating ELISA to determine the affinity of aIL-17 1B10 hybridoma for mIL-17. mIL-17 was coated onto ELISA plates and incubated with serially diluted antibody. $K_D$ was determined at coating concentration 123.46 ng/mL. Values are shown as OD at 450 nm.
The variable regions of the 1B10 antibody bear somatic point mutations compared to germline sequence

To investigate differences in sequence between the variable regions (VRs) of the hybridoma-derived 1B10 antibody and known germline gene fragments, we isolated RNA from hybridoma cells, amplified the genes of the immunoglobulin heavy (V<sub>H</sub>) and light (V<sub>L</sub>) and constructed a single-chain Fv (scFv). Expression of the scFv in HEK 293T cells as a fusion protein linked to a C-terminal Fc-γ1 domain allowed verification of mIL-17 binding by ELISA (data not shown). To examine the occurrence of specific somatic mutations, the nucleotide sequence of the variable regions were aligned to germline Ig sequence. Sequence comparison indicated the usage of gene segments belonging to the IgHV6 as well as IgKV12 gene families. While the V<sub>L</sub> sequence of the aIL-17 1B10 scFv showed differences in six base pairs that resulted in one silent and four replacement mutations, the V<sub>H</sub> segment revealed variance in five positions of evenly distributed silent mutations and six base pairs leading to amino acid replacement. Among the latter, two lie within CDR2 and encode the same amino acid substitution (Leu-to-Ala), the others are located in the V<sub>H</sub>-FR3 and each code for one replacement (Arg-to-Ser; Asn-to-Ser; Thr-to-Ala, and Thr-to-Ile) (Fig. 2). As it is generally assumed that germline encoded antibodies exhibit low affinities before they accumulated point mutations during affinity maturation, we hypothesized that mutating the aIL-17 1B10 V<sub>H</sub> genes towards germline would affect their affinity for the antigen (Kalinke, Bucher et al. 1996). Hence in a next step specific mutations were introduced into the hypermutated V<sub>H</sub> region of the aIL-17 1B10 scFv to revert the sequence to germline. The hypermutated and the germline V<sub>H</sub> regions were amplified along with the V<sub>L</sub> segment, joined and transferred into an expression vector as described, giving rise to full-length antibodies of the IgG2ακ isotype (aIL-17 1B10 hypermut and aIL-17 1B10 germline), identical to the isotype of the mAb originally isolated from the 1B10 hybridoma cell line.
Figure 2 | Comparison of V_L and V_H sequences of the aIL-17 1B10 scFv fragment to aligned germline VRs. The IL-17 specific 1B10 scFv was generated by isolation of total RNA from 1B10 hybridoma cells and amplification of V_H and V_L regions from cDNA, which were then joined by a flexible linker. Sequences were aligned to germline sequences available on the international ImMunoGene Tics Information system (IMGT) database. Depicted is the alignment of the nucleotide sequence (black) of the aIL-17 1B10 scFv with deduced amino acids shown in single-letter code (green) with framework (FR) and CDR regions (☐) as well as linker (☐). Positions in which nucleotide sequences of scFv and germline diverge are highlighted for replacement mutations (n) as well as for silent mutations (n). The corresponding germline amino acid is indicated (↵).

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Reversion of the V_H of the 1B10 hypermut IgG antibody to germline results in low affinity for mIL-17

Measuring the affinity of the interaction of mIL-17 with our recombinant aIL-17 antibodies was particularly challenging, as this cytokine is known to be an obligate homodimer and the occurrence of avidity effects represents an issue. However, as competition assays showed that our mIL-17 1B10 antibody bound to a single epitope, all binding assays were conducted under limiting coating conditions. With these measures, avidity effects were reduced to a minimum, as previously described for the measurement of the affinity of tanezumab for its ligand, the nerve growth factor (NGF), which adopts a cysteine knot fold structure, and thus shares a similar tertiary structure with the IL-17 family members (Abdiche, Malashock et al. 2008; Zhang, Angkasekwinai et al. 2011). As an initial study, the interaction was measured by ELISA and K_D values were calculated from ELISA titers measured by determining half-maximal optical density (OD) (Fig. 3A). While the aIL-17 1B10 hypermut bound
with very high affinity to mIL-17, with a $K_D$ value of $4 \times 10^{-11}$ M, no binding of the aIL-17 1B10 germline antibody could be detected. We then compared binding constants of the hypermutated and the germline antibody at significantly higher coating conditions, which assesses avidity rather than affinity and hence cannot be used to determine an exact $K_D$. Nevertheless we observed that backmutation of the $V_H$ resulted in a reduction of binding by a factor $10^6$ to a $K_D$ in the $\mu$M range. To verify these measurements and determine an exact $K_D$, we next performed a global kinetic analysis applying the Biacore T100 system. From previous Biacore experiments involving mIL-17 we learned that this cytokine was not stable enough to endure long measurement periods and repeated regeneration cycles when coated onto the chip surface. We thus proceeded with the global kinetic analysis of the antibody-cytokine interaction by flowing mIL-17 over aIL-17 1B10 hypermut or aIL-17 1B10 germline immobilized on a CM5 sensor at low coating concentrations to ensure univalent binding to the antigen (Fig. 3B). Despite the assessment of long dissociation periods, it was not possible to resolve the $k_d$ for the aIL-17 1B0 hypermut antibody. Following the 5% rule (Katsamba, Navratilova et al. 2006; Abdiche, Malashock et al. 2008), a off-rate ($k_d$) of $<10^{-5}$ s$^{-1}$ was suggested as an upper limit, which together with the measured association-rate ($k_a$) of $3.578 \times 10^5$ M$^{-1}$s$^{-1}$ resulted in a $K_D$ of $<28$ pM for the hypermutated 1B10 antibody. For the affinity measurement of aIL-17 1B10 germline we injected mIL-17 spanning a broad concentration gradient, titrated up to 10-times the $K_D$ value estimated by ELISA. Although the association as well as dissociation rates could be measured, surprisingly no fit exactly matching the theoretical binding kinetics could be found. We nevertheless used the 1:1 binding model that described the binding kinetics of the hypermutated antibody well and calculated a $K_D$ of $3 \times 10^{-7}$ M for the germline antibody as a rough estimate of the aIL-17 1B10 germline affinity. Indeed, this value fit quite well to the estimation obtained by ELISA.
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Figure 3 | Analysis of affinity of the aIL-17 1B10 hypermut and 1B10 germline recombinant antibodies for mIL-17. A | Limiting coating ELISA. ELISA plates were coated with mIL-17 and serially diluted antibody was applied. $K_D$ was derived at coating concentration 133.33 ng/mL and a value of $4 \times 10^{11}$ M was determined for aIL-17 1B10 hypermut. Values are given as OD at 450 nm. B | Global kinetic analysis of the interaction of mIL-17 with aIL-17 1B10 hypermut (top array) or aIL-17 1B10 germline (bottom array) coupled to a CM5 sensor utilizing Biacore T100. The aIL-17 1B10 hypermut antibody was analyzed measuring seven concentrations of mIL-17 ranging from 0.033 nM to 24.3 nM, giving an association rate constant ($k_a$) of $3.578 \times 10^5$ M$^{-1}$s$^{-1}$, while the dissociation constant ($k_d$) was set at <$10^{-5}$ s$^{-1}$ as dissociation could not be resolved, resulting in an estimated $K_D$ of <28 pM. For the aIL-17 1B10 germline antibody, mIL-17 concentrations spanning 2.5 – 160 nM were examined. As no satisfying fit could be found, an estimated $K_D$ of $3 \times 10^{-7}$ M was determined from the 1:1 binding model. Results are shown as time (s) plotted against resonance units (RU). In the overlay plots, sensorograms are presented as colored lines and simulated curve fits as black lines.
The fully hypermutated but not the germline 1B10 antibody neutralizes mIL-17 in vitro

