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Long term amelioration of established collagen-induced arthritis achieved with short term therapy combining anti-CD3 and anti-TNF treatments

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**Long term amelioration of established collagen-induced arthritis
achieved with short term therapy combining
anti-CD3 and anti-TNF treatments**

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biologie

par
Fabien Dépis
de
Paris (France)

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**UNIVERSITÉ
DE GENÈVE**

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**Doctorat ès sciences
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Thèse de *Monsieur Fabien DEPIS*

intitulée :

**" Long Term Amelioration of Established Collagen-induced
Arthritis Achieved with Short Term Therapy Combining
Anti-CD3 and anti-TNF Treatments "**

La Faculté des sciences, sur le préavis de Messieurs W. REITH, professeur ordinaire et codirecteur de thèse (Faculté de médecine, Département de pathologie et immunologie), I. RODRIGUEZ, professeur ordinaire et codirecteur de thèse (Département de génétique et développement), Y. DEAN docteur et codirecteur de thèse (NOVIMMUNE S.A. GENEVE, Département de recherche et expérimentation animale) et de Madame S. YOU, docteure (Induction et restauration de la tolérance immunitaire : stratégies d'immunointervention, Hôpital Necker, Enfants malades, Paris, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 30 janvier 2012

Thèse - 4401 -


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RESUME EN FRANCAIS

Le traitement des maladies auto-immunes représente un défi grandissant de santé publique. Ces maladies qui se développent sous l'influence de facteurs environnementaux et génétiques, consistent en une dérégulation de notre système de défense inné et adaptatif. Bien que l'arthrite rhumatoïde touche actuellement près d'1% de la population mondiale à l'heure actuelle il n'existe pas de traitement curatif de cette pathologie. Ainsi, afin d'obtenir des meilleurs résultats cliniques, les traitements actuels tendent à être administrés de manière plus précoce; le traitement par des thérapies anti-TNF en est d'ailleurs un exemple. Les combinaisons thérapeutiques représentent également une perspective intéressante dans le traitement des patients arthritiques.

Etant donné l'importance des cellules T dans l'initiation de l'arthrite, nous avons émis l'hypothèse qu'un traitement ciblant à la fois le TNF et les cellules T, administré rapidement après le diagnostic, devrait améliorer les bénéfices cliniques. Les études réalisées lors de cette thèse visaient à examiner l'efficacité d'un traitement combinant des thérapies anti-CD3 et anti-TNF dans un modèle expérimental d'arthrite rhumatoïde. Les résultats obtenus avec cette combinaison thérapeutique ont démontré un effet synergique, à l'instar de chaque traitement pris isolément. En effet cette synergie se traduit par le maintien d'une protection durable contre le développement de l'inflammation articulaire. .

Lors de cette étude, nous avons tout d'abord généré des anticorps monoclonaux spécifiques du CD3 et du TNF murins (anti-CD3 et anti-TNF). Par la suite, nous avons établi expérimentalement dans le modèle d'arthrite induite par le collagène chez la souris (Collagen-Induced Arthritis) les conditions nécessaires pour observer un gain thérapeutique lors d'un traitement ciblant simultanément le TNF et les cellules T. En cherchant à découvrir les

mécanismes sous-jacents, il s'est avéré que notre première hypothèse s'est révélée inexacte. En effet, nous avons exclu toute implication des cellules T régulatrices ainsi que des mécanismes impliquant le TGF- β et les interactions PD-1/PDL-1, pour finalement proposer un autre mécanisme d'action. Ainsi nous avons démontré que la combinaison de ces deux thérapies a permis d'éliminer les cellules T pathogéniques à la fois dans les organes lymphoïdes secondaires et dans les tissus enflammés. Par ailleurs ces résultats ont démontré la nécessité d'administrer de grandes quantités d'anti-CD3 pour éliminer suffisamment de cellules T pathogéniques afin d'obtenir une protection clinique sur le long terme. De plus, lorsqu'il est associé aux anti-TNFs, le traitement anti-CD3 assure la modulation d'un grand nombre de cellules T pathogéniques (demeurant silencieuses d'un point de vue fonctionnel), ainsi que leur élimination des sites inflammatoires, et ce de manière prolongée, permettant ainsi de restaurer « une balance immunitaire ».

Ce travail de thèse, a permis à la fois de mettre en lumière et de valider une nouvelle approche thérapeutique en s'appuyant sur un modèle reconnu d'arthrite rhumatoïde chez la souris, en utilisant des outils mimant les caractéristiques des anticorps actuellement utilisés en clinique chez l'homme. Ce traitement ouvre des perspectives intéressantes car des expériences préliminaires semblent indiquer que les effets thérapeutiques associés se maintiennent après disparition de la drogue dans l'organisme.

ABSTRACT

Treating autoimmune disorders is an escalating medical and social challenge in our community. Due to dysregulation of the innate and acquired immune systems, autoimmune diseases develop under the pressure of combined environmental and genetic influences. As about 1% of the world's population is afflicted by rheumatoid arthritis (RA), there is an urgent need for better therapies which originate from conducting relevant experiments in the laboratory before translating into clinical trials. Currently, there is no known cure for RA. However, early medical intervention has been shown to be important in improving outcomes with drugs such as anti-TNFs.

As T cells are also thought to play an important role in the initiation of RA, we hypothesized that early targeting of both TNF and T cells would result in better outcomes. The aim of the studies carried out in this thesis, therefore, was to examine the efficacy of combining anti-CD3 and anti-TNF therapies in experimental RA. Indeed, the results of the experiments demonstrate that a synergy can occur when using these treatments together, providing a longer term relief from inflammation and disease progression than of either monotherapy.

In order to carry out these experiments with the best chance of succeeding eventually in patients, relevant surrogate reagents were first generated and rigorously characterized. Next, the conditions were established using murine collagen induced arthritis (CIA) in which a therapeutic benefit was observed when targeting in parallel TNF and T cells. With this promising result, the mechanism underlying the effect was dissected and shown to be not related to the first hypotheses, that of inducing T regulatory cells or influencing through mechanisms involving TGF- β nor PD-1/PDL-1. In our experimental setting, the two therapy approach in the murine model had a significant effect on altering the pathogenic T cell

populations in the peripheral secondary lymphoid tissue and joints, immediately as well as in the longer term effect. The data leads us to conclude that high amounts of anti-CD3 are required to deplete enough pathogenic T cells to achieve clinical protection. Furthermore, in combination with the anti-TNF effect, anti-CD3 treatment ensures that the maximum of pathogenic cells are modulated (silenced), depleted from relevant environments and potentially in the long term, permits the system to reset.

Taken together, these results, generated using the gold standard model for RA and well characterized, relevant surrogates of currently used therapeutic antibodies, reveal and validate a new approach to achieve clinical remission of arthritis with a positive therapeutic outcomes that remains even in the absence of drug.

ABBREVIATIONS

Aa	Amino acid
ADCC	Antibody-dependent cell-mediated cytotoxicity
AICD	Antibody-induced cell death
APC	Antigen-presenting cell
A-SLC	A-synovial lining cell
APCA	Anti-citrullinated protein antibodies
BSA	Bovine serum albumin
B-SLC	B-synovial lining cell
bp	base pairs
CD	Cluster differentiation
CDC	Complement-dependent cytotoxicity
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund adjuvant
CH	Constant heavy chain
Da	Dalton
DC	Dendritic cell
dLN	Draining lymph node
DMARD	Disease-modifying anti-rheumatic drug
DNA	deoxyribonucleic acid
EAE	Experimental encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas Associated protein with Death Domain
FcγR	Cristallisable fragment gamma Receptor

Foxp3	Forkhead box P3
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GPI	glucose-phosphate isomerase
GWAS	Genome wide association study
HLA	Human leukocyte antigen
HAMA	Human anti-mouse antibodies
IBD	Inflammatory bowel disease
ICAM	Inter-cellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IL-1Ra	IL-1 receptor antagonist
iTreg	Induced-regulatory T cell
kDa	Kilodalton
LB	Lymphocyte B
LFA-1	Leukocyte Function-Associated Antigen-1
LN	Lymph node
LT	Lymphocyte T
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex

MMPs	Matrix metalloproteinases
mRNA	messenger ribonucleic acid
NK	Natural killer
NOD	Non-obese diabetic
nTReg	Natural regulatory T cell
PBS	Phosphate buffered saline
PD	Pharmacodynamic
PD-1	Programmed death-1
PDL-1	Programmed death-ligand-1
PK	Pharmacokinetic
PolyA	Polyadenylation site
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor (NF)- κ B ligand
RIP-1	Receptor interacting protein-1
RORγ-t	Retinoid-acid receptor-related orphan receptor gamma-t
SCID	Severe combined immunodeficiency disease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sIL-1Ra	Soluble IL-1 receptor antagonist
SLC	Synovial lining cell
sRANKL	Soluble receptor activator of nuclear factor (NF)- κ B ligand
sTNFR	Soluble tumor necrosis factor receptor (TNF)
T1D	Type-1 diabetes
TACE	Tumor necrosis factor (TNF)-alpha converting enzyme
TCR	T cell receptor
TGF	Tumor growth factor

Th	T helper
TNF	Tumor necrosis factor
TRADD	TNF receptor (TNFR) type 1-associated death domain (DD) protein
TRAF	TNF receptor (TNFR)-associated factor
Treg	Regulatory T cell
VH	Variable heavy chain
VL	Variable light chain

INTRODUCTION

A- RHEUMATOID ARTHRITIS (RA)

1/ PATHOGENESIS

1.1 Genetics

RA is a rheumatic disease with a large spectrum of age of onset, affecting approximately 1% of the population, progressively leading to pain, work disability, morbidity and death. The pathology reflects a chronic inflammatory, autoimmune disease affecting predominantly joints, but also to extraarticular locations including skin, eyes, heart, lung and nervous system. Older individuals and women are more prone to RA.

Association between RA and Human Leukocyte Antigen genes

Leukocyte Antigen (HLA) alleles, HLA-DR4 and HLA-DR1, were the first genetic factors to be associated with susceptibility to RA(Stastny, 1978a)(G. T. Nepom, Hansen, & Nepom, 1987)(Stastny, 1978b). The genes encoding HLA molecules are located in a region that was previously discovered for its contribution to the regulation of immune responses, the Major Histocompatibility Complex (MHC)(Benacerraf, 1981). Amongst the total genetic risk factors associated with RA, the contribution of MHC genes is evaluated at approximately 40%(C. M. Deighton, Walker, Griffiths, & Roberts, 1989)(Hasstedt, Clegg, Ingles, & Ward, 1994), placing MHC class II genes at the top of the genetic risk factors associated with RA.

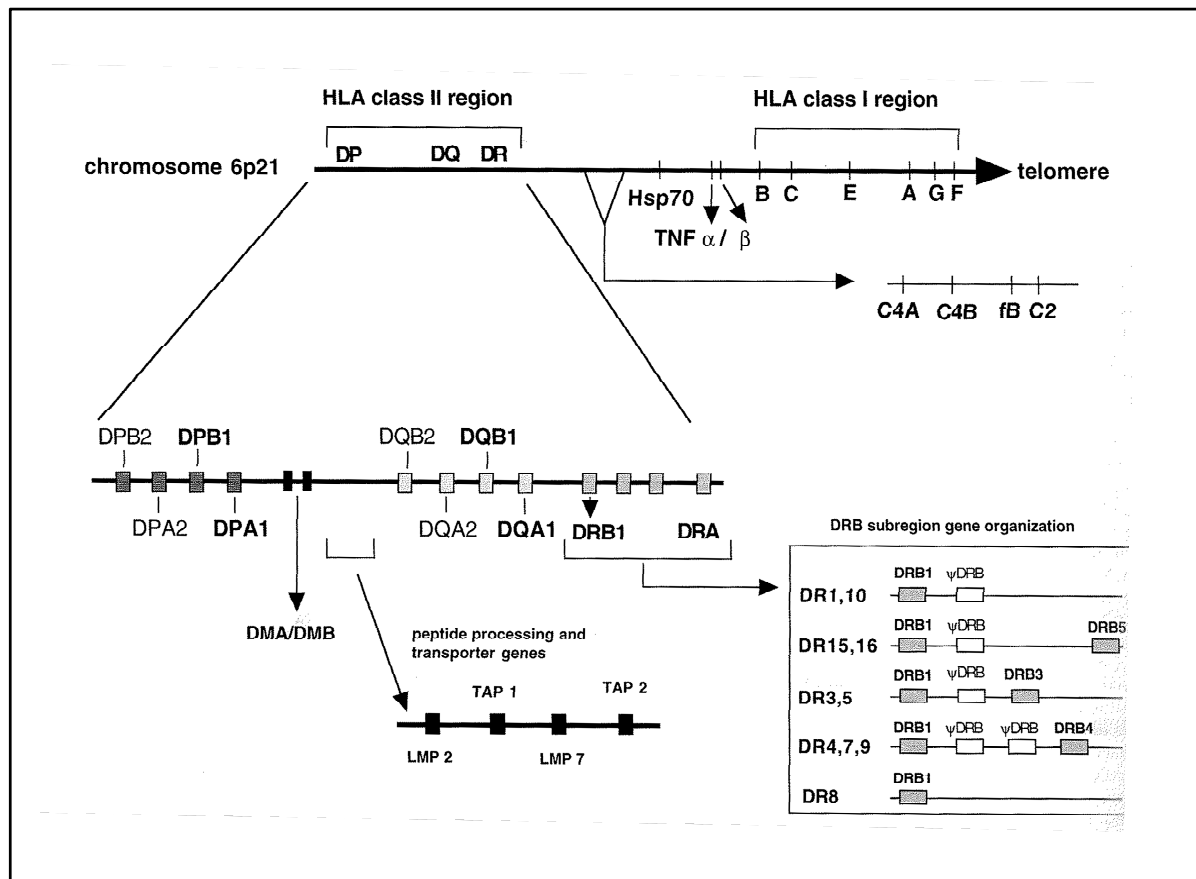


Figure 1. Illustrative representation of genes within MHC regions. Taken from (Clair, Pisetsky, & Haynes, 2004).

The MHC region is located on chromosome 6, harboring more than two hundred genes (“Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium,” 1999). In humans, the MHC region is divided into three subregions referred to as the central MHC region, the HLA class I region and the HLA class II region (Fig. 1). The genes within the class II region give rise to cell-surface class II proteins that are dedicated to present antigenic peptides to the antigen-specific receptors, i.e., the T cell receptors (TCR). MHC class II molecules are composed of alpha (α) and beta (β) chains noncovalently associated, being invariant (DRA) and highly polymorphic (DRB) chains, respectively. The HLA class II region is also divided into three subregions, DP, DQ, and DR (see Fig. 1), each of these harboring a variable number of α and β chain genes. Extensive

researches have been conducted to define which HLA alleles are firmly associated with RA. In the 1970s, Stasny and colleagues reported the association between the HLA-DR4 locus and RA(Stastny, 1978a)(Stastny, 1978b)(Stastny, 1976).

Each HLA molecule harbors a particular structure that preferentially bonds to certain anchor residues in an antigenic peptide. The genes encoding these HLA molecules are highly polymorphic and this characteristic has been maintained to provide enough diversity to cover the spectrum of potentially existing antigenic peptides. Indeed, this polymorphism affects mainly regions in the peptide binding cavity of HLA molecules and thus defines the specificity of what the immune system recognizes. In this regard, it is that some HLA alleles may be involved in autoantigen recognition, permitting development of autoimmune reactions and disease.

HLA-DRB1 association with RA: the shared epitope hypothesis

Stasny and colleagues identified several risk associated alleles in the HLA-DRB1 locus (Stastny, 1978a)(Stastny, 1978b)(Stastny, 1976), e.g, DRB1-0101, -0401, -0404, -0405, -1402, and -1001. Interestingly, in these alleles, a common cluster of amino acid sequences, are altered in the positions involved in peptide binding and presentation to the TCR. Peter Gregersen and colleagues advanced a hypothesis explaining the association between the HLA-DRB1 alleles and RA, referred to as, “the shared epitope hypothesis” (Gregersen, Silver, & Winchester, 1987). They proposed that the HLA-DRB1 alleles have a common amino acid sequence, referred to as “shared epitope”, corresponding to the hypervariable region of the HLA-DR molecule, i.e., the β chain. Furthermore, this “shared epitope” enabled the inappropriate recognition of the self antigens responsible for RA pathogenesis. Two major

explanations were then proposed to explain this shared epitope association with RA, both based on the role of HLA molecules in antigen presentation. The first hypothesis suggested that shared epitope-positive DRB1 alleles could confer an inappropriate capacity to present autoantigens to immune cells (Jane H Buckner & Nepom, 2002). This hypothesis is still difficult to demonstrate due to the lack of identification of the antigens clearly driving RA. The other hypothesis advanced a potent alteration of the thymic selection process leading to the generation of pathogenic peripheral TCR repertoire (Roudier, 2000). In addition, Roudier and colleagues proposed an alternative hypothesis explaining the association between HLA-DRB1 alleles and RA pathogenesis, based on the close proximity between these shared epitope sequences and viral antigens (Roudier, Petersen, Rhodes, Luka, & Carson, 1989). While the DRB1 locus, which is located in the MHC class II region can be one site, additional evidence suggest that other susceptibility genes may reside in other MHC regions. For example, the MHC class I region is divided into different subregions referred to as, HLA-A, HLA-B, HLA-C and others (Fig. 1). Based on HLA-A1-B8-DR3 haplotypes, it was reported that some of the susceptibility genes for RA were located in the central region of MHC (Jawaheer et al., 2002). Interestingly, this region contains genes encoding different cytokines including TNF-alpha, a major driver of RA. In this regard, some studies also reported high levels of TNF production in subjects harboring the HLA-A1-B8-DR3 haplotype (Lio et al., 2001).

Genome Wide Association Studies (GWAS)

GWAS have enrolled thousands of subjects enabling the identification of genes implicated in the susceptibility to autoimmune diseases including RA. The results from the GWAS confirmed the major contribution of MHC genes as genetic risk factor associated with susceptibility to RA (Delgado-Vega, Sánchez, Löfgren, Castillejo-López, & Alarcón-

Riquelme, 2010)(“Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls.,” 2007). The HLA-DRB1 gene was found to be the strongest genetic risk factor associated with susceptibility to RA, corroborating previous observations. In addition, since Stasny and colleagues work, GWAS studies have also contributed to provide supplementary HLA-DRB1 alleles associated with RA (Kochi, Suzuki, Yamada, & Yamamoto, n.d.). Non-MHC regions containing susceptibility genes were also identified, including the genes PTPN22, PADI4, and STAT4 (Fig. 2).

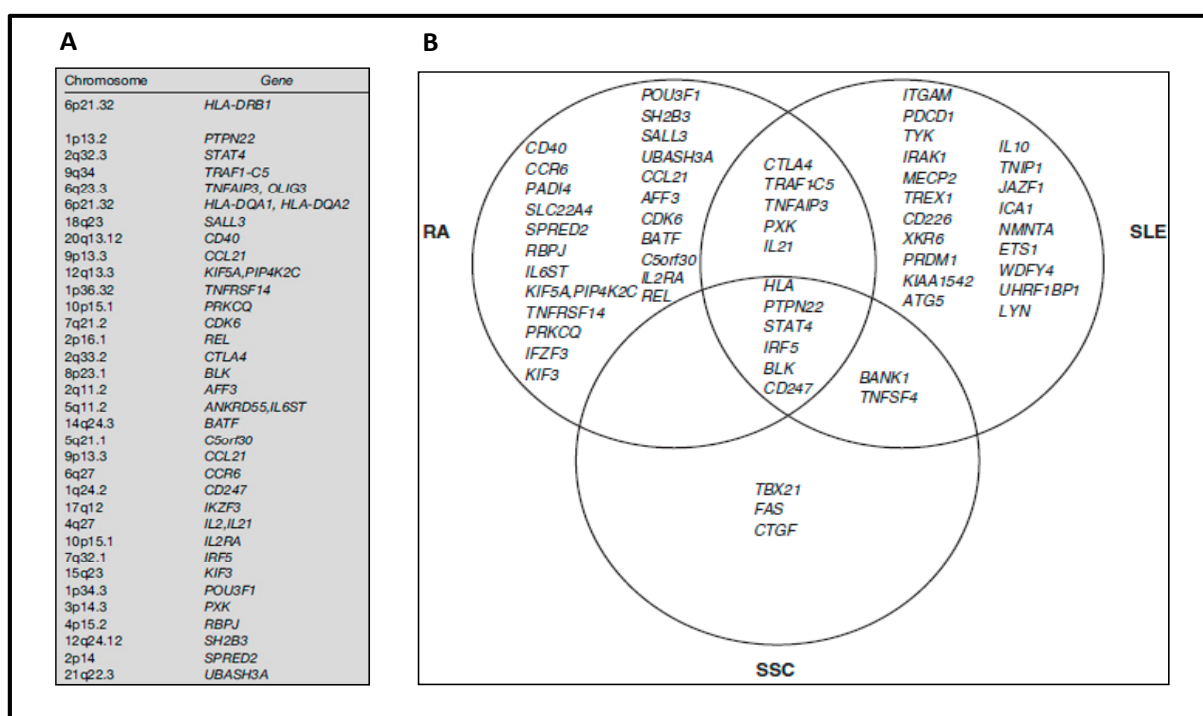


Figure 2. Susceptibility genes for autoimmune diseases including RA. Figure adapted from (Delgado-Vega et al., 2010).

Interestingly, these large scale studies provided evidence of important dissimilarities across different racial groups, suggesting that particular genetic risk factors may be restricted to subsets of patient populations. Indeed, for example, PTPN22, a phosphatase that regulates T cell activation, is only associated with RA in Europeans, while the association of PADI4 is

restricted to Asians (Delgado-Vega et al., 2010). These observations highly support the requirement of large scale population studies to cover the complex heterogeneity of the genetics underlying RA.

Difficulty to identify new gene risk factors associated with RA

The low relative risk ratio associated with RA susceptibility genes, as well as small sample sizes, make it difficult to discover new genetic risk factors. The GWAS provided a robust approach to overcome some of these limits, yet another level of complexity remains. This involves understanding of gene-gene as well as gene-environmental factor interactions. Smoking is an example of an environmental risk factor associated with susceptibility to RA, and an interaction between smoking and the HLA-DRB1 alleles indeed has been reported to increase susceptibility to RA. The current limited amount of knowledge in these interactions makes more complex the interpretations arising from genetic studies. The interactions amongst genes, with or without the impact of environmental factors, may explain the heterogeneity amongst patients. It also provides a strong rationale to pursue GWAS studies covering several groups of populations all over the world. A complete comprehensive picture of the genetics of RA will require a collaborative effort including geneticists, clinicians, statisticians, immunologists and other specialists.

As adaptive immune responses are involved in autoimmune diseases, including RA, the discovery of the specific antigens driving the disease could help to better understand RA pathogenesis and design improved therapies. Unlike some autoimmune diseases, in RA, the antigens driving the disease are not identified, which is likely to be due to the presence of a multitude of antigens with a low contribution to disease pathogenesis (Magalhães, Stiehl,

Morawietz, Berek, & Krenn, 2002) (listed in Table 1). Thus, research has mainly focused on understanding the cellular and molecular mechanisms driving the disease.

1.2 Cellular mechanisms

Pathophysiologically, RA is characterized by chronic inflammation leading to subsequent tissue destruction in multiple joints. This process can be driven by continuous exogenous triggers, i.e., bacterial or viral infections, and persistent T- and B-cell responses stimulated by an undiscovered autoantigen. The histopathological hallmarks of RA in joints are synovial hyperplasia, infiltration of inflammatory cells, e.g., neutrophils, macrophages, T cells or plasma cells, the presence of immune complexes and destruction of cartilage and bone (summarized in Fig. 3).

Normal synovium and joint morphology and architecture

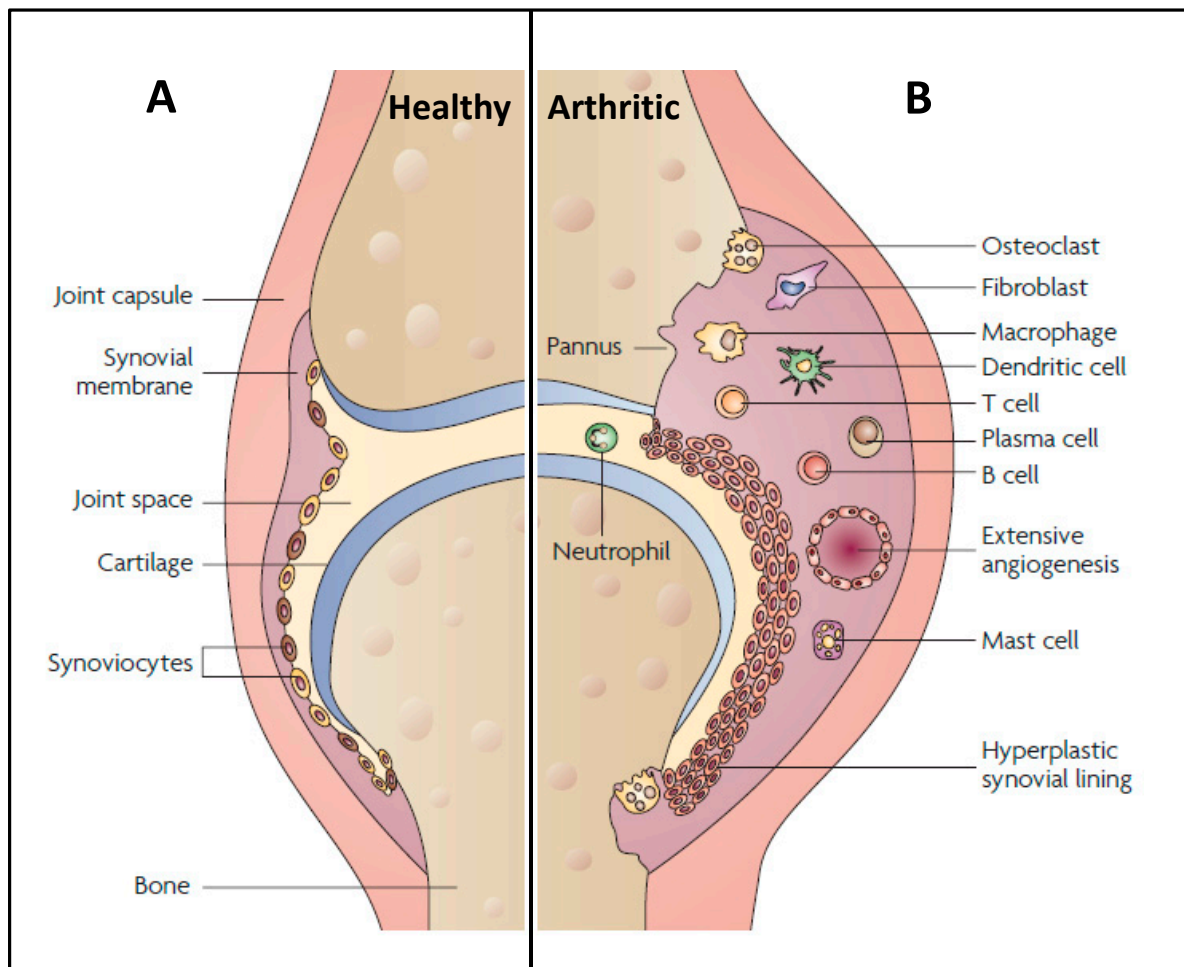


Figure 3. Schematic representation of joint morphology in healthy and arthritic conditions. This figure was adapted from (Strand, Kimberly, & Isaacs, 2007).

The synovial membrane, or synovium, is made of fibroblasts and macrophages, and lines the joint capsule, defining the intrarticular space where synovial fluid is produced and accumulates. The function of the synovium is to produce synovial fluid, cartilage, and other fibrous tissue, and permit nutrients to enter from the blood into the joint cavity. Unlike RA synovium, healthy synoviums contain thin walled synovial blood vessels that only poorly express adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1)(Hale et al., 1989).

Healthy synovium consists of a thin lining layer of one to two synovial lining cells (SLCs) (Fig. 3A). The SLCs includes two types of cells, macrophage-like type A SLC (A-SLC) that are also termed synovial macrophages, and the fibroblast-like type B SLC (B-SLC), also termed synovial fibroblasts (Burmester, Dimitriu-Bona, Waters, & Winchester, 1983). Synovial macrophages are of myeloid origin and derived from the bone marrow. They use the blood to migrate from the bone marrow to the joints. Synovial macrophages express MHC class II molecules conferring the capacity for antigen presentation. In healthy conditions, these cells clear the joint of debris and pathogens by phagocytosis, and participate in immune responses to resolve infections. However, during RA, synovial macrophages can be detrimental, promoting inflammatory responses and joint damage. In contrast, synovial fibroblasts do not have the capacity to phagocyte or present antigens. They are dedicated to producing collagen matrices and hyaluran, a lubricating proteoglycan essential for efficient articular movement.

Histopathology of the synovium in early stages of RA

Morphological features

The morphology of the synovium is altered during the inflammatory process in RA illustrated in figure 3B. In symptomatic joints, key histological features are SLC hyperplasia, vessel proliferation, edema, and infiltration of lymphocytes, i.e., mainly CD4⁺ memory T cells. In RA synovium, the SLC hyperplasia is the consequence of increased migration of synovial macrophage precursors (A-SLCs) from the bone marrow compartment, and exaggerated in situ proliferation of synovial fibroblasts (B-SLCs). As a consequence, the SLC layer enlarges dramatically and becomes more vascularized as compared to a normal synovium (FitzGerald, Soden, Yanni, Robinson, & Bresnihan, 1991). In addition, inflamed synovial tissues display higher expression levels of adhesion molecules than those expressed in normal synovium (Patel & Haynes, 2001) (Liao & Haynes, 1995). For example, ICAM-1 is up-regulated on the endothelium of RA synovial vessels, and this adhesion molecule is known to promote leukocyte extravasation by interacting with its ligand lymphocyte function-associated antigen-1 (LFA-1) expressed on leukocytes. These interactions promote B and T cell infiltration to the inflamed synovium. Other immune cells also infiltrate the synovium such as dendritic cells, plasma cells, and mast cells. In contrast, RA synovial fluid is infiltrated mainly by neutrophils and to a lesser extent by monocytes, T cells and dendritic cells. The process of SLC hyperplasia of the synovium results in an invasive structure referred to as pannus. Ultimately, in later stages, this chronic proinflammatory environment leads to degradation of articular cartilage and periarticular bone.

Synovial macrophages and fibroblasts

In RA synovium, macrophages substantially contribute to the local inflammatory process by stimulating pathogenic T cells, and secreting key proinflammatory cytokines including TNF, IL-1, and IL-6(Cooles & Isaacs, 2011). Synovial fibroblasts are also key cellular players activated, in a non-antigenic fashion, through stimulation by viral or bacterial triggers. Being highly proliferative, these cells are predominant in hyperplastic synovium(Müller-Ladner, Pap, Gay, Neidhart, & Gay, 2005). Synovial fibroblasts participate in the process of erosion by releasing degradative enzymes including collagenase and matrix metalloproteinases (MMPs). In addition, through their migratory capacity, activated synovial fibroblasts from RA synovium are able to invade unaffected joints as shown in a transfer model of arthritis using severe combined immunodeficient mice (SCID)(Lefèvre et al., 2009). These findings suggest that in RA, synovial fibroblasts are able to spread arthritis to previously healthy joints, thus, promoting the evolution from monoarticular to polyarticular forms of arthritis.

T cells, B-cells and citrulinated-antigens

Both their infiltration into inflamed synovia, and the genetic association between HLA class II molecules and susceptibility to RA, highlight the implication of T cells in RA pathogenesis. In the RA synovium there is a preponderant infiltration of oligoclonal CD4+CD28- T cells and CD45RO+ memory T cells(Namekawa et al., 2000)(Namekawa, Wagner, Goronzy, & Weyand, 1998), suggesting the existence of antigen-derived-T cell activation. Although, the precise antigen involved in RA remains elusive, several candidates in addition to collagen have been proposed. Table 1 provides a list of current autoantigens discussed in the field,

including HLA-derived peptides, proteoglycans, immunoglobulins and glucose-6-phosphate isomerase (GPI) (Matsumoto, Staub, Benoist, & Mathis, 1999).

Autoantigens defined by serum and B-cell analysis in RA and animal models	
Type/Nature	Specificity
Self antigen	Citrulline-containing peptides
	Keratin
	Perinuclear factor
	Savoy antigen
	Filaggrin
	Human leukocyte antigen
	Calpastatin
	Immunoglobulin (rheumatoid factor)
	Calreticulin
	Antineutrophil cytoplasmic antibody
	Antinuclear antibody
	Immunoglobulin heavy-chain binding protein/p68
	Heteronuclear ribonucleoprotein A2 (RA33)
	Glucose-6-phosphate isomerase
	Collagen type II
Cartilage (organ specific)	Chondrocyte antigen 65
	Large aggregating chondroitin sulfate proteoglycan (aggrecan)
	Human chondrocyte glycoprotein 39
	Cartilage oligomeric matrix protein
Non-self antigens	Bacterial heat shock protein

Table 1. List of autoantigens involved in RA and animal models. This Table was adapted from (Magalhães et al., 2002).

A few B cells are also found in RA synovium, potentially contributing to the activation of synovial T cells, as suggested by the protective effects afforded by B-cell depleting therapies in RA (Takemura, Klimiuk, Braun, Goronzy, & Weyand, 2001). The increased numbers of B

cells within RA synovium may result from B cells matured in the periphery, e.g., lymph nodes or spleen. Alternatively, the expanded synovial B cell population could also originate from ectopic germinal centers present in the synovium of approximately 20% of RA patients, referred to as synovial germinal centers (Takemura, Braun, et al., 2001) (Magalhães et al., 2002). Interestingly, as compared to germinal centers, synovial germinal centers contain higher numbers of T cells. Another characteristic is that CD8⁺ T cells accumulate in the mantle of synovial germinal centers and appear to contribute to their formation (Kang et al., 2002).

B cells also contribute to RA pathogenesis through the generation of autoantibodies, including antibodies specific to autoantigens, citrullinated-antigens, and other antibodies (i.e., rheumatoid factors). These autoantibodies mediate their pathogenic effect through immune complex-mediated mechanisms, e.g., antibody-dependent cell cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC).

Interestingly, evidence suggests that a posttranslational modification affecting autoantigenic peptides in RA could enhance their immunogenicity, i.e., the citrullination of proteins (Cooles & Isaacs, 2011). Citrullination is a posttranslational modification transforming the positively charged amino acid arginine into a neutral amino acid citrulline, i.e., citrulline being not directly encoded by DNA but resulting from citrullination of arginine. The measurement of autoantibodies directed against cyclic citrullinated peptide (anti-CCP) has demonstrated the association between anti-citrullinated protein/peptide antibodies (ACPAs) and RA. Indeed, the results of such studies conducted with RA synovial fluids reveals significantly higher titers of diverse citrullinated proteins as well as their corresponding specific antibodies compared to those of non-arthritic subjects (Snir et al., 2010).

Other recent findings have further supported “the shared epitope hypothesis” by demonstrating that some citrullinated peptides derived from autoantigens, e.g., vimentin, or fibrinogen, are recognized by HLA-DRB1-1001 molecules (James et al., 2010), i.e., molecules encoded by HLA-DRB1 alleles involved in “the shared epitope hypothesis”. Indeed, the antigen binding pocket of HLA-DRB1-1001 accommodates the citrullinated-autoantigenic peptides and not the original peptides harboring arginine residues. In addition, it has been reported that association of smoking with HLA-DRB1 'shared epitope' alleles is an example of gene-environment interaction, where smoking predisposes to generation of ACPAs, thus increasing susceptibility to RA (Mahdi et al., 2009).

Bone and cartilage erosion in RA

The bone remodeling process: bone formation versus resorption

Bone erosion is a characteristic feature of late stage RA. This process, which is mediated by osteoclasts, occurs in two sites (Pettit et al., 2001), i.e., the cartilage-pannus interface and the interface of invading synovial membrane with subchondral bone (S R Goldring & Gravallesse, 2000). In physiological conditions, bone structures undergo permanent remodeling processes, including both regeneration and degradation. Osteoblasts and osteoclasts are multinucleated cells of the myeloid lineage dedicated to bone-formation and bone-resorption, respectively (Boyce & Xing, 2007). In RA, the balance between normal bone resorption and remodeling is dysregulated, promoting osteoclastogenesis and leading to exaggerated bone resorption with subsequent joint destruction (Phillips, Aliprantis, & Coblyn, 2006).

Differences in bone resorption between healthy and RA conditions

Although bone resorption is necessary for bone homeostasis, this process is also responsible for bone erosion in RA. Interestingly, some differences distinguish the bone resorption process occurring in physiological conditions from that during RA. First, the source of receptor activator of nuclear factor-kappaB ligand (RANKL) that promotes osteoclast differentiation is of a different origin. In healthy conditions, osteoblasts represent the classical source of RANKL but in RA conditions, the major source of RANKL comes from synovial T cells and synovial fibroblasts(Gravallese et al., 2000). More importantly, during a physiological cycle of bone remodeling, the bone resorption ensured by osteoclasts is followed by a bone formation step that is absent in RA conditions(Steven R Goldring, 2002). This deregulation probably results from the aberrant production of factors promoting osteoclast differentiation. Inversely, another contribution could be an exaggerated synthesis of inhibitors of osteoblast differentiation.

Source of osteoclast inducing factors

The source of osteoclast differentiation factors originate from diverse cellular types. Activated synovial T cells directly promote osteoclast differentiation and activation by producing RANKL, as well as IL-17 and other osteoclast differentiation factors(S R Goldring & Gravallese, 2000). In addition, synovial T cells promote osteoclast differentiation indirectly by stimulating synovial fibroblasts and synovial macrophages to produce IL-1 and TNF, also leading to osteoclast differentiation and activation. Collectively, synovial T cells contribute to the process of bone destruction in advanced RA by inducing osteoclastogenesis directly or indirectly with subsequent activation of osteoclasts.

In advanced RA, the hyperplastic SLC layer also contributes to the process of bone erosion. Indeed, the resulting structure referred to as a pannus contains degradative enzymes, e.g., collagenase, matrix metalloproteinase (MMPs), and also proinflammatory cytokines, e.g., TNF, IL-1 α , IL-1 β , or IL-6, contributing to bone degradation(S R Goldring & Gravallesse, 2000). Thus, synoviocytes directly contribute to the process of cartilage degradation by secreting these degradative enzymes(Steven R Goldring, 2002).

SLC differentiation into osteoclasts

Another characteristic feature of bone resorption during RA comes from the origin of osteoclasts. In physiological conditions, osteoclasts derive from the bone marrow, but in RA, osteoclasts can also result from in situ transformation of synovial macrophages under the influence of the synovial proinflammatory milieu(S R Goldring & Gravallesse, 2000). In pathological settings, this additional source of osteoclasts favors the deregulation of bone homeostasis by promoting the bone resorption process, again ultimately leading to joint destruction.

1.3 Molecular mechanisms

At the molecular level, cytokines are the key players involved in RA pathogenesis. Cytokines are glycoproteins that mediate local intercellular communications, are crucial in many biological responses, i.e., cell survival, activation, proliferation, and death, leading to inflammation, and immunity. The cytokine family is divided into several subfamilies depending on their sequence, tridimensional structure and source (interleukins, lymphokines, monokines, interferons, and chemokines). Cytokines exert their biological function through binding to and subsequent activation of their receptors expressed at the cell surface. Cytokines

are produced by most cell types. Cytokines can be present as monomers as well as homodimers (IFN- γ , IL-10, or TGF- β), or even as homotrimers, such as TNF. In addition, different cytokines can associate to form heterodimers, e.g., IL-12-23, or LT- α/β . Most cytokines are secreted but some exist in membrane-bound forms (e.g., TNF and TGF- β). Interestingly, these membrane-bound forms of cytokines still conserve their capacity to signal through their receptor. Cytokines are involved in immune responses, and are pivotal in immune regulation. Indeed, they are referred to as proinflammatory or anti-inflammatory cytokines dependent on their capacity to promote or resolve inflammatory processes, respectively. In addition, some cytokines, referred to as chemokines, can also control the migration of cells via their chemotactic function. Collectively, cytokines harbor multiple functions, orchestrating physiological immune responses. However, the deregulation of the complex cytokine network can also have detrimental effect leading to autoimmune or inflammatory diseases.

Cytokines in leukocyte migration

In RA, peripheral leukocytes infiltrate synovium during the inflammatory process. To infiltrate, leukocytes undergo successive steps that are regulated by cytokines, i.e., adhesion, extravasation, and migration. The infiltration of leukocytes into tissues also results from chemoattractant gradients formed by chemokines, conditioning their migratory route. Cytokines also regulate the expression of adhesion molecules involved in the adhesion of leukocytes to the endothelium, required for their subsequent extravasation. For example, GM-CSF which is produced by several cell types including macrophages and T cells, promotes the migration of monocytes and neutrophils by up-regulating expression of the adhesion molecules CD11 and CD18. The phenomenon of extravasation is tightly regulated by a

complex balance of signaling. For instance, the proinflammatory cytokines TNF, IL-1, or IFN- γ positively promote extravasation, and, inversely, IL-4 and IL-10 inhibit this process.

Cytokines in RA: a complex cytokine network orchestrated by TNF

Initially, because of its accessibility, investigations of the cytokines involved in RA were conducted with synovial fluids. In this compartment, both IL-1 α and TNF are the predominant cytokines. Subsequently, these cytokines were detected in the synovium or synovial membrane of RA patients, from which tissue samples are more relevant but difficult to obtain (Baeten et al., 2000) (M Feldmann, Brennan, & Maini, 1996). Again, IL-1 α and TNF were also the predominantly expressed cytokines from these inflammatory sites.

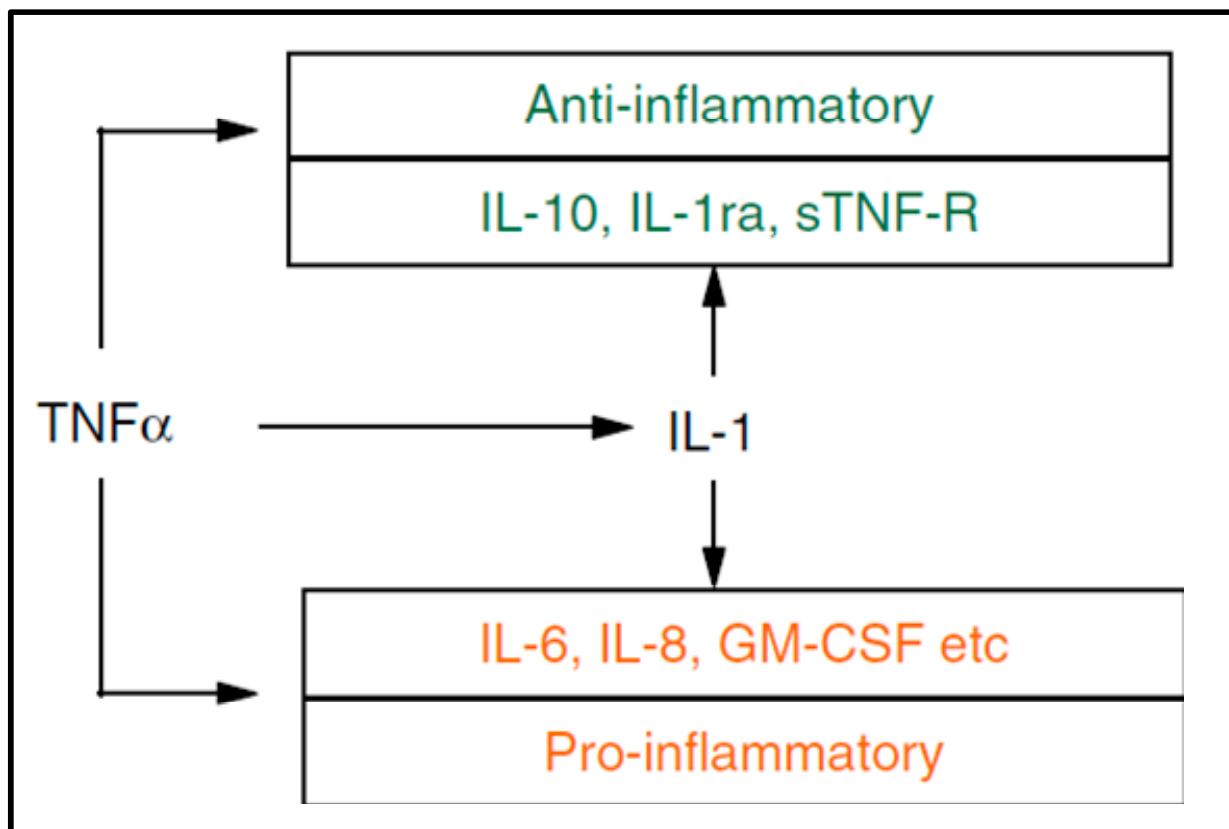


Figure 4. TNF-dependent cytokine cascade in RA. This figure was taken from (Marc Feldmann & Maini, 2008).

From clinical experience, a special focus was initially placed on IL-1 because of its importance in cartilage and bone degradation. However, the result from human TNF blockade studies revealed that TNF was the master regulator of the proinflammatory cytokine network driving inflammation in RA. Figure 4 illustrates the TNF-dependent cytokine cascade in RA. Indeed, with the use of neutralizing anti-TNF monoclonal antibodies (mAbs), it was observed that TNF was the major inducer of IL-1 in synovium (F M Brennan, Chantry, Jackson, Maini, & Feldmann, 1989). This finding was surprising as many other cytokines were known to also induce IL-1 (IL-1 itself, INF- γ , or GM-CSF). In addition, other proinflammatory cytokines, (IL-6, GM-CSF, or IL-8), as well as anti-inflammatory cytokines, (IL-10, and IL-4), were found to be under the control of TNF, as they were concomitantly reduced with use of an anti-TNF mAb (M Feldmann et al., 1996).

IL-6 is a multifunctional cytokine involved in immune regulation and inflammation. This cytokine is pathologically involved in RA (Mima & Nishimoto, 2009). IL-6 is known to play an important role during the physiological process of antibody generation, but in RA this function becomes detrimental regarding the contribution of autoantibodies in disease pathogenesis. IL-6 can also promote mechanisms contributing to inflammation, e.g., angiogenesis and vascular permeability, through the induction of vascular endothelial growth factor (VEGF). In addition, IL-6 is one of the osteoclast differentiation factors that induce exaggerated bone resorption with subsequent joint degradation.

Another cytokine important in RA pathogenesis is GM-CSF (Cornish, Campbell, McKenzie, Chatfield, & Wicks, 2009). GM-CSF is a growth factor that stimulates the differentiation of stem cells into granulocytes and macrophages, cells involved in RA pathogenesis. It activates macrophages, and up-regulates MHC class expression on RA synovial cells. In addition, GM-

CSF also promotes infiltration of inflammatory cells such as monocytes and neutrophils into the joints during RA through the induction of adhesion molecules.

More recently, a special emphasis has been put on IL-17 and its role in RA pathogenesis (Wim B van den Berg & Miossec, 2009). The IL-17 family is composed of six cytokines (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F). IL-17A and IL-17-F are the only ones that signal through the same receptor, with IL-17A being more potent. Several lines of evidence demonstrate the importance of IL-17A, also referred to as IL-17. In RA, IL-17 can be found in arthritic joints, it synergistically interacts with the proinflammatory cytokine network, including TNF, to exacerbate inflammation, and its neutralization or genetic deletion in animal models of RA alleviates disease severity. In addition, IL-17 contributes to synovial infiltration of immune cells by up-regulating the chemoattractant C-C motif chemokine ligand 20 (CCL20) in synoviocytes, in turn, attracting cells expressing the corresponding chemokine receptor CCR6 such as immature dendritic cells and Th17 cells.

