Insights into the biology and therapeutic implications of TNF and regulatory T cells

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Benoit L. Salomon[†]

Sorbonne Université, INSERM, CNRS, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), F-75013, Paris, France.

[†]email: benoit.salomon@inserm.fr.

Abstract

Treatments that block tumour necrosis factor (TNF) have major beneficial effects in several autoimmune and rheumatic diseases, including rheumatoid arthritis. However, some patients do not respond to TNF inhibitor treatment and some rare occurrences of paradoxical disease exacerbation have been reported. These limitations on the clinical efficacy of TNF inhibitors can be explained by the differences between TNF receptor 1 (TNFR1) and TNFR2 signalling and by the diverse effects of TNF on multiple immune cells, including $FOXP3^+$ regulatory T (T_{reg}) cells. This basic knowledge sheds light on the consequences of TNF inhibitor therapies on T_{reg} cells in treated patients and on the limitations of such treatment in the control of diseases with an autoimmune component. Accordingly, the next generation of drugs targeting TNF is likely to be based on agents that selectively block the binding of TNF to TNFR1 and on TNFR2 agonists. These approaches could improve the treatment of rheumatic diseases in the future.

[H1] Introduction

Tumour necrosis factor (TNF) is an inflammatory cytokine that is detected in the blood within minutes after an injury and has a major protective role in infectious diseases. In the late 1980s, TNF was detected in the joints of patients with rheumatoid arthritis (RA)[1,2]. A few years later, overexpression of TNF in transgenic mice was shown to induce autoimmune arthritis[3]. Agents that block this cytokine, termed TNF inhibitors, include monoclonal antibodies (mAbs) and soluble TNF receptors. Anti-TNF therapy was first tested in patients with sepsis without clear success and then repurposed for the treatment of RA in the early 1990s[1,2]. TNF inhibitors are now widely used and have greatly improved the medical care of patients with RA, juvenile idiopathic arthritis, psoriasis, psoriatic arthritis and ankylosing spondylitis. Five original TNF

inhibitors and numerous biosimilars have been approved, mostly for the treatment of arthritis, psoriasis or ankylosing spondylitis (**Table 1**). However, not all patients respond to TNF inhibitor treatment. One-third of patients with RA have to stop taking these drugs within the first year because of insufficient efficacy or adverse events[4]. About 20% of patients with psoriasis do not respond to treatment with a TNF inhibitor and around one-third of initial responders lose response over time[5]. Similar efficacy profiles are observed for patients with inflammatory bowel disease (IBD)[6]. Although this Review mainly focuses on the effects of TNF inhibitors in rheumatic diseases, particularly RA, I also discuss their effects and use in the treatment of other autoimmune and inflammatory diseases to illustrate the role and mechanisms of these agents in general.

Treatment with TNF inhibitors is also associated with adverse effects, such as infections, which are explained by the intrinsic anti-inflammatory effects of these agents. More intriguing (and counterintuitive) is the paradoxical exacerbation of pre-existing autoimmune disease or the development of new-onset autoimmune disease following TNF inhibitor therapy. Rarely, treated patients can develop lupus-like syndrome, vasculitis, antiphospholipid syndrome or sarcoidosis. For example, the reported prevalence of systemic lupus erythematosus (SLE) among recipients of TNF inhibitor therapy is 0.1–0.2%[7-9]. A few patients develop organ-specific autoimmune conditions, such as interstitial lung disease, optical neuritis, demyelinating neuropathy, multiple sclerosis (MS), psoriasis or autoimmune hepatitis, with the highest prevalence (2.00–5.00%) reported for psoriasis and the lowest (0.05–0.20%) for demyelinating disease. Several reviews have discussed in depth the spectrum of autoimmune diseases occurring in TNF inhibitor-treated patients[7-10], among which MS is of particular interest. In the late 1990s, before the increased risk of demyelinating neuropathy associated with TNF inhibitor treatment was known, two clinical trials investigated the efficacy of TNF inhibitors in MS. However, these drugs induced unexpected disease exacerbations that led to the worldwide contraindication of these drugs in these patients[11,12]. These observations sparked intense interest in elucidating why not all patients respond to TNF inhibitor therapy, developing biomarkers to predict response, and understanding why some treated patients develop paradoxical autoimmunity.

This Review focuses on the effects of TNF on inflammation and immunity. I describe the pro-inflammatory and regulatory roles of TNF, both of which are now well-established, and address the effects of this cytokine on diverse aspects of regulatory T (T_{reg}) cell biology, including their expansion, differentiation and suppressive function. Finally, I describe the effect of TNF inhibitors on T_{reg} cells and explore potential candidates for the next generation of drugs that target TNF or its receptors. Although TNF also has important roles in organogenesis and the

68 development of lymphoid organs, protection of tissues in the nervous system, heart and joints[13-15]

69 and inhibition of tumorigenesis[16], these topics are outside the scope of the present Review and

70 will not be considered.

71

[H1] The two Janus faces of TNF

73 TNF has complex regulatory and pro-inflammatory effects in diseases with an autoimmune

74 component[13-15], such as RA[17]. This cytokine is produced under various inflammatory conditions

75 by multiple cell types and exists in two forms: a soluble form that acts as a ligand, and a

76 membrane-bound form that can act as either a ligand or a receptor[18-20]. Furthermore, TNF can

77 induce multiple downstream signalling pathways[9] as a result of binding to two different

78 receptors, TNF receptor (TNFR) 1 and TNFR2, which are structurally related but have divergent

79 biological properties. TNFR1 is broadly expressed whereas TNFR2 is expressed mostly by T

80 cells, certain myeloid and endothelial cells and some cells of the central nervous system[21,22].

81 The next sections describe the distinct functions of TNFR1 and TNFR2 and discuss the

82 proinflammatory and anti-inflammatory effects of TNF on innate immune cells and

83 lymphocytes, and present information on the cellular source of TNF.

84

[H2] TNFR1 and TNFR2

86 TNF is one of the most potent pro-inflammatory cytokines[23], which explains the success of TNF

87 inhibitor therapy in diseases with an inflammatory component. However, the paradoxical

88 development or exacerbation of autoimmune disease in some patients treated with these drugs

89 reveals the anti-inflammatory aspect of this cytokine, which is partly explained by effects

90 downstream of TNFR2. Polymorphisms in *TNFRSF1B*, which encodes TNFR2, are frequently

91 observed in patients with rheumatic diseases (RA, SLE, ankylosing spondylitis and systemic

92 sclerosis) or IBD[24]. These mutations seem to alter the binding kinetics between TNF and TNFR2

93 and lead to inhibition of downstream NF-κB signalling, which suggests that TNFR2 signalling

94 has a protective role in these diseases[25]. A single nucleotide polymorphism in *TNFRSF1A*, which

95 encodes TNFR1, is specifically associated with an increased risk of MS. This allele results in the

96 expression of a novel soluble form of TNFR1 that binds to and blocks TNF, and therefore

97 mimics the MS-exacerbating effect of TNF inhibitor therapy[26]. Other mutations in *TNFRSF1A*

98 that cause TNFR1 misfolding and endothelium reticulum stress are found in patients with

99 periodic fevers[27].

100 The differential functions of TNFR1 and TNFR2 in rheumatic and autoimmune diseases

101 have been defined in mouse models. Generally, TNFR1-knockout mice have reduced disease

severity whereas TNFR2-knockout mice develop exacerbated disease (**Table 2**). In addition, treatment with either TNFR1 antagonists or TNFR2 agonists suppresses disease symptoms in mouse models of arthritis and in mice with experimental autoimmune encephalomyelitis (EAE), a model of MS, further supporting a pathogenic role of TNFR1 and a protective role of TNFR2 (**Table 2**). A pathogenic role of TNFR1 and a protective role of TNFR2 have also been observed in mouse models of IBD, at least during the chronic phase of the disease[15,28]. Thus, TNFR1 and TNFR2 seem to be pathogenic and protective, respectively, in some autoimmune and chronic inflammatory diseases.

[H2] Effects of TNF on innate immunity

[H3] Pro-inflammatory effects.

The pro-inflammatory effects of TNF on innate immunity involve several distinct mechanisms (**Figure 1**). TNF is one of the main drivers of acute inflammation because it activates endothelial cells, induces chemokine release and promotes intense and early (within hours) recruitment of neutrophils and monocytes, via both TNFR1 and TNFR2[29,30]. Acute inflammation is also attributed to the TNF-mediated activation of canonical NF-κB signalling, which leads to the early induction of inflammatory cytokines, including TNF itself, IL-6, IL-8 and IL-1β[14]. TNF also sustains inflammation through the activation of receptor-interacting protein kinase (RIPK) 1 and RIPK3, which promote necroptosis and the release of inflammatory compounds termed damage-associated molecular patterns (DAMPs)[31]. In addition, via TNFR1 signalling, TNF promotes innate immunity by favouring the maturation of dendritic cells[32,33].

[H3] Regulatory effects.

