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ACTIVE ANTI-TNF ALPHA IMMUNIZATION IN A MURINE MODEL OF RHEUMATOID ARTHRITIS. RELEVANCE TO HUMAN DISEASE

L'IMMUNISATION ACTIVE ANTI-TNF DANS LA POLYARTHRITE RHUMATOÏDE : DU MODELE ANIMAL A LA MALADIE HUMAINE

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LIST OF ABBREVIATIONS

ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
ACR	American College of Rheumatology
ADA	Anti-drug antibodies
Ag	Antigen
ANA	Antinuclear antibodies
Anti-dsDNA	Anti-double strand DNA antibodies
ANSM	Agence nationale de sécurité du médicament et des produits de santé
APC	Antigen presenting cell
ARMADA	Anti-TNF Research Study Program of the Monoclonal Antibody D2E7 in patients with Rheumatoid Arthritis trial
ASPIRE	Active-controlled Study of Patients Receiving Infliximab for the treatment of Rheumatoid arthritis of Early onset
ATTRACT	Anti-TNF Therapy in RA with Concomitant Therapy
AUC	Area under the curve
BCG	Bacillus Calmette-Guérin
CDC	Complement-dependent cell-mediated cytotoxicity
CDR	Complementarity-determining variable region
CH1	Constant region1 of IgG on heavy chains of immunoglobulins
CH2	Constant region2 of IgG on heavy chains of immunoglobulins
CH3	Constant region3 of IgG on heavy chains of immunoglobulins
CL	Constant region of IgG on hlight chains of immunoglobulins
CI	Cardiac insufficiency
CI	Confidence interval
CIA	Collagen-induced arthritis

c-IAP1	Cellular inhibitor of apoptosis-1
c-IAP2	Cellular inhibitor of apoptosis-2
COMET	COmbination of Methotrexate and *ETanercept* in active early rheumatoid arthritis
CRAT	Centre de Reinseignements sur les Agents Thératogenes,
CRI	Club Rhumatisme et Inflammation
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4)
DAS	Disease activity score
DAS28	28-joints disease activity score
DC	Dendritic cell
DD	Demyelinating disease
DMARDs	Disease-modifying anti-rheumatic drugs
EBV	Epstein-Barr virus
ELISA	Enzyme-liked immunoassay
ERA	Early rheumatoid arthritis *trial*
EULAR	European league against rheumatism
FADD	Fas-associated death domain
Fc	Fragment crystallyzable region of immunoglobulins
Fcγ	Fc-gamma receptor
FcNR	Neonatal Fc receptor
FOXP3	Forkhead box P3
FR	Framework region
GITR	Glucocorticoid-induced tumor necrosis factor receptor
HAQ	Health assessment questionnaire
HBV	Hepatitis B virus

HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
hTNFα	Human Tumor Necrosis Factor alpha
IDO	Indoleamine-2, 3 deaminase
IFNγ	Gamma interferon
IFX	Infliximab
IGRAs	Interferon-γ release assays
IKK	Inhibitor of NF-κB kinase
IL-1	Interleukin-1β
IL-1K	Kinoid of interleukin-1 β
IL-6	Interleukin-6
IL-6K	Kinoid of interleukin-6
IL-10	Interleukin-10
IL-17A	Interleukin 17A
IL-17F	Interleukin 17F
IL-23	Interleukin-23.
IL-23p19K	Kinoid of the p19 peptide of interleukin-23.
iTregs	induced/inducible regulatory T-cells
JNK	c-Jun N-terminal kinase
KLH	Keyhole limpet hemocyanin
LT	Lymphotoxin
LTBI	Latent tuberculosis infection
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MAP3K3	Mitogen-activated protein kinase kinase kinase 3
M-CSF	Macrophage colony-stimulating factor

MEKK-3	MEK kinase 3
MFI	Mean fluorescence intensity
MHC II	Major histocompatibility complex class II
MTX	Methotrexate
NNTH	Number-need-to-harm
nTregs	Natural regulatory T-cells
NYHA	New York heart association
p38 MAPkinase	p38 mitogen-activated protein kinase
p55-TNF Receptor	Tumor Necrosis Factor Receptor type 1
p75-TNF Receptor	Tumor Necrosis Factor Receptor type 2
PBS	Phosphate buffer solution
PPD	Purified protein derivative
RA	Rheumatoid Arthritis
RANKL	Receptor activator of nuclear factor κ-B ligand
RCT	Randomized controlled trial
RIA	Radioiummunoassay
RIP-1	Receptor-Interacting Protein-1
RORγt	Acid-related orphan receptor γ
ROR-C	Acid-related orphan receptor C
RR	Relative risk
SAES	Serious adverse events
SIR	Standardized incidence ratio
SODD	Silencer Of Death Domains
sTNF	Soluble Tumor Necrosis Factor
TAK1	Transforming growth factor β-activated kinase
TB	Tuberculosis

TEMPO	Trial of Etanercept and Methotrexate wit Radiographic Patient Outcome
tmTNF	Transmembrane Tumor Necrosis Factor
TNF-K	Kinoid of Tumor Necrosis Factor alpha
TACE	Tumor Necrosis Factor converting enzyme
TCR	T-cell receptor
Teff	Effector T-cell
TGF-β	Transforming growth factor β
TNFα	Tumor Necrosis Factor alpha
TNFR1	Tumor Necrosis Factor Receptor type 1
TNFR2	Tumor Necrosis Factor Receptor type 2
TNFRSF1A	Tumor Necrosis Factor Receptor superfamily 1A (TNF Receptor type 1)
TNFRSF1B	Tumor Necrosis Factor Receptor superfamily 1B (TNF Receptor type 2)
TRADD	Tumor Necrosis Factor Receptor Associated Death Domain
TRAF1	Tumor Necrosis Factor Receptor-Associated Factor 1
TRAF2	Tumor Necrosis Factor Receptor-Associated Factor 2
Treg	Regulatory T-cell
TST	Tuberculin skin test
TTG	Transgenic mouse for human TNFα
VEGF-K	Kinoid of vascular-endothelial growth factor
VZV	Varicella zoster virus

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FOREWORD

The advent of tumor necrosis factor alpha (TNFα) targeting drugs (anti-TNF) first opened the perspective of successful cytokine-targeting strategy in rheumatoid arthritis (RA), and subsequently in other diseases like Crohn's disease, psoriasis and spondyloarthritis. TNFα can be targeted with monoclonal antibodies (mAbs) or their fragments (infliximab, adalimumab, golimumab, certolizumab) or with fusion products carrying a TNFα soluble receptor (etanercept). Anti-TNF dramatically changed RA treatment over the last decade, giving unprecedented results in terms of disease control and structural damage prevention. By 2014 analysts forecast the entire class of ant-TNF to generate a $25 billion market. Nevertheless, current anti-TNF treatments showed several drawbacks as far as safety, efficacy and costs are concerned. Only 25 to 50% of anti-TNF-treated patients achieved remission in controlled clinical trials, and even lower remission rates are described in everyday practice. An approximately similar proportion reaches a functional status comparable to that of general population. Primary or secondary therapeutic failures on anti-TNFα drugs are not infrequent, and there is increasing evidence that the induction of anti-drug antibodies could be a major contributing factor to insufficient response to this class of therapeutics, at least in the case of anti-TNF mAbs. Despite the good risk/safety profile in selected patients, the overall risk of infection on anti-TNF treatment is increased. Direct and indirect treatment costs result in heavy economical burden for the community. These drawbacks of current anti-TNF treatments confirm that there is room for alternative ways to target this key proinflammatory cytokine.

Amongst these, active immunization against TNFα with TNFα kinoid (TNF-K) is a promising one. The chemically inactivated human TNFα (hTNFα) is coupled to a carrier protein (the keyhole limpet hemocyanine, KLH). This compound is capable of breaking B-cell tolerance to hTNFα, thereby inducing the production of polyclonal, neutralizing anti-hTNFα antibodies (Abs), avoiding the risk of anti-drug antibody induction. Importantly, TNF-K does not sensitize T cells to native hTNFα, and in the absence of specific T-cell help, the rupture of B-cells tolerance is transitory. Our group developed the proof of concept of TNF-K applicability in RA using the model of human

TNFα (hTNFα) transgenic mouse (TTg). TTg mouse develops a hTNFα-dependent spontaneous arthritis and is therefore the pertinent model to study the efficacy of a TNFα-targeting strategy.

In the present work we show the feasibility of this approach in a perspective of its applicability in RA. We demonstrate that the treatment of arthritic mice with TNF-K dramatically ameliorates the disease, that the production of anti-hTNFα Abs is time-limited and renewable by a boost dose of TNF-K. Conversely, native hTNFα does not induce anti-hTNFα Abs. Immunosuppressant treatments currently used in association to anti-TNF in clinical practice do not seem to impair TNF-K efficacy at inducing anti-TNF Ab response. We bring evidence that polyclonal and monoclonal anti-TNFα Abs share some key features in their mechanism of action. Both treatments induce the same modifications in regulatory T-cell populations. Moreover, serum level of both polyclonal and monoclonal anti-TNFα Abs is a major factor determining whether the treatment results in protection from joint inflammation and destruction. These results contributed to the development of active anti-TNF immunization in human disease; TNF-K recently entered phase II clinical trials in RA.

INTRODUCTION

CHAPTER 1

TNFα Targeting in Rheumatoid arthritis

Rheumatoid Arthritis (RA) is the epitome of immune-mediated inflammatory diseases in whose pathogenesis tumor necrosis factor alpha (TNFα) has been shown to play a role. The use of TNF-α agents in RA treatment dates back to1993, so that most of our knowledge on this class of therapeutics relies on data derived from clinical experience in RA.

1.1 TNFα AND TNFα RECEPTORS

TNFα and its receptors belong to the TNF–TNF-receptor superfamily, which currently includes >40 members with structural and functional analogies. Both the ligands and the receptors composing the superfamily are trimeric transmembrane proteins that are mainly (but not exclusively) expressed on immune system cells. The TNF–TNF-receptor superfamily has major role in inflammation, host defence against tumor and infections, organogenesis, apoptosis. Besides, they are involved in deleterious phenomena like septic shock and autoimmunity (1). TNFα is a cytokine synthesized as a membrane-bound protein, which subsequently trimerizes at the cell surface and that can be cleaved by the metalloproteases TNFα-converting enzyme (TACE) to release the soluble form. Both the transmembrane (mTNFα, a homotrimer of 26-kDa monomers) and the soluble form (sTNFα, a homotrimer of 17-kDa monomers) of TNFα are biologically active. TNFα is produced by many cell types in response to inflammation, infection, and other environmental stresses. These cells include immune system cells (like monocytes and macrophages, dendritic cells, B cells, T cells) , mesenchimal cells (like fibroblasts adipocytes, osteoblasts, mast cells) and epithelial cells(like keratinocytes, and mammary and gut epithelial cells) (1).

TNFα signals via two distinct receptors: TNFR1 (TNF Receptor type 1) (also called p55, or CD120a, or TNFRSF1A) and TNFR2 (TNF Receptor type 2) (p75or CD120b, or TNFRSF1B). Alike the cytokine, both TNF-receptors can be cleaved and released from the cell surface in soluble forms into the extracellular milieu where they act as non-signaling 'decoy' or 'sink' TNF-receptors. Like most superfamily receptors, TNFRs interact with more than one ligand of the corresponding superfamily, like lymphotoxin α (LTα)(2). Moreover, complexity is added to the system by the fact that mTNF can function both as a ligand and as a receptor. Reverse signaling through mTNF has been described in monocytes resulting in cellular activation via p38 MAPkinase (p38MAPK) pathway (3).

TNFα signaling regulates a number of critical cell functions including cell proliferation, survival, differentiation on one side, and apoptosis on the other (4).

1.1.1 Tumor necrosis factor receptor 1

TNFR1 is a ubiquitous receptor expressed on almost all nucleated cells. It is preferentially bound by soluble TNFα but can be activated by both soluble or membrane-bound cytokine (5). Both TNFR1 and TNFR2 have been shown to possess a pre-ligand-binding assembly domain that allows trimerization of the receptor, which is therefore already trimerized before TNF-binding (6). Binding of TNFα to TNFR1 triggers intracellular signaling. Two main signalization pathways were described in detail: the inflammatory pathway and the pro-apoptotic pathway.

Binding of the TNFα trimer to the extracellular domain of TNFR1 starts the pro-inflammatory pathway by releasing from the receptor the inhibitory protein silencer of death domains (SODD), which inhibits signaling, from the intracellular domain of TNFR1 in absence of TNFα ligation. The release of SODD permits the binding to TNFR1 of TNFR-associated death domain (TRADD)(7)

(which acts as an adaptor protein and recruits additional adaptor proteins, most importantly receptor-interacting protein-1 (RIP-1), and TNFR-associated factor 2 (TRAF2) (8). These adaptor proteins are then involved in activating the two key TNF proinflammatory TNFα downstream-signaling pathways:

1) The Nuclear factor-κB (NF-κB) pathway, via RIP-1 that recruits mitogen-activated protein kinase kinase kinase 3 (MAP3K3; also known as MEK kinase 3 [MEKK-3]) and transforming growth factor β-activated kinase (TAK1). These kinases activate the inhibitor of NF-κB kinase (IKK) that phosphorylates IκBα and IκBβ proteins. IκBα is then ubiquitinated and degradated (9), and therefore releases NF-κB subunits that translocates into the nucleus and evoke gene transcription. TNFR1 activates the so-called "classical" pathway of NF-κB activation, whose target genes include cytokines, chemokines, anti-apoptotic proteins, ligands and receptors involved in angiogenesis and in cell adhesion and migration (10).

2) The mitogen-activated protein kinase (MAPK) pathway, via apoptosis-signaling kinase-1 (ASK-1) another MAP3K. MAPK pathway results in activation of the c-Jun N-terminal kinases (JNKs) and p38 MAPK which, in turn activate the transcription factor activator protein 1

When the NF-κB pathway fails to be activated, TRADD and RIP1 associate with Fas-associated death domain (FADD) and caspase-8 and -10, thereby forming a complex that assembles in the cytoplasm and initiates apoptosis. Thus, the pro pro-apoptotic pathway seems to be alternative to the pro-inflammatory pathway, and the activation of one or the other would depend on the metabolic state of the cell. The pro-apoptotic pathway would be active only in cells with defective NF-κB signals that would be eliminated through TNF-induced apoptosis (11).

1.1.2 Tumor necrosis factor receptor 2

TNFR2 can bind both soluble and membrane-bound TNFα, nevertheless soluble TNFα shows a 30-fold faster dissociation rate from TNFR2 than from TNFR1 (5). Thus, it is assumed that only membrane bound TNFα can fully activate TNFR2. The expression of TNFR2 is inducible and restricted to hematopoietic and endothelial cells. It is found mainly on monocytes and macrophages but also on T-cells, B cells and NK cells (1).

TNFR2 signaling pathways are not as well characterized as TNFR1 and data form literature are not as compelling at demonstrating the functional properties of TNFR2. This can be due to the fact that many experiments were done using soluble TNFα and that only few experiments were performed in TNFR1-deficient cells, a necessary condition in order to confirm TNFR1-independent effects of TNFR2.

TNFR2 has been shown to activate non-canonical pathway of NF-κB activation (12) via TNFR-associated factor 1 (TRAF1) and 2 TRAF2, and cellular inhibitor of apoptosis-1 (c-IAP1) and 2 (c-IAP2 (13). In addition, TNFR2 activates p38MAPK and JNK pathways (14).

Despite the fact that TNFR2 lacks a death domain to interact with (like FADD for TNFR1), several reports describe the role of TNFR2 in apoptosis of various cells types, such as T-cells and myeloid cells (15, 16).

1.1.3 TNFR1–TNFR2 ‘cross talk’

Even if each receptor possesses specific modalities of activation and signaling, the ultimate biological effects of TNF depend even on TNFR1–TNFR2 cross talk. TACE-mediated enzymatic cleavage and shedding of the extracellular portion of TNFRs in the form of sTNFR2 and TNFR1 influences the concentration of soluble receptors and thus TNF-dependent biological effects. Due to its high-binding affinity to TNFα, the soluble form of TNFR2 in particular has been reported act as a ‘TNFα scavenger’ that inhibits interaction with signaling TNFRs. Moreover, the observation that

TNFα rapidly binds and dissociates from TNFR2 has given rise to a ligand-passing hypothesis, where sTNF that binds to TNFR2 is quickly released and passed to TNFR1, thereby augmenting TNFR1-mediated signals (17).

1.2 TNFα TARGETING IN RA: HISTORY

The rationale for the use of an anti-TNFα strategy in RA derives from some critical experiments that suggested TNFα as a possible therapeutic target in this disease, which we can summarize in an historical perspective as follows:

1) The demonstration of a cytokine, gamma interferon (IFNγ) as an inducer of major histocompatibility complex (MHC) class II molecules overexpression noted in rheumatoid synovial membrane and its role in murine arthritis models (18, 19) that first opened the stream of research on cytokines in RA pathogenesis

2) Immunohistology demonstrating the high expression of TNFα and TNFRs in rheumatoid synovial membrane (20, 21).

3) The blockade of TNFα in RA synovial cell cultures resulting in a reduced expression of other inflammatory cytokines (like interleukin-1β, IL-1) which lead to the concept of a TNFα-dependent cytokine cascade (22, 23).

4) The observation that TNFα transgenic mice spontaneously developed a destructive arthritis (24).

5) The efficacy of TNFα blockade with anti- TNFα monoclonal antibodies in collagen-induced arthritis in mice and rats (25).

All this knowledge constituted concept base for evaluating anti- TNFα therapy in patients with severe RA and insufficient response to existing disease modifying drugs.

In 1993, the report of the successful treatment of ten patients with long-standing refractory RA with the anti-TNFα chimeric monoclonal antibody cA2 (now called infliximab) (26) prompted the real

explosion of clinical studies on molecules targeting TNFα, first in RA, and subsequently in other immune-mediated inflammatory diseases like Crohn's, psoriasis, psoriatic arthritis and juvenile idiopathic arthritis.

1.3 NOMENCLATURE

Many different kinds of molecules have been developed targeting different cytokines or their receptors, the name of this agents summarizes the nature of the molecule and its action:

-mab means that the molecule is a monoclonal antibody or a fragment of monoclonal antibody

-ximab means that the monoclonal antibody (or the fragment) is chimeric

-zumab means that the monoclonal antibody (or the fragment) is humanized

-umab means that the monoclonal antibody (or the fragment) is fully human

-cept means that the molecule is a soluble receptor

- ra means that the molecule is a receptor antagonist

1.4 MOLECULES

Currently, 5 TNFα antagonists are available for RA treatment. Infliximab (Remicade®) is a chimeric human-murine anti- TNFα monoclonal antibody. Adalimumab (Humira®) is a human anti- TNFα monoclonal antibody. Etanercept (Enbrel ®) is a fusion protein between TNFα-receptor p75 (TNFR2) and the constant region (Fc) of a human IgG1 immunoglobulin. Certolizumab pegol (Cimzia®) is the fusion product of 2 TNFα-binding region of an anti- TNFα monoclonal antibody and a long chain of pegylate, which stabilizes the complex increasing its half-life. Golimumab (Simponi®) is the fully human evolution of infliximab.

Table 1 summarizes the main characteristics of the five agents.

Briefly, infliximab is a chimeric human-murine immunoglobulin comprising a murine Ag-binding region, which binds sTNF and mTNF, it is given intravenously q4-8 weeks, at a dose of 3-10mg/kg.

The use in association with methotrexate (MTX) is mandatory in RA, in order to reduce sensitization of immune system versus the murine component of the immunoglobulin. Nevertheless, studies that focused on the induction of anti-infliximab antibodies (Abs) did not consistently confirm a lower or less frequent production of anti-drug antibodies (ADA) when concomitant MTX is given (see after). Moreover, MTX might reduce ADA production by other mechanism than aspecific immunosuppression (27). Anti-infliximab antibodies have been implicated in primary but especially in secondary failure of infliximab treatment. Adalimumab is a fully human anti-TNFα immunoglobulin that is given subcutaneously q2w (or qw if necessary) at a dose of 40 mg. Due to its human origin it can be given in monotherapy without background MTX. Nevertheless, the production of anti-adalimumab Abs impairing efficacy has been described as well. Etanercept is a fully human fusion protein comprising two TNFR2 extracellular domains fused to a single human IgG1 Fc region containing the CH2, domain, the CH3 domain and hinge region, but not the Ch1 domain. It binds both TNFα and LT in their soluble and membrane-bound forms. Induction of anti-etanercept Abs has also been described in literature, but these ADA do not seem to reduce the efficacy of the drug.

Golimumab is a fully human IgG1 monoclonal antibody, and is the human evolution of infliximab. It is given subcutaneously monthly, at a dose of 50mg. Across the phase 2 and 3 trials of golimumab in RA, psoriatic arthritis and ankylosing spondylitis, anti-golimumab antibodies were found in 4% of patients. MTX concomitant treatment was associated with lower prevalence of ADA (2% of patients) vs. golimumab monotherapy (7%)(28). . Certolizumab pegol (Cimzia®) is the fusion product of a recombinant humanized Fab fragment conjugated to polyethyleng glycol (PEG), which stabilizes the complex decreasing proteolysis , reducing drug clearance thereby increasing its half-life. PEGylation might even reduce immunogenicity of certolizumab. Monotherapy with certolizumab was associated with anti-certolizumab Abs in 8, 1% of cases in the FAST4ward study (29). Certolizumab is given subcutaneously at a dose of 200 mg every 2 weeks (Table 1).

Table 1. Anti-TNF agents currently licensed for RA treatment

	Infliximab	Adalimumab	Etanercept	Golimumab	Certolizumab
Structure	Monoclonal antibody Chimeric	Monoclonal antibody Fully human	P75TNFR/Fc fusion protein Fully human	Monoclonal antibody Fully human	PEGylated Fab fragment Humanized
Ligand	TNFα	TNFα	TNFα, LTα3	TNFα	TNFα
Molecular weight	150 kDa	150 kDa	150 kDa	150 kDa	95 kDa
Half-life	8-10	10-14	3	12±3	14
Dosing route and frequency	Iv, every 6 to 8 weeks after loading at weeks 0, 2 and 6. Dose 3mg/kg up to 7.5 mg/kg	Subcutaneously every 2 weeks (up to weekly) at a dose of 40 mg.	Subcutaneously weekly at a dose of 50 mg.	Subcutaneously, monthly at a dose of 50mg.	Subcutaneously, every two weeks at a dose of 200mg, after loading of 400mg at weeks 0, 2 and 4

1.4.1 Differences in mechanism of action of anti-TNF agents

Even if TNF-agents seem to have similar efficacy in RA in terms of clinical control and protection from structural damage (see after), clinical data show some differences exist in terms of adverse events and treatment survival rates. Moreover, compared to monoclonal Abs, etanercept is ineffective in Crohn's disease, and less effective in sarcoidosis, in granulomatosis with polyangiitis and uveitis. Moreover, the risk of latent tuberculosis infection (LTBI) reactivation is lower with etanercept (see after).

Differences in molecular structure and ligand-binding properties between anti-TNF agents have been described that might be implicated in these clinical differences. With the exception of certolizumab, all licensed anti-TNF agents are IgG or IgG constructs. The main functions of IgGs are determined by their interaction with four classes of 'partners': i) the target antigen, ii) the Fc gamma receptors for IgG (FcγRs) iii) complement and iv) the neonatal Fc receptor (FcRn) (30).

Interaction with these partners might imply differences in mechanism of actions possibly resulting in differences in therapeutic effectiveness and safety.

i) *Antigen interaction*. Monoclonal Abs (mAbs) are bivalent and their specificity is directed to the monomeric subunit of the TNFα homotrimer. Thus, a single homotrimer could be bound to up to three mAbs, and each mAb can bind two homotrimers allowing the formation of large immune complexes. Conversely, certolizumab is monovalent and etanercept is functionally monovalent, since it has been shown to bind a single trimer of mTNFα or sTNFα in a 1:1 ratio (31). Binding of anti-TNF agents to mTNFα can activate reverse signaling resulting, depending on the cell type, in processes like apoptosis, cell activation or modulation of cytokine secretion profile. Each of the five agents can bind mTNFα, but their ability to activate reverse signaling differs. MAbs can cross-link mTNFα, and infliximab has been shown to induce suppression of T-cell proliferation, of cytokine production and to induce apoptosis. Etanercept, which is not able to cross link mTNFα homotrimers, does not share all of these features with infliximab (32).

Etanercept is the only anti-TNF agent that binds LTα. It has not been demosntrated that LTα-blockade could translate into higher clinical efficacy. Nevertheless, it was shown hat etanercept, but not anti-TNF mAbs, can reduce memory B-cells in peripheral blood and follicular germinal centers in RA patients, an action possibly linked to inhibition of LT-dependent lymphoid organogenesis (33).

ii-iv) *Fc interaction with Fc receptors and complement*. All anti-TNFs, except certolizumab, posses an Fc region of IgG1. Accordingly, infliximab and adalimumab and etanercept, but not certolizumab can mediate antibody-dependent-cell-mediated cytotoxicity (ADCC) and complement–dependent cytotoxicity (CDC) *in vitro* (34).

The presence of Fc mediates interaction with Fc receptors, which might be responsible for differences in pharmacokinetics and action of different anti-TNFs. The FcRn is expressed mainly on endothelial cells, and is known to transfers IgG from the mother to the fetus across the placenta. FcRn protects IgG from degradation, explaining the long half-life of this class of antibody in the

serum (35). Interaction with FcRn is a major determinant of plasma half-life of anti–TNF agents. It has been hypothesized that lower affinity for FcRn of etanercept might explain its shorter half-life compared to mAbs (2). FcγRs, expressed on a variety of immune cells, have critical roles in regulating the effects of Abs and immune complexes on cell function. The high-affinity IgGFc receptor I (FcγRI) binds monomeric IgG while lower-affinity FcγR II and III require multimeric immune complexes. Fcγ subtypes can mediate both stimulatory and inhibitory signals (36). Binding of Fcγ can enhance phagocytosis, induce ADCC, CDC and regulate cytokine and Ab production (34).

Moreover, the interaction with Fc might be implicated in the scavenging of immune complexes between TNF and the drug. In human TNFα transgenic mice, etanercept-TNFα complexes circulated for longer time and were less stable than those formed with mAbs (2) with possibly lower clearance of TNFα or release of TNFα to other sites. Larger immune complexes formed by bivalent mAbs might be more immunogenic and be involved in ADA induction for anti-TNF mAbs (37).

Other differences have been identified between anti-TNF agents. For instance, in mice collagen-induced arthritis (CIA) certolizumab penetrates better and shows longer persistence in inflamed paw vs. infliximab and adalimumab (38). Anti-TNF mAbs , and not etanercept, have been shown to induce a novel population of regulatory T-cells (Tregs) in responding RA patients (39, 40) (see after).

1.5 CLINICAL EFFICACY (CLINICAL TRIALS)

Given the feared consequences of a critical cytokine blockade numerous clinical trials were undertook before approval and commercialization of anti- TNF agents. These trials were aimed at evaluating clinical efficacy and safety of anti-TNF. For didactic purposes these two aspects will be treated separately. Closed and ongoing clinical trials on anti-TNF agents can be accessed at the following addresses: http://clinicaltrials.gov/ or http://www.who.int or http://www.actr.org.au/ .

The efficacy of the three commercialized anti-TNF agents has been evaluated in numerous clinical trials, the pivotal being: the ATTRACT (41-45) and ASPIRE (46, 47) trials for infliximab, the TEMPO (48-50), the ERA (51) and the COMET (52) trial for etanercept, and the ARMADA (53, 54), the study by Keystone *et al.* in 2004 (55) and the PREMIER (56-58) trial for adalimumab.

Even if each single study shows differences in design, studied population, inclusion criteria, and endpoints as compared to the others, there are even many common features, since the logic underlying the efficacy evaluation for all three drugs was the same. Therefore, the main purposes of the aforementioned studies can be roughly summarized in the following points. First: to compare the clinical response to anti-TNF with the gold standard traditional treatment for RA (MTX). Efficacy vs. MTX was evaluated for anti-TNF in monotherapy, were possible, or for anti-TNF in association to MTX itself. Second: to evaluate the radiographic and functional outcome of anti-TNF treatment over the follow-up time period. Third: to evaluate all, or some, of these parameters in early RA (with varying definitions of early RA). Each of the cited studies meets part of these requirements**.**

1.5.1 Infliximab

The ATTRACT study (Anti-TNF Therapy in RA with Concomitant Therapy) (41, 42), tested the efficacy of the association of different dose regimens of infliximab in association with MTX versus MTX, in RA patients refractory to MTX alone. Combination of infliximab and MTX was well tolerated and resulted in a significantly greater and sustained reduction of symptoms and signs of RA (clinical response, 51.8 percent vs. 17.0 percent) and a significantly higher improvement of quality of life vs. MTX. Radiographic scores of joint damage increased in the MTX group, but not in the groups receiving infliximab and MTX (mean change in radiographic score, 7.0 vs. 0.6). A sub analysis on the patients with early RA of the ATTRACT trial (43) showed that infliximab treatment resulted in higher inhibition of structural damage progression in those patients with early disease. Another sub analysis by Breedveld *et al.* (44) showed that baseline radiological damage was

associated with poorer physical function and less improvement in physical function after treatment, underlying the importance of early intervention.

Finally, a third sub analysis (45) showed that even the patients with no ACR clinical response showed a reduced radiological progression as compared to patients on MTX, suggesting that clinical activity and structural damage are partially dissociated.

The ASPIRE study (46, 47) (Active-controlled Study of Patients Receiving Infliximab for the treatment of Rheumatoid arthritis of Early onset) involved patients with early RA and showed that, combination therapy with MTX and infliximab provides greater clinical, radiographic, and functional benefits than treatment with MTX alone.

1.5.2 Etanercept

The TEMPO (Trial of Etanercept and Methotrexate wit Radiographic Patient Outcome) (48) is a three-year double-blind, multicenter study. It compared the association etanercept+MTX with either etanercept or MTX monotherapy, showing the superiority of the association vs. both monotherapy regimens as long as reduction of disease activity, improvement of functional disability, and retardation of radiographic progression are concerned at 1, 2 (49) and 3 years (50). A longitudinal analysis showed that patients receiving combination therapy were more than twice more likely to be in disease remission than those receiving either monotherapy.

The ERA study (51) compared the clinical and radiographic outcomes, in early aggressive RA, of etanercept or MTX monotherapy for 2 years, and showed that etanercept monotherapy was superior to MTX in reducing disease activity, arresting radiological progression and decreasing disability. A sub analysis of the ERA study showed that even in clinical nonresponders radiological progression was reduced compared to MTX group (59).

The COMET study (52) was the first study with an anti-TNF, which had clinical remission as primary endpoint. It compared remission and radiographic non-progression in patients with early RA treated with MTX monotherapy or with MTX plus etanercept. After 1 year 50% (95% CI 44-

56%) of patients taking combined treatment achieved clinical remission compared with 28% (23-33%) taking MTX alone. Moreover, 80% (75-85%) vs. 59% (53-65%), respectively, achieved radiographic non-progression. Given the high percentage of good disease control and hampering of radiological damage even in the MTX group, the study underlines the importance of early intervention in RA.

1.5.3 Adalimumab

The ARMADA trial (Anti-TNF Research Study Program of the Monoclonal Antibody D2E7 in patients with Rheumatoid Arthritis) (53)and its 4-year extension (54) showed the superiority of the association adalimumab+MTX versus MTX alone on disease activity and physical function. At 4 years 78%, 57% and 31% of patients had reached ACR 20 50 and 70response, respectively. Forty-three percent of patients where in remission according to EULAR (European league against rheumatism) response criteria (DAS28<2.6); 22% of patients had no functional impairment (health assessment questionnaire, HAQ=0); up to 81% of patients could reduce the dose of corticosteroid without reduction of efficacy. Another trial by Keystone *et al*. (55) evaluated the 52-week radiographic, clinical, and functional outcomes of the association adalimumab+MTX vs. MTX monotherapy. At week 52, the association showed superiority in all the endpoints. Adalimumab+MTX association proved a better clinical response and lesser radiographic progression even in early RA. In the PREMIER study (56) after 2 years of treatment, 49% of patients receiving combination therapy exhibited DAS28-defined disease remission (DAS28 <2.6), and 49% exhibited a major clinical response (ACR70 response for at least 6 months), rates approximately twice those found among patients receiving either monotherapy. A sub analysis of the PREMIER study (57) showed that after 2 years, the quality of life for patients with early RA treated with adalimumab plus MTX improved to reach the values of healthy general US population.

A Cochrane review meta-analyzed 6 clinical studies with Adalimumab (60) concluded for efficacy and safety of adalimumab in combination with MTX in the treatment of RA and reduction of radiographic progression at 52 weeks.

1.6 THERAPEUTIC STRATEGY WITH ANTI-TNF

Based on the results of RCTs, their pos-hoc analyses, metanalyses, open label trials, expert committees' opinion and cumulated clinical experience, many questions raise regarding clinical strategy for the use of anti-TNF

Several studies, like the Best trial (61-64) and the Swefoot trial (65) addressed the question of efficacy and utility of precocious use of anti-TNF agents compared to association of traditional DMARDs, in the context of different clinical strategies.

It is widely demonstrated that anti-TNFs produced an unprecedented clinical efficacy in the history of RA treatment. This effect is evident as an active control of disease activity, as confirmed by the percentage of patients achieving the different classes of ACR response and DAS/DAS28-defined remission. An inhibition and sometimes complete stop of structural damage evolution is evident, especially in case of association with MTX. The inhibition of structural damage has been demonstrated even in patients who did not achieve a satisfying clinical effect, suggesting an effect of anti-TNF independent from control of inflammation. Amelioration of health function and patient-based quality of life is evident as well. All these effects translate in positive cost-benefits, cost effectiveness, cost-utility analyses, which confirm anti-TNF treatment as a valuable strategy. Nevertheless, not only the efficacy, but even the costs and possible side effects of these treatments raise many concerns regarding their utilization strategy: we will address the main questions discussed in literature

1.6.1 When to start?

The proper timing of anti-TNF introduction is a critical point, posing on one side the problem of undertreatment of a condition possibly evolving toward structural damage and ultimately disability, and on the other, problems related to overtreatment and high costs.

Even if the progression of structural damage is linear and goes on along the course of the disease, an active control of disease activity in the early phases results in a better long term clinical control and in sloping of the linear trend of structural damage (66), but how early is early?

In most countries anti-TNF treatment can be started after insufficient response to MTX (or equivalent DMARD) at the highest dose and/or failure of other classic DMARDs associations.

For this reason, a tight clinical control possibly with DAS-driven adaptation of the treatment as suggested by studies like the TICORA (67) is warranted, in order to rapidly determine the degree of clinical response to classic DMARDs.

The BesT study (61) is a complex study, which yielded numerous sub analyses (62-64), and was the first study of therapeutic strategy with anti-TNF drugs. It compared 4 strategies of treatment, in early RA, a subsequent therapy (group1); a step-up combination therapy (group 2); an initial combination therapy with tapered high-dose prednisone (COBRA association) (group 3); initial combination therapy with MTX+infliximab (group 4). Initial combination therapy including either prednisone or infliximab resulted in earlier functional improvement and less radiographic damage after 1 year than did sequential monotherapy or step-up combination therapy. Initial treatment with infliximab resulted in the highest quality of life, highest productivity, but even highest medical costs. DAS-driven treatment adjustments were effective to suppress disease activity and damage progression in all groups.

A post-hoc analysis at 3 years (68) showed that initial treatment with infliximab+MTX resulted in lesser radiographic progression, better improvement of quality of life and more infliximab discontinuation as compared to later introduction on infliximab, so that the higher medical costs of 1-year analysis might be questioned.

In fact, the open problem is whether to start immediately anti-TNF treatment in case of diagnosis of RA.

The COMET study (52) showed 50% of clinical remissions and 80% radiologic non-progressions at 1 year with etanercept +MTX received at diagnosis of RA. Nevertheless, in MTX-only treated patients the respective values were elevated as well (28% and 59%), suggesting an advantage coming not only from drug choice, but from an aggressive clinical strategy. According to the results of the BesT study, in patients with recent onset rheumatoid arthritis receiving a COBRA combination (classic DMARDs association+ tapered dose of corticosteroids), systematic DAS-driven therapy results in significant clinical improvement and possible suppression of joint damage progression (69). Accordingly, the Swefoot (65) trial suggests that 3 months of MTX treatment followed, in case of non response, by 3 months of triple therapy with classic DMARDs (MTX+sulphasalazine+hydroxychloroquine) results in an overall 59% of patients achieving a good EULAR response at 6 months, confirming that aggressive and tightly controlled treatment with classic DMARDs leads to good results and identifies patients who might benefit of anti-TNF treatment with an overall delay of 6 months, which seems to be a reasonable standard.

1.6.2 When (and how) to switch?

Despite the unquestionable therapeutic advances introduced by anti-TNFs, more than 50% of patients in clinical trials fail to achieve at least an ACR50 response, data from national registries show that in everyday life even more patients fail to respond as compared to RCTs (70). Conversely, data derived from clinical practice in specialized centers suggest a higher than RCTs percentage of good responders (71). Nevertheless, a high number of patients loose efficacy (secondary failure or acquired therapeutic resistance) or experience adverse events during treatment (72).

According to national and international consensus statements, the response to anti-TNF treatment must be quantitatively evaluated for each patient; at week 12 of treatment an evaluation is made in order to assess its efficacy. A reduction of DAS 28≥1.2 or a DAS28 score≤2.6 is considered as a good response. A reduction of DAS28 between 0.6 and 1.2 or a final DAS score between 2.6 and 3.2 is considered a moderate response, while lower or absent reduction is considered as a therapeutic failure.

Amongst nonresponders to anti-TNF treatment one can distinguish between primary and secondary failures. A never-responder is considered a primary failure, while loss of a previous response is a secondary failure. A number of studies have shown that patients who fail on one anti-TNF may still respond well to either of the other two, (73), and even those failing on two, may still respond to a third. A survey of US rheumatologist showed that 94% of them had switched the patients from one anti-TNF to another (74).

The rationale of switching these agents might reside in differences in bioavailability, stability of the TNF-inhibitor complex or the occurrence of drug-neutralizing antibodies (75). The latter phenomenon seems to be strongly implicated in secondary failure to anti-TNF monoclonal antibodies (well established for infliximab and adalimumab) (76).

Even if some heterogeneity exists, mainly due to the quality of data on therapeutic switch, mostly derived from open label observational studies, some points can be drawn:

First: the number of switches reduces the efficacy (percentages of responders) of a new anti-TNF (77, 78) and the drug survival time.

Second: switching to another anti-TNF seems to be more efficacious if the reason for withdrawing the first anti-TNF was secondary failure or intolerance. In this sense are the results of the ReAct study (79), where patients were allowed to switch to adalimumab after failure of the two other anti-TNF: if the cause of switching was a secondary failure or intolerance, there was a better response to switch compared to primary failure. Other smaller studies suggest the same trend. Conversely, another study on switch from infliximab to etanercept, while showing that etanercept is effective in

patients who fail to respond to infliximab, suggests a higher response in primary nonresponders, even if the difference is not significant (80).

Third: there is an increased risk of adverse events under a second or third anti-TNF which is about twofold if the reason for switching was intolerance (81). Even if it has been demonstrated that the risk of infection is higher in the first year of treatment with a progressive reduction over time (82, 83), the switch to another anti-TNF is associated with a second increase in relative risk of infection (79, 84).

All these elements suggest that, especially in the case of primary failure or of intolerance, a class effect might be suspected, posing the rationale for introducing other biologic agents with another mode of action.

1.6.3 When to stop?

There are no official statements or guidelines regarding programmed withdrawal of anti-TNF agents, but some suggestions might be gathered from literature. In longstanding RA responding to anti-TNF, their withdrawal causes a clinical relapse (85). In early RA, the BesT study showed that 67 over120 patients treated with infliximab plus MTX (at a dose of 25 mg/week) from the start, kept a low disease activity in spite of infliximab withdrawal and MTX reduction to 10mg/week. In a small study by Quinn *et al*., a higher physical function and HAQ improvement of 12 months of infliximab plus MTX was maintained 1 year after infliximab withdrawal compared to MTX-only treated patients(86).

It is possible that the timing of anti-TNF introduction along the course of the disease might be important, as suggested even by the different percentages of therapeutic successes between early and long standing arthritis; further dedicated studies are anyway warranted on the subject before any clear suggestion for clinical practice can be established.

1.7 SAFETY

The evaluation of the safety of anti-TNF (and of any other drug) in RA is difficult for different reasons:

1) Single RCTs, whose sample size is chosen on the basis of expected efficacy, lack the statistical power to detect sparse, infrequent adverse events (AEs),
2) The time of observation might be insufficient to detect AEs developing on a longer time period than that of the study
3) Conversely, adverse events occurring early in the course of the follow up might induce to overestimate the overall risk
4) RA population has itself an increased risk of AEs like infection and malignancy
5) The contribution of other drugs like classic DMARDs and corticosteroids further complicates data interpretation

For this reason, most of the information we have on anti-TNF safety in RA derives from post-marketing surveillance (with the invaluable contribution of national registries) and from metanalyses and pooled analyses of RCTs. These approaches are surely useful, for instance post marketing surveillance allowed to detect the increased risk of tuberculosis reactivation, but have even some limitations. In the case of observational approaches, widespread use of a drug after approval for a significant amount of time is required to generate data that can be used for analysis. In addition, many biases may limit to safety assessments based on observational data (selection bias, lacking data concerning comorbidities or concomitant treatments, differences in study populations). On the other hand, metanalyses and pooled analyses, while keeping the limits of length of observation of original studies, are limited by heterogeneity of the studies themselves and possible under or overestimate of the events. Hereafter we will report the data of the literature regarding the possible AEs and the indication of the strategy according to the recommendations of the AFFSAP as reported on the website of the Club Rhumatismes et Inflammation (CRI) (www.CRI-net.com).

1.7.1 ANTI-TNF ALPHA AND MALIGNANCIES

TNFα is a critic cytokine in immunity against tumors; therefore TNF blockade has raised the concern of an increased risk of malignancies in treated patients.

1.7.1.1 Malignancies and RA.

Patients with RA appear to be at higher risk of lymphoma and lung cancer and potentially decreased risk for colorectal and breast cancer compared with the general population (87).

In addition, evidence has accumulated that RA disease activity is associated with the risk of lymphoma (88-90). A wide systematic review of articles on RA and lymphoma (91) from 1964 to May 2007 confirmed an approximate 2-fold increase in lymphoma incidence in patients with RA, with apparently no significant increased risk linked to treatment with classic DMARDs.

In the latter review, an increased lymphoma incidence with tumor necrosis factor-alpha inhibitors was suggested; however, follow-up in the studies considered was too short to definitively determine increased risk. Moreover, confounding by disease severity (patients with the most severe disease are treated with the strongest medications) in most studies makes the association between lymphoma risk and medications and/or disease severity difficult to assess.

This point needs to be taken into account when attempting to discriminate the potential effect of anti-TNF treatment on the risk of malignancies.

A metanalysis of RCTs conducted from inception to December 2005 with infliximab and adalimumab found a pooled OR for neoplasm of 3.3 (95% CI 1.2-9.1) and a number-need-to-harm (NNTH) for the studied population of 154 (95% CI: 91-500) over one year for one additional malignancy over a time period of 6-12 months (92). Importantly, the risk correlated with the dose of anti-TNF. No accumulation of malignancies with longer study duration was seen; this could be explained by an acceleration of pre-existing subclinical malignancies rather than induction, as suggested by the data of several national registries of biotherapies. To note, a follow-up report reduced the OR to 2.02 not reaching significance (95% CI 0.95–4.29) when additional trial data were added (93).

The same authors conducted a metanalyisis on etanercept RCTs from inception to December 2006, (94), that suggested an increased risk of malignancy for the molecule that did not reach significance (OR 1.84, 95%CI 0.79-4.28).

No particular time periods in etanercept treatment (0–6 months; 6–12 months and more than 12 months), nor different dose regimens (less than 50 mg/week and 50 mg/week or greater) was associated with an increased incidence of cancer.

A large observational longitudinal study on 13000 RA patients over 49,000 patient-years of observation in the years 1998–2005 showed increased OR for development of melanoma (R 2.3 (95% CI 0.9-5.4) and non-melanomaOR1.5 (95% CI 1.2-1.8) skin cancer in anti-TNF treated RA patients compared to controls.

A population-based study on three cohorts of RA patients (one prevalent, admitted to hospital 1990–2003 (n = 53 067 pts), one incident, diagnosed 1995–2003 (n = 3703 pts), and one treated with TNF antagonists 1999–2003 (n = 4160 pts)) showed an increased risk for lung cancer, non-melanotic skin cancer and lymphoma and a reduced risk for colorectal and breast cancer for all RA patients whether anti-TNF treated or not (95, 96).

As long as data from European national registries are concerned, they are somewhat consistent with the aforementioned results. Referring mainly to the data of the three registries of Germany, Sweden and UK that have agreed over a shared reporting system, after an early report of an 11.5-fold increase in the occurrence of lymphoma among patients treated with TNFα inhibitors (97) which disappeared when the data were included in a larger registry (95, 96) there have been no further reports of an increased risk of lymphoma. Currently available data do not suggest that anti-TNFα treatment confers an additional risk for lymphomas. Moreover, no difference in the overall risk of solid cancers between patients treated with anti-TNFα agents and other patients with RA was found (98).

Patients with a history of malignancy dating more than 5 yrs treated with biologics did not show any difference in the risk of recurrence or of a novel malignancy as compared to those treated with conventional DMARDs (99).

1.7.1.2 Guidelines for practice

In summary, literature suggests that the effect of biologic treatments on malignancy would be a sort of unmasking or facilitating effect more than a real induction of novel tumors, in the context of a disease like RA which confers itself an increased risk of malignancy, especially in uncontrolled, active disease. For this reasons efforts are made to eliminate any underlying hidden malignant or pre-malignant condition, before starting treatment and the follow up must systematically rule out any suspicion of novel malignancy. In case of a novel tumor the treatment will be withdrawn and a possible reintroduction after recovery might be discussed in each single case.

The ANSM (Agence nationale de sécurité du médicament et des produits de santé, formerly AFFSAPS) guidelines proscribe anti-TNF agents in case of underlying or previous malignancy dating less than 5 years. After this time interval of complete remission, an introduction can be discussed except in the case of lymphoma, where the tendency would be towards the choice of other biologics (e.g. rituximab).

1.7.2 INFECTIONS

As previously said, RA patients are at increased risk of infection; therefore general population is not the proper control group.

Single studies data with anti-TNFs in RA do not clearly show an increased incidence of infection. Nevertheless, metanalyses confirm that anti-TNF treatment is associated with increased risk of mild and severe infection (92).

Post marketing surveillance first detected an increased risk of tuberculosis that lead to the adoption of screening procedures before starting the treatment.

Dixon et al (83) reported that in the clinical practice setting the risk of serious infection with anti-TNF agents peaks in the first 3 months, with a subsequent progressive reduction of the risk. A recent metanalysis and pooled analysis of RCTs showed an increase in serious adverse events (SAES) (mainly infections) rate when anti-TNF were taken at a higher dose than that normally prescribed (100), and confirmed that the risk of SAES decreases over time (p=0.0351).These data suggest that the most valuable strategy before starting anti-TNF is a thorough screening for latent infection and possible exposure to sources of contagion. In France the CRI (Club Rhumatisme et Inflammation) suggests that patients on anti-TNF treatment should be strictly monitored for the emergence of rare and atypical infections just like other categories of immunodeficient patients like AIDS or cancer patients on chemotherapy. According to CRI guidelines anti-TNF treated patients should systematically undergo vaccination against influenza and pneumococcal infection. The indication of other vaccinations should be discussed in single patients. Vaccination with viable attenuated viruses should be avoided while on treatment or, in case of frequent travelers to endemic infection zones, performed before starting anti-TNF treatment.

1.7.2.1 Tuberculosis

Despite its high worldwide prevalence, tuberculosis (TB) incidence remains low in western countries, even if marked differences exist between countries (incidence rates ranging from 140 case for 100000 person-year in Romania, 18 in Spain, 5 in Sweden and USA). TB infection cannot in fact be detected in RCTs and a metanalysis of all RCTS and their extension could not detect any increased risk of TB infection on etanercept. An increased risk of activation of latent tuberculosis for infliximab was first demonstrated by post-marketing reports(101), leading to activation of national surveillance registries and promulgation in different countries of screening guidelines for latent tuberculosis infection (LTBI): in France the guidelines of the AFFSAP (now ANSM) are available on the website of the CRI (http://www.cri-net.com/). An intradermal reaction with PPD (purified protein derivative) (tuberculin skin test, TST) (0.1 ml , or 0.5 unities, lecture between 48 and 72 hours, positivity cut off at 5 millimeters) and a chest x-Ray is mandatory for all patients.