Anti-inflammatory cytokines, e.g. IL-4, and IL-10, are involved in homeostatic regulation contributing to the resolution of the inflammatory process before collateral damage occurs. These cytokines counteract the excessive effect of proinflammatory signals during sustained inflammation. In RA, as in other immune disorders, it is thought that an imbalance between the production of proinflammatory and anti-inflammatory cytokines contributes to disease initiation and maintenance. For example, IL-10 and IL-4, i.e, anti-inflammatory cytokines, can inhibit the production of both TNF and IL-1 in RA synovial tissue culture, when added exogenously (Chomarat et al., 1995). In addition, IL-10 can induce the production of soluble TNF receptors (sTNF-R), i.e, endogenous inhibitors of TNF, and down-regulate expression of the TNF receptor (TNF-R)(Joyce et al., 1994). Collectively, these examples suggest that anti-inflammatory cytokines can potentially control the inflammatory process during RA

pathogenesis. However, it appears that such immunoregulation is insufficient to control ongoing inflammatory processes in the patient.

The expression of several cytokine receptors was found in synovium obtained from RA patients. These include receptors specific for TNF (TNFRs), IL-1 (IL-1R), and IL-6 (IL-6R)(M Feldmann et al., 1996). Interestingly, the presence of soluble forms of these cytokine receptors were also detected, e.g., (sTNFR) and (IL-1Ra), which could provide additional inhibitory mechanisms involved in the immunoregulation taking place within RA synovium. Finally, complement factors were also detected in the synovium of RA patients, providing evidence that the complement cascade is activated locally(Neumann et al., 2002).

2/ THE MOUSE COLLAGEN-INDUCED ARTHRITIS (CIA) MODEL

Rationale for the use of animal models- advantages of animal model use

Our understanding of the pathogenesis of RA came in part from the use of animal models of arthritis(Moudgil, Kim, & Brahn, 2011). As discussed before, the pathogenesis of RA is multifactorial, involving complex networks of cellular and molecular immune processes. The direct dissection of the immunological mechanisms involved in the different phases of human RA, i.e, induction, maintenance, or remission of the arthritic process, is rendered difficult for both ethical and practical reasons. For example, in RA patients, homogeneous sample

collections are difficult to obtain as most subjects have received diverse treatments prior to biopsy, potentially influencing the disease course in different ways. As it is also difficult to precisely determine the onset of RA for each individual subject, the samples collected reflect different stages of the disease process, making more laborious the biological characterization of successive steps involved during arthritis development. In addition, most of the time interpretation can be challenging as there is an absence of relevant control tissue. Therefore, to aid in studying RA, animal models provide several advantages to study mechanisms behind its pathogenesis. Time courses of disease can be established, the access to relevant tissue is facilitated, and the existence of a large panel of transgenic mice as well as different models of arthritis enable us to ask a wide spectrum of relevant questions.

Development of the CIA model

Models can be divided into spontaneously arising or experimentally-induced forms of arthritis. Amongst the different choices, the experimentally-induced model of collagen-induced arthritis (CIA) in mice is the most widely used. Initially, CIA was found accidentally in 1977 by Trentham and colleagues, after immunization of rats with type II collagen (Trentham, Townes, & Kang, 1977). The immune responses were induced specifically by injection of exogenous collagen, which lead to cross-reactive responses directed against collagen of the host, resulting in an autoimmune disease process similar to that in RA. These initial observations have been translated into inducing a RA-like disease in mice as well as non human primates, the latter being helpful for lead drug candidate testing prior to entry into clinical trials.

Manifestation of arthritis in mouse model of CIA

There are two models of murine CIA, the homologous and the heterologous, driven by an initial immunization by self or foreign type II collagen, respectively. The heterologous mouse model of CIA is associated with a more acute, yet severe, and self-remitting form of arthritis. Both are commonly induced in DBA/1 mice that are known to harbor the MHC type H2-A^q, the mouse homologue of human HLA-DQ. Thus, these CIA models in DBA mice share a common genetic disease-susceptibility association of RA with MHC class II molecules, allowing the contribution of CD4⁺ T cells in the pathogenesis to be studied. Although, these animal models of RA are not the exact mirror of the human disease, they do present with most of the key immunological and pathological features e.g., severe polyarticular arthritis. Indeed, arthritic CIA mice are subject to an acute articular inflammation process associated with the characteristic hyperplasia and the formation of a pannus. As in humans, neutrophils infiltrate the synovial fluid, while macrophages, T and B cells mainly infiltrate the synovium. Initiated by T and B cell immunity, a proinflammatory network locally governed by TNF leads to, in the advanced stage, bone and cartilage erosion with subsequent joint destruction. In the CIA model, arthritis terminates with limited inflammation and sustained ankylosis. The pathogenesis of CIA also involves innate immunity and nonspecific stimuli, e.g., lipopolysaccharide, IL-1, or TNF can be used to synchronize the onset of CIA (L. A. Joosten, Helsen, & van den Berg, 1994). Collectively, in addition to the pathological and histopathological features shared by both RA and CIA, the presence of T cells infiltrating affected synovium and the genetic association between arthritis and MHC class II molecule, emphasize the contribution of T cell responses in both human RA and its mouse model.

Cytokines in CIA

CIA has been used to study pathogenic mechanisms as well as potential new therapies (R O Williams, 1998). In this regard, the generation of therapeutic mAbs specific to cytokines and their subsequent in vivo use in CIA contributed significantly to investigation of the role of cytokines in RA pathogenesis. In addition, in vivo administration of recombinant cytokines, and the use of mice genetically deficient for specific cytokines or cytokine receptors, have also substantially contributed to our understanding.

Kinetics of proinflammatory cytokines in CIA

During CIA, the results of blocking TNF or IL-1 before onset, at onset, and during the established phase, enabled the characterization of their relative contribution to different stages of the disease (L. A. Joosten, Helsen, van de Loo, & van den Berg, 1996) (W B van den Berg, 2001). TNF appears to be more predominant in early arthritis, inducing many proinflammatory cytokines including IL-1, and driving both inflammation and joint destruction. By contrast, IL-1, although present, is less important in the early phase yet plays a dominant role in advanced arthritis, driving cartilage and bone destruction. In CIA, the in vivo blockade of IL-1 with mAbs specific to both IL-1 α and β was also reported to inhibit cartilage destruction and inflammation, demonstrating that IL-1 can be another key player in established arthritis (W B van den Berg, Joosten, Helsen, & van de Loo, 1994). Additional kinetic studies, based on studying the synovium from CIA, confirmed the expression of TNF and IL-1 in early arthritis, but also demonstrated the presence of IFN- γ , TGF- β and IL-6 (W B van den Berg, 2001) (Müssener, Litton, Lindroos, & Klareskog, 1997). A more precise description of TNF's contribution in CIA will be discussed later.

The contribution of IL-6 in established CIA appears to be less remarkable than that of TNF. In CIA, two major lines of evidence support the contribution of IL-6 in disease pathogenesis. First, mice deficient for IL-6 are completely protected from developing CIA(Alonzi et al., 1998). Second, the use of neutralizing mAbs specific to IL-6 significantly delays the onset of CIA(Takagi et al., 1998). Although, in this model, IL-6 neutralization delays disease onset when administered prophylactically, the reduction in disease severity when assessing established arthritis is much less as compared to that of TNF blockade. These results suggest that IL-6 is downstream of TNF in the cytokine network driving established arthritis. IL-17 is a cytokine with proinflammatory function mainly produced by Th17 cells(Korn, Bettelli, Oukka, & Kuchroo, 2009). IL-17 has been observed in RA synovium, and thus has been investigated for its contribution in disease pathogenesis. In CIA, its neutralization with specific mAbs leads to reduced incidence and disease severity(Lubberts et al., 2004). The experiments provide evidence of a contribution in both early and later stage of the disease. Traditionally, it is thought that the involvement of T cells in arthritis pathogenesis is restricted to the induction phase of the disease. In contrast, the involvement of IL-17 in established arthritis highly supports a contribution of T cells in this later phase of the disease as well.

IFN- γ is a proinflammatory cytokine known to promote strong responses to certain types of pathogens (M Feldmann et al., 1996). For example, IFN- γ influences adaptive immune responses by up-regulating MHC class II expression and thus, the process of antigen presentation. In murine CIA, local injections of IFN- γ were associated with accelerated arthritis and increased severity(Mauritz et al., 1988). However, IFN- γ can also be associated with anti-inflammatory activity. Indeed, this aspect of its contribution in arthritis has emerged from studying CIA in mice that are deficient for IFN- γ (C.-Q. Chu, Swart, Alcorn, Tocker, & Elkon, 2007). These mice have increased IL-17 responses resulting in more severe arthritis

than their wild-type counterparts. This finding also suggests an immunoregulatory role of IL-17 by IFN- γ in arthritic conditions.

Anti-inflammatory cytokines in murine CIA

The cytokine network involved in murine CIA is also composed of anti-inflammatory cytokines, e.g, IL-4, IL-10, IFN- β , or TGF- β , aimed at counter-regulating the pathogenic effects of the pro-inflammatory cytokines. Continuous administration of IL-4 in murine CIA delays the onset of arthritis and slightly reduces disease severity(Horsfall et al., 1997). Although, IL-4 exerts modest effects on inflammation, its administration has more effect at inhibiting cartilage and bone erosion(L. A. Joosten et al., 1999)(L. A. Joosten, Lubberts, Durez, et al., 1997).

IL-10 is another immunoregulatory cytokine controlling arthritic pathogenesis. Indeed, in murine CIA, both blockade of IL-10 with specific mAbs(L. A. Joosten, Lubberts, Durez, et al., 1997) and the use of mice deficient for IL-10(Cuzzocrea et al., n.d.) showed accelerated and more severe manifestations of arthritis. It has been also reported that the anti-inflammatory cytokine TGF- β can contribute to reduce arthritis severity. Indeed, in murine CIA, the exogenous administration of recombinant TGF- β or the injection of anti- TGF- β mAbs led to amelioration or aggravation of arthritis, respectively(Thorbecke et al., 1992).

Similarly, the administration of IFN- β in murine CIA also ameliorated established disease(Triantaphyllopoulos, Williams, Taylor, & Chernajovsky, 1999). Collectively, these examples illustrate the potent immunoregulatory role of anti-inflammatory cytokines in arthritis pathogenesis. In summary, a deregulation of the balance between anti-inflammatory and pro-inflammatory cytokines, explains the establishment and maintenance of arthritis.

Complement involvement in CIA

As in patients with RA, complement factors have been found in synovium and synovial fluids of murine CIA (Linton & Morgan, n.d.). From these observations, it was further established that in murine CIA, the treatment with an anti-C5 mAb or soluble CR1, i.e. inhibitors of the complement system, induced a significant reduction in disease severity, emphasizing the contribution of the complement system in arthritis pathogenesis (Y. Wang, Rollins, Madri, & Matis, 1995) (Dreja, Annenkov, & Chernajovsky, 2000).

Overall scenario of arthritis pathogenesis in murine CIA

The bovine type II collagen (CII) is the inciting antigen in murine CIA. CII is injected into mice in association with adjuvants to stimulate the innate immune system, including Complete Freund adjuvant (CFA) or Incomplete Freund adjuvant (IFA). Together, they provoke T and B cell immunity specific to bovine collagen which ultimately cross-reacts with mouse collagen, subsequently leading to autoimmune responses. Following DC priming of T cells, the activated T cells provide help to B cells with collagen specific B cell receptors which then go on to differentiate into auto-antibody producing plasma cells. Once these auto-antibodies encounter type II collagen, present at high levels in joints, antigen-antibody complexes (i.e. immune complexes) are formed. In turn, these immune complexes permit Fc-gamma receptor bearing cells (e.g., synovial macrophages, DCs, neutrophils) to become activated locally leading to antibody dependent cytotoxicity (ADCC) and in parallel, provide the structure required for activation of the complement system leading to complement-dependent cytotoxicity (CDC). Indeed, the first inflammatory cells infiltrating the joint are neutrophils that migrate into the synovial fluid. This inflammatory process includes the early production of angiogenic factors by synovial cells, e.g. VEGF, IL-8, and MCP-1, and the

release of proinflammatory cytokines by synovial macrophages, e.g. TNF, IL-1. Together, these signals promote the homing from the periphery to the synovium of CD4⁺ T cells and B cells specific for collagen. Locally, a complex proinflammatory cytokine network, orchestrated by TNF, promotes an excessive cellular activation process leading to exaggerated cellular proliferation and the key histopathologic destructive features of synovitis, i.e. hyperplasia and pannus formation. Synovial cells, including activated synovial T cells, produce large amounts of osteoclast differentiation factors ultimately, deregulating the homeostatic bone remodeling process. These include RANKL, IFN- γ and IL-17. In the advanced stage of arthritis, activated osteoclasts promote bone resorption in favor of bone regeneration, leading to cartilage and bone degradation at the pannus-bone interface. The subsequent joint destruction is reflected in arthritic mice by abnormal rigidity of the bones of the joint (i.e., ankylosis , which results in reduced motility).

B- TNF AND ITS NEUTRALISATION IN ARTHRITIS

1/ TNF

1.1 TNF and TNFRs: structure and signaling

TNF

Carswell and colleagues were the first to identify the cytokine as they had found a soluble factor able to induce the necrosis of tumors in animals, and thus, gave the protein the name tumor necrosis factor (Carswell et al., 1975). The subsequent cloning of TNF by Goeddel and colleagues allowed production of the recombinant form of TNF, greatly contributing to its subsequent characterization (Pennica et al., n.d.). The nucleic sequence of TNF encodes a protein precursor of 26kda, harboring a signal peptide sequence enabling its insertion into the plasma membrane. TNF can form homotrimers both in membrane-bound and soluble forms. During inflammation, the membrane form of TNF is cleaved by a metalloprotease, i.e. TNF converting enzyme (TACE), releasing a truncated soluble 17kda form of TNF. Ultimately, both the soluble and membrane-bound homotrimeric forms of TNF are biologically active. TNF trimers signal through the same specific type I transmembrane receptors, that is TNFR1 and TNFR2, to induce diverse biological responses.

TNFR1 and TNFR2 signaling

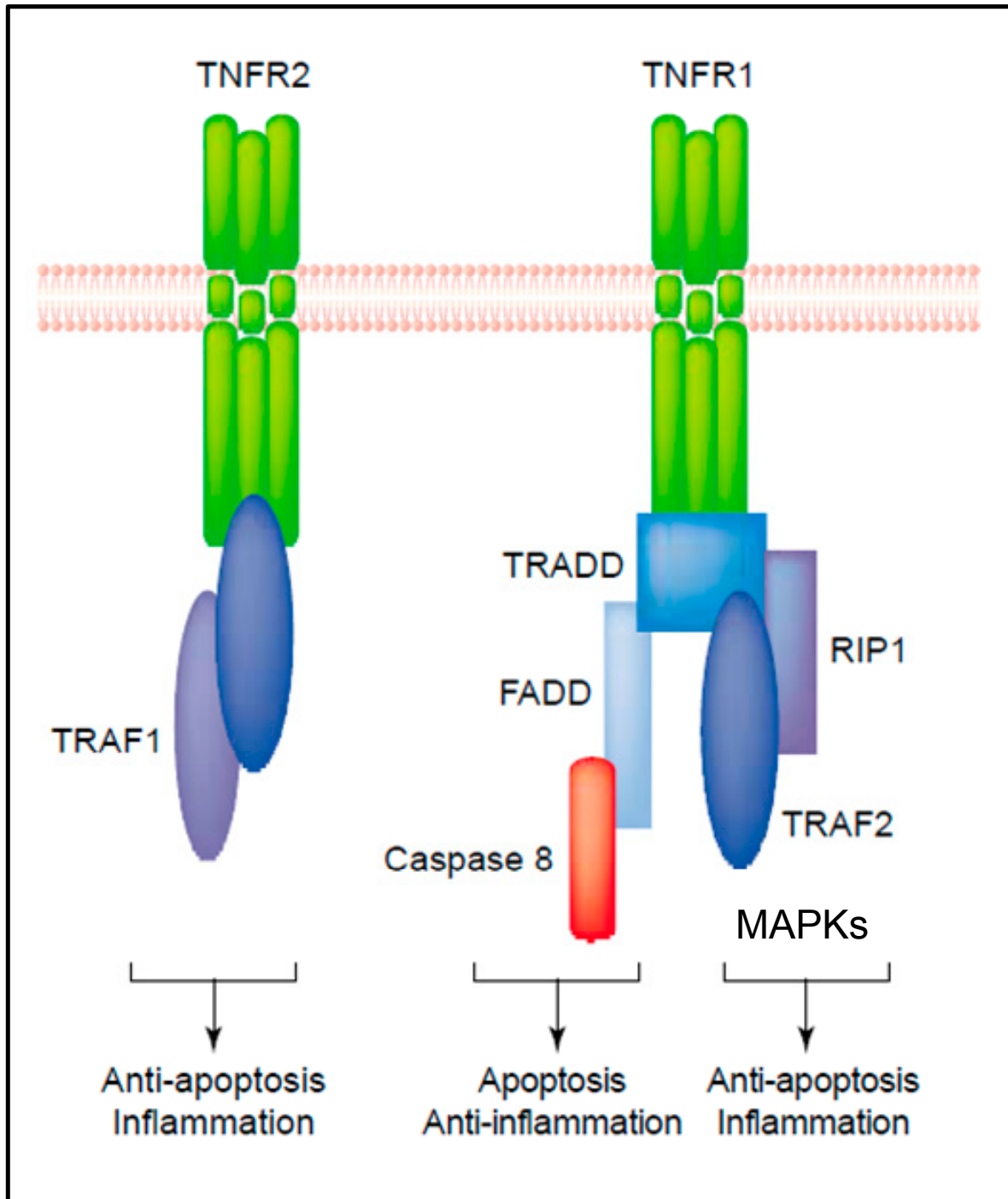


Figure 5. TNFR signaling. This Figure was adapted from (Baud & Karin, 2001).

TNF has two receptors, TNFR1 (also known as also termed p55-TNFR) and a slightly larger receptor, TNFR2 (also known as p75-TNFR) (Fig. 5). TNF triggers the trimerization of either TNFR1 or TNFR2. Similar to TNF, TNFRs also exist in both membrane and soluble forms, being cleaved by the TACE, i.e. the same protease as for TNF. The resulting soluble forms of TNFR1 and TNFR2 are natural inhibitors of TNF activity, referred to as sTNFR1 and sTNFR2, respectively.

TNFR1 is constitutively expressed on the surface of most cells and is considered as the major receptor by which TNF exerts its biological functions. Unlike the TNFR2, TNFR1 contains cytoplasmic death domains (DDs) that deliver TNF-induced apoptotic signals. Once TNF engages TNFR1, the death domains interact with the death domains of the TNFR-associated death domain (TRADD). This in turn leads to the sequential recruitment of additional adaptor molecules (summarized in Fig. 5) (i.e., receptor-interacting protein 1 (RIP1), TNFR-associated factor 2 (TRAF2), and the FAS-associated death domain (FADD)). FADD recruits and activates the caspase 8, pathway leading to apoptosis and anti-inflammatory responses. A second signaling pathway with opposite effects is initiated by TRAF2 which activates the cascade of mitogen activated protein kinases (MAPKs), leading to anti-apoptotic and inflammatory responses.

TNFR2 lacks DD domains, and thus does not induce apoptosis after TNF binding. Instead, the intracytoplasmic tail of TNFR2 recruits a heterodimeric complex composed of TRAF1 and TRAF2. Further downstream, activation of the MAPK pathway leads to anti-apoptotic and inflammatory responses (see Fig. 5). The MAPK pathway that induces inflammatory responses is commonly activated by both TNFR1 and TNFR2. For example, both transcriptional(Foey et al., 1998)(Rutault, Hazzalin, & Mahadevan, 2001) and translational levels(Swantek, Cobb, & Geppert, 1997) of TNF itself are regulated by this pathway, providing an auto-amplification loop of inflammatory signals organized around TNF.

1.2 TNF: biological function

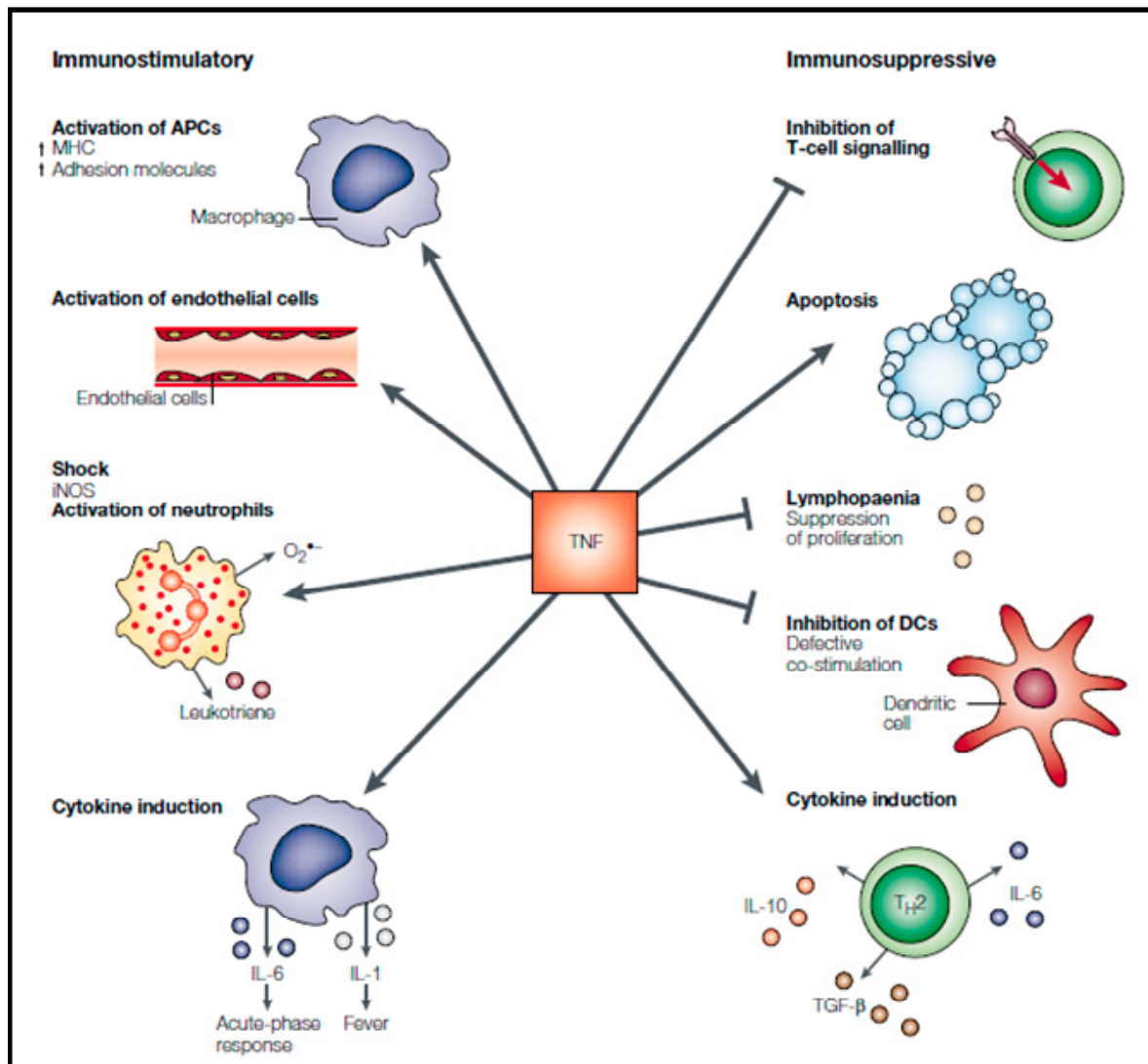


Figure 6. The wide spectrum of biological functions associated with TNF. This figure was taken from (O'Shea, Ma, & Lipsky, 2002).

Innate immune responses represent our first immunological barrier against infection. Amongst the different innate immune components, the cytokine TNF plays a key role, initiating a complex inflammatory process dedicated to eliminate invading pathogens. During

homeostatic conditions, this process is followed by counter-regulatory mechanisms aiming at resolving inflammation to prevent potential bystander destructive effects. Interestingly, TNF harbors both inflammatory and anti-inflammatory properties (see Fig. 6), certainly contributing in this sequential shift between initiation and resolution of inflammation. This point also reflects the complexity of the biological activities of TNF in healthy conditions. However, in pathological conditions such as in RA, inflammatory properties of TNF dominate, leading to a chronic inflammation with subsequent damage, as discussed later (O'Shea et al., 2002).

TNF induces biological responses by both secreted and membrane-bound forms, initiating different signaling pathways depending on the TNFR engaged, i.e, TNFR1 and TNFR2. Amongst its numerous immunostimulatory capacities, TNF can activate antigen-presenting cells (APCs) to initiate adaptive immune responses (Fig. 6). Indeed, it promotes both their ability to present antigens and their stimulatory capacities by up-regulating the expression of MHC and costimulatory molecules, respectively. In addition, by inducing the expression of adhesion molecules, TNF promotes the process of extravasation and migration controlling infiltration of inflammatory cells into target tissues. TNF is also involved in the production of proinflammatory cytokines and chemokines such as TNF itself, IL-1, IL-6, GM-CSF, and also chemokine (C-C motif) ligand-2 (CCL2), CCL3, and CCL5 by activating endothelial cells, macrophages, synovial cells, as well as other cell types (M Feldmann et al., 1996).

As mentioned, although it is accepted that TNF mediates pro-inflammatory effects, clear evidence also demonstrates that TNF can exert immunosuppressive functions. This dual function of TNF can be partly explained by both the timing and the duration of TNF exposure. For example, in murine diabetes (i.e. the Non-obese diabetic NOD model), TNF can either exacerbate or alleviate disease severity when given in adult mice or neonates, respectively (E. A. Green & Flavell, 2000) (Christen et al., 2001).

Another reported immunosuppressive function associated with TNF is its capacity to inhibit T cell responses during chronic exposure(Cope, 1998) (Fig. 6). Although, TNF is known to activate APCs, it was also reported that TNF can also inhibit mature dendritic cells, for example, by altering their capacity to present antigens or by inducing their apoptosis(O'Shea et al., 2002). Although, TNF mainly induces the production of inflammatory cytokines, it can also promote the production of anti-inflammatory cytokines (e.g, IL-10, IL-4, IL-6, and TGF- β) by different cell types including T helper (Th)-2 cells (Fig. 6).

Finally, as TNF triggers TNFRs to induce biological responses, the expression and functionality of TNFRs themselves also govern the activity of TNF. For example, patients harboring a mutation in the extracellular domain of TNFR1, i.e. a mutation preventing shedding of the receptor, suffer from the TNFR-associated periodic syndrome (TRAPS), which is associated with recurrent and periodic intense fevers(Aksentijevich et al., 2001).

To summarize, TNF is a proinflammatory cytokine with the capacity to exert pathogenic and protective functions depending on the timing and duration of exposure. However, in certain disease situations, TNF is the master cytokine driving and perpetuating inflammation.

2/ TNF AND ITS NEUTRALIZATION IN RA

2.1 Anti-TNF therapies: story, formats, and mode of action

TNF blockers

Therapeutic antibodies neutralizing TNF are widely used in the treatment of RA. The history of these mAbs starts in the early 1990s, originating from a collaborative work between Marc Feldmann and Ravinder Maini. At that time, starting from observations initiated in their laboratory, they pioneered the development of the first mAb specific to human TNF, referred to as Infliximab (Fig. 7A).

Initially, Feldmann and colleagues found that TNF was abundantly detected in the synovium of RA patients (C. Q. Chu, Field, Feldmann, & Maini, 1991). At that time, knowing the complexity of cytokine networks and their redundancy, it was not conceivable to neutralize a single cytokine to treat RA. To evaluate the extent to which TNF could contribute to regulation of the cytokine network in RA, they conducted *in vitro* experiments using synovial cell cultures. From these RA synovial cell cultures, they observed an abnormal prolonged production of pro-inflammatory cytokines, including TNF. Interestingly, when an anti-TNF mAb was added *in vitro* at the beginning of the culture, they reported that many proinflammatory cytokines were reduced (e.g., IL-1, IL-8, or GM-CSF) (F M Brennan et al., 1989) (Haworth et al., 1991). This experimental evidence suggested that TNF was at the top of a proinflammatory cytokine network in RA. In addition, the subsequent *in vivo* use of anti-TNF mAbs in murine CIA further supported these initial findings, providing the rationale to test the clinical impact of neutralizing TNF in RA patients.

From 1992 to 1998, the successful results obtained from clinical trials using TNF blockers in the treatment of RA provided strong evidence of the crucial role played by TNF in the pathogenesis of the disease. As a consequence, in 1998, etanercept (Enbrel, Immunex/Amgen), i.e., a soluble form of human TNFR2 engineered onto a Fc (depicted in Fig. 7B), became the first TNF blockers approved for RA indication. One year later, in 1999, infliximab (Remicade, Centocor) became the second TNF blocker approved in RA, being also the first anti-TNF mAb approved for this indication.

Formats of TNF blockers

The family of TNF blockers can be divided into two groups presented in figure 7. One group is composed of mAbs specific for epitopes present on human TNF, for example, infliximab (Remicade, Centocor), Adalimumab (Humira, Abbott), or Certolizumab Pegol (Cimzia, UCB Pharma). A second group consists of monomeric soluble forms of engineered chains of either p55 or p75 TNFR, including Etanercept (Enbrel, Immunex/Amgen).

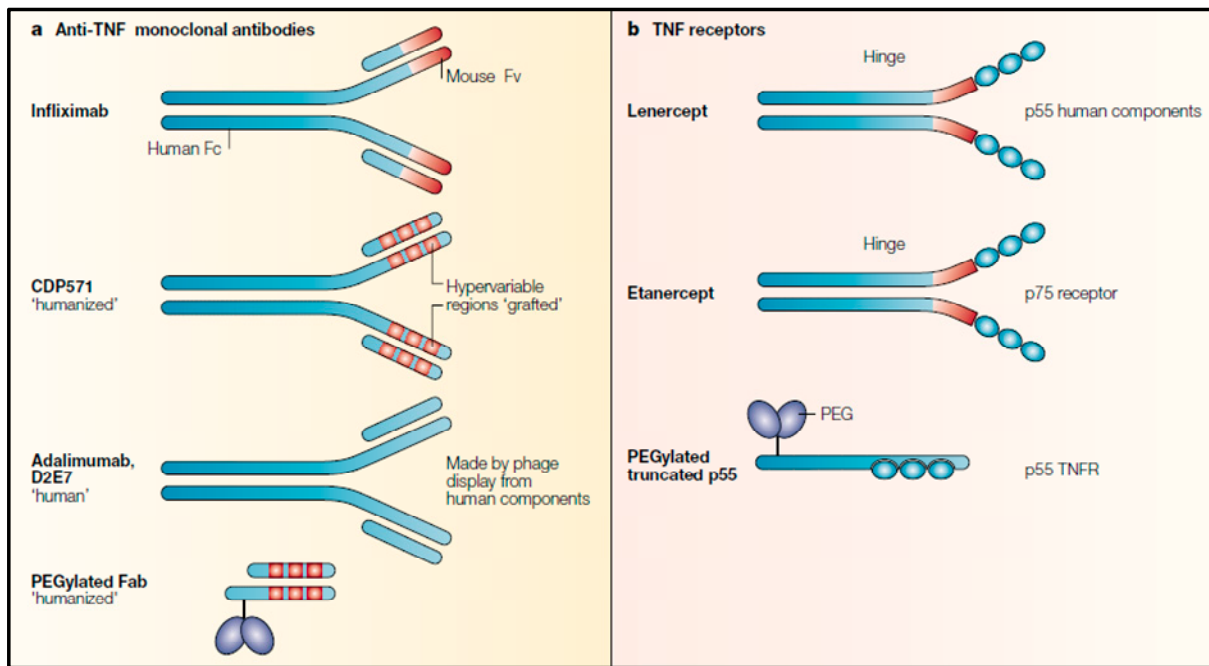


Figure 7. The different formats of TNF blockers. This figure was taken from (Marc Feldmann, 2002).

Etanercept

The generation of this TNF blocker was inspired by observation of the natural mechanism by which sTNFRs control TNF homeostasis. Indeed, once a cell bearing membrane forms of TNFRs becomes activated, in turn, the shedding of its receptors gives rise to sTNFRs, thus lowering the circulating levels of TNF. In this regard, due to the persistent pro-inflammatory activity of TNF in RA, it was suggested that the levels of natural sTNFRs present in RA settings were insufficient to counter-regulate the pro-inflammatory effects of TNF. Data from murine CIA demonstrated that the administration of recombinant forms of sTNFRs at disease onset reduced disease severity (Wooley, Dutcher, Widmer, & Gillis, 1993). Collectively, these observations provided the rationale to generate a recombinant form of sTNFRs with the aim to mimic its natural role. To fulfill this goal, Etanercept (Enbrel, from Immunex and Amgen) was engineered as a dimeric human TNFR p75-Fc fusion protein (Mohler et al., 1993).

Etanercept is composed of the Fc portion of a human immunoglobulin (Ig)-1 fused with the two extracellular domains of TNFR1 (see Fig. 7B), i.e. containing the ligand-binding domains specific to human TNF. This molecule has approximately the same molecular weight as IgG, and is a highly specific neutralizer of TNF as well as to the other natural ligand, lymphotoxin- α , initially termed TNF β . Interestingly, this characteristic indicates that Etanercept has potentially a broader spectrum of action than that of anti-TNF mAbs. However, currently the field considers that, as TNF is more active than lymphotoxin- α in RA pathogenesis, for this feature, Etanercept and anti-TNF mAbs have comparable therapeutic qualities. However, a differentiating factor is cell death as Etanercept is at best poor at inducing TNF-induced cell death (table 1). When compared to monomeric sTNRs, the dimeric structure of Etanercept enhances its avidity to TNF, thus, providing a considerably greater competitive inhibition of TNF. In addition, the presence of an Fc portion confers to Etanercept a prolonged half-life (attractive to patients from a dosing schedule perspective). Collectively, it can be concluded that Etanercept met the therapeutic aim to be a TNF blocker that mimics nature's own inhibitory mechanism provided by sTNFRs.

Infliximab

Amongst the family of TNF blockers, infliximab (Remicade, from Centocor) is the first anti-TNF mAb approved for RA. The original antibody producing clone was obtained as a hybridoma generated with cells from a BALB/c mouse immunized with recombinant human TNF(Knight et al., 1993). The therapeutic version of Infliximab was obtained by combining the variable sequences of the mouse antibody with the constant domains of human IgG1, resulting in a chimeric mouse/human IgG1 anti-TNF mAb (see Fig. 7A). Infliximab has a high binding capacity to both forms of TNF, and neutralizes by preventing its binding to

either of the TNFRs. In addition, infliximab is also able to dissociate preformed complexes between TNF and TNFRs. *In vitro*, it has been shown that infliximab mediates effector functions due to its Fc portion, by inducing cell lysis through ADCC and CDC (table 1).

Adalimumab

In 2002, yet another anti-TNF mAb was approved for the treatment of RA, referred to as Adalimumab (D2E7, Humira). The rationale to design this second generation of anti-TNF mAb was based on immunogenic concerns. Indeed, the underlying concept was that chimeric mAbs, like infliximab, containing foreign sequences would be progressively neutralized by humoral responses initiated by the host. To overcome this hurdle, a new anti-TNF was generated in a “fully human” format, i.e. Adalimumab. This “fully human” mAb was obtained by using human antibody libraries displayed *in vitro* on bacterial phage and then selected by *in vitro* panning on human TNF. Thus, unlike infliximab, the resulting Adalimumab should be indistinguishable from a natural human IgG1 and better tolerated by the patients’ immune system. Similar to infliximab, Adalimumab can bind both forms of TNF with high specificity and affinity, also preventing TNF binding to both TNFRs (Kempner, 2000).

Other TNF blockers have rapidly joined the developmental pipeline including Certolizumab Pegol (Cimzia, from UCB) (Fig. 7A), although some of these have been discontinued, such as CDP571 (Humicade, from Celltech). Today, the blockade of TNF by Infliximab, Adalimumab, or Etanercept is considered as the first line biologics treatment for RA, as well as other rheumatic diseases, e.g. psoriatic arthritis, ankylosis spondylitis and psoriasis.

Mode of Action of TNF blockers

The mechanisms by which TNF blockers work *in vivo* are not fully understood, which probably mirrors the complex functions played by TNF. However, several hypotheses currently exist as to the mode of action (MOA), which are summarized in figure 8.

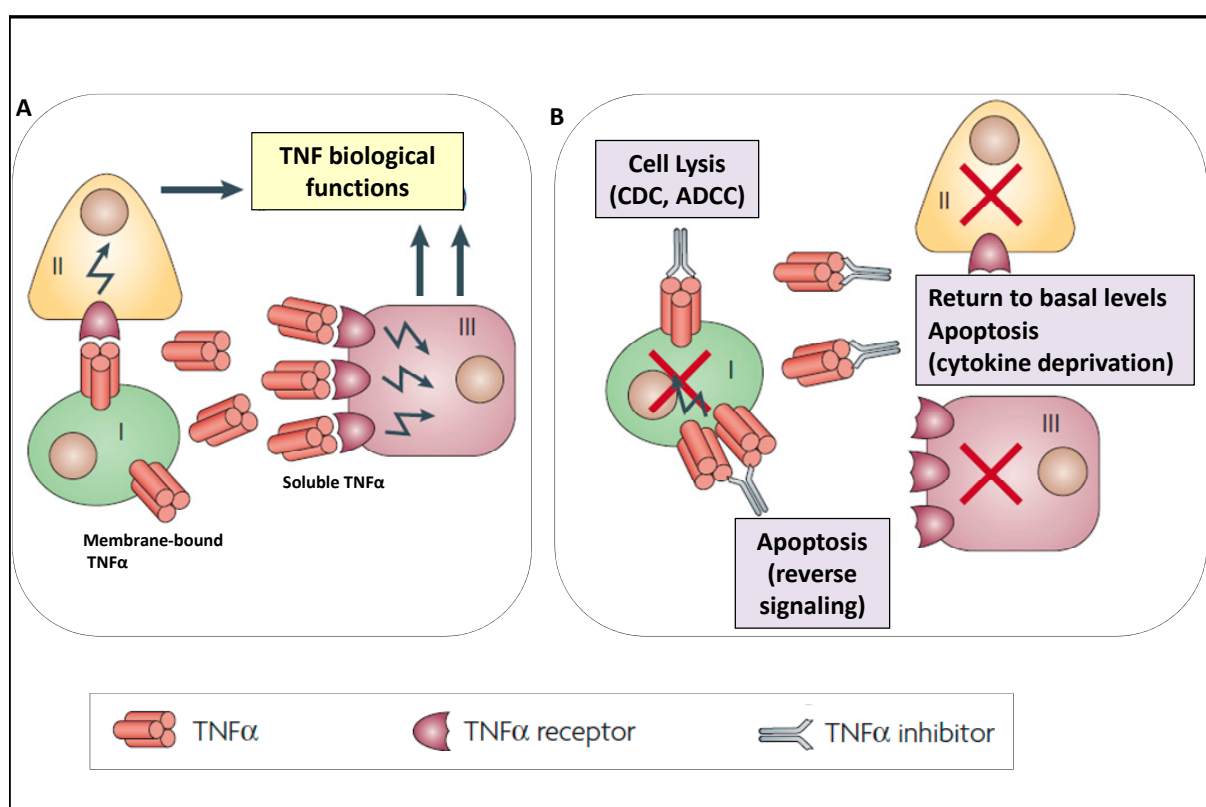


Figure 8. Potential mechanisms of action of TNF inhibitors. This figure was adapted from (Strand et al., 2007).

All TNF blockers, e.g, anti-TNF mAbs or engineered-TNFRs, can neutralize the activity of both soluble and membrane-bound forms of TNF (Fig. 8). The first explanation by which

anti-TNF mAbs exert an effect is based on TNF deprivation to dampen down inflammation, i.e., TNF deprivation can also result in cell death (Fig. 8B, right panel).

A second MoA occurs via the binding of anti-TNF mAb to membrane-bound forms of TNF expressed on the surface of target cells (Fig. 8B, lower panel), i.e., also referred to as transmembrane form of TNF. When this occurs, it can induce reverse signaling leading to cell death by caspase-mediated apoptosis. Once bound to transmembrane TNF, anti-TNF mAbs can also exert ‘non specific’ effector functions. Indeed, the Fc part interacts with Fc-gamma receptors expressed on the surface of many cells to mediate CDC and ADCC. Thus, anti-TNF mAbs induce cell death through apoptosis, CDC or ADCC (Fig. 8B, upper panel). A comparison of the effects of different TNF blockers as assessed with *in vitro* experiments is summarized in table 2.

Drug	Neutralizes soluble TNF α	Binds transmembrane TNF α	Inhibits transmembrane cytokine cleavage	Mediates		
				CDC/ADCC	Apoptosis	PMN death
Etanercept	+++	++	+/-	++	+/-	++
Infliximab	++	+++	++	+++	+++	+++
Adalimumab	++	+++	++	+++	+++	+++
Certolizumab pegol	+++	+++	+++	-	-	-
- to +++ represents relative potency. ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; PMN, polymorphonuclear leucocyte; TNF α , tumour-necrosis factor- α .						

Table 2. In vitro comparison of TNF inhibitors. This figure was adapted from (Strand et al., 2007).

2.2 TNF in RA is mirrored by the observed biological effects of its neutralization

Even in patients with persistent RA who had failed other treatments such as methotrexate, the administration of anti-TNF therapies causes a rapid amelioration of disease severity and pain, with an accompanying reduction in inflammation and joint swelling(Marc Feldmann, 2002). This emphasizes the significant contribution of TNF in RA pathogenesis. By assessing several biological parameters in sera and synovial biopsies from patients with clinical benefits, before and after anti-TNF treatment, it was reported that TNF was involved at numerous levels including the pro-inflammatory cytokine network, angiogenesis, immune cell infiltration into arthritic joints, as well as bone erosion(M Feldmann et al., 1996)(Marc Feldmann, 2002). These examples also illustrate how analysis of the biological consequence of neutralizing TNF in RA patients is useful for investigating the contribution of TNF in RA pathogenesis.

Altering the inflammatory network

The first and most striking biological observation reported after TNF blockade with anti-TNF mAbs is the quick and intense reduction in the expression of acute phase proteins such as C-reactive protein (CRP)(Elliot et al., 2008). CRP is regulated by cytokines, and its presence reflects on ongoing inflammatory process. After TNF neutralization, the reduction in serum levels of CRP parallels that of the pro-inflammatory cytokines (e.g., IL-6, IL-18, IL-8)(Elliot et al., 2008)(P Charles et al., 1999). In addition, serum levels of the anti-inflammatory cytokines, e.g. IL-10 and TGF- β , and soluble forms or cytokine receptors, e.g. sTNFRs and IL-1Ra, are also reduced, again pointing out the complexity of the cytokine loop controlled by TNF. More relevant to local inflammation, this decrease in the expression of pro-

inflammatory cytokines was also observed in rheumatoid synovium from such patients(Ulfgren et al., 2000). Altogether, these findings indicate that TNF regulates cytokine production both in the periphery and in inflamed tissue, emphasizing its key function in driving complex inflammatory networks during RA.

Adhesion molecules and cell infiltration

Deregulation of the balance between costimulatory and inhibitory molecules

Humoral autoimmunity, through the production of auto-antibodies by B cells with subsequent immune complex formation, is another mechanism involved in RA pathogenesis. The activity of cells including B cells is under the control of a permanent balance between costimulatory and inhibitory signals. Interestingly, in RA patients, peripheral B cells harbor unusual high levels of CD86 and relatively low expression of FcγR-IIb, corresponding to costimulatory versus inhibitory molecules, respectively(Catalán et al., 2010). Interestingly, these expression levels were reversed in RA patients treated by anti-TNF therapy. Collectively, these findings suggest that persistent TNF exposure in RA patients may contribute to RA pathogenesis by altering the equilibrium between costimulatory and inhibitory molecules expressed on circulating B cells, including autoreactive B cells.

Reduced infiltration of immune cells into arthritic joints

One of the key features of RA is an exaggerated proliferation of the cells forming the synovial membrane, leading to hyperplasia. The resulting hyperplastic tissues are also infiltrated by inflammatory mononuclear cells, mainly monocytes and T cells, and to a lesser extent B cells and DCs. It is thought that TNF contributes at different levels to the homing of immune cells from the periphery to synovium (M Feldmann et al., 1996). Evidence demonstrating the involvement of TNF in this process came from the observation of synovium from RA patients, before and after anti-TNF therapy. A drop in the number of synovial infiltrating cells is observed (M Feldmann et al., 1996). The analysis of synovium from RA patients revealed that TNF blockade resulted in reduced numbers of CD68⁺ macrophages, T cells, granulocytes, and B cells (P C Taylor, Peters, et al., 2000) (Tak et al., 1996). Furthermore, using labeled cells, i.e. indium-111-labeled granulocytes, it was demonstrated that the reduced cellularity of synovium can result from a reduced influx of immune cells into inflamed joints (P C Taylor, Peters, et al., 2000). Thus, the data suggests that TNF contributes to the observed increase in synovium cellularity by promoting the influx of immune cells into RA joints.

Reduced expression levels of adhesion molecules and chemokines

How does TNF actually contribute to leukocyte trafficking? The migration of leukocytes is a complex mechanism involving different successive steps, including rolling, adhesion and extravasation. Adhesion molecules play a central role in leukocyte trafficking, as *in vivo* blockade of these molecules significantly alters cellular migration. TNF induces the adhesion molecules VCAM-1 or E-selectin which are involved in mediating leukocyte adherence to endothelium. In this regard, in RA, it was hypothesized that TNF may up-regulate the

expression of adhesion molecules as well as the production of chemokines. Indeed, synovial biopsies of RA patients, collected before and after TNF blockade, demonstrated the reduction of leukocyte infiltration in joints with a parallel reduction in the expression levels of the adhesion molecules, VCAM-1, ICAM-1 and E-selectin, as well as reduced production of multiple chemokines including IL-8 and MCP-1 (P C Taylor, Peters, et al., 2000) (Tak et al., 1996). As these results correlate with an observed reduction in severity, it has been concluded that TNF-mediated leukocyte trafficking mechanisms are pivotal to the pathogenesis underlying RA.

Angiogenesis and VEGF

As compared to healthy synovium, RA synovium undergoes exaggerated proliferation. As a consequence, the rheumatoid synovium, being enriched in increased leukocytes and tissue mass, requires more nutrients than that of healthy synovium. Thus, the process of both angiogenesis and neovascularization occurs to provide the needed gas and nutrient exchange (Ewa M Paleolog, 2002). These processes consequently also contribute to the trafficking of inflammatory cells into the rheumatoid synovium.

Angiogenesis is driven by angiogenic factors including vascular endothelial growth factor (VEGF). Interestingly, in RA patients, serum levels of VEGF are significantly elevated compared to those of healthy subjects. TNF blockade reduces serum levels of VEGF in RA patients (E M Paleolog et al., 1998) (Ballara et al., 2001), illustrating another biological effect of TNF in RA.