In order to investigate whether the hypermutated and germline 1B10 antibodies neutralized IL-17 in vitro, we tested their ability to inhibit the mIL-17-induced secretion of the proinflammatory effector cytokine IL-6 from murine fibroblast cells. As shown in Fig. 4, the aIL-17 1B10 hypermut but not aIL-17 1B10 germline antibody was able to significantly neutralizing mIL-17 resulting in inhibition of IL-6 release from BALB/3T3 cells. This indicated that backmutation of the VH to germline sequence not only substantially reduced the antibody affinity but also abolished its neutralization capacity.

Figure 4 | In vitro inhibition of mIL-17 induced IL-6 release from murine fibroblast cells. Serial dilutions of aIL-17 1B10 hypermut, aIL-17 1B10 germline or total mouse IgG control were incubated with a constant amount of 20 ng/mL recombinant mIL-17 with 2 ng/mL TNF-α and added onto BALB/3T3 cells. IL-6 concentrations in supernatants were measured 48 h later by sandwich ELISA (BioLegend). Results are shown as OD at 450 nm.

Hypermutable but not the germline 1B10 protects from EAE

To assess, whether the affinity of the anti IL-17 1B10 antibodies not only correlated with the in vitro neutralization capacity but also the therapeutic potential in vivo, we tested the protective capacity of these mAbs in MOG\textsubscript{35-55} - induced EAE, a murine model of multiple sclerosis in which vaccination with Qβ-IL-17 has already been shown to have a protective effect (Rohn, Jennings et al. 2006). Groups of female C57BL/6 mice were either immunized with Qβ-IL-17 or treated twice weakly with 100 µg aIL-17 1B10 hypermut, aIL-17 1B10 germline or aKLH isotype matched
control antibody, and EAE was induced by injection of MOG_{33-55} in CFA and pertussis toxin. While the disease incidence was comparable in all groups, aKLH-treated mice developed severe signs of disease with scores reaching a plateau of 1.8 from day 23 on (Fig. 5). Similar to the polyclonal response induced in the Qβ-IL-17 vaccinated group, mice injected with aIL-17 1B10 hypermut showed not only a delay of disease onset but also significantly milder symptoms with average scores slowly increasing towards 0.8 on day 21 and eventually peaking with an average score of 1.28 on day 24 (p<0.0001 vs. aKLH). However, mice immunized with Qβ-IL-17 had an even stronger effect towards the end of the experiment and continued to display maximal scores of only 0.8 after day 21 (p<0.0001 vs. aKLH). In contrast, mice treated with aIL-17 1B10 germline failed to show any amelioration of the disease and even at times developed higher scores than the aKLH group. These results clearly demonstrate a correlation of the affinity of the aIL-17 1B10 antibody with protection against proinflammatory autoimmune diseases.

Figure 5 | AIL-17 1B10 hypermut but not aIL-17 1B10 germline shows protective effect in EAE. Female C57BL/6 mice (n = 10) were either administered twice weekly with 100 µg aIL-17 1B10 hypermut, aIL-17 1B10 germline or aKLH isotype control antibody (BD Pharmingen) starting one day before disease induction or immunized three times with Qβ-IL-17 in intervals of two weeks starting 36 days before induction. EAE was induced on day 0 by s.c. injection of 100 µg MOG_{35-55} peptide in CFA followed by an i.p. injection of 400 ng pertussis toxin. Starting on day 7, mice were monitored daily for the development of clinical signs of disease and scores were assigned according to the degree of paralysis of tail and limbs. Shown are mean values with SEM (p<0.0001 for aIL-17 1B10 hypermut vs. aKLH and Qβ-IL-17 vs. aKLH, two-way ANOVA).
RESULTS

All combinations of somatic mutations in the V<sub>H</sub> of the 1B10 antibody except germline sequence results in high affinity for IL-17

To determine whether there is in fact an antibody affinity threshold for the protection against IL-17-induced autoimmune diseases, we aimed to establish antibodies with intermediate affinities, lying between the ones determined for the fully hypermutated and the germline 1B10 antibodies. For set purpose we designed a panel of antibodies bearing intermediate numbers and combinations of somatic mutations, stepwise mutating the V<sub>H</sub> genes of the aIL-17 1B10 hypermut back to germline sequence. Mutated V<sub>H</sub> regions were amplified, joined with the V<sub>L</sub> sequence and transferred into an expression vector to generate full-length antibodies matching the IgG2ακ isotype (aIL-17 1B10 mut 1–14). The antibodies were expressed in HEK 293T cells and supernatants were screened by ELISA for IgG expression. Antibody affinity for mIL-17 was then measured under limiting coating conditions and $K_D$ values were derived from ELISA titers measured by determining half-maximal optical density (OD). Table 1 and 2 summarize the amino acids of the 1B10 V<sub>H</sub> region targeted by somatic hypermutations and the different combinations thereof used to create the mutant antibodies as well as the deduced affinities.