According to these guidelines systematic screening for LTBI must involve a detailed clinical examination and medical history gathering information about previous TB infection (treatments given before 1970 are considered ineffective for mycobacterium eradication), birth or extended living in areas of high TB prevalence, close TB contact, BCG vaccination. In case of imaging evoking radiological sequelae of TBC or in case of blistering reaction to TST the research of *Mycobacterium tuberculosis* in sputum and bronchial aspiration is mandatory. If LTBI is retained, prophylactic anti-TBC treatment should be started at least 3 weeks before anti-TNF. In case of active TBC a 4-drugs treatment must be carried out until the disease is completely eradicated before starting anti-TNF treatment. In individual cases anti-TNF could be started before complete eradication after collegial discussion of the benefit-risk balance of this attitude. Once the anti-TNF treatment has been started, careful follow-up for signs or symptoms of TBC (respiratory or other organ-related or constitutional) is mandatory.

Anti-TNF treatment must be stopped in case of incident TBC infection and complete eradicating treatment started. Anti-TNF treatment will not be reintroduced until 2 months after the end of the cure. Again, in selected cases the benefit/risk balance of precocious anti-TNF reintroduction can be discussed for selected cases.

Differences in TB risk with different molecules

To date the data of different national registries, even with their intrinsic limits (incomplete reports, lack of data like concurrent medication and underlying medical conditions), seem to agree in indicating a higher frequency of reactivation of LTBI for monoclonal antibodies vs. etanercept. The most recent data of the French RATIO register show a total standardized incidence ratio (SIR) of TB on anti-TNF of 12.2 [95% CI 9.7-15.5]. The SIR was higher infliximab (SIR 18.6 [95% CI 13.4-25.8]) and adalimumab (SIR 29.3 [95% CI 20.3-42.4]) than for etanercept (SIR 1.8 [95% CI 0.7-4.3]) (102).

A mathematical modeling of data on TB incidence (cases reported to FDA between 2003 and 2008) indicates that the clustering of events at the beginning of treatment corresponds to reactivation of

latent infection, while events randomly occurring along the treatments, correspond to progression of new infections. Modeling revealed an apparent median monthly rate of reactivation of LTBI by infliximab treatment of 20.8%, which was 12.1 times that with etanercept treatment (P<0.001). In contrast, the risk of progression of new *M. tuberculosis* infection to active TB appeared to be similar for both drugs (103). In animal models, monoclonal antibodies but not soluble receptor, decrease survival in chronic infection, while both increase bacterial burden and mortality in acute *M.Tuberculosis* infections (104). It should be stressed that most of TBC reactivation took place in patients that had not undergone prophylactic anti-TBC treatment when they should have had to. For this reason the screening protocol for LTBI has a major role, and even if lower incidence of TBC reactivation was found for etanercept maximal efforts are warranted to rule out LTBI for all anti-TNF treatments. We recently showed that in populations at high incidence of tuberculosis, in which the negative predictive value of diagnostic tests is reduced, maximal screening sensitivity is allowed by parallel lecture of TST and interferon-γ release assays (IGRAs). Even if IGRAs are more probably valid than TST, relying only on one immunological test, might miss patients at high risk of LTBI reactivation (105).

1.7.2.2 Chronic Viral Infections

Patients must be warned of the possibly increased rate of viral infections, and when possible the vaccination schedule must be updated before starting treatment. Any chronic viral infection and particularly HIV, HBV and HCV normally contraindicate treatment. Exceptions can be a slowly replicating HCV infection with tight control of aminotransferases and of viraemia at 1, 3, 6 months and at 1 year. To date, 15 cases of patients treated with anti-TNF treatment in the context of chronic HBV infection have been published, nine of them had a totally favorable outcome, amongst these 4 had antiviral lamivudine 100mg/day. This latter approach is advised after collegial discussion with a hepatologist.

In the case of HIV infection limited experience suggests a relative safety of the treatment but the approach remains extremely cautious.

Anti-TNF treatment might be associated with increased risk of activation of varicella zoster (VZV) (106) but data are somewhat heterogeneous. In case chronic infection by herpes simplex and VZV a cautious approach is therefore recommended, while no particular risk of reactivation of EBV seems to be elicited by anti-TNF treatment (107). No single specific viral infection has been described at a particularly increased rate under anti-TNF treatment.

1.7.3 OTHERS

1.7.3.1 Infusion- and injection-related

Infliximab infusion has been associated with hypersensitivity reactions, mostly urticaria, dyspnea, and/or hypotension, 2 hours of infusion. Some cases of serum sickness-like reactions have been observed in patients especially after initial therapy and when the treatment was reinstituted after a withdrawal. Serum sickness reactions were associated with marked increase in antibodies to infliximab, loss of detectable serum concentrations of infliximab, and possible loss of drug efficacy. Even if in post marketing experience, anaphylaxis and angioneurotic oedema have been rarely reported, the most common adverse reaction with adalimumab and etanercept is injection site reactions (erythema and/or itching, pain or swelling, rarely hemorrhage). Most injection site reactions are described as mild, lasting 3 to 5 days, and generally do not necessitate drug discontinuation.

1.7.3.2 Antinuclear antibodies (ANA) induction

During the course of anti-TNF treatments the development of ANA has been described (108), more frequently under infliximab treatment. This is a frequent phenomenon, regarding between 30 and 70% of patients in controlled studies, possibly due to lack of apoptotic signals due to TNF-TNFRI system blockade. Along the course of the treatment patients become positive and there is a progressive increase in titers of autoantibodies. Not only ANA are induced, but even specific autoantibodies like anti-dsDNA, anti-nucleosome and both of IgM and IgG classes. Nevertheless,

despite the high frequency of this phenomenon, the induction of a lupus-like syndrome is very rare, normally subsiding with anti-TNF withdrawal, and sometimes spontaneously resolving. After treatment withdrawal a progressive decrease in antibodies titers is the rule. According to CRI recommendations patients with ANA positivity or with overlap syndromes RA-other connective tissue disease do not formally contraindicate anti-TNF treatment, but a tighter monitoring is warranted. ANA titer monitoring has no place in patients' follow-up. Conversely, surveillance of clinical manifestations of lupus or lupus-like disease is warranted especially concerning fever, fatigue, or sign of cutaneous or hematological involvement, serositis or thrombosis.

1.7.3.3 Demyelinating diseases

In spite of efficacy of anti-TNF strategy in animal models of demyelinating disease (DD), its application in multiple sclerosis clinical trials resulted in an aggravation of the disease. This and the reports of cases of DD occurring during anti-TNF treatment lead to the formal contraindication of this class of therapeutics in case of multiple sclerosis and other DDs. Thereafter, DDs of the central and peripheral nervous system (demyelinating neuropathies) have been described as a complication of anti-TNF treatment. A prudent approach is advised in case of familial history of DD, the benefit-risk ratio must be pondered, an informed consent must be obtained and a careful neurological follow up must be carried out.

1.7.3.4 Cardiac insufficiency

TNF alpha is implicated in the pathogenesis of cardiac insufficiency (CI), with increased levels associated with reduced life expectancy. Nevertheless, the use of infliximab in CI'RCTs resulted in an increased mortality. The prevalence and incidence of CI seems to be increased in RA population. The effect of anti-TNF treatment on CI in RA has not been studied in RCTs but registry data don't suggest an increased occurrence or aggravation of CI in RA under anti-TNF treatment (109). NYHA class III and IV cardiac insufficiency (CI) is a formal contraindication for infliximab and

adalimumab therapy and a warning for etanercept. Milder CI does not contraindicate treatment but warrants for attentive follow-up and immediate withdrawal in case of aggravation.

1.7.4 USE OF ANTI-TNF IN PARA-PHYSIOLOGICAL CONDITIONS

1.7.4.1 Pregnancy

All anti-TNFs agents are categorized B as far as pregnancy is concerned. Even if animal experimental models and post-marketing surveillance do not suggest an increased risk of harm to the fetus, anti-TNF agents are currently contraindicated during pregnancy. Any ongoing treatment with these agents should stop before conception. In France according to the guidelines of the CRAT (Centre de Reinseignements sur les Agents Thératogenes, http://lecrat.org) a delay of 5 half lives of each drugs is advised before conception (2 months for etanercept and infliximab, 3 months for adalimumab). Shorter delays (3 weeks for etanercept, 2 months for infliximab and adalimumab) are suggested by the CRI based on benefit-risk analysis on literature data.

1.7.4.2 Nursing mothers

Even if it is not known whether anti-TNF agents are excreted in human milk or absorbed systemically after ingestion, anti-TNF treatment should be restarted only after nursing.

1.7.4.3 Elderly

Older age and lower functional status were shown to be negative predictors of response to anti-TNF in the Danish register (110). Nevertheless, no particular contraindications to anti-TNF treatment exist in elderly subject with RA. Even if a higher rate of AEs is described in RCTs for patients aged >65 years (92), no dosage correction is warranted for etanercept or adalimumab, while no particular suggestion exists for infliximab. The use of this class of therapeutics must, as a matter of course, take into account the comorbidities frequently associated in these patients, which may constitute themselves a real contraindication.

CHAPTER 2

Anti-drug antibodies on anti-TNF treatment in Rheumatoid Arthritis

2.1 ANTI-DRUG ANTIBODIES AGAINST ANTI-TNF AGENTS IN RA

At the beginning of the anti-TNF era, common notion was that anti-drugs antibodies (ADAs) induction was a rare event, and that the association of MTX treatment further reduced this event. For example, for only 8% of patient in the ATTRACT study ADA development was reported (111). Anti-TNF agents were given to patients and the mechanisms underlying their efficacy or their lack (or loss) of efficacy were not understood.

Several years later our comprehension has increased. In the meantime, ADA detection techniques have improved in sensitivity and specificity, and so far ADA have been detected in up to 40% of infliximab- treated and in 30% of adalimumab treated patients in some studies. Moreover, their presence is associated to lack or loss of therapeutic efficacy and adverse event occurrence on anti-TNF monoclonal Abs treatment. A recent metanalysis (112) confirmed that ADAs against infliximab or adalimumab result in a reduced response rate of 68% (RR of clinical response to treatment in ADA+ patients 0.32; CI: 0.22 to 0.48). Screening for human antidrug antibodies in clinical trials is now a regulatory requirement in Europe. The occurrence of ADA has been described in RA, Crohn's disease, ankylosing spondylitis and psoriatic arthritis. All monoclonal Abs whether chimeric or human have been shown to induce ADAs, but most of our knowledge in the field derives from studies on infliximab and adalimumab in RA.

2.2 ANTI- INFLIXIMAB AND ANTI-ADALIMUMAB ANTIBODIES IN RA

More than a decade of clinical experience has shown us that therapeutic failures with these two molecules are not infrequent. Patients can either have an absent or insufficient response from the

beginning of treatment (primary therapeutic failure) or they can respond initially and experience loss of therapeutic effect later (secondary failure).

The induction of ADA that increase anti-TNF clearance thereby reducing the drug half-life is recognized a major mechanism underlying the inefficacy of anti-TNFα monoclonal antibodies. ADAs are involved in primary failures and are probably a major cause of secondary failure.

The evidence supporting a major role of anti-infliximab and anti-adalimumab antibodies in therapeutic failure of these molecules in RA can be summarized as follows:

1) Most of the studies show a higher prevalence of ADAs in non-responders, compared to responders, in whom ADAs are rarely detected.
2) There is an inverse correlation between through drug levels and ADAs titers
3) There is a positive correlation between through drug levels and clinical response
4) Dose escalation has limited efficacy in case of insufficient clinical response to these drugs

2.2.1 Clinical response, drug trough levels and ADAs

2.2.1.1 Infliximab (IFX)

Wolbink et al. (113) prospectively followed up for 1 year 51 RA patients treated with 3mg/kg/8w IFX, measuring preinfusional serum through levels of IFX (by an enzyme-linked immunoassay, ELISA technique) and assessing anti-IFX antibodies with a radioimmunoassay (Abs reacting versus I^{125}-labeled pepsin-treated IFX). ADA were detected in 22 out of 51 patients; only 36% of them (8/22) were classified as EULAR responders compared to 69% of ADA-negative patients; moreover, ADA-positive patients had lower DAS improvement than ADA-negatives (mean ±SD decrease in the DAS28 1.9 ± 1.2 versus 0.9 ± 1.8; P <0.02).

Non-responders had higher ADA titers than responders (median 42 AU/ml, interquartile range 8–310 AU/ml vs. median 9 AU/ml, interquartile range 6–17 AU/ml, P <0.025). Three patients, all ADA-positive, had infusion-related reactions.

ADA were detected only in blood samples with undetectable serum trough levels of infliximab, and the development of ADA coincided with a decrease in serum trough levels of infliximab prior to ADA detection. The mean MTX dose was similar between patients with and without ADA: but, notably, only 3 patients didn't take concomitant medication and 2 developed ADA.

Another study by Bentzen et al (27) measured through IFX levels and anti-IFX antibodies (with a dedicated radioimmunoassay (RIA) assessing Abs reacting versus the whole I^{125}-labeled infliximab molecule) in 106 RA patients treated with IFX at 3mg/kg/q8w dose after the first 2 infusions (at 1.5 months), and at 3 and 6 months after the first infusion. The rate of ADA-positive patients rose from 13% at 1.5 months, to 30% and 40% at 3 and 6 months, respectively. Low through IFX levels at 1.5 months predicted ADA development at 3 and 6 months and subsequent therapeutic failure (at 6, <12 and >12 months according to the median through level of IFX). Detection of ADA at 3 months was associated with later IFX dose increases and discontinuation of the therapy. High pretreatment levels of CRP were associated with low through levels of IFX at 1.5 months. Being in the highest DAS28 quartile predicted the development of anti-IFX antibodies at 3 months. The percentage of ADA-positive patients was 40% (20/50) in patients taking concomitant MTX, and 50% (13/26) in those who were not taking it.

This study confirmed that a high percentage of patients regardless concomitant MTX treatment, develop anti-IFX antibodies, these antibodies are predicted by low preinfusional levels of IFX, which in turn, are predicted by high pre-treatment C-reactive protein (CRP) values. An active disease as confirmed by a high DAS28 score is also predictive of anti-IFX antibodies development.

The same group (114) subsequently compared the performance of their fluid-phase RIA to that of solid-phase cross-binding tests (whether ELISA or solid-phase RIAs) used in other studies to determine through IFX levels and detect anti-IFX antibodies. In the paper it is stated that the fluid-phase RIA would be superior to both ELISA and solid-phase RIA in terms of sensitivity and specificity.

The higher sensitivity would be related to the capability to detect ADA even in the presence of detectable levels of IFX, and the capability of fluid-phase RIA to detect Abs that are functionally monovalent.

The higher specificity would rely on the fact that fluid phase RIA is unaffected by rheumatoid factors (RF) (that might link infliximab Fc and be therefore dosed as anti-drug Ab), on the fact that the technique does not induce artifacts like neo-epitopes (that might occur when proteins are fixed on plastic surfaces), and that it does not detect low-avidity Abs that might be clinically irrelevant

2.2.1. 2 Adalimumab

The Dutch group of Wolbink first reported the case of a patient with moderate EULAR response to adalimumab, who underwent a secondary therapeutic failure after drug withdrawal for knee prosthesis implant. The subsequent lack of response was associated to the detection of ADA (formerly undetected in this patient) and with undetectable adalimumab through levels (115). Subsequently, the same group prospectively evaluated the incidence of ADA induction and its association with serum drug concentrations and clinical response in 121 adalimumab-treated RA patients in a 28-weeks follow-up (116).

During 28 weeks of treatment ADA were detected in 21/121 patients (17%). These patients showed less improvement in DAS28 disease activity compared to ADA-negative patients ($p = 0.001$). Moreover, 34% of EULAR non-responders had ADA vs. only 5% of good responders ($p = 0.032$). ADA-positive patients had lower serum adalimumab concentrations at 28 weeks than ADA-negatives (median 1.2 mg/l, vs 11.0 mg/l, $p<0.001$). Good responders had higher serum drug concentrations than both moderate ($p = 0.021$) and non-responders ($p = 0.001$).

In this study 52% of ADA-positive vs. 84% of ADA-negative patients had concomitant MTX ($p=0.003$).

This study first confirmed that, like for the chimeric molecule IFX, even adalimumab treatment can elicit ADA production; these antibodies are more frequently found in clinical non-responders and their presence is associated to low drug concentrations.

2.2.1.3 Comparison in immunogenicity: infliximab vs. adalimumab

Radstake *et al*. (76) evaluated the EULAR response at 6 months and its relation to trough drug levels and presence of ADA in 35 and 34 patients under IFX 3mg/kg/0q8w and adalimumab 40mg/q2w treatment, respectively. The technique to dose drugs through levels and ADA was fluid-phase RIA.

Through levels of anti-TNF drugs and ADA where detected with a fluid-phase radioimmunoassay using I^{125}-labelled TNF, IFX and adalimumab.

For IFX-treated patients there was the possibility to increase the dose to 5mg after 14 weeks in case of insufficient response, or to reduce the dosing interval. In 8 patients the dosing interval was reduced to 6 weeks, in one patient the IFX dose was increased to 5mg/kg/q8w. Twenty-one of the adalimumab-treated patients took part to the DE018 study and dose changing was not allowed. Of the remaining 14, 5 increased adalimumab dose to 40 mg/qw, 2 of them turned from moderate response at 3 months to good response at 6months.

At 6 months, 15 (43%), 6 (17%) and 14 (40%) of the infliximab-treated patients and 16 (47%), 8 (24%) and 10 (29%) of adalimumab-treated patients fulfilled the EULAR criteria for good, moderate and non-responders, respectively.

Clinical response correlated with IFX trough levels at 3 and 6 months in the IFX group (R=0.54, p=0.03), and adalimumab group (R=0.64, p=0.01).

To note, in the good, moderate and in non-responders to IFX at 6 months, the percentage of patients with detectable anti-IFX antibodies was 7, 50 and 100% respectively. In the moderate responders group the Abs were at moderate concentration, while in non-responders the levels were low in 7% of cases, moderate in 36%, high in 57%. None of the patient with detectable through IFX levels had

detectable anti-IFX antibodies, while conversely, all but 3 patients with anti-IFX antibodies, regardless of the antibody levels, there were no detectable trough levels of IFX, and in all these cases the drop in IFX levels corresponded to a raise in anti-IFX antibodies. No difference in mean MTX dosage in good moderate or non- responders was evident.

For the adalimumab group all non-responders had anti-adalimumab antibodies, and all but one had undetectable drug levels. In contrast to IFX, none of good and moderate responders had detectable anti-drug antibodies.

Interestingly, there was a 10% higher response rate in patients treated with adalimumab compared to their counterparts treated with infliximab, mirroring the percentage of patients who formed antibodies to the drug.

All moderate responders to adalimumab and 50% to IFX had detectable through drug levels and no detectable ADA. In these patients it is hard to conceive the presence of anti-drug antibodies below the detection threshold, given the high sensitivity of the radioimmunoassay; in these patients other mechanisms of non response are therefore probably implicated.

2.2.2 Drug trough levels and clinical response

Wolbink *et al.* (117) prospectively followed for 14 weeks 105 patients treated with 3mg/kg/8w IFX, and demonstrated that, at 14 weeks, EULAR responders had higher serum through levels of IFX compared to non-responders (IFX measured with an ELISA test measuring free IFX using recombinant human TNF). Even if high interindividual variability existed, the median (interquartile range) concentration of IFX fell from 22.3 (15.3–29.4) mg/l after 2 weeks, to 14.6 (7.3–22) mg/l after 4 weeks, to 2.8 (0.6–6.8) mg/l after 8 weeks.

Those with lower levels of IFX had even lower DAS28 improvement. By categorizing IFX trough levels in tertiles, it was shown that in the lowest tertile there were less EULAR responders and lower DAS28 improvement. Pretreatment CRP levels were negatively correlated with through IFX

levels at week 14after the start of treatment (Spearman rank correlation rs =20.43, p,0.001). Three patients had infusion reactions, and they all had undetectable through levels of IFX.

St Clair et al (118) in a post-hoc analysis of the ATTRACT study which comprised 4 dose regimens (3mg/kg each 8 or 4 weeks, 10 mg each 4 or 8 weeks), showed lower median through levels of IFX in the lowest dose regimen group (3mg/kg/8w), and more patients with undetectable through levels of IFX from week 22 to week 54. Even if there was no detectable difference in the number of patients achieving ACR20 response, this group had less patients achieving ACR50 and ACR70 response (and a trend towards increased rate of patients achieving ACR20, 50 and 70 with increasing dosage in the different groups). The ACR-N response was inversely correlated with trough levels of IFX at regression analysis. If the patients were divided in quartiles of IFX trough levels at week 54 the lowest quartile had the highest number of patients not achieving an ACR20 response, with higher rates of ACR50 and 70 responders with higher levels of IFX, even if this relation had some exceptions (i.e. some patients achieving an ACR70 response despite undetectable IFX through levels). Other regression analyses showed that reduction in CRP levels and less Sharp progression were correlated with higher trough levels of IFX. A pharmacokinetic modeling was used to predict the trough levels of IFX in the 3mg/kg/8w group whether the dose was increased of 100 mg or the dosing interval reduced to 6 weeks. Reducing the dosing interval increased median trough levels of IFX from 0.03 ug/ml to 2.146 ug/ml vs 0.416 ug/ml with increasing dosing.

2.2.3 Dose escalation

2.2.3.1 Partial efficacy of IFX dose escalation.

In an observational study by Sidiropoulos *et al.* on 68 insufficient responders to classic DMARDs, IFX was introduced and treatment dose was subsequently adjusted according to EULAR criteria of response (119). There were 20 drop-outs along the course of the study. In 50 patients an IFX dose escalation was necessary along a time period of 12 IFX infusions due to insufficient response, 12 of them were drop-outs. A significant reduction in mean DAS score (from 5.27 to 4.54, $p<0.002$) was

evident for the remaining 38 patients who underwent a dose escalation but only 10 of them (26%) improved their EULAR response category. These data suggest that dose escalation in insufficient responders to IFX might have a limited value.

Van Vollenhoven *et al.* more formally addressed the question of clinical response after dose escalation of IFX (120). They carried out a case-control study were the index cases were the patients whose IFX dose had been increased, and the controls were either patients who had not undergone a dose escalation or patients treated with etanercept.

As a comparator they used the DAS28 score attained when decision to switch was taken for the cases, and the worse DAS28 attained at any time point for the controls. In their analysis they showed that even if the escalation induced statistically significant reduction in DAS28 score, the improvement never exceeded the best improvement achieved before dose escalation. Even patients in the 2 control groups at a certain time point might improve from their worse DAS28 score to re-attain the best previously attained DAS28 score. The authors concluded that since similar improvements occurred in cases and controls, the impression of a "recaptured" clinical response following IFX dose escalation might probably due to a phenomenon of regression to the mean, i.e., since the IFX dose was increased at the highest DAS28 score attained until then by the patient, the DAS28 score was more likely to lower with time, independently of the treatment.

In a clinical trial of 12 months duration (121), 144 RA patients partial responders to 3mg/kg/q8w IFX where randomized to either keep the same dose or to undergo a dose escalation to 5mg/kg/q8w. At 12 months, the DAS28, its components and CRP levels did not differ between the two arms, while in the higher dose arm more non-serious adverse event were observed (28.7% vs 47.8%, p=0.023)

2.3 ANTI-TNF LEVELS, ADAs AND TREATMENT OUTCOME

The phenomenon of ADA induction appears to be inscribed in a rather complex network that links together TNFα levels in the inflamed tissues, trough anti-TNF drug levels and the sensitization of host immune system vs. the monoclonal molecule.

The first evidence that started to shed light on these interconnected phenomena came from the results of pilot studies with IFX in RA, which showed a trend towards an increased clinical response with increasing IFX dose from 3 to 10 mg/kg in the different treatment arms. A post-hoc analysis showed that clinical response to IFX was associated to higher trough levels of infliximab and that trough levels falling under the detection limit were associated to drug inefficacy and adverse event occurrence.

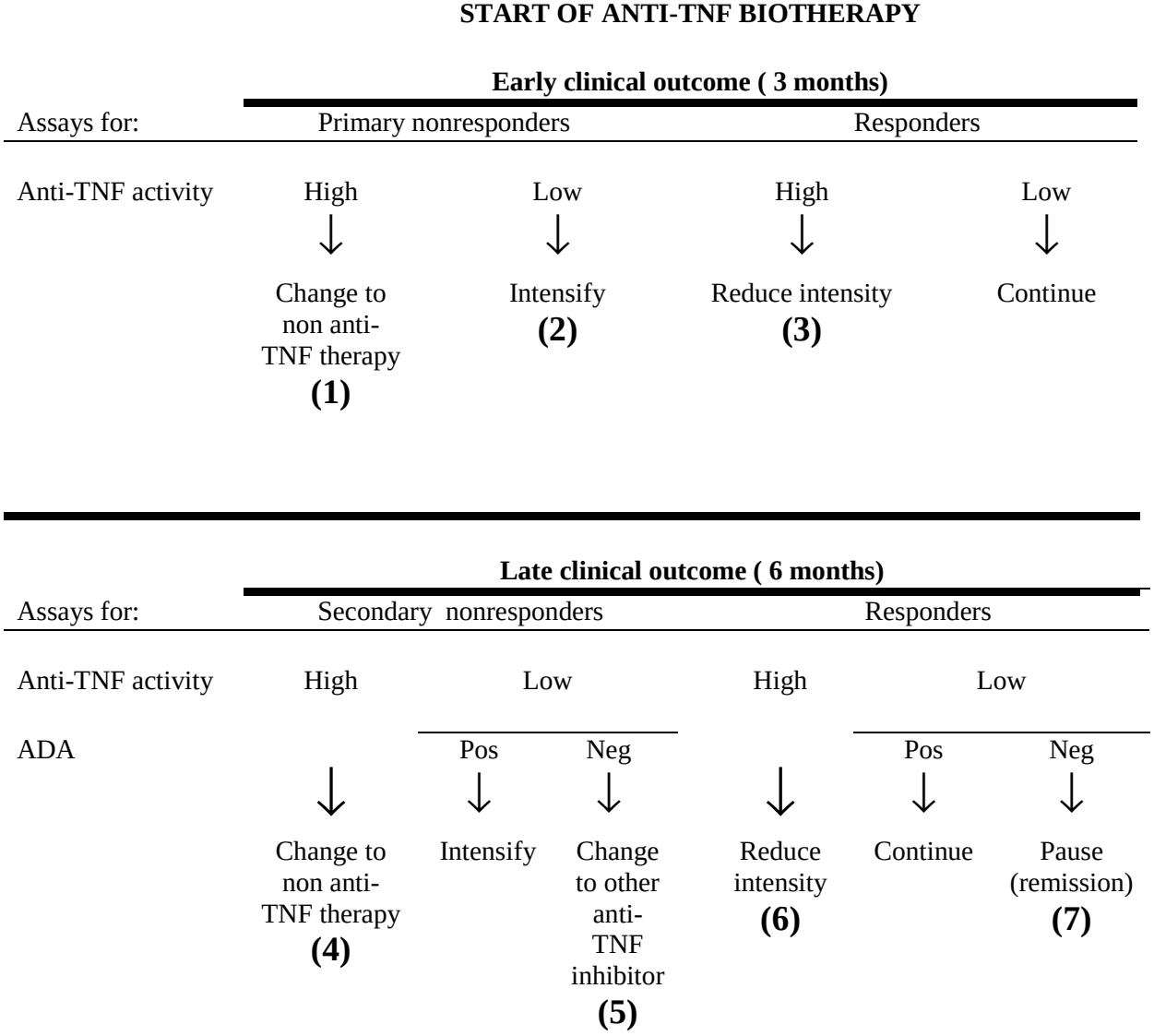
A metanalysis of randomized trials showed that patients with higher disease activity had less clinical improvement, and other studies that high pre-treatment CRP levels predicted lack or anti-TNF efficacy. Both the parameters can be a surrogate measure of pre-treatment TNFα levels in the inflamed tissues ("TNFα load"). A higher TNFα load would consume more anti-TNF drug. Thus, patients with higher TNFα production would have lower trough levels of anti-TNF agents. In these patients higher anti-TNF doses are necessary. The balance between basal TNFα production and anti-TNF trough levels would determine whether the host immune system will be sensitized or tolerized vs. the monoclonal antibody. Low levels of TNFα result in higher anti-TNF trough levels. A high foreign antigen (i.e. the anti-TNF drug) concentration would result in tolerance and no ADA production. Conversely, higher levels of TNFα result in low anti-TNF trough levels. The presence of foreign antigen at low concentration can elicit an immune response with ADA production.

The presence of ADAs (just like that of low trough anti-TNF levels) is associated with reduced efficacy and adverse events. ADAs inhibit the interaction between the monoclonal antibody and the target cytokine and accelerate the drug clearance, both phenomena contributing to reduce the drug efficacy (122). Monitoring of trough anti-TNF levels and ADAs in clinical practice might constitute a major step towards personalized medicine that would result in safer and more cost-effective

treatment. For example, in case of primary failure, if anti-TNF trough levels are high it would be useless to increase the dose of the drug, the best strategy would be to change the therapeutic target. Based on the concept of the link between anti-TNF trough levels, ADA induction and primary or secondary failure, Bendtzen (37) proposed a treatment algorithm for patients on anti-TNF mAbs (Figure1).

Other TNF blockers, like etanercept, do not seem to be concerned by ADAs: even if anti-etanercept antibodies have been described in one paper (123), they have never been linked to treatment outcome so far. Anti-etanercept Abs may be non-neutralizing and directed at the hinge region where recptor sequence joins Fc sequence to form a ‘neoepitope’(124). Nevertheless, even for etanercept treatment low serum levels are associated with lack of clinical response (125). Figure 2 summarizes all the potentially immunogenic sites on currently used anti-TNF agents. Our group developed an alternative TNF-blocking strategy based on active anti-TNF immunization, where polyclonal anti-TNF antibodies would be produced by the host, thereby potentially avoiding the risk of ADA induction (see after).

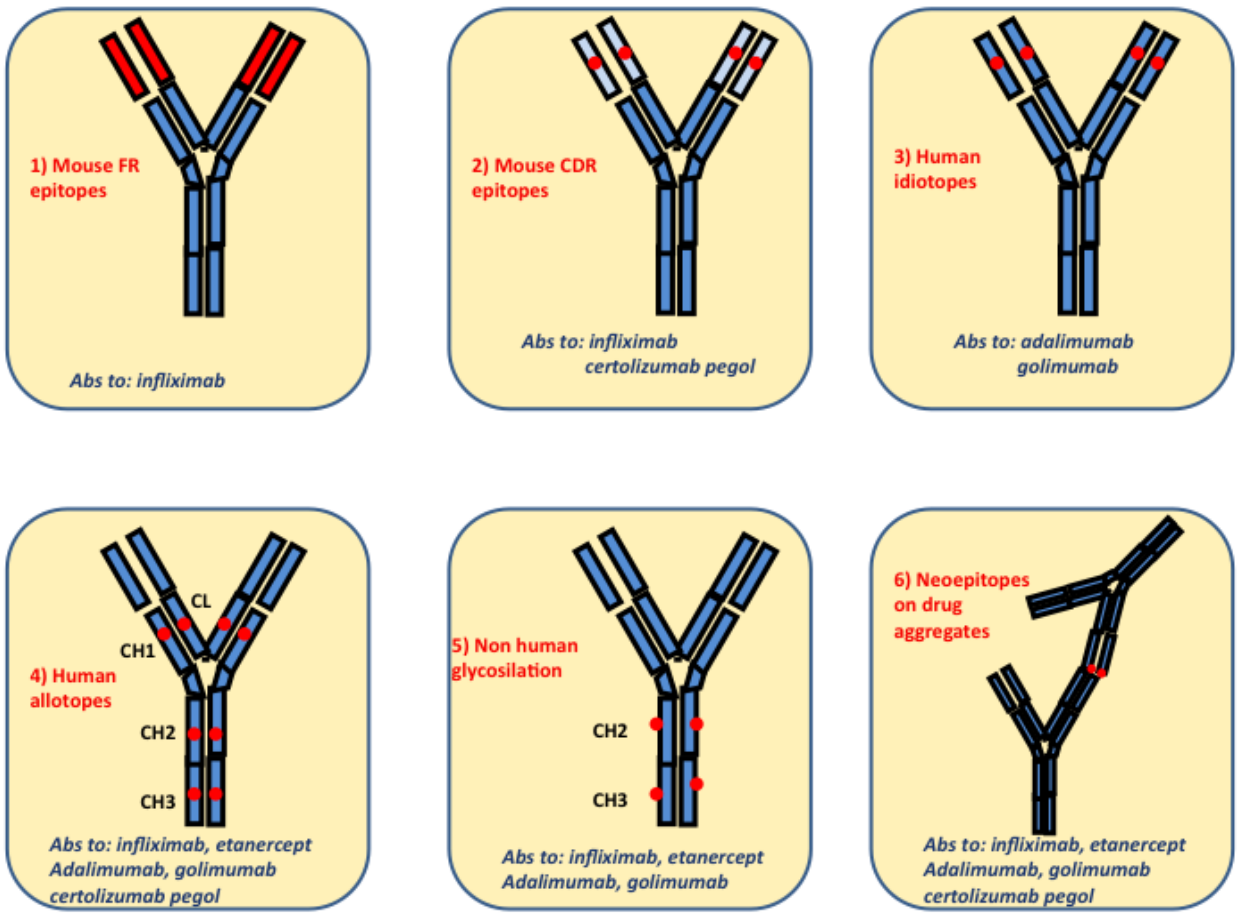
Figure 1. Proposed follow-up and treatment algorithm for patients on anti-TNF monoclonal antibodies



(1): Irrational to intensify therapy. Irrational to try another anti-TNF inhibitor. Early shift to effective therapy
(2): Evidence-based therapy
(3): Improved cost-efficiency
(4): Irrational to intensify therapy. Irrational to try another anti-TNF inhibitor. Shift to effective therapy
(5): Irrational to intensify therapy. Shift to effective therapy
(6): Improved cost-efficiency
(7): Continued drug use irrational and possibly dangerous. Improved cost-efficiency.

(Modified from Bendtzen, ref. 94)

Figure 2. Potential immunogenic sites on anti-TNF-α antibody constructs



Legend: 1: mouse epitopes on FR fragments in Fab region of chimeric drugs; 2: mouse epitopes ondrug CDR. 3: idiotopes on drug binding site on human drugs. 4: allotopes on constant regions of chimeric, humanized and human drugs. 5: neoantigens due to nonhuman glycosilation during drug engineering process. 6: neoantigens formed by drug aggregation. FR: Framework region CDR: Complementarity-determining variable region; CH1, CH2, CH3 and CL: Constant regions of IgG on light and heavy chains.

CHAPTER 3

Active anti-TNFα immunization in RA: the kinoid of human TNFα (TNF-K)

3.1 IS THERE A NEED FOR OTHER ANTI-TNF TREATMENTS?

Rheumatoid arthritis (RA) is the most common inflammatory rheumatic disease with a prevalence ranging from 0.3 to 1.5 % in the different populations (126). It is characterized by an invasive synovial proliferation that leads to joint damage with pain and loss of function, with precocious disability (127). RA patients have associated comorbidities leading to a mortality estimated at almost 2-fold that of general population (128). RA is therefore a huge public health problem resulting in high direct and indirect costs for the community (129).

In the last decade, tumor necrosis factor alpha (TNFα)-targeting agents have changed the treatment of RA, providing unheard of results in terms of disease clinical control and prevention of structural damage and consequent disability. TNFα can be targeted with monoclonal antibodies (mAbs) or their fragments (infliximab, adalimumab, golimumab, certolizumab) or with fusion products carrying a TNFα soluble receptor (etanercept). Anti-TNFα drugs first opened the perspective of a successful cytokine-targeting strategy in RA. Sales of the four anti-TNFα agents on the market in 2008 (adalimumab, infliximab, etanercept and certolizumab pegol) reached $16 billions. By 2014 analysts forecast the entire class of ant-TNF drugs to generate a $25 billion market, with growth driven by new entrants and continuing demand for the incumbents (source: EvaluatePharma®)(130). In 2008, TNF-alpha inhibitors accounted for 80 percent of rheumatoid arthritis drug sales in the United States, France, Germany, Italy, Spain, the United Kingdom and Japan (source: Decision Resources ®) (131) within a market that, for all biological therapies for RA, was estimated in 7 billion dollars in 2007 (source: Datamonitor® Research Store) (132).

Current TNFα-targeting strategies have nevertheless shown several drawbacks as far as safety, efficacy and costs are concerned. Despite the good safety/ efficacy profile in selected patients, the overall risk of infection and neoplasm seem to be increased RA patients treated with anti-TNFα mAbs compared to classic DMARDs (92). Primary and secondary failures are not infrequent, moreover less than 50% of responder patients in clinical trials attained disease remission (133). The treatment with anti-TNF blocking agents has high costs for the community (134). While some of these limitations, like the increased risk of infection and neoplasm are presumably related to the blockade of TNFα itself, others, like the high production costs, and the risk of anti-drug antibody (ADA) production with possible loss of efficacy and side–effects, are proper to current anti-TNFα agents, especially to monoclonal antibodies (122), and they might be possibly overcome by alternative anti-TNF blocking agents strategies.

An alternative way to target TNFα is active immunization, where a TNFα derivative can be used as the immunogen to develop an anti-TNFα active immunotherapy consisting in a vaccine (135). The immunogen must be capable of disrupting B-cell, but not T-cell, tolerance to TNFα, thereby eliciting the production of high titers neutralizing antibodies (136). This strategy allows the production of polyclonal autologous anti-TNFα antibodies potentially bypassing the risk of an anti xeno- or allogenic antibody response. Refining of ADA detection techniques allowed in fact to detect ADA in up to 40 and 30 % of infiximab (IFX) and adalimumab-treated patients, respectively (137). The presence ADA is associated with low trough drug levels, infusion-related reactions (for IFX) and therapeutic failure (76). Active immunization offers then the possibility to overcome this limitation.

The direct costs for anti-TNF blocking agents, together with the costs of drug administration, monitoring, and side-effect management, result in a heavy economical burden for the community (138), whilst active immunization strategy might potentially be a less expensive alternative. Finally, the long persistence of detectable anti-TNFα antibody titers induced by active anti-TNFα

immunization might warrant a less cumbersome administration scenario for the patient, with possibly higher treatment acceptance.

3.2 CHEMISTRY OF THE KINOID OF HUMAN TNFα

Our group established the preclinical proof-of-concept of active anti-TNFα immunization with a compound called human TNFα kinoid (TNF-K) in a TNFα-dependent animal model, the human TNFα (hTNFα) transgenic mice (TTG mice) (136, 139, 140). This lead to subsequently test TNF-K in a phase I/II clinical trial in Crohn's disease and in a phase IIa clinical trial in previously anti-TNFα treated RA patients on secondary therapeutic failure.

TNF-K belongs to a family of cytokine derivatives capable to act as anti-cytokine vaccines called "kinoids" (141). Their name and their preparation recalls those of the toxoids, detoxicated but still immunogenic products, derived from bacterial toxins by formalin treatment at 37°C for several days. At the beginning of the eighties, a detoxication procedure using glutaraldehyde instead of formaldehyde was described for the preparation of fully atoxic polymerized antigens with high immunogenicity (142). This technology with either glutaraldehyde or formaldehyde was then applied to cytokines in order to convert them into derivatives devoid of biological activity but capable, when administered to animals, of inducing anti-cytokines antibodies. These derivatives were called kinoids (143).

TNF-K is a heterocomplex of inactivated hTNFα and a carrier, the keyhole limpet hemocyanin (KLH). KLH is a heterogeneous copper-containing respiratory protein isolated from the mollusc *Megathura crenulata*, belonging to a group of non-heme proteins called hemocyanins. It consists of two subunits isoforms with a molecular weight of 390x 10^3 and 360 x 10^3 D, originating, respectively, two different oligomeric aggregates, KLH1 and KLH2. The molecular weight of the oligomers ranges from 4,500,000 to13,000,000. Due to its large size and its numerous epitopes KLH is capable of inducing a substantial immune response; its abundance of lysine residues for

haptens coupling, with a high hapten/carrier protein ratio, increases the likelihood of generating hapten-specific antibodies (144).

For preparing the heterocomplex, glutaraldehyde is used to couple hTNFα to the KLH carrier protein. KLH, and then glutaraldehyde, are added to a solution hTNFα treated with dimethylsulfoxide, in a mixture of one molecule of KLH and 40 molecules of hTNFα. After 45-min incubation at 4°C, the preparation is dialyzed against the working buffer and then treated with formaldehyde for 6 days at 37°C. Concentration and duration of aldehyde treatments have been adapted for hTNFα in order to obtain a strong and persistent inactivation of its biological activity. The unreacted aldehyde is quenched by addition of glycine (0.1 M), leading to complex stabilization. The excess aldehyde is eliminated by dialysis against Dulbecco's phosphate buffer solution (PBS) (136). KLH has been used in human mainly for immune antitumor therapy. Intravesical treatment of superficial bladder carcinoma with the KLH subunit product Immucothel® resulted in mean recurrence rate of 31% over a 26 months period (145). The mechanisms relies in the fact that the KLH disaccharide epitope Gal(β1-3)Gal Nac cross-reacts with an homologous epitope on bladder tumor cell surface. A vaccine consisting of a murine anti-idiotipic Ab (mimic a human melanoma antigen) conjugated with KLH was tested in a small group of patients with malignant melanoma (146). Recently, a sialyl-TN (STn)-KLH vaccine failed to demonstrate an increase in survival rate in a phase III study in breast cancer (147). KLH is used even as a generalized vaccine component. KLH-based vaccines have been developed for papillomavirus, tick-born encephalitis and *mycobacterium bovis* infections, and even for drugs of abuse like cocaine. To the best of our knowledge, despite good results in animal models none of them was subsequently tested in human (144).

3.3 PHARMACODYNAMICS OF TNF-K

It is assumed that TNFK is a heterocomplex in which KLH provides T epitopes and bears at its surface a high density of hTNFα preserved B-epitopes. The aim of carrier proteins is to promote

carrier-specific T-cell help to a B-cell polyclonal response (144). Given that a high number of hTNFα molecules are covalently bound to KLH, kinoid immunocomplexes will present a high density of hTNFα antigens in their native conformation to the antibody-producing B cells to cross-link specific B-cell receptors (141).

Transgenic hTNFα mouse (TTG), expressing hTNFα as a self antigen, is the only relevant model to study TNF-K induced anti-hTNFα antibody production (136). In all immunized mice in different study protocols, immunization with TNF-K induced specific anti-hTNFα antibodies as detected by ELISA (136, 139, 140). In a protocol where mice received three injections of TNF-K at days 0, 7 and 28, these antibodies, tested at day 122 after TNF-K first injection, appeared to belong mainly to IgG1 (52%) and IgG2a (48%), with negligible amounts of IgG3, IgM and IgE(136). Purified IgG from hyperimmune sera exhibited a high affinity for hTNFα with Kd ranging from $5x10^{-8}$ to 10^{-10} M and were able to block its interaction with the high affinity TNFRI (Kd of 0.6 nm)(148), resulting in undetectable circulating hTNFα in immunized mice.

Anti-hTNFα antibodies have neutralizing anti-TNFα effect as confirmed both *in vitro* by L929 cytotoxicity assay, showing cytotoxicity inhibition by hyperimmune sera at dilutions up to 10^{-4} , and *in vivo*, where purified IgG from sera of immunized mice prevented TNFα-galactosamine lethal shock in recipient mice(136).

TNFK is mixed at a 1:1 ratio with the phosphate saline buffer (PBS) and administered intramuscularly with the adjuvant ISA51® (Seppic, France). The latter is similar to Freund's incomplete adjuvant and is composed of a mix of mineral oil and a surfactant of the mono-oleate family; it is currently used in immunotherapy of cancer and infectious diseases (149). ISA51 is used in a 1:1 ratio with the mix TNFK-PBS to obtain a water-in-oil emulsion (141).

Different administration schedules have been tested in mice, involving 2 (at days 0 and 7), three (at days 0, 7 and 28) or four injections with dose regimens varying from 5 to 30 μg of TNF-K (136, 139, 140).

Whatever the exact administration schedule, all immunization protocols were able to induce anti-hTNFα antibodies in TTG mice. In a three injections scheme (30 +30+7 μg at days 0, 7 and 28), anti hTNFα antibodies were detectable at first bleeding as soon as five weeks after TNF-K first injection (139), they peaked at 6-8 weeks after first injection (136), with a more than 50% decline within 16 weeks.

In a protocol with three injections of TNF-K 4 μg at days 0,7,28 the anti-hTNFα Ab peak was at week 13 after TNF-K first injection, and a TNF-K boost given at week 17 induced a significant increase in neutralizing anti-hTNFα antibodies as soon as 3 weeks after the boost (140).

3.4 EFFECTIVENESS OF TNF-K IN ANIMAL MODEL

TNF-K immunization resulted in amelioration of TTG mice spontaneous arthritis, thereby posing the rationale for its use in RA.

When given before arthritis development, TNF-K markedly reduced the clinical severity of arthritis and resulted in less histological joint inflammation and destruction compared to control mice (136, 139).

In an experimental 3-injection protocol (day 0, 7 and 28) a highly significant difference in clinical and histological score was already evident when animals were sacrificed 6 weeks after the first injection, compared to controls. TNFK-immunized animals showed mild histological inflammation and no histological destruction. The co-administration of MTX did not modify the results (139).

When, with the same experimental protocol, the observation was prolonged up to 17 weeks, arthritis onset happened to be delayed of about 9 weeks compared to controls, and still low clinical and histological scores were found in immunized mice.

The therapeutic efficacy, its duration and the effect of a TNF-K boost were better evaluated in a subsequent experiment more resembling to a human disease scenario, since TTG mice were immunized after spontaneous arthritis onset (140). In a 12-week follow-up after TNF-K

immunization, arthritis was dramatically ameliorated, and clinical scores did not differ from those of mice treated with weekly infliximab at a dose of 1 mg/Kg over the same time period. These findings were corroborated by histology, showing low inflammation and no sign of cartilage destruction in immunized animals.

The observation was prolonged to 30 weeks after TNF-K first injection in order to study the duration of clinical effect and the kinetics of TNF-K-induced anti-hTNFα antibodies. After the initial amelioration, arthritis clinical score in immunized mice started to increase from week 12 after first injection, to the end of the experiment. This trend was reversed by a TNF-K boost given at week 17, before clinical degradation ensued. The worsening in clinical control of arthritis coincided with a decrease in anti-hTNFα Ab titers, while the TNF-K boost triggered a significant increase in Ab titers 3 weeks after its administration. Mild histological scores of joint inflammation, destruction and cartilage degradation at the end of the experiment, confirmed the long-term prevention of structural damage of TNF-K immunization.

3.5 SAFETY AND TOLERABILITY OF TNF-K

Some major safety issues are raised by the novel anti-TNFα approach of active immunotherapy, namely:

1) The delivered TNFα must be devoid of toxicity but still immunogenic. This is the case of TNF-K heterocomplex, where aldehyde treatment results in a hTNFα derivative satisfying these requirements. In all experiments conducted with TNF-K, no short-term toxicity linked to its administration and ascribable to hTNFα activity-related toxicity was detected. This was the case even in the limited experience in human.

2) Anti-TNFα vaccination must result in rupture of B-cell but not of T-cell tolerance (i.e. vaccination must not induce memory T-cells capable of recognizing the native cytokine). In fact,

the persistence of a T-cell population sensitized against a self-cytokine would result in a localized cellular response in its site of production.

This issue was addressed in an animal study where 6-8 weeks old TTG mice received 3 injections of TNFK (days 0,7,28 ± a boost at day 90) and were followed up for 120 days after the first injection. Our group showed that the splenocytes from TNFK-immunized TTG mice did not trigger any cell-mediated immune response to self-hTNFα, as tested by T-cell proliferation and IL-2 and IFN-gamma production in culture supernatants, whatever the administration regimen of TNF-K (136). The only detectable cellular response was directed against KLH. Conversely in Balb/C mice, TNF-K immunization induced anti-hTNFα cellular response, when hTNFα (a heterologous antigen for this strand) was administered.

