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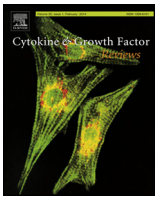

Mini review

TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future

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ABSTRACT

Tumor Necrosis Factor (TNF), initially known for its tumor cytotoxicity, is a potent mediator of inflammation, as well as many normal physiological functions in homeostasis and health, and anti-microbial immunity. It also appears to have a central role in neurobiology, although this area of TNF biology is only recently emerging. Here, we review the basic biology of TNF and its normal effector functions, and discuss the advantages and disadvantages of therapeutic neutralization of TNF – now a commonplace practice in the treatment of a wide range of human inflammatory diseases. With over ten years of experience, and an emerging range of anti-TNF biologics now available, we also review their modes of action, which appear to be far more complex than had originally been anticipated. Finally, we highlight the current challenges for therapeutic intervention of TNF: (i) to discover and produce orally delivered small molecule TNF-inhibitors, (ii) to specifically target selected TNF producing cells or individual (diseased) tissue targets, and (iii) to pre-identify anti-TNF treatment responders. Although the future looks bright, the therapeutic modulation of TNF now moves into the era of personalized medicine with society's challenging expectations of durable treatment success and of achieving long-term disease remission.

1. Introduction

This review summarizes the current state of knowledge on TNF/TNFR molecules and discusses the reagents currently being used to block TNF in the treatment of human diseases. It surveys the benefits and disadvantages of blocking TNF's broad range of biological activities *in vivo* and the reasons behind their therapeutic efficacy and limitations. This review will also debate the most recent developments in the use of TNF and anti-TNF agents: the search for ways to pre-identify treatment responders, and the status of the search for the "holy grail" of selective blockade of specific TNFR signaling pathways, not just *in vivo* but in selected cells or specific organs, for optimal disease treatment without the current known side-effects. Recent publications and an emerging worldwide culture of embracing personalized medicine suggest that this is not merely a laudable goal but will soon become standard practice. Here we review the discovery and development of anti-TNF agents in the treatment of human diseases: from mediators of cell death and inflammation to therapeutic giants – past, present and future, and oh what an exciting future!

2. The discovery of TNF and the initial use of cytokines in immunotherapy

TNF was discovered in 1975 as an endotoxin-inducible molecule that caused necrosis of tumors *in vitro* [1]. Soon after it was purified biochemically [2–4] and shown to be exquisitely cytotoxic for L929 cells [5,6] and synergistic with interferons [7,8]. TNF was quickly shown to be expressed by monocytes/macrophages and activated T cells, distinct from another cytotoxic

Abbreviations: ADA, adalimumab; CNS, central nervous system; CER, certolizumab; ETA, etanercept, etanercept; GOL, golimumab; Ig, immunoglobulin; IFX, infliximab, infliximab; LTα, lymphotoxin-α; mAb, monoclonal antibody; NF-κB, nuclear factor-κB; RA, rheumatoid arthritis; TNF, tumor necrosis factor TNF; TNFR, TNF-receptor.

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cytokine, lymphotoxin-α (LTα) [9–11]. Before long, these proteins were purified and characterized, the murine and human cDNAs were cloned [12–14], and thus began the exciting era of anti-TNF cytokine therapy.

Arguably the greatest interest in TNF came with the demonstration that TNF controlled tumor growth not only *in vitro* [5,6] but also *in vivo* [15]. Early reports demonstrated that monocyte-expressed TNF was capable of selective tumor cytotoxicity [16]. However, the rapidly improving molecular biology capabilities of that time quickly provided recombinant TNF, and the easy preparation and availability of lipopolysaccharide (LPS), otherwise known as endotoxin, meant that experimentally induced murine tumors could be treated *in vivo* either by TNF directly, and/or by LPS (endotoxin) – a biological inducer of TNF [1]. These studies convincingly demonstrated TNF's potent tumoricidal activity. However, with this early success, it was initially overlooked that the histopathological analysis had also revealed that the tumoricidal effects were both due to tumor necrosis and to tumor-associated capillary injury [15,17]. Spurred on by the hope of it being a non tumor-type specific anti-cancer therapeutic reagent, together with its apparent safety in rodent models of disease, recombinant TNF was quickly channeled into human clinical trials.

Phase I clinical trials began with recombinant human TNF monotherapy. In all cases, diverse dose-dependent acute toxicities were immediately evident, including fevers, chills, nausea (plus or minus vomiting), shortness of breath, tachycardia and hypotension [18–21]. Notably, these trials also reported that many patients surprisingly experienced noticeable confusion soon after infusion. Although a few patients experienced a transient benefit there were no long-lasting treatment responses and most patients eventually succumbed to their tumors – due in part to the trials' recruitment of patients with high tumor burden [18–21]. TNF was also administered after recombinant interleukin (IL)-2, but there was still no significant anti-tumor efficacy recorded [22]. Overall, some 18 monotherapy Phase I trials and 10 Phase II trials, and another 18 combination trials were performed – without any significant success (for a detailed review see [23]). Taken together, and now with the virtue of hindsight, the results of these trials revealed that the broad biological "side effects" (diverse physiological responses of TNF) far outweighed the preliminary indications of TNF's tumor cytotoxicity. Thus, the hopes of TNF being the great "tumor necrosis" factor and a cure for cancer were dashed, despite extensive trials and much analyses.

Simultaneously with these events there was also significant attention being paid to the observation that neutralizing antibodies to TNF (induced by passive immunization) protected mice against lethal TNF-mediated endotoxemia [24]. These studies were instrumental in proving that TNF is both potently tumorocidal, as well as being an essential mediator of inflammation. In fact, what quickly became evident was that TNF was a highly pro-inflammatory agent, both independently, and via its ability to induce expression of IL-6 [25,26]. These early findings represent the seminal studies that directly lead to the opposite approach of neutralizing TNF to inhibit inflammation. These were also the initial revelations of an incredibly diverse range of physiological functions of TNF. Today TNF is still widely regarded as arguably the most pleiotropic of all cytokines described in mammals, with activities spanning virtually every biological system from immune system physiology to neurobiology and beyond.

3. TNF and TNF-receptor (TNFR) molecules: complex interactions

TNF is a transmembrane 26 KDa protein expressed by activated monocytes/macrophages (including central nervous system (CNS) microglia), activated NK and T cells, but also by a diverse array of non-immune cells such as endothelial cells and fibroblasts [27,28]. The production of TNF mRNA is transcriptionally regulated, induced by nuclear factor-κB (NF-κB), c-Jun, activator protein-1 (AP1) and nuclear factor associated with activated T cells (NFAT), consistent with the presence of these transcription factor binding sites within the promoter region of the TNF gene [29]. Post-transcriptional mRNA regulation also occurs. This is largely by the actions of miRNAs and RNA binding proteins, such as specific 3′-untranslated region AU-rich elements, tristetraprolin and mRNA decay factors (for reviews, see [30,31]).

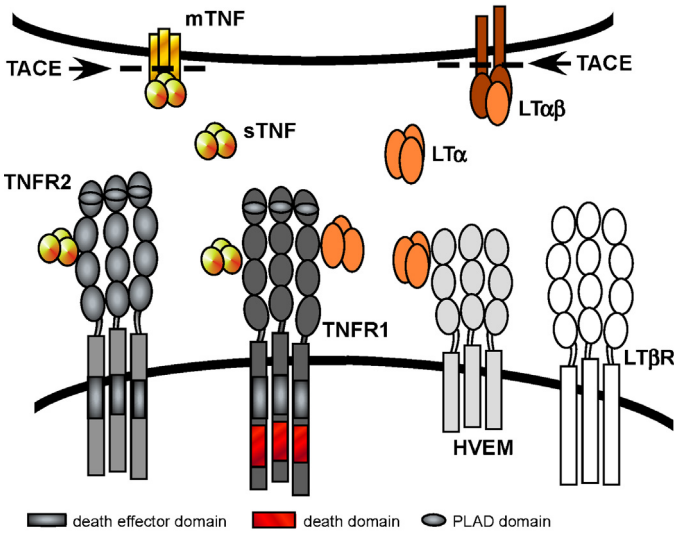

Fig. 1. TNF and TNFRs. Complex interplay between soluble and membrane bound TNF and LTα ligands, and their cognate receptors. Membrane TNF is cleaved by TACE to produce soluble trimeric TNF that binds TNFR1 and TNFR2. Membrane TNF also binds both TNFR1 and TNF2 molecules. Another TNF-ligand cytokine LTα is secreted as a homotrimer and/or found as a biologically active complex in association with membrane bound LT-β. LTα binds TNFR1 as well as the herpes virus entry mediator HVEM but not the LT-β receptor.

As a transmembrane protein expressed on the surface of cells, membrane TNF (also sometimes referred to as pro-TNF) is cleaved by a metalloprotease, TNFα-converting enzyme (TACE) [32,33]. This liberates a trimeric soluble cytokine – the 17 KDa soluble TNF (sTNF). This is the form of TNF found in blood plasma, i.e., the form that circulates throughout the body and confers TNF with its potent endocrine function – its ability to act at distant physiological sites, far away from the site of its synthesis. Both soluble and membrane TNF bind to two transmembrane receptor molecules: TNFR1 (also sometimes referred to a p55/p60) – a death-domain-containing protein, and TNFR2 (also known as p75/p80) [34] (see Fig. 1). Interestingly membrane TNF is a more potent ligand for TNFR2 [35], and while most cells express constitutive but low levels of TNFR1, only some cells express detectable surface TNFR2 [36]. However, the expression levels of TNFR proteins can be regulated by cytokines, especially by interferons [37,38], which explains, in part, the noted synergy between TNF and interferons [7,8].

