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How tumour necrosis factor blockers interfere with tuberculosis immunity

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Summary

Tumour necrosis factor (TNF) is a potent inflammatory cytokine that plays an important role in immunity to numerous bacterial infections, including *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB) in humans. Infliximab, adalimumab, certolizumab pegol and etanercept are anti-TNF agents used to treat a range of inflammatory/autoimmune diseases, such as rheumatoid arthritis. The use of some of these drugs has been linked to reactivation TB. In addition to blocking TNF-mediated immune responses, some anti-TNF drugs have been found to interfere with innate immune responses, such as phagolysosomal maturation and monocyte apoptosis, as well as cell-mediated responses, including interferon-γ secretion by memory T cells, complement-mediated lysis of Mtb-reactive CD8$^+$ T cells and increased regulatory T cell activity. This review summarizes some of the reported effects of TNF blockers on immune cell responses in the context of the observed clinical data on TB reactivation in patients on anti-TNF therapy.

Keywords: adalimumab, certolizumab, etanercept, golimumab, infliximab, mycobacteria

Introduction

Tumour necrosis factor-alpha (TNF) is a potent modulator of early inflammatory responses to a variety of physical, environmental, infectious and immunological stimuli. TNF is produced primarily as a type II transmembrane protein, arranged in stable homotrimers that can then be cleaved by the metalloprotease TNF alpha converting enzyme (TACE) to form the soluble form [1,2]. TNF binds to two receptors, TNFR1 and TNFR2. While both receptors can transduce anti-apoptotic, proinflammatory signals via activation of nuclear factor (NF)-κB or mitogen-activated protein kinase (MAPK); TNFR1 can also transduce apoptotic, anti-inflammatory signals via recruitment of Fas-associated death domain (FADD) and activation of caspase 8 [2]. sTNF and tmTNF can bind both receptors, although TNFR2, which is found typically on immune cells, can only be activated fully by tmTNF [3].

Clinical studies have associated the use of TNF blockers with progression from latent tuberculosis infection (LTBI) to tuberculosis (TB) disease [4,5]. This observation at the bedside was presaged by an abundance of laboratory data demonstrating a central role for TNF in TB immunity [6]. Several investigators have demonstrated that animals in which TNF has been neutralized were more susceptible to primary TB disease after infection with *Mycobacterium tuberculosis* (Mtb) [7,8]. It has been shown that treatment with soluble TNF receptor (sTNFR) can also cause disease in infected mice [9], while other studies demonstrate the central role of transmembrane TNF in protecting against disease in the mouse model [10,11]. TNF can support anti-TB immunity through the secretion of chemokines [12], up-regulation of adhesion molecules [13] and the induction of macrophage apoptosis [14]. While TNF blockers may therefore interfere with these important immune functions, other less predictable immune effects have been seen with these agents. In particular, TNF-blockers have been shown to diminish interferon (IFN)-γ effects [15–19] and stimulate apoptosis of key immune cells, including monocytes [20], CD4$^+$ T helper cells [21] and Mtb-reactive CD8$^+$ T cells [22]. Anti-TNF therapy is also associated with increased regulatory T cell (T_{reg}) function, which has been linked with susceptibility to TB [23–25].

TNF blockers

There are five licensed TNF blockers currently in clinical use: infliximab, adalimumab, certolizumab pegol, golimumab

and etanercept. Infliximab is a chimeric monoclonal antibody against TNF which comprises a human immunoglobulin (Ig)G1 Fc region and murine variable region, whereas adalimumab and golimumab are humanized monoclonal anti-TNF antibodies with human constant and variable regions. Certolizumab pegol is a pegylated humanized monoclonal anti-TNF Fab' fragment. Etanercept consists of two extracellular domains of the human TNFR2 fused to the Fc fragment of human IgG1. Between them, these TNF antagonists are used to treat a range of diseases, including rheumatoid arthritis, Crohn's disease, psoriatic arthritis, ankylosing spondylitis and ulcerative colitis [26].