In TNF-K001 study in Crohn's disease patients, stimulation of peripheral blood mononuclear cells (PBMC) of immunized patients with TNFα failed to induce T-cell proliferation (150).

3) The rupture of B-cell tolerance must be reversible. Our group demonstrated that when TTG mice were immunized with TNF-K before spontaneous arthritis appearance, anti-hTNFα antibodies peaked 6-8 weeks after TNFK first injection and had a >50% antibody titers decline within 12-16 weeks. This kinetics is ascribable to short life of B-cell memory in the absence of a specific T-cell help. A long-term study, where immunized TTG mice were monitored up to 30 weeks after TNF-K first injection, confirmed the results (140).

A similar kinetics, albeit with the limitation of study design and sample size, seems to be confirmed in human, based on the results of TNF-K001 study. In the 13 immunized patients anti-TNFα antibody titers were markedly reduced, and sometimes no longer detectable, within 12-15 weeks after first injection (150).

4) A raise in the levels of TNFα induced by other stimuli (infections, tumors) must not elicit the production of anti-TNF Abs after TNF-K immunization. This was demonstrated in a study where monthly administration of hTNFα to TTG mice failed to induce any raise of anti-hTNFα antibodies (140).

5) Ideally the “physiological” activity of hTNFα in normal tissues should be conserved (see points 2, 3 and 4).

3.6 CLINICAL TRIALS IN CROHN DISEASE AND RA

TNF-K was first administered in human in a phase I/II open-label dose escalation study on 13 patients with moderate to active Crohn’s disease, the TNF-K001 study (http://clinicaltrials.gov/ct2/show/NCT00808262). The administration schedule consisted of three injections of TNFK at day 0, 7 and 28 at doses of 60, 180 and 360 µg. Four patients received a fourth boost dose at 6 months. In all immunized patients anti-TNFα antibodies were detected, with a peak in titers between the fourth and the fifth week after first TNF-K injection, and a 50% reduction within 12 weeks. The boost at 6 month resulted in a new peak in antibody titers 3-4 weeks later (150).

As far as RA is concerned, a dose-finding phase II clinical trial has been carried out in RA patients on secondary failure of anti-TNF treatment (http://www.controlled-trials.com/mrct/trial/772671/TNFK003), in a multicentric, randomized double-blind trial vs. placebo on background MTX treatment. The primary goal was to demonstrate that active immunization with TNF-K was able to induce polyclonal anti-TNFα antibodies in RA patients previously treated with anti-TNFα monoclonal antibodies that had undergone secondary therapeutic failure (i.e. loss of clinical response). The development of antibodies against the TNF antagonist (ADA), at screening or on a sample taken since discontinuation of treatment, was an inclusion criteria, amended along the trial course and no longer necessary for patient inclusion. The study involved 40 patients with active RA. The trial protocol comprised 6 treatment arms, aiming to evaluate the safety and efficacy of three TNF-K dose regimens (90, 180 and 360 µg) administered (intramuscularly) according to one of two administration schedules, comprising two or three TNF-K administration for each dose regimen at day 0 and 28 or at days 0, 7 and 28, respectively. The primary outcome was anti-TNF Abs detection, secondary outcomes were the neutralizing activity of anti-TNF Abs, and DAS-28 based

clinical response. Global tolerance was good, and minor cutaneous reactions at injection site were the most frequently reported adverse event. No serious adverse events were reported at 52 weeks of follow-up after immunization. Anti-TNF Abs were detected in 50%, 75% and 91% of all patients that had received injections of 90, 180 and 360 µg, respectively. One hundred per cent of patients that had received three injections of 180 or 360 µg produced anti-TNF Abs vs. 67% of those that had received two injections. Only three-injections of 360 µg resulted in both 100% of patients producing Abs and in high Ab titers. Among the 21 patients that produced anti-TNFAbs a moderate or good EULAR response was found in 48% vs. 31% in those with no detectable Abs. In the former group C-reactive protein (CRP) showed a14% decrease vs. a 5% increase in the latter.

The results were considered positive in terms of tolerance and promising in terms of efficacy; the dose of 360 µg and the three dose regimen were retained as effective (151). A phase III clinical trial with TNF-K in RA is currently being planned.

3.7 TNF-K FOR RHEUMATOID ARTHRITIS: PROS AND CONS

The originality of the therapeutic strategy with TNF-K is high, meaning the possibility for a specific type of development in clinical situation. This active immunotherapy aims at reversibly vaccinate against TNFα. It belongs to anti-TNF agents' family. Contrasting with the already marketed anti-TNF agents, one can suppose that using TNF-K could have advantages in term of simplicity and frequency of injections. The effect would be likely quite long after each injection (several weeks). Moreover, TNF-K treatment should not be concerned by a reduction of effect due to ADAs: these antibodies are found in up to 40% of infliximab-treated and in 30% of adalimumab-treated patients. They reduce drugs therapeutic efficacy and are responsible for therapeutic failure and adverse reactions.

Another possible advantage is a lower economic burden for the community, since the costs of production of the kinoid would be presumably lower than those of marketed anti-TNF agents:

reduction of costs are currently requested in Northern countries, and appears as a necessary condition to treat TNF-driven diseases with targeted treatments in Southern countries. The access for the patients to expensive biological therapies is strongly limited, in many countries, by health authorities or other third party payers, and the choice of treatment will be more and more influenced by cost-effectiveness analyses. In this scenario, a less expensive alternative providing "value" and "value for money" in RA treatment would certainly be welcome.

If the safety and efficacy data suggested by the animal models are confirmed by ongoing human clinical studies, it is conceivable that TNF-K will have a considerable impact on RA treatment strategies.

The reversibility of anti-TNFα vaccination with TNF-K is a key condition of a favorable benefit/risk ratio. All preclinical studies show a bell curve of anti-TNF Ab levels and preliminary results in humans confirmed this point. Clinical trials should confirm these safety considerations when looking for adverse events. The lack of induction of immunological memory is also a major point, supported by results of both preclinical and clinical studies.

In summary, an important preclinical body of evidence (not inferior to that which first lead to test a monoclonal anti-TNFα antibody in 10 RA patients in 1992) supports the feasibility of anti-TNFα active immunization in TNFα–dependent human diseases. The efficacy in hTNFα transgenic mice spontaneous arthritis, the relevant model for TNFα inhibition, strongly supports its potential application in RA. The reversibility of anti-TNFα antibody levels increase, and the absence of memory T-cells induction, are both arguments in favor of a good safety profile. The results of an open-label study in Crohn's disease were first consistent with animal data regarding the kinetics of antibodies induction and decrease, and suggested a good tolerance. The results of a dose-finding double-blind, phase I/II study in RA provided more safety and efficacy information that opened way to the phase III of clinical development.

CHAPTER 4

Regulatory T-cells in RA

Regulatory T cells (Tregs) are critically important cellular mediators of peripheral immune tolerance. Current rheumatoid arthritis (RA) treatments are general (i.e. non antigen-specific) immune suppressant impacting on both pathogenic and physiological immune response. Treatment withdrawal usually results in relapse imposing longtime, possibly lifelong treatment, with potential adverse events for patients and high economical costs for society. The ultimate goal of therapy for patients RA and other autoimmune diseases would be restoring immune tolerance and allowing cessation of immunosuppressant therapy. Hence, Tregs could be an ideal target for therapies to induce durable remission. Even if the real role and function of Tregs in RA is still unclear, it has nevertheless been actively explored in the last decade, and there is increasing evidence that Tregs are involved in disease progression and in therapeutic remission. We will focus on the role of Treg populations in RA and on how current RA treatments impact Tregs. The potential of Treg-targeted treatment for RA treatment in the future will not be discussed.

4.1 REGULATORY T-CELLS PHENOTYPE AND FUNCTION

Tregs constitute 5–7% of CD4+ T cells in humans (152).Treg cells are defined based on phenotypic markers and functional assays that confirm their regulatory properties. Tregs are endowed with an autoreactive T-cell receptor (TCR) repertoire, and recognition of the antigen through the TCR is required to suppress immune responses. Tregs suppress immune responses through a variety of contact-dependent and contact-independent mechanisms (153, 154).

A major issue in literature is which are the phenotypic and functional markers to identify characterize Tregs.

The transcription factor forkhead box P3 (FOXP3), is critical for Treg**s** generation, peripheral maintenance and function (155, 156). Nevertheless, three main problems limit the use of FOXP3 expression alone to study Treg**s** in humans: first, under the influence of transforming growth factor β (TGF-β), CD4+ T-cells can transiently upregulate the expression of FOXP3 upon activation (157, 158)(see after). Moreover**,** in an inflammatory environment**,** regulatory cells expressing FOXP3 may lose regulatory capacities and differentiate into effector cells. Second, studies in mice have shown that DNA methylation status at the FOXP3locus are probably a better marker of a stable Treg rather that the mere protein expression of FOXP3 (159, 160). Third**,** and more importantly, Treg**s** cannot be selected for functional studies on the basis of FOXP3 expression, since FOXP3 is an intracellular protein. Thus, in current practice, the study of Tregs in patients with autoimmune diseases relies on use of cell surface markers to identify and isolate Treg**s** for functional studies. Since no known cell surface markers are expressed exclusively on Treg**s**, the best approach is to use a constellation of cell surface marker**.**

Tregs were originally identified as characterized by high expression of CD25, the alpha chain of IL-2 receptor. CD25 is not specific of Tregs: it can be expressed by activated memory T-cells, especially in autoimmune diseases. For this reason, the CD4+CD25^{high} population can also include effector T-cells (161). This contamination has, in some cases, misled investigators to believe that deficits in Treg**s** function existed where they did not and can be responsible for the heterogeneity of results between different studies.

Another feature of Treg**s** is the absence of production of IL-2. Even if in vitro FoxP3 cells can produce high amounts of IL-2, this seems not be the case in vivo.

A low expression of the IL-7 receptor CD127 on Tregs has been proposed as a feature to differentiate Tregs from activated Teff cells, and a group could show that low levels of CD127 expression in combination with CD4 and CD25 expression led to identify more than 95% of FOXP3+ T-cells endowed with high immunosuppressive activity (152). CTLA-4 (cytotoxic T-lymphocyte-associated protein-4) (see after), a surface molecule expressed on both Teff and Treg,

binds to CD80/CD86 on antigen presenting cells (APCs). Its ligation inhibits Teff proliferation and activates Tregs. Tregs even express molecules belonging to the TNF- receptor family, like GITR (glucocorticoid-induced tumor necrosis factor receptor), and CD 27. Agonist anti-GITR Abs inhibit the suppressor effect of Treg cells. The exact role of these and other markers, like Neuropilin-1, which is constitutively expressed on Tregs, remains to be determined.

4.2 THE TREG/TH17 BALANCE

A main feature of Tregs is that they are linked to pro-inflammatory Th17 cells via an alternative pathway of CD4+ T-cell differentiation, under the action of the pleyotropic cytokine TGF-β. Depending on the cytokine environment TGF-β is able to act to opposite effects.

This dualistic effect of TGF-β has been dissected in mouse models providing an elegant solution for linking the potentially pathogenic Th17 pathway with a potent counter-regulatory pathway that can control it (162-165). In humans the scenario is partially more complicated but substantially analogous.

Th17 cells and other effector T-cells, like Th1 and Th2, differentiate from antigen-naïve CD4+T-cell precursors, under the action of cytokines produced by innate immune cells that have recognized specific infectious agents. Depending on the pathogen, a distinct cytokine profile is induced leading to the differentiation of the T cell type best suited to control the specific pathogen

Figure 3. Patterns of differentiation of naïve Tcells

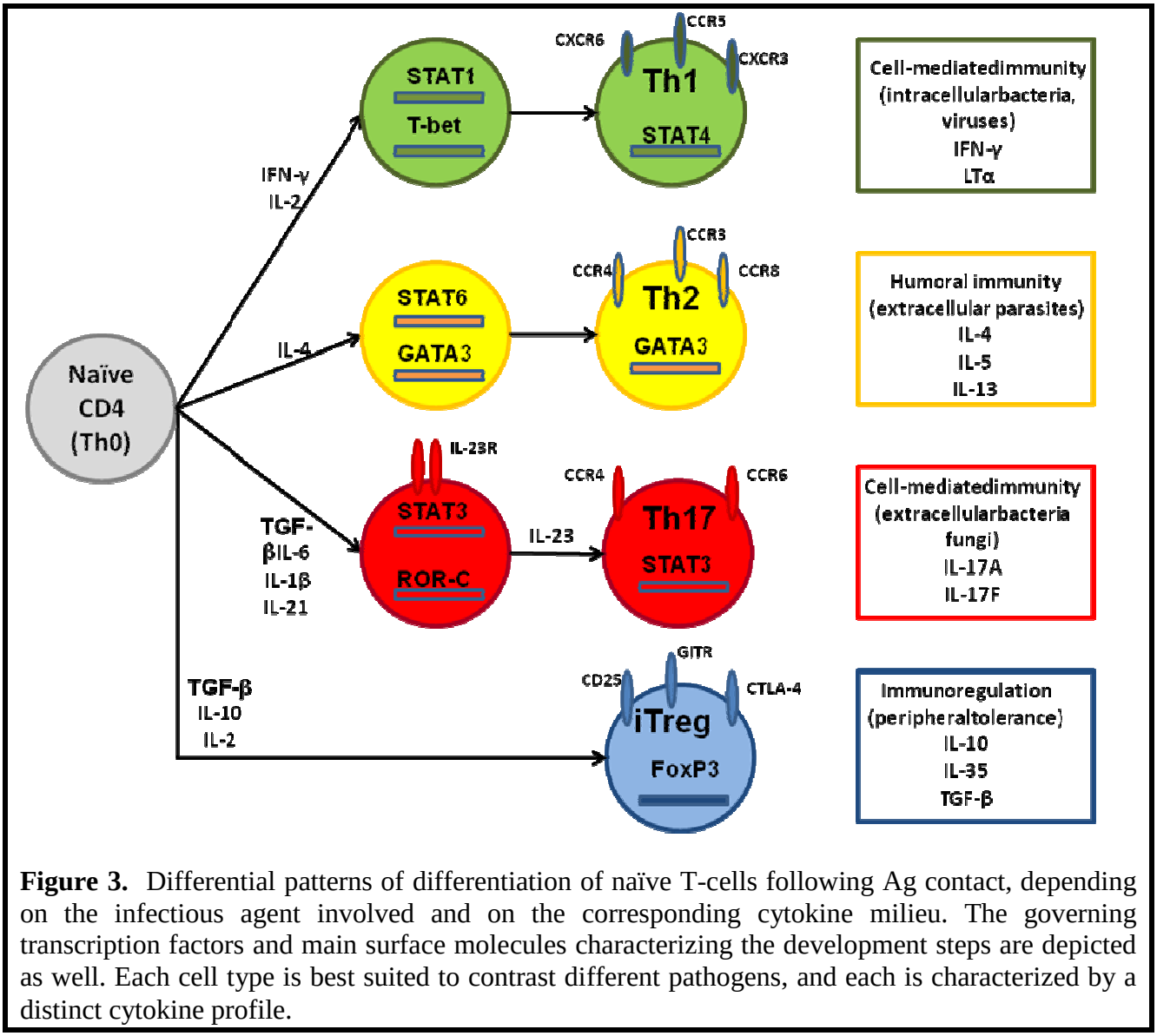


Figure 3. Differential patterns of differentiation of naïve T-cells following Ag contact, depending on the infectious agent involved and on the corresponding cytokine milieu. The governing transcription factors and main surface molecules characterizing the development steps are depicted as well. Each cell type is best suited to contrast different pathogens, and each is characterized by a distinct cytokine profile.

In the case of Th17, IL-6 produced by dendritic cells (and perhaps other cells in the local environment) acts in concert with active TGF-β to direct Th17 differentiation. Conversely, in the absence of IL-6, TGF-β promotes the differentiation of Tregs.

Tregs are defined by their master transcription factor, FOXP3, which is both necessary and sufficient to program Tregs development and maintenance. In mice, Th17 cells are defined by their own master transcription factor, a T-cell isoform of the retinoic acid-related orphan receptor γ, RORγt (166). The corresponding transcription factor in human is called ROR-C. Briefly, in vitro naïve T-cells stimulated with TGF-β alone upregulate both FOXP3 and RORγt, but they do not to express an appreciable level of IL-17 and progressively extinguish RORγt expression, as they differentiated into Tregs(167, 168). Conversely, early Th17 in vitro differentiation induced by TGF-β and IL-6 is accompanied by transient co-expression of RORγt and FOXP3, with FOXP3 extinguished as Th17 development progresses. In humans, other pro-inflammatory cytokines like IL-21 are necessary to suppress ROR-C expression. IL-23 is subsequently necessary to allow Th17 complete differentiation (169).

It seems therefore that FOXP3 and RORγt engage sort of antagonistic competition, whose outcome determines whether a cell would differentiate as a Treg or a Th17 cell. Figure 4 better details the reciprocal patterns of Th17 and Tregs differentiation

Figure 4. Alternative pathways of Treg and Th17 differentiation

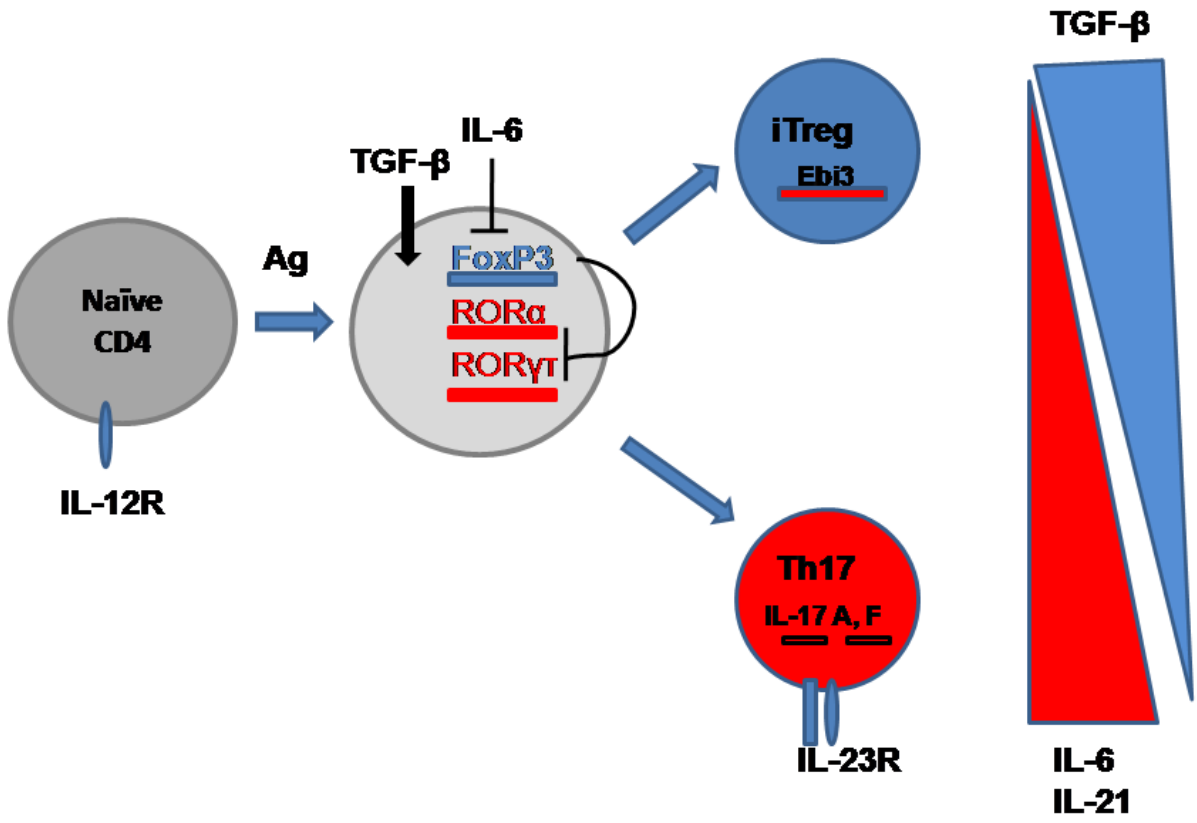


Figure4: In vitro model dissecting the alternative pathways of Treg and Th17 differentiation. Under the influence of high levels of TGF-β, FOXP3 inhibits the transcriptional activity of ROR transcription factors (RORα or RORγt both described in mice) by direct binding via FOXP3 exon 2 that posses a LQALL motif (similar to the LxxLL motif of other ROR co-activators and repressors). At lower doses, TGF-β cooperates with signals initiated by IL-6 (as well as by IL-21 and IL-23, which like IL-6 are all STAT3-activating cytokines) to overcome Foxp3-mediated repression of ROR genes. Th17-promoting cytokines may act through STAT3-dependent pathways to reverse the Foxp3-mediated repression of ROR genes. The downregulation of FOXP3 by STAT3 activation is takes place even in mice deficient for ROR transcription factors and seems therefore to be independent of RORα or RORγt.

4.3 REGULATORY T-CELLS IN RA

Data from literature concerning count and function of Tregs in RA are quite conflicting. One study reported higher number of CD4+CD25^{high} Tregs in the peripheral blood of RA patients (170) another recent study reported lower percentages of peripheral blood Tregs in RA vs. controls (171), while other studies did not find this difference (172-174). Tregs showing normal suppressive function in vitro have been identified in synovial fluid of RA patients (170, 173, 175). As far as suppressive activity is concerned, the studies are contradictory as well. Several papers reported no difference in suppressive capacity of Tregs in RA vs. healthy subjects (170, 171, 173), while one group repeatedly and consistently documented a defective suppressive activity of Tregs in RA (39, 172, 174) (see after). Tregs in RA display reduced CTLA-4 expression. Increasing CTLA-4 expression with phorbol ester treatment in vitro can restore suppressive activity on Th1 cells (174). These divergent results could reflect differences in the populations of patients, in the methods used to purify Tregs or in the way to perform the suppression assays. Moreover, activated Teff cells in the inflammatory milieu that characterizes RA, may become resistant to suppression, which could further perturb the results. Another key point is whether the defects in Tregs function reported in RA patients are directly involved in the pathophysiological process or are only consequences of the disease chronic inflammation.

4.4 CURRENT RA THERAPIES AND REGULATORY T-CELLS

Even if they were not originally designed as Treg-targeted treatments, research has shown that the majority of currently used biological treatments for RA might involve some level of Treg modulation.

4.4.1 Anti-TNF agents

Anti-TNF-a treatments constitute the first class of treatments developed to target a specific pathogenic molecule in RA. The simplest rational underlying the efficacy of this class of

therapeutics is that blockade of the early and highly pro-inflammatory cytokine blocks downstream TNF-dependent pathways dampening the immune response thereby controlling articular and systemic inflammation. However, evidence exists that anti-TNF drugs may exert at least part of their therapeutic effect via Tregs.

It has been shown that high concentrations of TNF-α can block the immunosuppressive functions of Tregs in vitro (176). The group of Ehrenstein first demonstrated that treatment of RA patients with infliximab resulted in increased peripheral count of CD25 ^{high} FOXP3+ cells, cells that were absent in non responders or in responders to MTX, which suggests that the presence of these cells was associated to, and maybe partially involved in successful TNF-α blockade. Further work of the same group showed that these cells corresponded to induced Tregs (iTregs) expressing low levels of L-selectin (CD62L) and that these cells displayed TGF-β and IL-10 dependent suppressive activity cytokine production by effector T-cells (39). Thus, successful infliximab treatment reversed the previously discussed defect in suppression of cytokine production that characterizes RA patients by inducing a new Treg population (iTregs). Conversely, the pre-existing natural Tregs, characterized by high expression of CD62L remained defective in their suppressive activity despite TNF-α blockade (39).

Adaptive or inducible Tregs (iTreg) have been classically described to arise in the periphery from naïve CD4+ CD25- cells after exposure to low doses of antigen (177). In vitro studies showed that naive T -cells could be directly converted to FOXP3+ expressing Treg cells by incubating them with TGF-β (178). Since, in the absence of an inflammatory environment, TGF-β is a major cytokine for differentiation of naïve Tcells into Tregs, it is conceivable that TNF-α blockade results in control of inflammation and notably of downstream cytokine production (amongst which, IL-6) allowing the action of TGF-β to induce naïve Tcells differentiation into Tregs and preventing differentiation into Th17 that would take place in the presence of TGF-β and pro-inflammatory cytokines.

In the work of Ehrenstein and coworkers, the addition of infliximab to purified CD4+CD25− T cells from active RA patients (but not from healthy controls) cells resulted both in a substantial increase in the percentage of CD4+FOXP3+ cells and in a significant increase in TGF-β production from these cells. The differentiation was TGF-β-dependent and was completely prevented by TGF-β blockade.

Moreover, iTregs suppressive activity was highly dependent on TGF-β and IL-10, dependent on cell contact, and almost abolished by blockade of both TGF-β and IL-10 in coculture studies

This reminds the compensatory mechanisms developed by Treg of CTLA-4-knockout mice (179) whose suppressive activity depends as well on both TGF-β and IL-10 (180). This is probably consistent with the observations that CTLA-4 accumulation on the surface of Tregs in patients with active RA is defective (174).

In fact, the question of whether anti-TNF treatments act on Tregs by virtue of specific TNF-α blockade, or rather because of non specific control of inflammation is still unanswered, and is directly linked to the question of whether TNF-α has a direct action on Tregs

It is known that TNFR2 is expressed on a proportion of Tregs in both mouse and humans (176, 181). Nevertheless data from literature are quite conflicting about the effect of TNF-α on Tregs.

One group showed that stimulation of the TNFR2 on Tregs from healthy subjects reduced their suppressive function, mirroring the defects of Tregs from active RA patients (176). The authors noted that both TNFR2-stimulated Tregs from healthy controls and unmanipulated Tregs from patients with active RA, showed a reduction in the level of FOXP3 expression. In longitudinal studies these defects and corresponding reduction in FOXP3 expression were reversed by infliximab treatment. Infliximab treatment concordantly decreased expression of TNF-receptor-2 and GITR on Tregs (176). Conversely, another group (181, 182) showed that TNF-α interaction with TNFR2 promotes Tregs function and expansion. Other studies failed to note any effect of TNFα on Tregs (172).

Our group studied the evolution of Tregs phenotype in a context of uncontrolled constitutive TNF-α overexpression, in the model of human-TNF-α transgenic mice. We could document that chronic exposition of Tregs to TNF-α resulted in progressively increasing expression of TNFR2. TNFR2 expression was further, but transiently, increased vs. untreated mice by infliximab or TNF-K treatment. After prolonged treatment, no difference in TNFR2 expression was detectable in treated vs. untreated mice (183). A recent paper confirmed reduced suppressive function of Treg populations in RA providing a clear-cut mechanistic explanation for TNFα action on Tregs (184). The authors showed that TNFα interaction with TNFR1 acts via the NFκB pathway to increase the expression of the protein phosphatase 1 (PP). PP1, in turn, dephosphorilates Ser 418 on FOXP3, thereby reducing its DNA-binding activity, which results in reduced suppressive function of Tregs. Adalimumab treatment restored Treg suppressive function in RA patients; this was associated with reduced PP1 expression and increased phosphorilation of FOXP3.

4.4.1.1 TNF blockade and Tregs/Th17 balance

The distinct T-helper cell subset, Th17, described both in mouse and human, is characterized by the production of the pro-inflammatory cytokine interleukin-17 (IL-17) and is governed by the specific transcription factor ROR (ROR-γt in mice and ROR-C in humans). Th17 are recognized as pivotal cells in the pathogenesis of RA and of several autoimmune diseases (185). IL-17 deficiency reduces mice experimental arthritis severity (186), while IL-17 overexpression aggravates it (187).

In RA patients, Th17 cells have been shown to be elevated in the periphery (188) and to contribute, in synergy with TNF-α and other proinflammatory cytokines, to synovial chemokine and cytokine production and to joint destruction (189). IL-17 is quite unique among the proinflammatory cytokines. In fact, in the peripheral blood of healthy individuals, a substantial proportion of memory Tregs (characterized by the phenotype FOXP3+CD45RO+) express the Th17 transcription factor ROR-C and can secrete IL-17 (190). Inflammation can impair the stability of FOXP3-expressing Tregs and exacerbate the tendency to secrete IL-17. Hence, in an appropriate inflammatory milieu, this highly proinflammatory cytokine can be produced by those FOXP3-expressing Tregs that are

supposed to control and reduce immunity and inflammation. The other unique feature of IL-17 is its high resistance to Treg cell–mediated suppression, probably due to its central role in immune response against infections, especially to *Mycobacterium tuberculosis* (191) and *Candida Spp.* (192).

Natural Tregs from healthy subjects are capable of suppressing IFN-γ production by Th1 cells but not IL-17 production by Th17 (193). Natural Tregs from RA patients with active disease lack suppressive activity on both Th1 and Th17, even after successful infliximab treatment (39).

Conversely, infliximab treatment induced the emergence of a novel population of induced Treg cells (iTregs) lacking the homing molecules CD62L and CCR7 and capable of inhibiting Th1 cytokines production (mainly IFN-γ) in a mechanism depending on both IL-10 and TGF-beta. More recently, the same group showed the emergence, in RA patients responding to adalimumab treatment, of a population of iTregs CD62L- (probably the same population as that induced by infliximab) capable to effectively suppress IL-17 production (40). This suppression does not require cell contact, is independent from TGF-β and IL-10 and seems to rely on inhibition of IL-6 secretion by monocytes (40). Interestingly, this population of iTregs was absent in patients with active RA, in non-responders to adalimumab and, more interestingly, even in patients responding to etanercept. These results suggest that monoclonal anti-TNF-α antibodies and etanercept might act through different cellular mechanisms despite the fact of sharing the same molecular target. Importantly, given the role of IL-17 in host defense against infection, blockade of IL-17 pathways by monoclonal anti-TNF-α Ab might justify the higher risk of Tuberculosis reactivation on monoclonal Ab treatment vs. etanercept (102).

4.4.2 Anti-IL-6 therapy

The only currently marketed IL-6 targeting treatment is tocillizumab, a humanized anti-interleukin-6 receptor monoclonal antibody. IL-6 exerts pleiotropic effects on numerous cells of the immune

system; it acts in concert with active TGF-β to direct Th17 differentiation, and directly attenuates the suppressive function of Tregs (194), therefore controlling the balance between regulation and inflammation. In mice, TGF-β induces the expression of FOXP3. In the absence of pro-inflammatory cytokines FOXP3 inhibits RORγt activation favoring Tregs differentiation (195). Conversely, in the presence of IL-6, FOXP3-mediated suppression of RORγt is abrogated, with predominant induction of Th17 cells (167). Th17/Tregs differentiation in humans is probably more complex than in mice, for example IL-6 and TGF-β are insufficient for Th17 induction (196-198). Hence, one possibility is that the efficacy of IL-6 targeting might rely on suppression of Th17 induction, favoring the emergence of adaptive Tregs.

In a recent paper Samson *et al*. (171) showed that tocillizumab treatment induced a significant decrease in disease activity associated with a significant decrease in the percentage of Th17 cells (from a median of 0.9% to 0.45%; P = 0.009) and an increase in the percentage of Treg cells (from a median of 3.05% to 3.94%; P = 0.0039). Our group studied the modification of Treg/Th17 balance on IL-6 blockade both in collagen-induced arthritis and in RA patients. We could document that in mice MR16-1 treatment (an anti-mouse IL-6R monoclonal Ab) changes the balance in favor of Tregs, and that these Tregs show an increased expression of CD39, an ectonucleotidase that hydrolyzes ATP, with documented suppressive activity on Th17 cells (199). Similarly, patients responding to tocillizumab treatment at 12 weeks had higher CD39+ Tregs counts vs. controls (200). CD39+ Tregs were first described as a subset of natural Tregs capable of suppressing Th17 cells (199). The mechanism of suppression by CD39+ Treg cells appears to require cell contact, and can be duplicated by adenosine, which is produced from ATP by the ectonucleotidases. Lower frequency and suppressive activity of CD39+Tregs has been documented in the peripheral blood of patients with frequently relapsing multiple sclerosis (201).

4.4.3 CTLA-4-Ig

CTLA-4 is a membrane-bound protein whose expression is constitutive on Tregs (202). CTLA-4 is conversely inducible on conventional T-cells that upregulate CTLA-4 following activation. On both cell types, CTLA-4 competes with its homologue CD28 for binding of their shared ligands CD80 and CD86 (B7-1 and B7-2) on antigen presenting cells (APCs). The main difference between the two cell types is that, on Treg CTLA-4 ligation leads to augmentation of function, while on conventional T cells ligation inhibits function (203). CTLA-4 can transduce reverse signals via CD80/CD86 to down-modulate the APC to become tolerogenic (204).

Abatacept, a soluble fusion protein between CTLA- and an IgG1 immunologlobulin (CTLA-4-Ig) is a treatment for RA, developed with the initial rationale that binding of CTLA-4 to the co-stimulatory molecules CD80/86 would hamper conventional T-cells activation by preventing second signaling from APCs. But abatacept might even directly convert CD4+ CD25- cells to Tregs in a TGF-β dependent manner. Alternatively, it could indirectly favor Tregs prevalence by inducing the production of indoleamine-2, 3 deaminase (IDO) by reverse B7 signaling on dendritic cells (DCs), thereby inducing tolerogenic DCs, that in turn would activate Tregs (205).

In mouse collagen-induced arthritis (CIA) model, treatment with CTLA-4-Ig was associated with an increased proportion of FOXP3+ Tregs. This finding, which could be replicated by the transfer of DCs pre-treated with CTLA-4-Ig, demonstrated the importance of tolerogenic DCs in this process. It is to note that it was possible to generate tolerogenic DCs despite the fact that DCs had been harvested from the inflammatory environment of active CIA, suggesting that this CTLA-4-based approach has the potential to overcome the pro-inflammatory environment (206). The authors could not determine whether the increase in Tregs was due to induction of a new Treg population or expansion of an existing one. Subsequent studies suggested that both scenarios are possible and may act synergistically (205, 207).

4.5 CONCLUSIONS

Regulatory T-cells are major actors in immune regulation, and alterations in their frequency and function have been documented in RA and other immune diseases. Successful treatment of RA with multiple-target currently used biologics has been associated with restored immune regulation, corresponding to increased frequency and/or function of regulatory cells.

The main still partially unanswered questions are:

1) How specific pathogenic processes in RA do affect Tregs function, i.e. whether altered Tregs functions exist that are specific to RA and to it(s) auto-antigen(s), or rather if the described defects result from chronic inflammation and immune activation.
2) To better define whether (and how) the pro-inflammatory cytokines and other molecules with known pathophysiological role in RA have a direct action on Tregs.
3) Whether the restored suppressive capacity observed on treatment is really part of the mechanism of disease control or is rather an associate phenomenon consequent to disease control.

Giving an answer to these questions is critical in order to define whether a directly Treg-based therapeutic approach in RA could be a valuable one.

OBJECTIVES

OBJECTIVES

Our group first developed the proof of concept of active anti-TNF immunization, in the model of transgenic mouse for human TNFα (TTG). TTG mouse develops a spontaneous, progressive, chronic arthritis from the age of 8-10 weeks that depends on uncontrolled production of hTNF. Immunization with the kinoid of TNF (TNF-K) (an heterocomplex formed by inactivated hTNF molecules conjugated to a carrier protein, the keyhole lympet hemocyanin KLH) allowed tolerance rupture and induced the production, by the immunized host, of polyclonal anti-hTNF Abs, endowed with neutralizing biological activity. Importantly, the rupture of tolerance was restricted to quiescent autoreactive B-cells, while no TNF-restricted T-cell response was elicited by TNF-K immunization.

Our work continued this stream of research and attempted to deepen the comprehension of the mechanisms of action of TNF-K, and to answer to relevant questions in order to substantiate active immunization as potential treatment tool in human disease, notably, rheumatoid arthritis.
The key objectives we dealt with in our research pathway were:

1) To demonstrate that TNF-K does not only exert a preventive effect, but is also curative on active established disease (i.e. it exerts therapeutic effect in mice with clinically developed arthritis). Another major point was the manageability of the treatment: the therapeutic effect needed to be reversible and re-inducible if necessary. Moreover, endogenous production of TNF should not induce anti-TNF Ab response by the host. **(ARTICLE 1)**

2) To determine whether the efficiency of TNF-K at inducing anti-hTNF Abs is impaired by immunomodulators commonly used as background treatment in RA patients on biologics, like MTX or corticosteroids. **(ARTICLE 2)**

3) To study the consequences of TNF-K treatment on the populations of regulatory T-cells and to compare them to those induced by treatment with monoclonal anti-TNF Abs infliximab. **(ARTICLE 3)**

4) To determine whether the efficacy of TNF-K treatment depends on the titers of anti-TNF Abs induced in individual mice. We also studied whether the association of a short course of fast-acting infliximab treatment to TNF-K before the raise of immunization-induced Ab polyclonal response, would result in more favorable structural evolution. **(ARTICLE 4)**

RESULTS

Article 1

Active immunization to tumor necrosis factor-alpha is effective in treating chronic established inflammatory disease: a long-term study in a transgenic model of arthritis.

Delavallée L, Semerano L, Assier E, Vogel G, Vuagniaux G, Laborie M, Zagury D, Bessis N, Boissier MC.

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In previous work our group had demonstrated the feasibility of active anti-TNF immunization in the model of human TNFalpha (hTNFα) transgenic mouse (TTG). TTG mice constitutively express hTNFα and develop spontaneous chronic progressive arthritis governed by the secretion of this major proinflammatory cytokine. Immunization of TTG mice with the kinoid of hTNFα (TNF-K) causes rupture of tolerance vs. hTNFα (an autoantigen for TTG mice) and the production of neutralizing polyclonal anti-hTNFα Abs. Immunization of TTG mice before the age of arthritis development (i.e. 8 weeks) significantly delays arthritis onset (of about 9 weeks) and reduces its severity. The transfer of immune sera form TNF-K immunized mice to naïve C57BL/6 recipient mice protected them from TNFα-galactosamine induced shock. Importantly, no cell-mediated immune response was detectable to self hTNFα, whilst a positive T-cell response was detected to KLH, the carrier protein (a xenoantigen for TTG mice)(136).

In the present paper we used the TTG mouse model to deal with crucial points to better define the efficacy and safety profile of TNF-K for the sake of potential development in human disease, notably rheumatoid arthritis. The objectives of our work were:

1) To demonstrate therapeutic effectiveness of TNF-K on TTG mice with full blown arthritis
2) To compare TNF-K effectiveness to that of a standard anti-TNF treatment (infliximab) with recognized therapeutic effect in the model.
3) To demonstrate that the clinical effect of TNF-K is time-limited and renewable on demand following a recall dose (boost) of TNF-K.
4) To demonstrate that challenging of immunized animals with native hTNFα cytokine does not elicit any anti-TNF response (i.e. absence of B-memory response to TNF).

To answer these questions we immunized TTG mice at the age of 15 weeks, after arthritis onset (three injections at weeks 15 16 and 19, respectively). We used phosphate-buffer (PBS) (administered on the same schedule) as negative control. Positive control mice were injected weekly infliximab during 12 weeks (from week 15 to week 27).

At twelve weeks after the primo injection (at the age of 27 weeks) PBS mice had developed clinically severe arthritis, with important inflammation and articular destruction at histology. Conversely, TNF-K-treated mice showed dramatically significant amelioration of arthritis at both clinical and histological analysis. These data confirm that TNF-K exerts therapeutic effect on clinically full blown arthritis, a necessary requisite to conceive its use in human disease.

In order to compare the long-term of TNF-K vs. infliximab, clinical observation was prolonged up to 45 weeks of age for infliximab-treated and a group of TNF-K-treated mice (total clinical observation duration: 30 weeks after primo injections). No significant difference was detectable in terms of clinical scores. Conversely, three injections of TNF-K at the beginning of the experiment resulted in less histological inflammation and destruction vs. a 12-week infliximab treatment, suggesting a more durable effect of TNF-K vs. infliximab. These results show that in TTG model the efficacy of three injections of TNF-K from week 0 to 5 is at least equal to that of a reference anti-TNF treatment.

During the 30-week follow-up of TNF-K-treated mice with serial blood draws, we observed that anti-hTNF Ab titers followed a bell-shaped curve with a peak at week 13 after primo injection and a subsequent decline. In parallel, we observed a worsening of clinical scores of arthritis, detectable from around week 23 after primo injections. Taken together, these data show that anti-TNF immune response is time-limited and that the decrease in anti-TNF Ab titers is followed by clinical worsening of arthritis.

To study whether anti-TNF Ab response is renewable, a group of TNF-K treated mice received a boost dose of TNF-K at week 23 after primo injection (when Ab titers were significantly lower vs. the peak). The boost dose elicited a novel raise in anti-TNF Ab titers. The raise in anti-TNF Abs was followed by amelioration of arthritis clinical scores at time-trend analysis in TNF-K boost-receiving mice. These results confirm that the time limited immune response and clinical effect of TNF-K immunization can both be renewed by a late boost dose of TNF-K.

A major safety concern for anti-cytokine immunization is the fear of potential sensitization of the host against the native cytokine (i.e. the induction of B-memory anti-cytokine response). To demonstrate the absence of B-memory anti-TNF response in TNF-K immunized mice, we challenged the mice with different doses of hTNF. hTNF was administered at week 23 after immunization, when anti-TNF Abs had significantly decreased vs. the peak. Doses of 10 to 100 ng of hTNF were not able to elicit any detectable anti-TNF Ab response in treated mice. Conversely, challenging of mice with KLH (the carrier protein in the TNF-K heterocomplex), elicited a raise in anti-KLH Abs in all treated mice.

These data confirm that TNF-K immunized mice are unable to mount any anti-hTNF response following contact with the native cytokine, whilst, conversely, a prompt Ab response takes place after challenge with antigens of the carrier protein. These results are consistent with previous ones that had shown that T-cell sensitization in TNF-K immunized mice is restricted to KLH antigens and does not involve hTNF.

Research article

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Active immunization to tumor necrosis factor-α is effective in treating chronic established inflammatory disease: a long-term study in a transgenic model of arthritis

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Abstract

Introduction Passive blockade of tumor necrosis factor-alpha (TNF-α) has demonstrated high therapeutic efficiency in chronic inflammatory diseases, such as rheumatoid arthritis, although some concerns remain such as occurrence of resistance and high cost. These limitations prompted investigations of an alternative strategy to target TNF-α. This study sought to demonstrate a long-lasting therapeutic effect on established arthritis of an active immunotherapy to human (h) TNF-α and to evaluate the long-term consequences of an endogenous anti-TNF-α response.

Methods hTNF-α transgenic mice, which spontaneously develop arthritides from 8 weeks of age, were immunized with a heterocomplex (TNF kinoid, or TNF-K) composed of hTNF-α and keyhole limpet hemocyanin after disease onset. We evaluated arthritides by clinical and histological assessment, and titers of neutralizing anti-hTNF-α antibody by enzyme-linked immunosorbent assay and L929 assay.

Results Arthritides were dramatically improved compared to control mice at week 27. TNF-K-treated mice exhibited high levels of neutralizing anti-hTNF-α antibodies. Between weeks 27 and 45, all immunized mice exhibited symptoms of clinical deterioration and a parallel decrease in anti-hTNF-α neutralizing antibodies. A maintenance dose of TNF-K reversed the clinical deterioration and increased the anti-hTNF-α antibody titer. At 45 weeks, TNF-K long-term efficacy was confirmed by low clinical and mild histological scores for the TNF-K-treated mice. Injections of unmodified hTNF-α did not induce a recall response to hTNF-α in TNF-K immunized mice.

Conclusions Anti-TNF-α immunotherapy with TNF-K has a sustained but reversible therapeutic efficacy in an established disease model, supporting the potential suitability of this approach in treating human disease.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease with an estimated prevalence of about 0.5% in the adult population. This disease, characterized by synovial membrane hyperplasia and immune cell infiltration, affects multiple peripheral joints and leads to destruction of bone and cartilage, inducing pain and disability. Although its precise etiology is still unknown, the pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-17, and more recently IL-23, have been shown to be critical mediators in the inflammatory process [1]. It has also been demonstrated that TNF-α mediates a wide variety of effector functions in RA, including the release of pro-inflammatory cytokines and chemokines, leukocyte accumulation, angiogenesis, and the

ANOVA: analysis of variance; CI: confidence interval; ELISA: enzyme-linked immunosorbent assay; hTNF-α: human tumor necrosis factor-alpha; IL: interleukin; IM: intramuscular; IP: intraperitoneal; KLH: keyhole limpet hemocyanin; mAb: monoclonal antibody; OD: optical density; PBS: phosphate-buffered saline; RA: rheumatoid arthritis; TNF-α: tumor necrosis factor-alpha; TNF-K: tumor necrosis factor kinoid; TTg: human tumor necrosis factor-alpha transgenic.

activation of endothelial cells, chondrocytes, and osteoclasts [2,3]. Based on the pivotal role of TNF-α in the pathogenesis of RA [4], two classes of biologic drugs to block this cytokine have been developed: a soluble TNF-α receptor (etanercept) and TNF-binding monoclonal antibodies (mAbs) such as infliximab, adalimumab, golimumab, or certolizumab [5,6]. Although they show a rapid and substantial therapeutic benefit in most patients, with a good safety profile, primary unresponsiveness and secondary escape phenomena are not uncommon [7]. Nonetheless, the tremendous success of TNF-α blockade by mAbs has sparked interest in developing alternative strategies for antagonizing TNF-α, such as gene therapy by electrotransfer [8], short interfering RNA [9], or active anti-TNF-α immunotherapy [10-13].

Active immunotherapy is based on the established principles of vaccination. The aim of such a strategy is to use immunization with a protein compound to generate high titers of neutralizing antibodies to a given antigen, which can be either a self-protein or an environmental non-infectious agent. Therapeutic immunization has produced promising results in several fields, and in the case of active immunotherapy against cytokines (AIC), the choice of the target cytokine is informed by the long-term experience with mAbs, receptors, or antagonists in inflammatory and autoimmune diseases [2]. Over the last decade, several active anti-TNF-α immunotherapies using mTNF-α derivates as the immunogen have been developed and tested in murine experimental models of RA [10,11,13].

More recently, with the aim of addressing diseases mediated by human TNF-α (hTNF-α), we developed an anti-hTNF-α compound called TNF kinoid (TNF-K), which is composed of biologicaly inactive but immunogenic hTNF-α conjugated to a carrier, keyhole limpet hemocyanin (KLH). We have tested TNF-K in hTNF-α transgenic (TTg) mice, which overexpress hTNF-α and develop an erosive polyarthritis that shares many features with RA [14,15]. This model is the only relevant model since anti-TNF antibodies generated by TNF-K target hTNF-α. Previously, we have shown that a prophylactic anti-hTNF-α immunization protected TTg mice OK from developing arthritis [12,16]. To determine the potency of this compound against established arthritis, we immunized TTg mice after the onset of arthritis. We studied the animals for a long time period to evaluate the duration of the potential disease-modulating activity of TNF-K. We showed that TNF-K immunization is efficacious against established arthritis and induces a transient TNF blockade with reversible effects on arthritis in TTg mice.

Materials and methods

Animals

Six- to nine-week-old male hemizygous TTg mice (1006-T) were purchased from Taconic Farms (Germantown, NY, USA) [14]. These mice are similar to Tg197 mice and develop a spontaneous arthritis at from 8 to 10 weeks of age [15]. All procedures were approved by the Animal Care and Use Committee of the University of Paris 13.

Reagents

We obtained hTNF-α kinoid (TNF-K), a protein complex of hTNF-α and KLH, as previously described [16]. Dulbecco's phosphate-buffered saline (PBS) was purchased from Eurobio (Les Ulis, France). ISA-51 adjuvant was obtained from Seppic (Paris, France).

Therapeutic and long-term effect of TNF-K active immunization

All treatments were started after the onset of arthritis, when TTg mice reached an average clinical score of 3 out of 12. The experimental protocol was as follows (Additional file 1). The control group consisted of eight mice treated with PBS emulsified in ISA-51 adjuvant (PBS group) at 15, 16, and 19 weeks of age. This group was followed for 12 weeks and then euthanized for ethical reasons. A group of 23 TTg mice received three primary intramuscular (IM) injections of TNF-K (4 μg) emulsified in ISA-51 (TNF-K group) at 15, 16, and 19 weeks of age. They were then randomly subdivided into two subgroups of eight and one subgroup of seven TTg mice. The first eight mice were euthanized at 27 weeks of age to compare the TNF-K immunized group with controls. At 32 weeks of age, the subgroup of seven mice received a maintenance dose of TNF-K emulsified in ISA-51 adjuvant, whereas the second subgroup of eight mice received, as a control, an injection of PBS emulsified in ISA-51 at the same time; both were followed until 45 weeks of age. In parallel, another group of eight mice was given weekly intraperitoneal (IP) injections of infliximab (1 mg/kg) from week 15 to week 27. At this time, infliximab was discontinued.