Whilst the molecules comprising the TNF/TNFR system are all well defined biochemically, the biological interactions of ligand and its receptors are not so simple. For example, complex models for soluble TNF (ligand) passing between receptors have been proposed [39], and TNFR-binding to membrane TNF is capable of resulting in "reverse signaling", that is, signaling back into the membrane TNF producing cells [40]. In this context the intracellular regions of membrane TNF can become phosphorylated [41] and signal transduction can result in the activation of NF-κB, *i.e.*, within the TNF-producing cell [42,43]. Thus, reverse signaling can lead to altered cytokine expression by the same TNF-producing cell [44–46]. On the other hand, membrane TNF reverse signaling by

TNFR1-expressing endothelial cells can also lead to resistance of monocytes to LPS, resulting in reduced production of IL-1, IL-6 and IL-10 [47]. In addition, overexpression of the receptors alone, either *in vitro* or *in vivo*, can spontaneously induce TNFR signaling independent of ligand [48]. This feature is used extensively in *in vitro* assays demonstrating functional TNFR signaling and is largely due to overexpression-mediated TNFR oligomerization. Furthermore, lymphotoxin (LT)-α (LTα) is another TNFR1 ligand. It too has high affinity for TNFRs but it usually acts quite independently of TNF (see Fig. 1). The complexity of TNF ligand/receptor interactions cannot be understated since LTα binds both to TNFR1 and another TNF-R family protein, the herpes virus entry mediator (HVEM). Moreover, LTα in complex with LTβ, binds to the LTβ receptor (for review on TNF, LTα and their receptors see [49]) (see Fig. 1).

4. TNF-induced TNFR signaling: diverse pathways of apoptosis and inflammation

A plethora of *in vitro* studies have revealed complex and divergent TNF-R signaling pathways. Generally speaking there are distinct TNFR-specific signaling pathways, which are extraordinarily complex, but which account for all aspects of TNF's ability to induce both cell death and/or co-stimulation and cell activation. Generally speaking the process begins via the association of TNFR1 or TNFR2 proteins forming trimers and this is required for TNF-binding. The trimers themselves are probably only transiently expressed on the cell surface, as they are notoriously difficult to visualize in that location (Gale A and Sedger LM, personal observations). Signaling-competent TNFR trimers undergo a conformational adjustment that requires the pre-ligand assembly domain (PLAD) which is located within the N-terminal cysteine-rich domain (CRD) of many TNFR-family molecules [50]. This PLAD-dependent TNFR trimer adjustment is thought to be required to permit TNF binding and ligand-induced receptor signaling [50]. On other hand, it is unclear if a PLAD-mediated conformational change is required for ligand-independent (overexpression induced) receptor signaling.

4.1. TNFR-induced cell death

TNF-induced cell death signaling is carried out by TNFR1 [51]. This requires the release of an intracellular TNFR inhibitor, the silencer of death domain (SODD) protein [52,53]. Essentially, PLAD-stabilized TNFR interactions permit the release of SODD and the recruitment of intracellular "death signaling inducing signaling complex" (DISC) proteins, including TNFR-associated death domain protein (TRADD), Fas associated protein with death domain (FADD) and the TNFR-associated factor (TRAF)-1 [54–57]. These proteins create a scaffold permitting the recruitment of additional proteins such as the initiator caspase, pro-caspase-8, which, when proteolytically cleaved, releases an active form of caspase-8 [58]. The freed, active, caspase-8 then enzymatically processes pro-caspse-3, -6, -7, and other cytosolic substrates, converting these executioner pro-caspases themselves into active enzymes [59]. The activation of caspase-3, in particular, is essential for TNF-induced cell death, as it targets a latent DNAse that degrades genomic DNA [60] thus causing apoptotic cell death; the caspase activated DNase (CAD) [60]. The protease activity of caspase-8 is tightly regulated by a negative inhibitor protein FLICE/caspase-8 inhibitory protein (cFLIP). cFLIP lacks a death-domain but contains a death-effector domain (DED) that permits its interactions with pro-caspase-8 as well as other DED containing proteins [61], thus preventing constitutive pro-caspase-8 recruitment to the TNFR1 DISC. The inhibitor of apoptosis proteins (IAPs) are also important regulators of TNFR-induced cell death. IAPs act by virtue of their direct interaction with TRAF2 [62,63]. Thus, TNF interaction with TNFR1 induces a caspase-dependent apoptotic cell death that is critically regulated by cFLIP and IAPs. Although TNF-induced cell death is well characterized, it does not often occur without provocation or cellular pertubation, that is, not unless there is some sort of aberration or inhibition of the cell cycle [64], protein synthesis, or altered cell metabolism [65]. As such, TNF is a powerful inducer of apoptotic cell death, but, as originally stated, usually only in transformed cells (cancer cells) [66,67], virus infected cells [68,69], biochemically imbalanced or stressed cells, not in most normal primary mammalian cells.

4.2. TNFR-induced NF-κB

In contrast to its name, TNFR signaling generally does not kill most cells, but instead, it results in the activation of NF-κB and/or several additional non-death signaling pathways. TNFR signals NF-κB activation for cell survival by recruiting TRADD and TRAF2, which results not only in the activation of NF-κB but also in signaling via mitogen activated protein kinase (MAPK) and c-Jun-terminal kinase (JNK) [70]. Here, TRAF2 interacts with MAPK kinases, that permits the activation of JNK, p38 SAP kinase and MAPK [71,72]. TRAF2 is therefore critical to TNFR-induced activation of NF-κB because TRAF2 and receptor interacting protein kinase (RIP) activate the inhibitor of NF-κB kinase (IKK), as well activating the IKK-activating kinase, NF-κB-inducing kinase (NIK) [73]. Upon the phosphorylation and ubiquitin-dependent degradation of IKK, NF-κB transcription factors translocate into the nucleus where they bind to DNA and function as transcriptional activators. Moreover, NF-κB itself can transcriptionally induce TNF, as well as TRAF1 and TRAF2 genes, and thereby further amplify TNF/TNFR signaling pathways [74]. Furthermore, the activation of JNK and its subsequent signaling activates transcription factors c-Jun, AP1 and ATF2 [75,76]. Hence, these pathways explain the ability of TNF to induce other inflammatory cytokines such as IL-6 and IL-8, and TNF's ability to induce [77] and synergize with interferons [8,78].

This classical NF-κB activation pathway reverts to a non-canonical, or alternate NF-κB signaling pathway in situations where TRAF2/3 or IAP are blocked [79]. Under these circumstances NIK abundance is stabilized, which allows NIK-dependent processing of NF-κB2 p100 [80–82]. [Note: the classical pathway proceeds first because the cIAP/TRAF2/3 complex constitutively degrades NIK in normal circumstances]. TNF and TRAF3 are important in activated T cells [83], where expression of an alternatively spliced form of TRAF3 (lacking exon 8) allows for non-canonical activation of NF-κB [84] and, while the mechanism(s) that control the differential regulation of TRAF3 alternate splicing are not known, it has recently been shown that T cell-specific TRAF3$^{-/-}$ mice produce twice the normal number of TNFR2-expressing T regulatory cells (Tregs) [85]. Nevertheless, taken together, TNF-induced NF-κB is important in inflammation since NF-κB is a global trans-activator of numerous pro-inflammatory cytokines, chemokines, and their receptors, and a critical regulator of leukocyte activation and function.

4.3. TNFR-induced ubiquitination and viral deviation

The full spectrum of TNF-signaling molecules involved in these pathways is actually quite broad and these pathways are described above in relatively simplified terms. In fact there are a number of additional, more recently described, proteins involved in TNFR signaling, such as HOIL-1, HOIP and Sharpin. These molecules are recruited to the TNFR-signaling complexes, where they function in the linear ubiquitination and degradation of RIPs and NEMO/IKKγ [86–90]. There are also a number of variations to these TNFR cell death and NF-κB pro-proliferation pathways. For example,

caspase-8 can cleave BID leading to a mitochondrial involvement in the apoptosis process [91–93]. Moreover, JNK activation is not always pro-proliferative, but can drive apoptosis through the cleavage of the BH3-only protein BID (at a different site to that which is cleaved by capase-8) leading to release of second mitochondrial-derived activator of caspase Smac/DIABLO and mitochondrial-mediated apoptosis [94,95].

Given the potent anti-viral activity of TNF [68,69], which is mediated through both TNFRs [96], one must also consider signaling in the context of virus infection. In this regard it is worth noting that many viruses have evolved to encode and express molecules that specifically inhibit almost every step of TNFR-induced apoptosis [97] and NF-κB signaling pathways [98,99]. Indeed most poxviruses viruses express a pan-caspase inhibitor such as CrmA [100,101], and certain herpes viruses encode a viral-FLICE that inhibits caspase-8 [102]. In cells infected with these viruses "normal" TNF/TNFR1 signaling is blocked [68,69] and TNFRs induce cell signaling via a RIP kinase-dependent cell death pathway that results in a form of TNF-induced cell death described as "programmed necrosis" [103].

4.4. TNFR-induced inflammation

A review of TNFR signaling must also consider that TNF ligation of TNFRs also leads to non-apoptotic and non-proliferative signaling pathways. These include acid and neutral sphingomyelinase pathways and the activation of 5-lipoxygengase and phospholipase A2 enzymes, that result in the production of arachindonic acid, 5-hydroxyeicosatetraenoic acid (5-HETE) and proinflammatory leukotrienes [104]. The sphingomylinase pathway leads to the production of diacyl glycerol and subsequently to the activation of protein kinase C, and eventually NF-κB (independent of TRAF-activated NF-κB [105,106]). This results in the expression of pro-inflammatory cytokines and chemokines but also in production of prostaglandins [107]. There is also the recruitment of a molecule known as Fas-associated with neutral sphingomyelinase (FAN), to a membrane proximal domain in TNFRs [108], and the actions of acid sphingomylinase that result in production of ceramides. Ceramides induce active cathepsin D, an aspartate non-caspase protease that can target BID [109,110]. Thus FAN connects the TNFR biology to the plasma membrane, and more specifically, to cytoskeletal re-organisation, filopodium formation, and macropinocytosis [111], and hence to processes of leukocyte migration [112]. Ceramide is also a powerful inflammatory intermediate involved in several cellular processes including cell migration, proliferation, and apoptosis (for review see [113]). Thus, the effects of TNF-induced TNFR signaling pathways are diverse, and varied in different cell types, and specific circumstances – explaining TNF's pleiotropic properties.