Surprisingly, all fourteen mutants, aIL17 1B10 mut 1 to mut 14, bound with insignificantly lower affinity to mIL-17 than the fully hypermutated antibody did. Since their $K_D$ values deviated only by factors 0.5 to 3 from the $K_D$ of aIL-17 1B10 hypermut, no Biacore measurements were conducted.

<table>
<thead>
<tr>
<th>Location in V&lt;sub&gt;H&lt;/sub&gt;</th>
<th>aIL-17 1B10 Hypermut</th>
<th>aIL-17 1B10 Germline</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>Nomenclature</td>
<td>AA</td>
</tr>
<tr>
<td>CDR2</td>
<td>Leu</td>
<td>Ala</td>
</tr>
<tr>
<td>FR3</td>
<td>Arg</td>
<td>Ser</td>
</tr>
<tr>
<td>FR3</td>
<td>Asn</td>
<td>Ser</td>
</tr>
<tr>
<td>FR3</td>
<td>Thr</td>
<td>Ala</td>
</tr>
<tr>
<td>FR3</td>
<td>Thr</td>
<td>Ile</td>
</tr>
</tbody>
</table>
## RESULTS

Table 2 | Affinity and substituted amino acids of aIL-17 1B10 antibody mutants

<table>
<thead>
<tr>
<th>Mutations inserted</th>
<th>Nomenclature</th>
<th>$K_D$ [M]*</th>
<th>Aminoacids</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Hypermut</td>
<td>$&lt; 28 \times 10^{-12}$</td>
<td>A B C, D E</td>
</tr>
<tr>
<td>1</td>
<td>Mut 1</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>V B C, D E</td>
</tr>
<tr>
<td>1</td>
<td>Mut 2</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>A W C, D E</td>
</tr>
<tr>
<td>1</td>
<td>Mut 3</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>A B X, Y E</td>
</tr>
<tr>
<td>1</td>
<td>Mut 4</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>A B C, D Z</td>
</tr>
<tr>
<td>2</td>
<td>Mut 5</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>V B X, Y E</td>
</tr>
<tr>
<td>2</td>
<td>Mut 6</td>
<td>$\sim 28 \times 10^{-12}$</td>
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<td>Mut 7</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>A B X, Y Z</td>
</tr>
<tr>
<td>3</td>
<td>Mut 8</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>V W X, Y E</td>
</tr>
<tr>
<td>3</td>
<td>Mut 9</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>V B X, Y Z</td>
</tr>
<tr>
<td>3</td>
<td>Mut 10</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>A W X, Y Z</td>
</tr>
<tr>
<td>2</td>
<td>Mut 11</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>V W C, D E</td>
</tr>
<tr>
<td>2</td>
<td>Mut 12</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>V B C, D Z</td>
</tr>
<tr>
<td>2</td>
<td>Mut 13</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>A W C, D Z</td>
</tr>
<tr>
<td>3</td>
<td>Mut 14</td>
<td>$\sim 28 \times 10^{-12}$</td>
<td>V W C, D Z</td>
</tr>
<tr>
<td>4</td>
<td>Germline</td>
<td>$\sim 3 \times 10^{-7}$</td>
<td>V W X, Y Z</td>
</tr>
</tbody>
</table>

* $K_D$ of aIL-17 1B10 hypermut and aIL-17 1B10 germline was determined from Biacore experiments. Affinity of mutants 1 – 14 is was measured by limiting coating ELISA and directly compared to aIL-17 1B10 hypermut.
4.2.4 DISCUSSION

In this study we attempted a systematic comparison of antibodies specific for a single epitope but differing in affinity to investigate the influence of antibody affinity on clinical efficacy. We immunized mice with our established VLP-based vaccine Qβ-IL-17 and isolated the very high affinity antibody aIL-17 1B10, which binds to mIL-17 with a $K_D$ of $<28$ pM. Based on the general concept of affinity maturation, which proposes that germline antibodies exhibit low affinities while late response antibodies are hypermutated and thus stronger binders, we reverted the aIL-17 1B10 sequence towards germline, expecting a reduction of its affinity. We indeed derived a very low affinity germline version from the same antibody, binding IL-17 with a $K_D$ of 30 µM. While the hypermutated antibody was neutralizing in vitro and protective in vivo, the germline antibody exhibited no capacity whatsoever to block mIL-17. By establishing antibodies with intermediate numbers of mutations, we aimed to generate a set of antibodies recognizing the same epitope on mIL-17, but binding mIL-17 with intermediate affinities in between the ones detected for aIL-17 1B10 hypermut and aIL-17 1B10 germline. Using these antibodies, we intended to compare their in vivo efficacy in murine models of inflammatory autoimmune diseases including IMQ-induced skin inflammation and CIA, to directly determine the relevance of antibody affinity for clinical efficacy. Thus to find that besides aIL-17 1B10 germline, all possible combinations and degrees of point mutations in the heavy chain variable region (V$_H$) generated antibodies with almost identical affinity as the parental aIL-17 1B10 hypermut was utterly surprising, especially since all construct were sequenced and certainly comprised the desired mutations. At this point there is no explanation to this observation other than that any single mutation found in the hypermutated V$_H$ chain is sufficient to fold or assemble the antigen-binding groove of the 1B10 antibody. The next step will be to repeat the procedure for the variable light chain (V$_L$), which differs in four amino acids from the germline sequence and hence, like the V$_H$, enables us to produce antibody variants carrying different combinations and levels of hypermutations. We thus are eager to see, whether this approach will give rise to antibodies with affinities lying between µM and pM values. In this case, we will progress to investigate their protective effect in vivo.
5 GENERAL DISCUSSION

To date, autoimmune disorders, such as rheumatoid arthritis and psoriasis, are estimated to affect a significant percentage of the population and diagnosis and treatment imposes a substantial burden on healthcare costs. Understanding disease-underlying mechanisms and developing effective therapies has thus been of pivotal importance. Uncontrolled and persistent immune responses against autologous antigens together with the loss of self-tolerance and a dysbalance of immune regulatory cells are today believed to be the main causes of systemic and tissue-specific inflammatory autoimmune disorders.