Antibody assay

From blood samples collected at different time points during the experiment and at sacrifice, sera were obtained and tested for anti-KLH and anti-TNF-α antibody titers and for anti-TNF-α antibody neutralizing capacity. Specific anti-hTNF-α and anti-KLH antibody titers were determined using direct enzyme-linked immunosorbent assay (ELISA) [12]. Precoated ELISA plates with 100 ng per well hTNF-α or KLH were incubated with serial dilutions of sera from immunized and control mice. Specific IgGs were detected by using horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed Laboratories Inc., now part of Invitrogen Corporation, Carlsbad, CA, USA). The optical density (OD) was measured at 490 nm for each well.

The neutralizing capacity was assessed by using the L929 cytotoxicity assay, reflecting neutralizing antibodies [12]. Briefly, mouse fibroblast L929 cell line (CCL 1) (American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cells were seeded in flat-bottomed 96-well

plates and grown to 95% confluence. After 21 hours of incubation at 37°C, serial dilution of serum with a 100% toxic hTNF-α dose was added on L929 cells with 1 μg/mL of actinomycin D. After 20 hours of incubation at 37°C, the medium was removed and replaced with MTS/PMS during 4 hours at 37°C. The OD at 490 nm was measured for each well. The neutralization titer was expressed as the reciprocal of the serum dilution that neutralizes 50% of hTNFα activity.

Evaluation of B-memory response after TNF-K immunization

Thirty-six TTg mice received three IM injections of TNF-K emulsified in ISA-51 adjuvant at 7, 8, and 11 weeks of age. They were then randomly subdivided in two subgroups of ten and two subgroups of eight TTg mice. Neutralizing anti-hTNF-α antibody titers were monitored every month. When a decrease of 50% of the neutralizing capacity of these antibodies was observed, mice were intraperitoneally injected with native hTNF-α (10 ng), native hTNF-α (100 ng), KLH (10 μg), or PBS (equivalent volume) 24 weeks after the primary injection. Four weeks later, these mice received IM injections of the same compound with the same doses. The mice were further followed for 10 weeks. The native hTNF-α doses were chosen based on previous results we obtained in a TNF-α-dependent lethal shock experiment, in which we showed that 1 μg of native hTNF-α injections in TTg mice sensibilized with D-galactosamine was enough to kill the mice [12].

Clinical and histological assessments

Blinded weekly monitoring of body weight and arthritis scores in all four limbs was started from the reception of the animals (9 weeks of age). Clinical severity of arthritis for each paw (fingers, tarsus, and ankle) was quantified by attributing a score ranging from 0 to 3: 0, normal; 1, slight redness and swelling; 2, pronounced edematous swelling of the entire foot; 3, joint deformity and rigidity [12]. The scores of each paw were summed, resulting in an arthritis score ranging from 0 to 12. The mean arthritis score on each clinical observation day was calculated for each treatment group.

For histological assessment of arthritis, all animals were sacrificed after 18-week or 36-week follow-up. Left forelimbs and right hind paws were collected, fixed with formol, decalcified, dehydrated, and included in paraffin blocks. Slides of 5 μm in thickness were made using a microtome. At least four serial sections were realized for each paw in order to obtain a reliable spatial evaluation of articular hints. Slides were then stained with hematoxylin and eosin or with safranin-O before microscopic observation (optical microscope). Synovitis and bone erosions were defined on slides stained with hematoxylin and eosin. Lesions were evaluated quantitatively on each slide using a 3-point scale ranging from 0 to 3, where 0 = normal articulation; 1 = slight inflammation and thickening of the synovium; 2 = mild thickening of the synovium and mild inflammation with invasion of the subsynovial area by inflammatory cells; 3 = severe inflammation and massive invasion of adjacent tissues by pannus [17]. Other sections were scored for loss of safranin-O staining as a measure of cartilage proteoglycan depletion using a scale from 0 to 3, where 0 = no depletion; 1 = depletion of staining and thinning down of the lateral superficial layer; 2 = depletion of staining and thinning down of the central superficial layer; 3 = severe and mostly complete depletion of staining in the superficial layer [18].

Statistical analysis

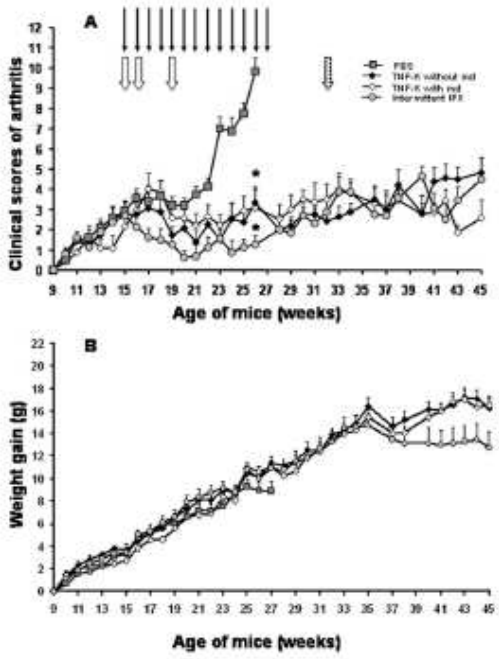
Data distribution was preliminarily checked by the Kolmogorov-Smirnov test. Serial measurements of clinical scores, body weight, antibody titers, and antibody neutralizing capacity were analyzed considering the area under the curve for each subject as a summary measure; these measures were then analyzed as raw data [19]. According to data distribution and number of groups, a parametric (analysis of variance [ANOVA], *t* test) or non-parametric (Kruskal-Wallis, Mann-Whitney) test was then performed. *Post hoc* comparisons were performed with the appropriate test according to data distribution (Student-Newman-Keuls for parametric data and Dunn test for non-parametric data). Clinical score time trend was analyzed by Spearman rho, and 95% confidence intervals (CIs) were given. Histological scores were compared with ANOVA or Kruskal-Wallis and their appropriate *post hoc* analysis according to data distribution. Differences in antibody titer at different time points were analyzed with repeated measures ANOVA due to normal distribution of data. Incidences of arthritis were compared using Fisher exact test with Yates correction. All statistics were performed with MedCalc statistical software version 10.4.8 (MedCalc Software bvba, Mariakerke, Belgium).

Results

Effect of TNF-K immunization in TTg mice on established arthritis

We investigated the potency of anti-hTNF-α immunization against established arthritis. To address this question, TTg mice, which develop spontaneous arthritis at around 8 to 10 weeks of age, were monitored for any signs of clinical arthritis from 9 weeks of age. When the mice exhibited an average clinical score of 3 (scoring range from 0 to 12; see Materials and methods), treatments were started for all of the mice. The control group (eight mice) was injected with PBS emulsified with ISA-51 adjuvant (PBS group) at 15, 16, and 19 weeks of age and developed severe arthritis over a 12-week period. At 27 weeks of age, these mice were euthanized for ethical reasons (Figure 1a). Compared with the control group, TNF-K immunized mice, receiving injections following the same time schedule, showed a dramatic improvement of the disease after immunization ($P < 0.05$ versus control group) (Figure 1a), demonstrating good efficacy of the TNF-K treatment against established arthritis. TNF-K immunized mice exhibited lower peak clinical scores and fewer inflamed paws than control animals (data not shown). The infliximab-treated group showed,

Figure 1



Clinical evaluation of human tumor necrosis factor-alpha transgenic (TTg) mice immunized with tumor necrosis factor kinoid (TNF-K) or phosphate-buffered saline (PBS) or treated with infliximab (IFX). TTg mice were immunized with TNF-K or PBS emulsified in ISA-51 adjuvant or were IFX-treated. All mice were monitored for clinical signs of arthritis and for weight for 18 or 36 weeks. **(a)** TTg mice received three primary injections at 15, 16 and 19 weeks of age (open arrows) of TNF-K (n = 15, open and closed diamonds) or PBS (n = 8, squares). At 32 weeks of age (shaded arrow), TTg mice received a maintenance dose (md) of TNF-K (n = 7, open diamonds) or an injection of PBS emulsified in ISA-51 adjuvant (n = 8, closed diamonds). Eight TTg mice (circles) received weekly intraperitoneal injections of IFX (bold arrows) from week 15 for a period of 12 weeks (until 27 weeks of age). **(b)** The weight gain of all groups is represented. Results are expressed as mean ± standard error of the mean. *$P < 0.05$ versus PBS.

as expected, a significant improvement of the disease (Figure 1a), with lower scores than the PBS group ($P < 0.05$ at week 27). Based on a comparison of clinical scores, the TNF-K immunized and infliximab-treated mice showed comparable efficacy, with no statistically significant differences, although the infliximab has a more rapid efficacy than TNF-K immunization. We did not observe significant differences in body weight in any studied group (Figure 1b).

We next investigated the histological efficacy of TNF-K vaccine. At 27 weeks of age, eight TNF-K immunized mice and all control animals were euthanized. We observed that the clinical assessment was corroborated by histological evaluation (Table 1). All control mice exhibited significant histological signs of arthritis, whereas all TNF-K immunized mice showed lower inflammation scores compared with the control group (Table 1 and Figure 2a, b). In regard to joint destruction, TNF-K immunized TTg mice did not exhibit any signs of cartilage damage while the control group showed extensive cartilage destruction ($P < 0.05$) (Table 1). We did not evaluate the histological efficacy of infliximab on TTg mice at 27 weeks of age. For histological arthritis, we observed specific diffusion and pale proteoglycan coloration by safranin-O, reflecting cartilage degradation for control PBS mice in comparison with TNF-K-treated animals (Figure 2c, d).

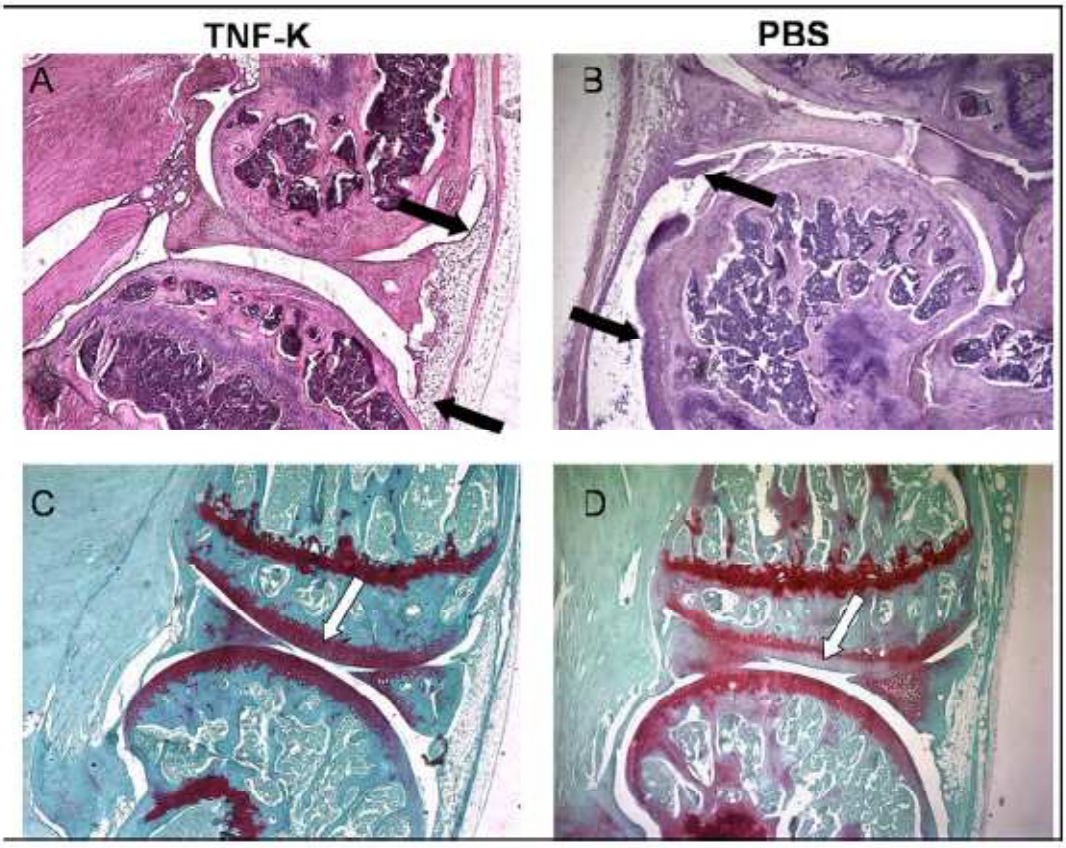
Reversibility of TNF-α blockade

As TNF-K treatment is able to improve established arthritis based on 12-week follow-up, we investigated the duration of its disease-modulating activity over a longer period. To explore this, we extended by 18 weeks the study of the TNF-K immunized TTg mice for a total study duration of 30 weeks after the first immunization. We observed that, at around 23 weeks of age, arthritis clinical scores started to increase slightly with time (Figure 1a). A time-trend analysis of the clinical scores of both groups having received the primary course of three injections of TNF-K from 21 to 32 weeks of age shows a positive correlation of clinical scores with the age of mice ($\rho = 0.194$, 95% CI 0.043 to 0.337, $P < 0.05$), demonstrating the transitory effect of anti-hTNF-α immunization (Figure 3a). Furthermore, we observed that, over this period, the number of inflamed paws of TNF-K immunized mice increased compared with that of TNF-K immunized animals sacrificed at 27 weeks of age ($P < 0.05$, data not shown). Histological comparisons were then made between groups of TNF-K immunized mice sacrificed at week 27 and those at week 45. This showed a mild progression of the disease over this 18-week period, with higher inflammation and destruction scores for all of the animals in the week 45 groups (Table 1).

Effect of a maintenance dose

We next investigated whether this flare in arthritis disease could be ameliorated by the administration of a maintenance dose (late boost) of TNF-K. Therefore, seven TTg mice that had received a primary course of three injections of TNF-K were administered a maintenance dose of TNF-K at 32 weeks of age. As a control, the remaining eight TTg mice that had received the primary course were injected with PBS emulsified in ISA-51 adjuvant. The arthritis clinical score curves decreased for mice that received the maintenance dose and increased for the controls (Figure 1a). The differential in clinical scores between the two groups did not reach statistical significance, and this was due to the small sample size related to effect size. (With an alpha error of 0.05 and a beta error of 0.2, a sample size of 22 mice would have been necessary for the detected difference to be statistically significant.) Never-

Figure 2



Examples of histological evaluation of tumor necrosis factor-alpha transgenic (TTg) mice immunized with tumor necrosis factor kinoid (TNF-K) or phosphate-buffered saline (PBS). Histological sections (magnification × 40) of the knees of TNF-K- or PBS-treated mice were prepared (see Materials and methods) and colored with hematoxylin and eosin **(a, b)** to observe synovial inflammation or with safranin-O **(c, d)** to observe cartilage degradation. For the histological sections of TTg mice immunized with TNF-K, inflammation (a) and destruction (c) were scored at 0; for the control group, inflammation (b) and destruction (d) were scored at 2. Black arrows show thickness and inflammatory infiltration of synovial membrane in (b) and a normal appearance in (a). White arrows show depletion of proteoglycan (a marker for cartilage destruction) in (d) and a normal ful-red staining in (c).

theless, clinical score time-trend analysis with Spearman rho showed a reduction of the scores for maintenance-dosed mice ($\rho = -0.249$, 95% CI -0.448 to -0.026, $P < 0.05$) and a deterioration for controls ($\rho = 0.405$, 95% CI 0.214 to 0.567, $P < 0.05$), supporting the efficacy of a maintenance dose of TNF-K in treating the late flare of arthritis (Figure 3b, c).

Histological inflammation and destruction were assessed at 45 weeks of age (Table 1). All of the immunized animals exhibited mild signs of histological inflammation and destruction of ankle and knee joints. As with the clinical scores, the differences between immunized animals that received the maintenance dose and those that did not were not statistically significant (Table 1).

We also compared the clinical efficacy of TNF-K active immunization with infliximab intermittent treatment on arthritis of TTg mice over this 18-week extension period. No statistically significant difference was detected between the two treatments (Figure 1a). However, as would be expected, the clinical scores of the infliximab group deteriorated over time since treatment was withdrawn at 27 weeks of age (Figure 3d).

Table 1

Histological evaluation of arthritis in human tumor necrosis factor (TNF)-alpha transgenic mice immunized with TNF kinoid

Group	Number of mice	Inflammation score	Incidence	Destruction score	Incidence
TNF-K (3 injections, sacrifice at week 27)	8	0.1 ± 0.1[a]	2/8[b]	0.0 ± 0.0[a]	1/8[c]
TNF-K (3 injections without maintenance dose, sacrifice at week 45)	8	0.6 ± 0.2[d]	6/8	0.2 ± 0.1[d]	3/8
TNF-K (3 injections with maintenance dose, sacrifice at week 45)	7	0.5 ± 0.1[d]	7/7	0.3 ± 0.1[d]	5/7
Intermittent infliximab (sacrifice at week 45)	8	1.4 ± 0.1	8/8	0.9 ± 0.2	7/8
Phosphate-buffered saline (sacrifice at week 27)	8	1.6 ± 0.1	8/8	0.9 ± 0.2	7/8

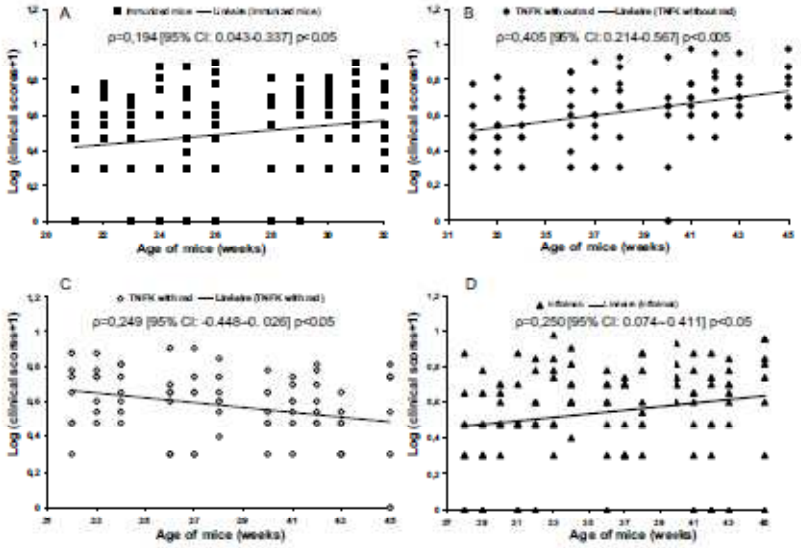
The incidence of inflammation/destruction as evaluated by histology is the number of mice with a score of inflammation/destruction of at least 0.25. Results are given as mean ± standard error of the mean. [a]$P < 0.05$ versus phosphate-buffered saline (PBS); [b]$P < 0.01$ versus PBS; [c]$P < 0.05$ versus PBS; [d]$P < 0.05$ versus infliximab. TNF-K, tumor necrosis factor kinoid.

We further examined the histology of infliximab intermittent-treated TTg mice sacrificed at 45 weeks of age. All of the mice from this group, treated with infliximab during 12 weeks, had developed severe inflammation and exhibited mild cartilage destruction of the joints 18 weeks after the infliximab withdrawal (Table 1 and Additional file 1). By comparison, TNF-K immunized animals, receiving or not receiving the maintenance dose, showed lesser inflammation and cartilage destruction compared with the infliximab group ($P < 0.05$) (Table 1).

Anti-TNFα antibodies after TNF-K immunization

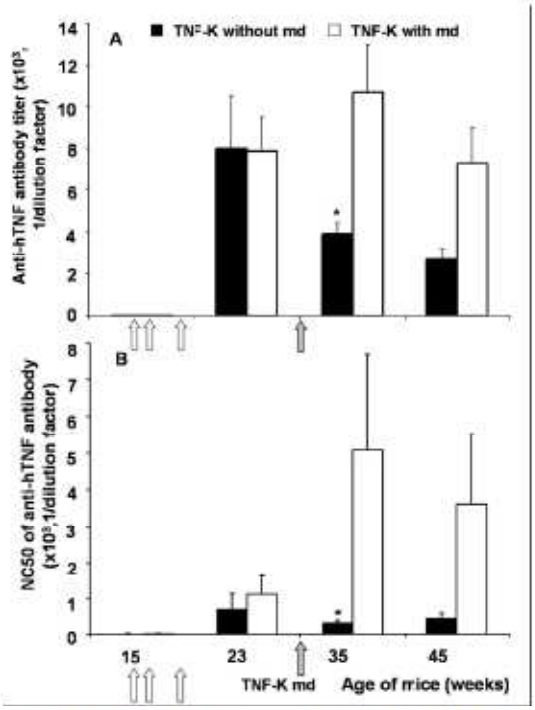
To evaluate the duration of the immune response after immunization with TNF-K in TTg mice, we assessed the titers and the neutralizing capacity of anti-hTNF-α antibodies in sera of

Figure 3



Clinical score time trend. The severity of disease evolution over time was analyzed using Spearman rank correlation. We correlated clinical scores with the age of the mice, expressed in weeks, and divided the study into two periods of time. **(a)** Correlation between week 21 and week 32 for all of the immunized mice (n = 15). We observed an aggravation of disease in all mice immunized with tumor necrosis factor kinoid (TNF-K) a couple of weeks after the last immunization. **(b)** Correlation between week 33 and week 45 for immunized mice not receiving the maintenance dose (md). We observed an aggravation of the severity of the disease. **(c)** Correlation between week 33 and week 45 for immunized mice receiving the maintenance dose. After the maintenance dose at 32 weeks of age, we observed an amelioration of the scores. **(d)** Correlation between week 28 and week 45 for infliximab-treated mice. The injections were stopped at week 27, and we observed an aggravation of the disease over time thereafter. CI, confidence interval.

Figure 4



Evaluation of anti-human tumor necrosis factor-alpha (anti-hTNF-α) antibody production in TNF-α transgenic (TTg) mice immunized with TNF kinoid (TNF-K). TTg mice were immunized at 15, 16, and 19 weeks of age (open arrows) with TNF-K. **(a)** Enzyme-linked immunosorbent assay of anti-hTNF-α antibodies. **(b)** The neutralizing capacity of the anti-hTNF-α antibody was evaluated on L929 cells and is expressed as the mean of the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity (NC50). Closed histograms represent mice that did not receive the TNF-K maintenance dose (TNF-K without md) at 32 weeks of age (shaded arrow). Open histograms represent mice that did receive it (TNF-K with md). Results are expressed as mean ± standard error of the mean. *$P < 0.05$.

TNF-K immunized TTg mice and of the PBS group. High levels of anti-hTNF-α antibodies were detected only in TNF-K immunized mice (Figure 4a). These antibodies were neutralizing as evaluated by L929 cytotoxic assay (Figure 4b). Mice receiving the maintenance dose at week 32 exhibited a significant increase in neutralizing anti-hTNF-α antibody titers as early as 3 weeks after the maintenance dose. Conversely, mice treated with PBS at week 32 showed a slow decrease in their neutralizing anti-hTNF-α antibody titers (Figure 4). At sacrifice, the neutralizing anti-hTNF-α antibody titers had decreased for both groups (Figure 4).

B-memory response against TNF-α after TNF-K immunization

We wished to evaluate the response of the immune system to native (that is, unmodified) hTNF-α after immunization with the TNF-K. We immunized TTg mice with TNF-K; once we observed a clear diminution of the neutralizing anti-hTNF-α antibody titer (Additional file 2), we injected native hTNF-α into the TNF-K immunized mice with a view to establishing whether this native hTNF-α injection induced an anti-hTNF-α response (Figure 5b, d). Control groups received injections of native KLH or PBS (Figure 5e-h). We observed that injections of native hTNF-α (10 or 100 ng) had no effect on titers of either neutralizing anti-hTNF-α antibody (Figure 5b, d) or anti-KLH antibody (Figure 5a, c). On the other hand, injections of KLH induced a dramatic increase in anti-KLH antibody titer (Figure 5e), indicating a recall response to KLH. Moreover, injection of KLH had no impact on the production of anti-hTNF-α neutralizing antibody (Figure 5f). PBS injections had no impact on the production of either anti-KLH or neutralizing anti-hTNF-α antibodies (Figure 5 g, h). Four weeks after injections by the IP route, each group of mice received IM injections of the same compound at the same dose. Anti-KLH antibody titers further increased while neutralizing anti-hTNF-α antibody titers remained stable over time (data not shown).

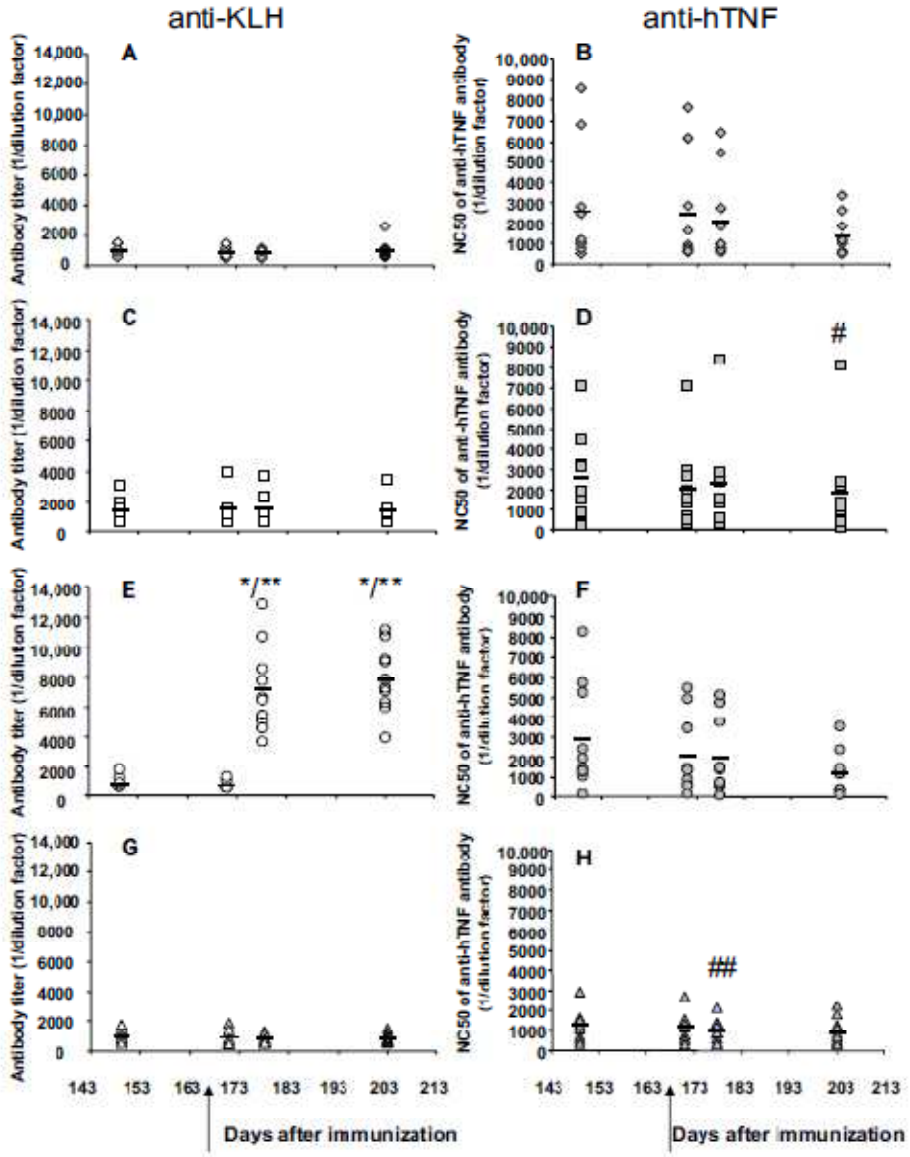
Discussion

In the present study, we show in a long-term follow-up that TNF-K immunization dramatically improves the disease status of clinically established arthritis. When the active immunization was administered after the onset of active disease, its beneficial effect, mediated by the production of a high titer of neutralizing anti-hTNF-α antibodies, was evident both in clinical symptoms and in the histological indicators for arthritis. Additionally, in these experiments, we evaluated the effect of TNF-α blockade over a long-term period and showed the long-lasting efficacy and the reversible effect of TNF-K immunization. Moreover, we present evidence that no B-cell memory response to native hTNF-α was induced by TNF-K immunization.

Active immunization has previously shown its efficacy in several experimental models of human autoimmune diseases, as well as other pathologies, using cytokines cross-linked to virus-like particles of the bacteriophage Qβ [13,20,21] or complexed with KLH (kinoids) [16,22,23]. The numerous clinical trials that have been performed or that are under way support both the feasibility and the safety of the use of active immunization against self-proteins in humans [24-27].

Major questions with our active anti-cytokine immunotherapy targeting TNF-α, a pleiotropic cytokine, are the depth and the duration of the TNF-α inhibition [2]. In contrast with the previous studies, the present one has been performed with a long-term clinical follow-up (over a 38-week period). Importantly, our present data show a decrease in anti-hTNF-α neutralizing

Figure 5



B-memory response after tumor necrosis factor kinoid (TNF-K) immunization. Thirty-six human tumor necrosis factor-alpha (hTNF-α) transgenic mice were immunized with TNF-K at 7 (day 0), 8 (day 7), and 11 (day 28) weeks of age. Bleeding was done every month from 12 weeks of age (day 35) until sacrifice. When we observed a decline of the anti-hTNF-α neutralizing antibody titer (closed symbols), we injected intraperitoneally (arrow) native hTNF-α (10 ng, n = 10, diamonds) **(a, b)**, native hTNF-α (100 ng, n = 9, squares) **(c, d)**, keyhole limpet hemocyanin (KLH) (10 μg, n = 10, circles) **(e, f)**, or phosphate-buffered saline (equivalent volume, n = 10, triangles) **(g, h)**. We studied the anti-KLH antibody titer (open symbols) and neutralizing anti-hTNF-α antibody titer (closed symbols) for 10 weeks (70 days). Each single plot represents the antibody titer of one mouse. The bold line represents the mean antibody titer at each time point. *$P < 0.001$ versus day 149; **$P < 0.0001$ versus day 171; #$P < 0.05$ versus day 178; ##$P < 0.05$ versus day 149. NC50, mean of the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity.

antibodies after a peak 8 weeks after immunization. At the same time, comparisons of histological scores of TNF-K-treated animals at week 27 and week 45 showed a slight progression over time of arthritides. These data support the hypotheses of both residual hTNF-α activity and the reversibility of the blockade of hTNF-α in vaccinated animals. Furthermore, a maintenance dose given 17 weeks after treatment initiation both increased the anti-hTNF-α neutralizing antibodies and ameliorated the course of disease, demonstrating that the immune system remains responsive to TNF-K immunization.

In the present study, we have also demonstrated the B-memory response to hTNF-α after TNF-K vaccination. When we stimulated the immune system of TNF-K immunized transgenic mice, we demonstrated that IP injection of KLH dramatically induced the production of new anti-KLH antibodies. This B-cell memory response to KLH was not accompanied by any increase of anti-hTNF-α neutralizing antibody titers. Furthermore, injections of native autoantigen hTNF-α after active immunization with TNF-K against hTNF-α did not induce the production of new neutralizing anti-hTNF-α autoantibodies, demonstrating no B-cell memory response to native hTNF-α. These data suggest that in physiopathological situations in which native hTNF-α production would be stimulated (for example, infections), it would not be thwarted by an immunization with TNF-K performed a long time before. Taken together, these data are consistent with the transient production and effect of neutralizing anti-hTNF-α antibodies after TNF-K immunization.

Finally, we demonstrated that TNF-K and infliximab have comparable efficacy measured by clinical parameters in our model. Moreover, once infliximab weekly injections were discontinued (at 27 weeks of age), infliximab-treated mice exhibited a worsening of arthritides over time following the withdrawal of infliximab. Histopathological scores of these animals were significantly higher than those of TNF-K immunized mice, with or without late maintenance dose.

Conclusions

Our data show that active immunotherapy with TNF-K induced a long-lasting improvement in an RA model. The occurrence of a disease flare in previously immunized mice, the bell-shaped neutralizing anti-hTNF-α antibody curve, the increase of anti-hTNF-α neutralizing antibodies after a maintenance dose, and the absence of evidence of *in vivo* B-cell memory response to native hTNF-α are all elements supporting a favorable benefit-risk ratio for such a strategy and a transient response against hTNF-α after TNF-K immunization. Further studies should be performed to evaluate the risk of infections or tumors under TNF-K treatment in dedicated models since their occurrences are a matter of debate in patients treated with passive immunotherapies against TNF-α [28,29].

Competing interests

GVo and ML are scientists with Neovacs SA (Paris, France), and DZ is a shareholder of Neovacs SA. TNF-K is patented and the patent is held by Neovacs SA. GVu is a scientist with Debiopharm SA (Lausanne, Switzerland). The other authors declare that they have no competing interests.

Authors' contributions

LD and M-CB shared responsibility for the study design and manuscript preparation and helped to interpret the data and to perform the animal experiments. GVo shared responsibility for the study design and helped to interpret the data. GVu and NB shared responsibility for the study design. LS shared responsibility for manuscript preparation and helped to interpret the data and to perform the statistical analysis. DZ shared responsibility for manuscript preparation. EA helped to perform the animal experiments. ML performed the ELISA and L929 cytotoxic assay. All authors read and approved the final manuscript.

Additional files

The following Additional files are available online:

Additional file 1

TNF-K immunization protocol scheme. Long-term follow -up of the experiment is represented by horizontal arrow with time expressed in week (from week 9, w9, to week 45, w45). Slashes represent discontinuation of time. A- Control group treated with PBS/ISA-51; B- TNF-K group; C- Intermittent infliximab group. The follow-up for each group (PBS, TNF-K and infliximab) is represented by a larger black line, with vertical black arrows at each time where treatment was given. IP injections, intraperitoneal injections.
See http://www.biomedcentral.com/content/supplementary/ar2897-S1.pdf

Additional file 2

Evolution of neutralizing anti-hTNF-α antibody titers during time, in TTg mice immunized with TNF-K. 36 TTg mice were immunized with TNFK at days 0, 7 and 28. Bleeding was done every month from day 38 post primo-injection to sacrifice. Results are expressed as mean ± SEM of all the sera of all the 36 immunized mice.
See http://www.biomedcentral.com/content/supplementary/ar2897-S2.pdf

Acknowledgements

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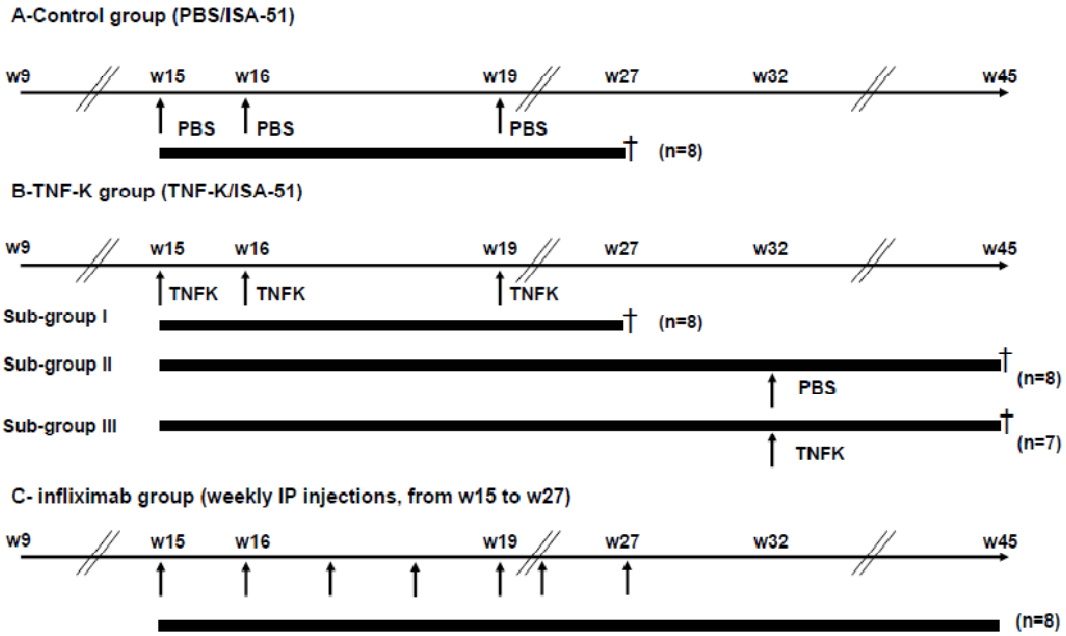
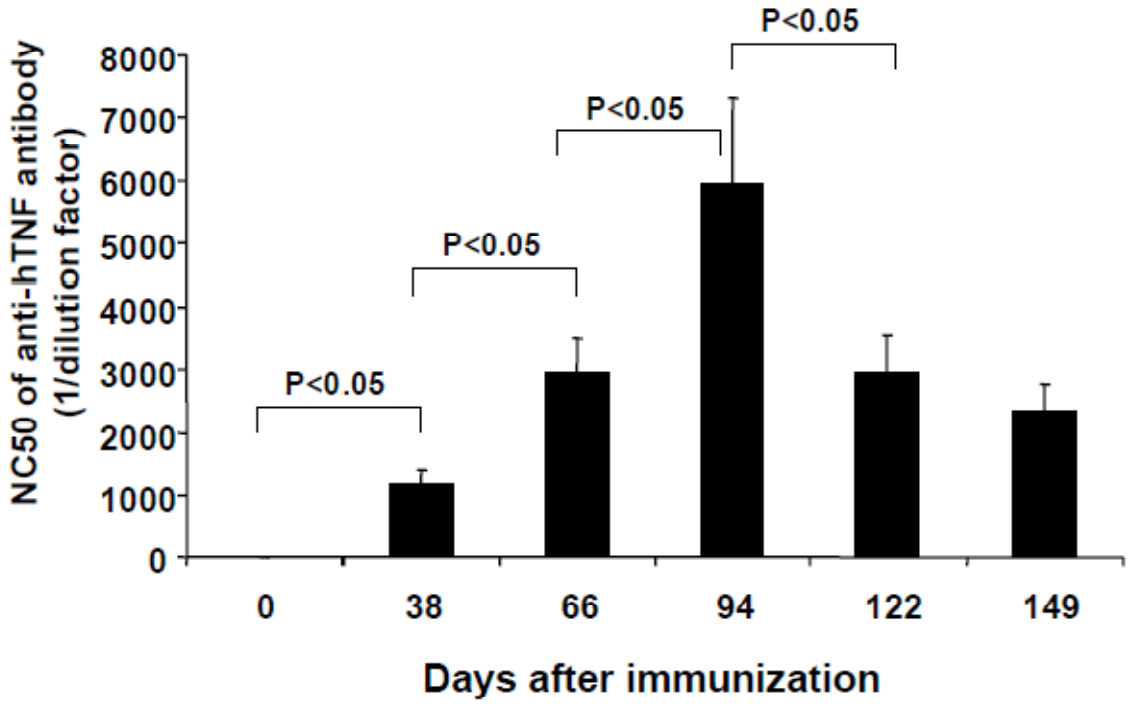
Additional file 1



Supplemental 1- TNFK immunization protocol scheme
Long-term follow-up of the experiment is represented by horizontal arrow with time expressed in week (from week 9, w9, to week 45, w45). Slashs represent discontinuation of time. A- Control group treated with PBS/ISA-51; B- TNF-K group; C- Intermittent infliximab group. The follow-up for each group (PBS, TNF-K and infliximab) is represented by a larger black lign, with vertical black arrows at each time where treatment was given. IP injections, intraperitoneal injections.

Additional file 2



Supplemental 2- Evolution of neutralizing anti-hTNF-α antibody titers during time, in TTg mice immunized with TNFK
36 TTg mice were immunized with TNFK at days 0, 7 and 28. Bleeding was done every month from day 38 post primo-injection to sacrifice. Results are expressed as mean ± SEM of all the sera of all the 36 immunized mice.

Article 2

Modulation of anti-tumor necrosis factor alpha (TNF-α) antibody secretion in mice immunized with TNF-α kinoid

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*Equal contribution

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In RA patients, anti-TNF treatments are usually administered in association to classic DMARDs, mainly MTX. This maximizes clinical response rate, which was shown to be higher vs. anti-TNF monotherapy for all marketed anti-TNF drugs (208). MTX and corticosteroids (CS) have been reported to potentially affect vaccination efficiency, even if data in rheumatologic conditions are heterogeneous (209). TNF-K is an anti-TNF treatment and, as such, it might potentially benefit from MTX and CS background treatment, with resulting increased clinical response rate. On the other side, TNF-K exploits vaccination principle, and therefore its efficiency at inducing therapeutic levels of anti-TNF polyclonal Abs might be impaired by concomitant immunosuppressant administration.

To determine whether MTX and CS affects TNF-K induced Ab production, we tested anti-TNF Ab response (in terms of Ab titers and TNF-neutralizing capacity) in Balb/c mice treated with either MTX (1mg/kg three times per week for nine weeks) or methylprednisolone (0.2mg/kg, same time schedule) started before or at the moment of TNF-K immunization.

We compared the individual AUC of Ab production for all individual mice in different treatment groups and we found that MTX or CS treatment did not result in significantly different anti-TNF Ab production. There was a high heterogeneity in Ab production, and methylprednisolone treatment started at the moment of TNF-K immunization resulted in numerically lower AUCs vs. PBS

(control group). Even if this difference was not statistically significant, we cannot rule out an effect of methylprednisolone on active anti-TNF immunization, even if the effect size is small. CS treatment might therefore potentially impair anti-TNF vaccination efficacy, but the effect size does not allow to conclude on this point at our sample size.

High heterogeneity was found even in neutralizing anti-TNF capacity of sera as tested by the L929 cytotoxicity assay. In this case, MTX administration (started before TNF-K) resulted in numerically lower anti-TNF neutralization capacity vs. PBS. Again, this difference was not statistically significant and the effect size small. Based on the detected difference, the minimal sample size required for this difference to be significant in an experiment involving only two groups would be 26 mice per group.

In summary, MTX and CS treatment does not seem to impair anti-TNF Ab production or neutralizing capacity at our sample size. A modest effect of both treatments, undetectable at our sample size, cannot be ruled out and needs to be confirmed in clinical series to evaluate its real importance for practice.

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Modulation of Anti-Tumor Necrosis Factor Alpha (TNF-α) Antibody Secretion in Mice Immunized with TNF-α Kinoid

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Tumor necrosis factor alpha (TNF-α) blockade is an effective treatment for patients with TNF-α-dependent chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, and psoriasis. TNF-α kinoid, a heterocomplex of human TNF-α and keyhole limpet hemocyanin (KLH) (TNF-K), is an active immunotherapy targeting TNF-α. Since the TNF-K approach is an active immunization, and patients receiving this therapy also receive immunosuppressant treatment, we evaluated the effect of some immunosuppressive drugs on the generation of anti-TNF-α antibodies produced during TNF-K treatment. BALB/c mice were injected intramuscularly with TNF-K in ISA 51 adjuvant. Mice were also injected intraperitoneally with one of the following: phosphate-buffered saline, cyclophosphamide, methylprednisolone, or methotrexate. Anti-TNF-α and anti-KLH antibody levels were assessed by enzyme-linked immunosorbent assay and the anti-TNF-α neutralizing capacity of sera by L929 bioassay. Our results showed that current treatments used in rheumatoid arthritis, such as methylprednisolone and methotrexate, do not significantly alter anti-TNF-α antibody production after TNF-K immunization. In contrast, the administration of cyclophosphamide (200 mg/kg) after immunization significantly reduced anti-TNF-α antibody titers and their neutralizing capacity.

Tumor necrosis factor (TNF) is a well-established therapeutic target in several chronic inflammatory diseases, including rheumatoid arthritis (RA), psoriasis, and Crohn's disease (1, 14). For treatment of RA, two classes of TNF-α-blocking agents have been developed so far: a soluble TNF-α receptor (etanercept) and TNF-binding monoclonal antibodies (MAbs) or MAb fragments, such as infliximab, adalimumab, golimumab, and certolizumab. These biologic drugs show rapid and substantial therapeutic efficacy in most patients and in experimental models (12). TNF-α is not the only compound involved in the pathophysiology of RA, and better disease control is often achieved when TNF-α antagonist therapy is associated with an immunosuppressant like methotrexate (MTX) (15, 16). Recent data have shown that anti-TNF-α treatment may counteract RA progression not only via the neutralization of soluble TNF-α but also by the modulation of T cell homeostasis. Indeed, infliximab treatment induces the reemergence of a discrete regulatory T cell subtype in RA patients and inhibits Th1 and Th17 accumulation in the joints (3, 4, 23, 24).

Current TNF-α-targeting strategies have several drawbacks. First, anti-TNF-α agents raised some concern because of the role of TNF-α in controlling infections and tumors. Second, primary and secondary failures are not infrequent: in clinical trials, less than 50% of responder patients attained disease remission (27). The risk of antidrug antibody (ADA) production, with possible loss of efficacy and side effects, is inherent in the use of current anti-TNF-α agents, especially monoclonal antibodies (2). Third, treatments with biologics have high costs for the community, which precludes their usage in some countries (12). Thus, there is a need to develop new drugs to neutralize TNF-α.

A promising alternative strategy consists of active immunotherapy against TNF-α, i.e., anti-TNF-α vaccination. This technique leads to the production of neutralizing polyclonal antibodies by the patient and avoids the possible loss of efficacy by production of antidrug antibodies. Over the last decade, several active anti-TNF-α immunotherapies using TNF-α derivates as immunogens have been developed and tested in experimental models of RA (5, 7, 8, 26). Immunogens must be capable of disrupting B cell but not T cell tolerance of self cytokines, thereby eliciting the production of neutralizing antibodies at high titers. Recently, we developed a heterocomplex vaccine, called human TNF-α kinoid (TNF-K), consisting of biologically inactive but immunogenic human TNF-α (hTNF-α) conjugated to a carrier protein, keyhole limpet hemocyanin (KLH) (20). Since antibodies generated by TNF-K immunization target only hTNF-α, we tested TNF-K in hTNF-α-transgenic (TTg) mice, which overexpress hTNF-α and develop a spontaneous arthritis at 6 to 8 weeks of age (19). In the TTg mouse model, we showed first that an early anti-hTNF-α immunization protected TTg mice from developing arthritis (9). We were subsequently able to show that TNF-K is efficacious against established arthritis, inducing a transient hTNF-α blockade with reversible effects on arthritis (10). These results contributed to the initiation of two clinical trials in Crohn's disease (EudraCT number 2010-019996-32) and RA (EudraCT number 2009-012041-35).

The objective of the present study was to further investigate the immune effect of TNF-K in the context of coadministration of immunosuppressant drugs. We first aimed at studying the effect on the response to the kinoid of coadministration with various immunosuppressant agents, such as MTX and corticosteroids,

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TABLE 1 Experimental design

Study part	Group	Immunosuppressant	Dose of TNF-K (μg) or immunosuppressant (mg/kg)[a]												
			Day −6	Day −4	Day 0	Day 2	Day 4	Day 7	3×/week for 9 weeks	Day 28	Day 35	Day 38	Day 41	Day 44	Day 49
I	A1	MTX	–	–	4	–	1	4	1	4					4
	A2	MTX	1	1	4	1	1	4	1	4					4
	B1	MP	–	–	4	–	0.2	4	0.2	4					4
	B2	MP	0.2	0.2	4	0.2	0.2	4	0.2	4					4
	C	PBS	/	/	4	/	/	4	/	4					4
II	D1	PBS			4			4		4	/	/	/	/	-
	D2	CYC			4			4		4	200	–	–	200	-
	D3	MP			4			4		4	5	5	5	5	-
	D4	MTX			4			4		4	2.5	2.5	2.5	2.5	-

[a] Immunization with TNF-K was by the intramuscular route, and administration of immunosuppressant was by the intraperitoneal route. BALB/c mice were sacrificed at day 70. Shading indicates TNF-K immunization; other columns show doses of immunosuppressant. –, no administration; /, PBS injection.

which are currently used in clinical practice during the treatment of RA and other TNF-dependent diseases. Then, we investigated the impact of a high dose of an immunosuppressant agent on the response to TNF-K immunization.