5. TNF *in vivo* biology – animal models and human diseases, what do they tell us?

5.1. TNF – a physiological mediator of inflammation

TNFs' stimulation of globally activating transcription factors such as NF-κB, and its signaling via bio-active lipids that induce arachidonic acid, 5-HETE and ultimately leukotrienes and prostaglandins, explain its effects on diverse cells within almost every human physiological system. They also explain TNFs powerful pro-inflammatory capacity, especially within immune cells capable of producing a cascade of downstream cytokines and chemokines. For example TNF promotes monocyte/macrophage differentiation [114,115], can enhance activated B cell proliferation [44] concomitant with an autocrine increase in TNFR expression [116,117]. It promotes the proliferation of fibroblasts [118,119] and is a powerful inducer of inflammation, often acting together with together with IL-1β [120]. Depending on the cell type it is produced by, or acts upon, TNF (with or without IL-1β) is a potent inducer of IL-6 [25,26] and the further production of TNF itself [121]. In fact circulating IL-6 is significantly elevated in healthy humans infused with recombinant human TNF, and/or TNF and IFNγ, even when administered locally [122,123], or during bacterial infection [124,125]. Together these cytokines are the central mediators of endotoxic shock which is physiologically regulated via the natural production of soluble IL-1 receptor antagonist (IL-1Ra) and/or soluble TNF-receptors (for review see [126]).

5.2. TNFR biology in genetically predetermined autoinflammation

The role of TNF inflammation was further confirmed by the observation that germline mutations in TNFR extracellular domains defines a family of dominantly inherited auto-inflammatory syndromes, known as "TNF-receptor associated periodic syndrome" or TRAPS [127]. Presenting with a range of symptoms, but predominately as unexplained episodes of fever and inflammation, TRAPS patients represent nature's version of a structure/function mutational experiment. A number of theories as to why germline TNFR extracellular domain mutations result in fever and autoinflammation have been proposed, including aberrant folding [128,129], spontaneous overexpression, aggregation and constitutive TNFR signaling [130]. However, abnormal ER retention [131], mitochrondrial reactive oxygen species [132], ER stress, and the stress response to "unfolded" or aggregated proteins also contribute to TRAPS pathogenesis [133]; for review see [134]. In most cases of TRAPS, fever and inflammation are reflected in elevated inflammatory mediators, of which TNF, IL-6 and IL-1β are central [135]. Consistent with this, most TRAPS patients respond well to treatment with TNF or IL-6 inhibitors [136,137], although not without complications or incomplete remission, and these treatments often fail to reduce the elevated levels of acute phase proteins (for reviews see [135,138]). Nevertheless, the findings that humans with naturally occurring TNFR mutations present with transient spontaneous fevers and inflammation represent the ultimate biological evidence that TNF and TNFRs are central components of inflammation.

5.3. TNF has direct anti-viral and anti-bacterial activity

TNF is one of the most potent anti-viral cytokines described to date. It acts alone or in synergy with interferons [68,139] and its anti-viral activity requires both TNFR1 and TNFR2 [96]. Here, TNF plays several hands simultaneously: it is required for inflammatory cell recruitment, acting largely through TNF-induced chemokine expression, and its ability to induce inflammatory mediators that act as potent chemoattractants for innate immune cells such as neutrophils, monocytes, natural killer cells and antigen presenting cells, including immature or tissue resident macrophages and dendritic cells [140–142]. TNF, through its potent activation of NF-κB, appears to be integral to the maturation of these myeloid cells into their functionally mature effector phenotypes [140–142]. For example, immature tissue resident dendritic cells require NF-κB to convert them into mature antigen presenting cells, that stimulate naïve T cells in nearby draining lymph nodes and initiate antigen-specific T and B cells responses. It also influences macrophage differentiation, promoting M1 phenotype cells, over the alternatively activated and largely tolerogenic M2 cell-subtype [143,144]. As a membrane bound molecule TNF provides B cell "help", thereby promoting antibody production [145]. TNF is also directly anti-viral via its direct induction of TNFR1-mediated apoptotic cell death [69]. In this scenario TNF is directly cytotoxic, specifically killing the virus-infected cells prior to maximal virus replication,

an effect that is amplified in the presence of interferon [68]. The potency of TNF-anti-viral activity is reflected in the fact that many human pathogenic viruses have evolved sophisticated strategies to specifically subvert various molecules in the TNF/TNFR axis [97]. Of these, the poxviruses are particularly noteworthy, evolving to encode viral TNFR-homologous genes that produces soluble TNFR "decoy" receptors [146]. In many ways these viral TNFR molecules can be considered the prototype of Etanercept (Enbrel®), binding to soluble TNF with high affinity [147–149] and inhibiting TNF's cytotoxicity and inflammatory properties [150].

5.4. TNF in neurobiology

TNF has incredibly broad biological effects that are far beyond the scope of this manuscript. Suffice that we briefly pay attention to this most under-appreciated area of TNF biology: the role of TNF in neurobiology. This is an exciting area of research that is only recently enjoying the spotlight, as neuroscience research rapidly expands and joins hands with immunology. Intriguingly, the TNF/IL-1β/IL-6 axis in LPS-challenge has been shown to also involve a neurological response, physiologically linking systemic inflammation with subsequent neurological and neuropsychiatric conditions [151]. Even peripheral inflammation, by LPS, Toll-like receptor (TLR) stimulation or TNF, induces increased local brain TNF expression in mice [152]. These findings represent the tip of the iceberg as there is now considerably attention being paid to the physiological role of TNF in the central nervous system (CNS), especially in psychological and neurological conditions. What is clear is that cytokines such as TNF, IL-1 and IFNγ are produced by glial cells in the CNS, but whether TNF plays a protective or pathological role appears to depend highly on the context (for reviews see [153,154]). Excess TNF is also implicated in neuronal toxicity acting syneristically with glutamate, albeit in neuronal cells *in vitro* [155]. Of particular note, however, is the recent demonstration that sympathetic neurons express membrane TNF that are capable of reverse signaling, which is important for neuronal growth and branching during post-natal development [156]. This study may be the first to provide a convincing example of a physiologically important role for soluble TNFR and TNFR reverse signaling independent of infection, inflammation or immunopathology [156]. There is also now an increasingly large bank of publications emerging that strongly implicate a role for TNF in conditions with cognitive impairment, bipolar disorder (especially during episodes of mania and/or depression), and in CNS tissue injury. Further detailed knowledge of the physiological role of TNF in normal CNS tissue is therefore urgently needed, especially given that the capacity to co-treat these conditions with TNF neutralization is being actively explored (discussed below).

6. Anti-TNF therapeutics – What are they?

Despite the broad dose-limiting toxicities preventing the use of TNF as a chemotherapy agent, the potential to block TNF in inflammatory disease has remained evident from the very early days. Moreover, the long functional half-life and *in vivo* safety of Ig immediately suggested that anti-TNF antibodies would ameliorate TNF-mediated inflammation. Several TNF-specific monoclonal antibodies (mAbs) and recombinant fusion proteins have been produced. Their development and human therapeutic uses are summarized below.

6.1. Infliximab (abbreviated here as IFX), trade name Remicade®

A human TNF-specific neutralizing antibody, infliximab (abbreviated here as IFX), trade name Remicade®, was developed in the late 1990's. This anti-TNF chimeric mAb reagent comprises the murine immunoglobulin (Ig) heavy (H) and κ light (L) chain variable (V) regions with specificity for human TNF, and human IgG1 Ig constant (C) regions [157] (see Fig. 2). IFX binds to soluble and membrane TNF, and when bound it prevents TNF from binding to its receptors; it therefore prevents ligand triggered TNF-R signaling [157,158]. IFX was highly successful therapeutically, even from the first of many clinical trials [159]; it is an effective inhibitor of TNF-induced inflammation in a range of human diseases, including the spectrum of rheumatic inflammatory diseases as well as Crohn's disease (see Table 1).

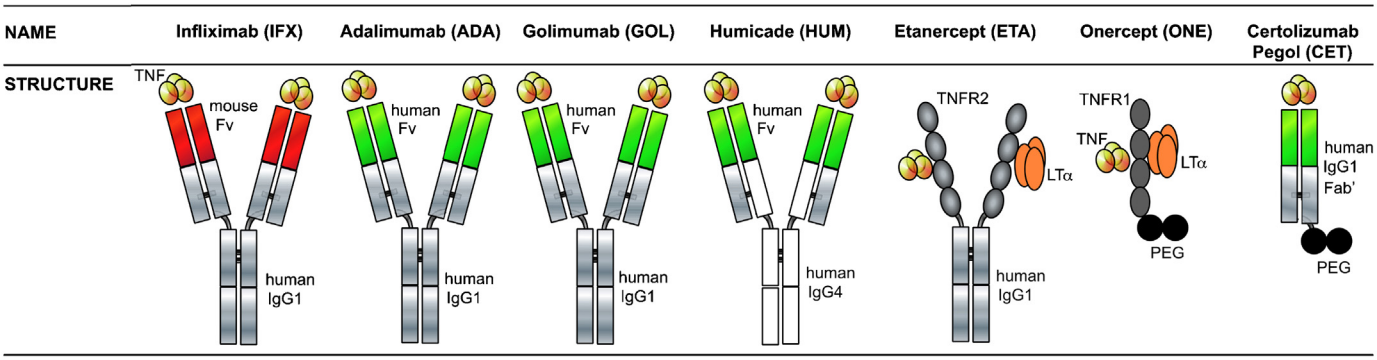

Fig. 2. Current anti-TNF biologics (including biosimilars) and their biological properties. Shown are chimeric mouse Fv (red) human Fc (gray) anti-TNF monoclonal Ig infliximab (IFX), and humanized or fully human Fv (green) anti-TNF monoclonal Ig adalimumab (ADA), golimumab (GOL) and humicade (HUM). TNFR-based TNFR2: human Ig Fc etanercept (ETA), pegylated recombinant extracellular TNFR1 onercept (ONE) and pegylated human IgG1 Fab' certolizumab pegol (CET).