The immunoregulatory functions of TNF are likely to involve multiple mechanisms (**Figure 1**). TNF might promote the extra-adrenal production of immunoregulatory glucocorticoids[34] and inhibit haematopoiesis[35]. TNF also stimulates innate immunosuppressive cells (via TNFR2) and activates mesenchymal stem cells, which produce increased levels of immunosuppressive prostaglandin E2 (PGE$_2$), as has been shown in synovial fluid from patients with RA[36,37]. TNF also promotes immunosuppression by favouring either the differentiation or the suppressive function of myeloid-derived suppressor cells via increasing their production of reactive oxygen species, arginase 1 and inducible nitric oxide synthase[38-42].

[H3] Effects on dendritic cells, monocytes and macrophages.

Although TNF seems to favour the production of T-bet and IL-12 by dendritic cells[43], other

136   studies suggest that the presence of TNF inhibits the production of p40 (the common chain of IL-

137   12 and IL-23) by dendritic cells, macrophages and monocytes[44-46]. These divergent findings

138   could be explained by differential actions of TNF depending on the maturation stage and type of

139   both antigen presenting cells and dendritic cell subsets.

140   In addition, TNF can either promote or inhibit macrophage activation, effects that are

141   both probably mediated by TNFR1 (reviewed elsewhere[14]). The early response of macrophages

142   to incubation with TNF, observed after a few hours, is both NF-κB-dependent and MAPK-

143   dependent and involves the expression of genes encoding various inflammatory molecules and

144   cytokines. This initial response is followed (after 24 hours) by a state of desensitization, also

145   called cross-tolerance or endotoxin tolerance. Desensitized (also termed tolerized) macrophages

146   are unable to produce inflammatory factors when stimulated by potent activators such as Toll-

147   like receptor ligands. The mechanism of desensitization involves NF-κB inhibition following

148   activation of glycogen synthase kinase 3 (GSK3) and TNF-induced protein 3 (TNFAIP3)[47].

149   Tolerized macrophages have a transiently reduced capacity to produce IL-12 and IL-23, which

150   are pro-inflammatory[46,48]. The physiological role of cross-tolerance is probably the prevention of

151   life-threatening inflammation in the context of overwhelming macrophage activation by

152   pathogens and Toll-like receptor ligands. Ultimately, after prolonged incubation with TNF,

153   specifically in the presence of type 1 interferons, macrophages overcome this desensitized state

154   and recover their inflammatory function by modifying their metabolism and epigenetic

155   status[49,50].

156

157   **[H2] Effects of TNF on lymphocytes**

158   *[H3] Pro-inflammatory effects.*

159   TNF can either promote or suppress immunity through its differential effects on lymphocytes

160   (**Figure 1**). The pro-inflammatory effects of TNF result from co-stimulation of T cells, mainly

161   via TNFR2. TNF activates NF-κB and AKT signalling pathways that lead to increased T cell

162   proliferation and survival, which are associated with increased levels of BCL2, BCLXL, IL-2

163   and survivin[51-56]. However, the co-stimulatory effect of TNF binding to TNFR2 on conventional

164   T cells seems to be of marginal importance compared with its strong effect on T_{reg} cells[57], which

165   is extensively discussed below.

166

167   *[H3] Regulatory effects.*

168   Although one report suggests that TNF promotes the expression of IL-10 by B regulatory (B_{reg})

169   cells[58], much more is known about the inhibitory effects of TNF on T cells. Prolonged exposure

to TNF attenuates T cell receptor signalling by impairing store-operated calcium influx[59,60] and also favours T cell exhaustion; in one report, TNF blockade during chronic infection with lymphocytic choriomeningitis virus abrogated the inhibitory gene expression signature[61]. TNF is able to induce activation-induced cell death via TNFR1 engagement[62]. Interestingly, TNFR2 signalling also seems to increase T-cell apoptosis by interfering with signalling pathways downstream of TNFR1[63]. However, TNFR2-dependent cell death might specifically occur in autoreactive T cells, which have altered TNFR2 signalling[25,62,64-68]. Cross-talk between TNFR1 and TNFR2 signalling is discussed in more detail in subsequent sections.

TNF also inhibits the differentiation of T_H17 cells by increasing IL-2 production[69], and decreases IL-17 production by conventional T cells and effector T_{reg} cells via activation of TNFAIP3[70,71]. This mechanism might explain the increase in numbers of T_H17 cells described in TNFR1-knockout mice or after treatment with TNF inhibitors in mouse models of RA and psoriasis[44-46,72,73]. A similar increase in T_H17 cells has been reported specifically in non-responding patients with RA treated with TNF inhibitors[44,74]. Interestingly, these non-responding patients showed a T_H1-mediated and T_H17-mediated immune response against the TNF inhibitor, which might have precipitated their lack of clinical response[75]. Finally, in the late 2000s, regulatory properties of TNF were proposed to result from its effects on T_{reg} cells. These mechanisms are extensively discussed below.

[H2] Cellular sources of TNF

Multiple cell types are able to produce TNF, but the immune cells that produce this cytokine in the highest amounts are myeloid cells and activated T cells[76]. The role of TNF produced by these two cell types in rheumatic and autoimmune diseases has been investigated using genetically modified mice with conditional knock-out of TNF only in myeloid cells or only in T cells. In mice with collagen-induced arthritis, conditional knockout of TNF in myeloid cells leads to reduced disease severity, showing that the TNF produced by these cells contributes to the pathology. By contrast, mice with TNF-deficient T cells have exacerbated arthritis, suggesting a protective role of the TNF produced by T cells[77]. Similarly, mice with EAE and TNF-deficient myeloid cells have attenuated disease, which is either delayed in onset or reduced in severity depending on the model[76,78]. Finally, the role of TNF produced by B cells has been analyzed in mice with TNF-deficient B cells. These mice have reduced arthritis and reduced levels of autoantibodies[77].

[H2] TNF structure and signalling

[H3] Soluble and transmembrane TNF.

Crystallographic studies show that trimers of TNF interact with trimers of either TNFR1 or TNFR2[79,80]. This trimeric association of the cytokine with its receptor is characteristic of the TNF superfamily and is critical for downstream signalling[63,81].

TNF is initially produced as a transmembrane molecule that can be processed by disintegrin and metalloproteinase domain-containing protein 17 (also known as TNF converting enzyme (TACE)) encoded by the *ADAM17* gene[82]. Thus, activated myeloid and T cells produce transmembrane TNF and secreted soluble TNF, which are both biologically active[77,83,84]. The role of soluble TNF in the pathophysiology of rheumatic and autoimmune diseases has been assessed in mice engineered to express a TNF protein that cannot be cleaved by TACE. Thus, these mice produce normal levels of transmembrane TNF but no soluble TNF[83]. Importantly, such mice do not develop EAE or arthritis, showing that soluble TNF but not transmembrane TNF contributes to these diseases[77,83,84]. By contrast, mice with global deletion of TNF (full knock-out) still develop EAE, which suggests that transmembrane TNF has protective effects in the disease[83].

Despite a similarly high binding affinity for its two receptors, trimeric soluble TNF triggers TNFR1 signalling much more efficiently than it does TNFR2 signalling[85]. Although this observation requires further confirmation, TNFR1 signalling is usually considered to be triggered by both soluble and transmembrane TNF whereas TNFR2 signalling is preferentially triggered by transmembrane TNF[86]. These observations suggest that soluble TNF (notably that produced by myeloid cells at the onset of a rheumatic or autoimmune disease) binds to TNFR1 to promote inflammation and precipitate the disease, whereas transmembrane TNF (probably that expressed by both myeloid and T cells) has regulatory effects mostly derived from triggering TNFR2. These observations might have implications for the use of TNF inhibitors. For example, etanercept (a TNFR2–Fc fusion protein) can efficiently block soluble TNF (as well as α3 and α2β1 lymphotoxins) but not transmembrane TNF, whereas anti-TNF monoclonal antibodies block both soluble and transmembrane TNF[82]. This concept also has implications for the design of next-generation TNF inhibitors, as discussed below.

[H3] TNFR1 and TNFR2 signalling pathways.

TNFR1 and TNFR2 signalling pathways are complex and have been extensively reviewed elsewhere[14,15,63,86]. Accordingly, only the pathways most relevant to this review are outlined here. Most of the available knowledge has been obtained in cell lines and non-immune cells and deserves further investigation to confirm its relevance in immune cells.

Upon binding of trimeric TNF to TNFR1, the cytoplasmic tail of the receptor recruits the adaptor protein TNFR1-associated death domain (TRADD) via its death domain. TRADD can then interact with other adaptor proteins, such as TNF receptor associated factor 2 (TRAF2), and kinases, such as receptor-interacting serine/threonine-protein kinase 1 (RIPK1) or cellular inhibitor of apoptosis (cIAP) 1 and cIAP2. The resulting molecular complex, named complex 1, is able to phosphorylate and ubiquitinate several other molecules, ultimately leading to potent activation of canonical NF-κB and MAPK pathways. Members of these pathways, such as c-Jun N-terminal kinase (JNK) and p38, in turn, activate AP1 complex[14,15,63,86]. This complex-1-dependent signalling pathway favours cell proliferation and survival. Alternatively, TNFR1 and TRADD interact with the Fas associated death domain (FADD) adaptors RIPK1 and RIPK3, forming the complex 2 interactome, which is able to induce cell death: either apoptosis (via caspase 8 activation) or necroptosis (via mixed lineage kinase domain-like (MLKL) protein activation)[14,15,63,86].