MATERIALS AND METHODS

Mice. Seventy-three female BALB/c mice (6 weeks old) were purchased from Janvier Laboratory (Le Genest-St-Isle, France). *In vivo* experiments complied with the recommendations for animal experimentation issued by the Institutes of Laboratory Animal Resources committee and by the local Ethics Committee on Animal Care and Experimentation. Mice were randomly distributed into nine groups of 8 mice each (except for group D1, which had 9 mice) and identified according to the study design described in Table 1. The first five groups of mice (A1, A2, B1, B2, and C) were included in part I of the study, while the last four groups (D1 to D4) were included in part II.

Immunogens and administration. Human TNF-α kinoid (TNF-K; 40 mg, scale of vaccine production; batch G) was provided by NeoVacs (Paris, France). TNF-K was emulsified with Montanide ISA 51 VG (36362Z; Seppic). TNF-K emulsions were aseptically prepared under a laminar flow hood and kept at 2 to 8°C for at least 1 h and no more than 4 h before injection. Animals were injected intramuscularly with 4 μg of TNF-K at days 0, 7, 28, and 49 for part I of the study and days 0, 7, and 28 for part II.

Bleeding. Blood was collected by retro-orbital sinus puncture at days −6, 32, 40, and 60 and by heart puncture at day 70 for anti-hTNF-α and anti-KLH antibody (Ab) titration, as well as anti-hTNF-α Ab neutralization assessment. The blood was directly transferred to gel separator tubes (Microvette, Sarstedt, France). The tubes were kept at room temperature for at least 20 min and then centrifuged at 1,800 × *g* for 10 min (at 4°C). Sera were stored at −80°C until use.

Immunosuppressant administration. Animals were injected intraperitoneally (IP) with the immunomodulators cyclophosphamide (CYC), methylprednisolone (MP), and methotrexate (MTX).

Study part I. To investigate the potential impediment to the induction of antibodies after immunization by immunomodulator coadministration, immunomodulators were administered at a chronic dose (MP, 0.2 mg/kg; MTX, 1 mg/kg) three times per week for 9 weeks (except one time missed for all groups) before and during the TNF-K immunization from day −6 or day 4 to day 67.

Study part II. To investigate the possibility of influencing the production of antibodies after immunization, several immunomodulators were administered in short courses at high doses 7 days after the last booster dose of TNF-K. Two (CYC) or four (MP and MTX) injections of immunomodulators or phosphate-buffered saline (PBS) were given. CYC (200 mg/kg) was injected at days 35 and 44. MP (5 mg/kg), MTX (2.5 mg/kg), and PBS were injected at days 35, 38, 41, and 44.

Serum analyses. Serum samples were analyzed for anti-hTNF-α- and anti-KLH-antibody titers by enzyme-linked immunosorbent assay (ELISA) and for neutralizing capacity by an L929 bioassay.

Specific anti-KLH or anti-hTNF-α Ab titers in sera were determined by a direct ELISA. Precoated ELISA plates with 1 μg/ml of KLH or hTNF-α were incubated with serial dilutions of sera from immunized and control mice. Specific IgG was detected by using peroxidase-conjugated rabbit anti-mouse IgG (Zymed, Carlsbad, CA). Endpoint titers were expressed as the mean dilution factors giving half-maximal absorbance. The ability of sera to neutralize hTNF-α activity was assessed by using the L929 cytotoxicity assay (13). hTNF-α pretreated with serial dilutions of tested sera was incubated for 18 h with L929 cells in the presence of actinomycin D (1 μg/ml), and the number of surviving cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt)–phenazine methosulfate (MTS/PMS) colorimetric assay. The neutralizing titer was expressed as the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity.

Statistical analyses. Data distribution was preliminarily checked by the Kolmogorov-Smirnov test. Since data did not always show a normal distribution, preliminary logarithmic transformation allowed the use of parametric tests for all data. Serial measurements of antibody titers and antibodies' neutralizing capacity were analyzed considering the area under the curve (AUC) for each subject as a summary measure; these measures were then analyzed as raw data (22) by one-way analysis of variance (ANOVA) with *post hoc* comparisons by the Student-Newman-Keuls test. The same methods were applied to analyze maximum and minimum antibody titers in each group. Differences in antibody titers and neutralizing capacity at different time points were analyzed with repeated-measures two-factor ANOVA. The data were analyzed for their variation over time (within-group variation, factor 1), for their variation across groups (between-group variation, factor 2), and for the interaction of the two factors. *Post hoc* comparisons were performed with the Student-Neuman-Keuls *t* test. All statistical analyses were performed with MedCalc statistical software, version 10.4.8 (MedCalc Software bvba, Mariakerke, Belgium). The significance threshold was set at 0.05.

RESULTS

Absence of influence of concomitant low doses of immunosuppressant on anti-hTNF-α and anti-KLH Ab levels. The first part of the study aimed at determining whether the effects of TNF-K immunizations were modified by concomitant and repeated immunosuppressive treatments in BALB/c mice.

As shown in Fig. 1A, high levels of anti-hTNF-α Abs were

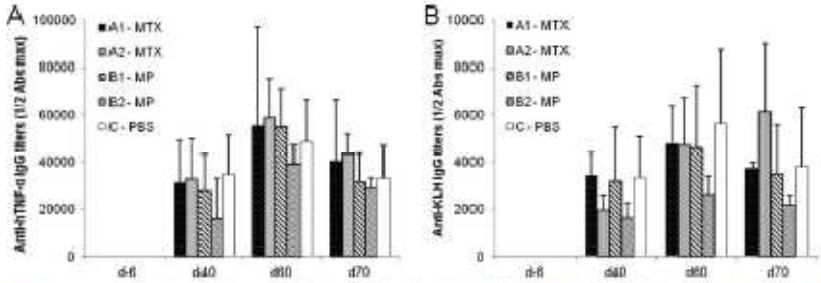

FIG 1 Effect of chronic immunosuppressant treatment on anti-hTNF-α (A) and anti-KLH (B) Ab titers in sera of TNF-K-immunized mice, showing a significant production peak at day 60 and a significant decrease at day 70 for all groups (within-group variation, $P < 0.001$), except for anti-KLH antibodies in group A2. BALB/c mice received four TNF-K immunizations (days 0, 7, 28, and 49). Chronic injections of low doses of immunosuppressants started either 6 days before (A2 and B2) or 4 days after (A1 and B1) the first injection of TNF-K. Anti-KLH and anti-hTNF-α Abs in sera were quantified by ELISA. Results are expressed as the dilution factor giving half-maximal absorbance (medians and interquartile ranges are reported).

detected in all groups, with a significant production peak at day 60 ($P < 0.001$ for within-group variation) and a significant decrease at day 70 for all groups ($P < 0.001$ for within-group variation). Levels of anti-hTNF-α Abs were not significantly affected by the different treatments versus the control PBS group (between-group variation and the two factors' interaction were not significant). A decrease in anti-hTNF-α IgG titers was noted between MP-treated groups at day 60, when MP treatment started prior to TNF-K immunization (day −6 versus day 2), that did not reach statistical significance.

As a control, levels of anti-KLH Abs were studied (Fig. 1B). High levels of anti-KLH Abs were detected in sera of immunized mice with a production peak at day 60 for all groups ($P < 0.001$ for within-group variation), except for group A2 (MTX). A significant decrease in anti-KLH Abs was observed at day 70 for all groups ($P < 0.001$ for within-group variation), except for group A2, without any difference in anti-KLH titers for MTX and MP versus PBS, whatever the dose or the time schedule applied (inter-group variation was not significant).

Analysis of the neutralizing capacity of anti-hTNF-α Abs in the sera of BALB/c mice undergoing repeated administrations of immunosuppressive treatments showed high titers of hTNF-α-neutralizing antibodies for all groups, with a production peak at day 60 ($P < 0.001$ for within-group variation) and a decrease at day 70 ($P < 0.001$ for within-group variation) for all groups (Fig. 2). No significant dose or time schedule effects of concomitant administration of either MP or MTX on neutralization capacity were observed. MTX-treated groups had lower NC_{50} values (i.e., the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity) than the PBS group, but these differences were not statistically significant. To estimate the effect size of the difference, we calculated the 95% confidence interval (CI) for the difference in the means of the two groups displaying the highest difference in NC_{50} values (groups A1 and C; both displayed a normal distribution of values). The 95% CI included 0 (−7,488 to 91,954), and the minimal sample size required for this difference to be significant in an experiment involving only these two groups would be 26 mice per group.

Influence of high doses of immunosuppressant on anti-hTNF-α and anti-KLH Ab levels. In the second part of this study, we aimed at determining whether a high dose of immunosuppressants significantly impacted the production of antibodies after TNF-K immunizations in BALB/c mice. As shown in Fig. 3A, high levels of anti-hTNF-α Abs were detected in sera of immunized mice with a peak at day 60 for the MP, MTX, and PBS groups ($P < 0.001$ for within-group variation), and then a decrease in anti-hTNF-α Ab was observed at day 70 ($P < 0.001$ for within-group variation). Conversely, mice receiving a high dose of CYC had a production peak at day 40 for anti-hTNF-α Abs ($P < 0.01$). They had overall lower Ab production throughout the experiment ($P < 0.01$ for AUC comparison) and an early decrease at days 60 and 70 compared to all other groups ($P < 0.001$ for within- and between-group variation). For MP and MTX treatments, a slight increase in Ab levels versus those in the PBS group that did not reach statistical significance was observed at days 60 and 70. We calculated the 95% CI for the difference in the means of the two groups displaying the highest difference in Ab levels (MP and PBS groups; both displayed a normal distribution of values). The 95% CI included 0 (−4,966 to 43,724).

As a control, levels of anti-KLH Abs were studied (Fig. 3B). High levels of anti-KLH Abs were detected in sera of immunized

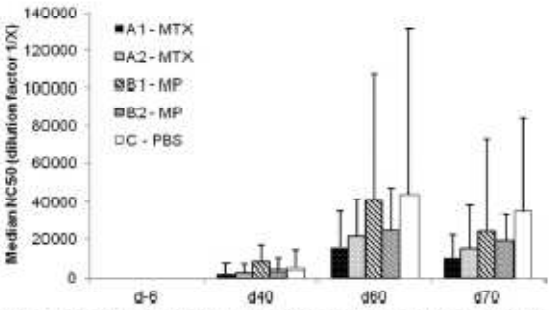

FIG 2 Effect of chronic immunosuppressant treatment on hTNF-α-neutralizing capacities in sera of TNF-K immunized mice, evaluated by an L929 bioassay. A significant production peak at day 60 and a significant decrease at day 70 were found for all groups (within-group variation, $P < 0.001$). Chronic injections of low doses of immunosuppressants starting either 6 days before (A2 and B2) or 4 days after (A1 and B1) the first injection of TNF-K did not induce significant reduction of neutralizing capacity. Neutralizing titers of individual mice are expressed as the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity (medians and interquartile ranges are reported).

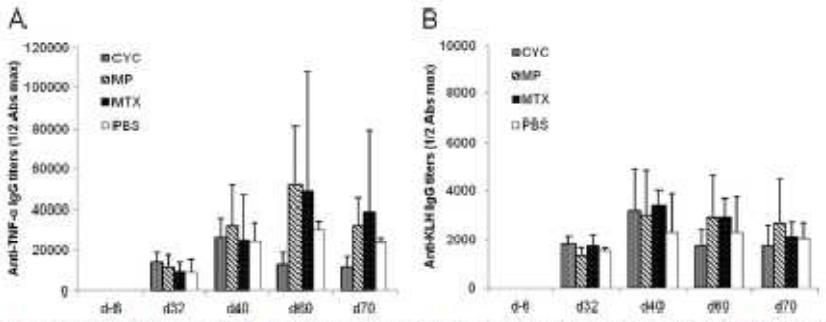

FIG 3 Effect of short-term immunosuppressant treatment on anti-hTNF-α (A) and anti-KLH (B) Ab titers in sera of TNF-K-immunized mice, showing an overall lower anti-hTNF-α Ab production in the CYC group ($P < 0.01$) and a significant reduction in Ab levels at days 60 and 70 compared to all other groups ($P < 0.001$). Mice received three TNF-K immunizations (days 0, 7, and 28) before high-dose immunosuppressant administration. Mice received 2 (CYC) or 4 (MP, MTX, and PBS) doses between day 35 and day 44. Anti-KLH and anti-hTNF-α Abs in sera were quantified by ELISA. Results are expressed as the dilution factor giving half-maximal absorbance (medians and interquartile ranges are reported).

mice, with a peak at day 40 for all groups ($P < 0.001$ for within-group variation). Then, a significant decrease in anti-KLH Abs was observed from day 40 to day 60 and from day 60 to day 70 for all groups ($P < 0.001$ for within-group variation). Administration of a high dose of immunosuppressant between days 35 and 44 had no effect on anti-KLH Ab levels compared to the PBS control group.

The neutralization capacity of anti-hTNF-α Abs was investigated with an L929 bioassay. As shown in Fig. 4, hTNF-α-neutralizing Ab was first detected at day 40, with a production peak at day 60 (except for the CYC group). Then, a decrease was observed at day 70 for all groups ($P < 0.001$ for within-group variation). A high heterogeneity in responses was observed for all groups. Mice receiving a high dose of CYC exhibited a significant strong decrease at day 60 and 70 of neutralizing capacity compared to all other groups ($P < 0.05$ and $P < 0.001$ at day 60 and at day 70 for within- and between-group variation, respectively). Administration of MP (5 mg/kg) or MTX (2.5 mg/kg) after TNF-K immunization did not affect the neutralizing capacity of anti-hTNF-α Abs.

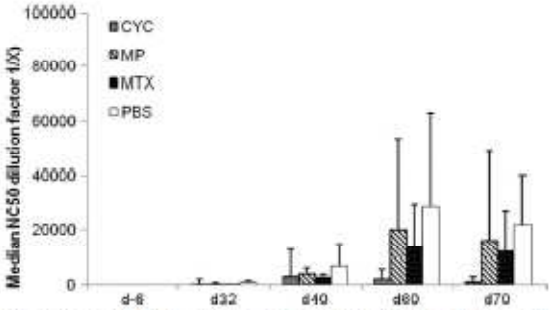

FIG 4 Effect of short-term immunosuppressant treatment on hTNF-α-neutralizing capacities in sera of TNF-K-immunized mice, evaluated by an L929 bioassay. An overall lower rate of production of anti-hTNF-α-neutralizing Ab was found in the CYC group ($P < 0.05$), with a significant decrease at day 60 and day 70, resulting in lower neutralizing capacity than in all other groups ($P < 0.001$). Neutralizing titers are expressed as the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity (medians and interquartile ranges are reported).

DISCUSSION

In the present study, we showed that anti-TNF antibodies induced by TNF-K immunization were not significantly influenced by a concomitant low-dose immunosuppressive treatment, such as methylprednisolone or methotrexate. Our study, performed in mice, is relevant to the current scenario of anti-TNF administration in humans; in most cases, corticosteroids and immunomodulatory agents are associated to enhance the clinical efficacy of the treatment. A potential side effect of immunosuppressive drugs a reduction in the response to vaccinations (17, 18, 29). It was of high interest to explore the influence of this class of treatments on the anti-TNF antibody response to TNF-K. We observed a slight modification of anti-TNF Ab levels in BALB/c mice treated with concomitant MTX or MP and TNF-K that was not statistically significant, despite the fact that the doses of MTX we used in mice were 10-fold higher than those used in RA patients (0.3 mg/kg/week). Similarly, despite the high doses of MTX we used, the difference in TNF-neutralizing capacity between MTX- and PBS-treated groups had a modest effect size.

The timing of the start of immunosuppressive treatment did not influence the production of neutralizing anti-hTNF-α Abs. Taken together, these results showed that the use of low doses of MP or MTX during TNF-K immunization does not alter titers of anti-hTNF-α Abs or their neutralizing capacity. This is consistent with a previous study performed with hTNF-α transgenic mice developing arthritis, in which no alteration in the production and neutralizing capacity was observed after TNF-K immunization in the presence of MTX (9). In this context, we could speculate that TNF-K should preserve its efficacy even in patients on concomitant disease-modifying antirheumatic drugs (DMARDs), such as MTX at the doses used in RA, or patients on steroids.

Another point addressed in our study was the reversibility of the anti-TNF-α Ab response to TNF-K. Previous studies consistently demonstrated a bell curve response of Abs to anticytokine vaccines with an ~12- to 16-week cycle of response (9, 25, 28, 30), slightly longer than the duration of action of infliximab in humans.

On the other hand, we show here that partial acute immunosuppression with high doses of CYC efficiently blocked at least in part the immunological response induced by TNF-K immunization in mice. Indeed, CYC significantly reduced both the levels of

anti-hTNF-α Abs and their hTNF-α-neutralizing capacity. This CYC inhibitory effect on Ab titers was not observed with the two other immunosuppressive agents (MP and MTX). Indeed, an increase, though not statistically significant, in production of anti-hTNF-α Abs was observed with both MP and MTX treatments, once again with a modest effect size. Furthermore, the neutralizing capacity was not affected by MP or MTX treatment. Moreover, this discrepancy in regulating anti-hTNF-α Ab production in the presence of CYC and MP or MTX treatments may be due to the implications of different modulatory molecular pathways of the immune response. MP is a glucocorticoid that exerts its anti-inflammatory effect by acting on selective genes, whereas MTX exerts its effect by two different pathways depending on the dose. High doses of MTX exert a cytostatic effect by blocking purine and thymidine synthesis, whereas low doses of MTX in humans (5 to 30 mg/week) inhibit inflammation via inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, leading to the accumulation of extracellular adenosine, which inhibits neutrophil activity. CYC is an alkylating agent that cross-links DNA and causes cytotoxicity in various cell types. In contrast to its well-known immunosuppressive effect at high doses (6), low doses of CYC are associated with immune response enhancement via specific targeting of regulatory T cells (21).

Our experimental results with CYC showed a pharmacological effect on Ab response. Even if high-dose CYC treatment, in order to reverse the effect of TNF-K immunization, is not applicable in a human clinical setting due to safety concerns, CYC served as a model of a drug with potent immunosuppressant activity. Further studies more specifically targeting memory B cells are needed to define a useful immunomodulator in RA patients treated with an active immunization strategy. Of note, recent data from antihumoral therapies in organ transplantation recipients have shown that proteasome inhibition by bortezomib depletes plasma cells, the source of Ab production (11). Additional studies to determine whether this drug might modulate Ab production after anti-TNF-α immunization could be warranted.

ACKNOWLEDGMENTS

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Article 3

Interplay between TNF and regulatory T cells in a TNF-driven murine model of arthritis

Biton J, Semerano L, Delavallée L, Lemeiter D, Laborie M, Grouard-Vogel G, Boissier MC, Bessis N.

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In this article we used the model of TTG mouse to study the effect of deregulated TNF production on Treg and Teff count, percentage and phenotype. We subsequently analyzed the modification in Treg populations after TNF-blockade with either infliximab or TNF-K.

The animal model allowed us to study the effect of TNF overexpression on Tregs from early age on in a dynamic, longitudinal fashion, and to better dissect the effect of TNF blockade.

In the presence of constitutive TNF production, but before arthritis clinical development, we documented reduced Treg percentage is in TTG mice vs. the wild type counterpart. With arthritis onset and later on a progressive increase in Treg percentages ensues and at the age of 24 weeks Treg proportions were no longer different from WT mice. Moreover Tregs progressively increased the expression of TNFR2.

These results suggest that TNF overexpression might reduce Treg populations, while later on, with TNF-dependent disease development there is an expansion in this population with increased expression of TNFR2 as to counteract inflammation.

TNF blockade with either infliximab or TNF-K increased Treg percentages vs. untreated mice. In the meantime we observed a modification of Treg phenotype with increased expression of TNFR2 and of CTLA-4.

On TNF blockade we even observed a significant increase in the percentage of cells lacking CD62L (the homing receptor for lymph nodes) or expressing it at very low levels. Moreover, suppression of Teff proliferation was higher in anti-TNF treated mice vs. untreated.

All these results, consistent with what shown in RA patients confirm that TNF-blockade is associated with the emergence of a population of Tregs with increased suppressive activity.

Like in RA patients, we showed that the expression of CTLA-4 and TNFR2 (activation markers for Tregs) increases with TNF blockade, whilst these cells express lower CD62L. The lack of the homing receptor for lymph nodes might define a population of Tregs with different homing capacity more suited to enter inflamed tissues and exert their regulatory activity.

It is interesting to note that exactly the same modifications of Treg populations take place with either passive monoclonal anti-TNF administration (infliximab) or with active anti-TNF immunization. Polyclonal and monoclonal anti-TNF Abs might therefore act via the same upstream cellular pathways.

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Interplay between TNF and Regulatory T Cells in a TNF-Driven Murine Model of Arthritis

Jérôme Biton,* Luca Semerano,*,† Laure Delavallée,* Delphine Lemeiter,* Marion Laborie,‡ Géraldine Grouard-Vogel,‡ Marie-Christophe Boissier,*,† and Natacha Bessis*

CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Treg) are involved in several autoimmune diseases, including rheumatoid arthritis. TNF-α blockers induce therapeutic benefits in rheumatoid arthritis via a variety of mechanisms. We aimed to characterize the impact on Treg of TNF-α overexpression in vivo and of TNF-α inhibiting treatments. We used human TNF-α transgenic mice as a model of strictly TNF-α–dependent arthritis. Our study showed that initial Treg frequency was lower in TNF-α transgenic mice than in wild-type mice. However, the course of arthritis was marked by elevation of Treg frequency and a dramatic increase in expression of TNFR2. Antagonizing TNF-α with either the anti-human TNF-α Ab (infliximab) or active immunotherapy (TNF-kinoid) increased the Treg frequency and upregulated CTLA-4, leading to enhancement of suppressor activity. Moreover, both anti–TNF-α strategies promoted the differentiation of a CD62L$^-$ Treg population. In conclusion, in an in vivo model of TNF-α–driven arthritis, Treg frequency increased with inflammation but failed to control the inflammatory process. Both passive and active TNF-α–inhibiting strategies restored the suppressor activity of Treg and induced the differentiation of a CD62L$^-$ Treg population. *The Journal of Immunology*, **2011, 186: 000–000.**

Rheumatoid arthritis is a chronic autoimmune disease involving T lymphocytes and whose hallmark is hyperplastic synovitis responsible for cartilage and bone destruction. Factors involved early in the disease process include proinflammatory cytokines such as TNF-α, IL-1, and chemokines. Among all of the cytokines involved in the disease process, TNF-α has a particularly important role in the cascade of pathogenic events in rheumatoid arthritis (RA). TNF-α acts within a complex network of cells and mediators of inflammation, as shown by the ability of IL-1β or IL-17 to induce TNF-α. The hypothesis that TNF-α drives much of the pathophysiology in the rheumatoid joint is supported by studies of TNF-α overexpression or TNF-α neutralization in animal models of RA (1, 2). The experimental results in animal models reflect findings from studies of TNF-α antagonism in patients with RA. Whereas previous medications used in RA were developed primarily based on serendipitous observations, TNF-α antagonists (mainly mAbs such as infliximab and soluble receptors such as etanercept) were the first rationally designed drugs and the first U.S. Food and Drug Administration–approved recombinant proteins ("biologics") for the treatment of RA. Although TNF-α antagonists provide substantial therapeutic benefits in most patients, primary unresponsiveness and secondary escape phenomena are not uncommon, indicating a need for alternative treatments (3). We recently developed a novel concept consisting of active immunotherapy to cytokines (4, 5). In this anticytokine immunotherapy strategy, the immunogen (TNF-kinoid [TNF-K]) induces the production of Abs that block the effects of the targeted cytokine. This strategy protected human TNF-α transgenic (TTg) mice against clinical and histological arthritis in short- and long-term experiments involving preventive (4, 5) or curative (6) immunotherapy.

TNF-α antagonists may act through mechanisms involving regulatory T cells (Treg), which exhibit the CD4$^+$CD25$^+$Foxp3$^+$ phenotype. Treg are essential for maintaining immune homeostasis, preventing autoimmunity, and limiting chronic inflammatory diseases. Treg act by preventing both the activation and the effector function of T cells that have escaped other mechanisms of tolerance (7, 8). Their central place in the maintenance of peripheral tolerance is underlined by the fact that Treg deficiency results in spontaneous autoimmunity in both mice and humans (9, 10). Furthermore, Treg play a pivotal role in preventing autoimmune diseases such as type 1 diabetes (11) and in limiting chronic inflammatory diseases such as asthma and inflammatory bowel disease (12, 13).

In patients with RA, Treg functions, including suppression of proinflammatory cytokine secretion by activated T cells and monocytes, are diminished compared with healthy individuals (14, 15). The regulatory role for Treg in experimental models of RA has been demonstrated in a few studies (16, 17). The link between TNF-α antagonists (infliximab and adalimumab) and Treg is that TNF-α antagonists normalize immune homeostasis by restoring the capacity of Treg to inhibit cytokine production and by conveying a suppressive phenotype to conventional T cells (14, 18). However, direct interactions between TNF-α and Treg have been

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Abbreviations used in this article: MFI, mean fluorescence intensity; RA, rheumatoid arthritis; TNF-K, TNF-kinoid; Treg, regulatory T cell; TTg, TNF-α transgenic.

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documented chiefly in in vitro studies, which have produced conflicting results. In a first study, TNF-α had no direct effect on Treg (14), but subsequent work showed that TNF-α inhibited the suppressive capacity of Treg via a TNFR2-dependent mechanism leading to downregulation of Foxp3 expression (19). More recently, TNF-α interaction with TNFR2 was shown to promote Treg expansion and function (20, 21).

Our aim in this work was to study T cell differentiation to the Treg phenotype in an in vivo model of TNF-α–driven disease, namely the hTNF-αTg model. This model is relevant for investigating the consequences of TNF-α overexpression on arthritides and the effect of TNF-α blockade. We show that Treg differentiation may constitute a critical mode of action of TNF-α blocking treatments such as infliximab and TNF-K immunotherapy.

Materials and Methods

Mice

Male transgenic mice (1006-T) aged 6–9 wk were purchased from Taconic Farms (Germantown, NY). These mice were produced using the microinjection construct previously used to generate the Tg197 strain (5). They were hemizygous for the hTNF-α transgene and maintained on a C57BL/6 background. All pups were genotyped by PCR, which established the presence of the hTNF-α transgene in all the animals. C57BL/6 mice aged 7–31 wk were purchased from Janvier (Le Genest-Saint-Isle, France) and used as controls. All procedures were approved by the Animal Care Use Committee of Sorbonne Paris Cité-Université Paris 13 (Bobigny, France).

Clinical and histological assessments

1006-T transgenic mice develop spontaneous arthritis, similar to the Tg 197 mice first described by Keffer et al. (1). Clinical arthritis begins to develop in 1006-T mice at ~8–10 wk of age (22). A blinded procedure was used to monitor body weight and arthritis in all four limbs. Clinical severity of arthritis in each limb was scored from 0 (normal) to 3 (severe inflammation with deformities) (23). For incidence determination, arthritis was defined as a score ≥1. The mean arthritis score on each clinical observation day was calculated in each treatment group. In each mouse, we recorded the maximal arthritis score as the highest score reached during the observation period. For histological analysis, the hind paws were dissected and processed as described elsewhere (24). Numerous sections were cut from each paw, and at least four sections per paw were examined. Slides were then stained with H&E before microscopic observation (optical microscopy). A blinded procedure was used to evaluate the lesions in each joint as described elsewhere (6), using a 4-point scale for synovitis (0–3, where 0 is normal and 3 indicates severe synovial proliferation or inflammatory cell infiltration) and a 4-point joint-destruction scale (cartilage, irregularities, and bone erosions). For prevalence determinations, histological inflammation or destruction was defined as an inflammation or destruction score ≥0.5.

Treatments

We obtained hTNF-α kinoid from Neovacs (Paris, France). Briefly hTNF-α (Boehringer, Austria) (1 mg/ml) in 0.5 EDTA/0.1 M phosphate buffer (pH 7.8) was treated with 1% DMSO for 30 min. Keyhole limpet hemocyanin, purchased from Intracel (Frederick, MD), was added, followed by glutaraldehyde. After 45 min incubation at 4°C, the preparation was dialyzed against the working buffer and then treated with formaldehyde. After

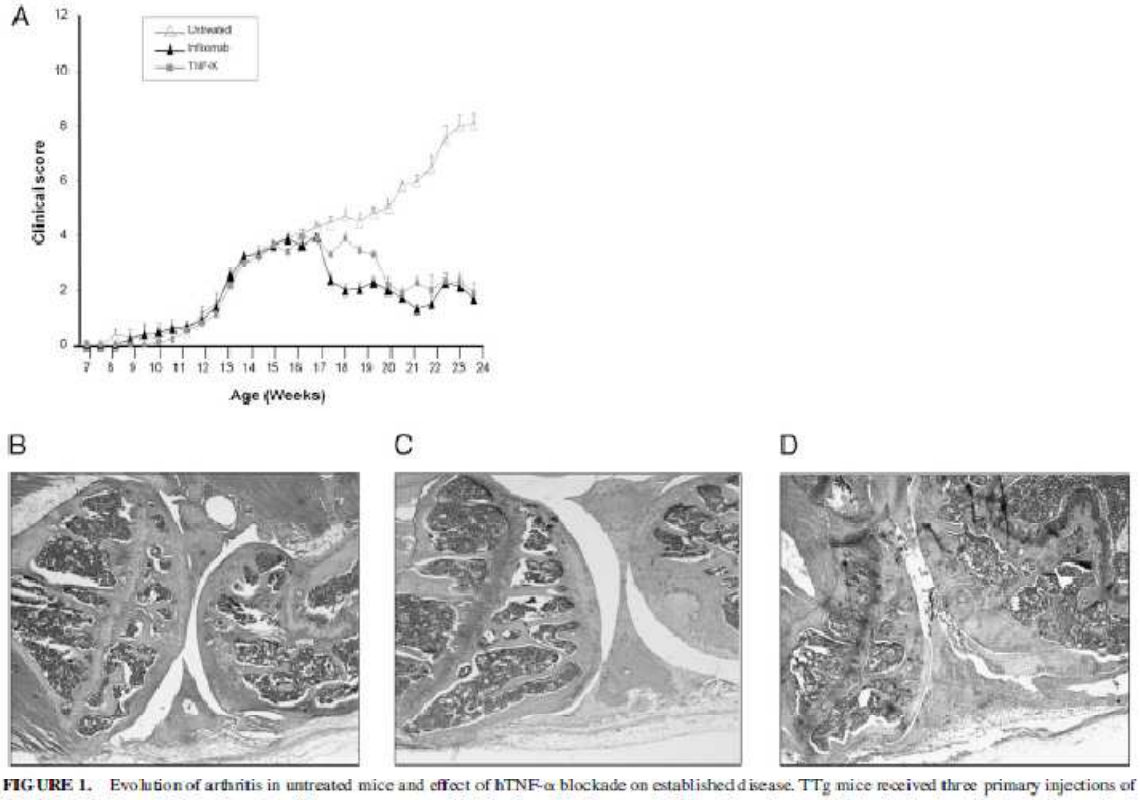

FIGURE 1. Evolution of arthritis in untreated mice and effect of hTNF-α blockade on established disease. TTg mice received three primary injections of TNF-K at 15, 16, and 19 wk of age ($n = 20$, gray squares) or infliximab weekly from week 15 to euthanasia ($n = 16$, black triangles). Control mice received no treatment ($n = 20$, open triangles). Because half the mice were sacrificed at 24 wk of age and the other half at 31 wk of age, *A* shows the change in clinical scores only until 24 wk of age. Data are expressed as means ± SEM for each group. $p < 0.0001$ for TNF-K versus untreated TTg, $p < 0.0001$ for infliximab versus untreated TTg. Examples are shown of histological evaluation of 24-wk-old TTg mice immunized with TNF-K (*B*), treated with infliximab (*C*), and untreated (*D*). Histological sections (original magnification ×40) of the knee were prepared (see *Materials and Methods*) and stained with H&E. Date represent one experiment representative of three similar experiments.

quenching with glycine (0.1 M) and subsequent dialysis against Dulbecco's PBS, the preparation was stored at 4°C (4).

Immunization involved i.m. injections of TNF-K emulsified in ISA51 (Seppic, Paris, France). Three injections were given (4 μg TNF-K) at 15, 16, and 19 wk of age. In parallel, other mice were given weekly i.p. injections of infliximab (10 mg/kg; Schering-Plough, Levallois Perret, France) from week 15 to week 24 or 31, when they were sacrificed.

Ab assays

Sera obtained from blood samples collected at different time points during the experiment were tested for anti–hTNF-α Ab titers and for anti–hTNF-α Ab-neutralizing capacity. Specific anti–hTNF-α titers were determined using a direct ELISA (4). The neutralizing capacity of sera was assessed using the L929 cytotoxicity assay reflecting neutralizing Abs (4).

Real-time quantitative RT-PCR

Total RNA was extracted from synovial tissue using the TRI-Reagent kit (Euromedex, Mundolsheim, France). Then, 5 μg RNA was primed with oligo(dT) and reverse transcribed into a 20 μl volume using SuperScript III RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR reactions were performed using PCR LightCycler FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN).

Foxp3 expression was measured by real-time PCR. The Foxp3 primers were 5′-CAGCCTGCCTCTGACAAGAA-3′ (forward) and 5′-GGGGGTTCAAGGAAGAAGAG-3′ (reverse). Each reaction mix contained 5 μl cDNA, 0.2 μM each primer, and LightCycler FastStart DNA Master SYBR Green I mix (Roche Molecular Biochemicals) to a final volume of 15 μl. For Foxp3 amplification, initial 8-min holds at 95°C were followed by 45 cycles of 10 s at 95°C, 5 s at 62°C, and 8 s at 72°C. Amplification was analyzed using LightCycler software (RealQuant, version 1.0; Roche, Indianapolis, IN).

Cell and tissue preparation

Leukocytes from the spleen were prepared using a homogenizer, and RBCs were lyzed in hemolysis buffer (NH_4CL, $KHCO_3$, and EDTA). Afferent and popliteal lymphes nodes were dissected out of the hind limbs, and leukocytes were prepared using a homogenizer. Blood was collected by heart puncture. Finally, the knees were dissected and opened and synovial tissue was removed from both knees and pooled for further processing and analysis.

Flow cytometry

For FACS, surface cells were stained with FITC-labeled anti-CD25 (clone 3C7), PE-labeled anti-TNFR2 (clone TR75-89) or PE-labeled anti–CTLA-4 (clone UC10-4F10-11) or PE-labeled anti-CD62L (clone MEL-14) and PerCP-Cy5.5–labeled anti-CD4 (clone RM4-5) (all from BD Biosciences, San Jose, CA). Cells were stained at 4°C in PBS containing 2% heat-inactivated FCS and 0.01 M sodium azide, incubated for 30 min with 2.4G2.3 mAb to block the Fc-γ receptors (BD Biosciences), and incubated for 30 min with appropriate dilutions of various mAbs coupled to FITC, PE, or PerCP-Cy5.5. The allophycocyanin-labeled anti-Foxp3 (clone FJK-16s) staining set (eBioscience, San Diego, CA) was used for intracellular staining according to the manufacturer's recommendations.

For intracellular cytokine staining, cells were stimulated for 5 h with PMA and ionomycin (Sigma-Aldrich, Saint Louis, MO). Brefeldin A (BD Pharmingen, San Diego, CA) was added for the last 4 h. For surface staining, cells were incubated with PerCP-Cy5.5–labeled anti-CD4 (clone RM4-5) for 30 min at 4°C in the dark and then washed. The cells were then permeabilized using Fixation/Permeabilization solution and stained with allophycocyanin-labeled anti–IFN-γ (XMG1.2) and PE-labeled anti–IL-17A (TC11-18H10) (all from BD Biosciences) for 30 min at 4°C in the dark.

Flow cytometry was performed on a four-color FACSCalibur (BD Biosciences, Mountain View, CA). Dead cells were excluded based on forward and side scatter characteristics. Reported statistical data are based on at least 1000 events gated on the population of interest. Results were analyzed using CellQuest Pro software (BD Biosciences). WEASEL version 2.3 (Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) was used for graphical representations.

Lymphocyte purification

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from the spleen were purified using a Treg isolation kit according to the manufacturer's protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). In brief, $CD4^+CD25^+$ T cells were isolated using a two-step procedure. First, $CD4^+$ T cells were isolated by

Table I. Clinical and histological parameters of arthritis in TNF-K– or infliximab-treated TTg mice

	Clinical Parameters					Histological Parameters			
	Onset (wk)	% Incidence	A_{max}	Clinical Score 24 wk	Clinical Score 31 wk	Inflammation 24 wk	% Inflammation Incidence 24 wk	Destruction 24 wk	% Destruction Incidence 24 wk
Untreated (n = 20)	9.6 ± 0.5	100 (10/10)	8.2 ± 0.3	8.1 ± 0.4	10.2 ± 0.4	2.7 ± 0.2	100 (10/10)	2.6 ± 0.2	100 (10/10)
TNF-K (n = 20)	10.1 ± 0.4[1]	100 (10/10)	4.5 ± 0.1[2,3]	1.7 ± 0.2[3]	2.1 ± 0.3[4]	0.3 ± 0.1[5]	50 (5/10)[5]	0.02 ± 0.02[4]	10 (1/10)[6]
Infliximab (n = 16)	8.7 ± 0.5	100 (8/8)	4.1 ± 0.1[3]	1.9 ± 0.4[3]	1.6 ± 0.3[4]	0.3 ± 0.2[5]	50 (4/8)[5]	0.03 ± 0.03[4]	0 (0/8)[6]

The mice are the same as in Fig. 1. Time at arthritis onset, mean maximum arthritis score (A_{max}), clinical scores at 24 and 31 wk of age, and inflammation and destruction scores are expressed as mean ± SEM: (1) $p < 0.05$, (2) $p < 0.01$ versus infliximab 24 wk TTg, (3) $p < 0.0001$ versus untreated 24 wk TTg, (4) $p < 0.0001$ versus untreated 31 wk TTg. The incidences of arthritis, inflammation, and destruction are expressed as percentages: (5) $p < 0.05$, (6) $p < 0.0001$ versus untreated 24 wk TTg.

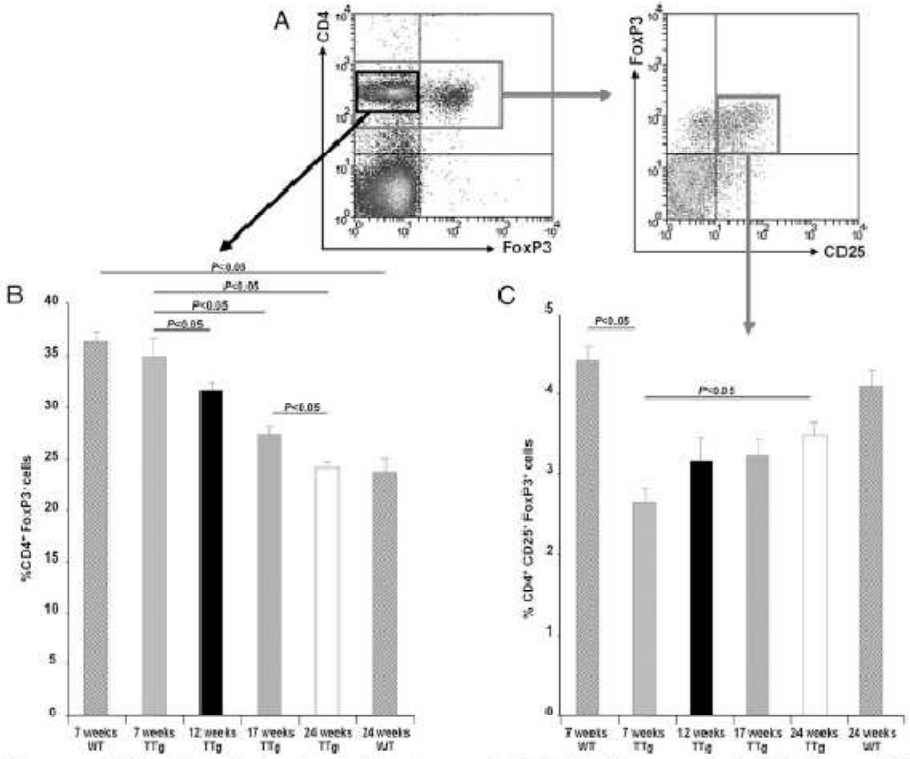

FIGURE 2. Percentages of Teff and Treg in the lymph nodes during the course of arthritis in hTNF-α transgenic mice. TTg mice were killed at the age of 7 ($n = 6$), 12 ($n = 6$), 17 ($n = 8$), or 24 wk ($n = 8$). All mice had been monitored for clinical signs of arthritis. Controls were 7-wk-old ($n = 6$) and 24-wk-old ($n = 6$) C57BL/6 wild-type mice (WT). Lymph node leukocytes were stained with fluorochrome-conjugated anti-CD4, anti-CD25, and anti-Foxp3. Teff and Treg were monitored in lymph nodes using flow cytometry. *A*, As shown on this representative dot plot, Teff cells were defined as $CD4^+Foxp3^-$ T cells and Treg as $CD4^+CD25^+Foxp3^+$ T cells. *B*, Teff percentages among lymph node leukocytes are given as means ± SEM for each group. *C*, Treg percentages among lymph node leukocytes are given as means ± SEM for each group.

negative selection using a mixture of biotin-conjugated Abs, anti-biotin microbeads, an LD column, and QuadroMACS (all from Miltenyi Biotec). Then, $CD4^+$ T cells were directly labeled with a PE-conjugated anti-CD25 Ab and anti-PE microbeads. The cell suspension was loaded onto an MS column, which was placed in the magnetic field of a MACS separator (OctoMACS; Miltenyi Biotec). The flow-through cells were collected and used as $CD4^+CD25^-$ cells, whereas the retained cells were eluted from the column and used as $CD4^+CD25^+$ Treg. To increase purity, two consecutive column runs were performed. Flow cytometry analysis showed that purity of the $CD4^+CD25^-$ and $CD4^+CD25^+$ cell-enriched fractions was 90–95% (data not shown).

Measurement of $CD4^+CD25^-$ effector T cell IFN-γ secretion

Spleen $CD4^+CD25^-$ (2.5×10^4) effector T cells (Teff) were cultured in RPMI 1640 with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 1 M HEPES, and 5 μg/ml of soluble anti-CD3 (clone 2C11) (BD Biosciences) in U-bottom 96-well plates. APCs (2.5×10^4) treated with mitomycin were added to the culture medium. The cells were then incubated at 37°C in a 5% CO_2 atmosphere. After 4 d culture, IFN-γ levels in culture supernatants were measured using commercially available ELISA kits (Quantikine; R&D Systems, Abingdon, U.K.) according to the manufacturer's instructions. The sensitivity of the cytokine assays was 2 pg/ml.

Assessment of Treg suppressive effect on $CD4^+CD25^-$ Teff

Spleen $CD4^+CD25^-$ Teff were prelabeled with 5 μM CFSE (Invitrogen) for 10 min. Then, CFSE-labeled Teff (2.5×10^4) were cocultured in RPMI 1640 with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 1 M HEPES, and 5 μg/ml soluble anti-CD3 (clone 2C11) (BD Biosciences) in U-bottom 96-well plates with Treg (2.5×10^4 or 1.25×10^4) to produce Teff/Treg ratios of 1:1 and 2:1, respectively. Controls were performed using non–CFSE-labeled Teff instead of Treg ($CD4^+CD25^-$; 2.5×10^4 or 1.25×10^4). APCs (2.5×10^4) treated with mitomycin were added to the culture medium. The cells were then incubated at 37°C in a 5% CO_2 atmosphere. After 4 d culture, the cells were stained with allophycocyanin-labeled anti-CD4 (clone RM4-5, BD Biosciences), and Teff proliferation was then determined for each Teff/Treg ratio using flow cytometry to measure the CFSE dilution. The values were compared with the control, in which Teff cells were cultured without Treg. The percentage of suppression was calculated as follows: % suppression = [(Teff prolif-

Table II. Frequency of Teff and Treg in the spleen during the course of arthritis in hTNF-α transgenic mice

	% Teff	% Treg
7 wk wild-type	20.40 ± 0.39	2.04 ± 0.09(2)
7 wk TTg	20.81 ± 0.72	1.36 ± 0.16
12 wk TTg	19.19 ± 0.93	1.73 ± 0.08
17 wk TTg	17.80 ± 0.89	1.59 ± 0.17
24 wk TTg	18.55 ± 0.52(1)	1.90 ± 0.15(2)
24 wk wild-type	15.07 ± 1.05	1.83 ± 0.15

Splenocytes from TTg mice were stained with fluorochrome-conjugated anti-CD4, anti-CD25, and anti-Foxp3. The mice are the same as in Fig. 2. Teff and Treg in the spleen were monitored using flow cytometry. Percentages of Teff and Treg among splenocytes are given as mean values +/− SEM.

(1) $p < 0.05$ versus 24 wk wild-type, (2) $p < 0.05$ versus 7 wk TTg.

eration without Treg-Teff proliferation with Treg)/Teff proliferation without Treg] × 100. Data were analyzed using CellQuest Pro software (BD Biosciences). IFN-γ levels were measured in the supernatants after 4 d culture using commercially available ELISA kits (Quantikine; R&D Systems) according to the manufacturer's instructions. The sensitivity of the cytokine assays was 2 pg/ml.

Statistical analysis

According to data distribution, a parametric test (ANOVA, Student test) or a nonparametric test (Kruskal–Wallis, Mann–Whitney *U*) with appropriate post hoc comparisons was used to compare data across the different groups. Clinical scores curves were compared with ANOVA. Categorical data were compared by a χ^2 test. All statistical analyses were performed using StatView version 5.0 software (Abacus Concepts, Berkeley, CA).