Table 1
List of currently available anti-TNF therapeutics and their approved indications.

CURRENT ANTI-TNF BIOLOGICS				
Drug name & structure	Brand name (Company and first aproval date)	Route/Half-life/co-therapy[a]	Approved disease indications	Website
Etanercept Recombinant fusion protein: Human TNFR2:IgG1-Fc	Enbrel® (Immunex/now Amgen (US), and Pfizer (UK)) FDA registered Nov.1998	s.c. injection, 2–4 weeks 25 mg vial 50 mg/mL	• Rheumatoid arthritis • Polyarticular juvenile idiopathic arthritis • Psoriatic arthritis • Ankylosing spondylitis • Plaque psoriasis	enbrel.com
Infliximab Humanized (chimeric) IgG1κ mAb	Remicade® (Centocor Ortho Biotech Inc. (US) and Janssen-Cilag Pty Ltd. (UK)). FDA registered Aug. 1998	i.v. infusion 4 weeks 100 mg vial	• Rheumatoid arthritis* • Psoriatic arthritis* • Ankylosing spondylitis • Plaque psoriasis • Crohn's disease (moderate/severe) • Pediatric RA & Pediatric Crohn's	remicade.com
Adalimumab Human IgG1κ mAb	Humira® (ABBVRIE Inc.) FDA registered Dec. 2002.	s.c. injection 2–4 weeks, 40 mg/0.8 mL syringe/vial	• Rheumatoid arthritis* • Psoriatic arthritis* • Plaque psoriasis • Active ankylosing spondylitis • Crohn's disease (moderate/severe) • Juvenile idiopathic arthritis (severe)	humira.com
Golimumab Human IgG1κmAb	Simponi® (Centocor Ortho Biotech Inc.) FDA registered Apr. 2009.	s.c. injection 2 weeks 50 mg injection	• Ulcerative colitis • Rheumatoid arthritis* • Psoriatic arthritis* • Plaque psoriasis • Ulcerative colitis	simponi.com
Certolizumab Pegol Pegylated-Fab' fragment of humanized IgG1κ mAb	Cimzia® (UCB Pharma SA) FDA registered Apr. 2008	s.c. injection 200 mg injection	• Rheumatoid arthritis* • Psoriatic arthritis* • Ankylosing spondylitis • Crohn's disease (moderate/severe)	cimzia.com
CTP-13 Humanized (chimeric) Infliximab biosimilar IgG1κ mAb	Remsima®; Infliximab (Celltrion Healthcare Inc) and Inflectra® (Hospira)	tba	• Rheumatoid arthritis* • Psoriatic arthritis* • Ankylosing spondylitis • Plaque psoriasis • Crohn's disease (moderate/severe) • Pediatric RA & Pediatric Crohn's	tba

PREVIOUS PIPELINE ANTI-TNF BIOLOGICS				
Drug name & structure	Brand name (Company)	Route	Targeted/disease indications	Major clinical trials
CDP571 Humanized IgG anti-human TNF mAb	Humicade (discontinued)	N/a	•Crohn's disease	[213,214, 351,352]
Onercept Pegylated dimeric extracellular Human TNFR1	Serono (discontinued)	N/a	•Crohn's disease	[353–358]

[a] Route of drug administration: sub-cutaneous injection (s.c.) and intravenous (i.v.).
Approved as monotherapy and/or with methotrexate (±MTX) for RA or psoriatic arthritis*, or for multiple myeloma combined with dexamethasone, as indicated.
Tba; to be announced.

6.2. Adalimumab (abbreviated here as ADA), trade name Humira®

ADA is another anti-TNF neutralising IgG that was first assessed in clinical trials in 2002. In this case the IgG was a fully human IgG1 (see Fig. 2) – theoretically minimizing the potential to elicit anti-mouse mAb Ig, specific to the murine Ig Fv component of IFX mAb. Phase I trials demonstrated safety, favorable pharmacokinetics, and efficacy for rheumatoid arthritis (RA), when administered with or without methotrexate [160–162].

6.3. CD571 (also known as Humicade®)

Soon afterwards CDP571 (Humicade®), a human IgG4 anti-TNF mAb was also developed (see Fig. 2). It too, bound human TNF with high affinity, and blocked TNFs cytotoxic activity. Known as Humicade® it was able to neutralize TNF in *in vivo* animal models, similarly to IFX and ADA, and yet it surprisingly failed to demonstrate efficacy in a clinical trial for Crohn's disease and was not developed further [163–166]. (Potential reasons for the failure of Humicade in the clinic may have subsequently become evident and are discussed below).

6.4. Etanercept (abbreviated here as IETA), trade name Enbrel®

A novel TNF-Receptor: Ig fusion protein was developed and FDA approved in 1998. ETA comprises the extracellular region of human TNFR2 expressed as a fusion protein with a C-terminal human IgG1 crystallizable fragment (Fc) domain [166] (see Fig. 2). This reagent was the first recombinant receptor:Ig fusion protein approved for therapeutic use in humans; it bound to human TNF with an affinity comparable to endogenous TNFR2, and blocked TNF's cytotoxicity and inflammatory capacity [163–166]. It was successful in clinical trials and is still broadly used in inflammatory diseases (see Table 1).

6.5. Golimumab (abbreviated here as GOL); trade name Simponi®

Ten years or more later, and patent protection for IFX, ADA and ETA aside, additional anti-TNF agents have now emerged. Golimumab (GOL), trade name Simponi®, is a fully humanized IgG1 anti-TNF (see Fig. 2) with an Ig Fc identical to IFX but an engineered human Fv Ig sequence [167]. It is effective in the treatment of rheumatoid arthritis (RA), psoriatic arthritis and ankylosing spondylitis, and even for RA patients who experienced little benefit or adverse events from IFX [167–169].

6.6. Certolizuman Pegol (abbreviated here as CERT): trade name Cimzia®

Certolizumab (CERT) is a pegylated dimeric Ig Fab'-domain of a TNF-specific IgG1 mAb (see Fig. 2). Of note, the PEG-component, which reduces immunogenicity and improves *in vivo* half-life [170], was specifically engineered for attachment to the C-terminus of the Ig Fab' in a manner that does not interfere with the Fab's TNF-specificity and TNF-neutralizing properties [171]. It has demonstrated efficacy, with or without co-immunosuppressant agents, for patients with moderate-to-severe Crohn's disease [172,173]. For recent excellent reviews on the properties of these newer anti-TNF agents see [174,175]; for a summary of the approved uses of all currently available anti-TNF biologics see Table 1.

6.7. Onercept (abbreviated here as ONE)

Several other biological anti-TNF reagents, such a onercept (ONE), a soluble recombinant human TNFR1 were also developed [176]. These reagents all demonstrated efficacy in early, *i.e.*, preclinical, animal models of inflammatory disease [177,178]. Although early data indicated these molecules were safe for *in vivo* administration, they exhibited relatively slow absorption rates, and short *in vivo* half-lives, and this appeared to correlate with minimal clinical efficacy in Phase II and III treatment trials of moderate-to-severe plaque psoriasis, even with pegylated versions for more favorable pharmacokinetics [179].

The anti-TNF agents IFX, ADA, ETA, GOL and CERT all comprise either high affinity human TNF-specific mAb or TNFR-extracellular IgFc engineered mAb domains, and as such, they all exhibit high-specificity for human TNF. Since ETA and ONE are literally TNFR proteins they also interact with LTα – similar to the native TNFRs (see Fig. 1). Note: LTα binds TNFR1, in addition to HVEM, but not TNFR2 [49], as shown in Fig. 1. Thus these reagents are **all** functionally capable of blocking TNF from binding to TNFR1 and/or TNFR2, either through steric-hindrance or because the antibody's epitope overlaps with the TNF/TNFR ligand/receptor interaction sites. They are, therefore, all high-affinity agents capable of neutralizing TNFs cytotoxicity *in vitro* and *in vivo*. Moreover, due to their *in vivo* safety and anti-inflammatory disease efficacy, IFX, ADA, ETA, GOL, and CERT are now licensed and approved for human therapeutic use worldwide; they are approved by the US Food and Drug Administration (FDA), the British Medicines and Healthcare Products Regulatory Agency (MHRA), and numerous other therapeutic regulator bodies such as the European Medicines Agency (EMA), the Australian Therapeutic Goods Administration (TGA), South African and Latin American and other health product regulatory bodies. They are approved for clinical use in the inflammatory diseases: RA and psoriatic arthritis, plaque psoriasis, ankylosing spondylitis, Crohn's disease and/or ulcerative colitis (see Table 1). Their consumer uptake depends essentially on pricing, availability and clinician preferences.

6.8. Biosimilars

A number of biosimilars (aka similar biological medicinal products), or "follow-on" anti-TNF drugs also now exist [180,181]. These are, by definition, "copy-reagents" produced by manufacturers who produce an analogous reagent by virtue of having access to the original innovator drug but without access to the original clone or manufacturing process (a trade secret protected by patent law). They are therefore theoretically identical in biological activity, yet they may differ in clinically inactive components, usually due to differences in manufacturing processes [180,181]; (biosimilars are distinct from "generic" pharmaceuticals – a term usually preferred for small-molecule inhibitor type drugs of identical active ingredient and equivalent quality, and usually sold without reference to the original brand-name). Through a streamlined process they must be assessed in at least one "non-inferior" clinical trial prior to FDA and EMA approval. The first of these is CTP-13 (Remsima®; Inflectra®). CTP-13 is an identical copy anti-TNF innovator mAb IFX. Recent reports of randomized double-blind, multi-center (multinational) trials found that CTP-13 is as effective as IFX in the treatment of active RA, when assessed by European League Against Rheumatism (EULAR) response criteria [182] and by change in disease activity scores, with similar pharmacokinetics in ankylosing spondylitis patients [183].