TNF and bone erosion

A key feature of advanced RA is cartilage and bone erosion, leading to subsequent joint destruction. In RA patients, TNF blockade is effective in stopping structural damage in the joint, indicating that TNF is also involved in bone damage during RA pathogenesis (Marc Feldmann, 2002). In RA, cartilage and bone damage result from a deregulation in bone homeostasis, resulting in an excessive bone resorption process, osteolysis, mediated by osteoclasts. One of the most important osteoclast differentiation factors is RANKL. TNF can induce the expression of RANKL on stromal cells, which in turn, interacts with RANK on osteoclast precursors and stimulates their differentiation into osteoclasts (Lam et al., 2002). In RA patients, serum levels of the soluble form of RANKL (sRANKL), which is biologically active, are higher than those of healthy subjects (Ziolkowska et al., 2002). More interestingly, in RA patients, levels of sRANKL are normalized after TNF blockade, supporting an indirect role of TNF in bone damage through induction of sRANKL.

At the molecular level, the process of bone resorption is mediated by several proteases including MMPs. TNF promotes this destructive process through the stimulation of synovial fibroblasts to produce MMPs. In RA patients, TNF blockade is associated with a reduction in serum levels of MMPs including MMP-1 and MMP-3 (F M Brennan et al., 1997). Collectively, these results demonstrate the implication of TNF in the process of bone degradation during RA.

In summarizing the literature cited in this section, it appears that the broad biological effects observed after TNF blockade in RA patients mirrors the capacity of TNF to interfere with multiple biological pathways involved in RA pathogenesis. Hence, this multifactorial consequence of unbalanced TNF signaling, together with the pharmaceutical mechanism of depleting cells via membrane bound TNF, explain why the neutralization of a single cytokine is sufficient to efficiently treat RA in so many individuals.

2.3 Limits of anti-TNF therapies

Although, anti-TNF therapies are considered effective and powerful to treat RA patients, their use remains limited (Marc Feldmann, 2002). There are considered to be two reasons for this. First, as approximately 40% of RA patients do not respond to anti-TNF therapy, forces other than TNF must contribute to disease chronicity. Second, where disease was initially attenuated by the anti-TNF treatment, with time, many patients become unresponsive to the medication. Indeed, this is why multiple anti-TNFs have been developed commercially as the chronic disease gets treated with one anti-TNF after another for decades. This dynamic scenario of treating with anti-TNF reagents is due to the eventual induction of neutralizing anti-drug antibodies (M Feldmann et al., 1996). In addition, although the strategy is overall seen as successful, at the individual level each patient responds differently, there are side effects and it does not have long-lasting effect.

This last feature is one of the most unsatisfactory and important limitation from a medical point of view. Anti-TNF therapy offers relief but is not a cure. Indeed, in all patients responding to the treatment, withdrawal of the anti-TNF reagents results in disease flares. This last point highlights the necessity of its chronic use to prevent the flare of the disease. The drawback related to chronic use is that increased susceptibility to infection is important with anti-TNF treatments (Keane et al., 2001). Different types of opportunistic infections associated with anti-TNF use have been reported including tuberculosis (Keane et al., 2001), histoplasmosis (J.-H. Lee et al., 2002), listeriosis (Keane et al., 2001), pulmonary aspergillosis (Warris, Bjørneklett, & Gaustad, 2001) and *Pneumocystis carinii* pneumonia (Keane et al., 2001). Amongst these opportunistic infections, the reported rate of tuberculosis remains the highest. Altogether, these examples of opportunistic infections reported after TNF blockade are not surprising given the role of TNF in host defense. As a consequence, a screen for patients with such latent infections is recommended to prevent their

reactivation during anti-TNF therapy and to control the rate by which this side effect occurs in the treated population.

Alternative therapies

Many RA patients treated with anti-TNF therapy do not respond at all or not well enough. Therefore, TNF is not the only factor potentially driving RA pathogenesis(Marc Feldmann, 2002). Disease heterogeneity is one explanation. Indeed, prior to treatment, some RA patients have low levels of synovial TNF present in biopsies and continued to be non-responsive to anti-TNF therapies(Ulfgren et al., 2000). In addition, genetic heterogeneity between RA patients could explain their relative sensitivity to anti-TNF therapy. In this regard, differences in TNF or TNFRs genes should be more investigated in RA patients treated with anti-TNF therapy. Another explanation comes from the existence of potent supplementary mechanisms sustaining the disease, involving cell types and/or molecules that are not fully controlled under TNF blockade. For example, amongst these additional players, the potent contribution of T cells has been proposed, as discussed later(Lacraz, Isler, Vey, Welgus, & Dayer, 1994). In this regard, the use of novel combination therapies targeting TNF as well as other biological targets could help to identify other mechanisms sustaining the disease, even under TNF blockade.

3/ TNF AND ITS NEUTRALIZATION IN MURINE CIA

Initially, in murine CIA, TNF blockade either with the use of mAbs specific to TNF or other TNF-specific inhibitors (e.g. engineered sTNFR), demonstrated the role of this cytokine in establishing disease(Thorbecke et al., 1992)(R O Williams, Feldmann, & Maini, 1992)(Piguet et al., 1992). Indeed, both inflammation and bone erosion were significantly inhibited in murine CIA when TNF blockers were given prior to disease onset. More relevant to human disease, it was also demonstrated when administered during established disease, but at high doses. Conversely, *in vivo* administration of TNF into CIA mice potentially accelerates disease onset and also increases arthritis severity(W. O. Cooper, Fava, Gates, Cremer, & Townes, 1992). In addition, the pathological role of TNF can be observed in a transgenic mouse model exhibiting increased endogenous production of TNF, in which TNF was sufficient to induce inflammation with erosive arthritis(Keffer et al., 1991). As expected, in these transgenic mice, TNF blockers also stopped the manifestation of arthritis, confirming clinical observations.

The kinetics of pro-inflammatory cytokines in arthritic joints is difficult if not impossible to study in humans as it involves the extraction of joint tissue containing cartilage. For this reason, the murine CIA model represents an alternative to investigate these questions. In a study conducted in murine CIA, amongst the proinflammatory cytokines detected in arthritic synovium during the disease course, TNF was the first expressed at disease onset, followed progressively, over the next days, by proinflammatory cytokines including IL-1 and IL-6(M Feldmann et al., 1996). This finding indicates that during the course of arthritis the proinflammatory cytokines expressed in arthritic synovium appear in a sequential manner rather than simultaneously. In this regard, as TNF is known to control the production of most of these cytokines, this once again provides an explanation as to why TNF blockade in

patients is effective, as it precedes that of the other proinflammatory cytokines, especially in new joints where the inflammatory process is initiating (Marinova-Mutafchieva et al., 1997).

C- T CELL SUBSETS AND THEIR NEUTRALISATION IN ARTHRITIS

1/ T CELL BIOLOGY AND T CELL SUBSETS

T cell development

T lymphocytes arise from lymphoid progenitors that are mainly generated in the bone marrow. Immature precursors of T cells migrate from the bone marrow to the thymus, where they undergo maturation involving rearrangement of genes encoding the T cell receptor (TCR), selection by affinity and modifications of the expression pattern of cell surface molecules, e.g. CD4 and CD8 (Spits, 2002).

Amongst these T cell precursors, referred to as thymocytes, only a subset will survive the thymic selection process leading to the development of mature naïve T cells. During this process, thymocytes harboring a TCR compatible with self-MHC molecules are first positively selected, but then, those with an autoreactive TCR die by apoptosis during a step referred to as negative selection. At the end of this maturation process, the surviving thymocytes lose either the CD4 or CD8 co-receptor expressed at the cell surface, to become CD8⁺ or CD4⁺ T cells, respectively. The key features of mature post-thymic T cells are the expression of a heterodimeric TCR associated with one of its co-receptors, i.e. CD4 or CD8, as well as a transmembrane multi-protein CD3 complex responsible for initiating TCR signaling.

These emerging T cells, which have undergone extensive gene rearrangements of their TCRs, provide the immune system with a peripheral TCR repertoire broad enough to recognize a wide spectrum of pathogens. Each T cell recognizes an antigen in the format of a digested peptide presented by MHC Class I or II expressing cell types, with the latter being restricted to APCs, such as DCs, B cells and macrophages. In general, both the nature and origin of the

antigen that is presented determine the class of MHC molecule involved in its presentation as well as the type of T cell response subsequently engaged, i.e. CD8⁺ versus CD4⁺ T cell responses. The most simplistic view is that MHC class I molecules, expressed by most cell types present peptides from intracellular self antigens of the host (for tolerance) and peptides from intracellular infecting pathogens (for immune surveillance and elimination) to CD8⁺ T cells. In contrast, MHC class II molecules, of which the expression is restricted to APCs, present antigens of extracellular origin to CD4⁺ T cells. Exceptions to this dogma exist, for example in cross priming, but will not be reviewed here.

The TCR can be composed of $\alpha\beta$ chains, defining the cell as an $\alpha\beta$ T cell, or γ and δ chains on $\gamma\delta$ T cells. Most $\gamma\delta$ T cells are localized in the epithelium, contributing to the first line of immune defense against invading pathogens. Two major features distinguish $\gamma\delta$ T cells from $\alpha\beta$ T cells. First, the $\gamma\delta$ TCR repertoire is relatively restricted in terms of diversity as compared to the repertoire of $\alpha\beta$ T cells. In addition, unlike $\alpha\beta$ TCRs, the $\gamma\delta$ TCRs can recognize antigen directly, i.e. independently of MHC molecules (Sciammas, Tatsumi, Sperling, Arunan, & Bluestone, 1994). Of the two, the subset of T cells that plays a well defined role in RA is the $\alpha\beta$ T cells.

CD4⁺, CD8⁺, Naïve and Memory T cells

$\alpha\beta$ T cells can be either CD8⁺ or CD4⁺. The main function of CD8⁺ T cells is to recognize and kill target cells by cytotoxic mechanisms (e.g., infected cells during infection or tumor cells). CD4⁺ T cells are dedicated to ‘helping’ and thus regulate the activity of other cells, especially immune cells. CD4⁺ T cells orchestrate adaptive immune responses through direct cell-cell contact and via the production of soluble cytokines and are referred to as T helper (Th) cells. CD4⁺ T helper cells can be divided into three cellular subsets referred to as Th1, Th2, and

Th17 cells based on a signature pattern of cytokine secretion and the expression specific transcription factors (see Fig. 9). In general, both Th1 and Th17 cells are associated with autoimmune diseases while Th2 cells are mainly implicated in driving allergic responses.

New naïve T cells emerge from the thymus every day and become activated after encountering their antigen. To be fully activated, a T cell requires two activation signals. The first is antigen-specific, due to the interaction between its TCR and a specific peptide-MHC complex, and the second signal is antigen-independent, resulting from costimulatory signals delivered through both soluble cytokines and cell-cell contact, (e.g., B7/CD28, CD40/CD40L). Once activated, CD4⁺ T cells proliferate and differentiate into effector Th1, Th2 or Th17 cells. While the majority of these activated effector cells are short-lived, a subset differentiates into a long-lived memory T cell. Compared to naïve T cells, memory T cells have the capacity to elicit rapid recall responses when re-encountering antigen. Memory T cells can also recognize peptide-MHC complexes presented by non professional APCs (i.e., other than DCs). Another feature of memory T cells is their capacity to be fully activated in the absence of secondary costimulatory signals, a major advantage certainly explaining their ability to elicit fast immune responses after antigen recognition. Finally, in addition to the effector T (Teff) cells that promote immunity, there is also another subset of T cells, referred to as Tregs (Fig. 9). In general, these cells have been shown to regulate the responses of the effector cells, potentially containing deleterious activity for the host's tissues. Indeed, Tregs are proposed to prevent autoimmunity by adapting both the strength and duration of effector responses through diverse tolerogenic mechanisms (as discussed later).

Th1, Th2, Th17, and Treg cells

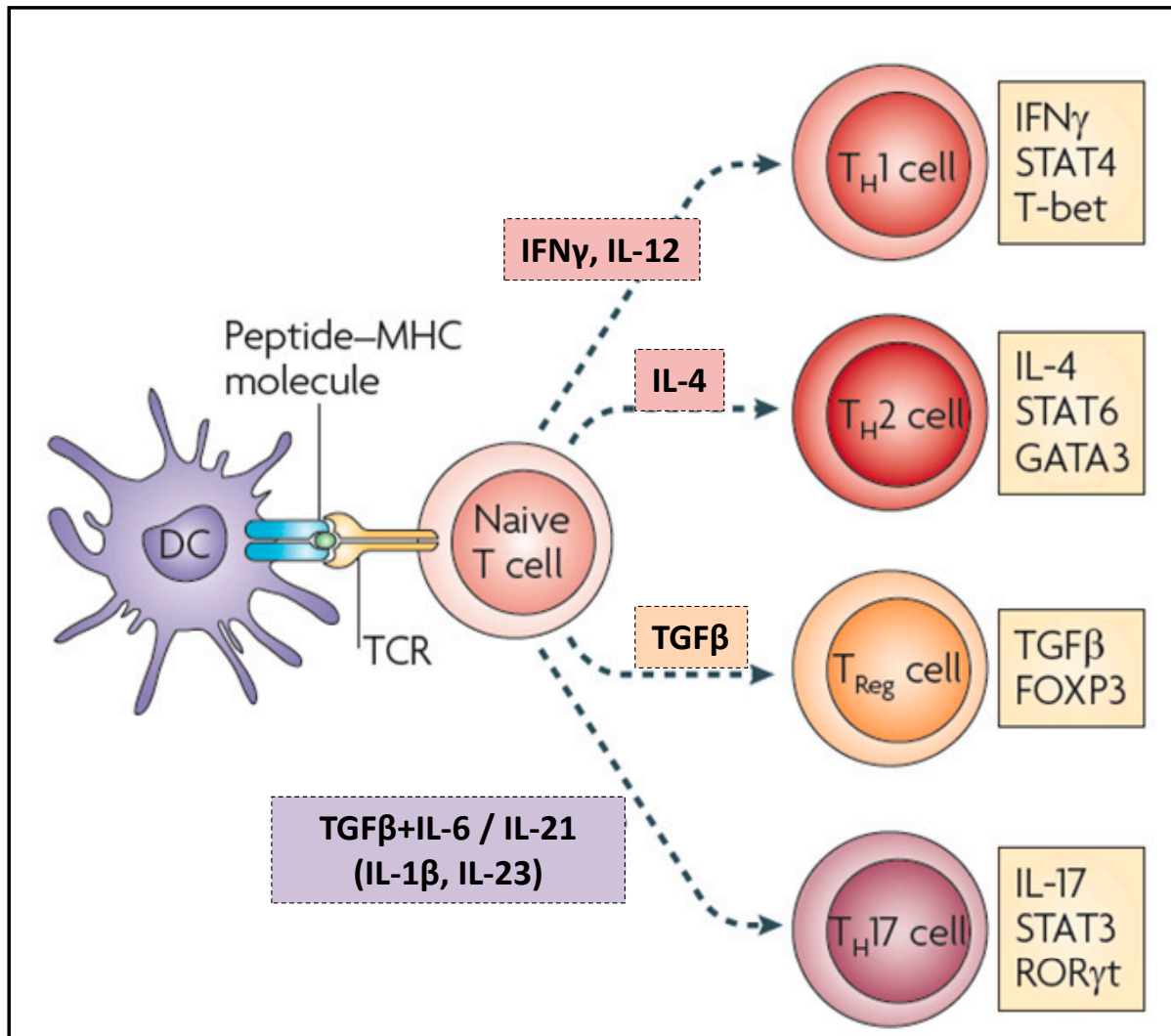


Figure 9. Differentiation of CD4⁺ T helper cell subsets. Adapted from (Zou & Restifo, 2010).

Th1 and Th2 CD4⁺ cells

The differentiation of CD4⁺ T cells into Th1 cells is dependent on the presence of IFN- γ and IL-12. The key properties of Th1 cells are based on their ability to produce high amounts of IFN- γ and their characteristic expression of STAT4 and the T-bet transcription factor (Fig. 9). Th1 cells expand in response to infections with intracellular pathogens, thereby mediating protective cellular immunity. Once activated, these cells promote inflammation by secreting high amounts of IFN- γ , and to a lesser extent, IL-2 and lymphotoxins. Th1 cells activate macrophages, stimulating both their phagocytic function and ability to present antigen.

Alternatively, CD4⁺ T cells can also differentiate into Th2 in the presence of IL-4. The key properties of Th2 cells are determined by their expression of the STAT6 and GATA3 transcription factors, and by their capacity to produce IL-4 in large amounts (Fig. 9). These cells can also produce other cytokines including IL-5, IL-6, IL-10, and IL-13. Th2 cells expand in response to extracellular pathogens, (e.g. during parasitic infestations)(Romagnani, 1997) and mediate humoral immune responses. In contrast to Th1 cells, Th2 cells negatively regulate the activation of macrophages by producing anti-inflammatory cytokines (e.g., IL-4 and IL-10).

In the process of T helper cell differentiation, cytokines are crucial factors regulating the fate of these cells. A simplified view is that IL-4 and IFN- γ promote the differentiation of CD4⁺ T cells into Th2 and Th1 cells, respectively. However, other factors also contribute to regulate T cell differentiation, (e.g. additional cytokines, costimulatory molecules and the strength and duration of TCR engagement with peptide-MHC complexes). In addition, IL-4 and IFN- γ compete by antagonizing their respective action on the Th1/Th2 axis of T cell differentiation. Indeed, elevated levels of IL-4 can block Th1 differentiation even in presence of IFN γ or IL-12. Inversely, the presence of IFN γ can counteract Th2 differentiation induced by IL-4.

Th17 CD4⁺ cells

For a several decades, Th1 and Th2 cells were thought to be the only T effector cells. Indeed, until about a decade ago, our view of the involvement of T cells in the pathogenesis of most animal models of autoimmune diseases was limited to Th1/Th2 cells (Korn et al., 2009) (e.g., Experimental encephalomyelitis (EAE), NOD, and CIA). However, the classical Th1/Th2 paradigm has been extended with the discovery of a third T helper cell subset that produces IL-17, referred to as Th17 (Annunziato, Cosmi, Liotta, Maggi, & Romagnani, 2009). The key features of Th17 cells are determined by their characteristic expression of the retinoic acid-related orphan receptor (ROR)- γ t and STAT3 transcription factors, and by their capacity to secrete IL-17, IL-22, and IL-23 (Fig. 9). In mice, the differentiation of CD4⁺ T cells into Th17 cells requires the presence of TGF- β plus IL-6 or IL-21. Interestingly, IL-23 is a key cytokine involved in both Th17 cell expansion as well as (although to a lesser extent) differentiation. Th17 cells are involved in the clearance of pathogens, and appear to exert complementary effector functions distinct from those of Th1 and Th2 cells.

T helper cells in autoimmunity and inflammatory disorders

The mechanisms that drive the differentiation of CD4⁺ T cells into a particular subset are multiple and complex. The proportion, timing and localization of the resulting differentiated T helper subsets, i.e. Th1, Th2, and Th17, is adapted to provide protective immune responses against pathogens. However, apart from their protective role, there is compelling evidence that these T helper cell subsets can also lead to pathologic immune disorders (Korn et al., 2009) (Dardalhon, Korn, Kuchroo, & Anderson, 2008). Indeed, for example, Th1 and Th17 cell subsets can also contribute to the pathogenesis of numerous autoimmune diseases and chronic inflammatory diseases, e.g. Crohn's disease, atherosclerosis, multiple sclerosis, and

RA, while Tregs maintain peripheral tolerance by down-modulating their activity via the production of anti-inflammatory cytokines and cell-cell contacts mechanisms(Wraith, Nicolson, & Whitley, 2004)(Askenasy, Kaminitz, & Yarkoni, 2008).

Regulatory T cells (Tregs)

Treg cells can express either CD4 or CD8 and are referred to as CD4⁺ Tregs or CD8⁺ T regs, respectively. Populations of CD4⁺ T regs represent the most abundant and best characterized regulatory T cell subset, i.e. corresponding to approximately 5-10% of circulating CD4⁺ T cells. In addition to natural Tregs (nTregs) that originate from the thymus, another Treg cell subset can be induced in the periphery, referred to as induced-Tregs (iTregs). Both nTregs and iTregs share key features, including expression of the forkhead boxP3 (Foxp3) transcription factor, high expression levels of CD25 and an inherent autoreactive TCR repertoire(Simons et al., 2010). This specificity for autoantigens enables Tregs to inhibit autoreactive effector cells in an antigen specific manner that initially requires cell-cell contacts(D. J. Campbell & Koch, 2011). In addition, the cytokines secreted by activated Tregs can provoke local effects not only restricted to the initial target cell. As a consequence, by secreting soluble anti-inflammatory cytokines, e.g. IL-10 and TGF- β , Tregs can also suppress immune responses in a non antigen-specific fashion. Initially, the tolerogenic functions of Foxp3⁺ Tregs were revealed in the case of the X-linked syndrome of immune dysregulation polyendocrinopathy and enteropathy (IPEX)(Bacchetta et al., 2006)(Wildin et al., 2001), in which mutations affecting the Foxp3 gene were associated with compromised Treg functions. In the IPEX syndrome, the absence of Tregs leads to an excessive polyclonal activation of Teff cells that become pathogenic by massively infiltrating tissues and promoting severe inflammation and tissue destruction. This example illustrates the requirement of functional Tregs for regulating emerging autoimmune diseases driven by uncontrolled activated T effector cells. Inversely,

any dysregulation in favor of Treg cells can also become detrimental, as excessive Treg function can lead to infection. In this regard, it is crucial to properly adapt the balance between effector and Treg cells over time.

2/ CONTRIBUTION OF DISTINCT T CELL SUBSETS TO ARTHRITIS PATHOGENESIS

2.1 T cell subsets in RA

Several lines of evidence support the involvement of T cells in both the initiation and perpetuation of RA. Briefly, the genetic association of RA with specific MHC class II alleles (P K Gregersen et al., 1987), the presence of infiltrating activated T cells in RA synovium as well as their ability to transfer arthritis when adoptively transferred into immunodeficient mice, demonstrate the contribution of T cells in RA pathogenesis. Although this T cell contribution was thought to be limited to the initiation phase of RA pathogenesis, the recent therapeutic success of some T cell targeted therapies has greatly contributed to support a role for T cells even in established disease, as discussed later.

CD4⁺ and CD8⁺ T cells in RA

CD4⁺ T cells play a major role in RA. Indeed, the genetic association between RA and MHC class II molecules emphasizes the preponderant contribution of CD4⁺ T cells (as compared to CD8⁺ T cells, to be discussed below). Locally, in RA synovium, CD4⁺ T cells are the major type of infiltrating T cell, where they orchestrate a number of inflammatory events, e.g. secretion of proinflammatory cytokines. Although several candidates for autoantigens driving RA have been proposed, e.g. collagen, HLA-derived peptides, glucose-6-phosphate isomerase (GPI), the precise nature of the antigen giving rise to RA pathogenesis remains elusive. As such, it is assumed that more than one autoantigen must drive the T cell immune responses involved in arthritis. As a consequence, a large effort has been placed on the characterization of both the phenotypes and function of CD4⁺ T cell subsets in RA.

CD4⁺ T cells are considered to be the major T cell subset involved in RA. As a consequence, the contribution of CD8⁺ T cells in RA has not been investigated as extensively. However, there is also evidence implicating CD8⁺ T cells in the disease process. For example, IFN γ -secreting CD8⁺ T cells were detected in synovial fluids from RA patients (Berner, Akça, Jung, Muller, & Reuss-Borst, 2000). It is likely that these cells contribute to the disease locally, as activated CD8⁺ T cells are known to both promote and sustain inflammation by secreting high amounts of proinflammatory cytokines (e.g., IFN γ , and TNF), and also by releasing cytotoxic molecules including perforin and granzymes. The formation of germinal centers (GCs) is generally restricted to secondary lymphoid organs, but in autoimmune diseases these structure can also develop abnormally in inflamed tissues to promote inflammation locally. In RA, ectopic GCs are found in the inflamed synovium, and it was reported that CD8⁺ T cells can also play an important role in both formation and function of such ectopic structures (Y. M. Kang et al., 2002) (U G Wagner et al., 1998). CD8⁺ T cell populations can expand during virus infections, and there are reports indicating that the expansion of virus-specific CD8⁺ T

cells is observed in RA patients(Tan et al., 2000). In this study, many of the oligoclonal CD8⁺ T cell populations detected in RA synovium were specific for epitopes of Epstein-Barr viral proteins, which may have contributed locally in arthritis pathogenesis.

Although these examples support a role of CD8⁺ T cells in RA pathogenesis, results from animal models of RA provided opposite conclusions. Indeed, unlike CD4-deficient mice, which were significantly less susceptible to arthritis, CD8-deficiency did not demonstrate any significant impact on arthritis induction or severity, further confirming the major contribution of CD4⁺ T cells at least in murine CIA(Ehinger et al., 2001). Another explanation could be that murine CIA is not a suitable model for the CD8⁺ T cell contribution seen in humans.

Th1/Th2/Th17 T cells in RA

The Th1/Th2 balance in RA: a dominance of Th1 responses

Initially, many models of autoimmune diseases were associated with Th1 effector cells, also termed IFN γ -secreting CD4⁺ T cells. As such, the involvement of Th1 cells was extensively investigated in RA. It has been reported that most, if not all, synovial T cell clones derived from RA patients correspond to the Th1 rather than the Th2 cell subset(Miltenburg, van Laar, de Kuiper, Daha, & Breedveld, 1992). This finding was further confirmed by the predominant detection of IFN- γ compared to IL-4 in histological section of synovial biopsies from RA patients(Cañete et al., 2000). By comparing the frequency of Th1 and Th2 cells in the circulation and synovial fluid, it was concluded that Th1 cells were preferentially found in RA synovial fluid, resulting in an imbalance in the Th1/Th2 ratio in favor of Th1 cells(van der Graaff, Prins, Niers, Dijkmans, & van Lier, 1999). During arthritis it is thought that Th1 cells are primed in the periphery and then migrate into joints to promote arthritis. In line with this

concept, it was demonstrated that blockade of ICAM-1, a key adhesion molecule involved in the cellular infiltration process, stopped the infiltration of Th1 cells into joints, resulting in an increased level of circulating Th1 cells that correlated with clinical benefits(Schulze-Koops, Lipsky, Kavanaugh, & Davis, 1995). This example also illustrates the importance of the migratory capacities of Th1 cells for the development of arthritis.

Another finding suggesting the importance of Th1 cells in RA is the protective effect of pregnancy on arthritis evolution. Surprisingly, it was reported that pregnancy improved the symptoms of RA in approximately 75% of the patients(Da Silva & Spector, 1992). Interestingly, during pregnancy, this amelioration is paralleled with a significant decrease in Th1 cell responses. However, the protection provided by pregnancy is transient and subsequent relapses are paralleled with an increase in Th1-mediated immunity. From these observations it was hypothesized that pregnancy induced a transient shift from Th1 to Th2 responses, thereby promoting the observed anti-inflammatory effects on the disease course. A study of the balance between peripheral Th1 and Th2 cells in the course of RA was conducted, and the results supported a ratio in favor of Th1 cells in RA conditions, further confirming a role for Th1 cells in aggravation of the disease(van der Graaff, Prins, Dijkmans, & van Lier, 1998).

These examples support the importance of Th1 cells in RA pathogenesis, suggesting that impaired Th2 responses in favor of increased Th1 responses promote persistent Th1-mediated inflammation leading to the chronic inflammations observed in RA. However, the simplistic view of RA as a purely Th1-driven autoimmune disease has been challenged by the recent emergence of a new T helper cell subset, Th17 cells.

Th17 cells in RA: emergence of a new pathogenic player

Recently, the role Th17 cells has been investigated in the course of RA(Leipe et al., 2010). The results from this study conducted indicated that both Th17 frequencies and activities correlate with disease severity. By assessing certain parameters in patients over time with clinical amelioration related to diverse treatments, it was concluded that the contribution of Th17 cells was important during the first signs of arthritis as well as during more advanced stages of the disease. Interestingly, unlike healthy subjects, RA patients have Th17 cells that are resistant to natural antagonist mechanisms, i.e., inhibition by IL-4 and IFN γ . These findings suggest that the Th17 cell population is abnormally promoted in RA patients, being involved in both early and established arthritis.

Th17 cells produce IL-17, a proinflammatory cytokine with a wide spectrum of action in RA pathogenesis. IL-17 promotes the production and secretion of proinflammatory cytokines by human macrophages and synovial cells, e.g., IL-1, TNF, IL-6, and IL-8(Fossiez et al., 1996; Jovanovic et al., 1998). IL-17 is also involved in the process of joint destruction, which is a feature of late stage arthritis. Indeed, IL-17 promotes osteoclastogenesis by up-regulating the osteoclast differentiation factor RANKL(Kotake et al., 1999), and also contributes directly to cartilage and bone degradation by inducing the synthesis of degradative enzymes(Chabaud, Lubberts, Joosten, van Den Berg, & Miossec, 2001). In this regard, initial positive results obtained during clinical trials conducted in RA patients treated with a neutralizing anti-IL-17 mAb, further supports the importance of Th17 cells in RA (M C Genovese et al., 2010).

Treg cells in RA

To counteract persistent and exaggerated inflammation mediated by pathogenic Th1 and Th17 cells, it has been proposed that Treg cells should function properly(C. a Notley & Ehrenstein,

2010) (Esensten, Wofsy, & Bluestone, 2009). For example, Tregs derived from non-arthritis subjects are known to inhibit both the proliferation of T effector cells and their ability to secrete proinflammatory cytokines such as IL-17 and IFN γ . However, T eff cells from RA patients were still able to produce these proinflammatory cytokines when autologous Tregs were added (Ehrenstein et al., 2004). From this observation it was not directly possible to distinguish between increased resistance of T eff cells to Treg inhibition and compromised functions of Tregs. However, in the same study, it was demonstrated that Tregs from healthy subjects were potent at inhibiting T eff cells from RA patients, indicating that Treg cells from RA patients were functionally defective in their ability to control T effector responses.

Interestingly, an explanation was proposed by the same team in a subsequent follow-up study. They reported a deficiency in the level of CTLA4 expression at the surface of Treg cells from RA patients compared to that of a healthy subject. As CTLA4 is an inhibitory molecule by which Tregs exert their suppressive effects, it was not surprising to find that those Tregs were compromised in their ability to control T eff cells. To demonstrate the involvement of CTLA4-deficiency in these observations, they artificially restored CTLA4 expression on Tregs from RA patients, and observed the restoration of Treg suppressive capacity (Flores-Borja, Jury, Mauri, & Ehrenstein, 2008).

In addition, chronic TNF exposure is known to inhibit Treg function, and as TNF is abundantly present in RA patients, it is likely that TNF is also responsible for the defect in Treg function specific to RA patients, as discussed later. Together, these findings indicate that RA is associated with an abnormal Treg suppressive function, particularly regarding IFN γ - and IL-17-secreting, Th1 and Th17 cells.

2.2 T cell subsets in murine CIA

As mentioned above, inducing CIA in CD4- or CD8-deficient mice revealed the predominant contribution of CD4⁺ T cells in the pathogenesis of this model. Amongst CD4⁺ T cells, IFN γ -secreting Th1 cells were initially considered as the dominant effector T cell population involved in the pathogenesis of CIA. As a consequence, until the discovery of Th17 cells, the CIA model was strictly considered as a Th1-mediated model.

Blockade of IFN γ and IL-12 to evaluate Th1 responses

Initially, the role of Th1 cells in CIA was addressed through the study of IFN γ , a hallmark of Th1 cell responses. In murine CIA, systemic injection of IFN γ at the time of immunization resulted in accelerated arthritis with increased severity (S. M. Cooper, Sriram, & Ranges, 1988). The same observations were made when IFN γ was injected locally into footpads (Mauritz et al., 1988). Conversely, *in vivo* prophylactic blockade of IFN γ with neutralizing mAbs inhibited arthritis induction in murine CIA. Together, these findings suggest a contribution of Th1 cells in the induction phase of CIA (Boissier et al., 1995). However, the pathogenic role of Th1 cells in this disease was controversial. Indeed, the use of neutralizing anti-IFN γ mAbs in already arthritic mice was associated with an aggravation of the disease (Boissier et al., 1995). As IL-12 is another proinflammatory cytokine associated with Th1 cell development and function, its role was studied. Interestingly, similar to IFN γ , IL-12 seems to play a dual role during the course of murine CIA. Indeed, it was reported that IL-12 promoted the induction of early arthritis, but also played a suppressive role during ongoing arthritis, supporting the controversy on the pathogenic role of Th1 cells in the disease (L. A. Joosten, Lubberts, Helsen, & van den Berg, 1997).

The IL-12p40 subunit is shared by IL-23, which promotes Th17 responses

Most of these conclusions were based on indirect studies in which IFN γ or IL-12 function was assessed with the use of corresponding deficient mice. Another explanation for these discrepancies is that, until IL-17 was discovered, quite late in the game, there was an underestimation of the pathogenic role of Th17 cell populations. For example, aggravated arthritis was observed in CIA mice rendered genetically deficient for either IFN γ or its receptor, and this surprising phenotype was associated with increased IL-17 responses, suggesting an additional role for pathogenic Th17 cells in arthritis pathogenesis (C.-Q. Chu et al., 2007)(Vermeire et al., 1997). Another example comes from the observation that CIA mice were protected from arthritis when rendered genetically deficient for the IL-12p40 chain gene, i.e., 'IL-12'-deficient mice(Gately et al., 1998). Using these mice it was initially thought that pathogenic Th1 cells were crucial to drive arthritis. However, this conclusion has been reversed by the finding that the p40 subunit is shared by IL-12 and IL-23. Indeed, unlike IL-12, which promotes Th1 responses, IL-23 is a crucial cytokine involved in the induction and maintenance of Th17 cells. IL-23 is composed of p40 and p19 subunits, while IL-12 consists of p40 and p35 subunits. As a consequence, to more accurately address the contribution of Th1 and Th17 cells in CIA, mice deficient for IL-12p35 and IL-23p19 were generated. Surprisingly, only mice deficient for IL-23p19 were protected from CIA, demonstrating that IL-23 and not IL-12 was critically linked to arthritis in this model(Murphy et al., 2003). These findings paved the way for a new role of Th17 cells in CIA pathogenesis.

Blockade of IL-17 to evaluate Th17 responses

The role of Th17 cells in murine CIA was first investigated by examining the importance of IL-17 in the disease process. As IL-17-deficient mice are protected from arthritis induction, it was concluded that IL-17 directly contributed to arthritis pathogenesis (Nakae, Nambu, Sudo, & Iwakura, 2003). In addition to a role of IL-17 in induction of the disease, the protective effects provided by neutralizing anti-IL-17 mAbs in established arthritis also demonstrated the importance of IL-17 in disease perpetuation (Erik Lubberts et al., 2004).

Th1 and Th17 cells during arthritis: a shift from Th1 to Th1/Th17

Together, these findings suggested that both Th1 and Th17 cells were involved in disease pathogenesis. However, as discussed above, the relative contribution of Th1 cells was also the subject of controversy, being protective or pathogenic depending of the study. For example, Chu and colleagues demonstrated that the resistance of C57BL/6 mice to CIA was explained by a protective role of Th1 cells that suppressed Th17 responses (C.-Q. Chu et al., 2007).

However, the situation is not so simple and it is important to take into consideration the kinetics of Th1 and Th17 cell responses during CIA. Indeed, it has been demonstrated that the production of Th1 and Th17 cytokines differs during the disease (Lamacchia, Palmer, Seemayer, Talabot-Ayer, & Gabay, 2010). Hence, it has been concluded that collagen-specific Th1 responses were predominant in early arthritis, whereas both Th1 and Th17 responses occurred in later stages.

In summary, in early stages of arthritis, pathogenic Th1 responses represent the dominating force driving arthritis. Their role then shifts from pathogenic to protective in later stages by potentially inhibiting Th17 cells. Indeed, pathogenic Th17 cell responses only expand in later

stages of arthritis. Consequently, when Th1 cytokines are neutralized *in vivo*, aggravation of the disease is associated with an expansion of Th17 responses(C.-Q. Chu et al., 2007).

Treg cells in murine CIA

As mentioned above, in both humans and mice, CD4⁺CD25⁺Foxp3⁺ Treg cells have been identified as cells that play a crucial role in preventing autoimmune diseases. For example, it is well known that the absence or dysfunction of Foxp3 is associated with compromised Treg function and subsequent manifestations of multi-organ autoimmune disease(Jane Hoyt Buckner, 2010). In murine CIA, although Tregs localize in inflamed joints and synovium, *in vitro* these cells have altered suppressive function compared to the same cells from non-arthritic mice(Kelchtermans et al., 2005). Interestingly, in arthritic mice, the altered ability of Tregs to suppress arthritogenic T cells can be successfully compensated for the adoptive transfer of functional CD25⁺ Treg cells(M. E. Morgan et al., 2005). Inversely, in murine CIA, CD25⁺ T cell depletion results in accelerated onset of arthritis(M. E. Morgan et al., 2005). Together, these results illustrate the importance of functional Treg cells for limiting arthritis in murine CIA.

3/ T CELL NEUTRALIZATION IN RA AND MURINE CIA

T cell-modulating strategy: from rationale to early techniques

Several lines of evidence indicate that T cells are involved in the pathogenesis of both human RA and murine CIA, providing a rationale to develop therapeutic strategies aimed at

neutralizing T cells during disease. Such therapies were designed to either eradicate or inhibit the activity of arthritogenic T cells. Initially, total lymphoid irradiation was conducted in RA patients but provided only limited beneficial effects with serious side effects (Strober et al., 1985). It is also relevant to note that most therapeutic agents that are efficacious in treating RA, e.g., cyclosporine, tacrolimus, leflunomide, and other disease-modifying anti-rheumatic drugs (DMARDs), also exert anti-inflammatory effect by inhibiting T cells. For example, it was reported that leflunomide preferentially inhibits Th1 cells, thus shifting the Th1/Th2 balance in favor of Th2 cells (Dimitrova et al., 2002). However, these agents are not specific for T cells, and a better understanding of T cell biology has permitted to generate more selective agents that specifically neutralize T cells. For example, therapeutic mAbs were generated against CD4. In addition, Abatacept (Orencia, Bristol-Myers Squibb) is in clinical trials and its mechanism of action is to deliver inhibitory signals that downregulate T cell activation, leading to T cell unresponsiveness, as described later.

Controversial effects of the first anti-T cell therapies: anti-CD4 mAbs

Species	Isotype	Designation	Trial type	Efficacy	Biologic effects
Murine	IgG2a	VIT4	Open-label pilot study ²¹⁴	N/A	CD4 depletion
Murine	IgG2a	M-T151	Open-label pilot study ²¹⁵	N/A	CD4 depletion
Murine	IgG2a	BL4	Open-label pilot study ²¹⁶	N/A	CD4 depletion; infusion reaction (1/7 patients)
Murine	IgG1	MAX.16H5	Open-label pilot study ²¹⁷	N/A	CD4 depletion; infusion reactions (4/10 patients); immunogenicity ²¹⁸
Murine	IgG1	B-F5	Phase II DBRCT ²¹⁹	Negative	CD4 depletion; infusion reactions
Chimeric	Human IgG1	cM-T412	Phase II DBRCT + MTX ²²⁰	Negative	Infusion reactions, prolonged CD4 depletion ²⁹
			Phase II DBRCT without MTX ²²¹	Negative	Immunogenicity ²²² ; coating of SF CD4+ cells correlated with efficacy ²²³
Primatized	Human IgG1	Keliximab	Phase I dose escalation ²²⁴	N/A	Transient depletion
			Phase II DBRCT without DMARDs ¹⁰	Positive	No depletion; efficacy correlated with CD4+ cell coating ⁷
			Phase II DBRCT with DMARDs ¹⁰	Negative	Depletion
Primatized	Mutated Human IgG4	Clenoliximab	Phase I ²²⁵	N/A	Non-depleting, antigen stripping
Humanized	Human IgG1	hIgG1-CD4 (4162W94)	Open-label pilot study ²²⁶	N/A	Non-depleting, infusion reactions
			Open-label dose escalation study ²²⁷	Positive	Moderate but transient depletion. Coating of blood and SF CD4+ cells correlated with efficacy
			Placebo-controlled repeat cycle study ¹⁵	Positive	Depletion. Skin rash (cutaneous vasculitis) at high dose
Humanized	Human IgG4	OKTcdr4a	Phase I DBRCT ³⁸	Transient efficacy	Non-depleting, reduction of T _H 1 activity in blood
			Phase I dose escalation ³⁹	N/A	Non-depleting
Fully human	Human IgG1	HM6G	Phase I DBRCT ¹⁵³	Negative	Non-depleting

DBRCT, double-blind placebo-controlled randomized clinical trial; DMARD, disease-modifying antirheumatic drug; Ig, immunoglobulin; MTX, methotrexate; N/A, not applicable; SF, synovial fluid.

Table 3. Summary of anti-CD4 mAbs used in RA. This table was taken from (Strand et al., 2007).

In animal models of autoimmune disease, anti-CD4 mAbs are potent at reversing established disease (Cobbald, Qin, Leong, Martin, & Waldmann, 1992). Although a wide spectrum of neutralizing anti-CD4 mAbs are available, the clinical benefits of treating RA patients were absent or very limited (Strand et al., 2007) (summarized in Table 3). It is possible that part of

the explanation comes from non-optimal dose-regimens, or the concomitant neutralization of the protective CD4⁺ T reg cells.

T cell costimulatory molecule blockade: success of Abatacept

Abatacept is a fusion protein containing the inhibitory molecule, CTLA4, fused to the Fc portion of an antibody (CTLA4-Ig). The rationale comes from the biological function of CTLA4 which is as a T cell inhibitory costimulatory molecule that downregulates T cell activation, leading to T cell nonresponsiveness and/or tolerance. The role of T cells in the pathogenesis of RA was controversial due to the observed failure to obtain clinical benefits by treating RA patients with anti-CD4 T cell therapies. However, the successful clinical amelioration observed in RA patients treated with Abatacept provided renewed interest of the involvement of T cells in RA pathogenesis (Linsley & Nadler, 2009). Briefly, Initially, Feldmann and colleagues observed that both administration of CTLA4-Ig at onset and during established murine CIA, significantly ameliorated disease severity (Webb, Walmsley, & Feldmann, 1996). In addition, to confirm the ability of CTLA4-Ig to inhibit T effector cell responses in murine CIA, their findings confirmed the active role of T cells in established arthritis, providing an alternative T cell therapy controlling efficiently CIA. As synovial T cells are known to contribute to joint destruction, another study evaluated the extent to which inhibition of T cell function by CTLA4-Ig could impact on this process *in vivo* (Kliwinski et al., 2005). As expected, they reported that the observed downregulation of T cells by use of CTLA4-Ig was associated with reduced bone erosion. Altogether, these findings indicated that inhibition of T cell responses by CTLA4-Ig in murine CIA was potent at reducing arthritis severity both clinically and histologically. In this regard, Abatacept, the therapeutic CTLA4-Ig, was then clinically used to treat RA patients. Interestingly, even in RA patients refractory to classic DMARDs treatments, or with inadequate responses to TNF blockers,

Abatacept provided significant clinical benefits(Kremer et al., 2005)(Mark C Genovese et al., 2005). Collectively, these findings support the utility of T cell targeted therapies in RA, thus, establishing a pathogenic contribution of T cells in RA.

4/ ANTI-CD3 IN THE TREATMENT OF AUTOIMMUNE DISEASES

4.1 Formats and mode of action (Pathogenic T cell killing, Tregs, TGF- β , IL-10, PD-1)

The CD3/TCR complex

T cells harbor a wide spectrum of TCR specificities covering a large panel of potential pathogenic antigens. Despite the existence of this broad TCR diversity, each T cell remains monospecific and harbors a single TCR specificity for a particular peptide-MHC complex. The TCR that confers the antigenic specificity also initiates the signaling cascade that leads to adapted cellular responses. However, the TCR by itself is not sufficient to induce signaling. Indeed, as illustrated in figure 10, TCR signaling requires the formation of complex involving CD3 molecules, linking antigenic recognition provided by the TCR, to subsequent signaling responses via molecules linked to CD3. The CD3 molecules are non-covalently associated to TCR on both CD4⁺ and CD8⁺ T cell surface, and consists of multiple signaling subunits organized in dimers, referred to as CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$ and CD3 $\zeta\zeta$. CD3 subunits contain signaling domains termed ITAMs, which recruit other signaling adapter molecules to initiate subsequent intracellular signaling. As shown in figure 10, depending on the context, TCR signaling initiated after engagement of the cognate peptide-MHC complex can deliver

stimulatory or tolerogenic signals. For example, positive thymocyte selection and effector T cell activation are cellular effects associated with immunogenic signals. Conversely, tolerogenic signaling is involved in thymic negative selection, anergy induction and Treg activation.

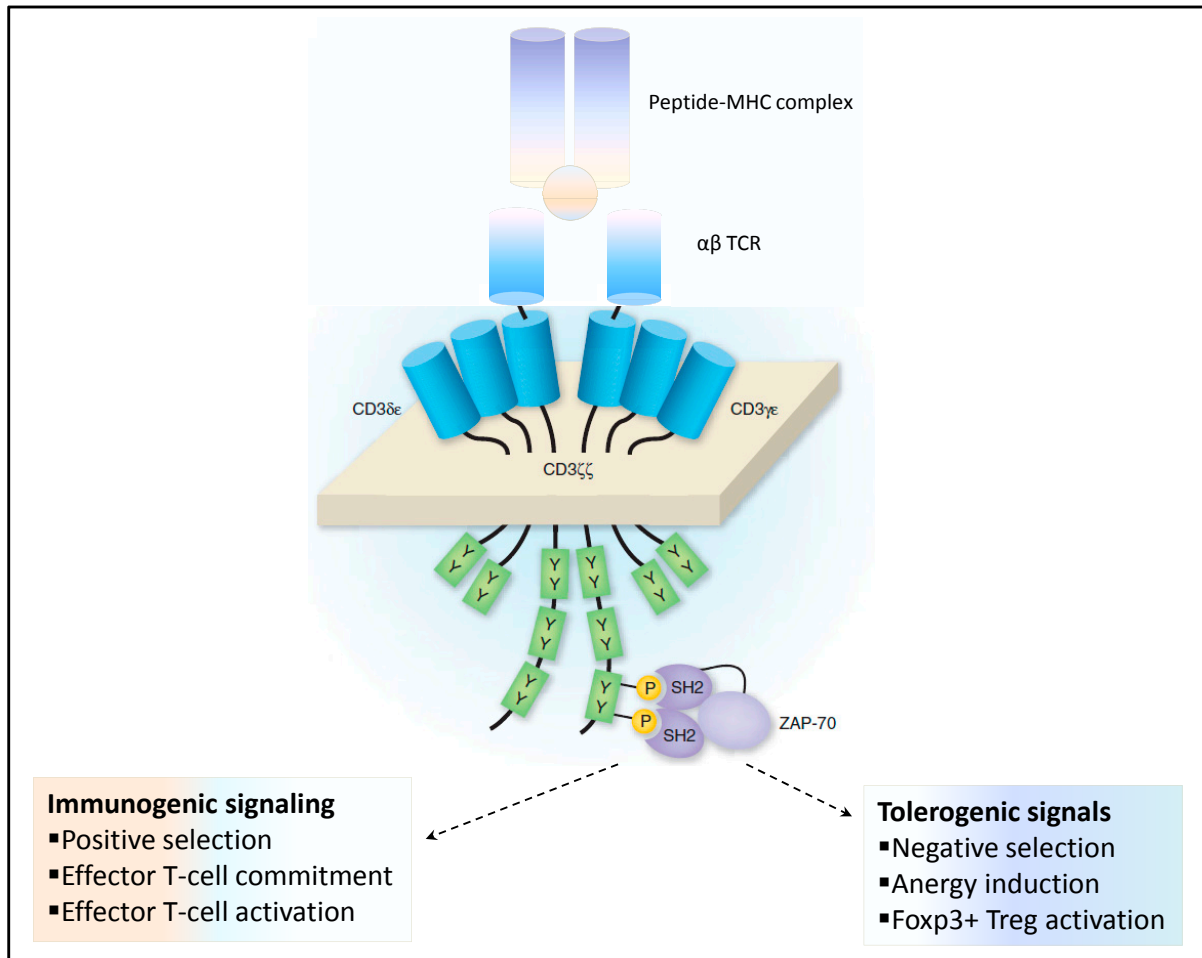


Figure 10. The CD3/TCR complex initiating signaling in the context of TCR-pMHC interaction. This figure was adapted from (Malissen, 2008).