Conventional therapies for autoimmune diseases have mainly relied on anti-inflammatory and immunosuppressive agents including glucocorticoids, cyclosporine A and cytostatica such as methotrexate and cyclophosphamide that constrain immune responses in general. While these medications have proven to be highly effective, the high dose and long-term treatment regimens that are required to obtain satisfying results and minimize relapse are often accompanied by severe adverse events. Not only does a potential overimmunesuppression promote opportunistic infections, but toxicity and the danger of relapse after discontinuing medication oppose the beneficial therapeutic effects (Bach 1993).

More recent approaches for the treatment of autoimmunity include the blockage of costimulatory pathways necessary for complete T cell activation. The chimeric fusion protein T-lymphocyte associated antigen 4 (CTLA-4) – Immunoglobulin (Ig) known as Abatacept for instance, directly counteracts CD28 mediated costimulation and thus inhibits T cell activation, as successfully shown in clinical trials for the treatment of RA and psoriatic arthritis. (Viglietta, Bourcier et al. 2008; Bathon, Robles et al. 2011; Mease, Genovese et al. 2011; Rosenblum, Gratz et al. 2012). However, preclinical studies revealed that this treatment seems to work best preventively in early phases of disease rather than ameliorating active disease and as the blockage is not specific to autoreactive T cells alone, it still renders patients susceptible to pathogenic infections. Other approaches aim at increasing the ratio of regulatory (T_{Reg}) to effector T lymphocytes, thereby pushing the suppression of immune responses against autologous antigens. Adoptive T_{Reg} therapy represents an ex vivo advance where host
T_{Reg} lymphocytes (CD4^{+}CD25^{+}Foxp3^{+}) are isolated, activated, expanded and then transferred back into the patient (Riley, June et al. 2009). Limiting factor to this method is the potential isolation of contaminating T cells due to the nuclear location of Foxp3 and the lack of adequate specific markers unique to T_{Reg}, thus not guaranteeing the infusion of a highly pure T_{Reg} population. Not even isolation using the cell surface marker CD25 yields more than 60-90% Foxp3^{+} T_{Reg}s. Also the unknown stability of transferred T_{Reg}s in humans may be of concern, as these cells may retain the potential to produce T_{H}17 or T_{H}1 effector cytokines, which could entail exacerbation of autoimmunity (Tang and Bluestone 2006; Voo, Wang et al. 2009; Lan, Fan et al. 2012; Rosenblum, Gratz et al. 2012). Nevertheless, adoptive T_{Reg} therapy has shown to be effective in clinical trials for the preventive treatment of acute graft-versus-host-disease (aGVHD) (Brunstein, Miller et al. 2011; Di Ianni, Falzetti et al. 2011). Another approach targeting the induction of antigen-specific T_{Reg}s relies on the clinical evidence that in allergen-specific immunotherapy (SIT), repeated allergen administration causes a strong and permanent suppression of the allergic reaction and an increase and activation of the T_{Reg} population (Mobs, Slotosch et al. 2010). However, this view has recently been challenged and allergen-specific antibodies may be of greater importance (Kundig and Bachmann 2010). In the context of autoimmunity, clinical studies investigating subcutaneous immunization with native or modified self-peptides have so far shown promising results (Muller, Monneaux et al. 2008; Sabatos-Peyton, Verhagen et al. 2010; Rosenblum, Gratz et al. 2011). Nevertheless, clinical data is still limited and repeated administration of proinflammatory autologous antigens bears the risk of enhancing autoimmune response (Rosenblum, Gratz et al. 2012).

Consequently, major efforts have been made to serve the need for more specific therapies without the risk of systemic immune suppression and improved tolerability. With increasing knowledge about the key-players causing autoimmune diseases and the technical advances made in engineering protein therapeutics, a new group of biologics including monoclonal antibodies and VLP-based vaccines have emerged as promising tools for targeting self-antigens in a wide range of diseases.
5.1 ADVANCES IN GENERATING THERAPEUTIC HIGH AFFINITY MONOCLONAL ANTIBODIES AND THEIR SUCCESS IN TREATMENT OF AUTOIMMUNE DISORDERS

Since the development of the hybridoma technology that allowed the production of murine monoclonal antibodies by Köhler and Milstein in 1975, tremendous technical advances on improving mAbs for therapeutic application in humans have been made (Kohler and Milstein 1975). Initial clinical studies using murine mAbs were setback by anti-mouse immune responses triggered in humans (Shawler, Bartholomew et al. 1985), resulting in short half-live, minimal efficacy and even toxicity (Li and Zhu 2010). However, progress in the development of recombinant DNA technologies over the past three decades enabled the engineering of antibodies bearing gradually reduced potentially immunogenic mouse components, transitioning from mouse via chimeric and humanized to fully human mAbs and antibody design has expanded its focus to improve efficacy, tissue accessibility and pharmacokinetic properties (Presta 2008; Hansel, Kropshofer et al. 2010). Emphasized by the fact that to date more than 20 mAbs are FDA approved for therapy in humans, with over 150 mAbs currently in clinical trials, monoclonal antibodies offer several advantages that explain their fast growing success as protein therapeutics. While humanization of the antibody variable regions diminished the issue of immunogenicity and long half-lives benefit infrequent administration regimens, their high antigen specificity, encoded in the variable regions of the Fab domain, enables effective target binding and neutralization (Hansel, Kropshofer et al. 2010). In contrast to this direct blockage of an antigen, other effector functions such as the destruction of target cells through antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) are mediated via the Fc region of the mAb. In the case of ADCC and ADCP, immune cells are recruited through the interaction with Fcγ receptors (FcγR), expressed on a variety of immune cells including NK cells and macrophages, or in the case of CDC, through binding to complement (Presta 2008). The type of effector function exerted depends on the antibody’s isotype and engineering of the constant domains thus offers the choice between isotypes promoting cell death such as IgG1, which is especially
desired in oncology, or isotypes less susceptible to recruiting effector cells, such as IgG4 (Hansel, Kropshofer et al. 2010). In addition, modification of a conserved glycosylation site at Asn 297 in the CH2 Fc region of IgGs, prone to interact with the neonatal Fc receptor (FcRn), can influence tissue penetration and antibody half-life (Yeung, Leabman et al. 2009). Furthermore, fine-tuning of the glycosylation pattern of the Fc domain can increase binding to FcγR and thus enhance ADCC (Nissim and Chernajovsky 2008; Jefferis 2009). Accordingly, antibody engineering has enabled and improved the targeting of a wide variety of molecules covering disease areas such as transplant rejection, autoimmune and infectious diseases as well as oncology (Hansel, Kropshofer et al. 2010).