6.9. Other agents: curcumin

One of the longest known natural anti-inflammatory agents is curcumin. It is chemically defined as (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dionediferuloymethanne), but better known as diferuloylmethane, and an abundant component of the spice turmeric. Curcumin is a naturally produced compound that inhibits TNF and other pro-inflammatory cytokines including TNF, IL-1β and IL-6; it is a broad-acting anti-TNF and anti-inflammatory nutraceutical, generally consumed orally via natural food spices and/or via medicinal preparations (for reviews see [184,185]). Despite its long-known medicinal anti-inflammatory activity, it is poorly soluble in aqueous solutions. However, it is pharmacologically well characterized and considered safe even at high concentrations [186,187]. Nearly 100 clinical trials for curcumin are currently listed at http://www.clinicaltrials.gov and due to its newly appreciated neuroprotective effects [188,189] it is also being actively investigated in several neurological conditions, including Alzheimer's disease [190].

6.10. Other agents: thalidomide

Thalidomide (brand names Immunoprin®, Talidex®, Talizer®, Thalomid®) is chemically defined as *RS*)-2-(2,6-dioxopiperidin-3-yl)-1*H*-isoindole-1,3(2*H*)-dione. It was initially developed in the early 1960's as a sedative and hypnotic agent [191], but it quickly became known for its anti-nausea properties and was consequently sold without prescription to thousands of pregnant women to treat "morning sickness". Soon afterwards, however, children were born with severe malformations, revealing its teratogenic capacity [192]. Amongst a wide spectrum of biological activities, thalidomide was also found to inhibit the production of TNF, primarily by suppressing TNF-induced NF-κB [193] (for review see [194]). It has therefore been reborn as an effective agent in the treatment of erythema nodosum leprosum (ENL, a complication of leprosy) and multiple myeloma [195]. Just like curcumin, discussed above, it has recently been reported to attenuate inflammation in CNS pathologies, such as Alzheimer's disease [196,197].

7. Anti-TNF therapeutics – how do they work?

All anti-TNF agents described above bind to soluble and membrane TNF with high affinity and specificity, and they all prevent TNF from binding to TNFRs (see Fig. 3, panel A and B). By blocking TNFR activation they prevent all of TNFs ability to induce inflammation, including all downstream mediators such as TNF/TNFR-induced IL-6 – a potent mediator of TNF-induced inflammation [25,26]. Other mechanisms that contribute to the biological activity of these anti-TNF reagents include their ability to bind to membrane TNF and hence to induce reverse signaling in membrane TNF-expressing cells [198–201] (see Fig. 3, panel C). For reagents that comprise Ig components, their ability to engage with IgG Fc receptors (FcR) provides a mechanism for their involvement in antibody-dependent cell-mediated cytotoxicity (ADCC), especially via myeloid-lineage cells and the Fc moiety permits complement-dependent cytotoxicity (Fig. 3, panel C). These properties are not shared by all current anti-TNF agents, in part because they do not all contain an FcR-binding region; this feature likely contributes, in part, to their varied effectiveness in disease modifying capabilities [202]. However, it's apparent that mechanisms of anti-TNF biologics are complex and not completely understood, for while IFX ameliorates both RA and Crohns's disease severity, ETA shows minimal or no efficacy in Crohn's – despite its effectiveness for RA and related rheumatic diseases [203,204]. One explanation is that IFX, but not ETA, results in production of the immunosuppressive cytokine IL-10 (which might occur through reverse signaling from membrane TNF [205]). However, this is unlikely to completely account for their differential effectiveness, since TNFR2 is also a potent agonist for membrane TNF [35] and ETA is a human TNFR2 fusion protein, and hence capable of inducing TNF reverse signaling, even if less so than anti-TNF Igs [206]. Alternative explanations are that IFX can induce T cell activation-induced cell death [207,208], or that anti-TNF antibodies inhibit T cell proliferation and cytokine secretion and induce regulatory macrophages via FcR interactions [209]. However, in a direct comparison study, IFX, ADA and ETA were all shown to induce monocyte and lymphocyte apoptosis to similar extents [171,210]. Perhaps more relevant to inflammatory bowel disease, where the microbiome (the population of microorganisms that inhabit gut, skin, mouth and elsewhere in the body) is strongly implicated in disease etiology, this study also demonstrated that IFX, ADA and CERT are able to inhibit LPS-induced monocyte IL-1β while ETA is less effective at doing so [171,210]. Consistent with this argument, membrane TNF reverse-signaling has been shown to downregulate LPS-induced TNF, IL-1 and IL-6 [47]. Thus, it is possible that the difference in clinical effectiveness in anti-TNF biologics in Crohn's reflects their relative ability to induce membrane TNF reverse signaling with concomitant engagement with FcR-bearing cells (Fig. 3 panel C). Finally, it should not be forgotten that ETA, and ONE, both comprising native TNFR extracellular proteins, can also bind and neutralize LTα as well as LTα1β2, and LTα2β1, with or without FcR interactions (see Fig. 3, panel D).

Another major difference between the anti-TNF molecules is that they are administered by different routes and have different *in vivo* pharmokinetics and pharmacodynamics [211,212]. Also, some molecules do not contain an IgG Fc-region (see Fig. 2) that prolongs their life-span in plasma, and even those that do, will not all bind FcR's. For example, the Ig Fc-region component of CDP571 (HUM) was specifically engineered for a lack of IgG FcR binding - a feature that may have unexpectedly contributed to its failure in the clinic [213,214]. Another feature of the antibody-based agents is that they have two TNF binding sites, which stands in contrast to ONE (a single recombinant pegylated extracellular TNFR) and CERT (a pegylated single dimeric Fab' Ig fragment). Thus, although these agents all bind and neutralize TNF with high specificity and affinity, they differ significantly in their relative bioavailability and "TNF:anti-TNF agent" stoichiometry. The importance of these differences in biological properties between anti-TNF agents is intriguing, and underscores the mechanisms of their action with respect to their use in diverse pathologies *in vivo*. Indeed, even within a single disease, it might be worth considering whether these differences are actually revealing new disease sub-stratifications. Taken together the biological effects of anti-TNF biologics can be summarized into three main effector functions: first, they mop up excess soluble TNF (and/or LTα), reducing the endocrine activity of these cytokines (Fig. 3, panels A). Secondly, they bind to membrane-bound TNF (and/or LTα/β) complexes and either block cell–cell contact and/or trigger reverse signaling (Fig. 3, panel B and C). Third, they can act as agonists on FcR-expressing cells (Fig. 3, panels C and D) – an effector function quite distinct from their capacity to neutralize TNF. In addition, TNFR2 is highly expressed on Treg cells [215], and a recent report demonstrates that TNF antagonism restores normal function to dysregulated Treg cells in patients with RA, in a dose-dependent fashion [216]. Moreover, phosphorylation of FOXP3 (a key transcription factor in Treg function) is downregulated by TNF [216]. Further research

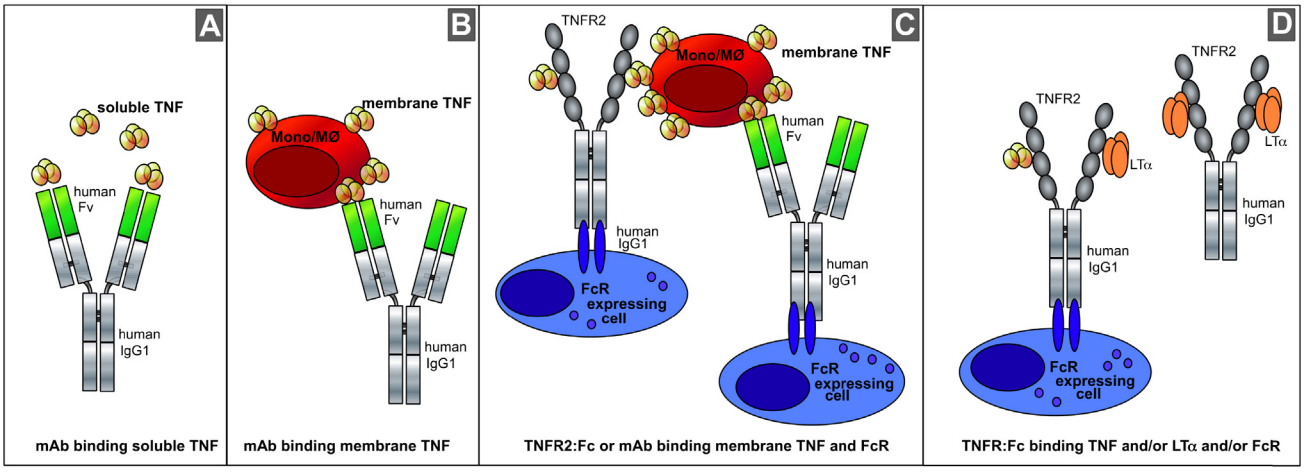

Fig. 3. Mechanisms of action of anti-TNF biologics. Anti-TNF biologics bind and neutralize soluble TNF (panel A) as well as membrane TNF (panel B). They also co-engage with Fc-R expressing cells, possibly with simultaneous engagement with TNF expressing cells (panel C). In addition, TNFR-based reagents can also bind and neutralize soluble or membrane bound forms of LTα cytokines (panel D).

will be required to determine if this also occurs with all anti-TNF biologics and in the treatment of other inflammatory conditions. Nevertheless, evidence is currently emerging that one of the main effector mechanisms of anti-TNF biologics is to critically control Treg cell function.

With respect to anti-TNF dietary compounds, curcumin appears to inhibit TNF transcriptionally, acting at several levels, but perhaps most importantly by inhibiting NF-κB [217,218]. It also broadly inhibits molecules known to be important in TNF-induced signaling, arachidonic acid metabolites such as phospholipase A2, cyclooxygenases and 5-lipoxygenase [219,220]. Thalidomide is similar in that, it too, inhibits TNF synthesis [193]. As discussed above, TNF is produced by diverse myeloid cells including dendritic cells, monocytes and macrophages including CNS microglia, and curcumin dramatically alters gene expression in all these cells [218,221–223]. Thus, curcumin and thalidamide are broad-acting anti-inflammatory agents that inhibit the production of TNF, amongst their other activities.