Within the CD3-TCR complex, emphasis is on the CD3 epsilon (CD3 ϵ) subunit that is crucial to initiate TCR-induced signaling. Indeed, neutralization of CD3 ϵ with specific mAbs is sufficient to inhibit T cell responses, by preventing TCR mediated responses or directly

inducing the death of the targeted T cell. Interestingly, almost all anti-CD3 mAbs are directed against the CD3 ϵ chain, and the reason is probably that CD3 ϵ represents a dominant antigenic epitope amongst other CD3 subunits. For the remainder of this section, CD3 ϵ is meant when CD3 is used, unless mentioned otherwise.

Interestingly, the biological effects promoted by anti-CD3 mAbs appear in a sequential manner over time (Lucienne Chatenoud & Bluestone, 2007). Indeed, anti-CD3 mAbs induce both short-term effects, at the time of administration, as well as prolonged effects that can be maintained even after antibody is cleared from the body. The mode of action (MOA) of anti-CD3 mAbs is thus complex, including different pharmacodynamic (PD) effects that occur sequentially after antibody treatment. As summarized in figure 11, the early PD effects include both CD3/TCR down-modulation (Fig. 11A) and T cell killing (Fig. 11B), followed by immunoregulatory mechanisms involving suppressive T reg cell populations (Fig. 11C).

Down-modulation of the CD3/TCR complex: T cell unresponsiveness

Upon *in vivo* administration, anti-CD3 mAbs bind to the CD3 ϵ subunit of the CD3/TCR complex expressed on the T cell surface. The targeted T cell becomes coated by these mAbs, inducing internalization of the CD3/TCR complexes, a mechanism also termed CD3/TCR down-modulation or antigenic modulation (Lucienne Chatenoud & Bluestone, 2007). As a consequence, the resulting T cells become blind for antigen recognition because TCR engagement with cognate peptide MHC complexes is prevented. This state of T cell unresponsiveness to antigenic stimulation is a mechanism also referred as anergy.

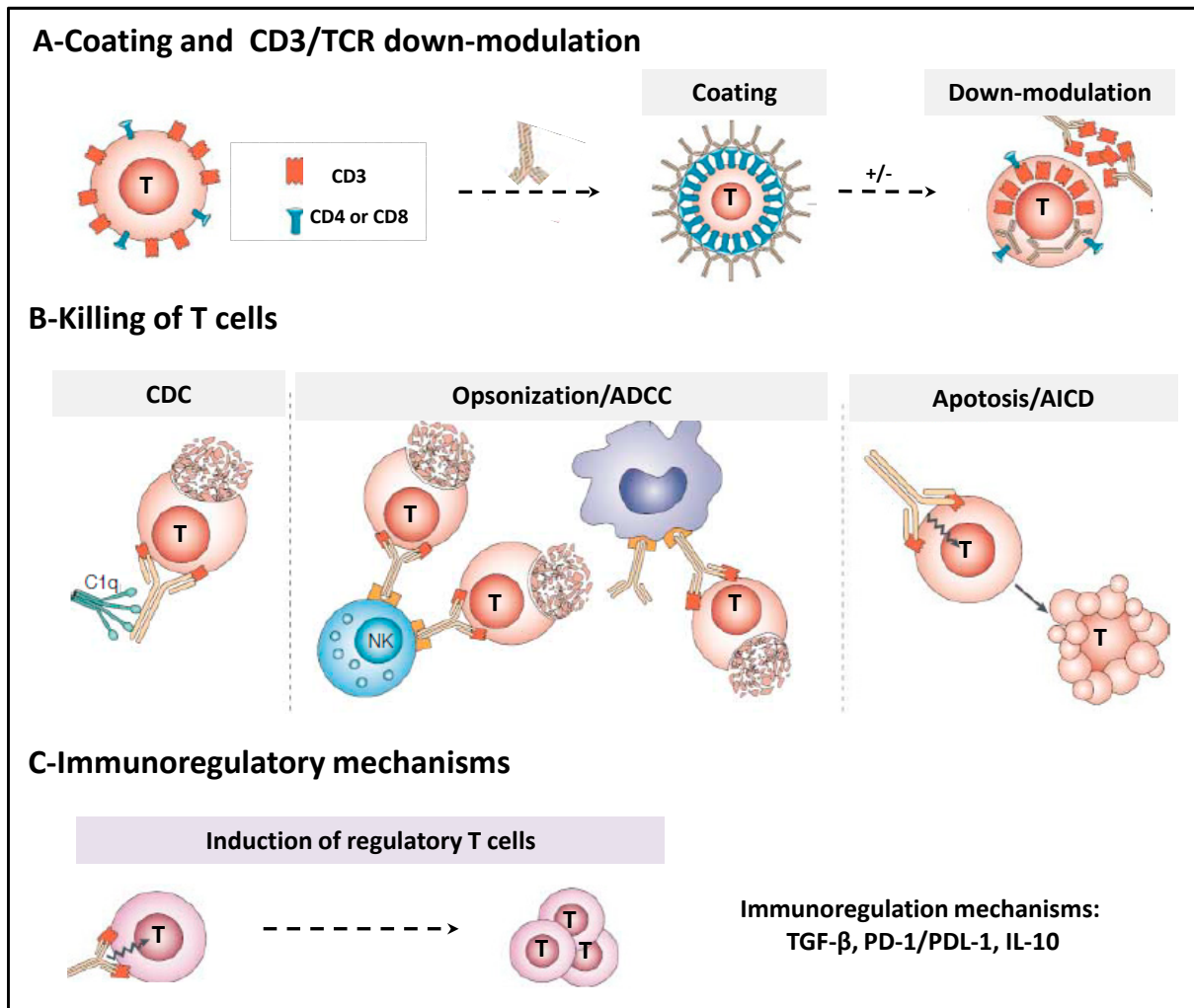


Figure 11. Mode of action of CD3-specific mAbs. This figure was adapted from (Lucienne Chatenoud, 2003).

Preferential killing of effector T cells while preserving Treg cells... a dream?

In addition to blocking T cell responsiveness, anti-CD3 mAbs can affect T cell responses by directly inducing the death of its target T cells (Lucienne Chatenoud & Bluestone, 2007) (Fig. 11B). Indeed, direct binding of anti-CD3 to a T cell can deliver intracellular signals leading to death by apoptosis, referred to as antibody-induced cell death (AICD). In addition, as the Fc part of the antibody can engage either complement or Fc γ R-bearing cells, bound anti-CD3

mAbs can also induce T cell death through complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC), respectively. Anti-CD3 mAbs potentially target any CD3-positive cell, including effector (Teff) and regulatory (Tregs) T cells, thereby promoting or preventing pathogenic T cell responses during autoimmunity, respectively. Interestingly, it has been observed that anti-CD3 mAbs could preferentially kill Teff cells when administered *in vivo*, while preserving Treg cell populations (Penaranda, Tang, & Bluestone, 2011). This interesting finding suggests that under certain conditions anti-CD3 mAbs treatment could reverse the balance between Teff and Treg cells by directly depleting autoreactive T cells. In this regard, the dose of anti-CD3 mAb used is critical. Low doses of the anti-CD3 antibody selectively depleted Teff cells, (e.g., 10µg per injection), but at higher doses, also depleted Treg cell populations, (e.g., starting from 100 µg per injection) (Lucienne Chatenoud & Bluestone, 2007). This observation exemplifies the necessity to use a dose of anti-CD3 mAb that depletes T cells in a selective manner (i.e., preferential killing of Teff cells rather than Treg cells).

Immunoregulatory mechanisms: introduction to Tregs, IL-10, TGF-β, and PD-1/PDL-1

A deregulated balance between Teff and Treg cell populations is a feature underlying many autoimmune disorders. As discussed, this balance can shift in favor of Treg cells when Teff cells are specifically depleted. An additional mechanism that could potentially amplify this shift is a concomitant expansion of Treg cell populations. Of particular interest is the fact that anti-CD3 mAbs are also known to induce differentiation and/or expansion of Treg cells, which remain still detectable after antibody clearance (Fig. 11C). As the presence of anti-CD3-induced Treg cells was concomitant with the observed long-term protection afforded in different animal model of autoimmune diseases (e.g., NOD and EAE), the contribution of

Treg cells in this prolonged protection was further investigated. Briefly, both Tregs cells and related immunoregulatory molecules including IL-10, TGF- β or PD-1/PDL-1, were found to orchestrate the long-term protection provided after anti-CD3 mAb treatment (Fig. 11C).

Formats of anti-CD3 mAbs: influence of the Fc part on their mode of action (MOA)

The different formats of existing anti-CD3 mAbs can be divided into Fc γ R-binders and non-Fc γ R-binders (Lucienne Chatenoud & Bluestone, 2007). The first anti-CD3 mAbs directed against human and mouse CD3 were Fc γ R-binders (i.e., OKT3 and 145-2C11 clones, respectively). Their ability to engage Fc γ R-mediated mechanisms was associated with a transient “flu-like syndrome”, provoked by a cytokine release storm. Indeed, once anti-CD3 mAbs bound to their cellular targets, the targeted T cells undergo a short and transient T cell activation process leading to the secretion of proinflammatory cytokines, including TNF, IL-6 and IFN γ , a process that is referred to as cytokine storm. To prevent such unwanted effects, non-Fc γ R-binding formats of anti-CD3 mAbs were generated. As expected, reduced levels of proinflammatory cytokines were secreted. The remaining question was to evaluate the extent to which the absence of Fc γ R-mediated mechanisms could impact on the long-term protection afforded by the classical Fc γ R-binding formats of anti-CD3 mAbs. Indeed, in addition to inducing cytokine release, engagement of the Fc part of these antibodies is also responsible for Fc-mediated depletion of T cells through CDC and ADCC mechanisms. As a result, non-Fc γ R-binding formats of anti-CD3 mAbs are likely to be less efficient at depleting T cells because they do not invoke CDC nor ADCC depleting mechanisms. Chatenoud and colleagues were the first to demonstrate that Fc-dependent mechanisms were not required for providing anti-CD3-induced long-term protection in a mouse model of type-1 diabetes (i.e., NOD mouse model) (L Chatenoud, Primo, & Bach, 1997). Similar observations were reported

by others in a mouse model of multiple sclerosis (i.e., EAE mouse models), further providing a rationale to develop non-Fc γ R-binding formats of anti-CD3 mAbs(Kohm et al., 2005).

Collectively, although non-Fc γ R binding anti-CD3 mAbs do not mediate Fc-mediated effector mechanisms (CDC, ADCC), they seem to be as potent as Fc γ R binding anti-CD3 mAbs at inducing long-term protection in several models of autoimmune disease.

4.2 Anti-CD3 mAbs in the clinic: formats and first indications

OKT3: from the discovery to its first use in patients

Anti-CD3 mAbs have immunosuppressive capacity through the T cell populations. Indeed, in humans, before the discovery of anti-CD3 mAbs, immunosuppressive agents were nonspecific, mainly inhibiting cellular proliferation. In the late seventies, Schlossman and colleagues generated the first anti-human CD3 mAb and named it OKT3, also referred to as Muromonab(Kung, Goldstein, Reinherz, & Schlossman, 1979). Muromonab is a mouse IgG2a neutralizing mAb directed against human CD3(Kjer-Nielsen et al., 2004; Salmerón, Sánchez-Madrid, Ursa, Fresno, & Alarcón, 1991). This antibody induces a transient but severe cytokine release when administered to humans. Furthermore, as it is a mouse antibody, Muromonab is highly immunogenic, leading to the production of neutralizing antibodies directed against the drug (i.e., human anti-mouse antibodies)(Sgro, 1995). Muromonab was successfully used in the clinic preventing transplant rejection(“A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. Ortho Multicenter Transplant Study Group,” 1985; Goldstein et al., 1986). However, the associated systemic cytokine storm limited its therapeutic use. As a consequence, to reduce both the

cytokine storm and immunogenicity associated with the in vivo use Muromonab, a new version of this antibody was generated, hOKT3g1-(ala-ala), also referred to as Teplizumab(Woodle et al., 1999). This improved version of Muromonab consists of a humanized mAb harboring mutations in its Fc portion that significantly reduce interactions with FcγRs, thus, rendering Teplizumab less activating.

Their successful use in T1D

In addition to transplantation, non-FcγR binding anti-CD3 mAbs were also used to treat T1D. Autoimmune diabetes is mediated by aggressive autoreactive T cells that gradually destroy the pancreatic beta-cells, the source of insulin. As T cells are important players in this disease, and considering the efficacy of non-FcγR binding anti-CD3 mAbs in reversing established disease in NOD mice, Herold and colleagues initiated the clinical use of Teplizumab to treat recently diagnosed T1D patients(Herold et al., 2002). The success of this first clinical trial was followed by the use of a new non-FcγR binding anti-CD3 mAb (i.e., ChAglyCD3 mAb, also referred to as Otelixizumab), which confirmed the clinical utility of non-FcγR binding anti-CD3 mAb to treat patients affected by recent-onset T1D(Keymeulen et al., 2005).

4.3 Anti-CD3 mAbs in mouse models of autoimmunity: T cell depletion, Tregs, TGFb, PD-1/PDL-1

A key feature of anti-CD3 mAbs is their unique ability to successfully reverse established disease in models of human autoimmune diseases such as T1D and Multiple sclerosis. In addition to reversing disease, anti-CD3 mAbs also provided long-term protection, preventing relapse even after their elimination from the body, thus making this treatment a potent new approach for curing of autoimmune disorders. These promising observations were at first difficult to explain, a reason that pushed several groups to investigate further the underlying mechanisms. Using the NOD mice, Chatenoud and colleagues pioneered our understanding on the *in vivo* mode of action of anti-CD3 mAbs.

Anti-CD3-induced Tregs

In the late eighties, Hayward and colleagues were the first to report a protective effect of anti-CD3 mAbs, demonstrating that its neonatal injection in NOD mice significantly reduced disease incidence (Hayward & Shreiber, 1989). This first study already suggested the therapeutic potential of anti-CD3 mAbs for treating NOD mice. Several years later, Chatenoud and colleagues were the first to demonstrate long-term anti-CD3-induced remission in adult NOD mice (L Chatenoud et al., 1997) (L Chatenoud, Thervet, Primo, & Bach, 1994). In these studies, a five day treatment with either FcγR binding or non-FcγR

binding anti-CD3 mAbs afforded long-lasting protection, ruling out a requirement for FcγR-mediated mechanisms.

In the pathogenesis of both the murine models T1D and NOD, the destruction of pancreatic islets results from autoreactive T cells specific for beta-cell antigens. As anti-CD3 mAbs induce immunosuppression of T cell responses, it was likely that a generalized T cell immunosuppression could explain the observed short-term protective effects. However, the persistence of the protective effect after clearance of the anti-CD3 mAb strongly suggested the existence of additional mechanisms providing long-lasting protection.

Interestingly, in NOD mice that were treated with anti-CD3 mAb, the immunosuppression was specifically restricted to islet antigens, as protected mice, while preserving the islet cells, rejected a MHC incompatible skin grafts (L Chatenoud et al., 1994). These observations suggested that, in addition to directly eliminating and/or inhibiting autoreactive T cells, antigen-specific tolerogenic mechanisms induced by anti-CD3 mAbs were also likely to be involved.

Tolerance can be ensured by T reg cell population. Interestingly, in NOD mice, it was demonstrated that anti-CD3 mAb treatment was associated with the peripheral induction of CD4⁺CD25⁺ Tregs cells and that these cells were responsible for the long-lasting protective effects (Belghith et al., 2003) (You et al., 2007). In addition, with use of adoptive transfer experiments into immunocompromised NOD-SCID mice, they demonstrated the ability of an anti-CD3 mAb to induce transferable T cell-dependent tolerance mediated by induced-Treg cells (Belghith et al., 2003) (You et al., 2007). Indeed, during the adoptive transfer experiments, diabetogenic CD4⁺CD25⁻ T cells alone were sufficient to transfer diabetes, but recipient mice were protected from disease when Tregs were added to these diabetogenic T cells.

Elucidation of the mechanisms by which anti-CD3-induced Treg cells function represents another important step in our understanding of the observed long-term protective effects. *In vivo*, Treg cells can function both through cell/cell contacts and soluble factors. Indeed, the contribution in this mechanism of the anti-inflammatory cytokine, TGF- β , as well as the inhibitory costimulatory molecules, PD-1/PDL-1, have been identified, as discussed in the next two sections.

Anti-CD3 and TGF- β

NOD mice

TGF- β is an anti-inflammatory molecule mainly involved in the resolution of inflammatory processes. Chatenoud and colleagues reported that TGF- β was involved in the tolerogenic effects provided by anti-CD3 mAb treatment in NOD mice (Lucienne Chatenoud & Bluestone, 2007). First, in protected mice, they observed that CD4⁺ T cells, restimulated *ex-vivo*, secreted elevated levels of TGF- β compared to those of untreated animals. Anti-CD3-induced CD4⁺CD25⁺ Foxp3⁺ Treg cells were shown to be the major source of TGF- β (Belghith et al., 2003) (You et al., 2007). Second, in mice protected by anti-CD3, neutralizing anti-TGF- β mAbs were used to assess the requirement of TGF- β in Treg cell function and disease protection. In mice protected by anti-CD3 mAb treatment, it was found that neutralization of TGF- β compromised the suppressive functions of Treg cells *ex-vivo*, and more interestingly, *in vivo* administration of anti-TGF- β reversed the protective effects afforded by anti-CD3 mAb treatment (Belghith et al., 2003) (You et al., 2007). Altogether, from a mechanistic point of view, these findings provided an explanation for the intriguing tolerogenic effects of anti-CD3 mAbs in the treatment of NOD mice. The proposed scenario was that in addition to inducing apoptosis of pathogenic T cells responsible at least for short-term protective effects,

anti-CD3 mAb treatment subsequently induced protective TGF- β -secreting CD4⁺CD25⁺Foxp3⁺ Tregs cells. However, with regard to the pivotal involvement of TGF- β in these tolerogenic effects, it is likely that sources of TGF- β other than Treg cells could also contribute to the protective effects afforded by anti-CD3 mAb treatment(Perruche et al., 2008).

EAE mice

The implication of TGF- β in the protective effects provided by anti-CD3 mAbs was also addressed in mice with EAE. In this model, similar to what was observed in NOD mice, anti-CD3 mAb treatment was potent at reversing established disease, whereas the use of neutralizing anti-TGF- β mAbs counteracted this effect(Perruche et al., 2008). Although previous findings from NOD mice identified Treg cells as the major source of TGF- β during the tolerogenic effects provided by anti-CD3 mAb, in mice with EAE it was demonstrated that phagocytes were the major producer of TGF- β (Perruche et al., 2008). As a consequence, pre-depletion of phagocytes prevented the protective effects afforded by anti-CD3 mAb treatment in EAE. In addition, by demonstrating the ability of phagocytes to produce high amounts of TGF- β in response to anti-CD3-induced T cell death, they proposed a model involving T cell death, phagocytes, TGF- β and Treg cells. In the proposed scenario, anti-CD3 mAbs induce apoptosis of CD4⁺ T cells. Apoptotic T cells become ingested by phagocytes, which then produce high amounts of TGF- β and thereby promote Treg cell induction. Based on these findings and those from NOD mice, TGF- β appears to be a key player in the long-term effects afforded by treatment with anti-CD3 mAbs(Lucienne Chatenoud & Bluestone, 2007). However, the finding that phagocytes are the major producers of TGF- β following anti-CD3 mAb treatment, suggest that we cannot exclude the possibility that Treg cell

induction simply mirrors the protective effects of TGF- β (Perruche et al., 2008). Although these findings suggest that anti-CD3-induced Treg cells are probably not the major source of TGF- β , other mechanisms mediated by Treg cells can also account for their protective role, such as interactions between programmed death (PD)-1 (PD-1) and programmed death-ligand (PDL)-1 (PDL-1)(Keir, Butte, Freeman, & Sharpe, 2008) (see Fig. 12).

Anti-CD3 and PD-1/PDL-1

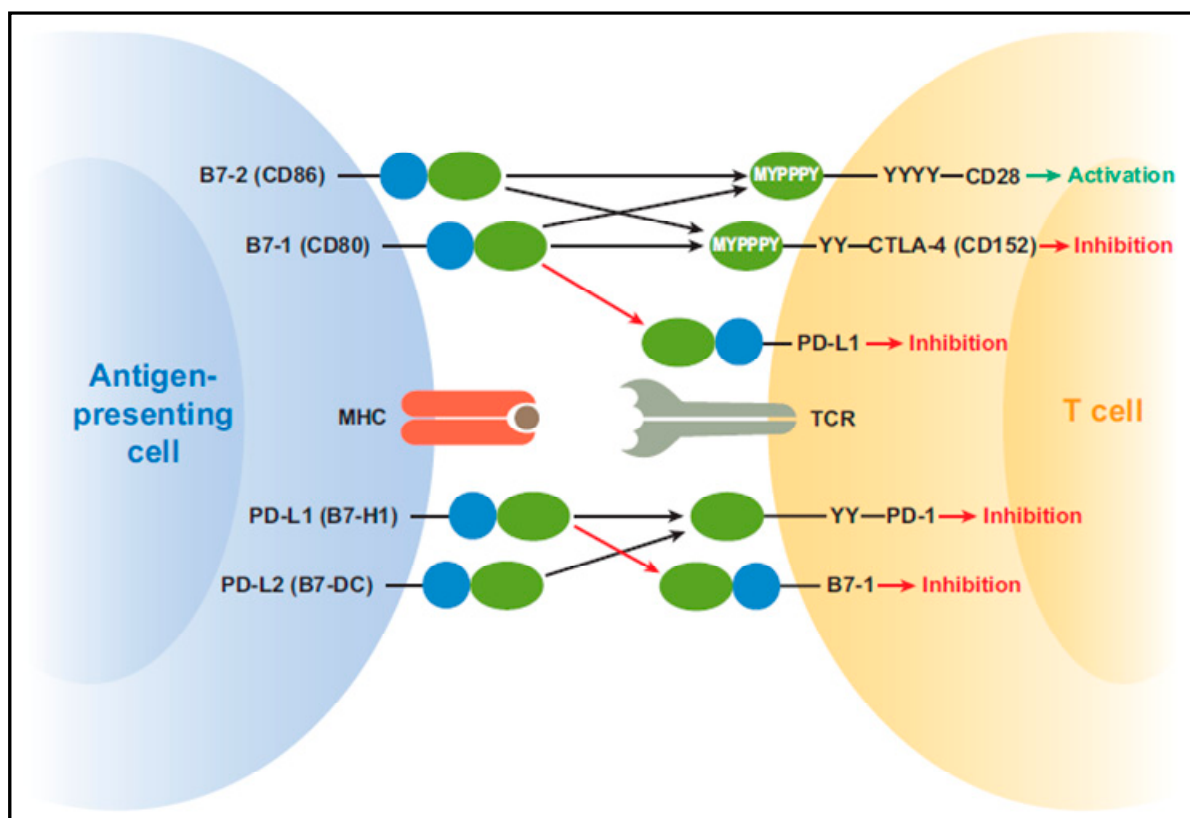


Figure 12. PD-1/PDL-1 inhibitory costimulatory molecules. This figure was taken from (Keir et al., 2008).

As mentioned, full T cell activation requires a first antigen-specific signal mediated by its TCR and a second non-antigen-specific signal. This second signal is mediated by costimulatory molecules, and the balance between activating and inhibitory costimulatory signals can either promote or attenuate T cell responses. Amongst members of the well-known B7-CD28 costimulatory family, PD-1/PDL-1 interactions that are illustrated in figure 12 deliver inhibitory signals that promote T cell tolerance(Keir et al., 2008).

Both PD-1 and its ligands, PDL-1 and PDL-2 are transmembrane proteins. Both PD-1 and PDL-1 can be found on the surface of different cells including activated T cells, B cells, activated monocytes and DCs (i.e., PDL-1 being also expressed on non-hematopoietic cells). The expression pattern of PDL-2 is more restricted, i.e., to DCs, macrophages and bone-marrow-derived mast cells.

The tolerogenic role of PD-1 was revealed by the observation that mice rendered genetically deficient for PD-1 exhibited accelerated autoimmunity in autoimmune-prone backgrounds(J. Wang et al., 2005). More precisely, PD-1 and PDL-1 are involved in both the induction and maintenance of tolerance, by inhibiting autoreactive naïve T cells but also subsequent T cell responses during antigen re-encounter. For example, in NOD mice it was reported that PDL-1 was upregulated in beta-cells(Liang et al., 2003), and neutralization of PD-1/PDL-1 interactions was associated with enhanced T cell responses with subsequent disease aggravation, suggesting a role for PD-1 and PDL-1 in T cell tolerance within tissues(Ansari et al., 2003; Keir et al., 2006; J. Wang et al., 2005).

Both PD-1/PDL-1 interactions and Treg cells contribute to maintain peripheral tolerance. As these molecules are expressed by Treg cells, Francisco and colleagues addressed the importance of PD-1/PDL-1 in the induction and the maintenance of Treg cell populations(Francisco et al., 2009). In their study, it was demonstrated that PD-1/PDL-1

interactions promote Foxp3 expression by induced-Treg (iTreg) cells and contribute to both the development and functional stabilization of iTreg cell populations.

Considering the contribution of anti-CD3-induced Treg cells to the induction of long-lasting tolerance in NOD mice, it was likely that, PD-1/PDL-1 interactions could also be involved in these protective effects. Interestingly, the group of Bluestone first reported that anti-CD3 mAb treatment failed to reverse disease in NOD mice rendered deficient for PDL-1 (Fife et al., 2006). Second, they also demonstrated that once anti-CD3-induced tolerance was established in NOD mice, *in vivo* administration of neutralizing anti-PDL-1 mAbs were sufficient to restore disease. Together, these findings demonstrated that PD-1/PDL-1 interactions are crucial for the maintenance of anti-CD3-induced tolerance afforded in NOD mice.

4.4 Anti-CD3 mAbs in the mouse model of CIA

As discussed, T cells are key players in arthritic pathogenesis. The contribution of T cells in murine CIA was essentially addressed by using neutralizing mAbs specific to T cell molecules. Surprisingly, in this model very few studies evaluated the effects of neutralizing T cell responses with mAbs specific for the CD3/TCR complex. A first study administering a non-FcγR binding anti-CD3 mAb (i.e., F(ab')₂ fragments of 145-2C11) around time of immunization demonstrated that T cell responses greatly contributed to the induction phase of the disease (C. Hughes, Wolos, Giannini, & Hirsch, 1994). They observed that prophylactic treatment of murine CIA with anti-CD3 mAbs was associated with T cell hyporesponsiveness resulting in a delayed and less severe onset of the disease.

As this study did not directly address the consequences of T cell neutralization in already established disease, a second study was conducted in the early nineties with anti-CD3 or anti-

TCR mAbs(Maeda et al., 1994). Surprisingly, although both mAbs were designed to inhibit T cell responses they exerted opposite effects. Treatment of murine CIA with Fc γ R binding anti-CD3 mAb (145-2C11) had limited but substantial protective effect on ongoing arthritis. In sharp contrast, the use of anti-TCR mAb in established arthritis exacerbated disease severity. These opposite results were difficult to explain as both antibodies were efficient at depleting T cells. In addition to differences inherent in their respective target specificity, two explanations were proposed to reconcile the opposing therapeutic effects of these two mAbs. First, as they did not know the IgG subclasses of the two clones, they suggested that a difference in Fc-mediated functions (CDC and ADCC) could account for their opposing effects on disease amelioration. Second, observing that the anti-TCR mAbs induced deeper and more sustained T cell disappearance, they hypothesized that unlike the anti-CD3 mAb, the anti-TCR mAb may have also depleted Treg cell populations. More recently, the group of Bluestone has recently demonstrated that *in vivo* administration of a non-Fc γ R-binder anti-CD3 mAb can selectively deplete pathogenic T cells while preserving Treg cells(Penaranda et al., 2011). Considering that the balance between T_{eff} and Treg populations is deregulated in favor of pathogenic T effector cells in autoimmune diseases, this last finding suggests that treatment with anti-CD3 mAb could potentially restore this balance towards increased regulation. In this scenario, the restoration of this balance can be achieved without concomitant induction and/or expansion of peripheral iTreg cells.

Given the ability of anti-CD3 mAbs to promote Treg cells *in vivo*, it is likely that treatment of murine CIA with anti-CD3 could both deplete pathogenic T cells and promote Treg cell induction/expansion. Recently, Notley and colleagues evaluated the extent to which the therapeutic use of Fc γ R-binding anti-CD3 mAbs (145-2C11) could impact on Treg cell populations and disease amelioration in CIA(C. a Notley, McCann, Inglis, & Williams, 2010). In this study, they observed a significant reduction in disease severity associated with

increased proportions of CD4⁺CD25⁺Foxp3⁺ Treg cells in the CD4⁺ lymph node T cell compartment. Although it was not addressed, it is likely that pathogenic Teff cells were also depleted in these mice. These findings demonstrate that T cells responses contribute to both early and established arthritis in the mouse model of CIA.

In addition to depleting pathogenic T cells, the *in vivo* administration of FcγR-binding format of anti-CD3 mAbs (1452-2C11) seems to significantly affect T cell responses in established arthritis, and promote the induction and/or expansion of Treg cell populations. However, to date, non-FcγR-binding anti-CD3 mAbs have never been used in established murine CIA. To overcome this limitation in our understanding of the biology of anti-CD3 mAbs in arthritis, it would be of interest to address the biological consequences of using this format of anti-CD3 mAbs in the therapeutic treatment of murine CIA.

AIMS OF THE THESIS

Both innate and adaptive immune components are involved in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA). RA is characterized by leukocyte infiltration and chronic inflammation of joints that induces synovitis and erosion of cartilage and bone. A widely used model of RA is collagen induced arthritis (CIA). In RA patients as well as in CIA, T cell infiltration and high levels of tumor necrosis factor (TNF) are observed in affected joints. T cells are thought to exert their effect during the induction phase of the disease while TNF, produced by activated macrophages, plays a critical role during the effector phase (Luross & Williams, 2001).

TNF blocking strategies produce a rapid clinical improvement in many patients with RA. However, some RA patients do not respond to TNF antagonists, and with time use of anti-TNF reagents becomes less effective in a substantial percentage of patients who primarily responded to this therapy. These observations underscore the complexity of the mechanisms contributing to chronic pathogenesis. Therefore, modifying both TNF and T cell responses in combination could lead to a synergy that provides long term relief by resetting the patient's immune system. A clinically proven way to alter T cell function is by the administration of anti-CD3 mAbs. The mechanism involves modulation of the CD3/TCR complex from the T cell surface and rapid disappearance of lymphocytes from the circulation. Anti-CD3 treatment in NOD mice has been shown to not only eliminate pathogenic effector T cells but also induce TGF- β dependent T regulatory cells (L Chatenoud et al., 1994) (You et al., 2007) (Perruche et al., 2008). However, the efficacy of anti-CD3 treatment in CIA remains controversial (Maeda et al., 1994) (C. Hughes et al., 1994), once again underlining the multi-factorial nature of this disease.

The aim of my thesis was therefore to study the dual targeting of an innate and adaptive immune element underlying an autoimmune disease, with RA as the prototype. This involved exploring the biological and clinical consequences of targeting TNF while also affecting the balance between different subsets of T cells, such as Th1, Th17 and Treg cells, by the administration of an anti-CD3 mAb. The combination of these two strategies achieved disease amelioration but not via the mechanisms that were originally hypothesized. A novel mechanism was revealed, thus providing the rationale to investigate further the induction of protection in other relevant models of human autoimmune diseases as well as to take the combination therapy approach into clinical development.

To study the biology of T cells and TNF during the pathogenesis of RA, the thesis project was divided into three main parts: (i) the generation and characterization of 2C11-Novi (a non-

FcγR binding anti-mouse CD3 mAb) and V1q-Novi (a neutralizing anti-mouse TNF mAb), (ii) the study of the *in vivo* effects on clinical disease of 2C11-Novi alone, V1q-Novi alone, and their combined administration in mice with CIA, and (iii) the mechanisms underlying the synergistic effect observed for the combined 2C11-Novi/V1q-Novi therapy .

1- Generation of mAbs specific for mouse CD3ε and mouse TNF

The first part of my thesis work consisted of:

- The generation of 2C11-Novi and V1q-Novi
- mAb production in gram amounts for *in vivo* studies
- Characterization of the mAbs *in vitro* and *in vivo*

These results are presented in Chapter I of the Results section.

2-Study the biology of administering an anti-CD3 mAb in the treatment of established arthritis using murine CIA as a preclinical model of RA

In murine CIA, I evaluated the *in vivo* consequences of an anti-CD3 monotherapy on T cell responses. Thi involved the assesement of:

- depletion of T cells *in vivo*
- antigenic modulation at the T cell surface (i.e. down-regulation of the CD3/TCR complex)
- T cell responsiveness following antigen-specific and non-antigen-specific stimulation
- clinical and histological scores

These results are presented in Chapters II and III Chapter I of the Results section.

3- Evaluate the therapeutic potential of combined targeting of TNF and T cells in established arthritis using the murine CIA model

I addressed, for the first time, the *in vivo* consequences of combining a non-Fc γ R binding anti-CD3 mAb with an Fc γ R binding anti-TNF mAb to treat ongoing arthritis in mice with CIA. Furthermore, I investigated the affected biology in order to propose a mechanism accounting for our observations. The experiments were conducted in three steps:

- **Identify an appropriate dose regimen to explore the potential of combined anti-CD3/anti-TNF therapy in mice with CIA:** (i) find a subtherapeutic dose regimen for the anti-TNF mAb, and then (ii) select the dose regimen for the anti-CD3 mAb that could provide the best therapeutic effect when combined with the subtherapeutic anti-TNF treatment.
- **Evaluate the long term efficacy of 2C11-Novi/V1q-Novi combination therapy on established arthritis:** the impact on disease was evaluated by monitoring clinical signs and symptoms as well as performing histology of the affected joints over 28 days.
- **Investigate the underlying mechanisms:** evaluate the ability of the combined therapy to (i) directly affect the pathogenic T cell pool, (ii) promote regulatory responses involving Treg cells and the participation of immunoregulatory molecules such as TGF- β and PD-1/PDL-1, as well as (iii) induce immunosuppressive mechanisms including antigenic modulation and non pathogenic T cell depletion.

These results are presented in in Chapters II and III of the Results.

RESULTS INCLUDING MATERIALS AND METHODS

**CHAPTER-I: GENERATION AND CHARACTERIZATION
OF MURINE SURROGATES FOR THERAPEUTIC ANTI-CD3
AND ANTI-TNF MAB THERAPIES**

ABSTRACT

Muromonab (Orthoclone OKT3), a murine anti-human CD3 α mAb, was the first therapeutic mAb to be approved for clinical use in humans. In 1986, it was approved by the FDA for the treatment of glucocorticoid-resistant rejection of allogeneic renal transplant. However, its clinical use is limited due to its capacity to induce a severe debilitating cytokine storm resulting from strong agonism of the CD3/TCR complex via Fc-Fc γ R interactions. To avoid these unwanted pharmacodynamic manifestations, we and others developed anti-CD3 mAbs with improved safety profiles. These non-Fc γ R binding mAbs have been tested clinically in various autoimmune diseases with overall disappointing outcomes. In rheumatoid arthritis or inflammatory bowel disease, an inflammatory component mediated by cytokines such as TNF plays important roles, thus, in these conditions we hypothesized that monotherapy with anti-CD3 may not be sufficient. In order to conduct meaningful *in vivo* studies aiming at evaluating potential synergism between anti-CD3 and anti-TNF mAb treatments, two murine mAbs were generated to act as surrogate therapies. The Fc engineered anti-mouse CD3 α mAb, 2C11-Novi, was shown to reproduce many pharmacological features of our non-Fc γ R binding anti-human CD3 α mAb therapy (i.e. Foralumab), including its pharmacokinetics. To determine the pharmacokinetics of 2C11-Novi in a sandwich ELISA-based bioanalytical assay, a neutralizing anti-idiotypic mAb, 2H2-Novi, was developed and used as capture reagent. Finally, a third murine mAb, V1q-Novi, was developed to act as surrogate for the anti-human TNF mAb therapies. Like Infliximab (Remicade) and Adalimumab (Humira), V1q-Novi was shown to bind Fc γ Rs and neutralize the biological activity of TNF, both *in vitro* and *in vivo*. In conclusion, this chapter describes the generation and characterization of relevant mAbs to conduct mechanistic *in vivo* studies aiming at exploring the mode of action of non-Fc γ R binding anti-CD3 mAbs either alone or in combination with anti-TNF mAb therapies.

INTRODUCTION

T cell activities are thought to be responsible for initiating and sustaining inflammation in a wide range of inflammatory diseases. As a result, strategies aimed at antagonizing pathogenic T cells have undergone intense investigation and the first therapeutic mAb to be approved by the food and drug administration was Muromonab (Orthoclone OKT3), a murine anti-human CD3 mAb(Kung et al., 1979)(Colvin & Preffer, 1991). Anti-CD3 mAbs are very strong immunosuppressors due to their capacity to rapidly induce transient (i) depletion of T cells and (ii) down-modulation of the CD3/TCR complex from the cell surface of remaining T cells(Lucienne Chatenoud & Bluestone, 2007). CD3 and TCR molecules are non-covalently linked and co-modulated following *in vivo* treatment with anti-CD3 mAbs, while other antigens such as CD4 or CD8 remain unaltered(Lucienne Chatenoud, 2003). Modulation of the CD3/TCR complex by anti-CD3 mAb is sometimes referred to as antigenic modulation. Muromonab has been shown to potently reverse and prevent acute allograft rejection(Goldstein et al., 1986). However, its clinical use in chronic diseases is limited due to its capacity to induce human anti-mouse antibodies and a severe debilitating cytokine storm resulting from a strong agonist activity on the CD3/TCR complex(Sgro, 1995). To avoid these unwanted pharmacodynamic (PD) manifestations, we and others developed improved anti-CD3 mAbs. The first generation of this novel class of improved anti-CD3 mAbs includes Teplizumab (HuOKT3 γ 1 Ala-Ala)(Woodle et al., 1999), Otelixizumab (ChAglyCD3)(Keymeulen et al., 2005) and Visilizumab (Nuvion)(Paul A Carpenter et al., 2002). These mAbs are humanized version of rodent anti-human CD3 mAbs to reduce their immunogenicity. In addition, they all have in common amino acid mutations in the CH2 domain of their Fc portion to reduce Fc γ R binding and the resulting cytokine release associated with cross-linking of the CD3/TCR complex via an Fc-dependent mechanism.

More recently we have generated the first representative of the second generation of improved anti-CD3 mAbs, Foralumab (NI-0401). Foralumab is the first fully human anti-human CD3 mAb that was obtained from transgenic mice harboring key gene sequences from unrearranged human immunoglobulin genes and inactivated mouse antibody genes. Foralumab's fully human format could reduce further its immunogenicity. In addition, the amino acid mutations introduced in the CH2 domain of Foralumab have been shown to abrogate the binding to FcγR *in vitro* (not shown).

These improved anti-human CD3 mAbs have been tested as mono- or add-on therapies in various autoimmune diseases, with mixed outcomes. In patients with new onset type 1 diabetes mellitus, while the early stage investigator sponsored trials with Teplizumab produced encouraging clinical results (Keymeulen et al., 2005) (Herold et al., 2002), its further development in Phase 3 randomized, placebo-controlled trials failed to meet the primary endpoints (Sherry et al., 2011). Furthermore, when conducting Phase 2a trials with Visilizumab or Foralumab in patients with inflammatory bowel disease (IBD), clinical benefit was deemed marginal in the context of the protocol used (Sandborn et al., 2010) (van der Woude et al., 2010). However, as compelling data exists from animal models and certain patient subpopulations that a treatment targeting CD3 will invoke clinical benefit (Lucienne Chatenoud & Bluestone, 2007) (J.-F. Bach & Chatenoud, 2011), where have we gone wrong? The failure to translate these findings into robust and broader clinical success is certainly multifactorial. At the preclinical level, the anti-mouse CD3 mAbs may not have been representative of the next generation of anti-human CD3 mAbs (J. Li et al., 2006) (Chao, Ma, Li, Park, & Law, 2009), the consequence being that the pharmacology may not yet have been optimally established for correct translation from bench to bedside. From a clinical perspective, for conditions such as RA or IBD, an inflammatory component mediated by cytokines such as TNF plays important roles. Thus, monotherapy with anti-CD3 mAb may

not be sufficient to treat RA. The superiority of a combination therapy with anti-CD3 and anti-TNF mAbs is supported by previous results in the CIA model(Malfait, Williams, Malik, Maini, & Feldmann, 2001).

In this chapter we describe the generation of 2C11-Novi, a murine surrogate mAb for Fc-modified anti-human CD3 mAb therapies. Importantly, here we show that 2C11-Novi reproduces both the PK and PD properties of its humanized/human counterparts. In addition, in order to study potential synergy between anti-CD3 and anti-TNF mAb therapies, an anti-mouse TNF mAb was generated to act as surrogate for therapeutic anti-human TNF mAbs such as Infliximab (Remicade) and Adalimumab (Humira).

MATERIALS AND METHODS

Mice and mAb treatment.

Studies were conducted in adult naïve Balb/c mice (Janvier Laboratories, Le Genest-St-Isle, France). Mice were housed in groups of 10. All animal experimentation was conducted according to license from the Swiss veterinary office for animal experimentation. All mAbs were administered intraperitoneally at doses indicated.

Induction and assessment of Collagen-induced-arthritis (CIA).

Please refer to Chapter II in the RESULTS section.

Generation of 2C11-Novi and V1q-Novi mAb reagents.

2C11-Novi is a chimeric mAb specific to mouse CD3 ϵ that contains a hamster variable domain and a mouse constant region which was mutated in the CH2 domain to reduce the binding to Fc gamma receptors (Fc γ Rs). The variable domain was derived from a well-characterized hamster anti-mouse CD3 ϵ mAb, clone 145-2C11(Leo, Foo, Sachs, Samelson, & Bluestone, 1987). Whole mRNA was extracted from a total of $2 \cdot 10^6$ 145-2C11 hybridoma cells using phenol chloroform (SIGMA, #P3803-100mL). Complementary DNA (cDNA) synthesis was carried out with reverse transcriptase kit (Amersham Bioscience, #27-9264-01) and using 3 μ g mRNA according to the manufacturer's instructions. Specific cDNA encoding the variable heavy (VH) and variable light (VL) chain sequences of 145-2C11 mAb were amplified using the set of primers described in Appendix 1A by PCR. Agarose gel electrophoresis of the resulting DNA fragments revealed bands of the appropriate sizes i.e. 405 and 396 bases pairs as deduced from the cDNA sequences for the variable heavy (VH) and variable light (VL) chains, respectively (Figure 1).

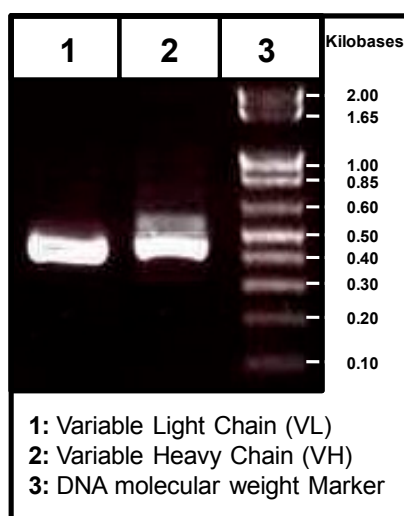


Figure 1. Agarose gel electrophoresis of fragments obtained from PCR amplification of the variable chains of 145-2C11 monoclonal antibody.

Amplified fragments corresponding to the VL and VH gene segments of 145-2C11 were introduced into two distinct cloning vectors (p-Light chain 2C11-Novi and p-Heavy chain 2C11-Novi) that contained the mouse kappa light chain and IgG1 heavy constant region, respectively (Figure 2). Hind III and Apa I restriction enzyme sites allowed for the cloning of the amplified fragments. The IgG1 heavy chain constant region was mutated at the EU position 265 from an aspartic acid to an alanine (D265A) by site-directed mutagenesis (QuickChange, Stratagen), primers used are detailed in appendix 1B. The resulting peptide sequences corresponding to VH and VL domains of 2C11-Novi are detailed in appendix 1C. The two cloning vectors mentioned above were then fused into a single double gene vector, i.e., double gene vector 2C11-Novi. Not I and Sal I restriction enzyme sites allowed for the cloning of these vector fragments. The resulting double gene expressing vector harbored a metabolic selection marker enabling the subsequent selection of 2C11-Novi producing cell clones (Figure 2).