Complex 1 and complex 2 are downstream effectors of TNFR1 signalling. Complex 1 is probably involved in most of the effects of TNF on dendritic cells and macrophages, including activation of inflammatory target genes and production of inflammatory cytokines. Complex 2 is involved in TNF-dependent, activation-induced cell death and the formation of inflammation-dependent DAMPs.

The signal transduction pathway downstream of TNFR2 lacks a death domain and involves different adaptors. Binding of transmembrane TNF to TNFR2 recruits TRAF1 or TRAF2 adaptors to this receptor, leading to activation of cIAP1 or cIAP2 kinases and activation of canonical and non-canonical NF-κB, JNK and AKT pathways that promote cell proliferation and survival[14,15,63,86-88]. These pathways are likely to be involved in the TNF-dependent activation of mesenchymal stem cells and myeloid-derived stem cells as well as T cell co-stimulation. TRAF2 recruitment to TNFR2 also decreases the amount of cytoplasmic TRAF2, which interferes with TNFR1 signalling by favouring the formation of (cell death-promoting) complex 2 to the detriment of (survival-promoting) complex 1[63]. This cross-talk between TNFR1 and TNFR2 signalling pathways seems to be responsible for TNFR2-dependent T cell death[89].

[H3] TNF reverse signalling.

Reverse (extracellular to intracellular) signalling induced by transmembrane TNF has been described but remains poorly documented. This phenomenon is only outlined here as it has been reviewed elsewhere[18-20]. In this context, TNFR1 or TNFR2 can act as ligands for transmembrane TNF, which can function as a cell receptor transducing a signal in several different situations.

272 For example, TNFR2-expressing T cells promote the increased expression of TNF in monocytes
273 and/or macrophages via transmembrane TNF, a phenomenon that has been observed in the joints
274 of patients with RA. Also, TNFR1-expressing endothelial cells induce cross-tolerance in
275 monocytes and/or macrophages via transmembrane TNF. Finally, TNF inhibitors are also able to
276 bind to transmembrane TNF and thereby induce the apoptosis of transmembrane TNF-expressing
277 cells; this phenomenon has been observed for instance in T cells and synovial macrophages from
278 patients with RA[90,91]. The mechanism of TNF reverse signalling involves increased intracellular
279 levels of calcium and TGF-β and activation of the MAPK–ERK pathway. However, the in vivo
280 relevance of reverse signalling is difficult to assess because this phenomenon has been poorly
281 described. I consider that reverse signalling might contribute to the spectrum of effects of TNF
282 and might have an important role in inducing cross-tolerance of macrophages and in the death of
283 transmembrane TNF-expressing cells induced by administration of TNF inhibitors.

284

285 **[H2] Integrative view of TNF functions**
286 Here, I present a simplified and integrated view of what I believe is the major role of TNF in
287 immunity (**Figure 1**). TNF is one of the most potent inflammatory cytokines owing to its
288 capacity to activate endothelial cells, neutrophils, macrophages and dendritic cells, leading to
289 leukocyte recruitment and massive release of inflammatory cytokines at sites of inflammation.
290 Most of these pro-inflammatory phenomena are mediated by TNFR1 signalling. Besides its pro-
291 inflammatory functions, TNF also has anti-inflammatory (regulatory) functions, although their
292 role and mechanisms in immunity are yet to be clarified. The regulatory functions involve TNF-
293 dependent activation of suppressive cells, such as mesenchymal stem cells, myeloid-derived
294 stem cells and of course T_{reg} cells (which are extensively discussed below). TNF might also
295 promote death or exhaustion of T cells and inhibit pathogenic T_H17 cells. Most of these
296 regulatory phenomena are mediated by TNFR2 signalling.
297 The end result of TNF blockade depends on the type of autoimmune disease present and
298 the timing of treatment. Blocking the interaction between TNF and TNFR1 led to increased
299 numbers of pathogenic T_H1 and T_H17 cells in mouse models of arthritis and psoriasis[44-46,72,73].
300 This increase was associated with exacerbation of psoriasis (as might logically be expected) but
301 surprisingly with attenuation of arthritis because this treatment also blocked the migration of
302 pathogenic T cells to the joints[46]. Similarly, patients with RA treated with TNF inhibitors have
303 increased levels of circulating T_H1 and T_H17 cells[44,74,75], which could explain some of the
304 paradoxical inflammation observed in a subset of these patients. The effects of TNF blockade
305 could also depend on the timing of treatment in relation to the course of disease. To investigate

the role of TNF signalling via TNFR2 in a model of collagen-induced arthritis, TNFR1-knockout mice were treated with TNF on either days 2–20 or days 22–40 after disease induction[92]. Interestingly, early TNF treatment led to disease exacerbation whereas late TNF treatment led to attenuation of arthritis. An opposite effect of TNF that is similarly dependent on the stage of disease progression has been documented in non-obese diabetic mice; TNF seems to exacerbate diabetes in young mice by activating dendritic cells and to attenuate it in adult mice by inhibiting conventional T cells and promoting T_{reg} cell activation[59,93-95].

[H1] Effects of TNF on T_{reg} cells

T_{reg} cells are master regulators of autoimmune diseases. Mice and humans that are genetically deficient in T_{reg} cells die soon after birth from a massive and systemic autoimmune syndrome, which reveals the critical role of these cells in the suppression of autoimmunity[96]. Functional or quantitative defects of T_{reg} cells have been reported in many human autoimmune diseases[97]. Other indirect evidence supports the concept that T_{reg} cells contribute to human autoimmune diseases. For instance, some biomarkers of disease activity, such as C reactive protein levels, are inversely correlated with T_{reg} cell proportion in patients with RA[98] or IBD[99,100]. Moreover, T_{reg} cell transfer seems to have beneficial effects in patients with various autoimmune diseases[101].

Transcriptomic analyses that compared T_{reg} with conventional T cells in lymphoid tissues showed that several members of the TNFR superfamily, including TNFR2, TNFR superfamily member 4 (OX40), TNFR superfamily member 9 (41BB) and TNFR superfamily member 18 (GITR) are included in the T_{reg} cell signature[102]. These molecules are further upregulated upon stimulation of either the T cell receptor (TCR) or T cell-specific surface glycoprotein CD28 and are therefore preferentially expressed by effector T_{reg} cells rather than resting T_{reg} cells[103,104]. At steady state, 30% of T_{reg} cells express TNFR2 and most of this subset are effector T_{reg} cells that have a stronger suppressive function in vitro than do TNFR2$^-$ resting T_{reg} cells[105,106]. Thus, TNFR2 belongs to the T_{reg} cell signature and is a marker of highly suppressive T_{reg} cells.

[H2] Effects on T_{reg} cell expansion

Expansion is defined as an increase in cell numbers, and results from a combination of increased proliferation, prolonged survival and phenotypic stability. TNFR2 signalling seems to expand T_{reg} cells by increasing all three of these factors.

Initially, TNF and/or TNFR2 co-stimulation were shown to increase T_{reg} cell proliferation in mice[22,107]. Our group and others showed that effector T cells, in particular T_H17 cells, are a major source of the TNF that induces this increase in the T_{reg} cell population in vivo[108-110].

340 Similar findings were obtained for human T_{reg} cells[84,111,112]. TNF can also substantially prolong

341 T_{reg} cell survival[103]. Indirect evidence indicates that TNFR2 signalling also maintains *FOXP3*

342 expression, which increases T_{reg} cell phenotypic stability and therefore their long-term

343 expansion[112-115].

344 In many of these in vitro studies, soluble TNF was capable of boosting T_{reg} cell

345 expansion. Although transmembrane TNF has a stronger effect than soluble TNF on induction of

346 TNFR2 signalling[85], strong evidence indicates that soluble TNF can indeed stimulate the

347 expansion of T_{reg} cells by binding to TNFR2. Furthermore, TNFR1 expression has not been

348 detected on T_{reg} cells (unlike TNFR2 expression)[22]. The expansion-promoting effect of soluble

349 TNF on T_{reg} cells was lost in TNFR2-deficient T_{reg} cells and when TNFR2, but not TNFR1, was

350 blocked[113]. Finally, treatment with TNF or TNFR2 agonists induced similar co-stimulation of

351 T_{reg} cells[111]. The capacity of soluble TNF to efficiently induce TNFR2 signalling could be

352 explained by the use of high concentrations of this cytokine or the presence of TNF aggregates

353 with crosslinking properties in the preparations. TNFR2 agonists, which are either multimers of

354 mutated TNF or mAbs that bind only to TNFR2 (discussed in more detail below), strongly co-

355 stimulate T_{reg} cells in both mice and humans[57,103,111,116-118]. In a study of pre-activated T cells,

356 TNFR2 co-stimulation strongly increased the proliferation of T_{reg} but had no effect on

357 conventional T cells[57]. The capacity of TNFR2 co-stimulation to promote T_{reg} cell expansion was

358 confirmed in vivo in animals treated with TNFR2 agonists[86,117,119-121].