8. Anti-TNF therapeutics – adverse events and side effects: a "chicken *versus* egg" scenario?

With over a decade's experience in the treatment of a spectrum of rheumatic and inflammatory diseases several adverse events have emerged. The most frequent of these are relatively minor adverse events: injection site reactions and infusion reactions. These are probably unavoidable, and in fact perhaps they are to be expected, given the modes of administration are sub-cutaneous injection or intravenous infusion, and the Ig-related properties such as FcR binding (ADCC, ACC), *etc.* For the most part these agents appear to be surprisingly well tolerated. The other obvious risk is the development of anti-drug antibodies. This was initially thought to be more likely for anti-TNF antibodies that contain murine Ig components such as IFX [224], but neutralizing and immune complex forming anti-drug antibodies can also arise during treatment with fully humanized mAbs such as ADA [225]. Anti-drug antibodies can result in the loss of clinical response [225,226] as well as other adverse drug reactions, even acute hypersensitivity (anaphlaxis) [227]. Interestingly, co-administration of immune suppressants, such as methotrexate, generally reduces the incidence of anti-drug antibodies [228]. It is also widely believed that sub-therapeutic doses of anti-TNF biologics contribute to the development of anti-drug antibodies, and hence that increasing drug dose simultaneously raises *in vivo* levels of drugs through "trough" times (between doses), whilst simultaneously reducing the risk of developing anti-drug antibodies; (for review see [229]). Careful therapeutic monitoring of objective measures of disease presentation, together with anti-drug antibody monitoring, are therefore clearly required to ensure ongoing efficacy and safety of current anti-TNF biologics, as no therapeutic administration of mAbs is considered to be completely without risk.

8.1. Anti-TNF induced imunosuppression and risks of infection

The immunoregulatory effects of TNF mean that anti-TNF biologics create an immunosuppressed individual, which is exacerbated by the concomitant use of additional disease modifying anti-rheumatic drugs (DMARDs), *e.g.*, methotrexate or sulfasalazine, *etc.* (anti-TNF agents are FDA-approved and generally prescribed for patients whose disease had not responded to first-line DMARDs; see Box 1). Similary, in Crohn's disease patients, anti-TNF agents are often used together with azathioprine. As a consequence, most patients receiving anti-TNF biologics are profoundly immunosuppressed. The clinical use of systemic anti-TNF biologics is therefore not infrequently associated with worsening symptoms of infection, especially chronic and persistent infection including tuberculosis, or latent viral infections, such as varicella-zoster (chickenpox) or herpes zoster (shinges) [230–233]. Cases of exacerbated legionella have also been added to this list [234–237] and reports of severe acute respiratory virus infections including new influenza and adenovirus infections are often reported [238–240]. The US FDA recommends cessation of IFX, ETA, *etc.*, with onset of symptoms of virus infection, particularly influenza and influenza-like illnesses.

Box 1. Indications for anti-TNF biologicals use in RA and/or related conditions [270]

- American classification criteria for a diagnosis of RA, or active ongoing RA defined as DAS score >5.1 with two measurements (minimum 1 month apart), or other relevant disease diagnosis.
- Failed standard therapy with at least two standard anti-rheumatic drugs (hydroxychloroquine, sulphasalazine, penicillamine, azathioprinem methotrezate, or leflumomide); minimum 6 months or <2 months treatment if due to drug intolerance or toxicity.

Exclusion criteria for anti-TNF biologicals in RA and related conditions [269,349,350]

- Women who are pregnant or breast-feeding.
- Active bacterial or viral infection (includes live virus based vaccinations).
- Septic arthritis of native joint.
- Septic arthritis of implant/prosthetic joint.
- New York Heart Association Grade 3 or 4 congestive cardiac failure.
- History of demyelinating disease or present diagnosis of Multiple Sclerosis.

With an increased risk of infection comes the issue of vaccination, especially vaccines comprised of live microorganisms. However, patients taking anti-TNF agents continue to mount adaptive immunity, including B cell production of neutralizing anti-influenza Ig, albeit with reduced numbers of circulating $CD27^+$ memory B cells and lower neutralizing anti-influenza Ig titers, *i.e.*, reduced vaccine immunity [241,242]. It also raises issues of "endogenous risk" within specific geographic locations and the endemic levels of microorganisms in different countries, together with varied healthcare capability and healthcare proximity/availability. Thus, while differences in the incidence of reactivation of tuberculosis have been reported, being higher with IFX and ADA (both anti-TNF antibodies) treatment than with ETA (a TNFR-Fc) [243,244] (due to differential inhibition of phagosome maturation and function [142,245]), the relative risk warrants closer consideration. For example, the relative endemicity of tuberculosis in Asia, means that the risks of tuberculosis reactivation are greater in Asia than they are in North America or Europe [246], and it is here that the differences between anti-TNF biologic appear to become clinically important. Nonetheless, as indicated above, it should be remembered that most of these patients experiencing exacerbated microbial infections are taking a powerful combination of immunosuppressive agents (for review see [247]), and thus these anti-TNF agents may be undeserving of their reputation for increased risk of infection entirely by themselves. Nonetheless, patients taking anti-TNF biologics are advised to be aware of the early symptoms of infection and to cease drug treatment when infection is apparent.

8.2. Hematological malignancies

There are also several reports of patients on anti-TNF biologics developing lymphomas and other hematological malignancies. These include reports of lymphomas (Hodgkin's lymphomas, B-cell

lymphoma of unknown subtype, peripheral T-cell lymphoma, unspecified lymphomas, and hepatosplenic T cell or gamma-delta T cell lymphoma) and acute leukemias [248–250]. As a direct consequence of the perceived increase in hematological malignancy and widespread use of these and other immunosuppressive agents, the WHO classification of tumors now includes the category "iatrogenic immunodeficiency-associated lymphoproliferative disease" [251]. Nevertheless the actual reported incidence of malignancy remains low in terms of relative risk expressed as person/years, and a statistically significant difference in lymphoma incidence is difficult to substantiate [250]. In fact a 2011 Cochrane review, which takes into consideration some 163 randomized controlled trials with over 50,000 participants, and 46 extension studies with 11,954 patients, concluded that the rate of lymphoma, and congestive heart failure, were not statistically significant [252]. However the risk of malignancy associated with anti-TNF's remains a concern because the statistical difficulty resides in the low overall incidence of spontaneous transformation [250]. This is consistent with murine studies where TNF gene knockout mice do not spontaneously develop tumors by 12 months of age (Sedger L., personal communication) and although differences in spontaneous tumor development are not always evident until moved onto a tumor suppressor gene deficient background [253], even p53- and TNF- double-deficient mice to not spontaneously develop tumors more frequently than p53-null mice [254]. Thus the role for TNF in tumor incidence appears to be in the development and regulation of immunity, but not in tumorogenesis *per se*, nor even in the first phases of tumor immunosurveillance [255]. Finally, it may be worth considering whether there is an increased risk of virally transformed tumors. This is an area of ongoing interest in cancer biology [256–258] and greater molecular interrogation of tumors in patients taking anti-TNF biologics is required to rule out this possibility.

8.3. Demyelinating events and neuropathies

As early as 2001 there were reports of demyelinating events that appeared to be associated with the use of IFX and ETA for RA [259]. Although the relationship to TNF blockade was unclear at the time, these cases were considered adverse events to anti-TNF treatment because they partially or completely resolved after cessation of treatment [259]. As a result of these cases, multiple sclerosis was quickly considered by a contraindication for the use of anti-TNF agents. While similar cases continue to be reported [260–262], it has been argued that the incidence of multiple sclerosis in patients receiving anti-TNF agents is similar to that which occurs in society in general [263,264]. Of particular note, the first comprehensive prospective study of demyelinating events has recently been published. Here, 77 patients received a full neurological examination including brain and spine magnetic resonance imaging (MRI) and electrophysiological tests *before* starting on anti-TNF biologics (IFX, ETA or ADA) for RA, psoriatic arthritis or ankylosing spondylitis. Of these, 2 patients were found to have lesions prior to anti-TNF treatment, and 4 developed neurological symptoms (MRI-confirmed demyelinating events), but overall the rate of neurological adverse events was 4% and not significantly different from the non anti-TNF group [265]. Taken together, one thing is clear: there is a need for careful monitoring of patients on anti-TNF treatments. Although it is unclear whether anti-TNF biologics constitute an *a priori* triggering event for demyelination, it has been suggested that there are several ways in which anti-TNF agents could be involved: (i) that they could regulate auto-reactive (self CNS-specific) pathogenic T and B cells, (ii) that they block TNF to alter downstream cytokine responses, (iii) that they can neutralize TNF systemically but not within the CNS, creating an artificially high local concentration of brain TNF (the so called "sponge effect"), or (iv) that they permit release of a latent or sub-acute CNS viral infection [266]. It is also possible that anti-drug antibodies and immune complexes are contributory to demyelination events, even if these Ig's are not directed to self CNS-specific antigens.

8.4. Potential impact on the cardiovascular system

Not long after the first phase of anti-TNF-biologics became widely used it was postulated that lowgrade chronic inflammation was related to progression of congestive heart failure. However, a clinical trial with IFX failed to demonstrate benefit in congestive heart failure, and in fact, quite unexpectedly, IFX at 10 mg/kg, was found to be associated with worsening condition and mortality [267]. Several additional case reports of worsening cardiac condition also emerged [268], and consequently caution is advised in using these drugs in patients with heart failure. In fact, the 2001 BSR guidelines state "patients should be carefully monitored for congestive cardiac failure, "whilst" being treated with any anti-TNF therapy. If symptoms and signs of congestive cardiac failure are stable, treatment should still potentially be discontinued if the benefits of the anti-TNF therapy are only limited" [269,270]. However, there are also studies that indicate that this effect is minimal, if indeed it is present, and it seems that the risk is associated with concomitant RA and not with the anti-TNF biologics *per se* [271]. One potential reason for this is that oxidative stress can be triggered by downstream metabolites of TNF signaling, such as arachidonic acid, and oxidative stress is strongly implicated in cardiovascular endothelium health [272]. It has also been argued that the reduction in inflammation and joint disease that anti-TNF agents bring to RA patients, yield an overall net benefit that reduces the risks of cardiovascular disease [273]. Also that TNF can influence plasma lipid profiles, but this too, remains controversial [274]. More research is therefore needed to determine the effects of anti-TNF biologics in cardiac disease, meanwhile, screening for cardiac risk factors is important for RA patients receiving current anti-TNF therapy.