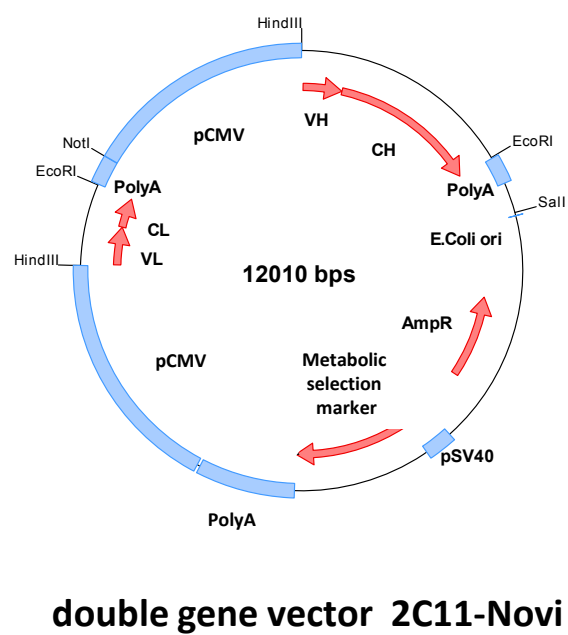
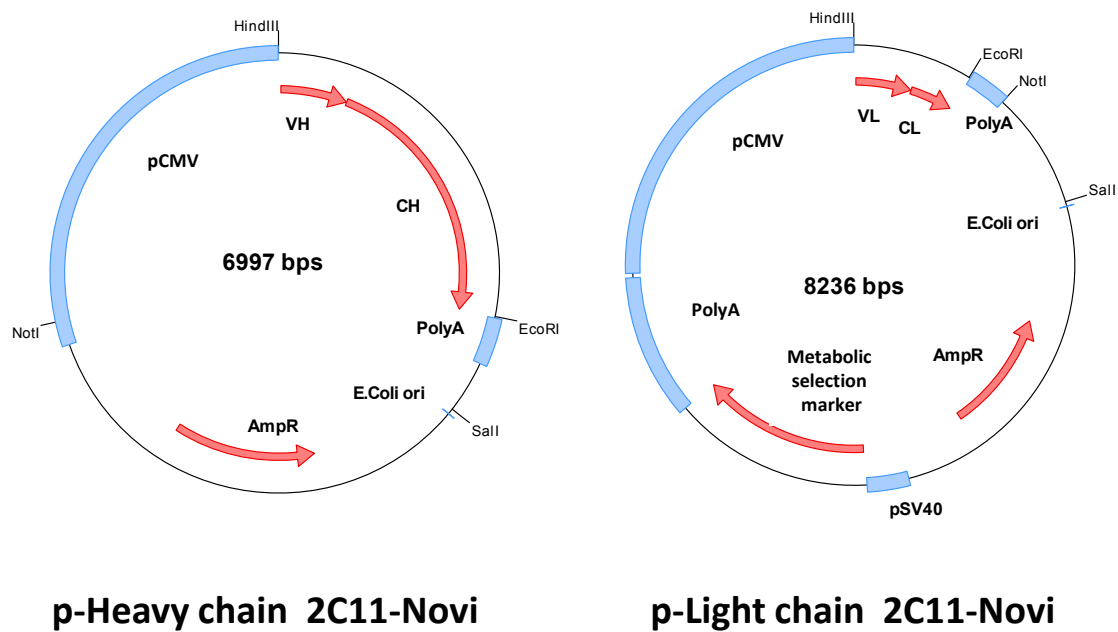


Figure 2. Schematic representation of the cloning vectors containing 2C11-Novi gene sequences.

CHO cells (1.10^7) were transfected by electroporation with 40 µg of linearized double gene vector 2C11-Novi and cultured in static flasks (T75, Nunc). Selective pressure was applied 24 hours later using a confidential selective agent in chemically-defined medium (CD-CHO, Invitrogen, #10743-029). Once the culture reached a density of 2.10^6 viable cells per mL, cells were adapted to growth in suspension in erlen flasks (Corning Life Science, #430422). Batch fermentation (10 liters) was operated in a 20 liter cellbag (GE Healthcare Lifesciences) using chemically-defined medium CD-CHO supplemented with the a confidential selective agent . After 10 days of culture, the supernatant was filtered by depth and sterilizing filtration (Sartorius Stedim, #3051445901E-SG) prior purification by protein G affinity chromatography (Mab Select Sure resin, GE Healthcare Lifesciences) using an AktaPurifer system. The concentration of purified 2C11-Novi was determined by measuring its absorbance at 280 nm by spectrophotometry.

The integrity and purity of the purified 2C11-Novi recombinant mAb were evaluated by SDS-PAGE (Figure 3), size exclusion chromatography combined with high performance liquid chromatography (SEC-HPLC) and limulus amoebocyte lysate (LAL) test (Charles River Endosafe Endochrome-K™ LAL test kit, #R1708K). Results are summarized in appendix 2.

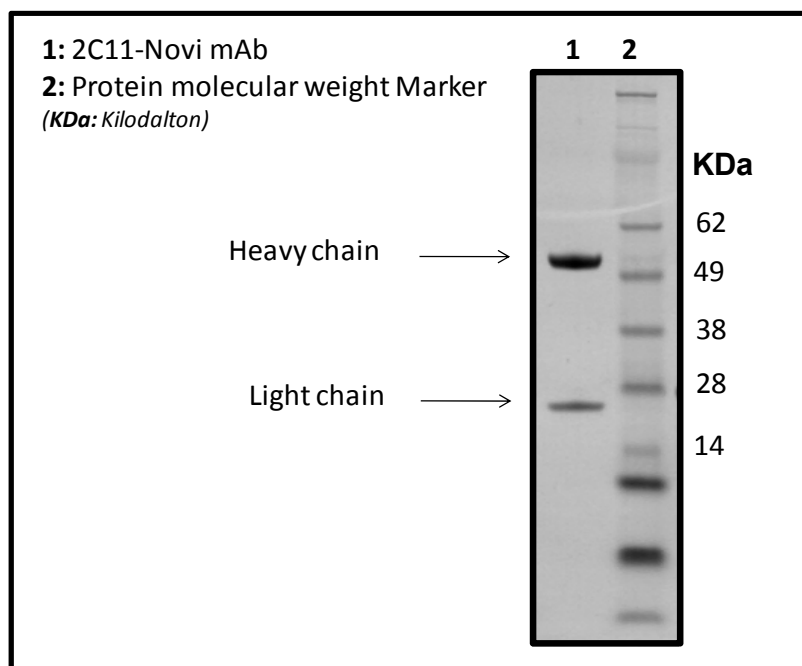
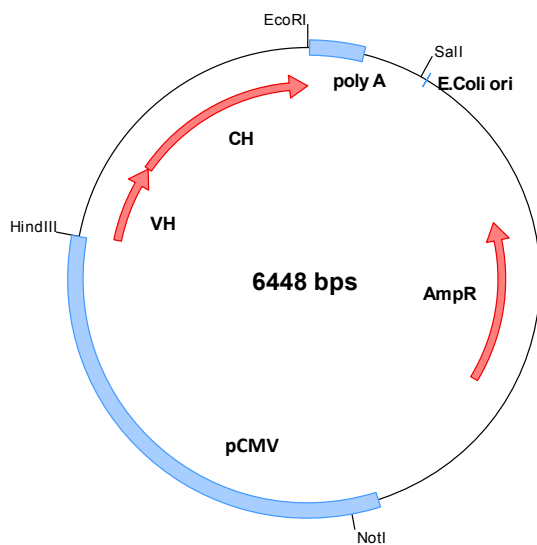


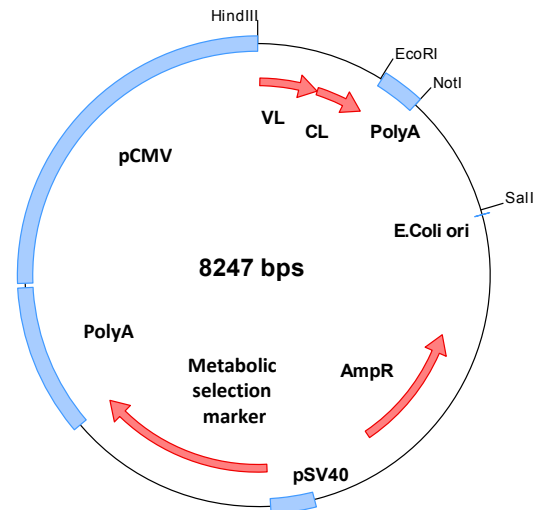
Figure 3. 2C11-Novi purified mAb profile after SDS-PAGE migration under denaturing conditions (β -mercapto-ethanol).

V1q-Novi is a chimeric mAb specific to mouse TNF that contains a rat variable domain and a mouse constant region which binds to Fc γ Rs. The variable domain was derived from a well-characterized rat anti-mouse TNF mAb, clone V1q(Echtenacher, Falk, Männel, & Krammer, 1990). Similarly to 2C11-Novi, whole mRNA was extracted from a total of $2 \cdot 10^6$ V1q hybridoma cells using phenol chloroform (SIGMA, #P3803-100mL). Complementary DNA (cDNA) synthesis was carried out with reverse transcriptase kit (Amersham Bioscience, #27-9264-01) and using 3 μ g mRNA according to the manufacturer's instructions. Specific cDNA encoding the VH and VL sequences of V1q mAb were amplified using the set of primers described in Appendix 3A by PCR. Agarose gel electrophoresis of the resulting DNA fragments revealed bands of the appropriate sizes i.e. 414 and 393 bases pairs as deduced from the cDNA sequences for the VH and VL, respectively (not shown). Amplified fragments corresponding to the VL and VH gene segments of V1q were introduced into two distinct

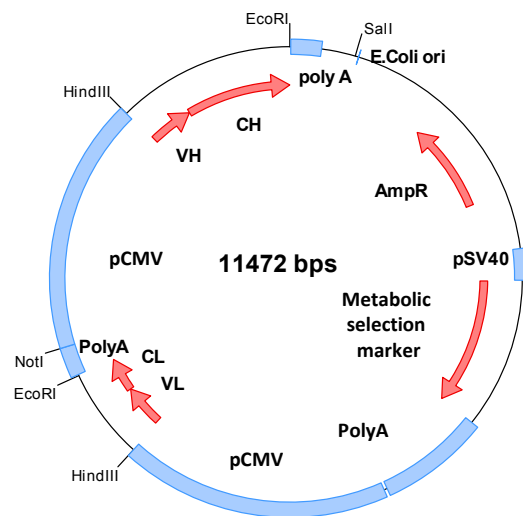
cloning vectors (p-Light chain V1q-Novi and p-Heavy chain V1q-Novi) that contained the mouse kappa light chain and IgG2a heavy constant region, respectively (Figure 4). The corresponding peptide sequences of both VH and VL domains of V1q-Novi are detailed in appendix 3B. Similarly to 2C11-Novi, Hind III and Apa I restriction enzyme sites allowed for the cloning of the amplified fragments. The two cloning vectors mentioned above were then fused into a single double gene vector, i.e., double gene vector V1q-Novi. Not I and Sal I restriction enzyme sites allowed for the cloning of these vector fragments. The resulting double gene expressing vector harbored a metabolic selection marker enabling the subsequent selection of V1q-Novi producing cell clones (Figure 4). Production, purification and subsequent quality analysis of V1q-Novi was performed similarly to that of 2C11-Novi (not shown).



p-Heavy chain V1q-Novi



p-Light chain V1q-Novi



double gene vector V1q-Novi

Figure 4. Schematic representation of the cloning vectors containing V1q-Novi gene sequences.

Generation of 2H2-Novi mAb reagent.

Naïve Balb/c mice (Janvier) received four subcutaneous injections on weeks 0, 1, 3, 7, of 50 µg of 2C11-Novi antibody dissolved in 300 µL RIBI adjuvant (Sigma Aldrich), and followed by a last hyperboost of 25 µg of 2C11-Novi antibody. A total of 45×10^6 lymph node (LN) cells from immunized mice and myeloma cells (Sp2/0) cells were resuspended in Cytofusion Medium (Cyto Pulse Sciences). Mixed cells were then transferred into a cuvette and electrofused using the electrofusion system Advanced PulseAgile® (Cyto Pulse Sciences, Model PA-4000/PA-101) with a single pulse (800 V, 1.4 MHz). Hybridoma cells were next incubated at 37°C, 5% CO₂ for two weeks in HAT selection medium supplemented with 1 µg/mL recombinant mouse IL-6, and the subcloning was performed using CLonePix^{FL} technology to ensure monoclonality. Resulting clones were screened in a series of *in vitro* assays to identify a mAb with three characteristics (Fig. 8). First, this mAb had to bind 2C11-Novi, 145-2C11 hamster IgG and 145-2C11 F(ab')₂ but not mouse and hamster isotype controls as assessed by ELISA (binding ELISA, Fig. 8B). Second, this mAb had to have the capacity to neutralize the binding of 145-2C11 to mouse T cell as assessed by flow cytometry (FACS, Fig. 8B and Fig. 8C). Third, this mAb had to have an isotype different from that of 2C11-Novi (i.e. IgG1) to allow detection of the latter using an anti-IgG1 mAb by ELISA (Fig. 8A). Two mAbs were identified to fulfill all of the above criteria i.e. 2G2 and 2H2. When used as coating reagent in the PK assay format described in Fig. 9A, both mAbs allowed quantification of 2C11-Novi in mouse plasma with excellent recovery (little or no matrix effect) and sensitivity (c.a. 4 ng/mL). Ultimately, the clone 2H2 was selected on the basis of a wider dynamic range (Fig. 9B).

Supernatants from 2H2-Novi hybridoma producing cells were filtered through a 0.22 µm filter (Millipore) prior purification by protein G affinity (Protein G Sepharose™ 4 Fast Flow, GE Healthcare) in a 50 mL tube (BD Falcon). The concentration of purified 2H2-Novi was

determined by measuring its absorbance at 280 nm by spectrophotometry. The integrity and purity of the purified 2H2-Novi mAb were evaluated by SDS-PAGE (not shown).

Pharmacokinetic assay specific to 2C11-Novi by ELISA.

Ninety six-well flat-bottom plates (Maxisorp-Nunc) were coated overnight at 4°C with 2 µg/mL 2H2-Novi anti-idiotypic antibody diluted in PBS. The content of the wells were flicked out and plates were blocked with PBS-2% BSA (Sigma Aldrich) for 1 hour at room temperature. Plates were washed once with PBS - 0.05% Tween 20. Diluted plasma samples, and 2C11-Novi standards were added into plates and incubated for 1 hour at 37°C. After four washes, bound-2C11-Novi mAbs were then revealed using a peroxidase labelled anti-mouse IgG1 mAb (BD Pharmingen, clone X56). Optical density (OD) was read at 450 nm using a plate spectrophotometer (Emax, Molecular Devices), and data were analysed with SoftMax Pro software.

Flow cytometry and measurement of cytokines.

For cell surface labeling, the following anti-mouse mAbs were used from BD Biosciences. Anti-CD3ε-PE (145-2C11), CD4-FITC (RM4-5), anti-CD8a-PerCP (53-6.7), anti-CD25-APC (PC61), anti-CD69-APC (H1.2F3), anti-TCR-PE (H57-597). Cells were incubated with Fc block (BD Biosciences) to reduce non-specific binding. For FcγR binding experiments, the cell surface expression of CD16/CD32 and CD64 on the RAW-264.7 cell line was confirmed using the PE-conjugated 2.4G2 and X54-5/7.1 mAbs, respectively. Serial dilutions of 2C11-Novi, V1q-Novi or the corresponding isotype controls were added to the RAW-264.7 cell line. Binding to FcγRs was detected using a DyLight 649 F(ab')₂ fragment goat anti-mouse IgG F(ab')₂ fragment specific (Jackson ImmunoResearch). The enumeration of circulating

CD4⁺ T cells was determined using TruCount tubes (BD Biosciences). Flow cytometry was performed using a FACS Calibur flow cytometer and analyzed with the CellQuestPro software (BD Biosciences). For cytokine quantification, two, four and six hours post-injection, mouse plasma were collected, and TNF, IL-6, and IFN- γ were quantified using the Milliplex mouse cytokine technology (Millipore).

CD3/TCR modulation.

CD3/TCR modulation was assessed on both CD4⁺ and CD8⁺ T cells using fluorescently-labeled anti-mouse CD3 (145-2C11) or anti-mouse TCR (H57-597) mAbs. Cells were ‘gated’ for CD4 or CD8 and then the CD3 or TCR cell-surface expression on CD4⁺ and CD8⁺ T cells analyzed by flow cytometry. The CD3/TCR modulation was presented in different ways. Sometimes the levels of CD3 or TCR expression (mean fluorescence intensity, mfi) was plotted on the same graph for treated and non-treated animals. Sometimes it was reported as levels of CD3 or TCR expression for treated animals relative to that of non-treated animals (%). In this case, antigenic modulation was calculated as follows:

$$CD3/TCR \text{ modulation (\%)} = [1 - (mfi \text{ treated} - \text{background mfi}^* / mfi \text{ non-treated} - \text{background mfi}^*)] \times 100$$

* The ”background mfi” corresponds to the level of T cell autofluorescence in mfi.

WEHI assay.

The capacity of V1q-Novi mAb to neutralize mouse TNF biological activity was evaluated *in vitro*. Prior to TNF stimulation (60 pg/mL), WEHI-164 cells were sensitized to TNF-induced

cell death using Actinomycin D (1 µg/mL). Inhibition of cell death was evaluated by measuring cell viability using the cell proliferation reagent WST-1 (Roche).

Statistical analysis.

Please refer to Chapter II in the RESULTS section.

Appendix 1. PCR primers and amino peptide sequences of 2C11-Novi.

A. Primers used to amplify the VH and VL genes from hybridoma 145-2C11 (ATCC, #CRL-1975).

5' primer: Leader 2C11 VH sens

AATTAAGCTTGCCGCCACCATGAACTCAGGACTCCAATTGG

3' primer: 2C11 VH anti-sens

GGAACCATGGTCACCGTCTCCTCAGCCAAAACGACACCCC

5' primer: Leader 2C11 VL sens

AATTAAGCTTGCCGCCACCATGAGGGCCCCTACTGTGTATCC

3' primer: 2C11 VL anti-sens

CCAAGCTGGAAATCAAACGGGCTGATGCTGCACCAACT

B. Primers used for site-directed mutagenesis (D265A) on CH2 domain of 2C11-Novi.

5' primer: D265A sens

CACGTGTGTTGTGGTAGCCATCAGCAAGGATGATC

3' primer: D265A anti-sens

GATCATCCTTGCTGATGGCTACCACAACACACGTG

C. Amino acid sequence of variable chains of 2C11-Novi.

VH-2C11-Novi

MEWSWVFLFFLSVTTGVHSEVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGMHWV
RQAPGRGLESVAYITSSSINIKYADAVKGRFTVSRDNAKNLLFLQMNILKSEDTAMY
YCARFDWDKNYWGQGTMTVSS

VL-2C11-Novi

MSVPTQVLGLLLLWLTARCDIQMTQSPSSLPASLGDRVITNCQASQDISNYLNWYQ
QKPGKAPKLLIYYTNKLADGVPSRFSGSGSGRDSSFTISSLESEDIGSYYCQQYYNYP
WTFGPGTKLEIKRADAA

Appendix 2. Composition and general properties of 2C11-Novi mAb product.

Property	Value
Formulation buffer	Phosphate buffer saline
Appearance	Clear, colourless liquid
Protein concentration	4.86 mg/mL
Purity	
• IgG monomer	94.55%
• Aggregate level	5.45%
• IgG fragments	Not Detected
• Endotoxin level	0.023 IU/mL <i>i.e. 0.005 IU/mg</i>
Extinction coefficient : $E_{1\text{ cm}}^{1\%}$	15.5 <i>i.e. 231'500 M⁻¹cm⁻¹</i>
Nominal theoretical molecular mass	148'891 Da

Appendix 3. PCR primers and amino peptide sequences of V1q-Novi.

A. Primers used to amplify the VH and VL genes from hybridoma cell lines producing the anti-mouse TNF mAb, clone V1q.

5' primer: Leader V1q VH sens

CACGAAGCTTGCCGCCACCATGGAATGGAGCTGGGTCTTTCTC

3' primer: V1q VH anti-sens

CCCCGGCAGCAAAACCGACTCCTCTGACACTGG

5' primer: V1q VL sens

CACGAAGCTTGCCGCCACCATGAGTGTGCCCACTCAGGTCCT

3' primer: V1q VL anti-sens

CCACGTCGTAGTCGTGCA AAGTCGAGGTCAAACCAGGGTTGAGG

B. Amino acid sequence of variable chains of V1q-Novi.

VH-V1q-Novi

MEWSWVFLFSLSVTTGVHSQVQLKESGPGLVQPSQTLSTCTVSGFSLTSYNVHWVR
QPPGKGLEWMGRMRYNGDTSYNSALKSRLSISRDTSKNQVFLKMNSLQTDDTGTTY
CTRDRFSWASYFDYWGQGVMTVSS

VL-V1q-Novi

MSVPTQVLGLLLLWLTDARCNIQLTQSPSLLSASVGDRVTLSCCKGSQNINFLAWYQ
QELGEAPKLLIYNTNSLQTGIPSRFTGSGSGTDYTLTISSLQPEDVATYFCYQYNNGNT
FGVGTKLELKRADAA

RESULTS

In order to evaluate the potential synergy between anti-CD3 and anti-TNF therapies in subsequent *in vivo* studies (Chapter 2), two murine surrogate mAbs were engineered. Their functional characteristics as compared to the human therapeutic equivalents are summarized in Table 1.

Engineering of 2C11-Novi as a murine surrogate for non-Fc γ R binding anti-CD3 mAb therapies

The anti-mouse CD3 mAb, 2C11-Novi, was obtained by combining the Fv sequences of 145-2C11(Leo et al., 1987), a hamster anti-mouse CD3 mAb, with the constant domains of murine IgG1, producing a chimeric hamster/mouse mAb (Fig. 5A). The sequences of the Fv portion of 145-2C11 were chosen as this mAb is directed against the epsilon chain of the mouse CD3/TCR complex(Leo et al., 1987). In addition, 145-2C11 has been shown to reproduce the immunosuppressive properties of muromonab, the only marketed anti-CD3 mAb. However, 145-2C11 binds to Fc γ R, triggering cytokine release as observed in patients treated with muromonab. Therefore, in order to generate a murine anti-CD3 mAb that can be used as surrogate for the new generation of anti-CD3 mAb therapies, murine IgG1 was selected as the Fc γ backbone and an alanine substitution was introduced at the amino acid position 265. This single amino acid mutation in the CH2 domain has been shown to drastically reduce the binding of IgG1 mAbs to Fc γ R(Nimmerjahn, Bruhns, Horiuchi, & Ravetch, 2005)(Baudino et al., 2008).

Pharmacodynamic characterization of 2C11-Novi

2C11-Novi was characterized both *in vitro* and *in vivo*. *In vitro*, 2C11-Novi had the same functional potency as 145-2C11, as assessed by measuring antigenic modulation by flow cytometry (Fig. 5B). This readout reflects the capacity to induce internalization of CD3/TCR

complexes from the surface of T cells. It is expressed as the percentage of CD3/TCR complex removed per T cell relative to baseline. As expected from the mutation introduced into the CH2 domain of its murine IgG1 Fc γ backbone, 2C11-Novi did not bind, even at high concentrations, to CD16, CD32 or CD64 which are expressed constitutively on the mouse macrophage cell line, RAW 264.7 (50 μ g/mL; Fig. 1C).

In vivo, anti-CD3 mAbs have been shown to induce a wide range of PD effects. Most of these are thought to be a consequence of T cell signaling resulting from a conformational change and/or cross-linking of CD3/TCR complex (Fig. 6). To evaluate the PD effects of 2C11-Novi *in vivo*, naïve DBA/1 mice were injected intraperitoneally with a single dose of 2C11-Novi or 145-2C11 using previously defined doses of 50 μ g and 20 μ g, respectively. First, the degree of antigenic modulation was assessed by measuring the levels of surface TCR expression of circulating and splenic CD4⁺ cells. After a single injection, 2C11-Novi and 145-2C11 similarly reduced, rapidly and almost completely, the levels of TCR expression at the surface of CD4⁺ T cells in blood (Fig. 7A) and spleen (Fig. 7B). In contrast, the kinetics of recovery of the CD3/TCR complex was strikingly different. For 145-2C11-treated mice, TCR expression levels had returned to baseline levels within 5 days post-treatment, whereas those of 2C11-Novi-treated mice remained low even 5 days after treatment. Next, we assessed whether 2C11-Novi induced T cell activation *in vivo*. Activation markers were analyzed on CD4⁺ splenocytes and cytokine release was measured in plasma. As expected, *in vivo* administration of a single dose of 145-2C11 induced a substantial increase in the proportions of CD69⁺ (Fig. 7C; mean \pm SEM at the peak, 91.2 \pm 2.3% 4h post-injection) and CD25⁺ T cells (Fig. 7D; mean \pm SEM at the peak, 79.9 \pm 2.8% 18h post-injection) in the spleen. In addition, it induced the release of the proinflammatory cytokines TNF (Fig. 7E), interferon- γ (IFN γ , Fig. 7F) and interleukin (IL)-6 (Fig. 7G); mean \pm SEM at the peak, 300 \pm 26 pg/mL 2h post-injection, 4642 \pm 227 pg/mL 6h post-injection and 14240 \pm 1419 pg/mL 4h post-injection,

respectively. 2C11-Novi was also able to activate T cells and induce cytokine release, albeit at a much lower level than 145-2C11. A consequence of anti-CD3 induced cytokine release is body weight loss. As shown in Fig. 7H, 2C11-Novi treated mice had no body weight loss while 145-2C11 treated mice had a significant reduction in body weight, at 72 and 96 h of 10.9% and 12.1%, respectively, as compared to baseline ($p < 0.0001$). These results concurred with the data obtained for cytokine released. Another *in vivo* feature of anti-CD3 mAbs is their capacity to induce a sharp reduction in T cell numbers in blood and tissues. We assessed the capacity of 2C11-Novi to induce lymphopenia and T cell depletion in tissues as compared to 145-2C11. As shown in Fig. 7I, 2C11-Novi induced a rapid profound and transient lymphopenia (96.5% average reduction of circulating $CD4^+$ cells as compared to baseline at 6h post-injection and 16.1% at 5 days post-injection). In the spleen, 2C11-Novi induced a delayed and less pronounced reduction in the number of T cells (Fig. 7J, 36.4% average reduction of $CD4^+$ splenocytes at 24h post-injection). In comparison, 145-2C11 induced a more profound reduction of $CD4^+$ T cell numbers in the spleen (60.8% average reduction of $CD4^+$ cells at 24h post-injection) that was more prolonged both in the blood and spleen (51.7% and 51.2% average reduction of $CD4^+$ cells at 5 days post-injection, respectively). Finally, 2C11-Novi also modulated, activated and reduced $CD8^+$ T cell numbers in a manner similar to that observed for $CD4^+$ T cells (data not shown).

Pharmacokinetic characterization of 2C11-Novi

To study the pharmacokinetics (PK) of 2C11-Novi, we sought to generate an anti-2C11-Novi idiotypic mAb with the ultimate goal to use it as capture reagent to specifically quantify 2C11-Novi in mouse plasma by sandwich ELISA. To this aim we immunized Balb/c mice with 2C11-Novi and screened clones in a series of *in vitro* assays to identify a mAb with three characteristics (Fig. 8A). First, this mAb had to bind to 2C11-Novi, 145-2C11 hamster IgG

and 145-2C11 F(ab')₂ but not to mouse and hamster isotype controls as assessed by ELISA (binding ELISA, Fig. 8B). Second, this mAb had to have the capacity to neutralize the binding of 145-2C11 to mouse T cell as assessed by flow cytometry (FACS, Fig. 8B and Fig. 8C). Third, this mAb had to have an isotype different from that of 2C11-Novi (i.e. IgG1) to allow detection of the latter using an anti-IgG1 mAb by ELISA (Fig. 9A). Two mAbs (2G2 and 2H2) were identified that fulfill all of the above criteria. When used as coating reagent in the PK assay format described in Fig. 9A, both mAbs allowed quantification of 2C11-Novi in mouse plasma with excellent recovery (little or no matrix effect) and sensitivity (c.a. 4 ng/mL). Ultimately, the clone 2H2 was selected on the basis of a wider dynamic range (Fig. 9B).

The assay was validated using plasma samples spiked with known concentrations of 2C11-Novi and a single ascending dose study was conducted in Balb/c mice (i.e. 0.25, 0.75, and 2.25 mg/kg, corresponding to approximately 5.5, 16.7 and 50 µg per mice, respectively). Single intravenous dose administration of 2C11-Novi showed good systemic exposure and distribution. The PK profiles exhibited a rapid increase after the injection, followed by a biphasic decrease in concentrations (Fig. 10A). The plasma PK over the dose range of 0.25 to 2.25 mg/kg were not dose proportionate based on C_{max} and AUC_{0-264h} suggesting the clearance was mediated by binding to the drug target, CD3 on T cells (not shown). The terminal half-lives of 2C11-Novi for the different dose groups ranged, on average, between 0.7 and 0.9 days. Interestingly, these PK properties are very similar to that of Foralumab in the clinic i.e. non-linear kinetics with terminal half life of 0.75 days after a single administration of 0.13 mg/kg (Fig. 10B).

Both the magnitude and duration of antigenic modulation on circulating CD4⁺ T cells increased in a dose-related manner (data not shown). Interestingly there is a tight correlation

between the rate of CD3/TCR 'demodulation' on circulating T cells and the decline in 2C11-Novi plasma concentrations (Fig. 11).

Engineering and characterization of V1q-Novi, a murine surrogate for anti-human TNF mAb therapies

The anti-mouse TNF mAb V1q-Novi is a chimeric mAb that was constructed by combining the Fv sequence of the rat mAb V1q(Echtenacher et al., 1990) with the constant domains of murine IgG2a. This Fv was selected as V1q neutralizes the biological function of TNF *in vivo*(Echtenacher et al., 1990). In addition, it has been shown to bind to both soluble and membrane-bound TNF(Gerspach et al., 2000), a feature shared by many TNF blockers(Gerspach et al., 2000)(T. Horiuchi, Mitoma, Harashima, Tsukamoto, & Shimoda, 2010). The IgG2a constant portion was selected as the effector function afforded by the Fc plays a role in the mechanism of action of therapeutic anti-TNF mAbs such as infliximab and adalimumab(Mitoma et al., 2008) (Table 1).

In order to characterize V1q-Novi, *in vitro* and *in vivo* functional experiments were conducted. *In vitro*, V1q-Novi inhibited TNF-mediated killing, in a dose dependent manner, of WEHI 164 cells, a murine fibrosarcoma cell line highly sensitive to TNF-induced cell death (Fig. 12A). In addition, V1q-Novi bound to RAW 264.7 cells in a dose dependent manner indicating that V1q-Novi interacts with FcγRs (Fig. 12B). Anti-TNF therapy is effective in reducing disease activity in patients with RA as well as in multiple preclinical models of RA including CIA(R O Williams et al., 1992)(Piguet et al., 1992). Therefore, we tested the capacity of V1q-Novi to inhibit disease progression in mice with established CIA. Arthritic mice were administered intraperitoneally from day 1 of disease onset with V1q-Novi at doses of 300 µg/mouse given once every 4 days. The clinical scores were evaluated every two days from day 1 (disease onset) to 23. The experiments demonstrated that V1q-Novi

inhibited arthritis progression in a statistically significant manner, confirming that V1q-Novi is functional *in vivo* (Fig. 12C).

DISCUSSION

Muromonab was the first therapeutic mAb to be approved for clinical use in humans (Colvin & Preffer, 1991). In 1986, it was approved by the FDA for the treatment of glucocorticoid-resistant rejection of allogeneic renal transplants (Goldstein et al., 1986). Muromonab is a mouse IgG2a mAb that is directed against the epsilon chain of the human CD3/TCR complex and that binds human FcγR via its Fc backbone. In the clinic, Muromonab induces both human anti-mouse antibodies and severe side effects resulting from cytokine release as a consequence of CD3/TCR cross-linking (Sgro, 1995). These side effects have prevented its clinical use in patients with autoimmune diseases. Several anti-CD3 mAb therapeutics have been developed to overcome unwanted the Muromonab-induced PD effects (Woodle et al., 1999) (Keymeulen et al., 2005) (Paul A Carpenter et al., 2002) (van der Woude et al., 2010) (Hirsch, Bluestone, DeNenno, & Gress, 1990). Like Muromonab, all of these are directed against the epsilon chain of the CD3/TCR complex. In order to reduce their immunogenicity these mAbs were engineered either as chimeric/humanized (i.e. Otelixizumab), fully humanized (i.e. Visilizumab and Teplizumab) or fully human (Foralumab) antibodies. In addition, all of them are mutated in the CH2 domain of their Fc backbone to reduce FcγR binding on leukocytes. Indeed, it has previously been shown that anti-CD3 mAbs exert their immunosuppressive and immunoregulatory effects largely via Fc-independent mechanisms (Lucienne Chatenoud & Bluestone, 2007).

This new generation of anti-human CD3 mAbs has been tested clinically in various autoimmune diseases(Lucienne Chatenoud & Bluestone, 2007). Reduced incidence of anti-drug antibodies and improved safety as compared to Muromonab were demonstrated in numerous clinical trials. However, efficacy wise, the results of the studies were disappointing. In patients with new onset type 1 diabetes mellitus, for example, Teplizumab failed to meet the primary endpoints in Phase 3 randomized, placebo-controlled trials(Sherry et al., 2011). Similarly, results from Phase 2a trials with Visilizumab and Foralumab in patients with inflammatory bowel disease (IBD) showed no clinically relevant therapeutic benefits(Sandborn et al., 2010)(van der Woude et al., 2010). The failure to translate compelling efficacy data generated in preclinical models of autoimmune diseases into clinical success is certainly multifactorial. At the preclinical level, the anti-mouse CD3 mAbs may not have been representative of the next generation of anti-human CD3 mAbs(J. Li et al., 2006)(Chao et al., 2009), the consequence being that the pharmacology may not yet have been optimally established for correct translation from bench to bedside.

The anti-human CD3 mAbs do not cross react with CD3 from standard animal laboratory species. Mechanistic studies in animal models of human diseases were therefore conducted with surrogate anti-CD3 mAbs. The first mAb of this kind is 145-2C11, a hamster anti-mouse CD3 mAb which reproduces the binding properties of Muromonab, i.e. specificity for the epsilon of the CD3/TCR complex and binding to FcγR. To act as surrogate mAb for the new generation of anti-human CD3 mAbs, the F(ab')₂ version of 145-2C11 has been used because it lacks the capacity to bind FcγR(Lucienne Chatenoud & Bluestone, 2007). However, due to a very short half-life *in vivo*, we and others considered that F(ab')₂ fragments were not appropriate surrogate molecules. To address these issues, as part of a “bedside to bench” process, we engineered an anti-mouse CD3 mAb, 2C11-Novi, to exhibit many of the

functional characteristics of Foralumab. 2C11-Novi is a chimeric form of 145-2C11 with a murine IgG1 Fc backbone mutated at a single amino position in the CH2 domain to abrogate FcγR mediated effector functions(Nimmerjahn et al., 2005)(Baudino et al., 2008).

In the present study we demonstrate that 2C11-Novi down modulates the CD3/TCR complex on mouse T cells and does not bind to FcγRs expressed on a murine monocytic cell line *in vitro*. *In vivo*, 2C11-Novi induced rapid and transient (i) antigenic modulation and (ii) reduced T cell counts in the periphery, as well as (iii) significantly less T cell activation as compared to 145-2C11. Importantly, 2C11-Novi shares these PD characteristics with Foralumab, which (i) modulates the CD3/TCR complex on human T cells, (ii) does not bind to FcγRs expressed on the human monocytic cell line THP1 and (iii) induces significantly less T cell activation as compared to Muromonab, *in vitro* (data not shown). *In vivo*, Foralumab has been shown to induce rapid, transient antigenic modulation and reduced T cell counts in the blood (data not shown).

FcγR binding anti-CD3 mAbs such as 145-2C11 and Muromomab, induce full activation of T cells by coengaging FcγRs and CD3 resulting in multivalent cross-linking and a conformational change of the CD3/TCR complex. Although Fc-modified anti-CD3 mAbs can no longer bind FcγRs they still have the potential to induce bivalent cross-linking and/or conformational change. This may explain why non/low FcγR-binding anti-human CD3 mAb therapies remain capable of inducing T cell activation and cytokine release after the initial dose in the clinic(Keymeulen et al., 2005)(van der Woude et al., 2010)(Herold et al., 2003)(Norman et al., 2000). Interestingly, 2C11-Novi reproduced this feature as it induced low but detectable levels of T cell activation and cytokine release including TNF and IL-6.

In order to further characterize 2C11-Novi, we sought to develop an ELISA-based bioanalytical assay to study its pharmacokinetics. 2C11-Novi recognizes a conformational epitope localized on the epsilon chain of the CD3/TCR complex which is particularly

challenging to express as a recombinant soluble antigen. Therefore, as capture reagent, we generated an anti-idiotypic mAb, 2H2-Novi, that was selected based on its ability to neutralize the binding of 145-2C11 to mouse T cells. 2H2-Novi is a mouse IgG2a mAb that can be coated on plates to specifically “capture” 2C11-Novi allowing its detection using an anti-mouse IgG1 antibody. Using this assay, the terminal half-life of 2C11-Novi was determined to be very similar to that of Foralumab i.e. between 0.7 and 0.9 days after a single parenteral administration. Interestingly, we observed a tight correlation between the clearance of 2C11-Novi in the plasma and the rate of CD3/TCR ‘demodulation’ on circulating T cells. These findings indicate that 2C11-Novi reproduces both the PK and PD characteristics of Foralumab.

From a clinical perspective the failure to translate preclinical results into clinical success for conditions such as IBD or RA, may be due to a dominant role played by an inflammatory component mediated by cytokines such as TNF. In other words, monotherapy with anti-CD3 mAbs may not be sufficient to treat RA and combination therapy with an anti-TNF mAb may increase the clinical benefit. Thus, in order to study potential synergy between anti-CD3 and anti-TNF mAb therapies, an anti-mouse TNF mAb was generated to act as surrogate for therapeutic anti-human TNF such as Infliximab (Remicade) and Adalimumab (Humira). For the surrogate anti-TNF mAb, we used the variable regions of V1q, a mouse anti-TNF mAb that neutralizes TNF *in vivo* and binds both the soluble and transmembrane forms of the cytokine (Echtenacher et al., 1990). However, as V1q is a rat IgD, it does not elicit effector functions which can be important for the therapeutic effects of anti-TNF therapies (Gerspach et al., 2000) (T. Horiuchi et al., 2010). Therefore, we produced a rat/mouse chimeric mAb, V1q-Novi, having the variable regions of V1q engineered onto a mouse IgG2a Fc backbone. A similar chimeric surrogate mAb has been shown to successfully neutralize TNF *in vivo* (Echtenacher et al., 1990) (Scott et al., 2003). We confirmed that V1q-Novi neutralizes

the biological activity of TNF, both *in vitro* and *in vivo*, and retains the capacity to interact with Fc γ R.

Taken together, the data described in this chapter indicate that 2C11-Novi and V1q-Novi are murine surrogates for anti-CD3 and anti-TNF mAb therapies, respectively. These mAbs share key functional characteristics with their human counterparts and represent relevant tools to study potential synergy between anti-CD3 and anti-TNF mAb therapies in autoimmunity.

FIGURES AND LEGENDS

TABLE 1

Table 1. Surrogate anti-mouse mAbs for therapeutic anti-CD3 and anti-TNF counterparts

Specificity	Generic name / Clone	Fv function	Fc format	Binding to FcR
Human CD3	Muromonab (OKT3) ^a	Binding to CD3 and modulation of the CD3/TCR complex	Mouse IgG2a	+
	Teplizumab, Otelixizumab, Foralumab ^b		Mutated human IgG1	Reduced
Mouse CD3	145-2C11		Hamster IgG	+
	2C11-Novi		Mutated mouse IgG1	Reduced
Human TNF	Infliximab, Adalimumab, Golimumab ^a	Binding to and neutralization of soluble and transmembrane TNF	Human IgG1	+
Mouse TNF	V1q		Rat IgD	-
	V1q-Novi		Mouse IgG2a	+

^a Marketed mAb therapies

^b Next generation anti-CD3 mAbs in clinical development

FIGURE 5

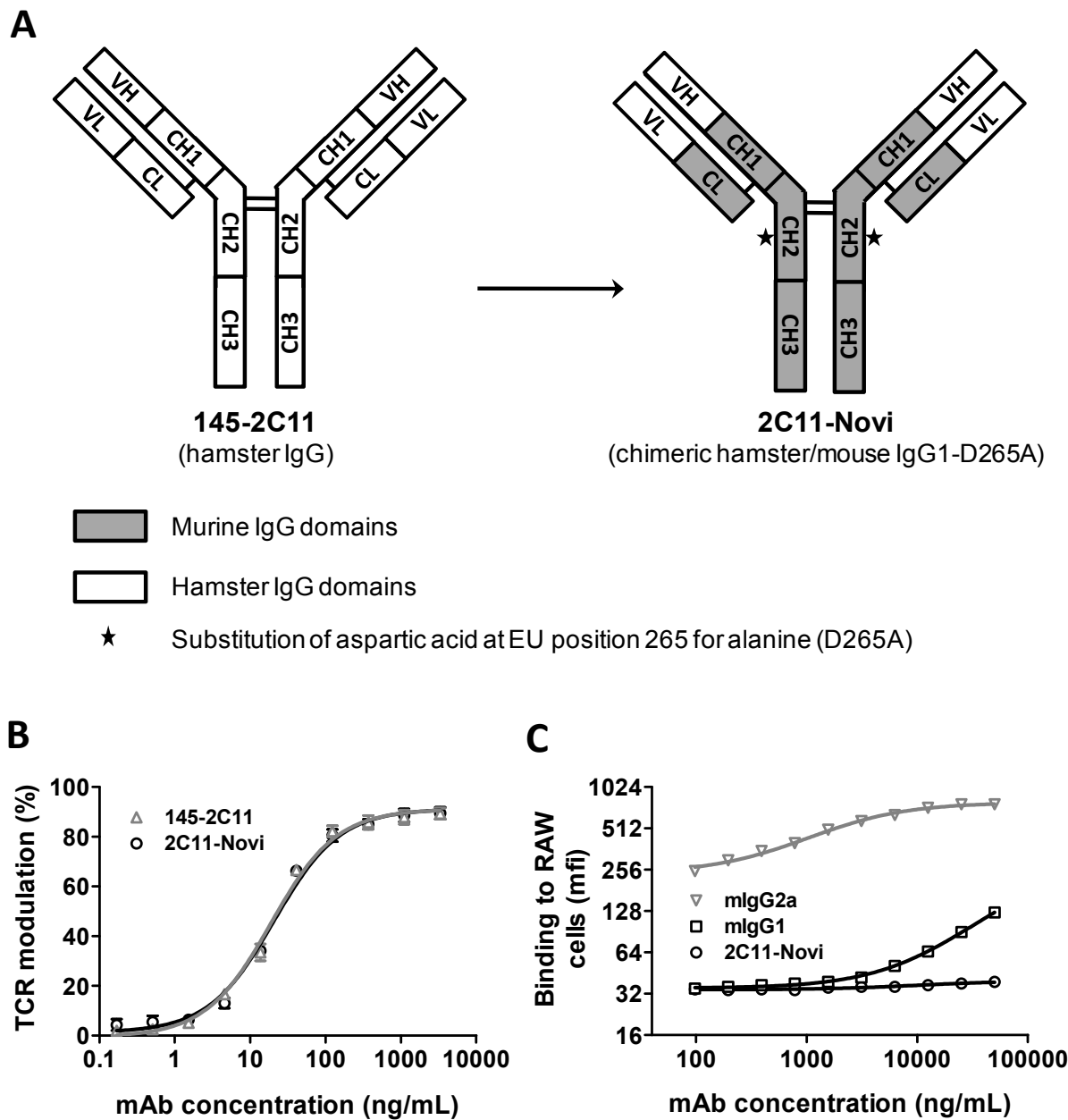


Figure 5: *In vitro* characterization of 2C11-Novi mAb.

A, Schematic representation of 145-2C11, a well-characterized hamster anti-mouse CD3 ϵ , and 2C11-Novi, a recombinant chimeric hamster/mouse anti-mouse CD3 ϵ IgG1 mAb with the D265A substitution. **B**, Antigenic modulation of CD3/TCR complex from the cell surface of mouse T cells assessed by flow cytometry. **C**, Binding to the RAW-264.7 cell line to evaluate Fc γ R engagement as assessed by flow cytometry. The following anti-mouse mAbs were used (from BD Biosciences); Anti-TCR-PE (H57-597), anti-CD16/CD32 (2.4G2) and CD64 (X54-5/7.1). For binding to Fc γ R: serial dilutions of 2C11-Novi or the corresponding isotype controls were added to the RAW-264.7 cell line. Binding to Fc γ R was detected using a DyLight 649 F(ab')₂ fragment goat anti-mouse IgG F(ab')₂ fragment specific (Jackson ImmunoResearch).

FIGURE 6

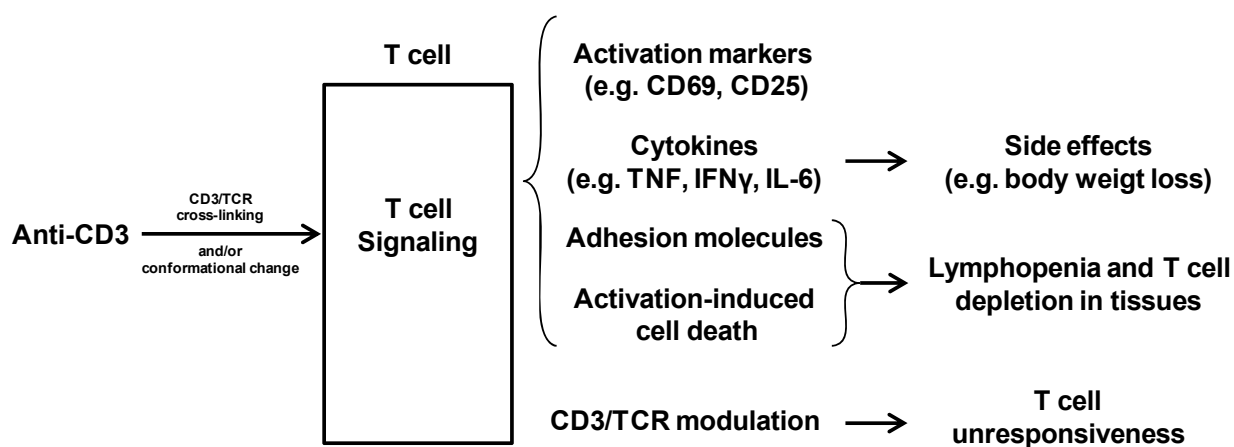


Figure 6: *In vivo* pharmacodynamic effects of anti-CD3 mAbs

Most PD effects induced by anti-CD3 mAbs are a consequence of T cell signaling events occurring after partial (non-Fc γ R binding anti-CD3) or full (Fc γ R binding anti-CD3) agonism of the CD3/TCR complex.