Results

Active and passive anti–hTNF-α immunotherapy improves established arthritis

As expected, clinical arthritis in untreated TTg mice was detected at 8 wk of age and arthritis severity increased over time (Fig. 1*A*). Fifteen-week-old TTg mice were given either classical hTNF-α–neutralizing mAb (infliximab) or active immunotherapy (TNF-K). At baseline, all mice had clinical scores >3. In mice immunized with TNF-K, anti–hTNF-α Ab was detected 5 wk after the first injection (Supplemental Fig. 1*A*). These anti–hTNF-α Abs inhibited hTNF-α bioactivity in vitro (Supplemental Fig. 1*B*). Infliximab-treated mice and TNF-K–immunized mice experienced

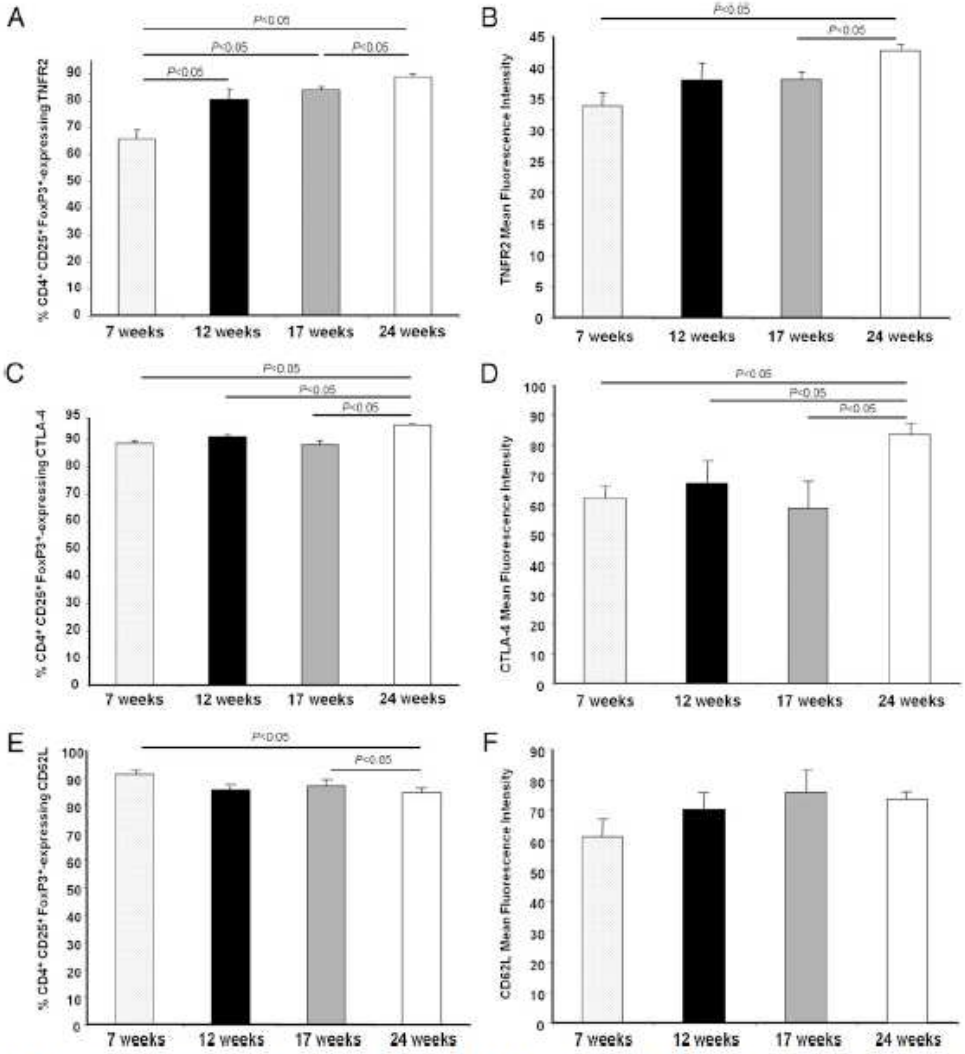

FIGURE 3. Treg phenotype in lymph nodes during arthritis in TTg mice. TTg mice were sacrificed at the age of 7 ($n = 6$), 12 ($n = 6$), 17 ($n = 8$), or 24 wk ($n = 8$). Controls were 7-wk-old ($n = 6$) and 24-wk-old ($n = 6$) C57BL/6 wild-type mice. The mice are the same as in Fig. 2. Lymph node leukocytes were labeled with fluorochrome-conjugated anti-CD25, anti-CD4, anti-Foxp3, and anti–CTLA-4 or anti-CD62L or anti-TNFR2. Expression of CTLA-4, CD62L, and TNFR2 was studied by gated $CD4^+CD25^+Foxp3^+$ T cells using flow cytometry. *A*, Percentage of Treg expressing CTLA-4, (*B*) CTLA-4 MFI on $CTLA\text{-}4^+$ Treg, (*C*) percentage of Treg expressing CD62L, (*D*) CD62L MFI on $CD62L^+$ Treg, (*E*) percentage of Treg expressing TNFR2, and (*F*) TNFR2 MFI on $TNFR2^+$ Treg. Data are expressed as means ± SEM for each group.

Table III. Treg phenotype in spleen during arthritis in TTg mice

	TNFR2		CTLA-4		CD62L	
	%	MFI	%	MFI	%	MFI
7 wk TTg	59.32 ± 5.35(2)	33.06 ± 1.33	91.54 ± 1.99(2)	51.46 ± 3.74(2)	89.95 ± 1.60	85.07 ± 18.66
12 wk TTg	68.99 ± 1.86(2)	30.33 ± 0.44	95.00 ± 0.95(2)	54.80 ± 2.76(2)	84.05 ± 2.43	66.36 ± 8.11
17 wk TTg	70.12 ± 2.30(2)	29.46 ± 1.07	94.91 ± 1.51	53.96 ± 9.09	79.10 ± 2.76(1)	60.51 ± 9.21
24 wk TTg	82.25 ± 1.94	34.35 ± 1.92	98.47 ± 0.46	74.97 ± 6.03	81.86 ± 1.91(1)	78.61 ± 9.25

The mice are the same as in Fig. 2. Spleen cells were labeled with fluorochrome-conjucted anti-CD25, anti-CD4, anti-Foxp3, and anti-TNFR2 or anti-CTLA-4 or anti-CD62L. Then, TNFR2, CTLA-4, and CD62L expression was studied by gated $CD4^+CD25^+Foxp3^+$ cells using flow cytometry. Percentage of Treg expressing TNFR2, TNFR2 MFI on $TNFR2^+$ Treg, percentage of Treg expressing CTLA-4, CTLA-4 MFI on $CTLA\text{-}4^+$ Treg, percentage of Treg expressing CD62L, and CD62L MFI on $CD62L^+$ Treg are given as means ± SEM.

(1) $p < 0.05$ versus 7 wk TTg, (2) $p < 0.05$ versus 24 wk TTg.

dramatic improvements in the disease compared with untreated mice (Fig. 1*A*). Although infliximab exerted its therapeutic effect more rapidly than did TNF-K, both treatments were effective. As expected, disease severity increased in untreated mice between 24 and 31 wk of age (Table I). Histological evaluation also indicated decreased joint inflammation and destruction in TNF-K– (Fig. 1*B*) and infliximab-treated (Fig. 1*C*) groups compared with untreated mice (Fig. 1*D*) (Table I).

hTNF-α overexpression modifies Treg frequency

We investigated whether in untreated TTg mice, in vivo systemic hTNF-α overexpression influenced the numbers and percentages of Treg (defined as $CD4^+CD25^+Foxp3^+$ cells) and Teff (defined as $CD4^+Foxp3^-$ cells) (Fig. 2*A*) in secondary lymphoid organs from untreated TTg mice during the course of arthritis. The total leukocyte count increased progressively from week 7 to week 24 in the lymph nodes ($24.3 \times 10^5 \pm 5.2 \times 10^5$ versus $38.1 \times 10^5 \pm 3.8 \times 10^5$, $p < 0.05$). In lymph node, Teff counts did not change significantly over time (data not shown) but their percentage decreased from week 7 to week 24 (Fig. 2*B*). However, a similar decrease was found in the wild-type mice (Fig. 2*B*), indicating that the cause was not hTNF-α overexpression. Compared to wild-type mice at the same age, 7-wk-old TTg mice had a lower percentage of Treg (Fig. 2*C*). Subsequently, Treg count (7 wk wild-type, $7.9 \times 10^4 \pm 1.7 \times 10^4$; 7 wk TTg, $6.1 \times 10^4 \pm 1.2 \times 10^4$(1); 24 wk wild-type, $8.9 \times 10^4 \pm 1.4 \times 10^4$(1); 24 wk TTg, $13.5 \times 10^4 \pm 1.0 \times 10^4$; (1) $p < 0.05$ versus 24 wk TTg) and percentage (Fig. 2*C*) increased in the lymph nodes of TTg mice but not in those of wild-type mice. In the spleen, Treg and Teff populations in TTg mice showed similar changes to those seen in the lymph nodes (Table II). Given that the percentage and the number of Tregs in the lymph nodes increased progressively during the course of arthritis, we assessed Treg in the synovium by measuring the level of Foxp3 mRNA expression. An increase, although nonsignificant, in Foxp3 mRNA expression between weeks 7 and 24 was detected (Supplemental Fig. 2). Taken together, our results in TTg mice show that hTNF-α overexpression leading to arthritis is accompanied by an initial Treg deficiency followed by an increase in Treg proportions.

Treg phenotype, but not suppressive activity, is modified during arthritis in hTNF-α transgenic mice

Because hTNF-α overexpression in untreated TTg mice led to an initial Treg deficiency followed by an increase in the lymph nodes and spleen, we characterized the Treg phenotype throughout arthritis development by evaluating the expressions of CTLA-4, CD62L, and TNFR2. As shown in Fig. 3*A* and 3*B*, CTLA-4 expression and mean fluorescence intensity (MFI) increased in Treg from the lymph nodes between week 7 and week 24, but it was also the case in wild-type mice. In contrast, percentage of Treg-expressing CD62L decreased slightly over time (Fig. 3*C*); however, the same was true in wild-type mice. As shown in Fig. 3*E* and 3*F*, lymph node Treg exhibited gradual and marked increases in TNFR2 expression (65.8 and 88.6% at weeks 7 and 24, respectively; $p < 0.05$) and in MFI during the course of arthritis. Similar results were observed in the spleen, except for TNFR2 MFI, which was similar in all groups (Table III).

To better characterize Treg activity during arthritis development in TTg mice, we evaluated the ability of Treg to suppress Teff proliferation and IFN-γ production. We found no significant difference in Treg suppressive activity across age groups at each of the Teff/Treg ratios studied either for proliferation (Fig. 4) or for IFN-γ secretion (data not shown).

hTNF-α blockade increases the Treg/Teff ratio in 24-wk-old TTg mice

We investigated whether the mechanism of action of passive and active anti–hTNF-α immunotherapy involved Treg in the hTNF-α–dependent arthritis model.

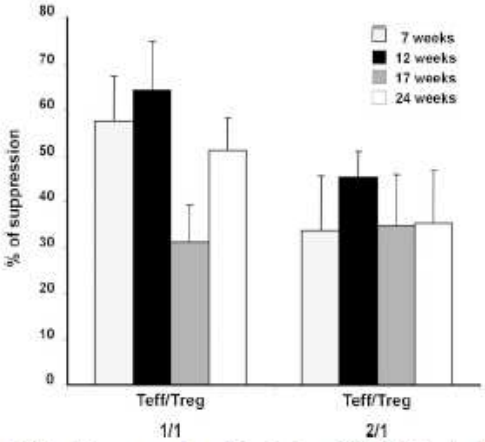

FIGURE 4. Treg suppressive activity during arthritis in TTg mice. TTg mice were sacrificed at the age of 7 ($n = 6$), 12 ($n = 3$), 17 ($n = 4$), or 24 wk ($n = 5$). $CD4^+CD25^+$ (Treg) and $CD4^+CD25^-$ (Teff) were isolated from the spleens of all mice. $CD4^+CD25^-$ CFSE-labeled T cells were cocultured with $CD4^+CD25^+$ Treg at ratios of 1:1 and 2:1 for 96 h, with 5 μg/ml soluble anti-CD3 and mitomycin-treated APCs. Teff proliferation was determined by flow cytometry measurement of CFSE dilution and was compared with Teff proliferation in the absence of Treg. The percentage of suppression was calculated as described in *Materials and Methods*. Results are expressed as mean ± SEM for each group.

At 24 and 31 wk of age, the total leukocyte count in lymph nodes was markedly decreased in TNF-K– or infliximab-treated mice (infliximab 31 wk, $16.3 \times 10^5 \pm 2.5 \times 10^5$(1); TNF-K 31 wk, $17.2 \times 10^5 \pm 2.5 \times 10^5$(1); untreated 31 wk, $67.2 \times 10^5 \pm 8.9 \times 10^5$; (1)$p < 0.05$ versus untreated 31 wk). This decrease in total cell number leads to a diminished lymph node Teff count (infliximab 31 wk, $5.3 \times 10^5 \pm 0.5 \times 10^5$(1); TNF-K 31 wk, $5.4 \times 10^5 \pm 0.5 \times 10^5$(1); untreated 31 wk, $12.5 \times 10^5 \pm 1.2 \times 10^5$; (1)$p < 0.05$ versus untreated 31 wk) and Treg count (infliximab 31 wk, $5.3 \times 10^4 \pm 0.8 \times 10^4$(1); TNF-K 31 wk, $5.3 \times 10^4 \pm 0.5 \times 10^4$ (1); untreated 31 wk, $16.5 \times 10^4 \pm 1.6 \times 10^4$; (1)$p < 0.05$ versus untreated 31 wk) in these treated mice at 31 wk. However, the percentages of lymph node Teff and Treg were higher in the TNF-K– and infliximab-treated mice than in the untreated mice (Fig. 5*A*, 5*B*), and these differences were more pronounced at 31 wk than at 24 wk. We determined whether the increases in both Teff and Treg percentages in lymph nodes seen in the TNF-K– or infliximab-treated mice modified the Treg/Teff ratio. The most interesting finding was that both of the anti–hTNF-α treatments increased the Treg/Teff ratio at 24 wk compared with untreated TTg mice (Fig. 5*C*). However, a decreased Treg/Teff ratio was observed at 31 wk in anti–TNF-α-treated mice, but percentages of Treg increase in those mice (Fig. 5*B*) and percentages of Teff dramatically increase in anti–TNF-α-treated mice at 31 wk (Fig. 5*A*). Collectively, these data suggest that the decreased Treg/Teff ratio is due to the more pronounced Teff percentage increase and not to a decreased Treg frequency. Overall, results in the spleen were similar to those in the lymph nodes for both Treg and Teff populations (Table IV).

hTNF-α blockade modifies Treg phenotype and enhances Treg suppressive activity

Because the improvement in arthritis produced by hTNF-α blockade was accompanied by an increase in Treg percentages, we investigated whether hTNF-α blockade also modified the Treg phenotype. As shown in Fig. 6*A*, TNFR2 expression (percentage and MFI) at 24 wk was higher in mice treated with infliximab or TNF-K compared with untreated mice. CTLA-4 expression (percentage and MFI) on Treg from the lymph nodes and spleen was also higher in 24- and 31-wk-old mice after both anti–hTNF-α treatments compared with untreated mice (Fig. 6*B*, Table V). Interestingly, the percentage of $CD62L^-$ cells within the Treg population was significantly increased in 31-wk-old mice treated with infliximab or TNF-K (Fig. 6*C*). Moreover, $CD62L^{low}$ Treg frequency among $CD62L^+$ Treg increased in infliximab and TNF-K–treated mice at 31 wk in the lymph node (Fig. 6*D*). Taken together, our results show that hTNF-α blockade by passive (infliximab) or active (TNF-K) immunotherapy induced CTLA-4 upregulation on Tregs and, most importantly, expansion of $CD62L^-$ Treg.

Because the improvement in established arthritis by hTNF-α blockade was accompanied by modifications in the Treg phenotype and an increase in the Treg/Teff ratio in 24-wk-old mice, we

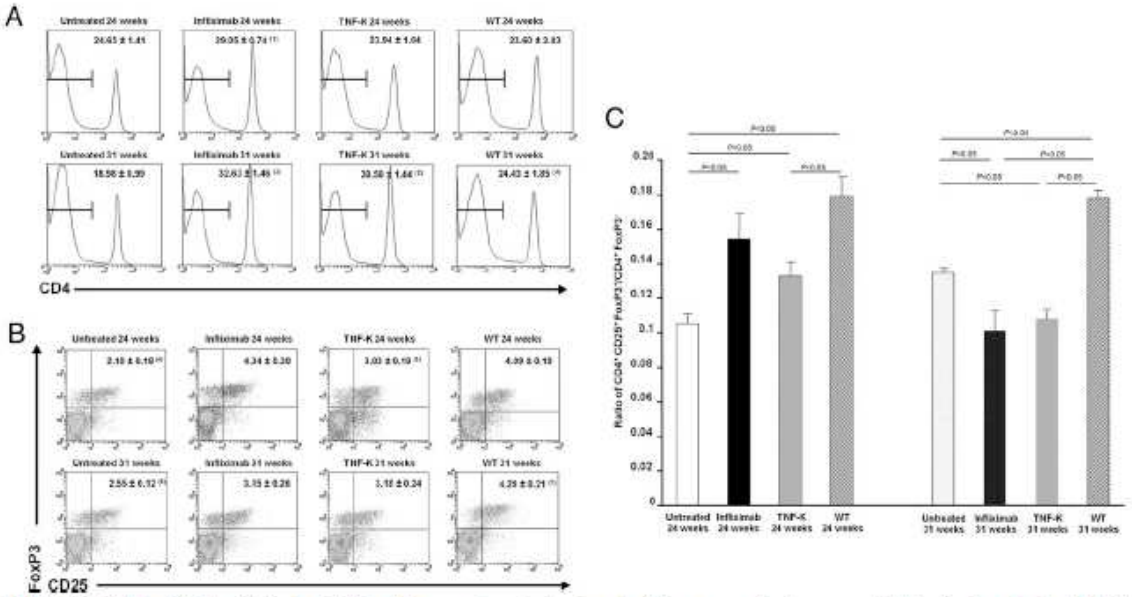

FIGURE 5. Effect of hTNF-α blockade on Teff and Treg percentages in lymph nodes. Three groups of mice were used: TTg mice immunized with TNF-K at 15, 16, and 19 wk of age ($n = 20$); TTg mice treated with infliximab ($n = 16$); and untreated mice ($n = 20$). Half the mice in each group were sacrificed at 24 wk of age and the other half at 31 wk of age. TTg mice are the same as in Table I. Wild-type mice (WT) used as controls were sacrificed at 24 ($n = 6$) and 31 ($n = 6$) wk of age. Lymph node leukocytes were stained with fluorochrome-conjugated anti-CD4, anti-CD25, and anti-Foxp3. As in Fig. 2*A*, $CD4^+$ $Foxp3^-$ and $CD4^+CD25^+Foxp3^+$ cells were monitored in lymph nodes using flow cytometry. *A*, Histograms are shown for one representative mouse in each group and the numbers are the percentages of Teff among lymph node leukocytes reported as mean ± SEM for each group. *B*, Dot plots are shown for one representative mouse in each group and the numbers are the percentages of Treg among lymph node leukocytes reported as mean ± SEM for each group. *C*, % Treg/% Teff ratios are given as means ± SEM for each group: (1) $p < 0.05$ versus WT 24 wk, untreated 24 wk, and TNF-K 24 wk; (2) $p < 0.05$ versus WT 31 wk and untreated 31 wk; (3) $p < 0.05$ versus untreated 31 wk; (4) $p < 0.05$ versus infliximab 24 wk and WT 24 wk; (5) $p < 0.05$ versus infliximab 24 wk, untreated 24 wk, and WT 24 wk; (6) $p < 0.05$ versus infliximab 31 wk, TNF-K 31 wk, and WT 31 wk; (7) $p < 0.05$ versus TNF-K 31 wk and infliximab 31 wk.

Table IV. Effect of hTNF-α blockade on splenic Teff and Treg frequencies

	Group	% Teff	% Treg	% Treg/% Teff Ratio
24 wk	Wild-type	15.07 ± 1.05(1)	1.83 ± 0.15(1)	0.122 ± 0.006
	Untreated	15.99 ± 0.55	1.41 ± 0.14(1)	0.091 ± 0.009
	Infliximab	19.84 ± 1.27(2)	2.41 ± 0.13	0.124 ± 0.012
	TNF-K	18.64 ± 1.26	2.02 ± 0.10(1)(2)	0.111 ± 0.008
31 wk	Wild-type	14.17 ± 1.15	1.56 ± 0.19(3)	0.110 ± 0.007(3)
	Untreated	16.43 ± 0.68	2.09 ± 0.06	0.129 ± 0.004
	Infliximab	16.92 ± 1.07	1.68 ± 0.20	0.118 ± 0.009
	TNF-K	17.80 ± 0.95	1.80 ± 0.10(3)	0.107 ± 0.005(3)

Splenic mononuclear cells from the mice used in Fig. 1 were stained with fluorochrome-conjugated anti-CD4, anti-CD25, and anti-Foxp3. Splenic Teff ($CD4^+Foxp3^-$ cells) and Treg ($CD4^+CD25^+Foxp3^+$ cells) were monitored using flow cytometry. Percentages of Teff and Treg among splenocytes and the % Treg/% Teff ratio are given as means ± SEM.

(1) $p < 0.05$ versus infliximab 24 wk TTg, (2) $p < 0.05$ versus 24 wk TTg, (3) $p < 0.05$ versus untreated 31 wk TTg.

characterized the biological suppressive activity of Treg on Teff after hTNF-α blockade. We found that Treg from 24-wk-old mice treated with infliximab or TNF-K induced greater suppression of Teff proliferation than did Treg from untreated mice (Fig. 7*B*, 7*C*), whereas no difference was seen for suppression of IFN-γ secretion (data not shown).

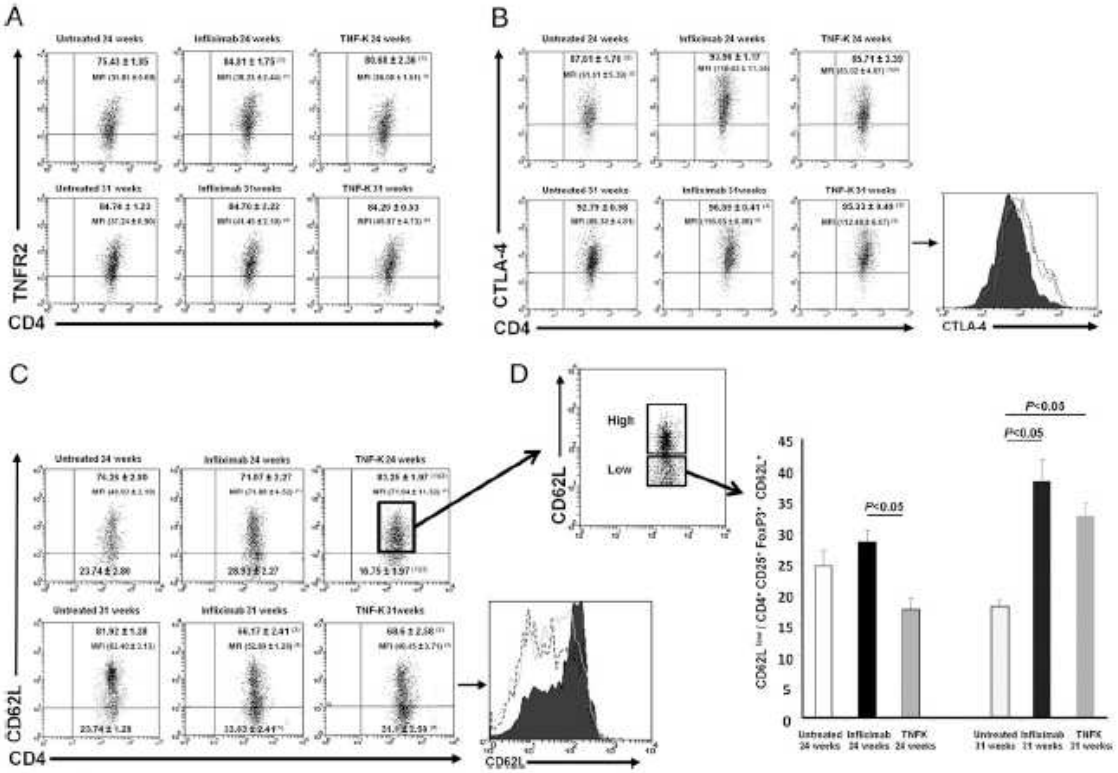

FIGURE 6. Effect of hTNF-α blockade on Treg phenotype in lymph nodes. Lymph node leukocytes from the mice used in Table I were labeled with fluorochrome-conjugated anti-CD25, anti-CD4, anti-Foxp3, and anti-TNFR2 or anti–CTLA-4 or anti-CD62L. Then, TNFR2, CTLA-4, and CD62L expression was studied by gated $CD4^+CD25^+Foxp3^+$ cells using flow cytometry. *A*, Dot plots are shown for one representative mouse in each group and the numbers are the percentages of Treg expressing TNFR2 and the MFI of TNFR2 on $TNFR2^+$ Treg, both given as means ± SEM for each group. *B*, Dot plots are shown for one representative mouse in each group and the numbers are the percentages of Treg expressing CTLA-4 and MFI of CTLA-4 on $CTLA\text{-}4^+$ Treg, both given as means ± SEM for each group. A histogram from a representative mouse in each group is shown; the shaded histogram represents the untreated 31-wk-old group, the gray line the TNF-K–treated 31-wk-old group, and the black dotted line the infliximab-treated 31-wk-old group. *C*, Dot plots are shown for one representative mouse in each group and the numbers indicate the percentage of Treg expressing CD62L and the MFI of CD62L on $CD62L^+$ Treg, both given as means ± SEM for each group. A histogram from a representative mouse in each group is shown; the shaded histogram represents the untreated 31-wk-old group, the gray line the TNF-K–treated 31-wk-old group, and the black dotted line the infliximab-treated 31-wk-old group. (1) $p < 0.05$ versus untreated 24 wk, (2) $p < 0.05$ versus infliximab 24 wk, (3) $p < 0.05$ versus untreated 31 wk. *D*, Frequency of Treg $CD4^+CD25^+Foxp3^+CD62L^{low}$ among $CD4^+CD25^+Foxp3^+CD62L^+$ cells. Data are expressed as mean ± SEM for each group.

Table V. Effect of hTNF-α blockade on splenic Treg phenotype

		TNFR2		CTLA-4		CD62L	
	Group	%	MFI	%	MFI	%	MFI
24 wk	Untreated	74.84 ± 2.90	30.42 ± 1.17	82.08 ± 1.93	56 ± 1.48	76.88 ± 1.22	70.75 ± 3.79
	Infliximab	81.25 ± 1.21(2)	33.58 ± 0.98(2)	97.05 ± 0.46(2)	109.52 ± 4.44(2)	80.84 ± 2.35	104.66 ± 27.57
	TNF-K	66.97 ± 2.99(1)	29.47 ± 0.92(1)	89 ± 2.84(1)(2)	73.89 ± 7.15(1)(2)	74.91 ± 2.73	55.49 ± 6.59
31 wk	Untreated	80.78 ± 1.80	35.96 ± 1.22	92.65 ± 0.74	80.90 ± 2.03	71.49 ± 0.94	64.30 ± 1.75
	Infliximab	86.05 ± 1.12	42.35 ± 1.78(3)	95.30 ± 0.90(3)	97.73 ± 8.67(3)	65.64 ± 6.23	55.20 ± 8.80
	TNF-K	82.91 ± 0.75	40.31 ± 1.58(3)	94.94 ± 0.36(3)	88.36 ± 2.37(3)	73.79 ± 1.12	72.44 ± 3.53

TNFR2, CTLA-4, and CD62L expression by gated $CD4^+CD25^+Foxp3^+$ from the spleen was studied using flow cytometry. The mice are the same as in Fig. 1. Percentage of Treg expressing TNFR2, TNFR2 MFI among $TNFR2^+$ Treg, percentage of Treg expressing CTLA-4, CTLA-4 MFI among $CTLA\text{-}4^+$ Treg, percentage of Treg expressing CD62L, and CD62L MFI among $CD62L^+$ Treg are given as means ± SEM.

(1) $p < 0.05$ versus infliximab 24 wk TTg, (2) $p < 0.05$ versus untreated 24 wk TTg, (3) $p < 0.05$ versus untreated 31 wk TTg.

hTNF-α blockade influences Th1 cells but not Th17 cells

RA is characterized not only by a Treg deficiency but also by an imbalance between proinflammatory Th1 and Th17 cells (25). To determine whether hTNF-α blockade acted via a mechanism involving Th1 or Th17 cells, we monitored the proportions of these cells in the lymph nodes and spleen. No modification was observed for Th17 (Fig. 8*D*), but we found a decrease in the percentage of Th1 cells among $CD4^+$ cells from lymph nodes at 31 wk of age in TNF-K-treated (nonsignificant) and infliximab-treated mice compared with untreated mice (Fig. 8*C*). To further investigate the involvement of Th1 cells in the effects of TNF-α antagonist therapy in TTg mice, we also assessed INF-γ production. Cultured Teff cells from TNF-K– or infliximab-treated 24-wk-old mice produced less IFN-γ than did those from untreated mice (Fig. 8*E*).

Discussion

A link exists between TNF-α and Treg, but its nature remains controversial and has been chiefly studied in vitro. In the current study, we used hTNF-α transgenic mice to characterize the Treg/

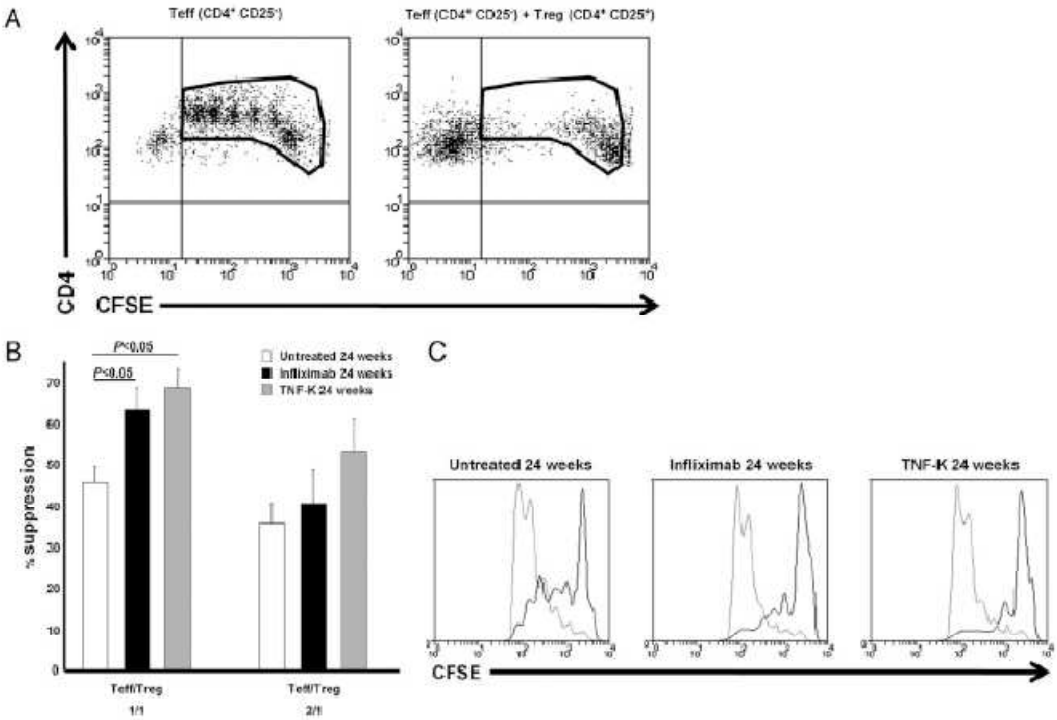

FIGURE 7. Effect of hTNF-α blockade on Treg suppressive activity. TTg mice immunized with TNF-K at 15, 16, or 19 wk of age ($n = 6$), TTg mice treated with infliximab ($n = 5$), and untreated mice ($n = 5$) were used. $CD4^+CD25^+$ (Treg) and $CD4^+CD25^-$ (Teff) were isolated from the spleen of all mice at week 24. $CD4^+CD25^-$ CFSE-labeled T cells were cocultured with $CD4^+CD25^+$ Treg at ratios of 1:1 and 2:1 for 96 h, with 5 μg/ml soluble anti-CD3 and mitomycin-treated APCs. *A*, Representative dot plot showing Teff proliferation among $CD4^+CFSE^+$ cells was determined by measuring CFSE dilution using flow cytometry and was compared with Teff proliferation in the presence of Treg. The percentage of suppression was calculated as described in *Materials and Methods*. *B*, Results are expressed as mean ± SEM for each group. *C*, Proliferation profiles of $CD4^+CFSE^+$ Teff cultured in the presence of Treg at 1:1 ratio (black line) or in the absence of Treg (gray line) are shown for one representative mouse in each group.

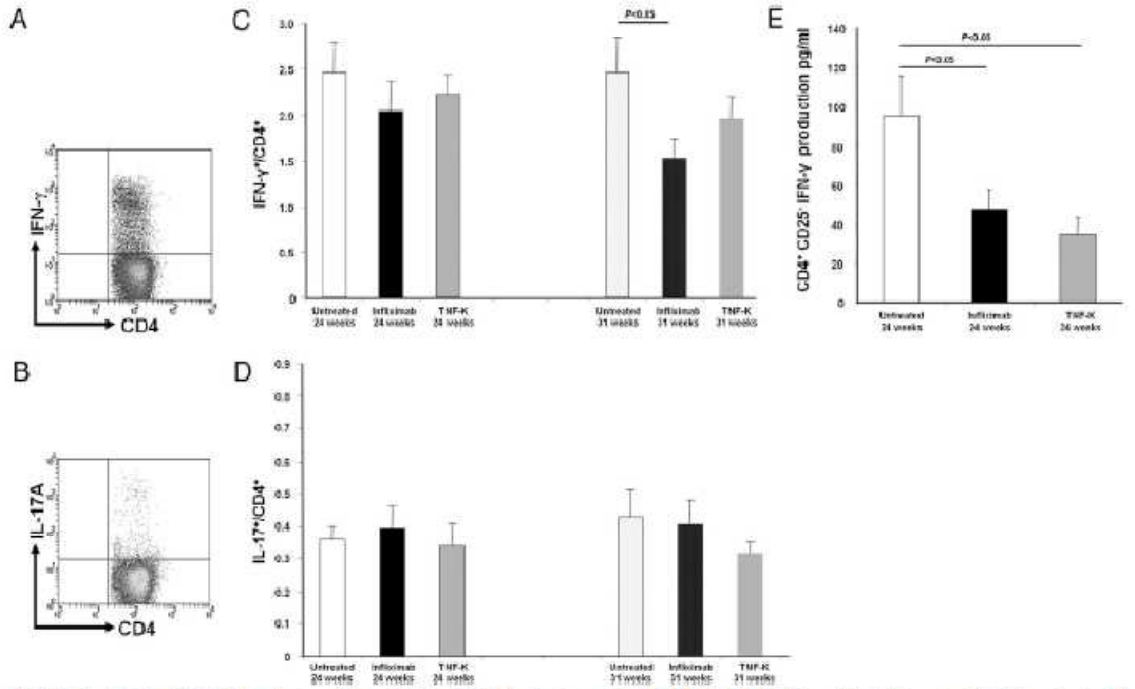

FIGURE 8. Effect of hTNF-α blockade on Th1 and Th17 cells. TTg mice immunized with TNF-K at 15, 16, or 19 wk of age ($n = 20$), TTg mice treated with infliximab ($n = 16$), and untreated mice ($n = 20$) were used. Half the mice in each group were sacrificed at 24 wk of age and the other half at 31 wk of age. Mice are the same as in Table I. Lymph node leukocytes were labeled with fluorochrome-conjugated anti-CD4, anti–IL-17, and anti–IFN-γ Abs. Th1 and Th17, defined as $CD4^+IFN\gamma^+$ (*A*) and $CD4^+IL\text{-}17^+$ (*B*) cells, respectively, were monitored in the lymph nodes using flow cytometry. Percentages of Th1 (*C*) or Th17 (*D*) cells among $CD4^+$ cells in lymph nodes are shown. Results are given as means ± SEM for each group. *E*, IFN-γ secretion by Teff from spleen. Results are given as means ± SEM for each group.

TNF-α link in vivo. TTg mice constitute a relevant model of severe RA and, moreover, a suitable system for studying the effects of human TNF-α antagonists including infliximab and new agents such as active immunotherapy to hTNF-α (4, 6) that are being tested in clinical trials (NCT00808262 and NCT01040715). A major finding from our study is that in vivo in animals exhibiting a proinflammatory state characterized by hTNF-α overexpression, Treg frequency is initially decreased compared with wild-type mice. Then, chronic inflammation development is accompanied by an increased frequency and a phenotype modification of Treg. In TTg mice, hTNF-α blockade by the conventional TNF-α antagonist infliximab or by anti–hTNF-α immunization improved the clinical manifestations of arthritis and induced modification in the amount and activation phenotype of Treg and, more importantly, enhanced their biological suppressive activity on Teff proliferation.

Overexpression of hTNF-α in TTg mice creates a proinflammatory environment that leads mainly to severe joint inflammation and destruction. We found that in TTg mice, although Treg percentages were consistently lower than in wild-type mice, they increased slightly over time. In keeping with this finding, administration of TNF-α to young adult NOD mice increased the number of $CD4^+CD25^+$ Treg in the spleen and thymus (26). In the RA synovium, most studies showed increased Treg counts. However, Treg counts in peripheral blood varied across studies (14, 27–30). These discrepancies can probably be ascribed to differences in RA patient selection and in the definition of Tregs, which hinder comparisons of results across studies. Interestingly, in patients with RA, a smaller percentage of Treg in peripheral blood was found compared with healthy controls only in a patient subgroup with early active RA (31).

Previous studies produced conflicting results regarding the effect of TNF-α on Treg phenotype and function in vitro and in vivo. In RA, TNF-α had no direct effect on Treg in one study (14), but in another study TNF-α inhibited the Treg suppressive effect via a TNFR2-dependent mechanism leading to downregulation of Foxp3 expression (19). Furthermore, in mice, TNF-α interaction with TNFR2 promoted Treg expansion and enhanced Treg function (20). Besides that, Treg are able to shed TNFR2, resulting in TNF-α inhibition (32). More recently, $TNFR2^+$ Treg from human peripheral blood were found to exhibit a stronger suppressive effect than $TNFR2^-$ Treg, suggesting that CD25 and TNFR2 coexpression might identify a Treg population characterized by greater potency compared with the $CD4^+CD25^{high}$ Treg population (21). In our study, arthritis development was accompanied by progressive and marked TNFR2 upregulation on Treg. We can hypothesize that this increased expression leads to TNFR2 shedding by Treg (32), leading to TNF-α neutralization, but insufficiently to inhibit inflammation. Nevertheless, Treg suppressive activity showed no change during the course of arthritis in TTg mice. Taken together, these data indicate that hTNF-α overexpression does not diminish the suppressive effect of Treg.

This study confirms our previous work showing that immunization against TNF-α is effective in treating established chronic inflammatory disease in TTg mice (4, 6), and hTNF-α blockade with infliximab or anti–TNF-α immunotherapy increased the frequency of Treg. In agreement with this finding, a study in RA patients showed that the percentage of $CD4^+CD25^+$ T cells in

peripheral blood was higher in responders to anti–TNF-α therapy than in patients with active RA (14). Moreover, the proportion of $CD4^+Foxp3^+$ cells among PBMC was increased in patients treated with infliximab (18). Regulation of the Treg/Teff balance is crucial to the control of immunity, and in our study hTNF-α blockade increased the Treg/Teff ratio in 24-wk-old mice. Furthermore, hTNF-α blockade had little effect on Th17 cells, whereas another study showed expansion of pathogenic Th17 cells in the lymph nodes and inhibited accumulation of these cells in the synovium (33). We found smaller proportions of Th1 cells among $CD4^+$ T cells, suggesting that Treg might not have been the only T cell subpopulation involved in the effect of TNF-α blockade. Importantly, in addition to Foxp3 Treg, other types of Treg can be induced from naive $CD4^+$ T cells in the periphery, such as TGF-β– and IL-10–producing Tr1 cells and TGF-β–producing Th3 cells. These various Treg types probably cooperate to regulate the immune response.

It is now well established that two distinct populations of Treg coexist, that is, natural Treg ($CD44^{low}CD62L^{high}CD103^-$) generated early in life in the thymus, and induced Treg ($CD44^{high}CD62L^{low}CD103^+$) developing from $CD4^+CD25^-Foxp3^-$ cells in a TGF-β–dependent manner (34). $CD62L^+$ Treg do not express chemokine receptors or homing molecules to inflammatory sites and probably play a major role in inhibiting the activation and proliferation of naive T cells in secondary lymphoid organs. On the other hand, $CD62L^-$ Treg can express different ligands for inflammatory selectins (E- and P-selectin) and chemokine receptors (CCR2, CCR4, and CXCR3) (35), which allows them to migrate to sites of inflammation. We found an increased frequency of $CD62L^{low}$ and of $CD62L^-$ Tregs in infliximab- and TNF-K–treated TTg mice. Consistent with this result, infliximab therapy in patients with RA gives rise to a Treg population that does not express CD62L (18). Those cells exhibited a stronger suppressive effect than did $CD62L^+$ Treg. However, this study showed that $CD62L^+$ Treg remained defective in RA patients after TNF-α antagonist therapy (18). It is therefore difficult to conclude that, in healthy patients, $CD62L^-$ Treg are more or less suppressive than their $CD62L^+$ counterparts. Taken together, these findings indicate that the differential regulatory capacities of the $CD62L^+$ and $CD62L^-$ subsets reflect differences in homing properties, rather than differences in suppressive capacity per se.

Another finding from our study is that hTNF-α blockade upregulated CTLA-4 expression by Treg in the lymph nodes. CTLA-4 participates in the suppressive activity of Treg since its blockade abrogated the suppressor function of Treg in mice (36) and in RA CTLA-4 deficiencies are associated with abnormal Treg function (37). Importantly, Treg phenotype modifications are accompanied by an increase ability of Treg to suppress Teff proliferation in mice treated with infliximab or TNF-K.

Overall, the results of our study support a link between TNF-α and Treg. They suggest that TNF-α may induce an initial defect in Treg, promoting the development of the inflammatory process that leads to arthritis. When chronic inflammation is established, Treg fail to control the harmful inflammatory response. Effects of hTNF-α blockade include an increase in the Treg population, stronger CTLA-4 expression, enhanced suppressive capacity, and differentiation of a $CD62L^-$ Treg population that is likely more able to migrate to the inflamed joints and to exert regulatory effects. Furthermore, our results show that TNF-K therapeutic action is accompanied by Treg modifications, similar to infliximab. Importantly, our results in an in vivo model of a strictly hTNF-α–dependent inflammatory context established that TNF-α can have different effects on Treg depending on the duration of exposure and disease state.

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Disclosures

The authors have no financial conflicts of interest.

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Article 4

Protection from articular damage by passive or active anti-TNFα immunotherapy in human-TNFα transgenic mice depends on anti-TNFα antibody levels

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It has been consistently shown in literature that trough serum levels of anti-TNF monoclonal Abs are a major determinant of treatment efficacy, whilst factors associated with reduced anti-TNF concentration (e.g. anti-drug antibodies, ADA) are associated with less (or absent) clinical response. In the present work we wanted to establish whether, in the same way, the titers of polyclonal anti-TNF Abs induced by TNF-K immunization are predictors of treatment efficacy.

In all the previous experiments on TTG mice with TNF-K we could show that after the primo injection a time lap of 4-5 weeks passed until any clinical effect on arthritis could be detected. This latency of action depends on the time necessary to sensitized B-cells to produce sufficient levels of anti-TNF Abs. For this reason, we wondered whether the co-administation of a short course of infliximab to TNF-K immunized mice would result in earlier TNF-blockade and higher long-term protection from articular damage.

To answer these questions we treated TTG mice with one of the following:

1) TNF-K (1 injections at week 0, 1 and 5),
2) Infliximab (1 weekly injection from week 0 to week 15, the end of the experiment
3) Co-administration of TNF-K (1 injections at week 0, 1 and 5), and a short course of infliximab (1 weekly injection from week 0 to week 5)

The two control groups received:

1) PBS on the same schedule as TNF-K administration. This was the negative control.

2) A short course of infliximab (1 weekly injection from week 0 to week 5). This was the control group for the co-administration arm

We could show that the co-administration strategy actually resulted in significantly more rapid clinical effect vs. solely TNF-K immunization. Nevertheless, we found that the co-administration arm had higher histological inflammation and destruction vs. TNF-K and infliximab monotherapy. Interestingly, the co-administration group had significantly lower titers of anti-TNF polyclonal Abs vs. TNF-K group.

In fact, in all TNF-K treated mice (both TNF-K and the co-administration group) a good and significant inverse correlation existed between anti-TNF Ab titers and histological scores (i.e. mice with high anti-TNF Abs titers had low or absent articular inflammation and destruction). Likewise, there was an inverse correlation between trough infliximab serum levels and histological scores in infliximab-treated mice.

We therefore hypothesized that the reason for the worse outcome in the co-administration group could be lower efficiency of TNF-K immunization at inducing anti-TNF Abs. When all TNF-K treated mice were analyzed, not on the basis of the treatment group, but based on anti-TNF Ab titers we could show that mice with high anti-TNFAb titers had significantly less inflammation and destruction vs. those with low titers.

Thus, we speculated that the reason for the worse efficiency of TNF-K immunization in the co-administration group could be that infliximab would bind to molecules of hTNFα on TNF-K. This binding would both prevent B-cells sensitization by TNF-K and accelerate clearance of immune complexes between infliximab and TNF-K.

We could indirectly support these hypotheses both by showing that infliximab efficiently binds to TNF-K *in vitro* and by showing that the co-administration group had undetectable serum levels of

infliximab vs. its own control group that had received infliximab on the same schedule (i.e. rapid elimination of immune complexes between infliximab and TNF-K in the co-administration group).

In summary, we showed that, just as shown in literature for anti-TNF monoclonal Abs, the titers of polyclonal anti-TNF Abs induced by TNF-K are determinants of treatment efficacy. Factors reducing anti-TNF Abs titers, like infliximab co-administration, reduce TNF-K efficacy and result in higher articular inflammation and destruction.

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Protection from articular damage by passive or active anti-tumour necrosis factor (TNF)-α immunotherapy in human TNF-α transgenic mice depends on anti-TNF-α antibody levels

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Summary

Active anti-tumour necrosis factor (TNF)-α immunization with the kinoid of TNF-α (TNF-K) induces polyclonal anti-TNF-α antibodies and ameliorates arthritis in human TNF-α (hTNF-α) transgenic mice (TTg). We compared the efficacy of TNF-K to that of infliximab (IFX) and of TNF-K and IFX co-administration, and evaluated whether the titres of anti-hTNF-α antibodies induced by immunization were a determinant of TNF-K efficacy. Forty-eight TTg mice received one of the following treatments: TNF-K immunization (TNF-K group); weekly IFX throughout the study duration (IFXw0–15); TNF-K plus weekly IFX for 4 weeks (TNF-K + IFX); and weekly IFX for 4 weeks (IFXw0–4); PBS. Animals were killed at week 16. Anti-hTNF-α antibody titres and clinical and histological scores were compared. All TNF-K immunized mice (TNF-K and TNF-K + IFX) produced anti-hTNF-α antibodies. Titres were higher in TNF-K *versus* TNF-K + IFX ($P < 0.001$) and correlated inversely with histological inflammation ($R = -0.78$; $P = 0.0001$) and destruction ($R = -0.67$; $P = 0.001$). TNF-K + IFX had higher histological inflammation and destruction *versus* TNF-K ($P < 0.05$). A receiver operating characteristic (ROC) analysis of anti-hTNF-α antibody titres identified the criterion cut-off value to discriminate most effectively between the TNF-K and TNF-K + IFX groups. Mice with high *versus* low titres had less histological inflammation and destruction ($P < 0.05$). In a model of TNF-α-dependent arthritis, protection from articular damage by TNF-K correlates with the titres of induced anti-hTNF-α antibodies. The co-administration of TNF-K and a short course of infliximab does not result in less articular damage *versus* solely TNF-K, due probably to lower anti-hTNF-α antibody production. These results are relevant for future development of active anti-TNF-α immunization in human disease.

Keywords: experimental arthritis, infliximab, rheumatoid arthritis, TNF-α transgenic mouse, tumour necrosis factor-alpha

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Introduction

The advent of tumour necrosis factor (TNF)-α-targeting drugs (anti-TNF-α) have changed the perspectives of rheumatoid arthritis (RA) treatment dramatically over the last decade, giving unprecedented results in terms of disease control and structural damage prevention [1]. Nevertheless, only 25–50% of anti-TNF-α-treated patients achieved remission in controlled clinical trials [2,3], and even lower remission rates are described in everyday practice [4]. An approximately similar proportion reaches a functional status comparable to that of the general population [5,6]. Primary or secondary therapeutic failures on anti-TNF-α drugs are not infrequent [7], and there is increasing evidence that the induction of anti-drug antibodies could be a major contributory factor to insufficient response to this class of therapeutics, at least in the case of anti-TNF-α monoclonal antibodies [8–11]. These drawbacks of current anti-TNF-α treatments confirm that there is room for alternative ways to target this key proinflammatory cytokine. Among these, active immunization against TNF-α with TNF-α kinoid (TNF-K) is promising [12,13]. The chemi-

cally inactivated human TNF-α (hTNF-α) is coupled to a carrier protein (the keyhole limpet haemocyanine, KLH). This compound is capable of breaking B cell tolerance to hTNF-α, thereby inducing the production of polyclonal, neutralizing anti-hTNF-α antibodies and avoiding the risk of anti-drug antibody induction [12]. Importantly, TNF-K does not sensitize T cells to native hTNF-α. In the absence of specific T cell help, the rupture of B cells tolerance is transitory, and within 12–20 weeks there is a greater than 50% decline in anti-hTNF-α antibody titres [14–16]. Our group developed the proof of concept of TNF-K applicability in RA using the hTNF-α model transgenic mouse (TTg) [17,18]. TTg mice develop an hTNF-α-dependent spontaneous arthritis and are therefore the pertinent model to study a TNF-α-targeting strategy. We were able to demonstrate the dramatic efficacy of TNF-K in TTg arthritis, with immunized mice showing mild clinical arthritis scores and prevention of histological joint inflammation and destruction compared to control mice [14–16].