9. What's next for the current generation of anti-TNF biologics? Can they be improved?

Anti-TNF biologics have arguably had stunning success globally, to the extent that some might argue that a "next generation" of reagents is not required. Indeed it is hard to think of another group of biologic-based drugs that has demonstrated greater efficacy worldwide, and especially for a broad spectrum of autoimmune and inflammatory diseases that once appeared for many patients to be refractory to standard immunosuppressant therapies. That these agents have changed the lives of so many people who were otherwise crippled by rheumatic diseases underscores their success, and their use in inflammatory bowel disease represents another chapter of their success. Still, the plethora of case reports and systematic analyses have documented varying degrees of efficacy, which remains an ongoing problem, and as many as one third of patients achieve little or no benefit. Moreover, the cost of these agents and their potential for significant side-effects indicates that there is an urgent need for the early identification of treatment non-responders. Thus a new generation of anti-TNF agents is still required.

The first requirement for better identification of treatment responders and non-responders is the development of strong objective guidelines for categorizing responsiveness. These must be definitively measured for easily assessment and be clinically meaningful. In the case of RA the presence of rheumatoid factor (RF) antibodies (antibodies that bind to IgG) has long been proposed to predict a more severe class of disease [275], and

antibodies to citrullinated fibrinogen stimulate macrophage TNF [276]. It has thus been suggested that RF and anti-citrullinated protein antibodies might be useful predictors of disease severity and indicators of anti-TNF responders [277]. However, this remains unconfirmed, and in fact, at least two studies refute their usefulness in identifying responders [278,279]. In contrast, a recent study claims to have identified a 24 protein biomarker signature of RA responders to ETA treatment [280], and there are differences in peripheral blood leukocytes in RA patients over controls, including mRNA-expressing $CD16^{+}$ granulocytes, NK cells and $CD14^{dim}$ monocytes [281]. Furthermore, analysis of circulating miRNAs can also reveal candidate biomarkers of treatment responders, and in fact, a 6 miRNA signature has recently been reported to potentially identify TRAPS patients responding to anakinra (IL-1R antagonist) [282]. Further validation of these potential biomarkers in independent and larger cohorts will be required to confirm the validity of these molecules as biomarkers of treatment responses.

In the case of Crohn's disease, a clinical activity score known as the "Crohn's disease activity index" (CDAI), or equivalent, can be used to objectively quantify symptoms. This includes a combination of CDAI, biomarkers such as serum C-reactive protein, serum drug trough levels, and the presence and quantification of anti-drug antibodies [283]. Indeed, despite the regulatory approval and success of anti-TNFs biologics in Crohn's disease, approximately 50% of patients do not respond to ADA. Thus the definitive identification of responders from non-responders becomes paramount in patient management. Of these, sub-therapeutic "drug trough" levels is thought to be critically linked to treatment outcomes/non-responsiveness [284–286]. Of particular interest, however, is a 2014 report demonstrating that expression of colonic membrane TNF is a possible identifier of anti-TNF therapeutic responders [287]. In this remarkable study of 25 patients, *in vivo* fluorescent colonoscopy imaging with GMP-prepared FITC-conjugated ADA, demonstrated that patients with high numbers of membrane TNF-positive mucosal immune cells strongly correlate with mucosal healing and ADA treatment efficacy [287]. The membrane TNF-expressing cells were mostly lamina propria CD14 macrophages and CD4 T lymphocytes [287]. This important report therefore provides a basis for rational identification of anti-TNF drug-responders, while simultaneously identifying dominant etiopathological elements of Crohn's disease. It also potentially reveals a mode of efficacy of anti-TNFs, *i.e.*, the ability of these bio-reagents to detect, neutralize and/or eliminate membrane TNF expressing cells. The obvious limitation for broad-scale use of this new knowledge, however, is the availability of confocal laser endomicroscopy, not to mention the difficulty in accessing skilled and time-consuming analysis of the image data it provides. Thus, there remains an ongoing critical need to develop novel serum biomarkers of drug responsiveness, not just in Crohn's disease, but for all rheumatic diseases for which anti-TNF biologics are utilized. Additional serum proteomic and circulating microRNA studies reflecting contemporary technologies and capabilities are therefore still needed.

Another approach to identifying anti-TNF drug-responders from non-responders is through pharmacogenetic analyses. Various studies of TNF biology revealed genetic polymorphisms in the TNF gene promoter sequence, including −1031T/C, −863C/A, −857C/T, −376G/A, −308G/A, −244G/A, −238G/A, +70C and +489G/A [27], where some are suggested to be linked to increased TNF production [288]. It stands to reason that increased TNF production may be linked to anti-TNF therapeutic success, *i.e.*, to sub-therapeutic anti-TNF doses, and thus these alleles represent a potential genetic basis for defining drug-responders. However, even with a clinical sub-types stratification analysis within specific inflammatory diseases, these studies have yielded mixed results [289–294]. It is probably fair to conclude that no single or simple combination of single nucleotide polymorphisms (SNPs) has been found that defines the failure of anti-TNF agents in non-responding patients. On the other hand, TNF promoter SNPs may define disease sub-type risks [295], and interestingly, additional non-TNF candidate alleles are emerging [289–294], including in genes such as NLRP3 and IFN [292,296] – i.e., in molecules involved in sterile inflammation [297,298] now additionally implicated in the auto-immune inflammation of Crohn's disease [299] and RA [292,296]. Similarly, polymorphisms in MAPK have also recently been implicated in anti-TNF treatment responsiveness in RA [300]. Time will tell if a particular spectrum of symptoms, a series of SNPs, and/or a panel of serum biomarkers can define anti-TNF treatment responders versus non-responders.

There are now seven FDA registered anti-TNFs biologics (including recently registered biosimilars), an IL-1 receptor antagonist (ankinra; Kineret®), and anti-IL-1β (Canakinumab®) and anti-IL-6-Receptor mAbs (tocilizumab; tradenames Actemra® and RoActemra®), all with considerable overlap in the clinical indications for FDA approved use in RA and other autoinflammatory diseases [301]. Defining which reagent is best suited to a specific and clinically defined disease type goes hand-in-hand with the ability to pre-identify drug non-responders. Arguably the most convincing current lead in identifying anti-TNF responders is the recent demonstration of locally high membrane TNF-expressing cells which correlates with anti-TNF efficacy in Crohn's patients, as mentioned earlier [287]. Indeed, it is tempting to consider what benefit might be afforded to Crohn's patients with low colonic TNF if they were treated with anti-IL-l neutralizing antibodies instead. Alternatively, other cytokine neutralizing mAbs might be beneficial, as it has also been demonstrated that anti-TNF non-responding psoriatic arthritis patients experience benefit from ustekinumab (Stelara®), a new IL-12/23 p40 neutralizing mAb [302,303]. Taken together these examples highlight the need for a more personalized approach to the treatment of inflammatory diseases, albeit without a current and proven directory for pre-defining successful treatments.

10. Next-generation anti-TNF agents: What are they? How will they be possible?

What are these next generation anti-TNF-agents? What will they look like? How can they achieve what the existing group of anti-TNF biologics fail to do in some patients, *i.e.*, achieve disease remission? The first thing a next-generation anti-TNF agent might do is accommodate an easier mode of delivery whilst maintaining pharmacokinetic goals, since all of the current agents are delivered by sub-cutaneous injection or intra-venous infusion, apart from the non-TNF specific curcumin and thalidomide (see Table 1). Oral delivery, although highly desirable, is extremely difficult – if not impossible – if the object is systemic suppression of TNF by large bio-molecules. Smaller, non-Ig-based molecules, are therefore likely to be required, albeit that this may also bring about the loss of the Fc-related effector functions, as discussed above.

10.1. Peptidomimics and PLAD only domain proteins

The first attempts at non antibody-based reagents comprised smaller molecules known as "peptidomimetics". These were simply peptides that represent the TNF contact region of TNFR1, designed from computer-simulations, and which function to block TNF from interacting with its receptor [304]. Next, with knowledge of TNFR dynamics came a "domain-only" protein called the "pre-ligand binding assembly domain" (PLAD). The PLAD resides within the N-terminus of TNFRs where it functions to permit TNFR homotypic interactions that facilitate ligand binding-induced TNFR signaling [50]. The domain is also required for virally

encoded TNFRs to interact with cellular TNFRs and thereby prevent TNF-induced cell death [305] – just one of many examples of how viral evolution generates functional mechanisms of potent inhibition and neutralization of TNF [97]. The PLAD itself is only approximately 44 amino acids in length and at least two groups have produced soluble recombinant human TNFR1 "PLAD-only" proteins [306,307]. At present there is ongoing disagreement about how PLAD-only proteins work. For example, the viral PLAD-containing TNFRs clearly subvert TNFRs by acting from an intracellular location since extracellular purified vTNFRs show no ability to interfere with cellular TNFRs [305,308]. In contrast, the extracellular recombinant TNFR1 PLAD-only proteins reportedly prevent TNF-induced L929 cell death [306] and its *in vivo* administration ameliorates arthritis in mice [307]. A TNFR1 PLAD-Ig-Fc fusion protein has also now been produced. It also reportedly reduces TNF-induced autoimmune inflammation and the expansion of Th17 cells in autoimmune disease in mice [309]. It currently remains unclear whether PLAD-only proteins require uptake from myeloid cells, such as inflammatory monocytes, and/or whether the discrepancies between viral and cellular PLAD proteins result from the viral proteins being homologous to TNFR2, compared to the recombinant human PLAD-only proteins being TNFR1. Nevertheless, although they show promise in animal models of disease, their delivery requires repeated injections. While they may be smaller than anti-TNF mAbs, the pharmacokinetics of PLAD-only proteins in humans is unknown and there is no advantage in the mode of delivery over the currently approved anti-TNF biologics.