FIGURE 7

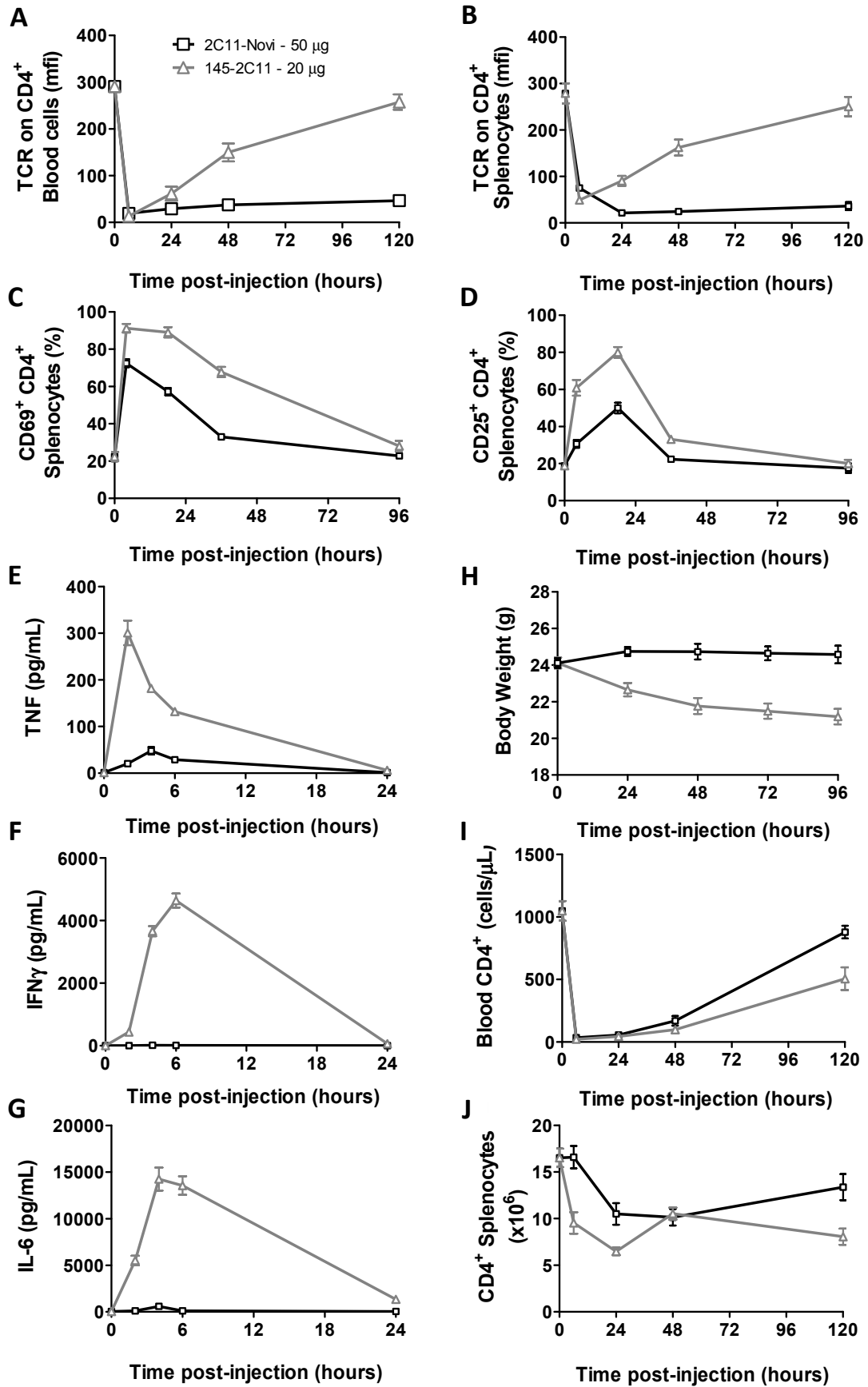
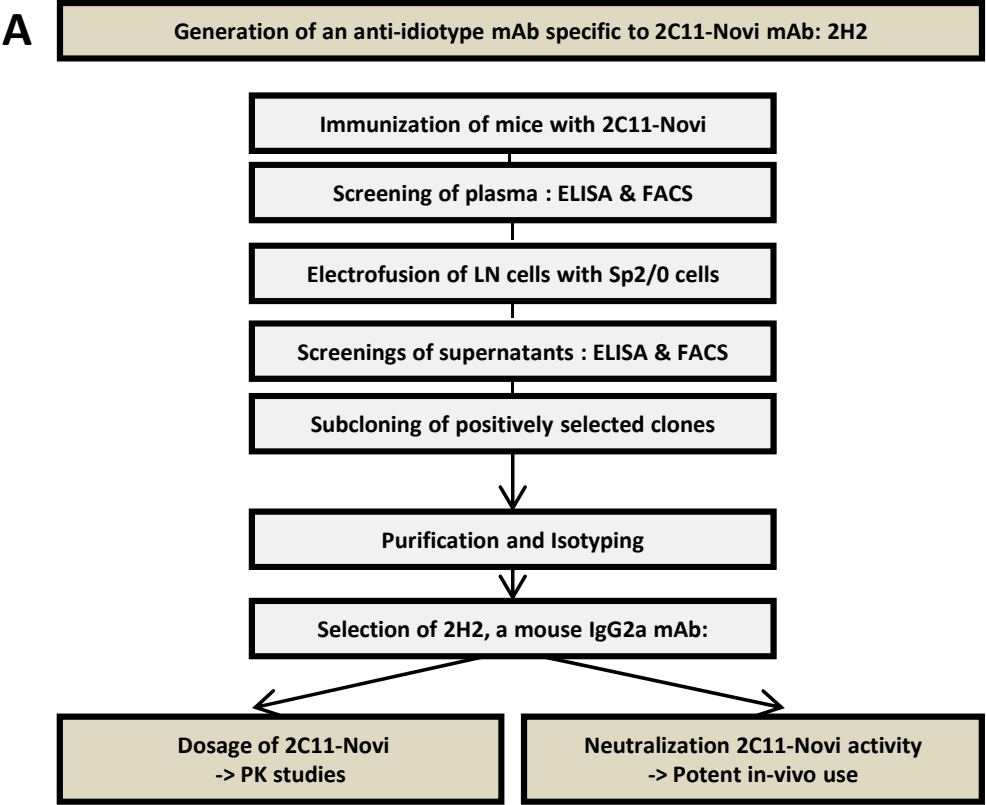


Figure 7: *In vivo* characterization of 2C11-Novi mAb as compared with 145-2C11 mAb.

A, TCR expression levels on circulating CD4⁺ T cells and **B**, CD4⁺ splenocytes as assessed by flow cytometry. **C**, Frequency of CD4⁺ splenocytes that express CD69 and **D**, CD25 as assessed by flow cytometry. **E**, Plasma levels of TNF; **F**, IFN γ and **G**, IL-6 measured using multiplex analysis. **H**, Body weight of the mice. **I**, Absolute count of circulating CD4⁺ T cells and **J**, CD4⁺ splenocytes. All *in vivo* analyses were performed after a single dose of 50 μ g 2C11-Novi (black open squares) or 20 μ g 145-2C11 (grey open triangles) administered into naive DBA/1 mice. Spleen and blood/plasma samples were collected at the indicated time points. Results presented are representative of experiments performed on five to six mice per group.

FIGURE 8



B

	Binding ELISA	FACS	PK ELISA
Clone tested	3840	94	7
Positive clones	1507	7	2
Clone Selected	94	7	1

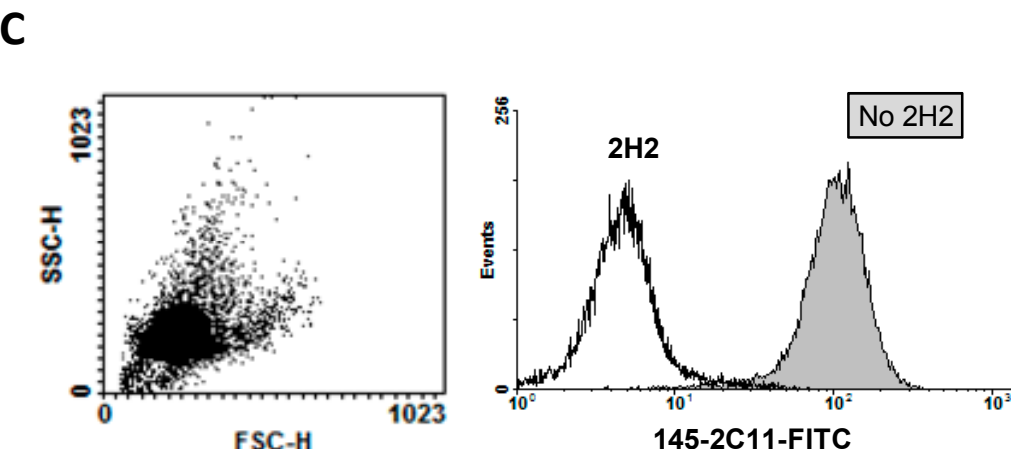


Figure 8: Generation of 2H2-Novi, a neutralizing anti-idiotypic mAb against 2C11-Novi

A, Overview of the successive steps during both generation and selection of an anti-2C11-Novi idiotype mAb. **B**, Summary of the screening process leading to selection of 2H2-Novi clone. Are represented the number of clones screened and positively selected through successive screening assays. ELISA assay tested each clone for binding to the variable region of 145-2C11, FACS assay tested for binding-inhibition of FITC-conjugated 145-2C11 (1 µg/mL) to mouse T cells. Ultimately, among the 7 clones that were identified to bind and neutralize 145-2C11, 5 had an IgG1 isotype and 2 were IgG2a i.e. 2H2 and 2G2. The anti-idiotypic, clone 2H2, providing the most sensitive PK assay specific for 2C11-Novi was further selected. **C**, The clone 2H2 inhibits the binding of 145-2C11 to the mouse T cell line 3DO-18.3.

FIGURE 9

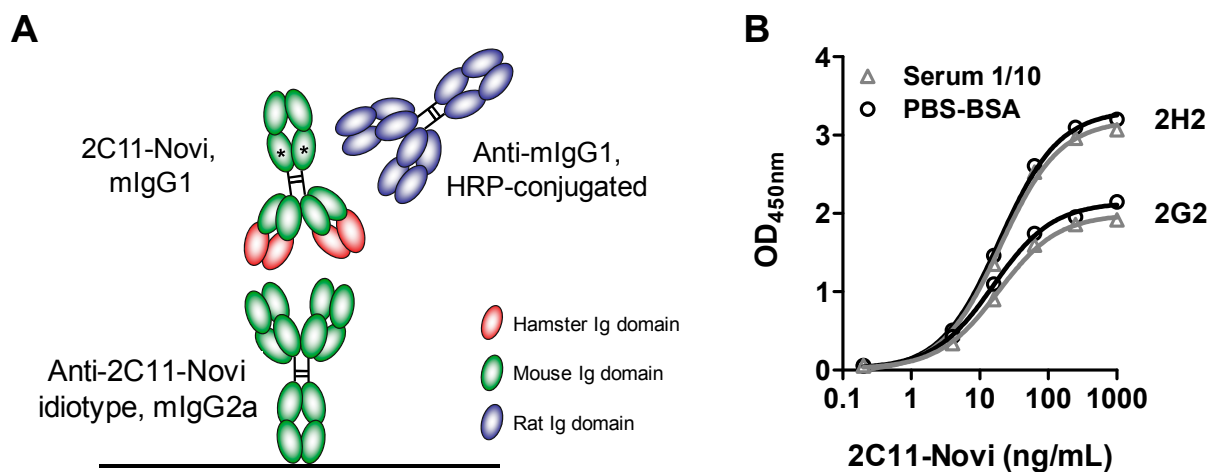


Figure 9: PK assay format and assay performance using 2G2 and 2H2-Novi anti-idiotypic mAbs.

A, Schematic representation of the ELISA-based bioanalytical assay developed to quantify concentrations of 2C11-Novi in mouse plasma. **B**, Mouse plasma samples (1/10) or PBS-BSA buffer spiked with known concentrations of 2C11-Novi were added to plates previously coated with immunobilized mouse anti-2C11-Novi idiotype mAb (clone 2G2 or 2H2-Novi, mIgG2a), and bound-2C11-Novi were then revealed using a peroxidase labelled rat anti-mouse IgG1 mAb.

FIGURE 10

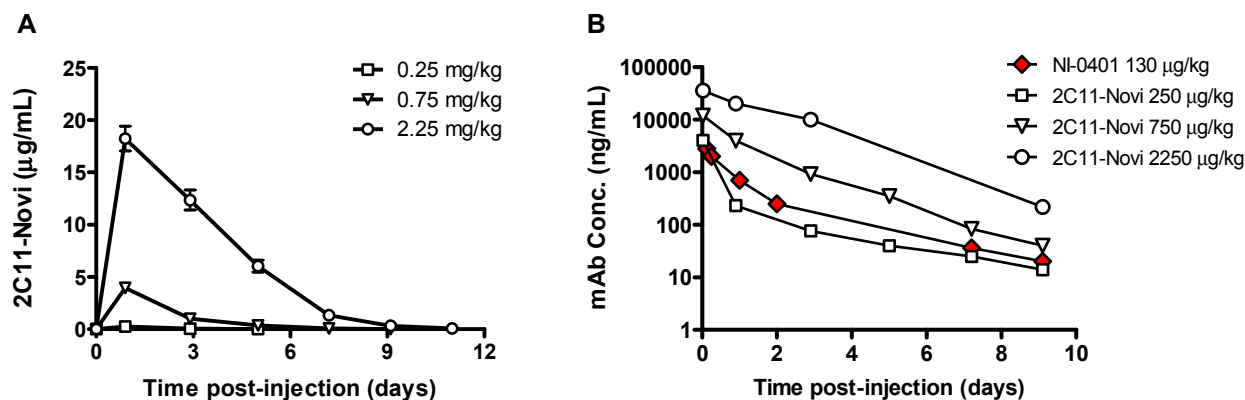


Figure 10: PK characterization of 2C11-Novi as compared with Foralumab (NI-0401).

A,B. Mice were injected intraperitoneally with a single dose of 2C11-Novi at 0.25, 0.75 or 2.25 mg/kg. Blood samples were collected at the indicated time point. Each mouse was bled twice and 3 mice were used for each time point. **A**, 2C11-Novi concentration-time profiles, linear scale and **B**, logarithmic scale. The NI-0401 concentration-time profile displayed in panel **B** was obtained from a patient with Crohn's disease treated with a single intravenous 2h infusion of 10 mg NI-0401 (130 $\mu\text{g/kg}$).

FIGURE 11

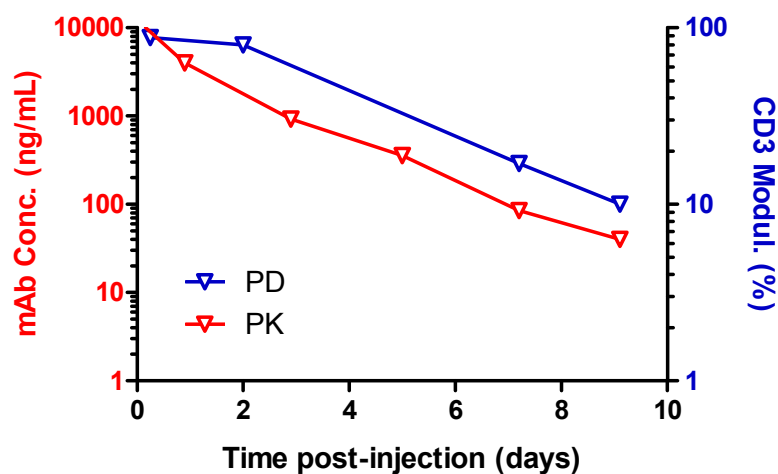


Figure 11: PK/PD profiles of 2C11-Novi.

The PK data correspond to plasma concentrations of 2C11-Novi and are plotted against the left vertical y-axis in red. The PD data correspond to CD3 modulation on CD4⁺ circulating T cells and are plotted against the right vertical y-axis in blue. Each data point represents the mean of 3 mice treated with a single intraperitoneal dose of 2C11-Novi of 750 µg/kg. Similar PK/PD profiles were observed for 250 and 2250 µg of 2C11-Novi per kg of body weight in mice as well as 130 µg of NI-0401 per kg of body weight in human.

FIGURE 12

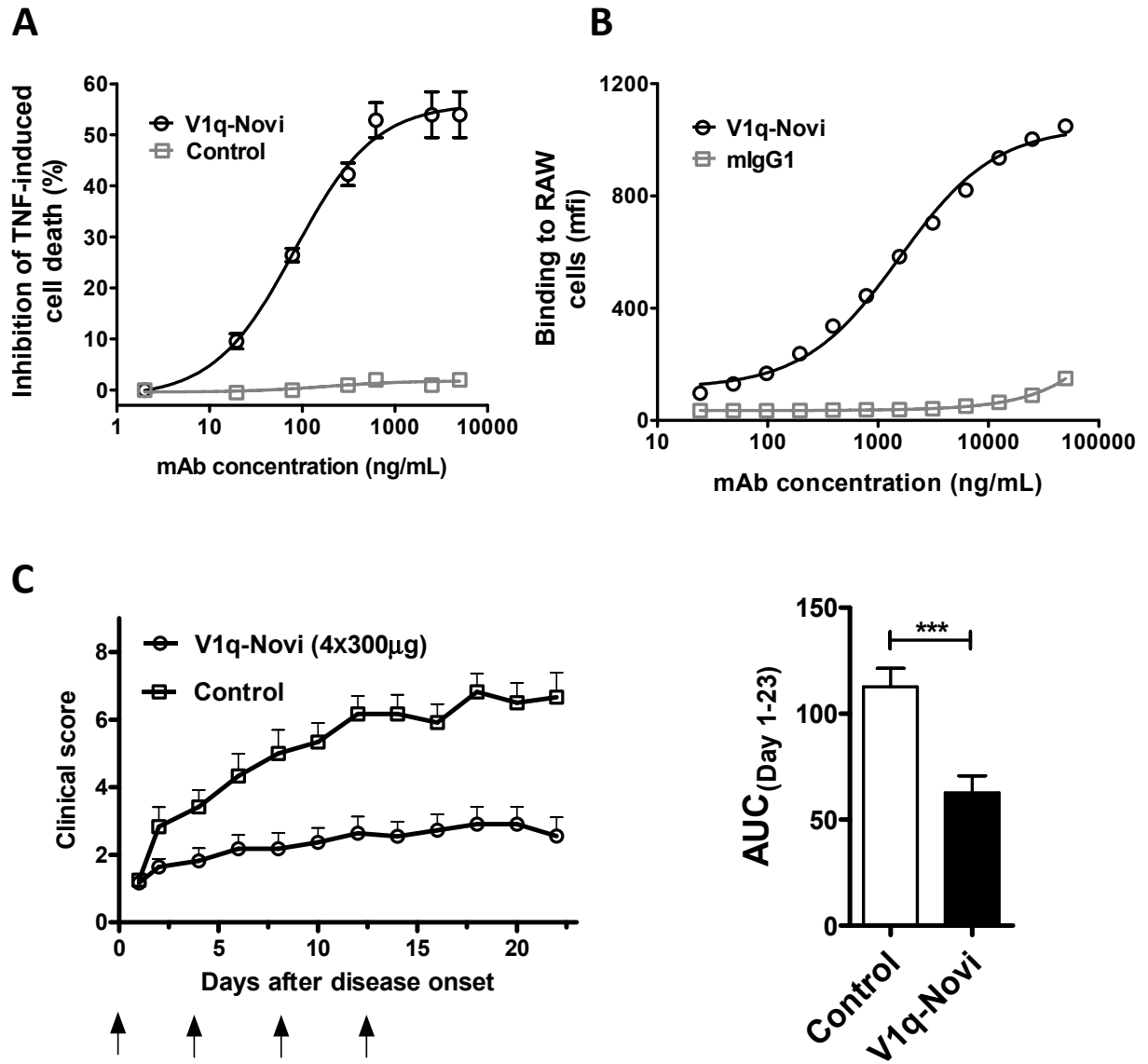


Figure 12: *In vitro* and *in vivo* characterization of V1q-Novi mAb.

A,B, *In vitro* characterization of V1q-Novi. **A**, Inhibition of TNF-induced WEHI-164 cell death and **B**, binding to the RAW-264.7 cell line to evaluate FcγR engagement as assessed by flow cytometry. **C**, *In vivo* characterization of V1q-Novi in arthritic male DBA/1 mice. The clinical scores were assessed over a 23-day period. Day 1 corresponds to disease onset i.e. the first day that clinical arthritis was observed. The anti-TNF mAb (V1q-Novi, 300 μg) was administered four times on day 1, 5, 9 and 13 (indicated by arrows). The control group represents mice treated with an isotype-matched control mAb (mIgG2a). Results presented are representative of experiments performed on 10 to 12 mice per group. The statistical significance of the difference between groups was calculated from area under the curve (AUC) for each mouse from day 1 to 23 (AUC₁₋₂₃).

**CHAPTER II: LONG TERM AMELIORATION OF
ESTABLISHED COLLAGEN-INDUCED ARTHRITIS
ACHIEVED WITH SHORT TERM THERAPY COMBINING
ANTI-CD3 AND ANTI-TNF TREATMENTS**

Arthritis & Rheumatism



Arthritis & Rheumatism

Long term amelioration of established collagen-induced arthritis achieved with short term therapy combining anti-CD3 and anti-TNF treatments

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NovImmune has developed the first fully human non-Fc γ R binding anti-human CD3 \square mAb, Foralumab (NI-0401). When conducting Phase 2a trials with Foralumab in patients with inflammatory bowel disease (IBD), clinical benefit was deemed marginal in the context of the protocol used (van der Woude et al., 2010). For conditions such as IBD or rheumatoid arthritis (RA), an inflammatory component mediated by cytokines such as TNF plays important roles. We hypothesized that monotherapy with anti-CD3 mAb may not be sufficient to treat IBD or RA. The superiority of a combination therapy with anti-CD3 (Fc γ R binding) and anti-TNF mAbs is supported by previous results in a preclinical model of RA (Malfait et al., 2001).

RA is a chronic inflammatory disease that involves cartilage and bone destruction leading to substantial loss of function and mobility if not adequately treated. The aim of intervention is to achieve clinical remission in order to limit the structural damage and physical disability. To this end, recent emphasis has been placed on aggressive treatment early in the course of disease. As the treatment paradigm is shifting towards early, robust and strong intervention, in addition to anti-TNF mAb treatment, improved strategies to target T cells, which play an important pathogenic role in the initiation phase of the disease, are warranted.

In this study, we demonstrate the potent synergy between non-Fc γ R binding anti-CD3 and anti-TNF therapies in an experimental model of CIA, i.e., murine CIA. To observe this unexpected finding, we used the clinically relevant 2C11-Novi and V1q-Novi surrogate mAbs previously described in Chapter 1. Interestingly, and perhaps in contrast to what the field would have expected based on the known mode of actions of anti-CD3 and anti-TNF mAb therapies, the mechanism is not via a contribution of Treg cells, TGF- β and/or PD-L1. Instead, we show that treating with a combination of anti-CD3 and anti-TNF mAbs result in a depletion of pathogenic IFN- γ and IL-17 secreting CD4⁺ T cells providing long lasting inhibition of disease progression with concomitant arrest of structural damage.

This scientific work constitutes a relevant preclinical proof of concept bringing new and strong evidence that anti-CD3/anti-TNF mAb therapy is an attractive strategy for the early and aggressive treatment of patients with RA.

Long term amelioration of established collagen-induced arthritis achieved with short term therapy combining anti-CD3 and anti-TNF treatments

Running Head: Anti-CD3 / anti-TNF combination therapy in CIA

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Financial interests: YD, MKV, WR and EH are shareholders in NovImmune SA., the discoverer and developer of NI-0401/Foralumab. YD, MKV and FD are inventors on patents filed by NovImmune SA relating to the clinical use of NI-0401/Foralumab. The other authors declare no competing interests.

ABSTRACT

Objective. The aim of rheumatoid arthritis (RA) treatment is to achieve clinical remission in order to limit structural damage and physical disability. To this end, recent emphasis has been placed on aggressive treatment early in the course of disease with drugs such as anti-TNFs. As T cells are also thought to play an important role in the initiation of RA, we hypothesized that targeting both TNF and T cells would result in better outcomes. The aim of this study was to examine the efficacy of combined anti-CD3 and anti-TNF therapies in experimental RA.

Methods. Two anti-mouse antibodies were developed as surrogate reagents for anti-TNF and anti-CD3 therapies. Collagen-induced arthritis (CIA) was induced in DBA/1 mice and antibodies were injected intraperitoneally alone or in combination at predetermined subtherapeutic doses. The frequency and number of pathogenic and regulatory CD4⁺ T cell subsets in the draining lymph nodes were determined to investigate the mechanisms of action.

Results. Strikingly, the combination of the two antibodies demonstrated a potent synergy in established CIA with long term inhibition of disease progression and protection from joint destruction. The results did not demonstrate any enhancement of CD25⁺Foxp3⁺ Treg cells but a significant depletion of pathogenic T effector cells.

Conclusion. A short combination therapy with anti-CD3 and anti-TNF efficiently depletes pathogenic T cells and affords long lasting inhibition of established CIA. This finding paves the way for clinical trials investigating anti-CD3/anti-TNF combination therapy in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic, painful and debilitating inflammatory disease that involves cartilage and bone destruction leading to substantial loss of function and mobility if not adequately treated. The aim of intervention is to achieve clinical remission in order to limit the structural damage and physical disability. To this end, recent emphasis has been placed on aggressive treatment early in the course of disease (1). In particular, tumor necrosis factor (TNF) antagonists are efficacious in reducing clinical and biological markers of disease activity and inhibiting the progression of structural damage in both early and late forms of RA. However, despite these positive effects, approximately 30% to 50% of patients do not respond to TNF antagonists. Furthermore, TNF antagonists do not provide long-term effectiveness in most patients.

Despite the unknown etiology of RA, numerous lines of investigation suggest that a T cell-mediated process is involved during the early phase of RA. Pathogenesis is associated with T cell receptor (TCR)-human leukocyte antigen (HLA) interactions, large numbers of infiltrating T cells and skewed TCR usage (2). As the treatment paradigm is currently shifting towards early, robust and strong intervention, improved strategies to target T cells in the initiation phase of the disease are warranted.

We hypothesized that combining a strong modifier of T cell responses in the presence of an anti-TNF neutralizing strategy could produce prolonged drug-free clinical remission. Indeed, the benefit of anti-TNF therapy has been shown to be enhanced by the use of anti-T cell therapy in a preclinical model of arthritis (3-6). However, this result was obtained using strong T cell depleting reagents, which are known to cause significant side effects.

For this study, we created an anti-mouse CD3 monoclonal antibody (mAb) specific for the epsilon chain of the CD3/TCR complex and mutated in its CH2 domain to reduce binding to Fc receptors (FcγR). For the anti-TNF mAb, we grafted the variable regions of V1q, a

neutralizing rat anti-mouse TNF, onto a mouse IgG2a backbone to confer effector functions. Using these murine anti-CD3 and anti-TNF antibodies, we observed that a short course combination therapy was associated with long-term inhibition of established collagen-induced arthritis (CIA). Anti-CD3/anti-TNF combination therapy efficiently depleted peripheral Th1 and Th17 pathogenic T cells, thus altering the accumulation of T cells in the joints.

MATERIAL AND METHODS

Mice and mAb treatment. Studies were conducted in eight-week-old male DBA/1J mice (Janvier Laboratories, Le Genest-St-Isle, France). Mice were housed in groups of 10. Animal experiments were conducted after obtaining the permission of the Swiss veterinary office for animal experimentation. All mAbs were administered intraperitoneally.

Generation of mAbs to mouse CD3 ϵ and TNF. The hybridoma clones 145-2C11 and V1q were obtained from the ATCC. The sequences encoding the heavy and light variable chain regions of 145-2C11 and V1q were cloned using conventional molecular techniques into plasmid vectors that encoded mouse IgG1,k and mouse IgG2a,k constant regions, respectively. The IgG1 heavy chain constant region was mutated at EU position 265 from aspartic acid to alanine by site-directed mutagenesis (QuickChange, Stratagen). The chimeric mAbs were produced in Chinese hamster ovary cells and purified from culture media by protein G affinity chromatography (Mab Select Sure resin, GE Healthcare Lifesciences) using an AktaPurifier system.

Induction and assessment of CIA. Lyophilized bovine collagen type II (CII, MD Biosciences, Zurich, Switzerland) was dissolved overnight at 4°C in 0.1 N acetic acid at 2 mg/mL and emulsified in an equal volume of CFA (2 mg/mL Mycobacterium tuberculosis; Difco, San Jose, CA, USA). Male DBA/1J mice were immunized intradermally with 100 μ L emulsion at the base of the tail (100 μ g bovine CII). After 21 day, mice received a secondary intradermal immunization with 100 μ g bovine CII emulsified in IFA. Disease onset was observed approximately 6 days after the second immunization. To assess the impact of the different therapies on ongoing arthritis, mice with swelling of at least one digit or paw were

recruited for treatment. Clinical scores were assessed immediately at recruitment and then every two days. CIA severity was graded by overall assessment of inflammation on individual paws, applying a scale ranging from 0 to 4. Each paw was graded according to the following system: 0, no inflammation; 1, swelling of at least one digit; 2, swelling of all the digits and inflammation of the paw; 3, severe inflammation of whole paw and digits or ankylosis; 4, necrosis. The sum of these four individual corresponds to the total clinical score, i.e. the maximal clinical score for an individual mouse is 16.

Histological studies. For each time point (day 15 and 29), at least 5 animals per group were euthanized, and joints were collected for histological assessment of synovial inflammation and cartilage damage. The joints were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 10 days and rinsed in PBS. Fixed tissues were decalcified in an EDTA-based decalcifying solution (Osteosoft, Merck, Darmstadt, Germany) for 3 weeks at room temperature. Tissue samples were rinsed in PBS, dehydrated, and embedded in paraffin blocks using an automated tissue processor (Microm, STP 120). Serial midsagittal sections (8 μ m thick) of the whole knee joint were stained with either Hematoxylin and Eosin (H&E) or Safranin-O and fast green. Histological sections were then graded by two independent observers blinded to the experimental groups. The following established scoring system for synovial inflammation was used: 0 = synovium with no hyperplasia to 3 = severe synovial hyperplasia; and for cellular infiltration in the synovium: 0 = no inflammation, to 3 = severe inflammation. To quantify the proportion of proteoglycan staining, whole knees were processed and one section every five was systematically used for analysis. For each section, the surface area of Safranin-O/fast green staining and the surface area of the whole cartilage were measured using Image J software. The data are presented as cartilage integrity, calculated as a percentage of Safranin-O staining relative to that of non arthritic mice.

For immunohistochemistry studies, knee joints were removed and immediately frozen in OCT embedding matrix and stored at -70°C. Tissue sections were obtained using an adhesive tape as previously described (7). Detection of CD4⁺ cells was performed using an anti-mouse CD4 mAb (H129.19, BD Biosciences) with a classical indirect immunoperoxidase technique.

Lymph node cell culture. In the course of CIA, popliteal, inguinal, brachial and axillary lymph nodes were extracted from arthritic mice six or fifteen days post-onset. Briefly, for each animal, lymph nodes were enzymatically digested in a solution of 1 mg/mL DNase and 2.5 mg/mL collagenase for 45 minutes at 37°C. A total of 1x10⁶ cells were then cultured in RPMI 1640 supplemented with L-glutamine, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol and 10% autologous serum, for three days in 96-round-well plate in the presence of bovine CII at 50 µg/mL final (MD Biosciences).

Flow cytometry. For cell surface or intracellular cytokine labeling, the following anti-mouse mAbs were used (from BD Biosciences unless stated). Anti-CD4-FITC (RM4-5), anti-CD8a-PerCP (53-6.7), anti-CD25-APC (PC61), anti-Foxp3-PE (FJK-16s, eBioscience). Cells were incubated with Fc block (BD Biosciences) to reduce non-specific binding. For intracellular staining, cells were treated with 50 ng/ml PMA, 500 ng/ml ionomycin and 3 mM monensin (Sigma-Aldrich) for 4 h at 37°C. Cells were incubated with anti-CD4 before fixation and permeabilization in Cytofix/Cytoperm (BD Biosciences). Intracellular IL-17A and IFNγ were detected using anti-IL-17A-PE (TC11-18H10) and anti-IFNγ-APC (XMG1.2), respectively. Flow cytometry was performed using a FACSCalibur flow cytometer and analyzed with the CellQuestPro software (BD Biosciences).

Statistical analysis. The data are displayed as mean \pm standard error of the mean (SEM). For all bar graphs, one-way analysis of variance (ANOVA) was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). P-values were adjusted using the Bonferroni correction to compensate for multiple testing. Statistically significant differences between groups are indicated as follows: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

RESULTS

2C11-Novi and V1q-Novi as surrogate mAbs and dose finding studies. In order to study potential synergism between anti-CD3 and anti-TNF therapies, two murine mAbs were engineered. Their functional characteristics as compared to the human therapeutic equivalents are summarized in Table 1. The anti-mouse CD3 mAb, 2C11-Novi, was obtained by combining the Fv sequences of 145-2C11, a hamster anti-mouse CD3 mAb (8), with the constant domains of murine IgG1 carrying an alanine substitution at amino acid 265 in the CH2 region, producing a chimeric hamster/mouse mAb which does not bind FcγR (9;10). The anti-mouse TNF mAb, V1q-Novi, is a chimeric mAb that was constructed by combining the Fv sequence of the rat mAb V1q (11) with the constant domains of murine IgG2a. Such chimeric anti-TNF mAb has already been developed by Centocor (3). (Document_not_found, n.d.) (Document_not_found, n.d.)^{>218}^{²¹⁸²¹⁸²¹⁸²¹⁸²¹⁸²¹⁸(Document_not_found) Document_not_found,}</sup>

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n.d.)²¹⁸²¹⁸²¹⁹²¹⁸²¹⁸²¹⁸²¹⁸²¹⁸²¹⁸²¹⁸ In vitro and in vivo characterization studies indicate that V1q-Novi and 2C11-Novi are relevant murine surrogates for anti-TNF and anti-CD3 mAb therapies, respectively (12).

To study a potential synergism between anti-CD3 and anti-TNF therapies in CIA, we performed dose-finding studies to identify doses of V1q-Novi and 2C11-Novi that provide suboptimal therapeutic effects. Anti-TNF therapy is effective in reducing disease activity in

patients with RA as well as in multiple preclinical models of RA, including CIA (13). As already described a chimeric mouse IgG2a V1q mAb inhibits arthritis progression when administered twice weekly from disease onset, at a dose of 300 µg/mouse⁶. Therefore, mice with established CIA were administered intraperitoneally from day 1 of disease onset with V1q-Novi at doses of 300 µg/mouse given once every 4 days. The clinical scores were evaluated every two days from day 1 (disease onset) to 23. As shown in Figure 1A (left panel) 4 injections of V1q-Novi inhibited arthritis progression through the entire study period. Therefore, the number of V1q-Novi injections was reduced to evaluate the efficacy of one (single administration of 300 µg/mouse on day 1) versus two injections (300 µg/mouse on day 1 and 5). The single dose was sufficient to inhibit disease progression for approximately 2 weeks. A subsequent single dose finding experiment at 30, 100 and 300 µg/mouse indicated that a single dose of 100 µg/mouse of V1q-Novi inhibited disease progression for less than 2 weeks (Figure 1A, middle panel). This dose was selected as a suboptimal dose of V1q-Novi. The classical treatment regimen for non-FcγR binding anti-CD3 mAbs, 5 consecutive daily injections of 50 µg/mouse (14) was used to study the therapeutic effect of 2C11-Novi in established CIA. As shown in Figure 1A (right panel) this treatment regimen did not provide long lasting therapeutic effect and was selected as suboptimal dose of 2C11-Novi.

Coadministration of subtherapeutic doses of 2C11-Novi and V1q-Novi provides long term inhibition of disease progression and protects from joint destruction in established CIA. A combination of a single dose of 100 µg V1q-Novi administered on day 1, and 5 doses of 50 µg of 2C11-Novi administered from day 1 to 5 of CIA onset was used to study potential synergy between anti-CD3 and anti-TNF therapies. The control groups were composed of mice treated with 2C11-Novi alone, V1q-Novi alone, or isotype-matched control mAbs. As shown in Figure 1B, the combination of suboptimal doses of 2C11-Novi and V1q-Novi

resulted in a long-term therapeutic effect that was highly significant from day 15 to day 29, as compared to the control groups (right bar graph, $p < 0.0001$).

Histological evaluation of the joints employing Safranin-O/fast green staining revealed extensive destruction of cartilage on days 15 and 29 (Figure 2) in arthritic versus non-arthritic mice. Most importantly, the combination of anti-CD3/anti-TNF exerted a chondroprotective effect. A statistically significant difference in the extent of structural damage was already observed on day 15 (Figure 2, left bar graph) but the effect was even more pronounced at day 29 (Figure 2, right bar graph, 58.2% cartilage integrity in the combination therapy group) when compared to the isotype controls, anti-CD3 only and anti-TNF only treated groups (15.5%, 22.6% and 19.9%, respectively; $p < 0.0001$).

Synergistic therapeutic effect of anti-CD3/anti-TNF therapy does not depend on classical immunoregulatory mechanisms. The induction of Treg cells could be a mechanism through which the combination therapy attenuated the severity of CIA. Indeed, both anti-TNF and anti-CD3 therapy alone have been shown to induce CD25⁺Foxp3⁺ Treg cells via TGF- β (14, 15). We therefore investigated whether the combination of anti-CD3 and anti-TNF increased the frequency of Treg in CIA. 145-2C11, the original hamster anti-mouse CD3 mAb, was used as positive control as it expands the frequency of CD4⁺ and CD8⁺ Treg in the lymph nodes of mice that develop CIA (16). As shown in Figure 3A, the percentage of CD4⁺ and CD8⁺ Treg increased in the lymph nodes of 145-2C11-treated mice by 2-3 fold, whereas there was no increase in the frequency of Treg in the 2C11-Novi, V1q-Novi or combination therapy-treated mice.

We next evaluated if other mechanisms of tolerance could be evoked despite the lack of detectable expansion of Foxp3⁺ T cells. *In vivo* neutralization of TGF- β or PD-L1 can abrogate the tolerogenic effects of anti-CD3 mAbs in NOD mice (14, 15, 17). We assessed

whether neutralizing TGF- β or PD-L1 in established CIA could reverse the therapeutic effects of anti-CD3/anti-TNF combination therapy. The neutralizing anti-TGF- β or PD-L1 mAbs were administered according to the treatment regimen previously described (14, 17). Consistent with previous reports, neutralization of TGF- β or PD-L1 alone in CIA exacerbated disease progression (18, 19). This finding demonstrates that both mAbs were functional *in vivo*. However, administration of blocking mAbs to TGF- β or PD-L1 did not modify the capacity of anti-CD3/anti-TNF therapy to inhibit the progression of CIA. These results indicate that the therapeutic effect of the anti-CD3/anti-TNF combination does not depend on immunoregulatory mechanisms previously associated with anti-CD3 or anti-TNF antibodies.

Synergistic therapeutic effect of anti-CD3/anti-TNF therapy is associated with marked depletion of pathogenic T cells. Another mechanism proposed for the tolerogenic effects of anti-CD3 mAbs is depletion of Teff cells (20). The depletion of activated T cells has been shown to play a role in the therapeutic effect of Infliximab (21). We therefore investigated the capacity of 2C11-Novi, V1q-Novi and the combination of the two mAbs to deplete CD4⁺IFN γ ⁺ (Th1) and CD4⁺IL-17⁺ (Th17) pathogenic (i.e. collagen-specific) T cells in CIA. As assessed using cells isolated from the draining lymph nodes on day 6 post disease onset, anti-TNF treatment alone resulted in a decreased number of collagen-specific Th1 and Th17 cells by 39.2% and 26.4% as compared to isotype-control treated mice, respectively (Figure 4A). The depletion of Th1 and Th17 pathogenic T cells was more pronounced in anti-CD3 treated mice with a reduction of 70.4% and 69.1%, respectively. Interestingly, an almost complete depletion of collagen-specific Th1 and Th17 cells (88.9% and 86.7%, respectively) was observed in mice treated with the combination therapy.

A possible mechanism of action for the depletion of pathogenic T cells by anti-CD3/anti-TNF combination therapy is represented schematically in Figure 4B. Following exposure to anti-

CD3 mAb, antigen-specific Teff cells either undergo apoptosis through the process of activation-induced cell death (AICD) or survive and express activation markers such as transmembrane TNF. The anti-TNF can then deplete pathogenic T cells resistant to anti-CD3 induced AICD by complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity and/or reverse signaling.

We next investigated the extent of inflammation and infiltrating T cells in the joints of arthritic mice treated with anti-CD3/anti-TNF mAbs. As illustrated in Figure 5A at day 15 a statistically significant reduction in cellular infiltration in the mice treated with the combination therapy was observed. The presence of T cells within the joints was assessed by immunohistochemistry. As depicted in Figure 5B, CD4⁺ T cells were detected in the pannus tissue of the inflamed joints of mice treated with the isotype controls, 2C11-Novi and V1q-Novi mAbs alone. In contrast, the mice that received the combination therapy presented no or very few CD4⁺ T cells (Figure 5B).

DISCUSSION

As a plethora of data generated using animal models of human diseases place T cells as contributors to the pathogenic mechanisms underlying autoimmunity (22-24), one would forecast that they represent an ideal cellular target for clinical intervention. As such, therapies aimed at modulating T cells have been developed, starting with Muromonab (Orthoclone OKT3), which is directed against the epsilon chain of the CD3/TCR complex. Muromonab potently reverses and prevents acute allograft rejection (25, 26). However, its clinical use in autoimmune diseases is limited due to its capacity to bind FcγR, thus triggering a debilitating cytokine storm (27). To avoid these unwanted pharmacodynamic manifestations, we and others developed improved anti-CD3 mAbs that lack FcγR binding (28). These mAbs have

been tested as mono- or add-on therapies in various autoimmune diseases, with mixed outcomes. In patients with new-onset type 1 diabetes mellitus, while the early stage investigator sponsored trials with Teplizumab (HuOKT3 γ 1 Ala-Ala) and Otelixizumab (ChAglyCD3) produced encouraging clinical results (29, 30), their further development in Phase 3 randomized, placebo-controlled trials failed to meet the primary endpoints (31). Furthermore, when conducting Phase 2a trials with Visilizumab (Nuvion) or Foralumab (NI-0401) in patients with inflammatory bowel disease (IBD), clinical benefit was deemed marginal in the context of the protocol used (32, 33). However, as compelling data exists from models and certain patient subpopulations that a treatment targeting CD3 will invoke clinical benefit (22, 34), where have we gone wrong? The failure to translate these findings into robust and broader clinical success is certainly multifactorial. At the preclinical level, the anti-mouse CD3 mAbs may not have been representative of the next generation of anti-human CD3 mAbs (35, 36), the consequence being that the pharmacology may not yet have been optimally established for correct translation from bench to bedside. From a clinical perspective, for conditions such as RA or IBD, an inflammatory component mediated by cytokines such as TNF or IL-6 plays important roles. Thus, monotherapy with anti-CD3 mAb may not be sufficient to treat RA. The superiority of a combination therapy with anti-CD3 and anti-TNF mAbs is supported by previous results in the CIA model (3). Our study demonstrates that this strategy is also effective with a novel, non-Fc-binding anti-CD3 mAb. The results suggest that the depletion of pathogenic Th1 and Th17 CD4⁺ T cells can be considered as a mechanism of action of the combination therapy, thus providing a novel rationale for the clinical application of this approach.

The pharmacodynamic characteristics of the next generation therapeutic anti-CD3 mAbs (Table 1) that we sought to reproduce in the surrogate mAb were to maintain the ability to efficiently induce CD3/TCR complex modulation yet present a lowered T cell activation

potential and provocation of cytokine release. Thus, the variable regions of 145-2C11 were selected as this mAb binds to the relevant chain of the complex, i.e. epsilon, mimicking the wanted level of modulation both *in vitro* and *in vivo*. To address the T cell activation and cytokine storm, others have resorted to using the F(ab')₂ form of 145-2C11 as it lacks the capacity to bind FcγR (22). However, due to a very short half-life *in vivo*, we considered that F(ab')₂ fragments were not appropriate surrogate molecules. Therefore, our surrogate mAb was engineered as a whole IgG with the variable regions of 145-2C11 grafted onto a mouse IgG1 Fc backbone mutated in a single amino acid rendering the molecule unable to bind and induce FcγR mediated effector functions (12). Similarly, for the antigen binding domains of the surrogate anti-TNF mAb, we used the variable regions of V1q, a mouse anti-TNF mAb that neutralizes TNF *in vivo* and binds both the soluble and transmembrane forms of the cytokine (11, 37). However, as V1q is a rat IgD, it does not elicit effector functions which can be important for the therapeutic effects of anti-TNF therapies (38, 39). Therefore, we produced a rat/mouse chimeric mAb, V1q-Novi, having the variable regions of V1q engineered onto a mouse IgG2a Fc backbone. A similar chimeric surrogate mAb has been shown to successfully neutralize TNF *in vivo* (3). We confirmed that V1q-Novi neutralizes the biological activity of TNF, both *in vitro* and *in vivo*, and retains the capacity to interact with FcγR (12).

CIA in rodents reproduces some of the pathological, histological and immunological characteristics of human RA (40-42). In mice, the susceptibility to CIA is linked to MHC Class II I-A^q haplotypes and likewise in RA patients, pathogenesis is associated with HLA-DR1 and HLA-DR4 MHC class II genotypes. In addition, in murine CIA and RA patients, TNF inhibitors block the evolution of arthritis. Thus, the CIA model is deemed to approximate well the clinical setting and was therefore used to study the therapeutic effect of combining our surrogate mAbs. Using this model, a short course treatment regime involving a

combination of anti-CD3 and anti-TNF mAbs synergistically and dose dependently inhibited the progression of established arthritis. The impact was remarkable as the effect was maintained for over 3 weeks after the end of the 5-day treatment period. By contrast to Fc binding anti-CD3 antibodies the mechanism does not involve an expansion of CD25⁺Foxp3⁺ Treg cells nor depend on TGF- β or PD-L1. We observed that anti-CD3/anti-TNF combination therapy efficiently depleted Th1 and Th17 pathogenic T cells in the periphery, a finding in line with a recent publication where an anti-CD3 therapy promoted tolerance by depleting pathogenic T cells (43). In the joints, treatment with the combination therapy was associated with a reduced number of CD4⁺ T cells. This alteration can be due to the fact that, either alone or in combination, T cells within the joint are directly depleted or migration into the joints is blocked. As it has been shown that an anti-TNF mAb inhibits migration of Th1 and Th17 cells to the joints in CIA (44), we speculate that pathogenic T cells are killed in the periphery, e.g. in secondary lymphoid tissues (draining lymph nodes and spleen).

Based on our work and recent findings of others (45), we propose the model shown in Figure 4B. During an autoimmune response, self-antigens are presented by antigen presenting cells (APC) which prime pathogenic effector T cell populations. In the presence of a non-Fc γ R-binding anti-CD3 mAb, a subset of this population will become CD3/TCR negative and partially activated thus sensitive to undergo AICD (46, 47). As shown in Figure 1B and 4A, the depletion of antigen-activated T cells by non-Fc γ R-binding anti-CD3 mAbs is incomplete and does not efficiently inhibit disease progression. The subset of pathogenic T cells that escape anti-CD3 induced AICD, being activated, may express transmembrane TNF, the precursor form of soluble TNF. As a one-two punch, the anti-TNF mAb could deplete the pathogenic T cells resistant to anti-CD3-mediated AICD via the mechanism of engaging transmembrane TNF (21, 38, 39, 48, 49).

In conclusion, we propose that additive biological mechanisms incurred by treating with a combination of anti-CD3 and anti-TNF mAbs result in a depletion of pathogenic Th1 and Th17 CD4⁺ T cells providing long lasting inhibition of disease progression with concomitant arrest of structural damage. These preclinical data argue for trials investigating short course treatment with anti-CD3/anti-TNF combination therapy as early treatment for patients with RA aiming at inducing longer lasting remission.