359 Although very little is known about TNFR2 signal transduction in T_{reg} cells,

360 transcriptomic analyses showed that binding of TNF to TNFR2 on purified mouse or human T_{reg}

361 cells induced a gene expression signature indicative of NF-κB pathway activation[103,122]. More

362 precisely, TNFR2 signalling induced nuclear translocation and binding of RelA to its DNA

363 target sequence, which suggests that the canonical NF-κB pathway is activated by TNFR2

364 signalling in T_{reg} cells. Importantly, the increased proliferation and prolonged survival of T_{reg}

365 cells induced by TNFR2 triggering was severely attenuated in RelA-deficient T_{reg} cells[103,104].

366 Some evidence also suggests that the non-canonical NF-κB pathway is also activated by TNFR2

367 signalling in T_{reg} cells but this observation has to be treated with caution because these assays

368 were conducted on a cell population with low T_{reg} cell purity[123]. Other data suggest that TNFR2

369 signalling induces activation of the MAPK pathway, notably via p38[124,125]. TNFR2-mediated co-

370 stimulation of T_{reg} cells also induced a glycolytic switch associated with activation of

371 mammalian target of rapamycin complex 1 (mTORC1) signalling via phosphoinositide-3 kinase

372 (PI3K), although the signalling pathway connecting TNFR2 to PI3K was not identified[57].

373   Overall, strong evidence indicates that the boost in T_{reg} cell numbers induced by TNFR2

374   signalling involves activation of the canonical NF-κB pathway. The role of the other signalling

375   pathways mentioned here requires further documentation.

376

377   **[H2] Effects on T_{reg} suppressive function**

378   The effects of TNF on the suppressive function of mouse and human T_{reg} cells have been

379   assessed in vitro (**Table 3**). The first of these studies showed no effect of low-dose (≤5 ng/ml)

380   TNF in human cells[98]. Five subsequent reports showed that treatment with TNF, usually at a high

381   dose (50 ng/ml), reduced the suppression of conventional T cell activation by human T_{reg}

382   cells[122,126-129]. By contrast, in vitro studies performed in mouse cells showed that the presence of

383   high amounts of TNF either had no effect or even increased T_{reg} cell-mediated suppression of

384   conventional T cell activation[22,107]. Moreover, other evidence also suggests that TNF does not

385   inhibit T_{reg} cell-mediated suppression of conventional T cells, and might even increase it. For

386   instance, administration of a TNFR2 agonist to mice with graft versus host disease (GvHD) or

387   collagen-induced arthritis promoted T_{reg} cell expansion and had a therapeutic effect[117,119,121].

388   Also, treatment of cultured T_{reg} cells with TNF increased their capacity to suppress colitis or

389   GvHD after transfer[103,130], whereas TNFR2-deficient T_{reg} cells had a reduced capacity to

390   suppress colitis or GvHD[114,131]. However, these observations provide only indirect evidence that

391   TNF either had no effect on or increased T_{reg} cell suppressive function in mice. Indeed, this

392   cytokine might influence other parameters of T_{reg} cell biology (such as proliferation, survival,

393   functional stability or migration). Interestingly, EAE was exacerbated in genetically modified

394   mice in which TNFR2 was ablated only in T_{reg} cells. Ablation of TNFR2 in T_{reg} cells seems to

395   decrease their suppressive function specifically in the inflamed central nervous system[132]. In this

396   context, the expression of TNFR2 by T_{reg} cells might be essential for their suppressive function

397   and their capacity to control EAE.

398   Our group also performed an analysis of the suppressive capacity of T_{reg} cells from

399   numerous different human donors under three different T cell activation conditions. We

400   consistently found that TNF (added either before or during the suppression assay) either had no

401   effect on or even slightly increased the suppressive activity of human T_{reg} cells[133]. The

402   preservation of T_{reg} cell suppressive activity after TNFR2 co-stimulation (achieved using a

403   TNFR2 agonist) in humans has also been confirmed[57].

404   Several factors might account for the contrasting findings in mouse and human cells.

405   First, as none of the available markers can exclusively characterize the population of human T_{reg}

406   cells, the purified T_{reg} cell populations used in some of these studies might still have some level

of contamination by activated conventional T cells, especially when only CD4 and CD25 expression was used to sort the cells[134]. Second, given the high inter-individual variability in T_{reg} cell phenotypes, responses to TNF and suppressive activity, it is important to collect data from a sufficiently large sample of individuals. Finally, a T_{reg} cell functional defect identified in a suppression assay could be due either to intrinsic T_{reg} cell dysfunction or to the presence of contaminating conventional T cells that are resistant to T_{reg} cell suppression. This last point is critical with regard to the effects of TNF. Indeed, in addition to its proliferation-promoting effect on T_{reg} cells, TNF not only increases the proliferation of conventional T cells[51,53,133] but also increases their resistance to T_{reg} cell-mediated suppression[135]. In several studies performed in human cells, TNF was present during the suppression assays and might act on any contaminating conventional T cells, which would impair the evaluation of T_{reg} cell suppressive function (**Table 3**). Accordingly, pre-incubation of the T_{reg} cells with TNF is appropriate before testing their capacity to suppress conventional T cells.

Another critical point is the choice of parameter used to assess the activation of conventional T cells. As TNF strongly increases T_{reg} cell proliferation (and possibly also cytokine production), measuring the activation of only the conventional T cells within the population is critical. This measurement can be done by analyzing fluorescent marker dilution or assessing intracellular cytokine production using flow cytometry techniques such as fluorescence-activated cell sorting (FACS). Researchers should not use thymidine incorporation or enzyme-linked immunosorbent assays (ELISA) to measure the proliferation or cytokine production of the whole cell population, which includes both conventional T cells and T_{reg} cells. For this reason, to accurately determine whether TNF alters the suppressive function of T_{reg} cells, we recommend that TNF is added only during the pre-incubation phase (that is, before the suppressive assay), and that activation of only the conventional T cells is measured by FACS. The absence of these two precautionary measures in some of the reports claiming that TNF inhibits T_{reg} cell suppressive activity in humans undermines their conclusions (**Table 3**).

To conclude, weak evidence indicates that TNF is able to either inhibit or increase the suppressive activity of T_{reg} cells. After careful analyses of the data from in vitro assays, I would say that TNF has no or only a minor effect on T_{reg} cell suppressive function in this context. However, this cytokine seems to have an essential role in stimulation of T_{reg} cell function in some conditions associated with inflammation.

The data derived from in vitro studies of mechanisms underlying the suppressive activity of T_{reg} cells reflect only the tip of the iceberg, as only two or three suppressive mechanisms have been analyzed in these studies to date. However, it is now well established that T_{reg} cells in vivo

441 are able to use a wide range of suppressive mechanisms depending on their tissue localization
442 and the type of inflammation present[136,137]. The suppressive activity of T_{reg} cells also involves
443 many different effector molecules. Some have been thoroughly studied and shown to be essential
444 for aspects of T_{reg} cell suppression, such as cytotoxic T-lymphocyte protein 4 (CTLA4) and IL-
445 10[138]. FOXP3 expression is also critical because its loss leads to loss of T_{reg} cell function[138], but
446 no single marker has been shown to easily quantify the level of T_{reg} cell suppression.

447 Several mechanisms have been suggested to explain how TNF might increase the
448 suppressive function of T_{reg} cells in mice. TNF promotes full differentiation of effector T_{reg} cells
449 by stimulating NF-κB, which might increase some of these cells' suppressive functions[103,104].
450 TNF also synergizes with IL-2 to increase the expression of CD25 (the IL-2 receptor α-chain)
451 and FOXP3[22,133]. Moreover, TNF increases the IL-2-induced phosphorylation of STAT5[22] and
452 limits the loss of FOXP3 expression in cultured cells by preventing re-methylation of the *Foxp3*
453 promoter[113,115]. Thus, TNF might increase T_{reg} cell suppression and stability by favouring both
454 phosphorylation of STAT5 and FOXP3 expression, which are key determinants of these T_{reg} cell
455 features[139,140]. Finally, TNF limits IL-17 production by T_{reg} cells by activating TNFAIP3[71].

456 Other mechanisms have been proposed to explain how TNF might decrease T_{reg} cell
457 function. TNF decreases FOXP3 expression by increasing the expression of deleted in breast
458 cancer 1 (DBC1) and miR-34a, which respectively promote FOXP3 degradation and reduce
459 *FOXP3* transcription and translation[128,141,142]. Alternatively, TNF might increase the expression
460 of serine/threonine-protein phosphatase PP1, which dephosphorylates FOXP3, thereby
461 decreasing its effect on T_{reg} cell suppressive function[126].

462

463 **[H2] Effects on T_{reg} cell differentiation**
464 The population of FOXP3$^+$ T_{reg} cells is composed of thymic T_{reg} cells, which acquire their T_{reg}
465 cell state during their development in the thymus, and peripheral T_{reg} cells, which acquire their
466 T_{reg} cell state during peripheral differentiation of mature naive conventional T cells. Finally,
467 induced T_{reg} cells can be differentiated in vitro from naive conventional T cells by TCR
468 stimulation in the presence of IL-2 and TGF-β. Thus, induced T_{reg} cells are the in vitro
469 counterpart of peripheral T_{reg} cells.