Based on the proof of concept established in this model and other preclinical and clinical studies, TNF-K entered clinical development for RA and a Phase IIa clinical trial is now terminated (Clinical trials.org identifier: NCT01040715). In all the experiments TNF-K showed a slower onset of clinical effect compared with a monoclonal anti-TNF-α antibody [infliximab (IFX)]. This latency results from the time necessary for antibody production by sensitized B cells. Conversely, two or three TNF-K immunizations over 4 weeks resulted in a longer-lasting clinical effect *versus* IFX given weekly for the same period [15,16].

The aims of the present study in TTg mice were: (i) to compare the efficacy of TNF-K to that of long-term IFX treatment and of the co-administration of TNF-K and IFX; and (ii) to determine whether the levels of anti-hTNF-α antibodies induced by TNF-K are correlated with articular damage and may therefore represent a prognostic factor for immunized mice.

Materials and methods

Mice

Forty-eight male hTNF-α hemizygous TTg, 4–8 weeks old, were purchased from Taconic Farms (Germantown, NY, USA). They were divided into four groups of 10 mice and one group of eight mice, and identified according to the study protocol described below. These mice develop a spontaneous arthritis between 8 and 10 weeks of age [17,18]. They were weighed and monitored for evidence of arthritis in the four paws throughout the duration of the experiment, and killed at week 16 after arthritis onset. All procedures were approved by the Animal Care and Use Committee of the University of Paris 13 (ethical approval ID: Ce5/2010/036).

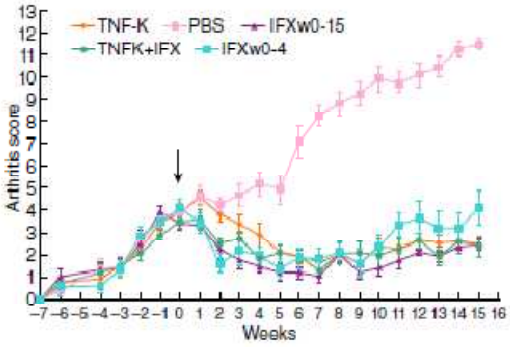

Fig. 1. Study protocol and arthritis scores. All treatments were started at week 0 (black arrow). The tumour necrosis factor-α-kinoid (TNF-K) group (orange diamonds) received three immunizations with TNF-K at weeks 0, 1 and 4 of the experiment. The phosphate-buffered saline (PBS) control group (pink squares) received three injections of PBS at weeks 0, 1 and 4. The infliximab (IFX)w0–15 group (purple triangles) received weekly injections of IFX from weeks 0 to 15. The association group TNF-K + IFX (green circles) received three immunizations with TNF-K at weeks 0, 1 and 4 and weekly injections of IFX from weeks 0 to 4. The IFXw0–4 group (blue squares) received weekly injections of IFX from weeks 0 to 4. The clinical score curves report the mean ± standard error of the mean of the clinical scores for each group at each observation.

Reagents

We obtained hTNF-α kinoid (TNF-K), a protein complex of hTNF-α and KLH, from Neovacs SA (Paris, France), as described previously [14,15]. Dulbecco's phosphate-buffered saline (PBS) was purchased from Eurobio (Les Ulis, France), ISA-51 adjuvant from Seppic (Paris, France) and IFX (Remicade®) from Schering-Plough (Levallois-Perret, France).

Study protocol

The study protocol is presented in Fig. 1. Week 0 is defined as the week when treatments were started. The treatment groups were: (i) immunization with TNF-K (TNF-K group), 10 mice; (ii) PBS as negative control (PBS group), 10 mice; (iii) weekly IFX throughout the experiment duration, from weeks 0 to 15 (IFXw0–15 group), 10 mice; (iv) immunization with TNF-K plus weekly IFX from weeks 0 to 4 (TNF-K + IFX group), 10 mice; and (v) weekly IFX from weeks 0 to 4 (IFXw0–4 group), eight mice.

TNF-K administration

Animals were injected intramuscularly with 10 µg TNF-K in a 1:1 emulsion with ISA-51 (100 µl) at weeks 0, 1 and 4 of the study.

PBS administration

Animals were injected intramuscularly with 100 µl of PBS in a 1:1 emulsion with ISA-51 (100 µl) at weeks 0, 1 and 4 of the study.

IFX administration

Animals were injected with IFX intraperitoneally, 10 mg/kg weekly, from weeks 0 to 15 or from weeks 0 to 4, according to the treatment schedule.

Blood samples

Blood samples were collected before the first treatment injection (week 0), then at weeks 5, 14 and at killing (week 16) for anti-hTNF-α and anti-KLH antibody titrations, IFX titrations and hTNF-α neutralizing capacity. Blood collection for each treatment group was performed just before treatment administration.

Clinical arthritis assessment

Weekly clinical assessment of arthritis was started from the receipt of the animals. Evaluations were carried out by an assessor unaware of assignment to treatment groups. Clinical severity of arthritis for each paw (fingers, tarsus and ankle) was quantified on a score ranging from 0 (normal) to 3 (severe inflammation with deformation) [16]. The score of each paw was summed, resulting in an overall arthritis score ranging from 0 to 12. The presented clinical score curves (Fig. 1) are based on mean arthritis score ± standard error of the mean (s.e.m.) at each clinical observation for each treatment group.

Histological arthritis assessment

All animals were killed at week 16. Right forelimbs and left hind limbs were collected, fixed, decalcified, dehydrated and set in paraffin blocks. Slides of 5 µm thickness were obtained. At least four serial sections were realized for each paw in order to obtain a reliable spatial evaluation of articular samples. Slides were stained with either haematoxylin and eosin or safranin-O before microscopic observation (optical microscope). Synovitis (articular inflammation) and bone erosions (articular destruction) were defined on haematoxylin and eosin-stained slides. Lesions were evaluated quantitatively on each slide using a three-point scale ranging from 0 to 3, where 0 = normal articulation; 1 = slight inflammation and thickening of the synovium; 2 = mild thickening of the synovium and mild inflammation with invasion of the subsynovial area by inflammatory cells; and 3 = severe inflammation and massive invasion of adjacent tissues by pannus [19]. Other sections were scored for loss of safranin-O staining as a measure of cartilage proteoglycan depletion, using a scale from 0 to 3 where 0 = no depletion; 1 = depletion of staining and thinning down of the lateral superficial layer; 2 = depletion of staining and thinning down of the central superficial layer; and 3 = severe and mostly complete depletion of staining in the superficial layer [19].

Antibody assays

Sera were obtained and tested for individual anti-TNF-α antibody titres and IFX trough levels from blood samples collected at different time-points during the experiment (weeks 0, 5 and 14) and at killing (week 16). Pooled sera from each group were tested for anti-KLH antibody titres and hTNF-α neutralizing capacity. Specific mouse anti-hTNF-α and anti-KLH antibody titres were determined using a direct enzyme-linked immunosorbent assay (ELISA). Precoated ELISA plates with 100 ng per well of hTNF-α or KLH were incubated with serial dilutions of sera collected from all groups. Specific immunoglobulin (Ig)G was detected by using horseradish peroxidase-conjugated (HRP) rabbit anti-mouse IgG (Zymed Laboratories Inc., now part of Invitrogen Corporation, Carlsbad, CA, USA). To each well, substrate solution was added (Fast OPD; Sigma, St Louis, MO, USA), then the reaction was stopped with H_2SO_4. The optical density (OD) was read at 490 nm. IFX titration was similar to mouse anti-hTNF-α titration except that we used HRP-conjugated mouse anti-human IgG1 (Zymed) instead of HRP-conjugated rabbit anti-mouse IgG. It is worth noting that HRP rabbit anti-mouse IgG does not cross-react with IFX.

The neutralizing capacity of anti-hTNF-α antibodies was assessed by L929 cytotoxicity assay, as described elsewhere [14]. Briefly, mouse fibroblast L929 cell line (CCL1) (American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cells were seeded in flat-bottomed 96-well plates at $2{\cdot}10^4$ cells per well. After 18 h of incubation at 37°C, serial dilution of serum with 2·5 ng/ml hTNF-α dose was added on L929 cells with 1 µg/ml of actinomycin D. After 18 h of incubation at 37°C, [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt]/phenazine methosulphate (MTS/PMS) was added for 4 h at 37°C. The OD at 490 nm was read for each well. The neutralization titre was expressed as the mean of the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity (NC50).

Antigenicity test

Binding of IFX to TNF-K was measured by direct antigenicity assay. Briefly, eight serial dilutions of TNF-K or hTNF as control were coated onto plates in PBS buffer at a starting dilution of 1000 ng/well. After plate saturation, targeted hTNF-α epitopes were detected using biotinylated IFX and revealed by streptavidin–HRP (Southern Biotech, Bir-

mingham, AL, USA). After 6 min of o-phenylenediamine (OPD) incubation, absorbance was read at 490 nm.

Statistical analysis

Data distribution was preliminary checked by the Kolmogorov–Smirnov test. According to data distribution, values are expressed as mean and standard deviation or median and interquartile range. Serial measurements of clinical scores were analysed considering the area under the curve (AUC) for each mouse as a summary measure; these measures were then analysed as raw data [20]. According to data distribution and number of groups, a parametric [analysis of variance (ANOVA), *t*-test] or non-parametric (Kruskal–Wallis, Mann–Whitney) test was then performed. *Post-hoc* comparisons were performed with the appropriate test according to data distribution (Student–Newman–Keuls for parametric data, corrected Mann–Whitney test for non-parametric data). For individual anti-hTNF-α titres and IFX levels, we calculated both the AUC and the geometric mean. Both measures were highly correlated ($R = 0{\cdot}91$); however, given the reduced number of blood samples, for subsequent analysis we chose to rely only on the geometric mean, which was therefore used as summary measure, in a *t*-test for comparison of anti-hTNF-α titres for the TNF-K *versus* TNF-K + IFX groups and for IFX levels for the IFXw0–15 *versus* IFXw0–4 and for TNF-K + IFX *versus* IFXw0–4 groups, respectively. Pooled anti-KLH titres and anti-TNF-α neutralizing activity values were analysed by Kruskal–Wallis test with appropriate *post-hoc* analysis. Histological scores are given as the arithmetic mean of all articular site scores for each mouse, and were compared with the Kruskal–Wallis test, with *post-hoc* comparison with the corrected Mann–Whitney test. The criterion value to discriminate between TNF-K-immunized groups on the basis of the geometric mean of anti-hTNF-α antibody titres was calculated with receiver operating characteristic (ROC) curve analysis. Pearson's correlation was used to correlate histological scores and the geometric mean of anti-hTNF-α antibody titres for each TNF-K-treated mouse, and to correlate the histological scores and the geometric mean of serum levels of IFX for each IFX-treated mouse. All statistical analyses were performed with MedCalc software version 10·4 (MedCalc Software bvba, Mariakerke, Belgium).

Results

Effect of TNF-K, IFX and their co-administration on arthritis

When the mice exhibited an average clinical score of 3 (scoring range from 0 to 12; see Materials and methods), treatment was started for all mice (week 0). We waited until each mouse exhibited sign of arthritis in at least one paw and the mean clinical score for each cage was at least 3 (scoring range from 0 to 12; see Materials and methods), then we started treatment for all mice (week 0). As expected, the PBS control group developed severe arthritis rapidly over a 15-week period. Conversely, all treatment groups showed evident amelioration of arthritis *versus* the PBS group (Fig. 1). We observed a rapid clinical effect mainly in the IFX-treated groups, while in a subsequent phase we also observed a clinical amelioration in the TNF-K group, and an aggravation in the group that received IFX only during the first 4 weeks (IFXw0–4).

We therefore conducted the clinical score curve analysis along three time-periods: throughout the whole duration of the experiment, for the initial part of the experiment (from weeks 0 to 7) and for the final part of the experiment (from week 10 to killing). For the overall experiment duration, the analysis was aimed to determine whether one treatment produced lower clinical scores throughout the study duration. There was overall significant variability ($P < 0{\cdot}001$), with all active treatments showing lower scores compared to PBS groups ($P < 0{\cdot}05$ for all *post-hoc* comparisons). Despite the qualitative impression of a difference between active treatment groups, with IFXw0–4 appearing less effective (Fig. 1), no significant difference could be detected in terms of AUC for a particular treatment, due probably to lack of statistical power. For the initial part of the experiment, the analysis of the curves from weeks 0 to 7 confirmed the rapid clinical efficacy of IFX *versus* TNF-K. The clinical scores of the PBS and TNF-K group were higher compared to those of all IFX-treated groups (IFXw0–4, IFXw0–15, TNF-K + IFX) ($P < 0{\cdot}05$). This confirmed that TNF-K treatment has a slower onset of action compared to IFX, and that the co-administration of IFX with TNF-K can overcome this latency. For the final part of the experiment, at week 10 of the experiment all IFX- and TNF-K-treated groups had similar clinical scores ($P = 0{\cdot}8$). At killing, the group that received IFX during the first 4 weeks (IFXw0–4) displayed significantly higher clinical scores *versus* the IFXw0–15, TNF-K + IFX and the TNF-K groups ($P < 0{\cdot}05$), while no difference was detectable between IFXw0–15 and both the TNF-K-treated groups. This confirms the longer-lasting clinical effect of TNF-K immunization given three times from weeks 0 to 4 compared to IFX given during the first 4 weeks. The best clinical control of arthritis could be assured only by long-term treatment with IFX (group IFXw0–15) or by TNF-K immunization (TNF-K and TNF-K + IFX groups).

Effects of TNF-K, IFX and their co-administration on histological inflammation and destruction

Histological scores of the different treatment groups at week 16 are presented in Fig. 2, ordered from the lowest to the highest. All treatment groups had significantly lower scores of histological inflammation *versus* PBS ($P < 0{\cdot}05$). The TNF-K group was the group with the lowest scores and

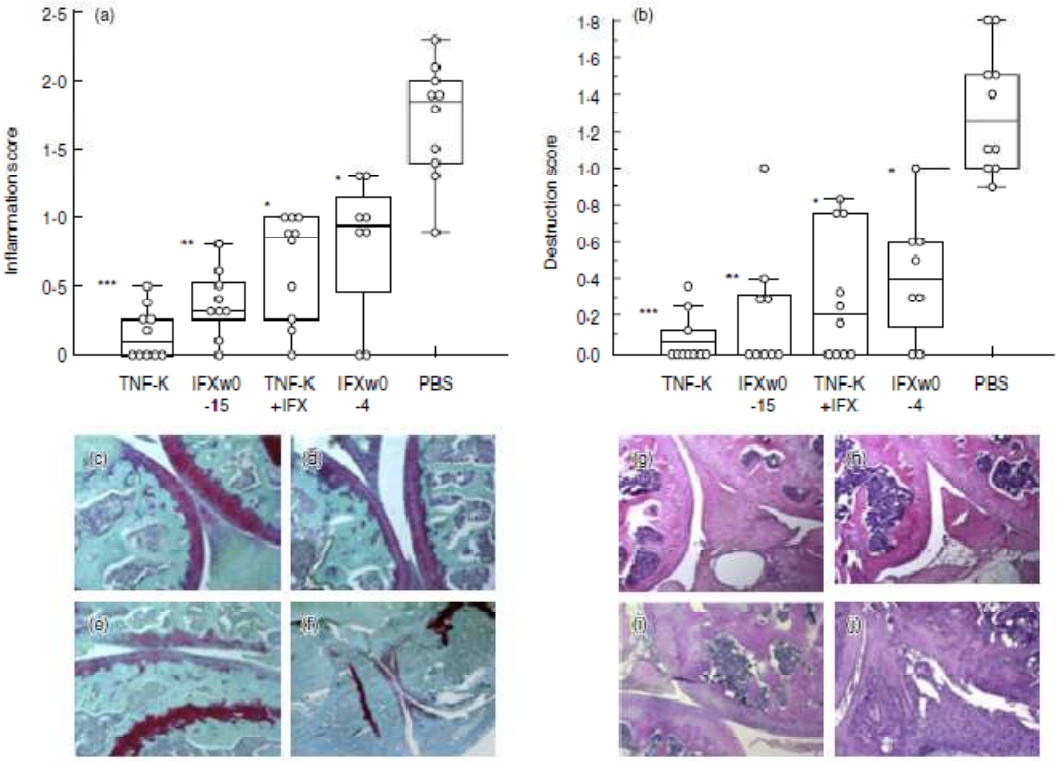

Fig. 2. (a,b). Box-and-whisker plot with all data (arithmetic mean of all articular site scores for each mouse) plotted for each treatment group of histological inflammation (a) and destruction (b) scores [haematoxylin-eosin (HE) staining], compared for each group. All groups had lower scores than the phosphate-buffered saline (PBS) control group. The tumour necrosis factor-α-kinoid (TNF-K) group had significantly lower scores *versus* the PBS, infliximab (IFX)w0–4 and TNF-K + IFX groups for both inflammation and destruction scores. $^{*}P < 0{\cdot}05$ *versus* PBS; $^{**}P < 0{\cdot}05$ *versus* PBS and IFXw0–4; $^{***}P < 0{\cdot}05$ *versus* PBS, IFXw0–4 and TNF-K + IFX. Representative examples (c–j) of histological section for each treatment group stained, respectively, with safranin O (c–f) for cartilage–proteoglycan depletion and with haematoxylin and eosin (g–j) for articular inflammation and destruction. The TNF-K and the IFXw0–15 groups show good protection from cartilage and proteoglycan depletion (c,d) and from histological inflammation (g,h). The PBS group displays marked cartilage depletion (f) and synovial inflammation and destruction (j). In some mice in the TNF-K + IFX group, moderate cartilage depletion (e) and synovial inflammation and destruction (i) were detected.

the only one that displayed significantly lower scores *versus* the TNF-K + IFX and IFXw0–4 groups ($P < 0{\cdot}05$ for both comparisons) (Fig. 2a). Similarly, all treatment groups had significantly lower scores of histological destruction *versus* PBS ($P < 0{\cdot}05$), with TNF-K displaying significantly lower scores *versus* both the TNF-K + IFX and IFXw0–4 groups ($P < 0{\cdot}05$) (Fig. 2b). Histological destruction scores with safranin-O staining showed a similar distribution (not shown). Representative examples of histological sections are shown in Fig. 2c–j.

Anti-hTNF-α antibody titres induced by TNF-K immunization

Because the co-administration of IFX and TNF-K (TNF-K + IFX) did not result in lower clinical and histological scores compared to either treatment alone, we investigated whether this might be due to lower efficiency of TNF-K immunization at inducing anti-hTNF-α antibodies in this group. Despite the fact that anti-hTNF-α antibodies were detected in all mice, mice in the TNF-K + IFX group had lower anti-hTNF-α antibody titres *versus* those in the TNF-K group ($P < 0{\cdot}05$) (Fig. 3a,b). Conversely, the two groups did not differ in terms of anti-KLH antibody titres ($P = 0{\cdot}7$) (analysis conducted on pooled sera for each group, data not shown). To confirm a link between anti-hTNF-α antibodies and histological damage, we subsequently investigated whether the anti-hTNF-α antibody titres were correlated with histological scores. For this purpose, the geometric mean of anti-hTNF-α antibody titres at three different blood samplings was calculated for each mouse. We then tested the correlation between the

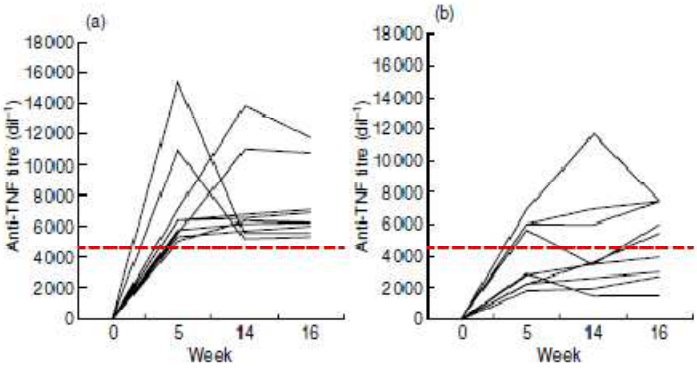

Fig. 3. Anti-human tumour necrosis factor (hTNF-α) antibody titres in tumour necrosis factor-α-kinoid (TNF-K) (a) and TNF-K + infliximab (IFX) (b) groups as detected by enzyme-linked immunosorbent assay (ELISA). The graph shows antibody titres in the three different blood samples for each mouse. The TNF-K group had higher titres than TNF-K + IFX ($P < 0{\cdot}01$). A receiver operating characteristic (ROC) curve analysis on the geometric mean of anti-hTNF-α antibody titres for each mouse identified the value of 4211 as the cut-off value to discriminate between the two groups (dotted line). Nine mice in the TNF-K group and four mice in the TNF-K + IFX groups had higher anti-hTNF-α titres than the cut-off.

geometric mean of anti-hTNF-α antibody titres and the histological scores for each mouse, finding an inverse correlation with all histological scores (Fig. 4). This correlation was good and significant for both inflammation [correlation coefficient (R) = −0·78; P = 0·0001] (Fig. 4a) and destruction scores (R = −0·67; P = 0·001) with haematoxylin and eosin (Fig. 4b) and moderate and significant for safranin O destruction scores (R = −0·51; P = 0·01, not shown).

Because anti-hTNF-α antibody titres were correlated inversely with histological scores of inflammation and destruction, and as the TNF-K + IFX group had lower anti-

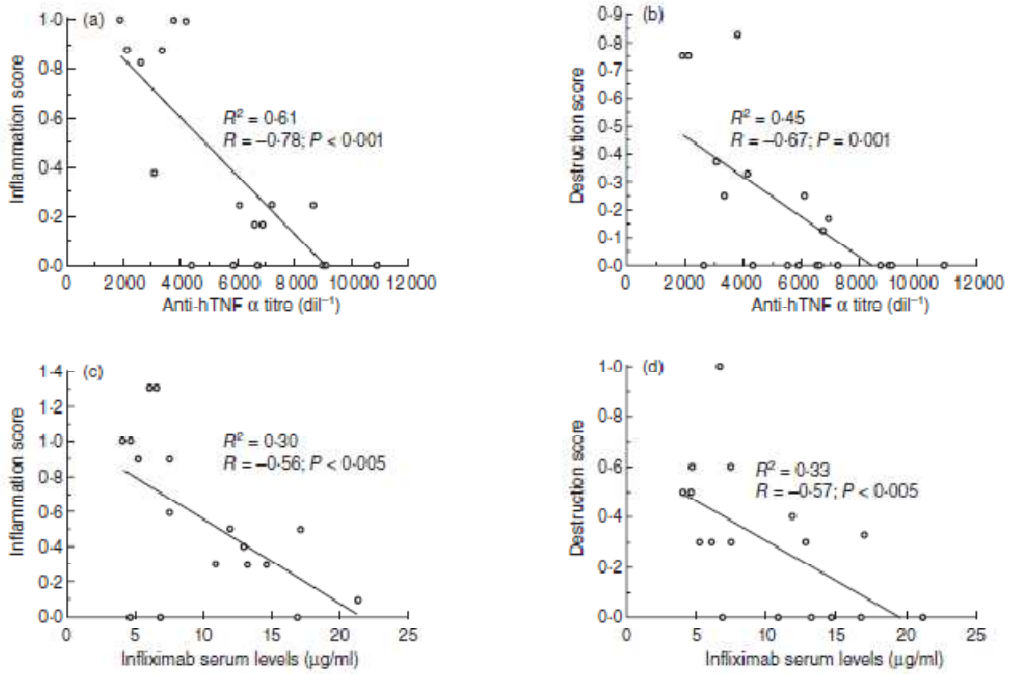

Fig. 4. (a,b) Tumour necrosis factor-α-kinoid (TNF-K)-treated mice [TNF-K and TNF-K + infliximab (IFX) groups]: correlation (with scatter diagram and regression line) between the geometric mean of TNF-K-induced anti-hTNF-α antibody titres and histological inflammation scores (a), and histological destruction scores (b) for each individual mouse. (c,d) IFX-only treated mice (IFXw0–15 and IFXw0–4 groups): correlation between the geometric mean of IFX trough serum levels and histological inflammation scores (c) and histological destruction scores (d) for each individual mouse.

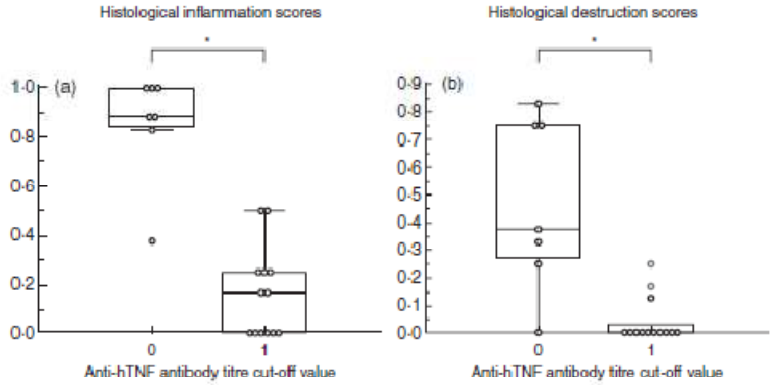

Fig. 5. Box-and-whisker plot with all data (arithmetic mean of all articular site scores for each mouse) plotted of histological inflammation (a) and destruction (b) scores [haematoxylin-eosin (HE) staining] categorized on the basis of anti-human tumour necrosis factor (hTNF-α) antibody production: higher (1) or lower (0) than the cut-off value of anti-hTNF-α antibody geometric mean that best allows to discriminate between TNF-K and TNF-K + infliximab (IFX) groups (4211) (see Fig. 4). Mice with higher anti-hTNF-α antibody titres had both lower inflammation and destruction scores. *$P < 0{\cdot}05$.

hTNF-α antibody titres *versus* TNF-K, we aimed to determine whether this factor might have accounted for the higher histological scores of the TNF-K + IFX group. We performed a ROC curve analysis on the geometric mean of anti-hTNF-α antibody titres in order to identify the criterion cut-off value to discriminate most clearly between the TNF-K and TNF-K + IFX groups in terms of antibody production. Nine mice in the TNF-K group and four of the mice in the TNF-K + IFX group had higher antibody titres than the cut-off (4211 dil^{-1}) (Fig. 3). We then compared the histological scores for mice having higher or lower antibody titres than the cut-off, and we showed that mice with higher antibody titres had significantly lower inflammation and destruction scores (with both haematoxylin and eosin and safranin O staining) ($P < 0{\cdot}05$ for all differences; Fig. 5).

The hTNF-α neutralizing capacity was evaluated by L929 cytotoxic assay at weeks 5, 14 and 16 on pooled sera from each group. The pooled sera from the TNF-K group displayed higher hTNF-α neutralizing capacity *versus* those from the TNF-K + IFX group ($P < 0{\cdot}05$) (not shown). Conversely, the TNF-K and IFXw0–15 groups displayed similar neutralizing capacity.

IFX serum levels and histological scores

As expected, the geometric means of IFX serum levels were higher for all mice in the IFXw0–15 group *versus* all those in the IFXw0–4 group ($P < 0{\cdot}001$). The geometric mean of the serum levels of IFX showed inverse correlation with histological inflammation ($R = -0{\cdot}56$; $P < 0{\cdot}05$) and destruction scores with haematoxylin and eosin ($R = -0{\cdot}57$; $P < 0{\cdot}05$) (Fig. 4c,d), and with safranin O staining ($R = -0{\cdot}56$; $P < 0{\cdot}05$) (not shown).

Lower efficiency of TNF-K immunization in the TNF-K + IFX group

We hypothesized that the lower production of anti-hTNF-α antibody in the group that received TNF-K and IFX co-administration might be due to binding of IFX to the molecules of TNF-α exposed by the TNF-K, with consequent hindered interaction between TNF-K and B cell receptors and/or formation of immune complexes between TNF-K and IFX, with subsequent higher clearance of both. To evaluate this hypothesis, we assessed whether IFX would bind to TNF-K in a direct antigenicity test. As shown in Fig. 6, IFX binds to TNF-K, which confirms that the molecules of hTNF-α exposed by the TNF-K keep expressing the conformational epitope recognized by IFX, even after chemical inactivation and coupling to KLH. We also observed that the serum levels of IFX in the TNF-K + IFX

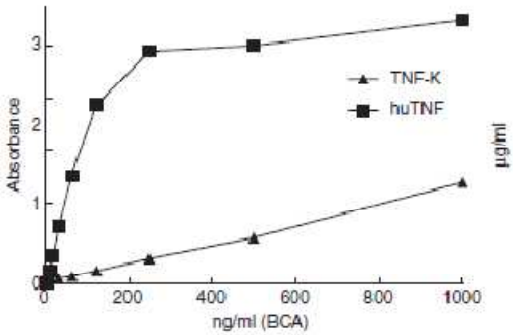

Fig. 6. Binding of infliximab to tumour necrosis factor-α-kinoid (TNF-K) (direct antigenicity).

group at the three blood samplings were always below the detection threshold, while the IFXw0–4 group, which had received IFX on the same schedule, had detectable levels of IFX at day 35 ($P < 0.001$ for geometric mean comparison between the two groups).

Discussion

In this study we show that the histological efficacy of TNF-K correlated with the titres of anti-hTNF-α antibodies induced by active anti-hTNF-α immunization. The co-administration of a short course of IFX with TNF-K overcame the latency in TNF-K clinical effect, but did not result in lower histological damage *versus* either strategy alone. Both TNF-K and long-term IFX treatments resulted in comparable clinical control of arthritis and prevention of histological inflammation and damage. IFX had a more rapid clinical effect than TNF-K, but this did not result in less histological damage at the end of the study (week 16). In addition, the TNF-K group was the only one to show significantly lower histological scores compared to the group that received IFX over 4 weeks and *versus* the group that received both TNF-K and IFX. In all TNF-K immunized mice (both TNF-K and TNF-K + IFX groups), histological destruction and inflammation scores showed a significantly good inverse correlation with the titres of anti-hTNF antibodies, and mice with higher serum levels of anti-hTNF-α induced by TNF-K immunization had significantly lower histological scores. This provides evidence for a dose–response effect of anti-hTNF antibodies induced by TNF-K, which explains the efficacy of active immunization. A similar correlation between IFX serum levels and histological scores was found in IFX -treated mice. It is noticeable that, in TTg mice, we have demonstrated previously the importance of anti-hTNF-α antibody generated after TNF-K immunization, as the transfer of sera from TNF-K immunized mice to naive mice induced strong protection from TNF-α-galactosamine-induced shock [14]. The anti-TNF-α inhibition capacity of sera (with K_d ranging from 5×10^{-8} M to 10^{-10} M [14]) was not higher than those of IFX (K_d from 10^{-9} to 10^{-12} M [21]) or adalimumab (K_d between 5·8 and 8·7 10^{-11} M [22]), which is reassuring concerning potential excessive TNF-α inhibition on TNF-K treatment.

Co-administration of active (TNF-K) and passive (IFX) anti-hTNF-α immunization overcomes the delay in therapeutic activity of TNF-K, but the group that received the co-administration did not fare better than the groups receiving either treatment alone with regard to global clinical scores of arthritis. Moreover, this group had higher histological scores *versus* either treatment alone (with differences that reached significance *versus* TNF-K group). This worse histological effect may be due to the fact that in the TNF-K + IFX group, and specifically in six of 10 mice, lower levels of anti-hTNF-α antibody were detected compared to the group that received only TNF-K. When histological scores were compared after categorization based not on the group of treatment, but on the titres of anti-hTNF-α antibody, the mice with low titres had significantly higher histological inflammation and destruction *versus* all other TNF-K-treated mice (from both TNF-K and TNF-K + IFX groups).

Thus, the TNF-K + IFX-treated mice were protected at the beginning of the experiment by IFX administration over the first 4 weeks while, later on, when the effect of IFX subsided, they had lower protection from anti-hTNF-α antibody induced by TNF-K immunization compared to the TNF-K group. Conversely, anti-KLH antibody titres did not differ between the two groups, suggesting that the lower efficacy of immunization is not dependent on the lack of T cell help. As TNF-K and IFX were administered together, we can speculate that IFX might have bound some molecules of hTNF-α onto TNF-K. This binding could prevent anti-TNF-α-specific B cell activation or accelerate clearance of immune complexes between IFX and TNF-K, or both. To partially support this hypothesis, we demonstrated that IFX binds efficiently to TNF-K *in vitro*, and that the process of chemical inactivation and coupling of hTNF-α to KLH does not alter the epitope recognized by IFX on the hTNF-α molecule. Moreover, the mice in the TNF-K + IFX group had undetectable serum levels of IFX, while in the IFXw0–4 group (which had received IFX on the same schedule) the drug was detectable. This supports a possible higher elimination of IFX in the former group, due possibly to the formation of immune complexes between IFX and TNF-K. The main limit of the study is that the co-administration strategy was tested with only one anti-TNF-α agent (IFX). It would be of interest to test the clinical and histological effect of the co-administration of TNF-K with other anti-TNF-α treatments, such as adalimumab, etanercept or certulizumab pegol. Clinically, it would be important to determine whether or not the lack of efficacy of the co-administration is IFX-restricted.

The results of this study bring two important elements for the future development of active anti-TNF-α inhibition in RA. First, the histological efficacy of active immunization, comparable to that of a long-term treatment with a standard anti-TNF-α drug, depends upon the amount of induced anti-hTNF-α antibodies. Secondly, the association of a passive (IFX) and active anti-TNF-α treatment allows a more rapid disease control compared to TNF-K, but does not result in less articular damage. The identification of prognostic factors of therapeutic success and the effect of the co-administration of passive and active anti-TNF-α immunization are major issues of interest for the further potential clinical development of TNF-K in human disease.

These results were obtained in the TTG mouse, which develops a spontaneous erosive polyarthritis dependent mainly on deregulated constitutive production of hTNF-α. As anti-TNF antibodies generated by TNF-K target hTNF-α

and not murine TNF-α, this model is the only relevant one to test the efficacy of an anti-hTNF-α treatment.

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Disclosure

TNF-K is patented and the patent is held by Neovacs SA (Paris, France). G.G.V., E.B. and O.D. are scientist employees with Neovacs SA. M.C.B. has been a consultant for Neovacs SA and his laboratory has received unrestricted research grants from Neovacs, Pfizer, UCB Pharma and Roche. The other authors declare that they have no competing interests.

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DISCUSSION

1 DISCUSSION

This work was centered on the steps of development of active anti-humanTNFα immunization with the TNFα kinoid (TNF-K) necessary, after the proof of concept, to justify kinoid applicability in clinical practice for RA treatment.

The detailed steps of the process can be summarized as follows. First, we demonstrated that TNF-K immunization is as effective as monoclonal anti-TNFα Abs in animals who have already developed clinical arthritis, a scenario that best resembles to that of patients, who are treated when their disease is fully developed.

Second, we could demonstrate that anti-TNFα Ab response is transitory and follows a bell-shaped curve. In the same way, clinical amelioration of arthritis is followed by a worsening of the disease, corresponding to a decline in serum anti-TNFα Abs. An additional administration of TNF-K induces another boost of antibody production and a reduction in clinical scores of arthritis. Conversely, challenging of the animals with the native cytokine could not induce any anti-TNFα Ab response.

Third, we demonstrated that immunosuppressive treatments currently used in association with biologics in RA treatment do not impair the polyclonal anti-TNFα response induced by TNF-K immunization. Fourth, we could show that cellular mechanisms associated to (and probably underlying) successful anti-TNF blockade in a setting of high TNFα production, are the same for both passively administered monoclonal anti-TNFα Abs and kinoid-induced, actively synthesized, polyclonal anti-TNFα antibodies. Fifth, we could demonstrate that the efficacy of TNF-K treatment depends on the titers of anti-TNFα Abs induced by immunization, providing a clear dose-response effect for active immunization. Sixth, we could demonstrate that the association of infliximab to TNF-K reduces the efficacy of both treatments, and that this reduced efficacy is, again, associated with lower anti-TNFα Abs production.

1.1 Active anti-cytokine immunization: the principle

The principle of anti-cytokine vaccination is to design molecules capable of triggering a humoral immune response versus a cytokine with a recognized pathogenic role in a given disease. The most used vaccines use either the self-protein coupled to a carrier (type I A vaccination), or a modified form of the protein, engineered to include neo-epitopes (type I B)(210). Essential safety requirements for anti-cytokine vaccination are the reversibility of the humoral response and the fact that vaccination must not induce T-cell response directed against the cytokine. The latter would result in localization of cell-mediated immunity in the site of cytokine production with potential deleterious effect. In a phase I clinical trial of anti-β-amyloid protein vaccination for Alzheimer's disease, vaccinated patients died for acute encephalitis due to intracerebral localization of T-cell mediated response (211). Type IB vaccination with modified recombinant murineTNFα molecules containing foreign immunodominant T-helper epitopes was capable to protect mice from TNFα-induced cachexia and ameliorated CIA (212). Nevertheless, the only safety details provided in the paper was the reversibility of anti-TNFα Ab response (that lasted 22 weeks) in immunized mice. To the best of our knowledge, this approach was not pursued further. Type IA vaccination exploits the conjugation of the target cytokine with a carrier protein. A major point is the choice of the carrier that needs to link a high number of cytokine molecules (or of peptides) displayed in a repetitive manner so to efficiently cross-link B cell receptors and elicit a strong and long-lasting autoantibody response.

Cytos biothech developed two anti murine -TNFα vaccines, constituted of multiple copies of the entire TNF-α molecule or of the 20-aminoacid N-terminal peptide, covalently linked to virus-like particles of the bacteriophage Qβ. Both vaccines protected mice from CIA, but the peptidic vaccine allowed selective recognition of only soluble TNFα and did not increase susceptibility to *listeria spp.* infection or the risk of *mycobacterium tuberculosis* reactivation (213). A press release

in 2007 reported the results of a phase I/IIa study with an anti human TNFα vaccine (CYT007-TNFQb) for the treatment of psoriasis (214). Nevertheless, to the best of our knowledge, the study was not subsequently published in a peer-reviewed scientific journal, and the development of anti-TNF vaccination strategy no longer appears in the pipeline of the company.

In type IA vaccination the ideal carrier protein should promote carrier-specific T-cell help to a B-cell polyclonal response against the hapten (215). A major advantage of TNF-K consists in the choice of the carrier: the keyhole limpet hemocyanine (KLH). KLH belongs to a group of non-heme proteins called hemocyanins. It aggregates to form oligomers whose molecular weight ranges from 4,500,000 to13,000,000. KLH binds a high number of human TNFα (hTNFα) molecules and presents a high density of hTNFα preserved B-epitopes in their native conformation to the antibody-producing B cells to cross-link specific B-cell receptors. Moreover, due to its large size and its numerous epitopes, KLH is capable of inducing a substantial immune response against the carrier. Our group could demonstrate (136) that immunization of TTG mice with TNF-K resulted in polyclonal neutralizing anti-TNF Ab production (i.e. B-cell response against self hTNFα,) that delayed and ameliorated arthritis. Conversely, no T-cell-mediated immune response was detectable to self-hTNFα, whilst a positive T-cell response was detected to KLH only. These results were fundamental in order to pursuit the development of TNF-K strategy.

1.2 TNF-K proof of concept and development: the (pertinent) animal models.

The choice of the animal model depends on the scientific question one attempts to answer. TNF-K is the kinoid of *human* TNFα, thus the main purpose was to demonstrate that TNF-K can break tolerance towards self-TNFα and treat a TNFα-dependent disease. The relevant the required model is model in which human TNFα is both an autoantigen and the molecule that governs the expression of clinical disease. For this reason the pertinent model is TTG mouse which constitutively express hTNFα as a self-antigen and develops a TNF dependent spontaneous chronic progressive arthritis. A hTNFα transgenic mouse, the Tg197, was first developed by Kollias and coworkers (24). We

used another strain of hTNFα transgenic mice developed and commercialized by Taconic (Germantown USA), called TTG. TTG mice are hemyzygous for the hTNFα transgene on a C57BL/6 background. In these mice the development of arthritis is less rapid compared to Tg197. The arthritis developed by TTG mice is chronic, progressive, proliferative and erosive, thereby reproducing the main feature of human disease.

Nevertheless, the disease is dependent only on TNFα and downstream pro-inflammatory cytokines pathways and does not allow to reproduce and study the role of adaptive immunity in RA (even if this does not exclude that TNFα blockade might work via upstream involvement of cellular actors like regulatory Tcells, see after).

The mouse genetic background is important in this model: when TTG mice are obtained on a DBA/1 background, that confers higher susceptibility to arthritis, the disease is more rapid and severe.

Another limit of TTG model is that the mice keep expressing murine TNFα, which limits the usefulness of the model to study the effect of TNF blockade on the risk of infection or tumor.

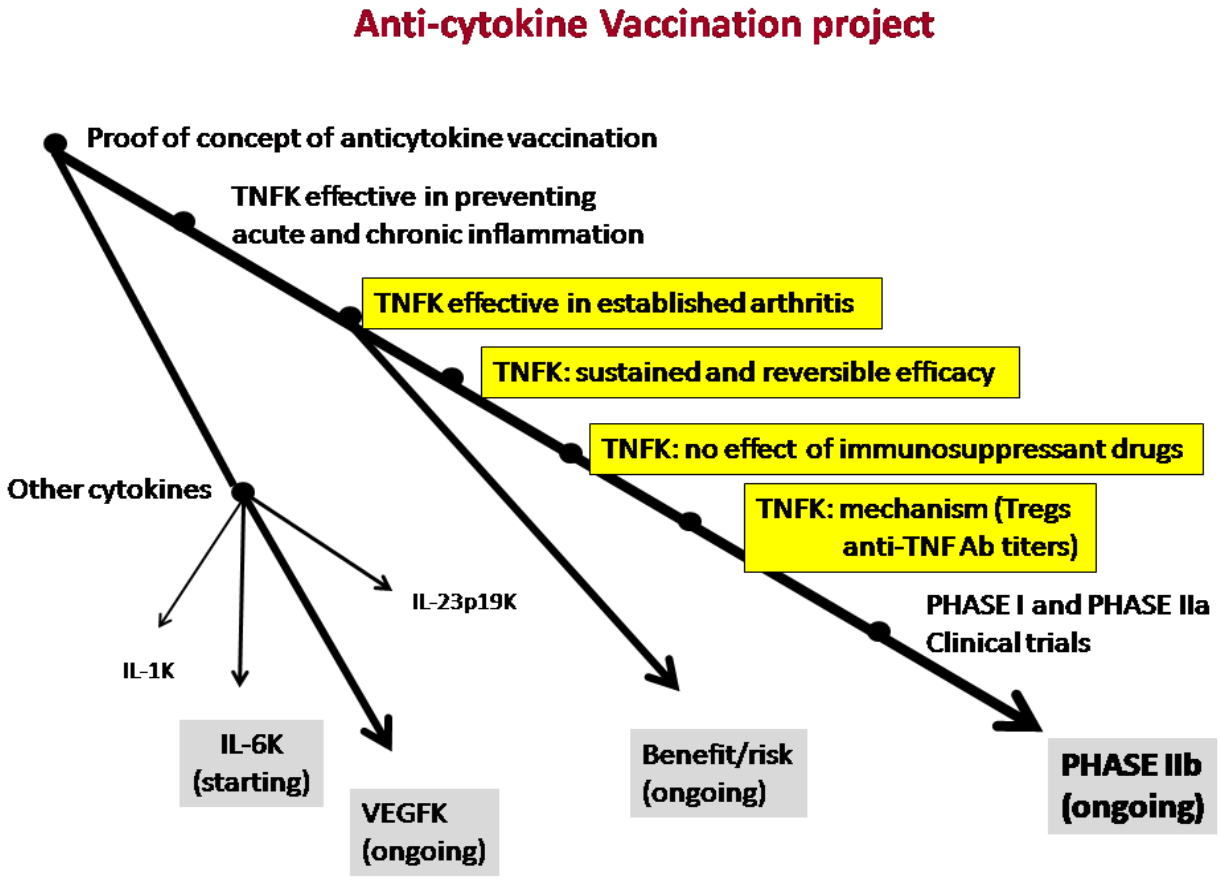
Mouse collagen induced arthritis is the most used model of autoimmune inflammatory arthritis. In this model, inflammation depends on murine proinflammatory cytokines, for this reason its usefulness in the development of TNF-K was limited. Our group used CIA in the proof of concept of TNF-K to demonstrate that TNF-K induced anti-TNFα Abs do not cross react with murine TNFα and that TNF-K is ineffective to treat CIA.

In the second article presented in the thesis, we focused on the evaluation of the effect of immunosuppressant treatment on anti-TNFα Ab production. In this case we were not interested in disease control or in the rupture of tolerance vs. a given autoantigen, but only on the magnitude of humoral immune response. For this reasons we could use Balb/c mouse, a strain characterized by strong Ab production, typically used for the production of polyclonal and monoclonal Abs.

1.3 Active anti-TNFα immunotherapy: from bench to bedside

Figure 5 summarizes the steps of the development of the anti-cytokine vaccination strategy.

Figure 5. Steps of development of the anti-cytokine vaccination project in our laboratory



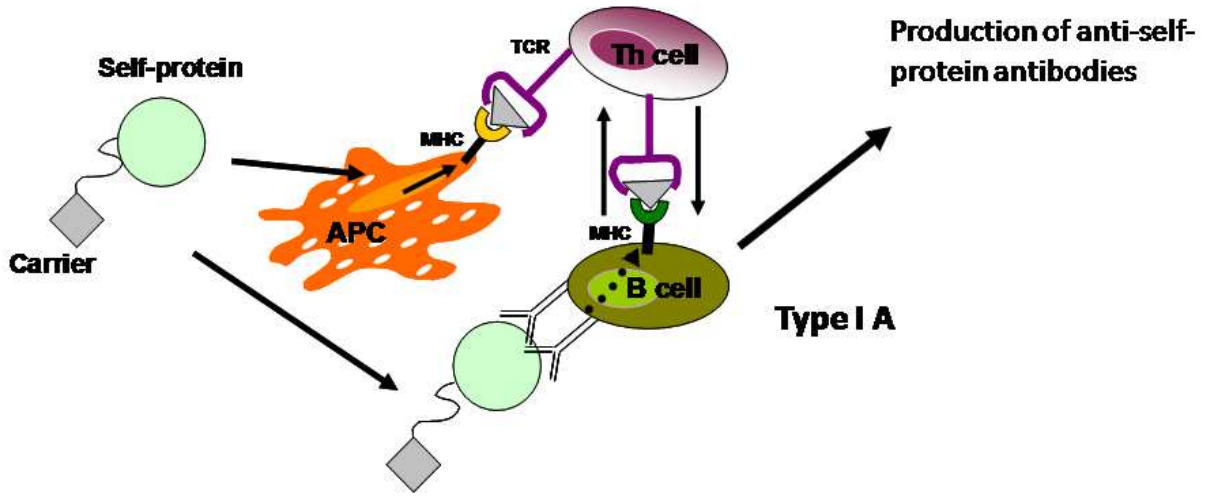
The main project (main arrow) is that of the kinoid of human TNFα (TNF-K). The main development steps (black dots) are depicted. In yellow boxes the subject addressed in this thesis work are highlighted. In grey boxes ongoing projects are depicted. IL-1K: kinoid of interleukin-1. IL-6K: kinoid of interleukin-6. VEGF-K: kinoid of vascular-endothelial growth factor. IL-23p19K: kinoid of the p19 peptide of interleukin-23.

The first experience of this thesis work, aimed to test whether TNF-K immunization, that had proved efficacy in preventing arthritis development in human TNF transgenic mouse, could be used as a therapeutic tool in human disease, in other words whether TNF-K would keep its efficacy when given once arthritis had developed. Another still unexplored question related to the safety of TNF-K treatment was whether B-cells sensitized vs. TNFα by TNF-K immunization would survive as

memory B-cells capable of novel anti-TNFα response without need for T-cell help in case of subsequent antigen meeting.

We could actually demonstrate, in a long-lasting experiment, that fully developed arthritis is effectively ameliorated by TNF-K treatment and that this efficacy is comparable to that of an already marketed anti-TNFα treatment (infliximab). At week 17 after immunization anti-TNF Ab titers had significantly declined vs. week 12. Accordingly, time-trend analysis of arthritis clinical scores from week 17 on showed an aggravation of the disease in immunized animals. Conversely, animals receiving a maintenance dose of TNF-K showed an increase in anti-TNFα Ab titers that was followed by a decrease in clinical scores. The implications of these results are valuable as far as both safety and efficacy are concerned. Reversibility of TNFα blockade is a major safety requirement for clinical use, while the possibility to renew the clinical effect of the treatment allows its long-term use in a chronic disease. As far as safety is concerned another major issue is that of immunological memory. Kinoid immunization strategy avoids T cells sensitization that would result in accumulation of cell-mediated immune response in the site of antigen production, which would be deleterious. T cells are only sensitized to epitopes of the carrier protein the KLH; no TNF epitope is recognized by T-cells. As shown in fig 6, T-cells only provide help to tolerized TNF-specific B-cells that are themselves sensitized to TNF by antigen contact and T cell help. Nevertheless, the question of possible persistence of memory B cells potentially capable of anti-TNF response was still open. We could actually demonstrate that TNF-K treated mice, challenged with increasing concentrations of TNFα (from 10 to 100 ng) are incapable of mounting any anti-TNF Ab response.