FIGURE 1

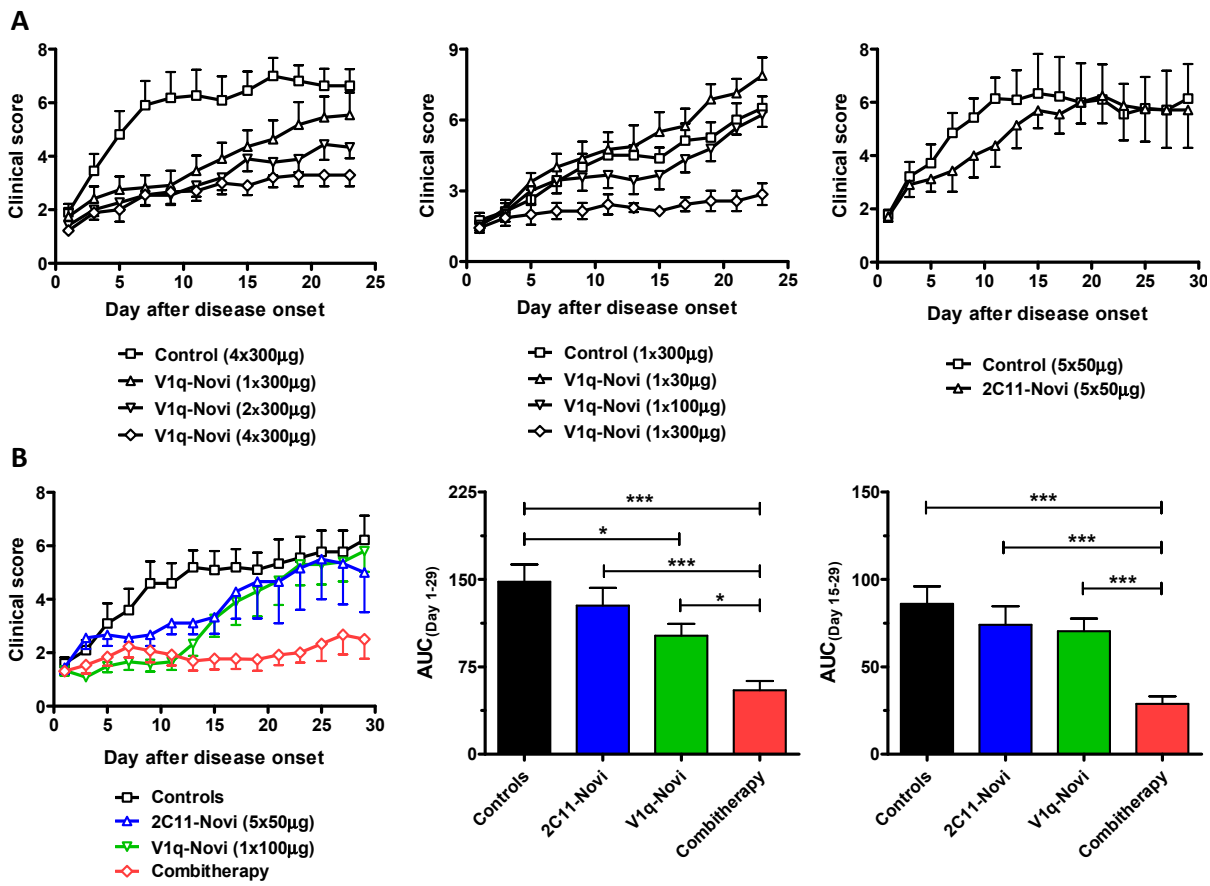


Figure 1. A short course of treatment combining suboptimal doses of 2C11-Novi and V1q-Novi affords long term inhibition of disease progression in established CIA.

Arthritis was induced in male DBA/1 mice by immunization with bovine CII. Changes of clinical scores over time are shown. Results are representative of experiments performed with 10 to 12 mice per group. **A**, V1q-Novi and 2C11-Novi dose finding studies. Arthritic mice were treated with 1 (day 1), 2 (day 1 and 5) or 4 (day 1, 5, 9 and 13) doses of 300 µg V1q-Novi (left panel) or single doses of 30, 100 and 300 µg V1q-Novi on day 1 (middle panel). For 2C11-Novi, mice received 5 consecutive daily injections of 50 µg (right panel). The control group represents mice treated with isotype-matched control mAbs (mIgG2a or mIgG1) administered as indicated. **B**, Arthritic mice were treated from day 1 of onset with a single 100 µg dose of V1q-Novi, 5 consecutive daily injections of 50 µg 2C11-Novi, V1q-Novi/2C11-Novi combination (combitherapy) or isotype-matched control mAbs (1x100 µg mIgG2a and 5x50 µg mIgG1). The statistical significance of the difference between groups was calculated from the area under the curve (AUC) for each mouse over the 4-week treatment period (AUC₁₋₂₉) and the last 2 weeks of the treatment period (AUC₁₅₋₂₉).

FIGURE 2

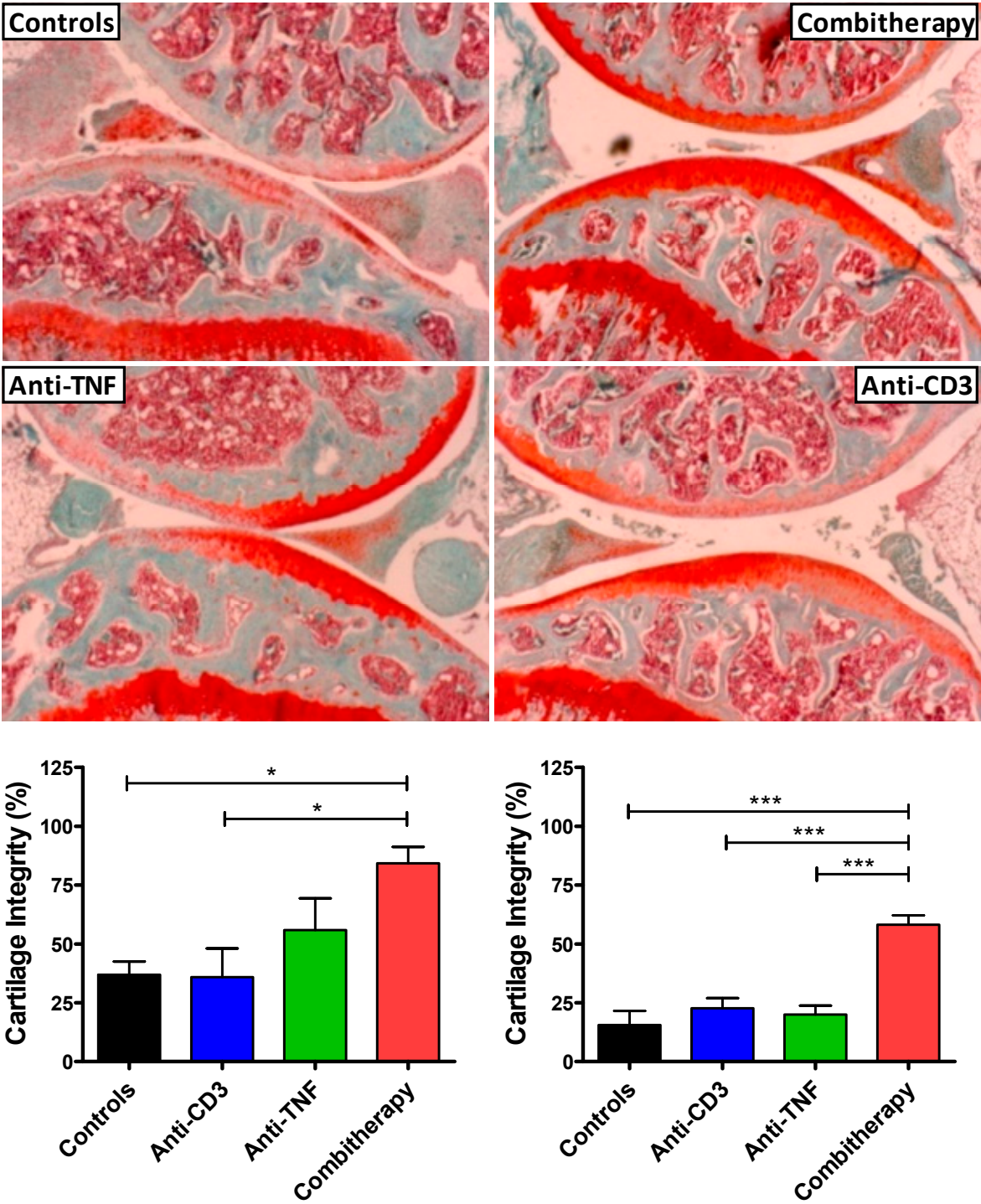


Figure 2. Combination therapy with anti-CD3 and anti-TNF in established CIA protects from joint destruction. Arthritic mice were treated as described in Figure 1B. The animals (6-7 mice per group) were euthanized, and joints were collected for histological assessment of synovial inflammation and cartilage damage. Representative images of Safranin-O/fast green stained paraffin-embedded tissue sections of knee joints are shown. Tissue sections were scored for the extent of cartilage integrity on day 15 (bottom left panel) and day 29 (bottom right panel). Data are presented as cartilage integrity, calculated as a percentage of Safranin-O staining relative to that of non arthritic mice.

FIGURE 3

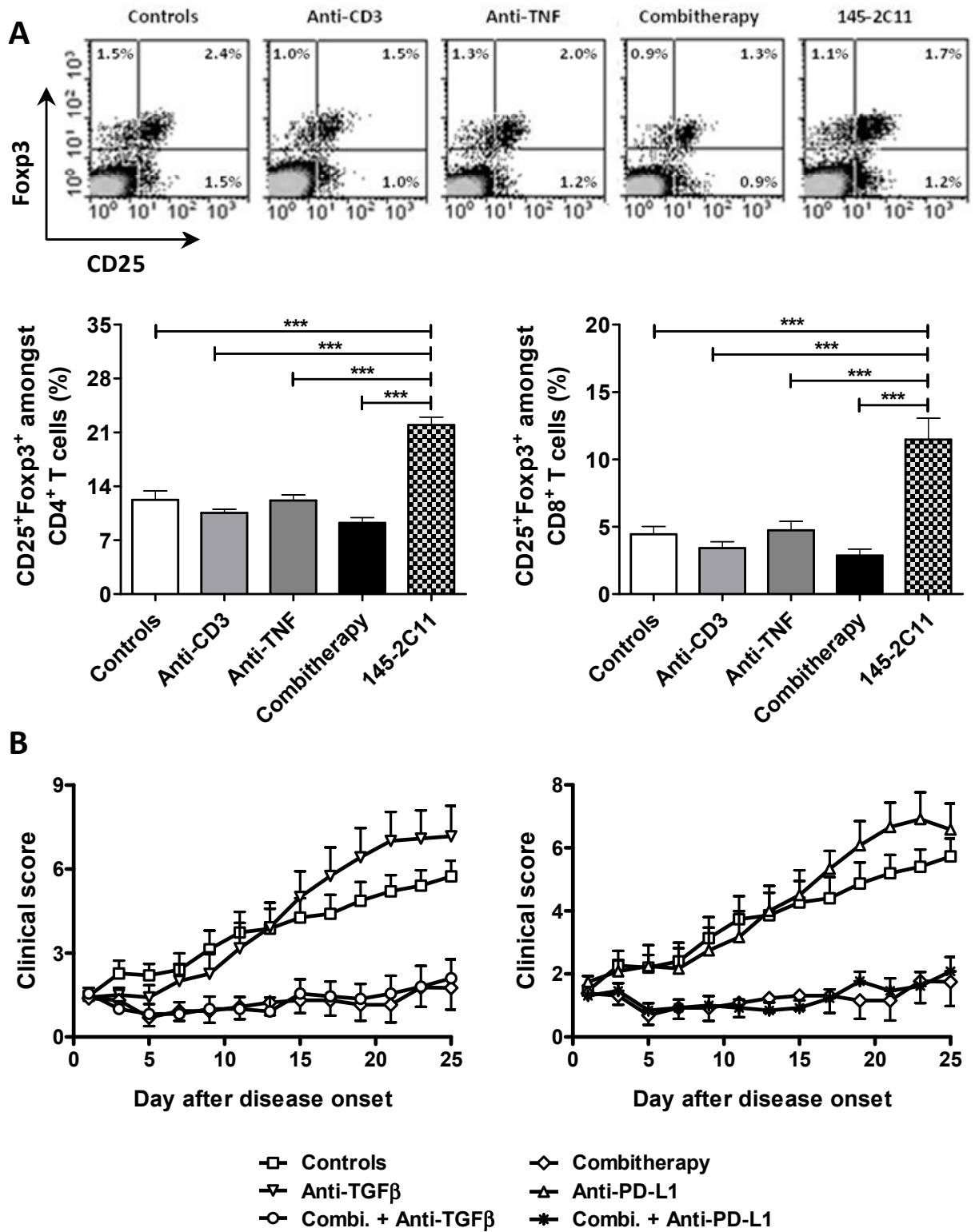


Figure 3. Long term inhibition of established CIA with anti-CD3/anti-TNF combination therapy does not depend on classical immunoregulatory mechanisms. **A**, Arthritic mice were treated as described in Figure 1B. An additional group of mice received a single 20 μ g dose of 145-2C11 used as positive control for induction of Treg. Mice were euthanized on day 6, 15 and 25 after disease onset and lymph nodes collected for evaluation of CD4⁺ and CD8⁺ T cells positive for CD25 and Foxp3 by flow cytometry. Representative dot plots showing Foxp3 and CD25-expressing cells gated on lymph node cells on day 15 are displayed. The bar graphs represent the percentage of Foxp3 and CD25-expressing cells gated on CD4⁺ (left panel) and CD8⁺ T cells (right panel) on day 15. **B**, Arthritic mice were treated from day 1 of disease onset with neutralizing mAbs to TGF- β (1 mg every 2 days, left panel) or PD-L1 (0.5 mg on day 1 and 0.25 mg every 2 days thereafter, right panel) alone or plus V1q-Novi/2C11-Novi combination therapy. Disease severity was evaluated from day 1 to day 25 and the statistical significance of the difference between groups was calculated from the AUC for each mouse over the 25-day treatment period (AUC₁₋₂₅). For all graphs, the control group represents mice treated with isotype-matched control mAbs as follows: 1x100 μ g mIgG2a and 5x50 μ g mIgG1. Results are representative of experiments performed with 10 to 12 mice per group.

FIGURE 4

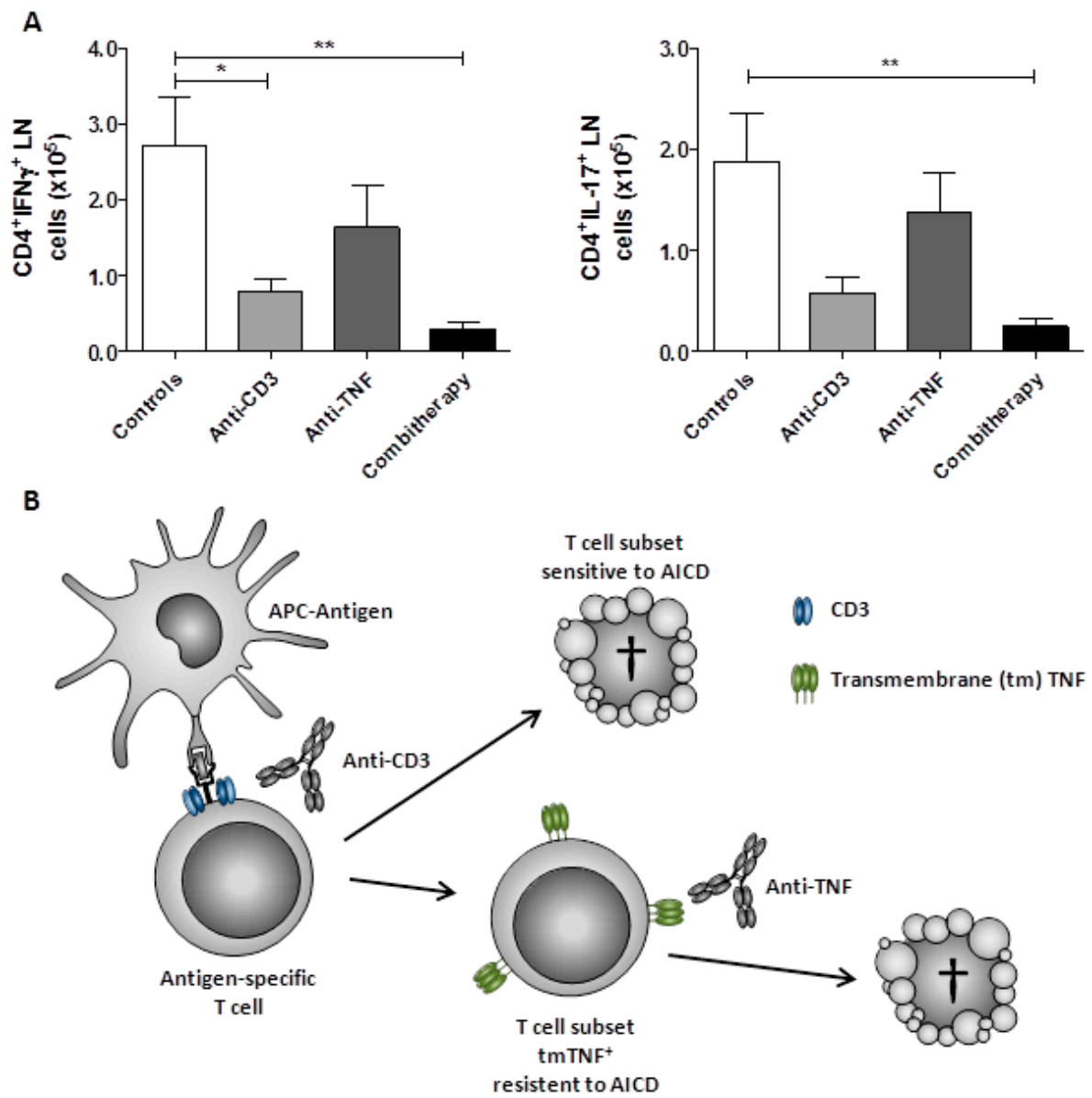
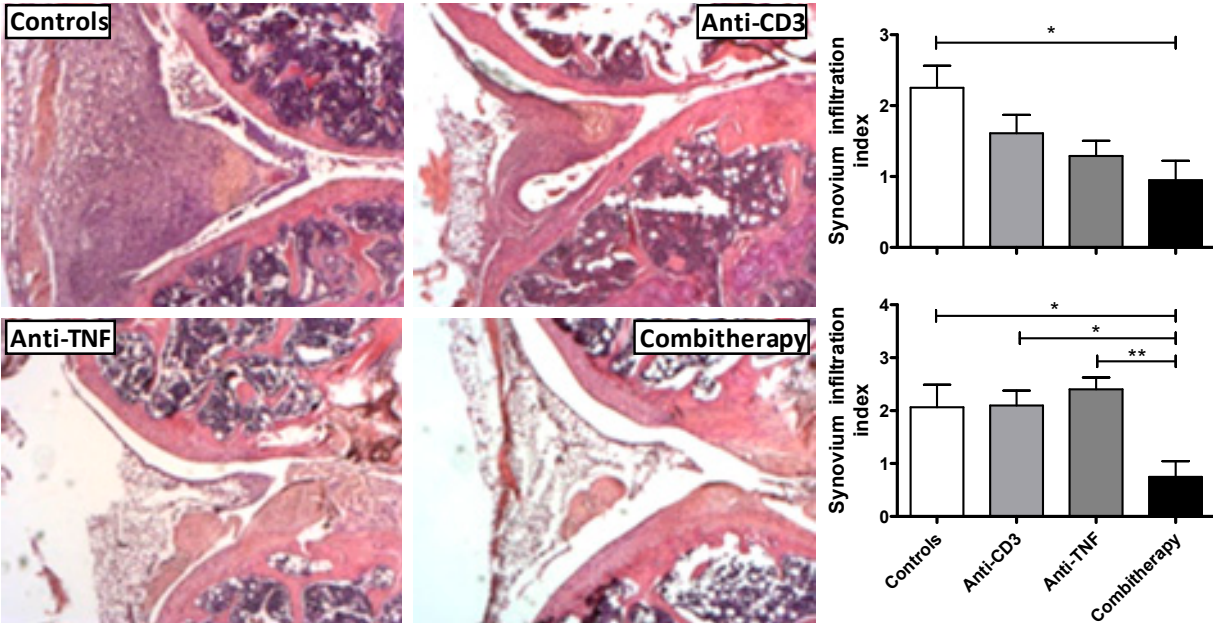


Figure 4. Anti-CD3/anti-TNF combination therapy effectively depletes Th1 and Th17 pathogenic T cells. Arthritic mice were treated as described in Figure 1B. **A**, Mice were euthanized on day 6 after disease onset and CD4⁺ lymph node cells isolated for intracellular cytokine expression by flow cytometry after stimulation with bovine CII for 3 days and restimulation with PMA/ionomycin. The bar graphs represent the absolute number of CD4⁺ T cells expressing IFN γ (Th1, left panel) and IL-17 (Th17, right panel). Data are representative of two independent experiments performed on 5 to 6 mice per group. **B**, Schematic representation of a possible mechanism of action for the depletion of pathogenic T cells by anti-CD3/anti-TNF combination therapy.

FIGURE 5

A



B

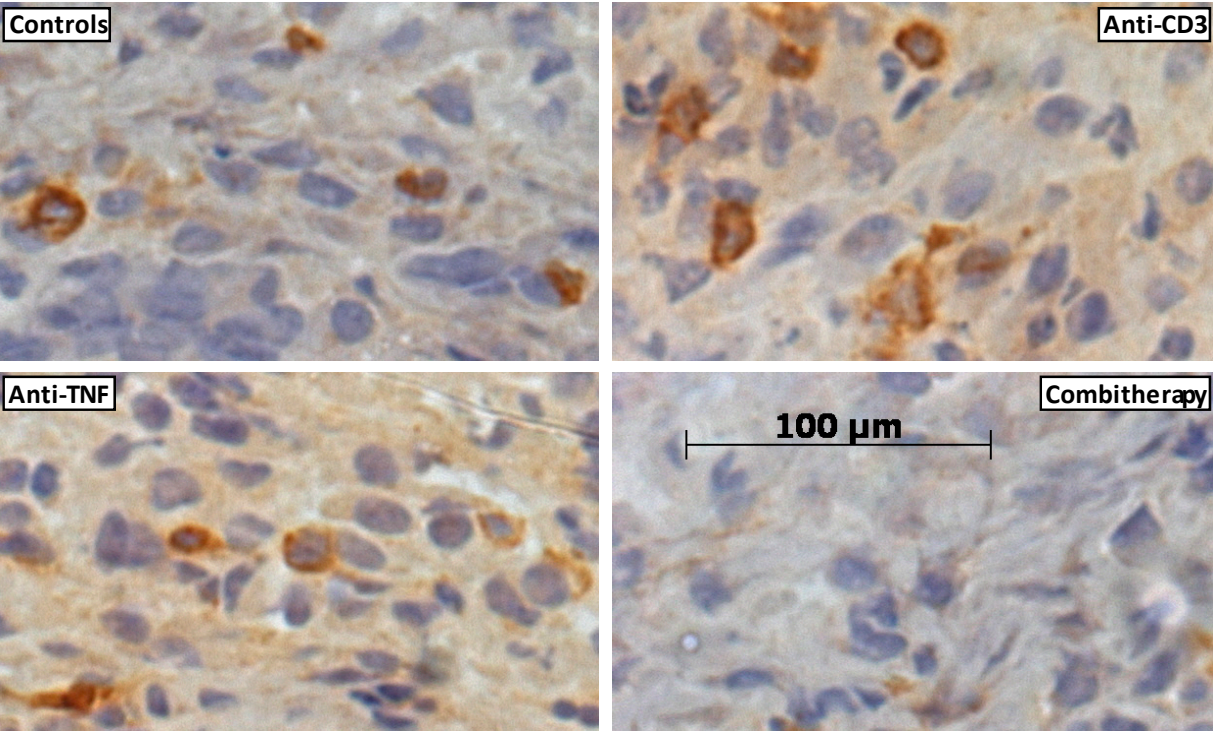


Figure 5. Anti-CD3/anti-TNF combination therapy is associated with reduced cell infiltration in the joints including T cells. Arthritic mice were treated as described in Figure 1B. **A**, On day 15 and 29, knee joints were processed for histological analysis. Representative images of H&E stained paraffin-embedded tissue sections are shown for day 15. At least 6 sections per knee joint from 6-7 mice per group were scored for the extent of inflammation on day 15 (top bar graph) or day 29 (bottom bar graph). **B**, Immunohistochemical staining of CD4⁺ cells (brown labeling) in the pannus tissue of knee joints using frozen tissue sections obtained from mice on day 15 post disease onset. The scale bar shown in the bottom right picture represents 100 μ m. All pictures were taken at the same magnification (x400).

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**CHAPTER-III: ANTI-CD3/ANTI-TNF THERAPEUTIC
EFFICACY IN ESTABLISHED CIA IS NOT A
CONSEQUENCE OF PROLONGED PERIPHERAL T CELL
IMMUNOSUPPRESSIVE MECHANISMS**

ABSTRACT

In a previous study, we have demonstrated that short term therapy combining anti-CD3 (2C11-Novi) and anti-TNF treatments is associated with long term amelioration of established collagen-induced arthritis (CIA) in mice. A series of experiments was conducted to investigate whether immunoregulatory mechanisms known to be associated with anti-CD3 and anti-TNF monotherapies were involved. The results did not demonstrate any enhancement of CD25⁺Foxp3⁺ Treg cells nor a contribution of TGF- β but a significant depletion of pathogenic T effector cells. In this study, we investigated whether the immunosuppressive pharmacodynamic (PD) effects of anti-CD3 mAbs could contribute to the mechanisms of actions. First, we showed that the treatment regimen of 2C11-Novi (5 x 50 μ g) that is required to obtain an optimal long lasting therapeutic effect is associated with long term T cell depletion and antigenic modulation in secondary lymphoid organs. Second, we confirmed that the neutralizing 2H2-Novi anti-idiotypic mAb can accelerate the return to baseline of the transient *in vivo* immunosuppressive PD effects of 2C11-Novi, presumably by accelerating its clearance from the plasma. Third, using 2H2-Novi we showed that the disease activity remained significantly lower than that of control mice, despite the absence of T cell immunosuppression (immunosuppressive PD effects of anti-CD3 mAbs) at the end of the 4-week study period. Taken together, these findings demonstrate that long lasting T cell immunosuppression induced by 2C11-Novi is not required for the long term inhibition of CIA progression afforded by anti-CD3/anti-TNF combination therapy.

INTRODUCTION

Patients with rheumatoid arthritis (RA) in whom the response to anti-tumor necrosis factor (anti-TNF) therapy is inadequate have several therapeutic options (Buch, 2010), such as switching to an alternative anti-TNF agent (C. Deighton, 2009) or initiating treatment with Abatacept (Mark C Genovese et al., 2005), a T cell co-stimulation modulator. Both therapeutic options have been proven to be effective in clinical studies and we hypothesized that dual targeting of both TNF and T cells in established arthritis could result in better clinical outcomes. We have previously shown that a short course treatment with a non-Fc γ R-binding anti-CD3 mAb administered in combination with a low dose of anti-TNF mAb inhibited progression of collagen-induced arthritis (CIA) for a long period of time (up to three weeks post-treatment, see Chapter 2, Fig. 1 and Fig. 2).

This result was not due to an expansion of CD25⁺Foxp3⁺ Treg cells and did not depend on TGF- β or PD-L1 as one might have expected (Chapter 2, Fig. 3). Indeed, both anti-TNF and anti-CD3 therapy alone have been shown to induce CD25⁺Foxp3⁺ Treg cells (Tregs) via TGF- β (Nadkarni, Mauri, & Ehrenstein, 2007) (C. a Notley et al., 2010) (Ke, Jiang, Sun, Kaplan, & Shao, 2011). Instead, we found that anti-CD3/anti-TNF combination therapy efficiently depletes pathogenic Th1 and Th17 cells in the periphery and altering the accumulation of T cells in the joints (Chapter 2, Fig. 4 and Fig. 5). Because both anti-CD3 mAb treatment alone and anti-TNF mAb treatment alone have been shown to deplete activated T cells (Penaranda et al., 2011) (P A Carpenter et al., 2000) (Mitoma et al., 2008), we proposed that additive biological mechanisms recruited by treating with a combination of anti-CD3 and anti-TNF mAbs result in a more complete depletion of pathogenic Th1 and Th17 CD4⁺ T cells, thereby providing long lasting inhibition of disease progression with a concomitant arrest of structural damage.

In addition to immunoregulatory functions involving Tregs and/or TGF- β , anti-CD3 mAbs are potent immunosuppressive agents that can exert immunosuppression via the depletion of non-pathogenic T cell subsets and antigenic modulation. We have previously shown that a single administration of 2C11-Novi in naïve DBA mice results both in antigenic modulation and in reduced T cell numbers (Chapter 1, Fig. 4). In this study we sought to investigate the contribution of 2C11-Novi-mediated immunosuppression to the mechanism of action of anti-CD3/anti-TNF combination therapy.

To achieve this aim we performed a 2C11-Novi dose response study in arthritic DBA mice treated with a single dose of 100 μ g V1q-Novi. We demonstrated that the dose of 2C11-Novi required for prolonged therapeutic protection is associated with long term immunosuppression of peripheral T cells. Therefore, we used 2H2-Novi to neutralize the excess levels of 2C11-Novi in order to reverse its pharmacodynamic effects after the end of the 5-day treatment period. Using this strategy we showed that in absence of 2C11-Novi-mediated immunosuppression at the end of the 4-week study period, disease activity in the anti-CD3/anti-TNF combined treatment group remained significantly lower than in the control group. In conclusion, long lasting T cell immunosuppression mediated by 2C11-Novi is not required for the long term inhibition of CIA progression afforded by anti-CD3/anti-TNF combination therapy.

MATERIALS AND METHODS

Mice and mAb treatment.

Studies were conducted in eight-week-old male DBA/1J mice (Janvier Laboratories, Le Genest-St-Isle, France). Mice were housed in groups of 10. All animal experimentation was conducted according to license from the Swiss veterinary office for animal experimentation. All mAbs were administered intraperitoneally at indicated doses.

Induction and assessment of Collagen-induced-arthritis (CIA).

Please refer to Chapter II in the RESULTS section.

Flow cytometry.

For cell surface labeling, the following anti-mouse mAbs were used from BD Biosciences. Anti-CD3 ϵ -PE (145-2C11), CD4-FITC (RM4-5), and anti-TCR-PE (H57-597). Cells were incubated with Fc block (BD Biosciences) to reduce non-specific binding. Flow cytometry was performed using a FACS Calibur flow cytometer and analyzed with the CellQuestPro software (BD Biosciences).

CD3/TCR modulation.

Please refer to Chapter I in the RESULTS section.

Lymph node cell restimulations.

In the course of CIA, popliteal, inguinal, brachial and axillary LNs were extracted from arthritic mice fifteen days post-onset (d15). For each animal, lymph nodes were enzymatically digested in a solution of 1 mg/mL DNase and 2.5 mg/mL collagenase for 45 minutes at 37°C.

LN CD4⁺ cells were isolated using a CD4⁺ isolation kit according to the manufacturer's instructions (Miltenyi Biotech, Bisley, UK). The purity of the CD4⁺ population, as assessed by FACS Calibur flow cytometer, was above 85%. A total of 2x10⁵ purified CD4⁺T cells were then cultured in RPMI 1640 supplemented with L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and with 10% autologous serum, for three days in 96-round-well plate in the presence of Concanavalin A (ConA, 5 μg/mL). Supernatants were collected for cytokine analysis, and cells were incubated for the last 18 hours with ³H-thymidine (1 μCi/well) to quantify cell proliferation (expressed in counts per minute, cpm). The cytokines Interferon-γ (IFN-γ), Interleukin-17 (IL-17), Interleukin-6 (IL-6), and Tumor necrosis factor (TNF) concentrations were quantified by multiplex technology (Luminex).

Statistical analysis.

Please refer to Chapter II in the RESULTS section.

RESULTS

I- The therapeutic effect of anti-CD3/anti-TNF combitherapy is anti-CD3 dose dependent (*Fig. 1, and Fig. 2*).

In order to further explore the underlying mechanisms of action of 2C11-Novi/V1q-Novi combination therapy in established arthritis, we investigated the contribution of 2C11-Novi induced immunosuppression (Fig. 1). We first conducted a dose response study of 2C11-Novi combined with a single low dose of V1q-Novi. Briefly, arthritic mice were treated with a single injection of 100 µg of V1q-Novi alone or in combination with two, three or five daily consecutive injections of 50 µg of 2C11-Novi. Although all combination therapies inhibited disease progression in a statistically significant manner over the 4-week study period, a clear dose response relationship was observed between disease severity and the number of 2C11-Novi injections (Fig. 2A-B). Lymph nodes from some of the mice were collected prior to and 5, 14 and 28 days after treatment to evaluate both the number of peripheral T cells and the level of TCR expressed at their cell surface. Interestingly, we observed an inverse correlation between the number of 2C11-Novi injections and the number of CD4⁺ T cells in lymph nodes (Fig. 2C). Similarly there was an inverse correlation between the number of 2C11-Novi injections and the level of TCR expression on the remaining CD4⁺ T cells (Fig. 2D).

Collectively, the above results suggest that, in addition to depletion of antigen-specific T cells, 2C11-Novi-induced depletion of non-pathogenic T cell, as well as CD3/TCR modulation, may contribute to the long lasting therapeutic effects of the anti-CD3/anti-TNF combination therapy observed in CIA.

II-The dose of 2C11-Novi required for the therapeutic effect of anti-CD3/anti-TNF combitherapy is associated with peripheral T cell unresponsiveness (*Fig. 1, and Fig. 3*).

The main consequence of antigenic modulation is the inability of T cells to respond to further antigenic stimulation. In order to assess whether the dose regimen of 5x50 µg 2C11-Novi, either alone or in combination with V1q-Novi, is associated with peripheral T cell unresponsiveness, a new series of in vivo experiments was conducted as described in chapter 2, Fig. 1. Consistently with previous results, the synergistic therapeutic effect of combined 2C11-Novi/V1q-Novi was manifested by a long term inhibition of disease progression that was highly significant from day 15 to day 29, as compared to the control groups (data not shown). As a consequence, we selected day 15 as a relevant time point for dissecting T cell responses in these animals. On day 15 of arthritis, we harvested lymph nodes to evaluate the levels of antigenic modulation on peripheral T cells and their ability to proliferate and secrete proinflammatory cytokines in response to polyclonal T cell activation using Concanavalin A (ConA) (Fig. 3). As observed in the previous experiment (Fig 2C) there was an almost complete disappearance of CD3/TCR complex from the surface of lymph node CD4⁺ T cells from mice treated with either 2C11-Novi alone or with 2C11-Novi/V1q-Novi combined therapy (Fig. 3A). In addition, these cells were unresponsive to ConA stimulation in terms of proliferation (Fig. 3B) and proinflammatory cytokine secretion, including IFN γ , IL-17, TNF and IL-6 (Fig. 3C).

As expected, these results demonstrate that antigenic modulation inversely correlates with the capacity of T cells to respond to antigenic stimulation. In addition, it shows that the dose of 2C11-Novi required for the therapeutic effect of anti-CD3/anti-TNF combitherapy (5x50 µg), which is associated with long lasting antigenic modulation, induces profound peripheral T cell unresponsiveness 2 weeks post disease onset.

III-The therapeutic effect of anti-CD3/anti-TNF combitherapy is not a consequence of long lasting peripheral T cell unresponsiveness caused by antigenic modulation (*Fig. 4, and Fig. 5*).

Interestingly, despite long lasting antigenic modulation and profound peripheral T cell unresponsiveness, treatment with 2C11-Novi alone does not significantly inhibit CIA progression (Fig.1, chapter 2). This observation suggests that antigenic modulation and the associated T cell unresponsiveness, either does not play any role in the long lasting therapeutic effects of the anti-CD3/anti-TNF combination therapy, or is not sufficient. Because there is a tight correlation between the anti-CD3 plasma concentrations and the levels of antigenic modulation (Chapter 1, Fig 8), we thought that the 2H2-Novi anti-idiotypic could be used in vivo to rapidly reverse the 2C11-Novi mediated CD3/TCR modulation and study its role in the anti-CD3/anti-TNF therapeutic effects.

We first checked whether 2H2-Novi could neutralize 2C11-Novi induced antigenic modulation in vivo. Naïve DBA mice were injected with a single intra-peritoneal dose of 50 µg 2C11-Novi with or without 50 µg of 2H2-Novi. As expected, the single dose of 50 µg 2C11-Novi induced an almost complete antigenic modulation on lymph node CD4⁺ T cells (Fig. 4A-B). In contrast, when 50 µg of 2C11-Novi and 2H2-Novi were injected concomitantly, no significant reduction in the levels of TCR molecules expressed at the surface of LN CD4⁺ T cells was observed, as compared to that of untreated animals (Fig. 4A-B). We then conducted a 2C11-Novi multiple dose study to assess whether 2H2-Novi could accelerate CD3/TCR reexpression on T cells. Naïve mice were injected with 5 doses of 50 µg 2C11-Novi, from day 1 to day 5, and a single dose of 250 µg 2H2-Novi on day 6 (Fig. 5C). Three days later, blood and lymph nodes from the mice were collected to measure the resulting levels of TCR expression on CD4⁺ T cells. As expected, injections of 2C11-Novi alone induced an almost complete antigenic modulation (Fig. 4D). Interestingly, in mice

treated with 2H2-Novi, the levels of TCR expression on peripheral T cells were back to approximately 80% of the baseline value (Fig. 4D). These results demonstrate that 2C11-Novi mediated antigenic modulation can be rapidly reversed after administration of 2H2-Novi in vivo.

This neutralizing anti-idiotypic mAb was next used to study the requirement for antigenic modulation and the associated T cell unresponsiveness in the therapeutic effects of anti-CD3/anti-TNF combination therapy. To achieve this aim, arthritic mice were treated with the combination therapy as previously described, and 2H2-Novi was administered on day 6 (single dose of 250 µg, Fig. 5A). Lymph nodes were collected for flow cytometry analysis prior to and 5, 14 and 28 days after the first injection. The results confirmed that treatment with 2H2-Novi can rapidly reverse 2C11-Novi induced antigenic modulation in vivo. At days 14 and 28 no antigenic modulation was observed in the 2H2-Novi+combitherapy treated group while it was approximately 90% and 50% in the combitherapy treated group at these time points, respectively (Fig. 5B). In addition, 2H2-Novi treatment accelerated the rate of T cell reappearance in the periphery (Fig. 5C). Indeed, CD4⁺ T cell count in the lymph nodes were back to baseline in the 2H2-Novi+combitherapy treated group on day 28 while it was approximately 60% of the baseline value in the combitherapy treated group (Fig. 5C). Interestingly, despite the absence of 2C11-Novi-mediated immunosuppression at the end of the study period (day 28) in the 2H2-Novi+combitherapy treated group, disease activity remained significantly lower than in untreated mice ($p < 0.01$, Fig. 5D).

Based on the above findings, we conclude that long lasting T cell unresponsiveness caused by antigenic modulation is not required for the long term inhibition of CIA progression afforded by anti-CD3/anti-TNF combination therapy.

DISCUSSION

2C11-Novi-induced prolonged immunosuppression is not sufficient for long term therapeutic effects afforded by anti-CD3/anti-TNF combination therapy

2C11-Novi was engineered as a whole IgG molecule with a mouse Fc fragment to act as a murine surrogate for anti-human CD3 mAb therapies. These characteristics confer the desired long half-life to 2C11-Novi when administered parenterally in mice (Chapter 1, Fig. 7 and Fig. 8). As a consequence 2C11-Novi induced long lasting immunosuppressive pharmacodynamic effects in vivo (Chapter 1, Fig. 8). Indeed, a single intraperitoneal administration of 50 µg 2C11-Novi induced almost complete antigenic modulation for approximately 5 days and it took over 9 days for the CD3/TCR expression levels on T cells to return to their baseline value. These findings incited us to investigate whether 2C11-Novi-mediated immunosuppression could contribute to the therapeutic effects of 2C11-Novi/V1q-Novi combination therapy in mice with established CIA.

In this study we have shown that the anti-CD3/anti-TNF therapeutic efficacy in established CIA is 2C11-Novi dose dependent. Interestingly, the dose of 2C11-Novi required to obtain a long lasting inhibition of disease progression (5 x 50 µg) is associated with profound T cell depletion and prolonged antigenic modulation. Both, 2C11-Novi alone or the 2C11-Novi/V1q-Novi combination induced similar levels of antigenic modulation associated with comparable peripheral T cell unresponsiveness at day 15 after disease onset. At this time point, in contrast to combined therapy with 2C11-Novi/V1q-Novi, treatment with 2C11-Novi alone did not inhibit disease progression. Therefore, from these initial observations, it can be concluded that prolonged 2C11-Novi-induced immunosuppression may be necessary but is not sufficient for the long term therapeutic efficacy of 2C11-Novi/V1q-Novi combination therapy in mice with established CIA.

2H2-Novi neutralizes 2C11-Novi in vivo and reverses its pharmacodynamic effects

As shown in Chapter 1, there is a tight correlation between the plasma concentration of 2C11-Novi and the degree of antigenic modulation on peripheral T cells. Therefore, from the kinetics of CD3/TCR expression on CD4⁺ T cells in lymph nodes of mice treated with 5 x 50 µg of 2C11-Novi, it can be inferred that the mAb is not completely cleared from the plasma at the end of the 4-week study period.

We wondered whether the 2H2-Novi anti-idiotypic mAb could be used to bind free 2C11-Novi mAbs in vivo and accelerate its clearance. To achieve this aim, we conducted experiments to demonstrate whether 2H2-Novi could neutralize 2C11-Novi in vivo and reverse its pharmacodynamic effects. The results demonstrate that a 1:1 dose of 2H2-Novi can rapidly neutralize the biological activity of 2C11-Novi in vivo. This finding validates the use of 2H2-Novi as a strategy to investigate whether prolonged antigenic modulation is required for the long lasting therapeutic effect of combined 2C11-Novi/V1q-Novi therapy in arthritis.

Using a single dose of 250 µg of 2H2-Novi administered 24h after the last injection of 2C11-Novi, we showed that both antigenic modulation and reduced CD4⁺ T cell numbers in lymph nodes returned to baseline values 4 weeks after the beginning of the 2C11-Novi/V1q-Novi combined therapy in arthritic mice. Despite the absence of 2C11-Novi-mediated immunosuppression at the end of the 4-week study period, disease activity remained significantly lower than in control mice. These results demonstrate that long lasting T cell immunosuppression induced by 2C11-Novi is not required for the long term inhibition of CIA progression afforded by 2C11-Novi/V1q-Novi combination therapy.

Conclusions

Anti-CD3 antibodies have been described to induce tolerance, which can be defined as a lack of a destructive immune responses towards a given (auto-)antigen in the absence of ongoing immunosuppressive therapy. We have previously shown that a short course treatment with a non-Fc γ R binding anti-CD3 mAb administered in combination with a single low dose of anti-TNF mAb was associated with long-term inhibition of established collagen-induced arthritis (CIA). We suggested that that this apparent tolerogenic effect was due to an efficient depletion of peripheral Th1 and Th17 pathogenic T cells. In order to further explore the underlying mechanism of action of 2C11-Novi/V1q-Novi combination therapy in established arthritis, we investigated the contribution of 2C11-Novi induced immunosuppression. The dose of 2C11-Novi required for the long term therapeutic effects of 2C11-Novi/V1q-Novi combitherapy is associated with long lasting immunosuppression. In this study, using 2H2-Novi to neutralize the biological activity of 2C11-Novi, we could show that long lasting immunosuppression is not required. Therefore depletion of pathogenic T cells is likely to be the main mechanism by which anti-CD3/anti-TNF exert tolerogenic effects in established arthritis.

FIGURE LEGENDS

FIGURE 1

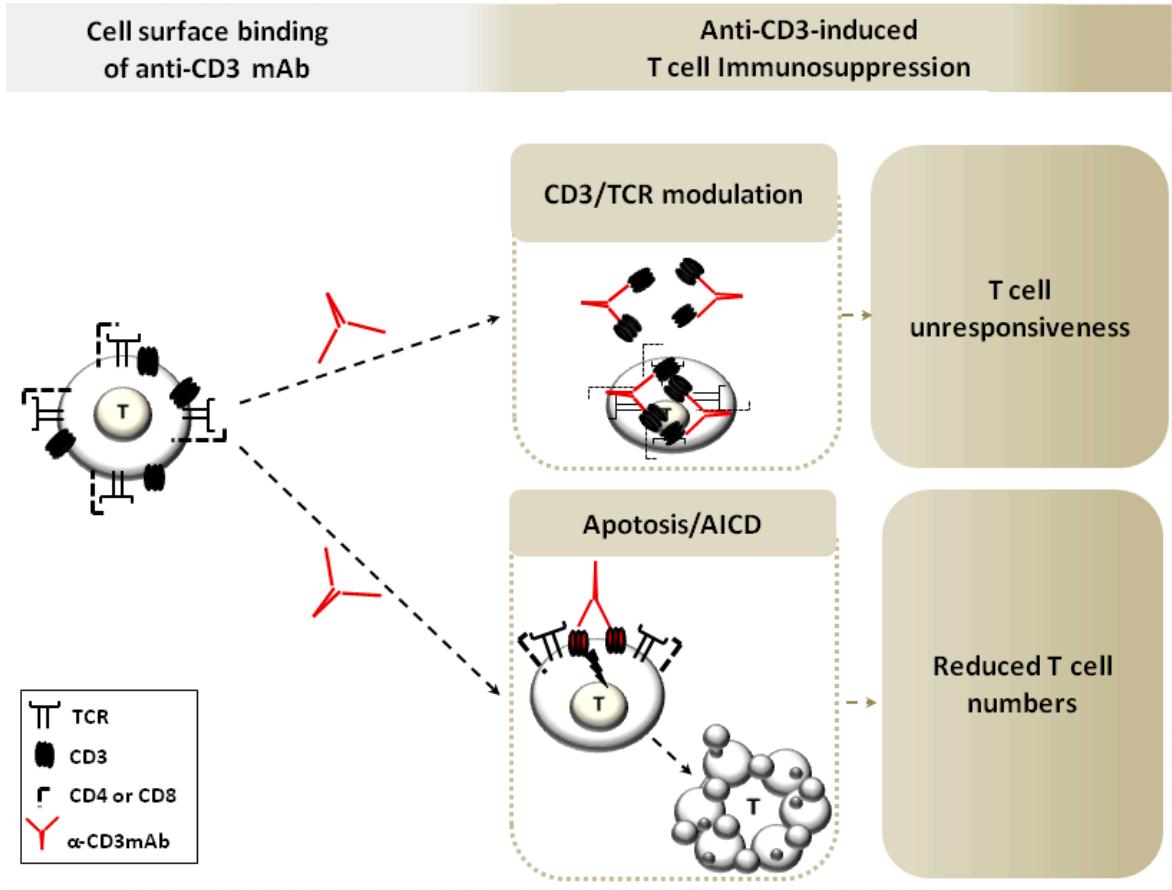


Figure 1. Overview of anti-CD3-induced T cell immunosuppressive mechanisms.

Anti-CD3 mAbs exert immunosuppression via two main pharmacodynamic effects on T cells i.e. modulation of the CD3/TCR complex from T cell surface, also referred to as antigenic modulation, (upper panel) and depletion of T cells (lower panel). Antigenic modulation consists in the internalization of the CD3/TCR complex that progressively disappears from T cell surface. This mechanism, which is reversible upon antibody clearance, renders T cell unresponsive to further antigenic stimulation. In addition, binding of anti-CD3 mAbs to CD3/TCR complex on T cells induces a partial T cell activation that can result in T cell death by apoptosis through a mechanism referred to as antibody-induced-cell-death (AICD) (lower panel).