470 However, whereas TNF alone has no effect on thymic T_{reg} cell differentiation,
471 experiments in mice show that TNF inhibits the differentiation of induced T_{reg} cells, whereas
472 treatment with TNF inhibitors increased the differentiation of induced T_{reg} cells[143,144]. This
473 inhibitory effect of TNF was also observed on peripheral T_{reg} cells in vivo. In mice with EAE,
474 injection of anti-TNF or anti-TNFR2 mAbs at the time of disease induction led to reduced

disease severity, which was associated with an increased proportion of T_{reg} cells and evidence of increased peripheral T_{reg} cell differentiation[144]. Two other papers do not support this observation and even suggest that the TNF–TNFR2 axis promotes the differentiation of induced T_{reg} and peripheral T_{reg} cells[28,73]. However, the design of these two studies meant that contaminating natural T_{reg} cells were present in the starting inoculum, and thus treatment with TNF might boost the expansion of these contaminating cells rather than increase the differentiation of induced T_{reg} cells[28,73]. TNF does not seem to affect thymic T_{reg} cell differentiation at steady state, because mice lacking TNFR2 have normal thymic T_{reg} cell numbers. However, ablation or neutralization of TNFR2 combined with ablation or neutralization of two other members of the TNFR superfamily, namely OX40 and GITR, led to reduced differentiation of thymic T_{reg} cells[145]. Overall, whereas the effect of TNF on T_{reg} cell differentiation is still open to discussion, an excess of TNF seems to impair the differentiation of induced T_{reg} cells and peripheral T_{reg} cells in mice.

In humans, the inhibition of T_{reg} cell differentiation by TNF was first observed in patients with RA. TNF inhibitor treatment increased the in vitro differentiation of induced T_{reg} cells derived from patients with RA but not those from healthy controls[146]. This observation explained why blood samples from patients with RA treated with infliximab had an increased proportion of T_{reg} cells, which might result from increased differentiation of peripheral T_{reg} cells[98,146]. Other members of the TNF family, such as 41BB, OX40 or TNFR superfamily member 25 (also known as death receptor 3 (DR3)), can also inhibit the differentiation of induced T_{reg} cells[147-149]. These observations suggest that a shared mechanism is involved, perhaps implicating NF-κB, PI3K or MAPK pathways. IFNγ produced by T cells following TNFR co-stimulation has also been proposed to inhibit the differentiation of induced T_{reg} cells. Alternatively, the increased activation of the PI3K–AKT pathway resulting from TNFR signalling could lead to reduced activation of phosphorylated SMAD3, which transactivates *Foxp3* expression in mouse induced T_{reg} cells[144].

[H2] Overall effects of TNF on T_{reg} cells

In summary, TNF has multiple negative and positive effects on T_{reg} cell biology, most probably resulting from TNFR2 rather than TNFR1 signalling (**Figure 2**). The best-characterized of the positive effects of TNF are increased T_{reg} cell proliferation and expansion. TNF also seems to promote T_{reg} cell survival in vitro, although the relevance of this effect in vivo is difficult to evaluate. The TNF-dependent increases in T_{reg} cell proliferation and survival are at least partially dependent on RelA and activation of the canonical NF-κB pathway. Involvement of p38 and

PI3K–AKT pathway activation has also been suggested but requires further investigation. Finally, weak evidence indicates that TNF increases the stability and suppressive function of T_{reg} cells, a phenomenon that might be partially due to TNF signalling synergizing with IL-2 signalling and with phosphorylation of STAT5. Other reports suggest a negative effect of TNF on T_{reg} cell biology in vitro. Whether this cytokine truly has a negative effect on T_{reg} cell function is questionable. By contrast, TNF seems to increase T_{reg} cell suppressive function in vivo, at least in some inflammatory contexts. However, the evidence of an inhibitory effect of TNF on differentiation of induced T_{reg} cells is fairly solid and might involve the PI3K–AKT pathway (**Figure 2**).

[H1] T_{reg} cells in RA

As T_{reg} cells have an important role in the suppression of autoimmunity, numerous studies have attempted to identify whether these cells have a quantitative or functional defect in patients with autoimmune diseases. Major drawbacks of these studies include the use of sample sizes too small to account for interindividual variability and the absence of a specific marker for human T_{reg} cells, which has led to the utilization of different combinations of markers. As a result, the literature is full of conflicting data.

I present here the main findings on T_{reg} cell proportion and function in patients with RA. This disease is particularly interesting as T_{reg} cells can be obtained from both the blood and joints (the target tissue of the disease), which are easily accessible for analysis. Data obtained in other autoimmune diseases are also included where relevant.

[H2] T_{reg} cell proportion

Contrasting findings have been reported in studies of the proportion of T_{reg} cells in the blood of patients with RA receiving conventional immunosuppressive treatment (the effects of TNF inhibitors are discussed below). Among studies that compared patients with RA with healthy control individuals, four described a decreased T_{reg} cell proportion[75,150-152], five found no difference[98,126,146,153,154] and one found an increased T_{reg} cell proportion in the patients with RA[155].

Most studies that have analyzed both blood and synovial fluid of patients with RA concluded that the proportion of T_{reg} cells was higher in synovial fluid than in blood, and remained stable over time in individual patients[152,155-157]. The T_{reg} cells isolated from synovial fluid seem to be bona fide T_{reg} cells because they exhibit *FOXP3* promoter demethylation. Also, the phenotype of these T_{reg} cells indicates that they have an activated status[157]. The synovial fluid

543 of patients with RA contains high amounts of IL-6, TNF and IFNγ, low levels of IL-17A, IL-10

544 or IL-13 and does not contain IL-1[126,157]. Which of these factors is responsible for the increased

545 proportion and activation of synovial T_{reg} cells remains unclear. However, IL-6 is not likely to be

546 involved, because this cytokine (which is produced by joint fibroblasts) induces

547 transdifferentiation of T_{reg} cells into highly pathogenic T_H17 cells in a mouse model of

548 autoimmune arthritis, a phenomenon that might also take place in patients with RA[158]. IL-6 also

549 induced proteasomal degradation of FOXP3 and loss of the suppressive activity of T_{reg}

550 cells[159,160]. We do not know much about the effect of IFNγ on T_{reg} cells. Therefore, the activation

551 and/or expansion of T_{reg} cells in the synovial fluid of patients with RA is likely to be caused by

552 high local levels of TNF.

553

554 **[H2] T_{reg} cell function**

555 Compared with T_{reg} cells obtained from the blood of healthy control individuals, T_{reg} cells

556 isolated from the blood of patients with RA were shown to have similar suppressive activity in

557 one study[155] and decreased suppressive activity in another[128]. In a third study, the capacity of

558 these cells to suppress conventional T cell proliferation was maintained but their cytokine

559 production was reduced[98]. Contrasting findings have also been reported for the suppressive

560 activity of T_{reg} cells isolated from the synovial fluid of patients with RA. Several studies showed

561 that synovial fluid T_{reg} cells from patients with RA were as active, or were more active, than

562 blood T_{reg} cells from either patients with RA or healthy control individuals in terms of

563 suppression of proliferation or IFNγ production[152,155-157]. Another publication reported that

564 synovial fluid T_{reg} cells from patients with RA had decreased suppressive activity[126].

565 Importantly, these studies noted considerable variation between patients, with T_{reg} cells from

566 some individuals but not others showing a high level of suppression[157]. This observation might

567 explain the contrasting results and further emphasizes the importance of generating data from at

568 least 7–10 different patients, which was not the case for most of these studies.

569   Firm conclusions are difficult to draw because the available evidence does not provide a

570 clear picture of whether T_{reg} cells in the blood of patients with RA have similar proportions and

571 functions to those of healthy control individuals. The situation is a little bit clearer for synovial

572 fluid T_{reg} cells, which seem to be present at an increased proportion in patients with RA.

573

574 **[H2] Effects of TNF inhibitors**

575 *[H3] T_{reg} cell proportion*

The proportion of T_{reg} cells in the blood has been analyzed in many studies of patients with RA 3–6 months (typically 3 months) after initiation of TNF inhibitor treatment. In studies of infliximab-treated patients with RA, the T_{reg} cell proportion increased[75,98,115,146,151] after treatment (**Table 4**). In studies of patients with RA treated with either adalimumab or etanercept, the T_{reg} cell proportion was either increased[150,154,161] or unchanged[153,154,162] (**Table 4**). This T_{reg} cell increase was more often observed in responding than in non-responding patients.

Moreover, in studies of infliximab-treated patients with Crohn disease or IBD (**Supplementary Table 1**), the T_{reg} cell proportion was also either unchanged[99,163] or increased[100,115,163-168]. Some of the studies in patients with IBD or Crohn disease also analyzed the kinetics of this treatment-related increase in T_{reg} cell proportion. In a study of patients with Crohn disease, the increase was transient and only occurred after the first injection[165]. In two studies of patients with IBD, the increase occurred 2 weeks after the first injection and was maintained for ≥22 weeks[100,166], whereas in another study in patients with Crohn disease no increase was detected after 1 week but an increase was detected at week 24 in patients who had low T_{reg} cell proportions before treatment[99] (**Supplementary Table 1**).

Two studies in patients with uveitis[169,170] and one in patients with ankylosing spondylitis[171] showed an increase in the T_{reg} cell proportion after TNF inhibitor therapy. However, one study in patients with juvenile idiopathic arthritis observed no difference[172] and one in patients with sarcoidosis observed a decrease in the T_{reg} cell proportion[173] following TNF inhibitor therapy (**Table 4**).