In the context of inflammation and autoimmunity, cytokines are key regulators of immune responses and therefore represent promising targets for mAb therapy (Kopf, Bachmann et al. 2010). Indeed, blocking proinflammatory cytokines such as IL-1, IL-6 and TNF-α has been intensely studied and clinically approved mAbs such as the fully human aTNF-α Adalimumab (Humira, Abbott) or the aIL-1β Canakinumab (Ilaris, Novartis) have shown promising results in clinical studies for RA, psoriasis and Muckle-Wells Syndrome (van de Putte, Rau et al. 2003; Chakraborty, Tannenbaum et al. 2012). Another proinflammatory cytokine that gained a lot of attention in the past years is IL-17, as it is implicated with inflammatory diseases including RA, psoriasis and MS. Several IL-17 neutralizing mAbs have so far been successfully tested in animal models as well as clinical trials. Secukinumab (AIN457, Novartis) for instance, a fully human aIL-17 mAb, is the most prominent example and has recently completed Phase II clinical trials for RA, Crohn’s disease, RR-MS and psoriasis with positive outcomes and has commenced Phase III for several of these indications, while Phase II studies for ankylosing spondylitis and noninfectious uveitis are still ongoing (Hueber, Patel et al. 2010). In this manner, two Phase II studies have been communicated to demonstrate an improvement of symptoms of 75% in up to 81% of patients suffering from psoriasis (Novartis media release Oct 24 2011). The humanized aIL-17 mAb Ixekizumab (LY2439821, Eli Lilly) is being investigated for treatment of RA and psoriasis and has so far shown similar results with a reduction of chronic plaque psoriasis severity of at least 75% in more than 80% of subjects in Phase II studies (Leonardi, Matheson et al. 2012). In addition Amgen’s AMG 827, a fully human aIL-17R mAb has as well completed Phase II for RA and psoriasis, whereas Roche’s RG4934 has been cut after Phase I. Overall these results
corroborate IL-17 as promising target for the treatment of inflammatory autoimmune diseases.

While blocking cytokines holds certain limitations such as the fact that due to the heterogeneous nature of several chronic inflammatory disorders this therapy might be ineffective in certain individuals and blockage of upstream cytokines may compromise the immune system and render subjects susceptible to infections, mAb therapy in general faces particular disadvantages (Kopf, Bachmann et al. 2010). Besides the previously mentioned adverse immune reaction towards chimeric and other non-human mAbs and the inconvenience of repeated injections, production costs are a major issue. As mAbs are large multimeric molecules containing several posttranslational modifications, production in a eukaryotic system is a requirement. Taken together with the fact, that administration of a rather large amount of antibody is required to reach clinical efficacy, the resulting extensive production and sophisticated purification methods under good manufacturing practice (GMP) conditions all lead to high production costs. In addition, the large size of mAbs and the presence of the Fc region present a limiting factor for tissue and extracellular matrix penetration. Thus, if treating diseases other than hematological disorders, antibody engineering must balance optimal pharmacokinetic and effective tissue penetration and retention (Chames, Van Regenmortel et al. 2009). Especially the diffusion through solid tissues such as tumors or the penetration of the BBB is limited, with later enabling only estimated 0.1% of systemic administered mAbs to enter the CNS, a rate which may only be higher if the BBB is disrupted (Petereit and Rubbert-Roth 2009; Fontoura 2010).

Overall, cytokine-targeted monoclonal antibody research and therapy has gained us insight into the complexity of cytokine networks and their role in inflammatory and autoimmune diseases. Moreover, initial studies in humans have revealed efficacy and safety issues and by validating targets paved the way for other, more promising therapeutics that avoid the current limitations of mAbs, most notably active immunization with therapeutic virus-like particle (VLP)-based vaccines.
5.2 IMMUNODRUGS

Active vaccination against self-molecules such as cytokines is an emerging field of drug development, which has so far shown promising results in preclinical and clinical studies for the treatment of diverse malignancies such as cancer, allergy, inflammatory autoimmune diseases and chronic viral infections (Bachmann and Dyer 2004). The potency of the immunogenicity of VLPs is further emphasized by the current success of several FDA-approved therapeutics such as Gardasil (Merck and Co) and Cervarix (GlaxoSmithKline) targeting human papilloma virus (HPV) or Engerix (GlaxoSmithKline) and Recombivax HB (Merck and Co) against hepatitis B infection (Jennings and Bachmann 2009; Roldao, Mellado et al. 2010). Immunodrugs in particular are therapeutic vaccines comprised of autologous antigens, such as IL-17, covalently coupled to the surface of VLPs that are potent inducers of neutralizing autoantibody responses. VLPs themselves are viral multiprotein structures, primarily derived from RNA bacteriophages, that lack replicative capacity and hold highly immunogenic features (Chackerian 2007). By mimicking viral repetitive surface structures they are capable of eliciting strong B cell responses, incorporation of natural TLR7/9 ligands, specifically host-derived ssRNA or CpG, induces innate immunity through activation of PAMP-receptors and their particulate structures efficiently target them to APCs, where they promote antigen-specific IgG2a responses (Bachmann, Rohrer et al. 1993; Jegerlehner, Maurer et al. 2007; Jennings and Bachmann 2009).