Figure 6. Type IA anti-cytokine vaccination



T-dependent antigens derived from the carrier protein are processed and presented to T-helper cells (Th) by antigen presenting cells. Dimerization of B-cell receptors on B-cell membrane by the hapten (self protein) promotes, together with the help provided by activated Th cells, the activation of hapten-specific quiescent B cells. This results in the production of polyclonal Ab against self-cytokine. T-cell response is restricted to the carrier protein, while B-cell response is directed against both hapten- and carrier-derived antigens.

Another requirement for potential applicability in clinical practice was that the effect of immunization would not be unpaired by immunosuppressive treatment. Immunosuppressive treatments used in RA have been reported to potentially reduce vaccination efficacy. Most of the data in literature concern influenza vaccination and pneumococcal vaccination. Pneumococcal vaccine is a polysaccharide, whereas the influenza vaccine is a protein antigen. MTX monotherapy is not associated with decreased response to influenza vaccination while, conversely, it seems to impair responsiveness to pneumococcal vaccination (216). Data concerning corticosteroids use in rheumatologic conditions and vaccination efficacy are heterogeneous. Four studies of influenza vaccination in adult patients, mainly with systemic lupus erythematosus, showed impaired immune responses vs. healthy controls. Conversely, five other studies concerning both lupus and RA patients did not find any difference. Nevertheless, small sample sizes limited the power of these

studies to detect small differences. Moreover, corticosteroids were often given in combination with other immunosuppressive agents, limiting the interpretation of isolated corticosteroid effects on immune response (209).

The influence of corticosteroids on polysaccharide pneumococcal vaccine responses has not been evaluated in patients with chronic rheumatic diseases. Nevertheless, in chronic obstructive pulmonary disease or asthma, corticosteroid therapy did not compromise the immune response (217).

In our work, we tested whether Ab response differed in mice receiving MTX or corticosteroids vs. controls.

We checked both anti-hTNF-α and anti-KLH Ab production at different time points from TNF-K immunization. We subsequently compared the AUC (area under the curve) of Ab titers at different time points for each individual mouse in different treatment groups. Even if remarkable differences in Ab production were evident for each individual mouse, whatever the treatment group, we could not detect any significant difference in TNF-K induced Abs in either corticosteroid or MTX- treated mice. Methylprednisolone treatment started before TNF-K immunization was associated with the highest variation of Ab titers. Even if at the group level this did not result in significantly lower anti-TNF Ab titers, an effect at individual level might be undetected due to low power, and does not rule out the possibility that, in individual patients on corticosteroids, TNF-K immunization might be less efficient (i.e. result in lower titers of induced Abs).

1.4 Articular damage and TNFα blockade

Bone erosions in RA depend chiefly on synovial inflammation, and uncontrolled synovitis almost inevitably results in articular erosions. DMARD treatment and even control of synovial inflammation with corticosteroids limits erosion development. Anti-cytokine treatment, and notably anti-TNF agents, control erosion more efficiently than classic DMARDs. The reason for better anti-erosive effect of anti-TNF treatment might be double. On one side, the reason might be due to

deeper control of synovial inflammation by anti-TNFα treatment vs. classic DMARDs. Saleem *et al.* (218) showed that patients in clinical remission on MTX still displayed some sign of synovial inflammation at power doppler ultrasonography. On the other side, TNFα (and downstream cytokines in TNFα pathways, like IL-6) exert direct effects on osteoclasts differentiation and activation, and selective TNF blockade may therefore exert additional anti-erosive effect vs. that caused by aspecific control of synovial inflammation on classic DMARDs. This would support the notion that the inflammatory and the erosive processes might be at least partially disconnected. In fact, a sub analysis of the ATTRACT study confirmed lower erosions development vs. MTX even infliximab-treated patients that did not achieve clinical response (45). Additionally, upstream regulatory mechanism evidenced on anti-TNFα treatment might even be involved in anti-erosive effect of anti–TNFα agents. TNFα-blockade with infliximab was shown to increase the expression of CTLA-4 on Tregs. Binding of CTLA4 to the cell surface receptors CD80 and CD86 on osteoclast precursors arrests further differentiation of these cells into osteoclasts, even in the presence of the stimulatory factors M-CSF (macrophage colony-stimulating factor) and RANKL (receptor activator of nuclear factor κ-B ligand) (219).

The major role of TNFα in driving joint erosion is confirmed in TTG model, in which uncontrolled human TNFα production results in chronic synovitis, with invasive pannus formation and, ultimately, erosive joint damage. In this context, we could study the effect of selective TNFα blockade on joint histological damage. Data from literature consistently showed, for all anti-TNFα agents, that attainable serum levels of the drugs are major determinants of clinical response to treatment in RA and other TNF-driven diseases like, Crohn's and ankylosing spondylitis. No studies had a sufficiently long follow-up to evaluate the implications of trough anti-TNFα levels on histological damage in RA.

In TTG model we were able to study the relationship between serum anti-TNFα Ab levels and histological inflammation and damage. We found that, in TNF-K-treated mice, the titers of

polyclonal anti-TNFα Abs were inversely correlated with histological scores of joint inflammation and destruction. The same correlation was found with trough levels of infliximab, in infliximab-treated mice (220).

The correlation was tight and highly significant, supporting a large effect-size, which pleads in favor of an evident biological phenomenon underlying these results. This establishes a clear-cut link between the titers of anti-TNFα Abs and their final biological effect (prevention of articular inflammation and damage). Importantly, this link was confirmed for both TNF-K and infliximab. Moreover, we could demonstrate that the co-administration of infliximab and TNF-K hindered the efficacy of vaccination and reduced serum levels of both infliximab and polyclonal anti-TNF Abs, which resulted in lower protection from histological damage vs. either treatment alone. Thus, passive and active anti-TNFα treatments seem to depend on the same factor (the level of monoclonal or polyclonal Ab levels) for their efficacy. Given the central role of articular TNFα on cytokine pathways and cells involved in articular erosive process, it seems presumable that higher articular levels of anti-TNF drugs may more efficiently counteract the effect of the "load" of tissular TNFα thereby limiting downstream and upstream (see after) events that depend on TNFα.

1.5 Upstream cellular mechanisms in TNF-K immunization. Effect on Treg populations

In our work (183) we studied the modifications induced by kinoid treatment in Treg populations with particular attention to percentage, numbers and phenotype of these cells. Previous work form other groups had demonstrated that in RA patients Treg might be reduced vs. healthy control (171) or show reduced suppressive activity (39). Efficacious TNFα-targeting with infliximab in RA patients was reported to be associated with restored Treg suppressive function and phenotype modification. In particular the restored suppressive capacity was due to the emergence of a population of Treg characterized by absent or low expression of CD62L. CD62L (L-selectin) is a

homing receptor" for lymphocytes that allows them to enter secondary lymphoid tissues via high endothelial venules permitting Treg to localize into the T cell area of the lymph node. Ligands for CD62L are CD34 on endothelial cells and Gly-CAM1 expressed in lymph nodes high endothelium venules. Tregs with low or absent CD62L expression have therefore different homing properties. Since their emergence is associated with successful arthritis treatment, it is tempting to say that these cells probably acquire a homing phenotype that better allows them to enter the joint and exert their suppressive activity.

In our work, we used the model of TTG mouse to study the effect of excessive TNFα production on Treg and Teff count, percentage and phenotype. In the presence of constitutive TNFα production, but before arthritis clinical development, we documented reduced Treg percentage vs. the wild type (WT) counterpart. With arthritis onset, and later on, a progressive increase in Treg percentage ensues and, at the age of 24 weeks, TTG and WT no longer differed in Tregs percentage. Treg phenotype even changed over time, with progressively and significantly increased expression of CTLA-4, and reduced CD62L; these modifications were shared with the WT counterpart, while unique to TTG mice Treg was a progressive increase in the percentage of Tregs expressing TNFR2 and in TNFR2 MFI. TNFα signaling via TNFR2 has been reported to induce both an inhibition of Treg suppressive capacity (via reduction of FoxP3 expression) (176) and a promotion of Treg expansion and function (181, 182). Thus, this increased expression could be interpreted both as a sign of reduced suppressive capacity in a context of TNFα over expression, or conversely as a counterregulatory mechanism attempting to control excessive inflammation. Nevertheless, TTG Tregs did not show reduced suppression of IFNγ production by TH1 cells vs. the WT counterpart.

TNFα blockade with either monoclonal Ab (IFX) or TNF-K increased Tregs percentages in both lymph nodes and spleen. Concomitantly, CTLA-4 expression increased in Tregs and there was an expansion of Tregs subsets CD62L- and $CD62L^{low}$ testifying a change in homing properties of Tregs induced by TNF blockade. Suppression of Teff proliferation was higher in anti-TNFα treated mice vs. untreated TTG. In summary, and consistently with results in RA, TNFα-blockade in TTG

mice resulted in increased percentages of Tregs, those Tregs expressed more CTLA-4, no or less CD62L and had higher suppressive activity vs. untreated mice. Importantly, these modifications were shared by both infliximab and TNF-K treatment, suggesting that the action of monoclonal and polyclonal anti-TNF Abs on Tregs is the same. These modifications are not merely induced by TNFα blockade itself, since etanercept treatment does not seem to lead to any modifications in Tregs percentages phenotype or functional profile (40).

1.5.1 TNFα, TNFα blockade and Tregs

Controversies are arisen in literature concerning the potential effects of TNFα on Tregs. TNFα seems to activate Tregs in culture when the judgment criterion is Treg proliferation. Conversely, when Teff cytokine production is measured in Treg-Teff co-culture the net effect of TNFα is increased proinflammatory cytokines production, which has been interpreted as a result of reduced suppressive activity. Nevertheless, in the latter case the effect of Treg activation might be masked by parallel TNFα-induced activation of Teffs, with consequent lower sensitivity of Teffs to Treg-mediated suppression. The hypotheses to explicate the effect of TNFα on Treg and teff co-cultures span from more rapid action of TNFα on Teffs vs. Tregs or a dose-response effect of TNF that would activate Teff at lower concentrations and Tregs only at higher concentrations. A third possible explanation would be that yet indentified soluble or cellular factors might mediate TNF effect on Tregs and that the contribution of this unknown variable would not be controllable in the different experiments and be responsible of the contradictory results (221).

Conversely, consistent results from our group and others confirm that Treg-dependent suppressive activity increases on TNFα-blockade in both RA patients and TTG mice. This activity is due to the induction of a CD62L- inducible Treg population (iTreg) that emerges when, in a context of high TNFα production, the cytokine is blocked by anti-TNFα Abs.

It is therefore conceivable that TNFα activates only natural Tregs, as a counterregulatory mechanism for inflammation. Conversely, TNFα might inhibit the induction of iTregs (222).

Thus, RA would be characterized by an intrinsic deficit of nTregs, which are not sufficiently activated by TNFα (in fact they cannot inhibit Th1 nor Th17 cells). TNFα blockade with either monoclonal or polyclonal anti-TNF Abs would allow the induction of iTregs that are endowed with suppressive activity on Th1 and Th17 cells, and are therefore capable of controlling the inflammatory process. A recent paper confirmed the lack of Treg suppressive activity in RA and first provided a mechanistic explanation to link this defect to TNFα overexpression. The authors could show that TNFα induced dephosphorilation of the master Treg transcription factor FOXP3. Dephosphorilated FOXP3 has lower DNA-binding activity. Infliximab treatment restored Treg suppressive function and was associated with increased FOXP3 phosphorilation vs. pretreatment (184).

2 PERSPECTIVES

Hereafter we will discuss potentially relevant question for future development of TNF-K strategy

2.1 Clinical efficacy and place of TNF-K in therapeutic strategy

Based on the results of the phase IIa study, a phase IIb trial better powered to evaluate tolerance and efficacy in RA patients is planned to start before the end of 2013. The dose of 360 µg and the three dose regimen (given on a background MTX treatment) were retained as the most immunogenic resulting in 100% of patients producing anti-TNFα Abs and in persistently high titers on a 52- week follow-up period. No serious adverse events were reported. The research agenda is now centered on the demonstration of clinical efficacy. The design will presumably be that of a double-blind placebo-controlled trial with background MTX treatment in insufficient responders to MTX.

If the trial were to provide favorable efficacy-safety profile a legitimate question would be that of the place of TNF-K in the therapeutic rheumatologic strategy.

TNF-K would promise to be an alternative anti-TNF approach and its place would presumably be

reserved, at least at the beginning to patients failing on classing DMARDs treatment. Presumably, responders to anti-TNF agents undergoing secondary failure due to ADA production could be the ideal candidates to TNF-K treatment (see after).

TNF-K was tested on background MTX treatment in order to adhere to current good clinical practice that would consider placebo comparison unethical in a clinical trial. Nevertheless, further studies are warranted in order to establish whether, alike other anti-TNF treatments, MTX co-administration would result in higher clinical efficacy vs. monotherapy. Moreover, despite our favorable results in animal models, additional data in human are needed concerning potential attenuation of TNF-K immunization by concomitant MTX (and possibly corticosteroid) treatment. Conversely, since TNF-K strategy should be potentially devoid of the problem of ADA induction, MTX would have no role in reducing immunogenicity of polyclonal anti-TNF Abs.

Association of biological therapies was quite deceiving in clinical practice. Anti-TNF treatment in association with anakinra (223), abatacept (224) or rituximab (225) did not result in increased efficacy, while it increased the burden of serious adverse events, mainly infections. In TTG model we found that the association of infliximab to TNF-K, in order to obtain rapid TNF blockade, reduces the capability of TNF-K to induce an effective polyclonal anti-TNF response. Infliximab binding to TNF-molecules on TNF-K impaired the vaccination process. Even if this phenomenon was not analyzed for other ant-TNF drugs, it is hard to conceive the association of TNF-K to other anti-TNF treatments in clinical practice. Moreover, our results should warn that careful wash-up of other anti-TNF treatments is mandatory before TNF-K immunization in order to avoid molecular interactions between anti-TNF drugs and TNF-K molecules.

Oral kinase inhibitors, like tofacitinib, are being tested in association with anti-TNF treatments in phase III studies. Favorable results of this association might plead for potential association with other anti-TNF treatments. Even if tofacitinib does not seem to reduce the efficiency of anti-pneumococcal and anti-influenza vaccines (226), interference of oral kinase inhibitors on the process of active anti-TNF immunization cannot be ruled out.

2.2 Safety concerns

The limited clinical experience (one phase 1 study in Crohn's disease and two phase II studies in RA and Crohn's respectively) with TNF-K pleads in favor of overall good tolerance. Major points supporting the safety of kinoid strategy are the bell-shape curve of Ab response observed in both animal models and in human and our results confirming the absence of T-cell or memory B-cell response against TNFα. Additional studies confirming the latter points in human are warranted.

Conversely, an aspect that could not be explored in TTG model was that of susceptibility to infections on TNF-K treatment. TNFα-blockade results in higher reactivation of latent tuberculosis and overall increased burden of infections (227). TTG mouse disease depends on uncontrolled hTNFα production and is therefore a good model to study the efficacy of the strategy. Nevertheless, the production of murine TNFalpha in response to pro-inflammatory stimuli is not affected by kinoid treatment, which renders the TTG model irrelevant for the study of the immune response against infections. For this purpose, our group developed a kinoid of murine TNFα that is currently studied in a model of TB infection. This will allow to better dissect the effect of TNF-K on immunity against infections, and to compare it to that of other -TNF drugs.

2.3 TNF-K administration

Compared to currently used anti-TNFα drugs, a less cumbersome administration scenario is conceivable for TNF-K, due to longer lasting anti-TNFα protection. Compliance to treatment is conditioned by the administration route and the patients' preference comes into play as a determinant of compliance to treatment. Even if no standard, routinely used measure of satisfaction exists in the rheumatology literature, predictors of treatment adherence are available from several studies suggesting that patients prefer subcutaneous over iv administration of the TNFα inhibitors and prefer to receive treatment at home (228, 229).

All pharmaceutical companies are developing subcutaneous delivery systems for biological drugs. TNF-K would be administered subcutaneously. Caution will presumably impose hospital administration, even if phase I and II studies did not describe any serious reaction following TNF-K administration. No data are available in literature concerning patient preference on frequency of treatment administration; it is therefore hard to foresee whether TNF-K that would be administered less frequently than currently marketed drugs, would be better accepted by patients or not.

Costs

The access for the patients to expensive biological therapies is limited, in many countries, by health authorities or other third party payers, and the choice of treatment is more and more influenced by cost-effectiveness analyses. An advantage of TNF-K over currently marketed anti-TNF would be presumably lower production costs, with considerable lower economic burden for the community. Lower costing therapeutic alternatives would surely be welcome in northern countries and might event potentially make anti-cytokine treatment affordable in southern world countries (230).

2.4 No induction of anti-drug antibodies

Adherence to treatment is a function of prolonged efficacy and lack of adverse events during treatment course as well. Literature suggests that the rate of therapy retention is the highest for etanercept followed by adalimumab and then by infliximab (231). Immunogenicity plays a major role in determining the vanishing or therapeutic efficacy of monoclonal Abs. In this case considerable advantage might be provided by TNF-K treatment that would not be limited by ADA production.

2.5 Are all anti-TNFα created equal? Anti-TNFα Abs vs. etanercept.

In clinical practice, monoclonal anti-TNF Abs and etanercept are considered almost interchangeable with regard to clinical and radiologic effectiveness. This is true if the evaluation criterion consists merely in the percentage of patients achieving clinical response or no radiological progression.

Nevertheless, some substantial differences have been recognized from the beginning. For example, etanercept is totally ineffective in inflammatory bowel diseases, and is less effective in uveitis, vasculitides and sarcoidosis compared to monoclonal Abs (2). Moreover, etanercept is associated with lower risk of tuberculosis reactivation vs. monoclonal Abs (102).

When it comes to effectiveness, some differences might exist as well. A recent paper compared the rates of clinical response to etanercept or adalimumab in 407 RA patients. The percentages of patients reaching low, minimal disease activity and remission did not differ between treatments. Nevertheless, if ADA treated-patients were categorized based on anti-adalimumab Abs (ADA) production, ADA-negative patients had significantly higher percentages of clinical response vs. etanercept (232). Mc Govern *et al*. (40) demonstrated that successful adalimumab treatment in RA is associated with the induction of Tregs capable of suppressing Th17 cells in an IL-6-dependent manner. Interestingly, in patients responding to etanercept no Treg induction, nor Th17 suppression, could be documented. Thus, the induction of regulatory cells and the blockade of IL-17 pathways might be responsible for the higher suppression of immunity to infection, notably to tuberculosis. In the meantime, the same mechanism might underlie a different clinical effect of monoclonal Abs. Nevertheless, immunogenicity resulting in ADAs is an intrinsic major limitation of monoclonal Abs. It is interesting to note that TNF-K induced the same Tregs modifications that did monoclonal anti-TNF treatment (183), with the potential advantage that ADAs should not be a concern. Active anti-TNF immunization could then potentially provide the advantages of monoclonal anti-TNF treatments without incurring their major limitation.

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APPENDICES

REVIEWS

Review N°1

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Review

Anti-cytokine vaccination: A new biotherapy of autoimmunity?

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ABSTRACT

Anti-cytokine vaccination is an innovative strategy of targeted, active immunotherapy with potential application in autoimmune diseases. The principle is to design molecules capable of triggering a humoral immune response versus a cytokine with a recognized pathogenic role in a given disease. The most used vaccination approach is based on self-protein coupled to a carrier. This strategy proved particular efficacy in models of TNF-α-dependent diseases, and promising results come from recent clinical trials in rheumatoid arthritis and Crohn's disease. The benefit/risk ratio and long term safety of anti-cytokine vaccination need to be determined to further develop this therapeutic strategy.

Contents

A large body of evidence confirms that anti-cytokine vaccination can now be considered as a serious alternative immunotherapy. The main targets are those cytokines involved in the chronic inflammatory process. Type I vaccination consists of using the protein of interest to engineer a vaccine [1]. The main approach that has been developed consists in linking the self-protein to a foreign carrier protein in order to provide adequate help to activate auto-reactive B-cells. Vaccination with such a hetero-complex, made of a biologically inactive cytokine and a carrier protein, induces a carrier-specific Th-cell proliferation that helps the self-cytokine-specific B cells to produce auto-antibodies. The carrier can be synthetic virus like particles (VLPs), keyhole limpet haemocyanin (KLH), or ovalbumin (OVA) [2–4].

1. Vaccination against TNF-α using TNF-α kinoid

The most consistent approach, to date, has been developed in rheumatoid arthritis, a chronic disease with overexpression of TNF-α in the joints. TNF-α blockers such as anti-TNF-α monoclonal antibodies or TNF-α soluble receptor proved efficacy in about 75% of patients (responders). Nevertheless, only 25 to 50% of anti-TNF-α treated patients achieved remission in controlled clinical trials [5,6], and even lower remission rates are described in everyday practice [7]. The vaccination against human TNF-α (hTNF-α) was developed using TNF-α kinoid (TNF-K), a heterocomplex of KLH and the entire molecule of hTNF-α; it induced production of high titers of neutralising anti-human TNF-α antibodies [8]. In hTNF-α transgenic mice, which develop severe arthritis from 8 to 10 weeks of age, vaccination with TNF-K protected from clinical and histological arthritis in both short and long-term experiments [9,10], even when the vaccination was performed after the onset of arthritis [11]. Indeed, TNF-K-vaccinated mice first showed a clinical and histological improvement and then, several weeks after TNF-K primo-injection, a clinical worsening paralleled by a decrease of anti-hTNF-α antibodies titer (bell curve). Both clinical worsening and anti-hTNF-α antibodies titer decline were reversed by a maintenance dose of TNF-K. Additionally no B-cell memory response to hTNF-α was induced by TNF-K immunization. Indeed, injection of native hTNF-α after immunization with TNF-K did not induce the production of neutralizing anti-TNF-α auto-antibodies [11]. More recently, we demonstrated that TNF-K immunization in this model resulted also in

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expansion, activation and differentiation of T regulatory cells [12]. Based on these data, confirming the reversibility and repeatability of TNF-K vaccination, a phase IIa clinical trial led by Néovacs (Paris, France) was performed in RA patients experiencing TNF-α-antagonist secondary failure (http://www.neovacs.fr/). The trial is completed at present and the main goals were achieved: TNF-K had good safety profile in the 40 involved patients and induced anti-TNF-α auto-antibodies in the patients that received the highest dose. These data are promising. A phase IIb/III trial is ongoing in RA. Another clinical trial in Crohn's disease, a chronic disease with overexpression of anti-TNF-α, is also ongoing.

2. Vaccination with peptides of cytokines

The same concept of using a carrier to present the targeted antigens to the immune system was used with peptides of cytokines. The principle is to couple peptides of cytokines, chosen within critical domains of the entire cytokine. An anti-inflammatory effect has been observed in experimental models of arthritis with peptides of IL-1β (potent inflammatory cytokine), TNF-α, or IL-23 (IL-17 inducing and pro-inflammatory cytokine) [13–17].

3. Vaccination with kinoids of VEGF and Interferon-α

The technology of kinoid was extended to other cytokines. VEGF is a key cytokine involved in angiogenesis [18,19]. Recent data in arthritis show the anti-inflammatory effect of an active immunization with VEGF kinoid in collagen-induced arthritis in mice [20]. In systemic erythematosus lupus (SLE), an Interferon-α signature is the hallmark of the disease. Based on preliminary experimental results, a clinical trial recently showed that in SLE patients, vaccination with IFN-α kinoid resulted in anti-Interferon-α antibodies [21,22].

4. Conclusion

Since cytokines play a major role in homeostasis, some concerns have to be pointed out, representing important points for further development of this strategy. The first is the mid and long-term safety of such an approach; in an initial analysis, the question of the persistence of the anti-cytokine antibodies has to be ascertained. A bell-curve over a reasonable period of time (few weeks or months) comparable to that of passive immunotherapy should represent a guarantee. In other cases, the harmlessness of the anti-cytokine antibodies should be demonstrated. Another major point is the T-cell response to the target cytokine. In order to prevent the deleterious effect of a cell-mediated immune response in the site of persistent over-expression of a given cytokine it seems in fact mandatory to select methods that do not enhance the cytokine-specific T-cell response.

Take-home messages

- Active immunotherapy is feasible in models of chronic inflammatory auto-immune diseases.
- Vaccination against TNF-α is effective in experimental models of arthritis.
- In a phase IIa trial in rheumatoid arthritis patients non-responders to anti-TNF-α monoclonal antibodies, vaccination against TNF-α results in production of anti-TNF-α antibodies.
- The concept of vaccination against cytokines is developed in many diseases with many targeted cytokines: VEGF, Interferon-α, IL-17, IL-23, and IL-1.

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Review N°2

Expert Opinion

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Kinoid of human tumor necrosis factor-alpha for rheumatoid arthritis

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Introduction: Anti-TNF-α drugs have dramatically changed treatment of rheumatoid arthritis (RA) in terms of both clinical control and articular damage prevention. Despite this, they hold some important drawbacks, such as frequent therapeutic failures and high costs. Anti-TNF-α active immunization, with a therapeutic vaccine against TNF-α, is a promising alternative anti-TNF-α targeting strategy, potentially devoid of treatment limitations of some of current anti-TNF blocking agents.

Areas covered: This review covers the preclinical proof-of-concept of anti-TNF-α vaccination with the kinoid of human TNF-α (TNFK) and analyzes the body of evidence forming the rationale for the application of this strategy in RA and other TNF-α-dependent diseases. We describe the theoretical bases of anti-TNF-α active immunization and of experimental data supporting the applicability of TNFK to human disease in terms of both safety and efficacy.

Expert opinion: Based on preclinical efficacy and safety data supporting its feasibility in a Phase I - II trial in Crohn's disease, anti-TNF-α vaccination with TNFK has entered the phase of clinical development and promises to be a valuable anti-TNF-α targeting strategy in human disease. The focus is made in the first clinical trial in RA (Phase II) on the efficacy in active RA patients having developed antibodies against anti-TNF mAbs.

Keywords: anti-cytokine vaccination, anti-TNF-α, kinoid, rheumatoid arthritis, TNFK

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1. Introduction

Rheumatoid arthritis (RA) is the most common inflammatory rheumatic disease with a prevalence ranging from 0.3 to 1.5% in different populations [1]. It is characterized by an invasive synovial proliferation that leads to joint damage with pain and loss of function, with precocious disability [2]. RA patients have associated co-morbidities leading to a mortality estimated at almost twofold that of general population [3]. RA is, therefore, a huge public health problem resulting in high direct and indirect costs for the community [4].

2. Overview of the market

TNF-α-targeting agents brought a revolution in the treatment of RA, providing unheard of results in terms of disease clinical control and prevention of RA structural damage and consequent disability. TNF-α can be targeted with mAbs or their fragments (infliximab (IFX), adalimumab, golimumab, certolizumab) or with fusion products carrying a TNF-α soluble receptor (etanercept). Anti-TNF-α drugs first opened the perspective of a successful cytokine-targeting strategy in RA. Sales of the four anti-TNF-α agents on the market in 2008 (adalimumab, IFX, etanercept and certolizumab pegol) reached $16 billions. By 2014, analysts forecast the entire

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Box 1. Drug summary.

Drug name	Kinoid of human TNF-α
Phase	Phase II clinical trial, pre-registration
Indication	Rheumatoid arthritis
Pharmacology	Active immunization (vaccination) against the pro-inflammatory cytokine TNF-α
Route of administration	Intramuscular
Pivotal trials	TNFK001 (http://clinicaltrials.gov/ct2/show/NCT00808262) TNFK003 (http://www.controlled-trials.com/mrct/trial/772671/TNFK003)

class of anti-TNF drugs to generate a $25 billion market, with growth driven by new entrants and continuing demand for the incumbents (source: EvaluatePharma®) [5]. In 2008, TNF-α inhibitors accounted for 80% of RA drug sales in the US, France, Germany, Italy, Spain, the UK and Japan (source: Pharmacor®) [6] within a market that, for all biological therapies for RA, was estimated at $7 billion in 2007 (source: Datamonitor® Research Store) [7].

Current TNF-α targeting strategies have nevertheless shown several drawbacks as far as safety, efficacy and costs are concerned. Despite the good safety/efficacy profile in selected patients, the overall risk of infection and possibly neoplasm is increased in RA patients treated with anti-TNF-α mAbs compared to classic DMARDs [8]. Primary and secondary failures are not infrequent; moreover, < 50% of responder patients in clinical trials attained disease remission [9]. The treatment with anti-TNF blocking agents has high costs for the community [10]. While some of these drawbacks such as the increased risk of infection and neoplasm are presumably related to the blockade of TNF-α itself, others, such as the high production costs, and the risk of anti-drug antibody (ADA) production with possible loss of efficacy and side effects, are proper to current anti-TNF-α agents, especially mAbs [11], and might be possibly overcome by alternative anti-TNF strategies.

An alternative way to target TNF-α is active immunization, where a TNF-α derivative can be used as the immunogen to develop an anti-TNF-α active immunotherapy consisting in a vaccine [12]. The immunogen must be capable of disrupting B cell, but not T cell, tolerance to TNF-α, thereby eliciting the production of high titers neutralizing antibodies [13]. This strategy allows the production of polyclonal autologous anti-TNF-α antibodies potentially bypassing the risk of an anti xeno- or allogenic antibody response. Refining of ADA detection techniques allowed in fact detecting ADA in up to 40 and 30% of IFX and adalimumab treated patients, respectively [11]. The presence of ADA is associated with low trough drug levels, infusion-related reactions (for IFX) and therapeutic failure [14]. Active immunization offers then the possibility of overcoming this limitation.

The direct costs for anti-TNF blocking agents, together with the costs of drug administration, monitoring and side effect management, result in a heavy economical burden for the community [15], while the active immunization strategy might potentially be a less expensive alternative. Finally, the longer persistence of detectable anti-TNF-α antibody titers induced by active anti-TNF-α immunization draws a less cumbersome administration scenario for the patient, with possibly higher treatment acceptance.

3. Chemistry and preparation of the TNF-kinoid

The preclinical proof-of-concept of active anti-TNF-α immunization with a compound called kinoid of human TNF-α (TNFK) has been established in a TNF-α-dependent animal model, the human TNF-α (hTNF-α) transgenic mice (TTG mice) [13,16,17] (**Box 1**). This has led to subsequent testing of TNFK in a Phase I clinical trial in Crohn's disease. A Phase II clinical trial in previously anti-TNF-α treated RA patients having developed ADA is currently ongoing.

TNFK belongs to a family of cytokine derivatives capable of acting as anti-cytokine vaccines called 'kinoids' [18]. Their name and preparation recalls those of the toxoids, detoxicated but still immunogenic products, derived from bacterial toxins by formalin treatment at 37°C for several days. At the beginning of the 1980s, a detoxication procedure using glutaraldehyde instead of formaldehyde was described for the preparation of fully atoxic polymerized antigens with high immunogenicity [19]. This technology with either glutaraldehyde or formaldehyde was then applied to cytokines in order to convert them into derivatives devoid of biological activity but capable, when administered in animals, of inducing anti-cytokine antibodies. These derivatives were called kinoids [20]. TNFK is a heterocomplex of inactivated hTNF-α and a carrier, the keyhole limpet hemocyanin (KLH).

KLH is a heterogeneous copper-containing respiratory protein isolated from the mollusk *Megathura crenulata* belonging to a group of non-heme proteins called hemocyanins. It consists of two subunits isoforms with a molecular mass of 390 × 10^3 and 360 × 10^3 D, originating, respectively, two different oligomeric aggregates, KLH1 and KLH2. The molecular mass of the oligomers ranges from 4,500,000 to 13,000,000. Due to its large size and its numerous epitopes KLH is capable of inducing a substantial immune response; its abundance of lysine residues for haptens coupling, with a high hapten: carrier protein ratio, increases the likelihood of generating hapten-specific antibodies [21].

For preparing the heterocomplex, glutaraldehyde is used to couple hTNF-α to the KLH carrier protein, KLH, and then glutaraldehyde, are added to a solution of hTNF-α treated with dimethylsulfoxide, in a mixture of 1 molecule of KLH and 40 molecules of hTNF-α. After 45 min incubation at 4°C, the preparation is dialyzed against the working buffer and then treated with formaldehyde for 6 days at 37°C.

Concentration and duration of aldehyde treatments have been adapted for hTNF-α in order to obtain a strong and persistent inactivation of its biological activity. The unreacted aldehyde is quenched by addition of glycine (0.1 M), leading to complex stabilization. The excess aldehyde is eliminated by dialysis against Dulbecco's phosphate buffer solution (PBS) [13].

4. Pharmacodynamics

It is assumed that TNFK is a heterocomplex in which KLH provides T epitopes and bears at its surface a high density of hTNF α preserved B epitopes. The aim of carrier proteins is to promote carrier-specific T-cell help to a B-cell polyclonal response [21]. Given that a high number of hTNF-α molecules are covalently bound to KLH, kinoid immunocomplexes will present a high density of hTNF-α antigens in their native conformation to the antibody-producing B cells to crosslink specific B-cell receptors [18].

TTG mouse, expressing hTNF-α as a self antigen, is the only relevant model to study TNF-induced anti-hTNF-α antibody production [13]. In all immunized mice in different study protocols, immunization with TNFK induced specific anti-hTNF-α antibodies as detected by ELISA [13,16,17]. In a protocol where mice received three injections of TNFK at days 0, 7 and 28, these antibodies tested at day 122 after TNFK first injection, appeared to belong mainly to IgG1 (52%) and IgG2a (48%), with negligible amounts of IgG3, IgM and IgE [13]. Purified IgG from hyperimmune sera exhibited a high affinity for hTNF-α with K_d values ranging from 5×10^{-8} to 10^{-10} M and were able to block its interaction with the high affinity TNFRI (K_d of 0.6 nM) [22], resulting in undetectable circulating hTNF-α in immunized mice.

Anti-hTNF-α antibodies have a neutralizing anti-TNF-α effect as confirmed both *in vitro* by L929 cytotoxicity assay, showing cytotoxicity inhibition by hyperimmune sera at dilutions up to 10^{-4}, and *in vivo*, where purified IgG from sera of immunized mice prevented TNF-α-galactosamine lethal shock in recipient mice [13].

5. Pharmacokinetics

TNFK is mixed at a 1:1 ratio with the PBS and administered intramuscularly with the adjuvant ISA51® (Seppic, France). The latter is similar to Freund's incomplete adjuvant and is composed of a mix of mineral oil and a surfactant of the mono-oleate family; it is currently used in immunotherapy of cancer and infectious diseases [23]. ISA51 is used in a 1:1 ratio with the mix TNFK PBS to obtain a water in oil emulsion [18].

Different administration schedules have been tested in mice, involving two (at days 0 and 7), three (at days 0, 7 and 28) or four injections with dose regimens varying from 5 to 30 µg of TNFK [13,16,17].

Whatever the exact administration schedule, all immunization protocols were able to induce anti-hTNF-α antibodies in TTG mice. In a three injection scheme (30 + 30 + 7 µg at days 0, 7 and 28), anti hTNF-α antibodies were detectable at first bleeding as soon as 5 weeks after TNFK first injection [16]; they peaked at 6 – 8 weeks after first injection [13], with a > 50% decline within 16 weeks.

In a protocol with three injections of TNFK 4 µg at days 0, 7 and 28, a TNFK boost given 12 weeks after the TNFK first injection induced a significant increase in neutralizing anti-hTNF-α antibodies as soon as 3 weeks after the boost [17].

TNFK was first administered in humans in a Phase I – II open label dose escalation study on 13 patients with moderate to active Crohn's disease, the TNFK001 study (http://clinicaltrials.gov/ct2/show/NCT00808262). The administration schedule consisted of three injections of TNFK at days 0, 7 and 28 at doses of 60, 180 and 360 µg. Four patients received a fourth boost dose at 6 months. In all immunized patients, anti-TNF-α antibodies were detected, with a peak in titers between the fourth and the fifth week after first TNFK injection, and a 50% reduction within 12 weeks. The boost at 6 months resulted in a new peak in antibody titers 3 – 4 weeks later [24].

As far as RA is concerned, a dose-finding Phase II clinical trial is currently ongoing in RA patients previously treated with anti-TNF agents having developed ADA. The primary goal of this trial is to demonstrate that active immunization with TNFK is able to induce polyclonal anti-TNF-α antibodies in RA patients previously treated with anti-TNF-α mAbs who underwent a secondary therapeutic failure (i.e., loss of clinical response) and have developed ADA. Among the inclusion criteria of these patients having an active RA is the positivity of antibodies against a TNF antagonist at screening or on a sample taken since discontinuation of IFX and/or adalimumab (http://www.controlled-trials.com/mrct/trial/772671/TNFK003)

6. Effects in animal models

TNFK immunization has proven its efficacy in the spontaneous arthritis of TTG mice thereby posing the rationale for its use in RA.

When given before arthritis development, TNFK markedly reduced the clinical severity of arthritis and resulted in less histological joint inflammation and destruction compared to control mice [13,16].

In an experimental three injection protocol (days 0, 7 and 28), a highly significant difference in clinical and histological score was already evident when animals were sacrificed 6 weeks after the first injection, compared to controls. TNFK immunized animals showed mild histological inflammation and no histological destruction. The co-administration of methotrexate did not change the results [16].

When, with the same experimental protocol, the observation was prolonged up to 17 weeks, arthritis onset

happened to be delayed by 9 weeks compared to controls and still low clinical and histological scores were found in immunized mice.

The therapeutic efficacy, its duration and the effect of a TNFK boost were better evaluated in a subsequent experiment more resembling to a human disease scenario, as TTG mice were immunized after spontaneous arthritis onset [17]. In 12 weeks follow-up after TNFK immunization, arthritis was dramatically ameliorated, and clinical scores did not differ from those of mice treated with weekly IFX at a dose of 1 mg/kg over the same time period. These findings were corroborated by histology, showing low inflammation and no sign of cartilage destruction in immunized animals.

The observation was prolonged to 30 weeks after TNFK first injection in order to study the duration of clinical effect and the kinetics of TNFK-induced anti-hTNF-α antibodies. After the initial amelioration, arthritis clinical score in immunized mice started to increase from week 12 after first injection to the end of the experiment. This trend was reversed by a TNFK boost given at week 12, before clinical degradation ensued. The worsening in clinical control of arthritis coincided with a decrease in anti-hTNF-α antibody titers, while the TNFK boost triggered a significant increase in antibody titers 3 weeks after its administration. Mild histological scores of joint inflammation, destruction and cartilage degradation at the end of the experiment confirmed the long-term prevention of structural damage of TNFK immunization.

7. Safety and tolerability

Some major safety issues are raised by the novel anti-TNF-α approach of active immunotherapy, namely:

i) The delivered TNF-α must be devoid of toxicity but still be immunogenic, and this is the case of the TNFK heterocomplex, where aldehyde treatment results in a hTNF-α derivative satisfying these requirements. In all experiments conducted with TNFK, no short-term toxicity linked to its administration and ascribable to hTNF-α activity-related toxicity was detected [13,16,17]. This was the case even in the limited experience in humans.

ii) The anti-TNF-α vaccination must result in rupture of B-cell but not of T-cell tolerance (i.e., vaccination must not induce memory, T cells capable of recognizing the native cytokine). In fact, the persistence of a T-cell population sensitized against a self-cytokine would result in a localized cellular response in its site of production.

iii) This issue was addressed in an animal study where 6 – 8 weeks old TTG mice received three injections of TNFK (days 0, 7, 28 ± a boost at day 90) and were followed up for 120 days after the first injection. Our group showed that the splenocytes from TNFK-immunized TTG mice did not trigger any cell-mediated immune response to self hTNF-α, as tested by T-cell proliferation and IL-2 and IFN-γ production in culture supernatants, whatever the administration regimen of TNFK [13]. The only detectable cellular response was against KLH. Conversely in Balb/C mice, a TNFK-induced anti-hTNF-α cellular response was detected when hTNF-α (a heterologous antigen for this strain) was administered.

iv) In TNFK001 study in Crohn's disease patients, stimulation of PBMCs of immunized patients with TNF-α failed to induce proliferation.

v) The rupture of B-cell tolerance must be reversible. Our group demonstrated that when TTG mice were immunized with TNFK before spontaneous arthritis appearance, anti-hTNF-α antibodies peaked 6 – 8 weeks after TNFK first injection and had a > 50% antibody titers decline within 12 – 16 weeks. This kinetics is ascribable to short life of B-cell memory in the absence of a specific T-cell help [13]. A long-term study where immunized TTG mice were monitored up to 30 weeks after TNFK first injection immunization confirmed the same results [17].

vi) A similar kinetics, albeit with the limitation of study design and sample size, seems to be confirmed in humans, based on the results of TNFK001 study. In the 13 immunized patients anti-TNF-α antibody titers were markedly reduced, and sometimes no longer detectable, within 12 – 15 weeks after first injection.

vii) A raise in the levels of TNF-α induced by other stimuli (infections, tumors) must not elicit the production of anti-TNF antibodies after TNFK immunization. This was demonstrated in a study where monthly administration of hTNF-α to TTG mice failed to induce any raise of anti-hTNF-α antibodies [17].

viii) Ideally, the 'physiological' activity of hTNF-α in normal tissues should be conserved (see points ii, iii and iv).

8. Conclusions

An important preclinical body of evidence (not inferior to that which first led to test a monoclonal anti-TNF-α antibody in 10 RA patients in 1992) supports the feasibility of anti-TNF-α active immunization in TNF-α-dependent human diseases. The efficacy in TTG mice spontaneous arthritis, the relevant model for TNF-α inhibition, strongly suggests its potential application in RA. The reversibility of anti-TNF-α antibody levels increase and the absence of memory T-cells induction are both arguments in favor of a good safety profile. The first results of an open-label study in Crohn's

disease are consistent with animal data regarding the kinetics of antibodies induction and decrease, and a good tolerance is suggested. A dose-finding randomized trial, ongoing at the present time in RA, will presumably provide more relevant safety and efficacy information determining whether or not TNFK will access the Phase III of clinical development.

9. Expert opinion

We are presently at an early phase of clinical development, as Phase II studies are ongoing in RA and Phase I – II in Crohn's is not ended, yet. The expert opinion is consequently based on proof-of-concepts experiments in preclinical and pharmacodynamics studies in mice that allow formulating some hypotheses.

The active immunotherapy with TNFK aims to reversibly vaccinate against TNF-α. Unlike the already marketed anti-TNF-α agents, one can suppose that using TNFK could have advantages in terms of simplicity and frequency of injections. The effect would probably be quite durable after each injection (several weeks or months). Moreover, TNFK treatment is not concerned by a possible reduction of effect due to ADA. These antibodies, found in up to 40% of IFX-treated and in 30% of adalimumab-treated patients, reduce the therapeutic efficacy of the drugs and are responsible of therapeutic failures and adverse reactions. So, the ADA-positive patient might be a specific clinical situation in which TNFK administration could be warranted.

Another advantage is a lower economic burden for the community as the costs of production of the kinoid would be presumably lower than those of current anti-TNF-α agents. Cost reductions are currently requested in developed countries and appear as a necessary condition for treating TNF α dependent diseases with targeted treatments in developing countries. The access for the patients to expensive biological therapies is strongly limited in many countries by health authorities or other third party payers, and the choice of treatment will be more and more influenced by cost-effectiveness analyses. In this scenario, a less expensive alternative providing 'value' and 'value for money' in RA treatment would certainly be welcomed.

If the safety and efficacy data suggested by animal models are confirmed by ongoing human clinical studies, it is conceivable that TNFK will have a considerable impact on RA treatment strategies. Not only TNFK promises to be a direct competitor of passive anti-TNF-α immunotherapies, but also future scenarios might be conceived, including combination or sequential treatment with both passive and active TNF-α-targeting strategies.

The reversibility of anti-TNF-α vaccination with TNFK and lack of induction of immunological memory versus the native cytokine are the key conditions for a favorable benefit:risk ratio. All preclinical studies show a bell curve of anti-TNF-α antibodies levels and preliminary results in humans confirm this point. The administration of TNF-α to TNFK-vaccinated animals fails to induce an anti-TNF-α response and, in addition, the persistence of residual levels of active TNF-α is probably sufficient to protect the host against infection and tumors. Nevertheless, all these safety considerations, based on animal models data, will have to be confirmed in ongoing and future clinical trials in humans.

Declaration of interest

M-C Boissier has been a consultant for Neovacs, Inc. and his laboratory has received research grants from Neovacs, Pfizer, UCB Pharma and Roche. This manuscript was written without any interactions outside co authors. The other authors declare no conflict of interest.

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RESUME
Les traitements utilisés pour le blocage de la cytokine pro inflammatoire TNFα (anticorps monoclonaux ou récepteurs solubles) ont révolutionné la prise en charge de maladies telles que la polyarthrite rhumatoïde (PR), mais montrent des limites en termes d'efficacité, effets secondaires et coûts. L'immunisation active par le kinoïde du TNFα humain (TNF-K) est une stratégie alternative de ciblage du TNFα, qui exploite le principe de la vaccination pour induire l'hôte à produire des anticorps (Ac) polyclonaux anti-TNFα. Nous montrons la faisabilité de cette approche dans un modèle d'arthrite qui reproduit les caractéristiques essentielles de la PR et qui dépende de la production déréglée de TNFα : la souris transgénique pour le TNFα humain (hTNFα). Nous montrons que le traitement des souris arthritiques par TNF-K améliore nettement la maladie, que la production d'Ac anti-hTNFα est limitée dans le temps et renouvelable par une dose de rappel de TNF-K. Au contraire, le hTNFα natif n'induit pas d'Ac anti-TNFα. Les traitements immunosuppresseurs ne semblent pas limiter l'efficacité du TNF-K. Nous apportons des preuves en faveur d'une homogénéité de fonctionnement entre les Ac polyclonaux et monoclonaux anti-TNFα. En fait, les deux traitements induisent les mêmes modifications des populations cellulaires de cellules T régulatrices. En outre, les taux sériques d'Ac monoclonaux et polyclonaux anti-TNFα sont le principal facteur qui détermine si le traitement protège ou pas de l'inflammation et de la destruction articulaire. Ces résultats ont contribué à faire avancer le développement de cette stratégie jusqu'à la phase II d'expérimentation clinique dans la PR.

L'IMMUNISATION ACTIVE ANTI-TNF DANS LA POLYARTHRITE RHUMATOÏDE : DU MODELE ANIMAL A LA MALADIE HUMAINE

ACTIVE ANTI-TNF ALPHA IMMUNIZATION IN A MURINE MODEL OF RHEUMATOID ARTHRITIS. RELEVANCE TO HUMAN DISEASE

SUMMARY
Current anti-TNFα treatments (monoclonal antibodies or soluble receptors) radically changed the treatment of rheumatoid arthritis (RA) and other TNFα-related diseases, but even show several drawbacks as far as safety, efficacy and costs are concerned. Active immunization with human TNFα kinoid (TNF-K) is an alternative anti-TNFα strategy that exploits vaccination principle in order to induce the production of polyclonal anti-TNFα Abs by recipients. We show the feasibility of this approach in a disease model that mimics the main features of human RA and that depends on deregulated TNFα production: the human TNFα transgenic mouse. We show that the treatment of arthritic mice with TNF-K dramatically ameliorates the disease, that the production of anti-hTNFα Abs is time-limited and renewable by a boost dose of TNF-K. Conversely, native hTNFα does not induce anti-hTNFα Abs. Immunosuppressant treatments do not seem to impair TNF-K efficacy. We bring evidence that polyclonal and monoclonal anti-TNFα Abs share some key features in their mechanism of action. Both treatments induce the same modifications in regulatory T-cell populations. Moreover, serum level of both polyclonal and monoclonal anti-TNFα Abs is a major factor determining whether the treatment results in protection from articular inflammation and destruction. These results contributed to the development of active anti-TNF immunization in human disease; TNF-K recently entered phase II clinical trials in RA.