Some general conclusions can be drawn from these data. Most publications described an increase in the proportion of T_{reg} cells in blood after TNF inhibitor therapy. Discrepancies between some studies could be due to the following factors: first, infliximab seems to induce an increase in the T_{reg} cell proportion more consistently than either adalimumab or etanercept. Second, a T_{reg} cell increase seems to be more consistent among patients who responded to TNF inhibitor treatment. The type of concomitant medications might also matter. For instance, although methotrexate monotherapy induces an increase in T_{reg} cell proportion[150], combination therapy with methotrexate and a TNF inhibitor provided an optimal increase in T_{reg} cells in vitro[174]. Also, steroid treatment might increase T_{reg} cell proportion and function[175,176]. Finally, as discussed above, technical factors related to the way that T_{reg} cells were purified might influence the conclusions of these studies. Some activated conventional T cells (which also express CD25) are likely to contaminate the population identified as T_{reg} cells. Thus, the findings of these studies have to be considered carefully because the level of conventional T cell contamination could differ between healthy control individuals and patients with rheumatic or autoimmune

disease, or before and after TNF inhibitor treatment. Use of the CD45RA (naive T cell) or CD45RO (memory T cell) markers, in addition to CD25 or CD127, would help to limit the risk of such contamination[134].

Several mechanisms by which T_{reg} cells might increase after TNF inhibitor treatment are supported by experimental evidence. First, T_{reg} cells might increase because treatment with TNF blockers such as infliximab favour the differentiation of peripheral T_{reg} cells[146]. Second, T_{reg} cells might increase because treatment with anti-TNF mAbs such as adalimumab augments the expression of transmembrane TNF on monocytes, which then triggers T_{reg} cell expansion via TNFR2 signalling. Thus, anti-TNF mAbs that are intended to inhibit TNF might paradoxically increase its activity[84]. The preferential expansion of activated T_{reg} cells rather than resting T_{reg} cells in patients receiving anti-TNF mAbs supports this hypothesis[100]. In patients with RA[151] or IBD[168], T_{reg} cells that are more sensitive to spontaneous apoptosis than are those of healthy control individuals, might be present at an increased proportion in patients treated with TNF inhibitors because they are protected from cell death by this therapy. In patients with IBD, TNF inhibitor therapy blocks T_{reg} cell migration to inflamed tissues, which results in increased T_{reg} cell levels in blood and decreased levels in the intestinal mucosa[166]. Finally, TNF inhibitor therapy leads to a decrease in inflammatory cytokine levels and pathogenic T cells while sparing T_{reg} cells in patients with Crohn disease[161] or ankylosing spondylitis[165,171]. Therefore, this treatment might target conventional T cells in preference to T_{reg} cells, thereby explaining the relative increase in the T_{reg} cell proportion within the population of CD4$^+$ T cells. As the increased proportion of blood T_{reg} cells following TNF inhibitor treatment is an in vivo phenomenon that occurs over a long time period, determining which of the above-described mechanisms is most relevant remains a challenge.

[H3] Suppressive function.

Treatment with mAb TNF inhibitors affects not only the T_{reg} cell proportion but also their suppressive function. Early work showed that T_{reg} cells from patients with RA obtained before the initiation of TNF inhibitor therapy had a poor capacity to suppress cytokine production by conventional T cells, and that the suppressive activity of these T_{reg} cells was restored following anti-TNF treatment[98]. These functional T_{reg} cells resulted from either the generation of new peripheral T_{reg} cells following infliximab treatment[146] or from the expansion of differentiated T_{reg} cells following adalimumab treatment[84]. These restored T_{reg} cells were even able to suppress pathogenic T_H17 cells, unlike the T_{reg} cells of healthy control individuals[154]. Dysfunction of T_{reg} cells obtained from the blood or synovial fluid of patients with RA and restoration of their

644 suppressive function after TNF inhibitor treatment (infliximab) were also confirmed in two other

645 studies[126,128]. Restoration of functional blood T_{reg} cells after TNF inhibitor treatment has also

646 been described in patients with IBD[164].

647   TNF inhibitors based on mAbs seem to act, at least in part, by restoring the functional

648 T_{reg} cell compartment. By contrast, etanercept is likely to act by suppressing conventional T cells

649 and/or rendering them sensitive to the suppressive effects of T_{reg} cells[172,177]. In another study, T_{reg}

650 cells obtained from patients with Crohn disease were shown to be functional even before

651 initiation of infliximab treatment[178]. However, the T_{reg} cell purification strategy used in this

652 paper meant that activated conventional T cells might have contaminated the population of T_{reg}

653 cells, thereby resulting in an inaccurate measurement of the suppressive activity of genuine T_{reg}

654 cells. Therefore, the conclusions of this report have to be interpreted with caution.

655   In summary, the beneficial effects of TNF inhibitor therapies could be due to either

656 restoration of fully functional T_{reg} cells or to an increased susceptibility of conventional T cells

657 to the suppressive effects of T_{reg} cells.

658

659 ### [H3] T_{reg} cell biomarkers of response.

660 The development of biomarkers to identify the 20–30% of patients with RA or IBD who will not

661 respond to TNF inhibitor therapy is highly desirable, and some T_{reg} cell-related biomarkers are

662 potential candidates. In some studies, an increase in the T_{reg} cell proportion after TNF inhibitor

663 treatment was observed only in patients who responded to this therapy (**Table 4**). Thus, the T_{reg}

664 cell proportion before TNF inhibitor treatment has been proposed as a predictive biomarker to

665 predict treatment response. However, patients with IBD who respond to TNF inhibitors could

666 have T_{reg} cell proportions before therapy that are either higher[99,100] or lower[178] than those of non-

667 responding patients. Moreover, in patients with ankylosing spondylitis[171,174] or RA[171,174], the T_{reg}

668 cell proportion before TNF inhibitor therapy was not predictive of treatment efficacy.

669   As discussed above, a possible mechanism for the observed increase of T_{reg} cells upon

670 mAb TNF inhibitor treatment is binding of the mAb to transmembrane TNF on myeloid cells,

671 leading first to its increased expression and then to a boost in T_{reg} cell numbers mediated by

672 TNFR2 signalling[84]. As the expression of transmembrane TNF on monocytes can be readily

673 assessed by flow cytometry, the capacity of adalimumab to provoke an increase in T_{reg} cell

674 numbers in a 3-day culture has been used to identify which patients with RA would respond to

675 this treatment[174].

676   In summary, pretreatment T_{reg} cell proportion does not seem to be a reliable biomarker of
677 response to anti-TNF therapies. The expression of transmembrane TNF on myeloid cells as a
678 biomarker of treatment response deserves to be confirmed in other studies.
679

680 **[H1] Next-generation drugs targeting TNF**
681 The putative mechanisms underlying non-response and paradoxical autoimmunity to TNF
682 inhibitor treatment could be explained by the regulatory aspect of TNF. Blocking TNF is
683 associated with an increased risk of impairing the activity of some suppressor cells, including
684 T_{reg} cells, or increasing the activation of autoreactive T cells. The overall effect of these
685 treatments is likely to depend on the specific autoimmune disease present, its stage and severity,
686 and on genetic and environmental factors unique to each patient. At the time of treatment, if TNF
687 has a dominant inflammatory and pathogenic role, TNF inhibitors will be beneficial. By contrast,
688 if TNF has a dominant regulatory and protective role, TNF inhibitors will be detrimental.
689   Given that most of the pro-inflammatory properties of TNF are due to TNFR1 signalling
690 induced by soluble TNF and most of the regulatory properties of are due to TNFR2 signalling
691 induced by transmembrane TNF, the next generation of TNF inhibitors might preferentially
692 target TNFR1 or TNFR2[14,25,82,86,88,179,180]. Two types of TNFR-specific agents have been
693 proposed: mAbs and so-called TNF muteins, which are forms of this cytokine harbouring
694 mutations in the receptor-interacting domains[181-183].
695

696 **[H2] Selective TNFR1 antagonists**
697 The capacity of TNFR1 antagonists to block the pro-inflammatory interaction of TNF with
698 TNFR1 has been investigated in mouse models of autoimmune diseases.
699   Both mAbs and TNF muteins have been developed that have potent TNFR1 antagonist
700 activity and a strong therapeutic effect in mouse models of autoimmune diseases (**Table 5**). For
701 example, the mAb DMS5540 was as effective as etanercept in the treatment of collagen-induced
702 arthritis. In addition, the effects of DMS5540 on immune cells were superior to those of
703 etanercept, as DMS5540 induced T_{reg} cell activation and reduced the activation of conventional
704 T cells, a phenomenon not observed with etanercept[184]. Several anti-TNFR1 mAbs (namely
705 atrosab, trivalent nanobody TNFR1 silencer (TROS) and HM1097) were able to suppress
706 EAE[185-187]. Finally, the muteins XPro1595 and R1antTNF had therapeutic effects in arthritis or
707 EAE; when these agents were compared with etanercept, they sometimes demonstrated
708 improved efficacy[188-192].
709

[H2] TNFR2 agonists

As TNFR2 signalling stimulates the expansion of T_{reg} cells, TNFR2 agonists such as the mAbs MR2-1 and another unnamed version are interesting candidates for improving T_{reg} cell therapy in autoimmune diseases (**Table 5**). When added to human T_{reg} cell cultures, these mAbs promote the expansion and improve the stability and purity of T_{reg} cells over time[111,118].