Compared to mAbs, VLP-based vaccines offer manifold advantages. First of all, large-scale vaccine production under GMP conditions is generally inexpensive, as simple expression systems including bacteria, yeast and insect cells can be used. In addition, only doses of several hundred micrograms of vaccine need to be administered in intervals of months to years to reach clinical efficacy in humans, whereas mAb therapy requires frequent injections of doses up to >100 mg (Kopf, Bachmann et al. 2010). While immunity achieved with the vaccine has shown to be long-lived, the induction of autoantibodies still is reversible as proven in clinical studies among others for vaccines against hypertension or nicotine addiction, thereby fulfilling crucial safety requirements for the targeting of self-molecules (Maurer, Jennings et al. 2005; Ambuhl, Tissot et al. 2007; Tissot, Maurer et al. 2008).
Furthermore, vaccines induce polyclonal immune responses, which is likely to contribute to faster immune-complex clearance versus potential stabilization of a cytokine-antigen with a mAb recognizing a single epitope, all provided clinically relevant antibody amounts of sufficient high affinity are generated (Montero-Julian, Klein et al. 1995; Phelan, Orekov et al. 2008).

5.3 **THE ROLE OF ANTIBODY AFFINITY IN PROTECTION AGAINST AUTOIMMUNITY**

The improvement of mAb-affinity has gained vast interest in the field of antibody engineering. Natural recombination of gene segments and somatic hypermutation increase an antibody’s affinity during *in vivo* affinity maturation but are limited to distinct gene regions, mutagenesis hot spots and substitutions of single nucleotides (Altshuler, Serebryanaya et al. 2010). However, the development of techniques of artificial random mutagenesis such as error prone PCR and DNA-, chain- or CDR shuffling, targeted mutagenesis and selection methods such as ribosome-, mRNA-, phage- and cell-display have lead to the design of mAbs with extreme affinities, reaching equilibrium binding constants up to the femtomolar range (Hawkins, Russell et al. 1992; Stemmer 1994; Neri, Carnemolla et al. 1997; Jermutus, Honegger et al. 2001; Marks 2004; Igawa, Tsunoda et al. 2011). In the context of IL-17, a recent example is given by the *in vitro* affinity maturation of a fully human anti-IL-17 monoclonal antibody using random mutagenesis of CDR3 loops, where the substitution of seven amino acids resulted in a 30-fold increased affinity (Gerhardt, Abbott et al. 2009). These developments rely mainly on the general assumption that the higher the antibody affinity, the better the clinical efficacy and pharmacokinetic properties and the more antibody doses can be reduced, meanwhile ignoring the fact that changing the sequence of a fully human antibody might cause generation of T\(_H\) cell epitopes. Basic immunization studies using small organic groups, so called haptens, to model the binding kinetics of antibody epitopes demonstrated that somatic hypermutation resulted in antibody affinity improved up to two orders of magnitude (Eisen and Siskind 1964; Weigert, Cesari et al. 1970; Nossal 1992). The development of hybridoma technology has allowed the extension of these experiments from
complex polyclonal mixtures of antibodies to monoclonal antibodies with single pharmacokinetic properties, which soon confirmed these results and further proposed a transition over time to antibodies with increased on-rate constants (Weigert, Cesari et al. 1970; Wysocki, Manser et al. 1986; Foote and Milstein 1991; Foote and Eisen 1995). Nevertheless, it soon became apparent, that increased affinity does not always correspond to increased efficacy. Immunization studies with hen egg-white lysozyme analyzed hybridoma-derived mAbs isolated at different time points during the immune response and demonstrated that transition from early to late immune response was accompanied by an increase in the number of antibody clones available. In addition, the antibodies underwent an organizational change of specificity patterns, which did not correlate with a significant change in overall avidity, suggesting the absence of affinity maturation (Newman, Mainhart et al. 1992). A few years later, the group of Zinkernagel supported the relevance of these finding by presenting studies addressing protection against infection by a cytopathic virus, vesicular stomatitis virus (VSV), with their findings again challenging the basic concept of affinity maturation. As opposed to the significant affinity changes observed with the anti-hapten immune responses, these data revealed that even germline antibodies isolated from primary immune response could bind with high affinity, which was sufficient for protection against lethal disease and that there was no significant improvement in binding, once hypermutation has occurred, most likely due to the repetitive expression pattern of antibody determinants on viruses (Roost, Bachmann et al. 1995; Kalinke, Oxenius et al. 2000; Hangartner, Zinkernagel et al. 2006). Furthermore, it was demonstrated that in vitro neutralizing activity and avidity of the VSV mAbs correlated well with specific neutralization titers but were independent of in vivo protection. In addition a minimal avidity threshold was postulated, above which protection depended solely on a minimum serum concentration (Bachmann, Kalinke et al. 1997). Interestingly, Foote and Eisen suggested that the threshold for the affinity of antibodies generated under physiological conditions results for one by the on-rates being limited by the diffusion coefficients of the reactant molecules, which would not be influenced by somatic mutations changing the structure of the antigen-binding site (Northrup and Erickson 1992; Raman, Jemmerson et al. 1992; Foote and Eisen 1995). If in addition a limit was set on the off-rates due to fact that the interaction time with the antigen above a certain threshold does offer further advantages on downstream signalling and effector processes, this was to result in an affinity ceiling of around $10^{10} \text{ M}^{-1}$. They proposed,
that while B cells producing antibodies with affinities above this threshold do occur, they would not offer any biologically relevant advantage for antigen selection (Foote and Eisen 1995). Findings from another study investigating the affinity dependence of the B cell response later supported this hypothesis on set affinity ceiling (Batista and Neuberger 1998). Interestingly, the above mentioned studies on VSV infection, observing discrepancies between parameters defining in vitro and in vivo activities of neutralizing antibodies, attributed these differences to the distinct physicochemical properties of mouse serum and tissues and in vitro utilized buffered saline conditions (Bachmann, Kalinke et al. 1997). Similar to the theory of Foote and Eisen it was suggested that diffusion of antibodies in the blood and tissues was substantially slower in vivo than in vitro, thus setting a kinetic limit above which faster on-rates were insignificant. Based on this it could thus be postulated that the affinity ceiling for neutralizing antibodies is even lower in vivo than it is in vitro. In addition to the above discussed immunization studies, experience from clinical trials also indicates that an increased antibody affinity is not necessarily accompanied by improved efficacy and dose reduction. Therapeutically active levels of mAbs are usually in the range of 1-10 µg/mL in patients; a value that is relatively independent of the affinity of the antibodies involved. By way of example, two established anti-cytokine antibodies modulating IL-1β activity, Xoma052 (Gevokizumab, Xoma) and Ilaris (Canakinumab, Novartis), differ in affinity by orders of magnitude, as Xhoma052 binds to human IL-1β with an equilibrium binding constant (K_D) of 300 fM whereas Ilaris binds with a K_D of 35–40 pM. Nevertheless, the doses tested clinically are fairly similar. However, it should be kept in mind that these antibodies recognize different epitopes, which may blur the result. (Alten, Gram et al. 2008; Dhimolea 2010; Owyang, Issafras et al. 2011; Chakraborty, Tannenbaum et al. 2012). Indeed, a systematic comparison of the influence of antibody affinity on clinical efficacy using antibodies specific for a single epitope has never been performed and is the central topic of this thesis.