FIGURE 2

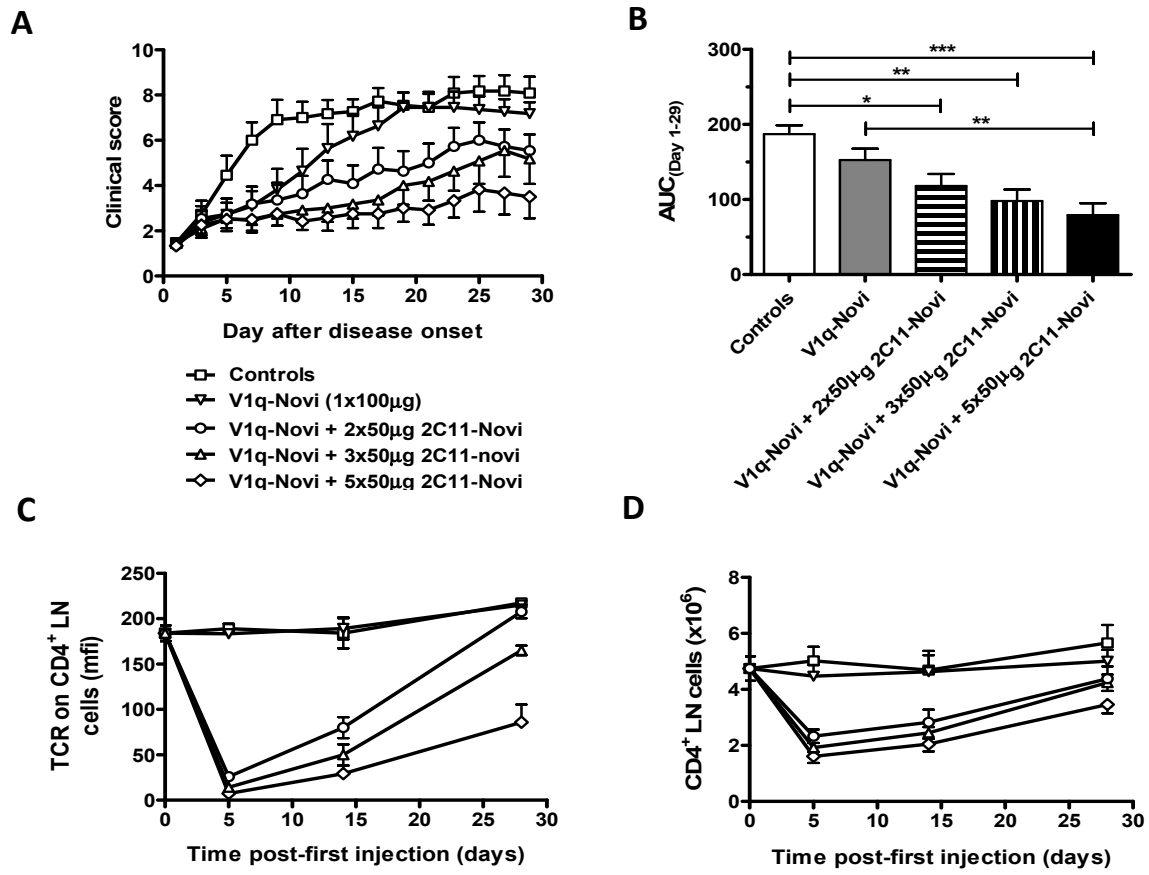


Figure 2: The therapeutic effect of combined anti-CD3/anti-TNF in CIA is anti-CD3 dose dependent. A-D, Arthritic mice were treated on day 1 of disease onset with a single dose of 100 µg of V1q-Novi in combination with 0, 2, 3 or 5 consecutive daily injections of 50 µg 2C11-Novi. The control group represents mice treated with isotype-matched control mAbs administered as that of the combitherapy group (1x100 µg mIgG2a and 5x50 µg mIgG1). Results are representative of experiments performed on 10 to 12 mice per group. **A,** Evolution of the clinical scores over time (4 weeks). **B,** The statistical significance of the difference between groups was calculated from AUC for each mouse. **C,** Levels of TCR expression on CD4⁺ T cell in the lymph nodes analyzed by flow cytometry prior to and 5, 14 and 28 day after the first mAb injection. **D,** Absolute CD4⁺ T cell counts in lymph nodes prior to and 5, 14 and 28 day post-first mAb injection.

FIGURE 3

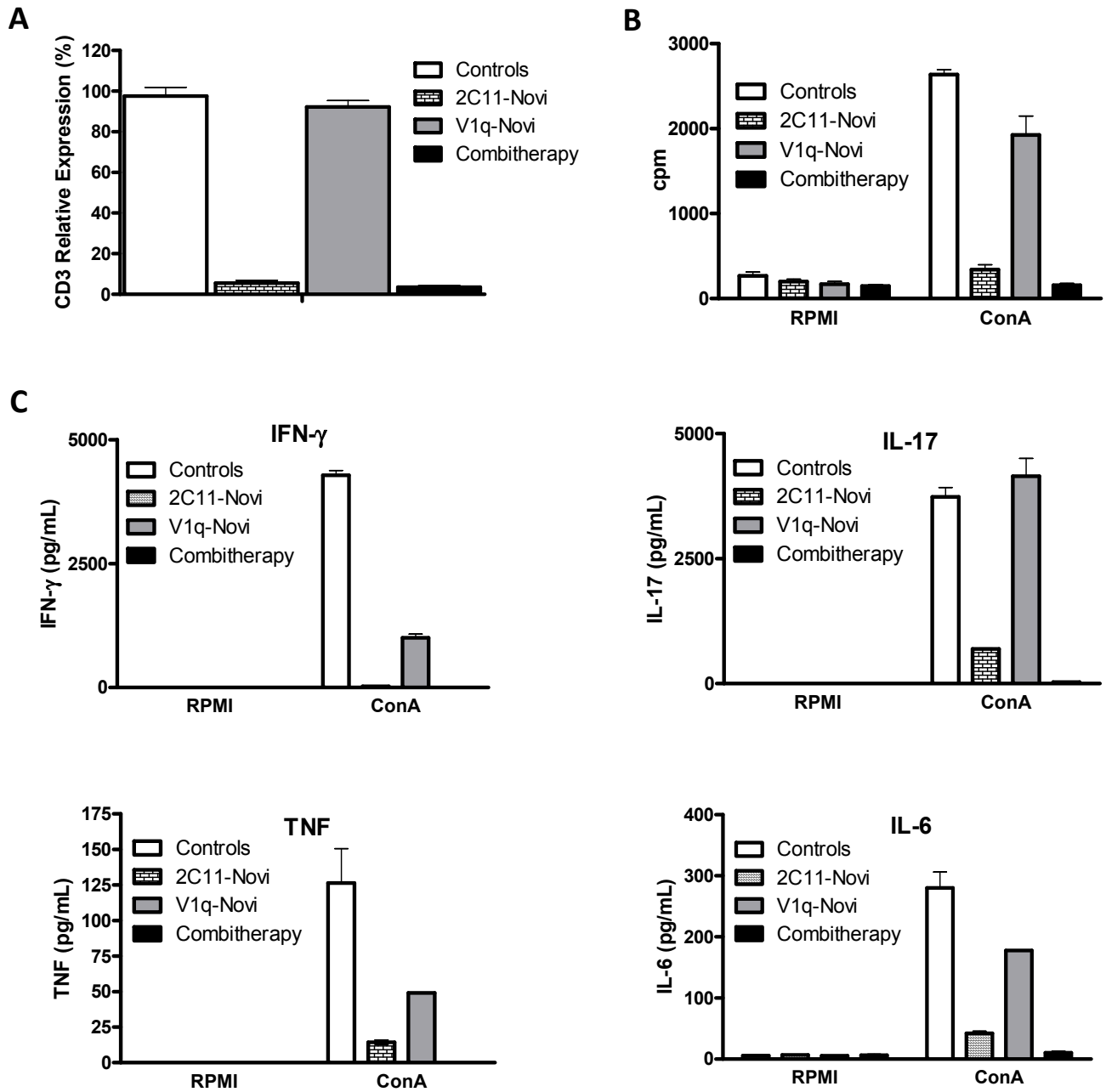


Figure 3: Dissection of peripheral T cell unresponsiveness induced by CD3/TCR modulation.

A-D, Arthritic mice were treated from day 1 of onset with a single 100 µg dose of V1q-Novi (anti-TNF), 5 daily consecutive injections of 50 µg 2C11-Novi (anti-CD3), V1q-Novi/2C11-Novi combination (combitherapy) or isotype-matched control mAbs (controls as described in Fig. 2). Mice were euthanized on day 15, and the lymph nodes were processed for subsequent analysis. **A**, Relative expression of CD3ε on CD4⁺ T cells (normalized to untreated animals). **B**, Proliferative responses of purified CD4⁺T cells stimulated by Concanavalin A (ConA) (5µg/mL). **C**, Cytokine secretion pattern of lymph node cells in response to ConA stimulation (5µg/mL). Interferon-γ (IFNγ), Interleukin-17 (IL-17), Interleukin-6 (IL-6), and Tumor necrosis factor-α (TNF) concentrations were quantified by multiplex technology (Luminex). Values are mean +/- SEM of at least eight individual mice pooled per group and are representative of two independent experiments.

FIGURE 4

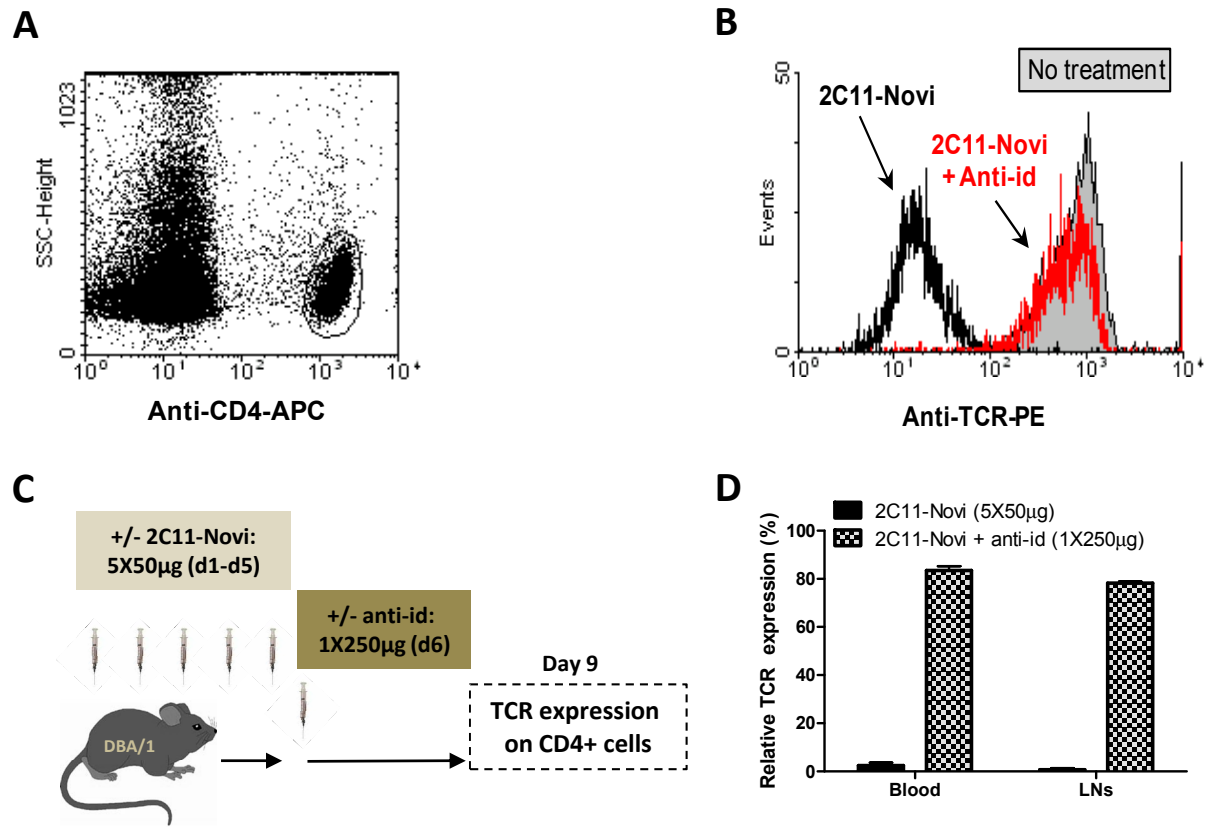


Figure 4: The anti-idiotypic 2H2 neutralizes 2C11-Novi in vivo.

A-B, Mice were injected with a single intra-peritoneal dose of 50 µg of 2C11-Novi with or without 50 µg of 2H2-Novi (Anti-id). Blood samples were collected 24h later for flow cytometry analysis. **A**, Representative dot plot used for the gating of CD4⁺ cell population from blood samples. **B**, Representative histogram including the resulting levels of TCR expressed on circulating CD4⁺ cells. **C**, Schematic representation of the study design. Five daily consecutive injections of 50 µg of 2C11-Novi (d1-d5) followed or not by a single administration of 250 µg of 2H2-Novi (Anti-id) (d6). Blood and lymph node cells were collected on day 9 for flow cytometry analysis. **D**, Relative TCR expression on circulating CD4⁺ T cells measured by flow cytometry using an anti-TCR mAb (normalized to untreated animals).

FIGURE 5

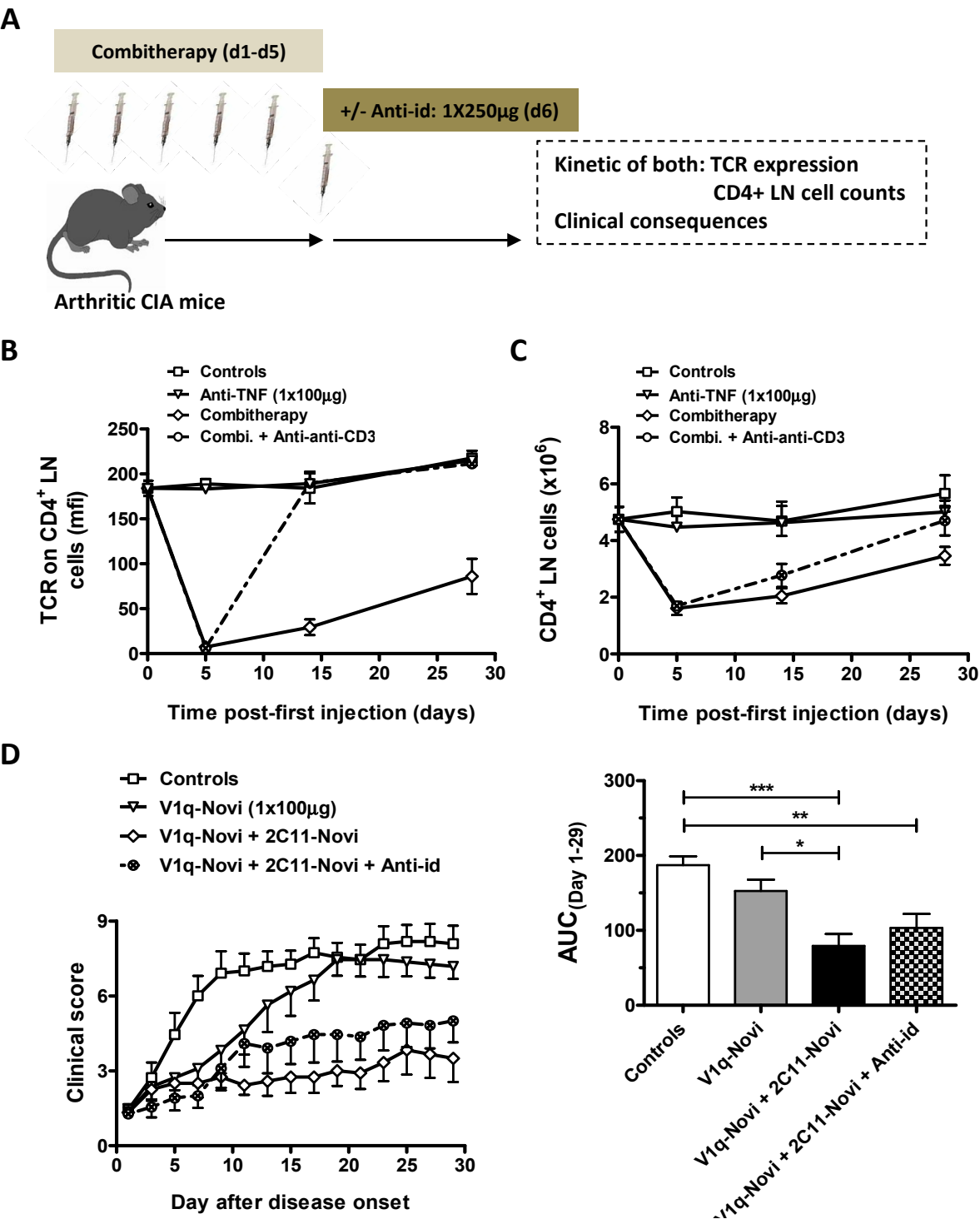


Figure 5: Synergistic therapeutic effect of anti-CD3/anti-TNF combined therapy in established CIA is not a consequence of prolonged immunosuppression. **A**, Schematic representation of the study design. Arthritic mice were treated on day 1 of onset with a single dose of V1q-Novi (100 μ g) (not shown), the V1q-Novi/2C11-Novi combined therapy (1x100 μ g V1q-Novi and 5x50 μ g 2C11-Novi) (d1-d5) or the combined therapy plus a single 250 μ g dose of the neutralizing anti-2C11-Novi mAb (Anti-id) administered on day 6. The control group represents mice treated with corresponding isotype-matched control mAbs. **B-C**, Prior to, and 5, 14 and 28 days post-first mAb injection, mice were euthanized to collect lymph nodes for flow cytometry analysis. **B**, Levels of TCR expression on CD4⁺ T cells. **C**, Absolute count of CD4⁺ T cells. **D**, Evolution of the clinical scores over time (4 weeks) (left panel). Results are representative of experiments performed with 10 to 12 mice per group. The statistical significance of the difference between groups was calculated from AUC for each mouse (right panel).

DISCUSSION AND CONCLUDING REMARKS

Studies conducted in animal models of human diseases contribute to a better understanding of the mechanisms driving disease pathogenesis, but also represent a valuable tool to evaluate the clinical potential of future therapeutics. For example, the murine CIA was initially used to validate the therapeutic use of anti-TNF mAbs to treat RA patients (M Feldmann et al., 1996). Using surrogate mAbs, the mode of action (MOA) and the benefit-risk ratio associated with future therapeutic mAbs can be evaluated in these animal models. Thus, the surrogate mAb of a therapeutic mAb must be specific for the corresponding mouse target and produce functional characteristics similar to that of their medicinal counterpart. However, as the emergence of improved versions of therapeutic mAbs occurs, the former anti-mouse reagent is often no longer a representative 'homologue'. This is the situation for the next generation of anti-CD3 mAbs. In addition, the accumulation of mechanistic knowledge once a protein is neutralized in patients may reveal characteristics needed that were not demonstrated by the originally used anti-mouse mAb. This is the case for the original clone of V1q mAb that is specific to mouse TNF.

The pharmacodynamic characteristics of the next generation of therapeutic anti-CD3 mAbs (Table 1) that we sought to reproduce in the surrogate mAb, 2C11-Novi, were to maintain the ability to efficiently induce CD3/TCR complex modulation yet present a lowered T cell activation potential and provocation of cytokine release. Thus, the variable regions of the original 145-2C11 mAb (Leo et al., 1987) were selected as they bind to the relevant chain of the complex, i.e. epsilon, mimicking the wanted level of modulation both *in vitro* and *in vivo*. Interestingly, others have used the F(ab')₂ form of 145-2C11 as it lacks the capacity to bind FcγRs (Lucienne Chatenoud & Bluestone, 2007), thus reducing both the T cell activation and cytokine storm associated with the 'whole' 145-2C11 mAb. However, due to a very short half-life *in vivo*, we considered that F(ab')₂ fragments were not appropriate surrogate molecules. Therefore, our surrogate mAb was engineered as a full IgG with the variable

regions of 145-2C11 grafted onto a mouse IgG1 Fc backbone mutated in a single amino acid rendering the molecule unable to bind FcγRs and to trigger the Fc-associated effector functions of IgG(Baudino et al., 2008)(Nimmerjahn et al., 2005) (Chapter 1).

For assessing TNF biology, we used the variable regions of V1q, a mouse anti-TNF mAb that neutralizes TNF *in vivo* and binds both the soluble and transmembrane forms of the cytokine(Echtenacher et al., 1990)(Scott et al., 2003). However, as the commercially available V1q is a rat IgD, it does not elicit effector functions which are now thought to be an important feature of the therapeutic effects of anti-TNF therapies(Mitoma et al., 2008)(Gerspach et al., 2000)(T. Horiuchi et al., 2010) (38, 39). Thus, we engineered a rat/mouse chimeric mAb, V1q-Nov1, to have the variable regions of V1q on the mouse IgG2a Fc backbone. We confirmed that V1q-Nov1 neutralizes the biological activity of TNF, both *in vitro* and *in vivo*, and has the capacity to interact with FcγRs (Chapter 1).

Thus in order to start to ask biologically relevant questions that would be relevant for translation into RA patients, these two new formats of existing anti-mouse mAbs were optimized and characterized prior to commencement of the pivotal experiments. The data show that both *in vitro* and *in vivo* these mAbs present the characteristics we aimed at producing (presented in Chapter 1). In addition, the 2C11-Nov1 and V1q-Nov1 mAbs will be available as tools for future *in vivo* nonclinical studies.

Therapeutic effects classically associated with use of anti-CD3 mAbs monotherapy: why not observed in CIA mice?

T cells contribute to the pathogenesis of autoimmune diseases including, for example, RA or Type I diabetes. The success of anti-CD3 therapy in the field of organ transplantation already demonstrated that a clinically proven way to alter T cell function was by the administration of anti-CD3 mAbs (Goldstein et al., 1986). Anti-CD3 mAbs act through both direct and indirect mechanisms that can result in long term tolerogenic effects in the murine models of NOD, EAE and atherosclerosis (Lucienne Chatenoud & Bluestone, 2007) (Steffens et al., 2006). For example, in NOD mice, anti-CD3 mAbs directly eliminate pathogenic effector T cells, but also contribute to the maintenance of tolerance to beta islet cells by inducing TGF- β dependent Treg cells. Consistently, TGF- β has also been shown to be involved in the tolerogenic effects induced by anti-CD3 mAbs in EAE (Perruche et al., 2008) and a murine model of atherosclerosis (Steffens et al., 2006). Thus, it was hypothesized that, as T cells also contribute to CIA pathogenesis, it was likely that anti-CD3 monotherapy would also provide such protective effects in this model. However, in our study with our new reagent, i.e. 2C11-Novi, therapeutic administration of the anti-CD3 mAb alone did not significantly ameliorate nor alter the course of CIA for long term (Chapter 2). Therefore, it was surprising to us that Notley and colleagues recently observed that a single dose of an anti-CD3 mAb, had a long term effect on established arthritis (C. a Notley et al., 2010). However, their study was different from ours in two ways: first, the reagent, i.e. the 145-2C11 used is Fc γ R-binding, inducing significant T cell activation and secondly, technically, as the authors evaluated the clinical scores for only ten days after antibody injection. Indeed, repeating the conditions of their experiment in our lab, with the 145-2C1, we were able to confirm the published observation, but more surprisingly, we also observed a progressive flare of the disease resulting in a clinical arthritis comparable to that of control animals from day 15 to day 28 post-disease onset (data not included in thesis), contrasting and challenging the conclusions of Notley and colleagues (C. a Notley et al., 2010). Altogether, these data demonstrate that the

therapeutic use of anti-CD3 mAbs alone is not truly sufficient to ameliorate established murine CIA. Thus, other mechanisms are dominating the orchestration of the inflammation, and potentially masking the expected effect of an anti-CD3 treatment. Therefore, we hypothesized that in the pathogenesis of CIA, TNF, which plays a dominant role in the effector phase of the disease (M Feldmann et al., 1996), could mask the importance of pathogenic T cells in established disease, in turn, rendering T cell neutralization insufficient by itself to ameliorate ongoing arthritis. Indeed, it is likely that inflammatory effector mechanisms driven by TNF can progressively dominate when compared to T cell responses in established arthritis. The dominating TNF role hypothesis is supported by the observation that, anti-TNF monotherapy using V1q-Novi controlled articular inflammation in already arthritic mice with CIA, while anti-CD3 monotherapy was not efficient in that (Chapter 2).

Another question to reflect on from our results is why are T cells detected in joints of mice treated with anti-CD3 monotherapy despite the capacity of this mAb to deplete T cells in the peripheral lymph nodes? The most attractive hypothesis is that anti-CD3 mAbs gain access to the cellular compartment more effectively than into the synovium of joints. Indeed, we inject doses high enough to saturate the target in the blood and thus cells in the circulation for several weeks e.g., prolonged antigenic modulation presented in Chapter 3, and the lymphatic circulation has a direct interface with secondary lymphoid tissues. And thus, 2C11-Novi is first removed in the compartment of the blood, then in the lymph nodes and spleen with only minimal amounts available to arrive in the sites of articulation which have a poorer system of vasculature. To formally address this question, we could quantify the amount of 2C11-Novi from joint extracts of mice treated with 2C11-Novi. Another alternative explanation is that the T cells within the joint are different from their peripheral counterparts in that they have become resistant to anti-CD3-induced cell death, as discussed later.

TNF and T effector cell responses in CIA

In addition to directly promoting articular inflammation, TNF can also negatively affect T cells responses. Indeed, it was reported that chronic exposure of T cells to TNF reduced their ability to proliferate and secrete cytokines both *in vitro* and *in vivo* (Cope et al., 1994). More recently, Notley and colleagues have demonstrated that TNF dampened down Th1 and Th17 responses in arthritis through the p40 arm of IL-12 and IL-23 (C. A. Notley et al., 2008). They observed that anti-TNF treatment of murine CIA resulted in an expansion of pathogenic Th1 and Th17 cells in the draining LNs. To explain this finding, the authors proposed that somehow TNF neutralization alters the egress of T eff cells from LNs resulting in a local accumulation. Indeed, this protective mechanism consisting in sequestering pathogenic T effector cells in the periphery has been observed by Fagete and colleagues in murine CIA by neutralizing chemokines (Buatois et al., 2010). In our study, however, using V1q-Novi in murine CIA, we did not observe any increase in the number of Th1/Th17 cells in LNs. Inversely, we observed a substantial reduction in the number of these cells in mice treated with V1q-Novi alone. The seemingly opposing results between the two laboratories are most likely due to our anti-TNF mAb that may be more efficient at inducing T cell killing after its binding to transmembrane TNF.

Synergism during dual targeting of both TNF and T cells in established CIA

As anti-TNF mAbs were given chronically by Notley and colleagues to block the egress of pathogenic T effector cells in the LNs of mice with CIA, i.e., 300 µg of TN3-19.12 mAb was given every three days to neutralize TNF all along the study period, it would be interesting to address the consequence of antibody withdrawal on the future migratory behavior of these T cells (C. A. Notley et al., 2008). Importantly, they also demonstrated that these pathogenic T

cells accumulating in the periphery still preserved their ability to transfer arthritis, the same observation published by Fagete and colleagues (Buatois et al., 2010), i.e. when the drug is removed, the T cells emerge and disease returns in force. Therefore, as these pathogenic T cells still represent a potent risk for future flare of arthritis, additional therapies aiming at neutralizing T cell responses while blocking TNF could represent a valuable strategy to be tested in the treatment of established arthritis. Indeed, a previously conducted study demonstrated that anti-CD4/anti-TNF combined treatment synergistically ameliorated established CIA (R O Williams, Mason, Feldmann, & Maini, 1994). However, at that time, several scenarios were proposed but no clear mechanism was found to explain the synergistic effects resulting from this combined therapy. First, as they observed reduced titers of antibodies specific to the anti-TNF mAb used, they proposed that penetration of anti-TNF mAbs into joints could be greater under this combined therapy, thus probably resulting in a better neutralization of TNF within joints. Second, unlike anti-TNF monotherapy, they hypothesized that combination of anti-CD4/anti-TNF mAbs should also block pathology caused by other inflammatory mediators that are dependent on CD4⁺ T cells. A few years later, another study also reported the clinical benefits from dual targeting of TNF and T cells in established arthritis (Malfait et al., 2001). For this, they used the FcγR-binding anti-CD3 mAb 145-2C11 combined with an anti-TNF mAb in a chronic relapsing mouse model of CIA (Malfait et al., 2001). Although this study clearly demonstrated both clinical and histological improvement, it did not address the underlying mechanisms. Surprisingly, in their publication, there is an absence of control animals treated with 145-2C11 alone. Given that the FcγR binding format of anti-CD3 mAb used was known to induce morbidity when administered at high doses in mice, we speculate that this could explain the lack of including a control group of mice treated with the anti-CD3 mAb alone in their publication. In our experience they have used a lethal dose of 145-2C11, i.e., 100 μg. Interestingly, combined

with an anti-TNF there was no mortality reported. This observation is consistent with studies showing that TNF is the main cytokine responsible for the anti-CD3 side effects (Lucienne Chatenoud, 2003). By neutralizing TNF Chatenoud demonstrated that most anti-CD3 induced side effects could be prevented (Lucienne Chatenoud, 2003).

In our studies, we demonstrated, for the first time, the therapeutic potential of combining a non-FcγR binding anti-CD3 mAb with an anti-TNF mAb in murine CIA. To study potential synergism, dose finding studies were performed first in order to identify doses of V1q-Novi and 2C11-Novi that, when administered individually, provide suboptimal therapeutic effects. V1q-Novi produced a dose dependent amelioration of the clinical readouts, and a single injection of 100 μg was chosen as the sub-therapeutic dose to use in the combination experiments. As for the anti-CD3, the treatment regimen, established in the NOD mouse model of 5 consecutive daily injections of 50 μg/mouse was tried and, as it did not provide long term therapeutic effects in murine CIA, was selected to evaluate the potential of the combined therapy.

Although each monotherapy provided respectively some of the effects afforded by anti-CD3/anti-TNF combined therapy, the results of the combination therapy were more effective than either monotherapy alone. For example, we observed an initial neutralization of TNF within joints of mice with CIA treated with either V1q-Novi or with the combined therapy (not shown), ultimately, resulting in the blockade of ongoing articular inflammatory process, i.e. at least up to day 6 of arthritis. On the other hand, we also observed a significant depletion of pathogenic T cells in the periphery of mice treated with either 2C11-Novi alone or with the combined therapy (Chapter 2 and not shown). However, the intensity of these effects were different. For instance, we observed that murine CIA treated with anti-CD3/anti-TNF combined therapy resulted in a more profound reduction in the number of pathogenic T cells

in the periphery than that observed when treated with the anti-CD3 mAb alone. In addition, and probably the most relevant for the clinical scoring was that only mice treated with the combined therapy showed no or very few infiltrating CD4⁺ T cells in the joints. Taken together, we proposed that anti-CD3/anti-TNF combined therapy neutralized TNF within joints while efficiently killing pathogenic T cells (in the periphery and/or in joints), thus preventing articular inflammation and subsequent long term development of arthritis.

Pathogenic Th1/Th17 cells in the treatment of murine CIA with anti-CD3/anti-TNF combined therapy: balance between peripheral/local killing and prevention of T cell migration into joints

During the process giving rise to arthritis, T cell responses are continuously initiated in the periphery before the cells migrate into the target tissues. In this regard, it is conceivable that both the killing of pathogenic T cells in the periphery and in arthritic joints would provide better clinical outcomes. In our study, amongst the overall reduction in the number of CD4⁺ T cells, we observed a greater reduction in the number of collagen-specific Th1/Th17 cells in LNs from mice treated with the combined therapy compared to that of mice treated with 2C11-Novi alone. From this observation, we hypothesize that some pathogenic T cells that escaped anti-CD3-induced cell death, i.e., (AICD), were ‘captured’ and killed by the additional anti-TNF treatment. Anti-TNF mAbs are known to induce T cell death via the mechanism of engaging transmembrane TNF (Mitoma et al., 2008) (Gerspach et al., 2000) (T. Horiuchi et al., 2010), and to test our hypothesis, we are in the process of demonstrating that T cells that are resistant to anti-CD3-induced AICD express transmembrane TNF. However, as this precursor form of soluble TNF is quickly cleaved from T cell surface, it may be difficult to detect the latter, especially in physiological conditions. To overcome this

limitation, we will try to inhibit the enzyme responsible for its cleavage, i.e. TACE. If technically feasible and the data corroborates the hypothesis, the next experiment will be to demonstrate that these cells can be effectively killed after addition of V1q-Novi mAb.

As we observed no or very few T cells in joints from mice treated with the combined therapy, we hypothesize that the absence of T cells locally could result from local depletion of T cells and/or altered migration of T cells from periphery to joints. In contrast, T cells still accumulated in joints from mice treated with 2C11-Novi given alone, suggesting that these T cells may have become resistant to anti-CD3-induced cell death locally. In line with the scenario previously proposed for the killing of LN T cells, we hypothesized that in joints of mice treated with the combined therapy, T cells that were resistant to anti-CD3-induced cell death, could be killed by the additional anti-TNF mAb therapy.

Alternatively, we could also consider the consequences of the different treatments on the migratory behavior of pathogenic T cells. Anti-CD3 therapy alone did not alter T cell migration into arthritic joints as T cells still accumulated within joints of mice treated with the anti-CD3 mAb alone. Inversely, it is well established that anti-TNF therapy inhibits the migration of pathogenic T cells from periphery to joints (Peter C Taylor, Peters, et al., 2000) (C. A. Notley et al., 2008). Therefore, we proposed another scenario based on the capacity of both anti-CD3 and anti-TNF mAbs, to kill T cells and to prevent their migration into joints, respectively. We proposed that anti-TNF transiently stopped T cell migration into joints while anti-CD3 mAbs killed T cells accumulating in the periphery. However, this model is still speculative, and other experiments should be conducted to test this hypothesis. For this, experiments could be carried out to demonstrate the ability of chronic treatment with V1q-Novi to induce the accumulation of pathogenic T cells in the draining lymph nodes. If correct, a second experiment to determine if the additional administration of anti-CD3 mAbs could deplete these accumulated pathogenic T cells could be performed.

To conclude, in murine CIA when treated with anti-CD3/anti-TNF combined therapy, it is possible that both scenarios may occur simultaneously, i.e., local depletion of pathogenic T cells resistant to anti-CD3-induced cell death and altered migration of T cells from periphery to joints, ultimately limiting the generation of pathogenic Teff cells that results in a reduced number of T cells within the joints of these mice.

Requirement of high amounts of 2C11-Novi combined with anti-TNF to obtain better clinical amelioration in murine CIA: probably needed to achieve enough T cell depletion?

Initially, we observed that the therapeutic effect of the combined anti-CD3/anti-TNF treatment in murine CIA was anti-CD3 mAb dose dependent as the best clinical amelioration was observed with the higher dose of the anti-CD3 mAb (Chapter 3).

Another question that our results provoke is why higher amounts of anti-CD3 mAb must be combined to anti-TNF mAb to achieve better clinical outcomes? The explanation may lie in the ability of anti-CD3 mAbs to deplete T cells. Indeed, in this dose response study we also observed that reduced numbers of LN CD4⁺ T cells correlated with better clinical benefits. From this observation, we propose that high amounts of 2C11-Novi mAb when combined with V1q-Novi invoke a greater combined T cell depletion (in the peripheral tissue and/or joints) to provide a long term clinical amelioration of CIA. Thus, high amounts of 2C11-Novi must be combined with an anti-TNF mAb to reach sufficient levels of pathogenic T cell depletion. To test further this hypothesis, simultaneously both the clinical scores and the extent of depletion of pathogenic Th1/Th17 cell in LNs from mice treated with increasing amounts of 2C11-Novi combined to V1q-Novi could be evaluated. In parallel, the resulting extent of T cells detected in joints would be evaluated. Together, these experiments would

help to evaluate the clinical importance of depleting pathogenic T cells in the periphery and/or in joints of mice with CIA treated with anti-CD3/anti-TNF combined therapy.

During the characterization of our non-FcγR binding anti-mouse CD3 mAb, we observed that 145-2C11 mAb was more potent at inducing T cell depletion *in vivo* (*not shown*). Interestingly, both anti-CD3 mAbs have been used to treat established murine CIA. One single injection of 145-2C11 was potent at inhibiting ongoing arthritis, which was probably due to an effective depletion of pathogenic T cells (C. a Notley et al., 2010). By contrast, administration of 2C11-Novi that depleted less T cells compared to 145-2C11, was less potent at inhibiting clinical arthritis. Taken together, these findings would suggest that the extent of T cell depletion ensured by an anti-CD3 mAb probably determines its potential efficacy in the treatment of established arthritis. If this hypothesis is correct, it could be anticipated that high amounts of Fc-modified anti-human-CD3 mAb should be used in the treatment of RA patients. This is presumably the reason that we had to combine high amounts of 2C11-Novi with anti-TNF mAbs to achieve better clinical protection in murine CIA. In other words, higher amounts of 2C11-Novi are required to deplete enough pathogenic T cells early in the course of disease. This hypothesis is even more probable if we consider T cell migration from periphery to joints as a dynamic process still occurring after disease onset in murine CIA. In this case, during the five consecutive days of administration of 2C11-Novi, it is likely that the amount of anti-CD3 injected was not sufficient to induce a quick and appropriate level of pathogenic T cells depletion after the first injection. As a consequence, some pathogenic T cells that escaped anti-CD3-induced T cell death in the periphery during the first days of the treatment eventually migrated into joints to induce arthritis locally. To test this hypothesis, at disease onset the cumulative amount of 2C11-Novi classically injected over the five days of the regimen protocol (i.e. 250 μg) should be injected in one dose and the extent of T cells accumulated within joints evaluated over time.

Collectively, our data demonstrated the requirement to combine high amounts of 2C11-Novi with anti-TNF in established arthritis to achieve long term clinical remission.

Consequences of high doses of 2C11-Novi combined with anti-TNF mAbs in CIA: prolonged PK of 2C11-Novi resulting in long lasting antigenic modulation and T cell unresponsiveness

Importantly, anti-CD3/anti-TNF combined therapy also resulted in persistent antigenic modulation on T cells that was still observed in mice with CIA up to three weeks after the end of the treatment period (Chapter 3). Therefore, as we have recently observed in naïve mice that pharmacokinetics of 2C11-Novi correlates with antigenic modulation, it can be inferred from this observation that 2C11-Novi was not fully cleared from the plasma at the end of the four-week study period (Chapter 1). This was probably due to the high amounts of 2C11-Novi injected in these mice, i.e., five daily consecutive injections of 50 µg. As a major challenge in clinical development is to achieve clinical remission after drug clearance, we next address whether clinical protection afforded by anti-CD3/anti-TNF combined therapy would be maintained after 2C11-Novi withdrawal. To address this question, different strategies could be envisaged. For example, we initially thought to prolong the study period to enable a physiologic clearance of 2C11-Novi to occur in these mice. However, because the manifestation of clinical arthritis does not exceed several weeks in mice with CIA, it was not relevant to prolong the duration of clinical observation of these mice. Alternatively, to accelerate drug clearance we also thought to reduce the initial amount of antibody injected. However, we did not choose to reduce the amount of anti-CD3 mAbs in the combined therapy as it resulted in reduced clinical efficacy. Ultimately, to address this question, our strategy was to use 2H2-Novi, a potent neutralizing anti-idiotypic mAb specific to 2C11-Novi in order

to accelerate the clearance of 2C11-Novi *in vivo*. By administrating 2H2-Novi in mice previously treated with the combined therapy, we demonstrated that *in vivo* neutralization of 2C11-Novi was still associated with a significant clinical protection. Therefore, we concluded that prolonged exposure of free 2C11-Novi in these mice was not required for long term clinical protection, thus, probably providing a potential new approach to achieve drug-free remission in arthritis.

The prolonged exposure of T cells to 2C11-Novi induced a persistent antigenic modulation that resulted in T cell unresponsiveness in mice treated with the combined therapy (Chapter 3). From this observation, we hypothesized that prolonged antigenic modulation induced by excess of 2C11-Novi was required for the long lasting therapeutic effect of combined 2C11-Novi/V1q-Novi therapy in established arthritis. However, we next demonstrated (with the anti-anti-CD3 mAb, 2H2-Novi) that inducing experimentally the early re-expression of the CD3/TCR complex *in vivo* had little impact on the disease score in that there remained a significant clinical protection. We thus concluded that prolonged antigenic modulation induced by 2C11-Novi was not required for the long lasting therapeutic effect afforded by the combined therapy. Nevertheless, in these experiments we did not address whether collagen-specific T cells re-expressing CD3/TCR were still responsive to further antigenic stimulation. It will be interesting to address the question of prolonged unresponsiveness of pathogenic T cells as another tolerogenic effect induced by the combined therapy after anti-CD3 mAb withdrawal, i.e., induction of an anergic state. For this, for example, mice developing CIA could be treated with the combined therapy, then the 2H2-Novi administered, rapidly reversing 2C11-Novi antigenic modulation *in vivo*, and evaluate the responsiveness of antigen-specific T cells *ex vivo* from these mice. Non-antigen specific stimulation could be performed *in vitro* to evaluate the extent of generalized T cell immunosuppression occurring in these treated mice.

Together, these findings and future experiments could help to better understand both qualitatively and quantitatively the consequences of anti-CD3/anti-TNF combined therapy on T cell responsiveness after 2C11-Novi clearance.

Perspective for additional tolerogenic effects probably afforded by the combined therapy after 2C11-Novi withdrawal: investigate consequences on T reg cell development/function

In addition to inducing anergy of pathogenic T cells, 2C11-Novi could also promote long term tolerogenic effects by expanding regulatory T cell responses to counteract that of the pathogenic T cells. Indeed, the suppressive function of Treg cells isolated from RA patients is compromised, and the absence of controlling pathogenic T cell populations due to ineffective Treg cells certainly contributes to disease maintenance(Ehrenstein et al., 2004)(Nadkarni et al., 2007). In this regard, therapies promoting the development/restoration of functional Tregs should be beneficial in the treatment of RA patients, i.e. by restoring the balance between regulatory and effector T cell responses. Interestingly, both anti-CD3 and anti-TNF monotherapies promoted Treg cells by either inducing their development and by restoring their suppressive function, respectively. Indeed, for example, the clinical use of anti-TNF therapy restored the suppressive functions of T regs in RA patients(Ehrenstein et al., 2004)(Nadkarni et al., 2007), and the therapeutic use of 145-2C11 induced Treg cell expansion in murine CIA(C. a Notley et al., 2010). As a consequence, we initially thought that anti-CD3/anti-TNF combined therapy would promote T reg development or function in murine CIA. Surprisingly, no expansion of a Treg cell population was observed. In addition, as antigenic modulation affected all T cell populations including Tregs during the study period, it was unlikely that Tregs would have been able to exert any antigen-specific

suppressive function in these conditions. For this reason, as long as 2C11-Novi induced antigenic modulation persists, it may be difficult to directly investigate the consequence of this treatment on the suppressive function of Treg cells. However to address this question, 2H2-Novi could be administered in these mice to accelerate clearance of 2C11-Novi, ultimately, restoring CD3/TCR expression on T cells and then evaluate the expansion or competency of the Treg cells. Alternatively, adoptive transfer experiments could also be envisaged to address this question.

Together, these future experiments would further illustrate the *in vivo* consequences of combining a non-FcγR binding anti-CD3 mAb with an anti-TNF mAb on the suppressive function of Treg cells. From a therapeutic point of view, these findings would help to better evaluate the potential of this treatment to provide long lasting tolerogenic effects mediated by Tregs after 2C11-Novi clearance.

Recently, it has been demonstrated that a single injection of 20 µg of 145-2C11, was potent at inducing the expansion of Treg cells in murine CIA(C. a Notley et al., 2010). In contrast, neither 2C11-Novi alone or combined with anti-TNF mAb reproduced these effects in murine CIA (Chapter 2). Part of the explanation may originate from the high cumulative amount of 2C11-Novi administered in these mice, i.e., 250 µg. We propose two reasons for why such elevated amounts of anti-CD3 mAbs injected into mice with CIA could affect Treg cell populations *in vivo*. First, as we observed a reduced number of Treg cells in LNs from these mice, it is possible that 2C11-Novi killed a broad spectrum of T cells including Treg cells *in vivo*. Due to the prolonged pharmacokinetics of 2C11-Novi *in vivo*, another consequence of injecting high amounts of this mAb into mice is a long lasting antigenic modulation that also affects the Treg cell population. For this reason, it is likely that the resulting Treg cells were unable to respond to antigenic stimulation *in vivo*, and thus, preventing the latter to proliferate

in vivo. As a consequence, regarding the dose regimen of 2C11-Novi we administered into murine CIA, it is not surprising that Treg cell population did not expand *in vivo*.

Although we did not observe Treg cell expansion, potentially for reasons mentioned above, it would be interesting to investigate whether Treg cells could expand in a delayed manner after the clearance of 2C11-Novi. Indeed, as the combined therapy induced important T cell depletion in murine CIA, with time the space left due to this depletion could allow a future re-population of Treg cells by the resulting homeostatic pressure.

PD markers associated with the clinical efficacy of anti-CD3/anti-TNF combined therapy in the treatment of established arthritis in CIA mice

In addition to the clinical readouts of the treatment, other parameters can be monitored during the therapeutic use of anti-CD3 mAbs, such as those considered pharmacodynamic (PD) markers, e.g., T cell counts, antigenic modulation. The present work based on anti-CD3/anti-TNF combined therapy in murine CIA also permits the evaluation of these relevant PD markers and determines their level of association with clinical outcome. For example, in this study, we observed that the analysis of both antigenic modulation and T cell counts in the blood of mice with CIA were not predictive of clinical efficacy. Indeed, unlike anti-CD3/anti-TNF combined therapy, anti-CD3 therapy alone did not afford statistically significant amelioration of arthritis despite similar levels of both antigenic modulation and circulating T cell numbers observed in both groups. More interestingly, key features were associated with mice treated with the combined therapy only. For example, a profound reduction in the number of collagen-specific Th1/Th17 cells in LNs, as well as no or very few T cells detected in their joints.

To conclude, as about 1% of the world's population is afflicted by RA, there is an urgent need for better therapies which originate from conducting relevant experiments in the laboratory before translating into clinical trials. Taken together, our results, generated using the gold standard model for RA and well characterized, relevant surrogates of currently used therapeutic antibodies, reveal a new approach to achieve clinical remission of arthritis with a positive therapeutic outcome that remains even in the absence of drug. This work demonstrated the potential clinical utility of the combination of anti-CD3 mAbs with anti-TNF therapies in established arthritis. In addition, as TNF is not the only proinflammatory cytokine involved in the pathogenesis of RA (e.g. IL-6 and IL-1), it is possible that their neutralization in combination with anti-CD3 mAb therapy could also result in synergistic therapeutic effects. In this regard, in preliminary experiments, I have shown that simultaneous treatment with anti-CD3 and anti-IL-6 mAb therapies synergistically inhibit the progression of established murine CIA (not shown in the thesis). Along this line, Ablamunits and colleagues have recently demonstrated synergistic reversal of T1D in NOD mice with anti-CD3 mAb treatment in combination with interleukin-1 blockade (Ablamunits et al., 2011). Collectively these findings validate the combined targeting of adaptive and innate immune systems, using anti-CD3 and anti-cytokine mAb therapies, as a very attractive strategy for the treatment of autoimmune diseases.

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