Two additional TNF muteins with human TNFR2 agonist activity (TNF07 and TNC-scTNF$_{R2}$) have been generated[116,193] and TNF07 has been shown to promote T_{reg} cell activation in vitro. In the future, mAbs or TNF muteins with TNFR2 agonist activity might be used to improve cell culture methods used to generate T_{reg} cell preparations for use in cell therapy. This notion is supported by mouse studies showing that adding TNF or a TNFR2 agonist to T_{reg} cell cultures increased the capacity of these cells to suppress colitis[103,130] or GvHD[103,130] after their reintroduction in vivo[103,130].

The capacity of TNFR2 agonists to stimulate T_{reg} cells in vivo has been tested in mouse models. Treatment with either of two TNF muteins with TNFR2 agonist activity (STAR2 and EHD2-sc-mTNF$_{R2}$)[194,195] induced in vivo T_{reg} cell activation and expansion[117,120] that was associated with prevention or amelioration of arthritis[119,121], EAE[132] or GvHD[117]. These agents also protected the central nervous system of treated animals from inflammation and neuronal injury induced by chronic nerve constriction or drug treatment, respectively[195,196].

Whereas classical immunosuppressive drugs aim to suppress autoimmunity by neutralizing pathogenic cells, an alternative approach is based on increasing the expansion or suppressive capacity of T_{reg} cells. A prototype of this new class of drugs is IL-2[197]. Our group showed that administration of low-dose IL-2 boosts the proliferation of T_{reg} cells and induces remission of type 1 diabetes mellitus in non-obese diabetic mice[198,199]. Low-dose IL-2 is now being investigated as a treatment for other autoimmune diseases in multiple clinical trials. One study has investigated this treatment in 14 different autoimmune diseases, including RA and ankylosing spondylitis (NCT01988506). TNFR2 agonists are another type of drug that are able to boost the number or function of T_{reg} cells. No clinical trial has so far investigated the use of TNFR2 agonists to treat an autoimmune disease. However, bacillus Calmette–Guérin (BCG) vaccine can induce TNF release without secondary effects, thereby providing an indirect way to trigger TNFR2 signalling. BCG vaccine has been tested for efficacy in type 1 diabetes (NCT00607230 and NCT02081326).

[H1] Conclusions

TNF has a long and fascinating yet chaotic history. This cytokine was discovered in the mid-

1970s and named for its effect as a tumour cell killer. Major milestones in its history include its cloning in the mid-1980s, the discovery that TNF binds to two receptors, that its signalling transduction is highly complex (and still remains to be fully explored), and that it has multiple effects at steady state.

TNF is now known to be one of the most important inflammatory cytokines. Although TNF is critical for beneficial immune responses, the realization that TNF is also harmful in many autoimmune diseases led to the great success of TNF inhibitors and ultimately to the flowering of research into other biological therapies. The regulatory role of this cytokine is also important to consider. Here again, the mechanisms underlying the immunosuppressive activity of TNF are complex. However, one of its main features seems to involve the expression of transmembrane TNF on myeloid or T cells, which interacts with TNFR2 on T_{reg} cells to boost their proliferation and maybe also their stability and suppressive function.

The inflammatory and regulatory roles of TNF are both essential to take into account in the design of future generations of TNF inhibitors. Preclinical studies have shown that selective antagonists of TNFR1 inhibit the inflammatory action of TNF whereas selective agonists of TNFR2 boost T_{reg} cell numbers and potentially also improve their function. Therefore, TNFR1 antagonists and TNFR2 agonists could be beneficial in future treatments of several diseases with an autoimmune component.. In the future, biotechnology and pharmaceutical companies are expected to work hand in hand with academic laboratories towards the successful translation of these fascinating observations into the clinic.

Competing interests

B.S. declares that he received consultancy fees from HiFiBio Therapeutics regarding the applications of TNFR2 agonists and antagonists in cancer and autoimmunity.

Peer review information

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Key points

- Tumour necrosis factor (TNF) is a major inflammatory cytokine that has deleterious effects in several rheumatic and autoimmune diseases, as attested by the success of TNF inhibitor therapy.

- Some patients do not respond to TNF inhibitors and others develop paradoxical autoimmune exacerbations that can be explained by the immunoregulatory role of TNF.

- The pro-inflammatory and anti-inflammatory properties of TNF are largely segregated by the capacity of this cytokine to bind to TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), respectively.

- The anti-inflammatory effects of TNF are explained by its capacity to increase the proliferation, stability and suppressive function of FOXP3^{+} regulatory T cells via TNFR2 signalling.

- Antagonists of TNFR1 and agonists of TNFR2 constitute a new generation of drugs that might be more effective and have fewer adverse effects than classical TNF inhibitors.

798

Table 1. Clinically approved TNF inhibitors in the USA and Europe

Drug	Molecule	Biosimilars	Approved rheumatic disease indications[a]
Etanercept	Human TNFR2—IgG1–Fc fusion protein	Benpali, Erelzi, Nepexto	RA, JIA, psoriatic arthritis, plaque psoriasis, AS
Infliximab	Humanized chimeric anti-TNF IgG1/κ mAb	Remsima, Inflectra, Flixabi, Ixifi, Zessly, Avsola	RA, psoriatic arthritis, plaque psoriasis, AS
Adalimumab	Fully human anti-TNF IgG1/κ mAb	Exemptia, Adfrar, Amjevita, Cyltezo, Amgevita, Solymbic, Imraldi, Cyltezo, Halimatoz, Hefiya, Hyrimoz, Hulio, Idacio, Kromeya, Hadlima, Abrilada, Amsparity	RA, JIA, psoriatic arthritis, plaque psoriasis, AS, hidradenitis suppurativa, non-infectious uveitis
Certolizumab pegol	PEGylated human Fab fragment of anti-TNF mAb	NA	RA (Europe only), psoriatic arthritis
Golimumab	Fully human anti-TNF IgG1/κ mAb	NA	RA, psoriatic arthritis, AS

799

800 [a]Disease indications for biosimilars can differ from those of the original drug and depend on the

801 countries where they are registered. AS, ankylosing spondylitis; JIA, juvenile idiopathic arthritis;

802 mAb, monoclonal antibody; NA, not applicable; RA, rheumatoid arthritis.

803

804

Commenté [JM1]: Caroline: Check against table 1 in the published paper below for overlap:

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This table might need to be adjusted to avoid overlap (or else might need permissions).

(see handover sheet for one idea of alt layout)

805 Table 2. Pathogenic and protective roles of TNFR1 and TNFR2 in models of rheumatic and

806 autoimmune diseases

Mouse model	*TNFR1* knockout	*TNFR2* knockout	TNFR1 antagonist	TNFR2 agonist	Refs
Collagen-induced arthritis	Attenuated	Exacerbated	Attenuated	Attenuated	200,201,184,119,121
Antigen-induced arthritis	ND	Exacerbated	ND	ND	201
DTHA	ND	Exacerbated	ND	ND	115
Arthritis in TNF-transgenic mice	Attenuated	Exacerbated	ND	ND	202
EAE	Attenuated	Exacerbated	Attenuated	Attenuated	28,187,203-205,28,187,191,203,204,206,207,208,132

807 DTHA, delayed-type hypersensitivity arthritis; EAE, experimental autoimmune

808 encephalomyelitis; ND, not determined.

809

810

811 Table 3. Effect of TNF on T_{reg} cell function in vitro.

T_{reg} cell population	Culture condition(s)	n	TNF added	Assay type	Effect of TNF on T_{reg} cell suppression	Refs
Human CD4$^+$CD25^{hi}	Soluble anti-CD3 and anti-CD28 mAbs	6	Before or during	Cytokine FACS	No change	[98]
Human CD4$^+$CD25^{hi}	Coated anti-CD3 mAbs	6	Before	Proliferation ^{3}H	Decreased	[128]
Human CD4$^+$CD25$^+$	HBV e-antigen	7	None	Proliferation ^{3}H	Decreased	[127]
Human CD4$^+$CD25$^+$	Coated anti-CD3 mAbs	NR	During	Proliferation FACS, cytokine ELISA	Decreased	[129]
Human CD4$^+$CD25^{hi}	Coated anti-CD3 mAbs	3	Before or during	Proliferation FACS	Decreased	[122]
Human CD4$^+$CD25^{hi}CD127^{low}	Coated anti-CD3 and anti-CD28 mAbs	5?	Before	Proliferation FACS	Decreased	[126]
Human CD4$^+$CD25^{hi}CD127^{low} CD45RA$^-$	Coated anti-CD3 and anti-CD28 mAbs; APC and soluble anti-CD3 mAbs; APC and coated anti-CD3 mAbs	28	Before or during	Proliferation FACS	No change or increased	[133]
Mouse CD4$^+$CD25$^+$	APC and soluble anti-CD3 mAbs	6	Before or during	Proliferation FACS	No change or increased	[22]
Mouse CD4$^+$CD25$^+$	APC and soluble anti-CD3 mAbs	3	Before	Proliferation FACS	Increased	[107]

812 APC, antigen presenting cells; ELISA, enzyme-linked immunosorbent assay; FACS,

813 fluorescence-activated cell sorting; n, number of healthy individuals; NR, not reported; TNF,

814 tumour necrosis factor.