In order to address the question of relevance of anti-cytokine antibody affinity for clinical efficacy, we have generated two very high affinity antibodies for IL-17, aIL-17 2E1 and aIL-17 1B10, both exhibiting affinities in the pM range, as determined via Biacore measurements. These antibodies are derived from IgG antibodies induced by immunization of mice with our established Qβ-IL-17 vaccine and thus are reliable
representatives of the heterogeneous polyclonal antibody population induced by the vaccine. As it is generally assumed that germline encoded antibodies against proteins such as cytokines have relatively low affinities before they have accumulated point mutations during affinity maturation, we hypothesized that mutating the antibody genes towards germline would affect their affinity for IL-17.

Indeed, antibody aIL-17 1B10 exhibited a 10'000-times lower affinity with a equilibrium binding constant ($K_D$) of 30 µM if its heavy chain variable region ($V_{H}$) was mutated back to germline and was thus used to further pursue this project. We could show that the hypermutated but not the germline 1B10 antibody was capable of neutralizing mIL-17 in vitro, as determined by the assessment of the inhibition of IL-6 release from IL-17 stimulated murine fibroblast cells. In accordance, we found that only the hypermutated 1B10 antibody was able to reduce disease severity and onset in EAE, an animal model for multiple sclerosis. As in total, the germline Ig sequence differed from the hypermutated antibody in six point mutations corresponding to five amino acid changes in the $V_{H}$, we proceeded to produce mutants containing different levels and combinations of the hypermutations to produce a set of antibodies with a single specificity but varying affinities. We then aimed to use these antibodies to compare their in vivo efficacy in EAE and other murine models of autoimmunity such as psoriasis and rheumatoid arthritis to directly determine whether an affinity threshold for clinical efficacy exists. We thus generated all combinations of somatic mutations in the aIL-17 1B10 $V_{H}$ and investigated their affinity by ELISA. Surprisingly, we found that all mutants except the aIL-17 1B10 germline resulted in similar high affinity for mIL-17 as the parental antibody aIL-17 1B10 hypermut. Even though it is evident that a stepwise reduction of single somatic mutations in the amino acid sequence does not automatically predict a gradual change or loosening of the specific conformation of the antigen-binding site and correlated decrease of binding strength, it is astonishing that only the complete removal of all hypermutations in the $V_{H}$ translates into a reduction in affinity, which in addition differs to such a great extent from the affinity of the hypermutated version. To completely understand this process, crystal structures of the different antibodies in complex with IL-17 would have to be investigated. In a next step, the light chain variable region ($V_{L}$) will be mutated back to germline, even though it is often speculated that the $V_{H}$ is more important than the light chain for recognition of protein antigens. The $V_{L}$ of the aIL-17 1B10 hypermut differs in four amino acids from germline sequence and thus also
offers the possibility to construct a panel of mutant antibodies with distinct combinations of mutations variants, which will hopefully translate into distinct affinities, enabling us to proceed to test the antibodies in vivo.

Interestingly and in contrast to the aIL-17 1B10 antibody, the germline version of the aIL17 2E1 antibody, which differed from the hypermutated antibody in only three amino acids located in the $V_h$ and possessed a light chain in germline configuration, bound mIL-17 stronger than expected, with an equilibrium binding constant ($K_D$) of 0.5 nM. This result raised the question of the nature and affinity maturation-state of antibodies induced by our vaccines in general and whether this germline antibody itself might contribute to protection, similar to the germline anti-VSV antibodies found in the studies by Roost et al. In fact, in vivo neutralization assays revealed that blocking mIL-17 with the aIL-17 2E1 germline mAb significantly reduced the biological activity of this cytokine, as determined by measuring CXCL-1 and IL-6 release in sera of immunized mice stimulated with mIL-17, comparable to the neutralization capacity of its hypermutated counterpart (aIL-17 2E1 hypermut). Furthermore we then tested the two antibodies in different models of autoimmune inflammatory diseases for their protective competence. We could show, that the aIL-17 2E1 hypermut as well as the aIL-17 2E1 germline antibody reduced disease severity in an IMQ-induced skin inflammation model for psoriasis to the same level as in IL-17-deficient mice, confirming the complete neutralization of the cytokine with both our antibodies. Similarly, we demonstrated that the germline as well as the hypermutated 2E1 antibody significantly reduced disease severity and incidence in the CIA model for rheumatoid arthritis compared to mice treated with a control antibody. These results indicate that for a fraction of antibodies induced with our VLP-based vaccines, affinity maturation may result in slightly higher affinities, but that the affinity of their germline version is already sufficient for protection against disease and that hypermutation may not necessarily offer significant advantages for cytokine binding and neutralization. We will perform dose titration experiments to look further into this issue.