10.2. Small molecule inhibitors – is this still an achievable goal?

The question still remains open as to why small molecule inhibitors of TNF or TNFR have been slow to emerge, especially with the availability of powerful contemporary computer modeling software. This area is still potentially the most profitable, since small-molecules inhibitors are likely to be far cheaper to produce than mAb or Ig-Fc-based fusion proteins. Early reports of small molecule inhibitors of TNF/TNFR were intriguing; 4 compounds that bound reversibly to TNFR1 in the dark, but irreversibly in light, have been reported [310]. Structural studies indicated that these molecules interact with sites on TNFR that prevented TNF association with TNFR1 [310]. That the interactions are relatively weak and reversible in the dark meant they were not particularly attractive for further clinical development – although they confirmed that a potential target site is the regions on TNFRs that interact with TNF. Another early report of a small molecule inhibitor preventing TNF binding to TNFR, actually interacts with TNF at amino acid Tyr-119 (which residues deep within TNF trimer) and not with TNFR itself [311]. This intriguing molecule disrupts the formation of TNF trimers [311] which implies that TNF exists in an equilibrium between monomeric and trimeric TNF, at least at some stage in its production and maturation. Unfortunately there have been no further reports of any of these compounds, suggesting that they have insufficient physiological potency, or that there are issues with solubility or toxicities in physiological conditions.

A novel chemically synthetized anti-TNF compound has recently been reported, C87, that binds directly to TNF and prevents TNFR signaling [312]. It was found from an initial screen of approximately 90,000 compounds that interact with a 7-amino acid region comprising the TNF-interacting site on TNFR1. C87 is a potent inhibitor of TNFR-1 mediated activation of caspase-8, phosphorylated JNK and NF-κB [312]. It prevents L929 cell production of TNF-induced cytokines and chemokines and has been trialed in animal models, where it reportedly inhibits LPS-induced hepatic inflammation in mice [312]. This is one of only a few reports of a small-molecule inhibitor of TNF with *in vivo* efficacy. The remaining challenge is to determine if it is pharmacologically stable and non-toxic with longer-term use. In an alternative approach, at least three groups have developed small molecule inhibitors of the membrane TNF releasing enzyme TACE. All are soluble at physiological pH and effective at reducing LPS-induced TNF, and rodent arthritis (when administered orally) [313–315]. However, with limited recent publications, the state of clinical development of these molecules is unknown. While the hunt continues for new small molecules inhibitors, one cannot forget thalidomide, which is effectively a soluble, orally administered, small molecule that inhibits TNF synthesis. As stated above, thalidomide inhibits the production of TNF, but also IL-6 and IL-1, acting by inhibiting certain pathways of NF-κB and MyD88 signaling [194]. Taken together these examples prove that it is possible to specifically design and synthesize small-molecule inhibitors of TNF, TNFR, or TACE, yet none are presently available or approved for therapeutic use. Thus the goal remains to find small molecule inhibitors of TNF that are biologically active after oral administration, present *in vivo* at pharmacologically meaningful concentrations, and that are physiologically well tolerated; a combination of challenges that may yet turn out to be too difficult to meet.

10.3. The potential for cell specific drug targeting

TNF-expressing cells are implicated in most inflammatory diseases and conditions. For example CD14-dim monocytes and tissue macrophages, as well as granulocytes and natural killer cells can be detected by biotinylated-IFX in RA patients [316]. Furthermore, their numbers are diminished after IFX treatment [316]. This begs the question as to how the current anti-TNF biologics, especially the antibody-based reagents, might be specifically targeted to inflammatory cells, leaving normal TNF-mediated physiological functions unaffected in non-immune cells. There is also the issue of self-perpetuating immunopathology driving further TNF production, for example, through the production of anti-drug antibodies and their potential ability to opsonize and/or aggregate and thereby activate monocytes and other FcγR expressing cells [317]. Of interest is the recent report of the use of immune modifying microparticles for the specific elimination of inflammatory monocytes [318]. On this basis one can envisage the potential for future liposome- or microparticle- mediated targeting of inflammatory monocytes/macrophages, *i.e.*, for the specific delivery of anti-TNF biologics to block the production of TNF by phagocytic monocytes.

Also attracting increasing attention, is the exciting use of dual-specific engineered mAbs, with at least two such reagents already FDA approved for human clinical use: catumaxomab (Removab®; EpCAM/anti-CD3) – approved for malignant ascites, and blinatumab (BiTE®; anti-CD19/anti-CD3) approved for acute lymphoblastic leukemia and lymphoma [319]. Precisely how they would work *in vivo*, and whether they prove to be safe, and tolerated long-term (due to immunogenicity concerns), is yet to be fully determined, as there are already reports of anti-drug antibodies to these unnatural Igs, and other complications, but this will become evident with more time and clinical experience [320,321]. Suffice to say they both bring T cells to tumors cells, and mediate ADCC through FcR binding [319]. Newly re-engineered, dual-specific, anti-TNF antibodies could similarly offer the possibility of TNF neutralization in specific cell types. Finally, antibody engineering also offers the potential for altered *in vivo* half-life or effector function via differing affinities for FcR [322].

10.4. TNF-blockade in neurological systems – the next frontier

The blood–brain barrier of endothelial cells constitutes a physiological boundary preventing efficient entry of therapeutic

antibodies into the CNS. One of the more exciting developments in this area is the potential use of Fc Ig targeting to neonatal Fc Receptors (FcRn) which permits Ig/mAb transcytosis across the blood–brain barrier [323,324] and thus providing opportunities for therapeutic antibody-mediated TNF neutralization within the CNS. Of note, a TNFR2 fusion protein has been engineered for expression as a transferrin-receptor-specific Ig constant-domain tag, *i.e.*, specifically for delivery across the blood–brain barrier, into the CNS [325,326]. It has already been used in mice to neutralize pathology associated with ischemic stroke [326]. These findings are consistent with the fact that (i) TNF is high in CSF and serum of humans after stroke [327], (ii) that TNF transgenic rats are more susceptible to ischemic stroke [328,329], and (iii) that the engineered transferrin-receptor specific-mAb-TNFR2 fusion protein can be detected within the CNS after intravenous or subcutaneous injection [330]. Another approach for entry into the CNS might be to harness cell-penetrating peptides [331], but this has not yet been explored in the context of anti-TNF biologics, possibly because of the relatively large size of Ig-based biomolecules. Nevertheless this approach might be significant with smaller, more selective, anti-TNF peptidomimetics.

On the other hand tag-specific CNS-targeting may not always be necessary, since perispinal injection of radiolabelled-ETA can be detected within the cerebrospinal venous system within minutes [332]. Indeed, it has been reported that perispinal ETA produces multiple effects in Alzheimer's patients, including improved cognitive function, mood, memory and motor function [332]. However, independent confirmation of these intriguing responses will require a double-blind trail. Peripsinal ETA has also been reported to relieve neuropathic pain such as sciatica [333–335], although this also requires replication in controlled trials. Nevertheless, TNF blockade with IFX inhibits nociceptive pain responsiveness in mice [333], and it had long been a conundrum that RA patients report feeling considerably better long before their joint damage has had time to heal – a key factor in patient's perceptions of therapy success [336]. These findings are not surprising considering that TNF synthesis inhibitors curcumin and thalidomide can both offer relief for neuropathic pain [337–340]. The application of anti-TNF biologics for CNS-related pathologies such as Azlheimer's disease is the current frontier. TNF is now also strongly implicated in playing a role in the biology of depressive and bipolar disorders [341–344]. If successful, then the search for non-injectable anti-TNF inhibitors will become increasingly important given the need for an aging dementia-affected patient population being able to self-administer medications. Careful dosing and monitoring will also be required – the role of TNF in normal neurobiology is paramount to health; over-inhibition may be detrimental given that genetic TNFR-deficiency exacerbates Alzheimer's-like symptoms in mice [345]. Indeed, one envisages that it is of paramount importance in brain more than any other tissue, that anti-TNF agents achieve the fine balance of inhibiting inflammation while permitting essential functions for normal tissue homeostasis and health.

11. Summary: an exciting future

It is now some 15 years since ETA was first FDA approved to ameliorate inflammation in the treatment of methotrexate-refractive RA. Currently five TNF-specific monoclonal antibodies are also approved to treat inflammatory disorders. Despite the well-documented risks associated with their use, and the fact that they still cost between $US17,000 and $25,000 per patient/year [346], they have been proven to provide a significant health benefit to many. Not only are they broadly successful in ameliorating inflammation in several disease settings, but also their off-label use is still expanding [332]. Looking back, one of the most remarkable and perhaps unexpected revelations that has come from the large-scale use of anti-TNF biologics, is that they have taught mankind as much about normal biology as they have about immunopathology. The current challenges are (i) to better pre-identify or predict the non-responders, *i.e.*, prior to treatment, (ii) to better tailor drug delivery to permit normal physiological effects of TNF in non-diseased tissues, and (iii) to develop more selective anti-TNF agents that block only select aspects of TNFR signaling. Finally, with respect to RA, there is also now considerable evidence that mechanisms of etiopathology change over time [316] and this is also likely to be true for other inflammatory diseases. Thus more careful patient monitoring over time may implicate the need for dose adjustments and/or for changes in the anti-cytokine therapies being delivered – several neutralizing mAbs are now available. This highlights, once again, the need for more personalized medicine, as well as ongoing medical education for clinicians who prescribe these reagents. It also indicates that anti-TNF biologics, although effective, are expensive and infrequently bring complete and durable disease-free remission. Arguably, the biggest, morally compelling challenge, is to use the current knowledge to develop more affordable anti-TNF agents; ones that will be both effective and available to all independent of financial status [347]. With significant successes already realized in RA and related autoinflammatory disorders [206,348], anti-TNF agents move bravely into a new era of personalized medicine and the search for better treatments for chronic neurological diseases.

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