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Table 4. T_{reg} cell proportions in blood before and after TNF inhibitor therapy

Study population	TNF inhibitor (concomitant medications)	Sampling time points[a]	T_{reg} cells			Refs
			Cell population	Pre-treatment (proportion)[b]	Post-treatment (proportion)	
27 patients with RA; 8 healthy controls	Infliximab (NSAIDs, methotrexate)	Baseline, 1.5 and 3.0 months	$CD4^+CD25^{hi}$	Same	Increased from baseline; increased in responders vs nonresponders	98
17 patients with RA; 15 healthy controls	Infliximab (NSAIDs, methotrexate)	Baseline and 3.0 months	$CD4^+CD25^{hi}$	Decreased[c]	Increased[c] from baseline	151
31 patients with RA; 20 healthy controls	Infliximab (NSAIDs, methotrexate)	Baseline and 4.0–6.0 months	$CD4^+FOXP3^+$	Same	Increased from baseline	146
40 patients with RA; 10 healthy controls	Infliximab (methotrexate, salazopyrin, hydroxychloroquine, steroids)	NR	$CD4^+CD25^+FOXP3^+$	Decreased	Increased from baseline and in responders vs nonresponders	75
10 patients with RA; 10 healthy controls	Adalimumab (NSAIDs methotrexate, steroids)	Baseline and 3.0 months	$CD4^+CD25^{hi}$	Same	No change from baseline	153
50 patients with RA; 15 healthy controls	Adalimumab or etanercept (NR)	NR	$CD4^+FOXP3^+$	Same	Increased from baseline; increased in responders vs nonresponders to adalimumab; no change from baseline with etanercept	154
48 patients with RA	Adalimumab or etanercept (methotrexate, leflunomide)	Baseline, 1.5 and 3.0 months	$CD4^+FOXP3^+$, $CD25^{hi}127^{low}$	ND	No change from baseline, no difference between responders and nonresponders	162
20 patients with RA; 10 healthy controls	Etanercept (methotrexate)	Baseline and 3.0 months	$CD4^+CD25^{hi}FOXP3^+$	Decreased	Increased from baseline	150
33 patients with RA	Etanercept (methotrexate)	Baseline, 3.0 and 6.0 months	$CD4^+CD25^+FOXP3^+$	ND	Increased from baseline	161
16 patients with RA	Infliximab or etanercept[d] (NR)	Baseline and 3.0 months	$CD4^+CD25^+127^{low} FOXP3^+$	ND	Increased from baseline	115
7 patients with JIA	Etanercept (NSAIDs, methotrexate)	Baseline and 1.0–5.0 months	$CD4^+FOXP3^+$	ND	No change from baseline	172
222 patients with AS; 68 healthy controls	Infliximab or etanercept (NSAIDs)	Baseline and 6.0 months	$CD4^+CD25^{hi}FOXP3^+$	Decreased	Increased from baseline; increased in responders versus nonresponders	171
46 patients with sarcoidosis; 26 healthy controls	Infliximab (NR)	Baseline, 3.5 and 6.0 months	$CD4^+CD25^{hi}$	Increased	Decreased from baseline	173
12 patients with uveitis	Adalimumab (NR)	Baseline, 1.0 and 6.0 months	$CD4^+CD25^{hi}127^{low} FOXP3^+$	ND	Increased from baseline	169
16 patients with uveitis; 15 healthy controls	Infliximab (NR)	Baseline, 4.0–27.0 months	$CD4^+FOXP3^+$	Same	Increased from baseline[e]	170

[a]Baseline (before initiation of TNF inhibitor treatment). [b]In patients versus controls. [c]Absolute

number. [d]Three patients also received golimumab, adalimumab or certolizumab. [e]Versus patients

820 treated only with ciclosporine or colchicine. AS, ankylosing spondylitis; JIA, juvenile idiopathic

821 arthritis; ND, not determined; NR, not reported; NSAID, nonsteroidal anti-inflammatory drug.

822

Table 5. Therapeutic effects of drugs targeting TNFRs in autoimmune disease models

Agent	Structure	Therapeutic efficacy	Refs
	Antagonists of TNFR1		
DMS5540	Bispecific anti-TNFR1 and anti-albumin mAb	Arthritis (CIA)	[184]
ASTROSAB	Humanized anti-TNFR1 IgG1 mAb, mutated in the Fc fragment to abrogate complement and immune complex activation	EAE	[185]
TROS	Trivalent nanobody comprising two mAb domains binding to TNFR1 and one mAb domain binding to albumin	EAE	[186]
HM1097	Hamster IgG	EAE	[187]
XPro1595	Dominant-negative PEGylated TNF muteins that interact with soluble TNF to form inactive heterotrimers, which have low binding and signalling activity	Arthritis (CIA) and EAE	[188,190,192]
R1antTNF	PEGylated TNF mutein that binds specifically to TNFR1 without signalling activity	Arthritis (CIA) and EAE	[189,191]
	Agonists of TNFR2		
MR2-1	Mouse mAb against human TNFR2	Increased expansion and stability of T_{reg} cells; not tested in vivo	[57, 118]
Unnamed	Mouse mAb against human TNFR2	Increased expansion and stability of T_{reg} cells; not tested in vivo	[111]
TNF07	Human TNF mutein trimer	Increased expansion of T_{reg} cells; not tested in vivo	[116]
TNC-scTNF$_{R2}$	Human TNF mutein trimer	Not tested in vitro or vivo	[193]
STAR2	Mouse TNF mutein nanomer	Increased expansion, survival and function of T_{reg} cells; effective in CIA, EAE and GvHD	[121,103,117,120,132]
EHD2-sc-mTNF$_{R2}$	Mouse TNF mutein hexamer	Increased expansion of T_{reg} cells; effective in EAE and CIA	[119,208]

824 CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; GvHD,

825 graft versus host disease; mAb, monoclonal antibody; TNF, tumour necrosis factor; TNFR, TNF

826 receptor.

827

828 **Figure 1. The proinflammatory and anti-inflammatory activities of TNF are driven by**

829 **effects on innate and adaptive immunity.** Tumour necrosis factor (TNF) is a major

830 proinflammatory cytokine (top panel) that activates both innate (left side) and adaptive (right

831 side) immunity. TNF promotes recruitment of leukocytes, favours the production of other

832 proinflammatory cytokines, activates neutrophils and participates in co-stimulation of

833 conventional T cells. TNF also has regulatory activities (bottom panel) such as inhibition of

834 haematopoiesis, increased glucocorticoid production, activation of suppressive cells (such as

835 mesenchymal stem cells (MSC) and myeloid-derived suppressor cells (MDSC)) or altering the

836 function of dendritic cells (DCs) and macrophages. TNF also regulates immunity by promoting

837 IL-10-producing B cells, inducing T cell apoptosis, altering T cell receptor (TCR) signalling,

838 inhibiting T_H17 cell differentiation and boosting numbers and function of regulatory T (T_{reg})

839 cells. APC, antigen-presenting cell; FLS, fibroblast-like synoviocyte; HSC, haematopoietic stem

840 cell.

841

842 **Figure 2. The overall effects of TNF on regulatory T cells.** Most of the effects of tumour

843 necrosis factor (TNF) on regulatory T (T_{reg}) cells are due to induction of TNF receptor 1

844 (TNFR2) signalling, which is probably preferentially mediated by transmembrane TNF rather

845 than soluble TNF. Signal transduction downstream of TNFR2 that does not involve kinase

846 activity involves TNF receptor-associated factor (TRAF) adaptor proteins. Multiple downstream

847 signalling pathways lead to positive (left) and negative (right) effects on T_{reg} cell biology.

848 TNFR2 signalling strongly induces T_{reg} cell proliferation and has a moderate survival-promoting

849 effect on T_{reg} cells; both of these effects depend on RelA and probably also on the activation of

850 p38, AKT and mTORC1 by phosphorylation (p). Weak evidence indicates that TNF also

851 promotes the stability and suppressive function of T_{reg} cells, perhaps via TNF-induced protein 3

852 (TNFAIP3)and signal transducer and activator of transcription 5 (STAT5) signalling pathways.

853 In addition to these positive effects of TNF, the negative effects of this cytokine are clear in

854 relation to the inhibition of induced T_{reg} cell differentiation (which involves phosphoinositide 3-

855 kinase (PI3K) and/or phosphorylated RACα serine/threonine-protein kinase (AKT) pathway

856 activation). Weak evidence suggests that TNF induces T_{reg} cell dysfunction, perhaps via a

857 mechanism involving deleted in breast cancer 1 (DBC1), microRNA 34a (miR-34a) and

858 serine/threonine-protein phosphatase PP1. Arrow thickness and box shading intensity is

859 proportional to the importance of the effect or the likelihood that a given molecule is involved in

860 the pathway.

861

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