While the polyclonal population of antibodies induced with the Qβ-IL-17 vaccine consist of antibodies with heterogeneous affinities during early immune response, possibly ranging from nM to pM $K_D$s, we conclude that the affinity threshold for protection must be $<0.5$ nM in the animal models tested so far and that artificial improvement of mAb affinities above this threshold does not necessarily result in
better protection in mAb-therapy, but rather produces unreasonable higher costs of development and may unduly change the sequence of the Ab to introduce $T_H$ cell epitopes. Even though antibodies raised by Qβ-IL-17 vaccination apparently did not reach the artificially constructed antibody affinities of fM equilibrium binding constants, the efficacy of their polyclonal response is indisputable.

### 5.4 Conclusion

Collectively, the present study investigates the role of antibody affinity for protection against autoimmune inflammatory diseases, based on the analysis of two murine high-affinity anti-IL-17 antibodies generated with our established vaccine Qβ-IL-17 and their deduced versions in germline configuration. In the first section the antibody 2E1 is described. Its germline counterpart was found to maintain a surprisingly high affinity in the nM range and, like the hypermutated mAb, was neutralizing and protective in vivo. Our results suggest that the affinity of germline antibodies targeting cytokines might be sufficient for protection against autoimmune inflammatory diseases, thus questioning the clinical significance of the industry’s effort to enhance mAb affinity up to femtomolar levels. In addition, these results indicate that there is no immunological tolerance for IL-17 at the B cell level and spontaneous IL-17-specific antibody responses, as found in Aire-deficient patients, can be driven by an compromised $T_H$ cell tolerance. In the second part we describe the antibody 1B10, of which the germline version exhibited a very low affinity and was neither neutralizing in vitro nor protective against EAE. By introducing intermediate levels of mutations into the heavy chain, we expected to generate a panel of mAbs with intermediate affinities lying between µM, as found for the germline mAb, and pM, as resolved for the hypermutated mAb. By testing the protective capacity of these mAbs in animal models of autoimmune disorders, we aimed to define the exact affinity threshold required for in vivo protection. Surprisingly, all 14 mutants displayed as high affinities, as the hypermutated 1B10 mAb, leaving the question open whether other mAbs with additional germline-directed light chain mutations will exhibit intermediate affinities and thus enable us to determine an affinity threshold.
6 REFERENCES


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of Th2 cells and inhibition of Th1 cell-specific factors." Cell research 16(1): 3-10.


## 7 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ADCP</td>
<td>Antibody-dependent cellular phagocytosis</td>
</tr>
<tr>
<td>aGVHD</td>
<td>Acute graft-versus-host-disease</td>
</tr>
<tr>
<td>AIA</td>
<td>Antigen-induced arthritis</td>
</tr>
<tr>
<td>AID</td>
<td>Activation-induced deaminase</td>
</tr>
<tr>
<td>Aire</td>
<td>Autoimmune regulator</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>APS-I</td>
<td>Autoimmune polyendocrine syndrome type I</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activation factor</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCR</td>
<td>B lymphocyte antigen-receptor</td>
</tr>
<tr>
<td>BCSFB</td>
<td>Blood cerebrospinal fluid barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>CBAD</td>
<td>C/EBPβ-activation domain</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity-determining region</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding proteins</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CMC</td>
<td>Chronic mucocutaneous candidiasis</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine dinucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>CR</td>
<td>Constant region</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony-stimulating factor</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>ECAM</td>
<td>Endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystalline</td>
</tr>
<tr>
<td>FcεR</td>
<td>Fcε receptor</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fcγ receptor</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead winged-helix transcription factor</td>
</tr>
<tr>
<td>FR</td>
<td>Framework region</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA binding protein 3</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase 3β</td>
</tr>
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</table>
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HxKymez</td>
<td>Histone-(x) Lysine-(y) z-methylation</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IkBζ</td>
<td>Inhibitor of NF-κB-ζ</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-17</td>
<td>Interleukin-17</td>
</tr>
<tr>
<td>IL-17R</td>
<td>Interleukin-17 receptor</td>
</tr>
<tr>
<td>IMQ</td>
<td>Imiquimod</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IS</td>
<td>Immune system</td>
</tr>
<tr>
<td>iT(_{\text{Reg}})</td>
<td>Induced regulatory T cell</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LAP</td>
<td>Liver-enriched activator protein</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Leukocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LIP</td>
<td>Liver-enriched inhibitory protein</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor- κB</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NK cells</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>nTReg</td>
<td>Natural regulatory T cell</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated pattern</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PPMS</td>
<td>Primary progressive MS</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
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<tr>
<td>P.tx.</td>
<td>Pertussis Toxin</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-like helicase</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoid-related orphan receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RR-MS</td>
<td>Relapsing remitting MS</td>
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<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>SEFIR</td>
<td>SEF/IL-17R</td>
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<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
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<tr>
<td>SIT</td>
<td>Allergen-specific immunotherapy</td>
</tr>
<tr>
<td>SLC</td>
<td>Surrogate light chain</td>
</tr>
<tr>
<td>SPMS</td>
<td>Secondary progressive MS</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen presentation</td>
</tr>
<tr>
<td>TBX21</td>
<td>T-box transcription factor 21</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>$T_{FH}$</td>
<td>Follicular helper T cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>$T_H$</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TI</td>
<td>T cell independent</td>
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<td>TILL</td>
<td>TIR-like loop</td>
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<td>TIR</td>
<td>Toll/IL-1R</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
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<tr>
<td>$V_H$</td>
<td>Variable region of the Ig heavy chain</td>
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<tr>
<td>$V_L$</td>
<td>Variable region of the Ig light chain</td>
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<tr>
<td>VLA4</td>
<td>$\alpha_4\beta_5$-Integrin</td>
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<td>VLP</td>
<td>Virus like particle</td>
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<tr>
<td>VR</td>
<td>Variable region</td>
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<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
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8 ACKNOWLEDGEMENTS

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9 CURRICULUM VITAE

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