There are significant differences in the pharmacology of these agents, particularly between the monoclonal antibodies and etanercept. Etanercept binds only trimeric sTNF, while infliximab and adalimumab bind both monomeric and trimeric sTNF [26]. While etanercept has a higher affinity for sTNF than all the monoclonals, it also releases TNF more quickly, with more than 90% of bound cytokine released after 2–3 h, while infliximab forms much more stable complexes [27]. In addition, infliximab binds tmTNF more strongly than etanercept, which also dissociates from tmTNF at a similar rate to sTNF [27]. Moreover, etanercept binds sTNF and tmTNF at a ratio of 1:1, while infliximab and adalimumab can bind two TNF molecules at a time, allowing the formation of immune complexes and cross-linking of tmTNF [26,27]. Such differences in the pharmacology of TNF blockers may help to explain the different propensity they have to cause reactivation of TB [28].

Abnormal clinical phenotypes

The report in 2001 from the *New England Journal of Medicine* [4] showed us that TB associated with infliximab had a tendency to reactivate after 11 or 12 weeks of therapy, and the disease followed an unusual course. Specifically, extrapulmonary disease occurred in about 50% of patients and disseminated disease in about 10% of patients (compared to 15% and less than 1% in immunocompetent people, respectively). Moreover, infliximab has been shown to have similar effects on immunity to other granulomatous diseases [31].

Interestingly, the pattern of TB disease after treatment with TNF blockers is similar to that seen in other immune deficiency settings, such as human immunodeficiency virus (HIV) infection. The working hypothesis of TB reactivation in this setting is that the integrity of the granuloma, which is normally intact and contains latent bacilli, becomes compromised [32,33]. This has been demonstrated in mice given anti-TNF treatments [34,35] and has been reported in patients taking TNF blockers or who have HIV disease [4,36]. However, patients who develop TB in this setting may also have granulomas that appear well organized. Thus, it is important that scientists research further the mechanisms of compromise seen in the setting of TNF blockade, which results in TB reactivation. In this review we report on some cellular experiments that address this issue. Research into how these agents cause progression from LTBI to TB disease will undoubtedly improve our understanding of TB, and potentially unveil new therapeutic targets. In addition, such research can also inform us on how to render these treatments safer.

Cellular effects of TNF blockers

It is clear from a wide range of studies that TNF blockers have significant effects on immune cells, both in terms of activation status and on intracellular responses to infection. These effects impact upon both innate and adaptive immune responses to Mtb (and other pathogens) and provide an insight into the specific roles of TNF in maintaining immunity in LTBI and in reactivation of the disease. Here we discuss research on a number of these effects, including phagosome maturation, apoptosis and T cell activation.

Phagosome maturation

Fusion of Mtb-containing phagosomes with lysosomes (phagosome maturation) results in acidification of the phagosome and exposure of the contents to lysosomal hydrolases, as well as reactive oxygen and nitrogen species [37]. A number of mycobacterial genes have been linked with the inhibition of this phagosome maturation, although only two products have been characterized at least partially [38] (Fig. 1). Lipoarabinomannan (LAM), a constituent of the mycobacterial cell well, inhibits production of phosphotidylinositol-3-phosphate (PI3P) by the type III phosphotidylinositol-3 kinase (PI3K) vps34 [39–42]. This, in turn, prevents recruitment of early endosomal antigen 1 (EEA1) and other Rab5 effectors to the phagosomal membrane, thus inhibiting recruitment of Rab 7 and fusion with late endosomes and lysosomes. Similarly, secreted acid phosphatase M (SapM) is a mycobacterial PI3P phosphatase that dephosphorylates PI3P on the phagosome, again preventing recruitment of EEA1 [43]. However, activation of macrophages with IFN-γ induces maturation and acidification of mycobacteria-containing phagosomes, leading to increased intracellular killing by macrophages *in vitro* [44,45]. Recently, we have demonstrated that this IFN-γ-induced increase in phagosome maturation is inhibited in human macrophages treated with TNF blockers [46].

Infliximab, adalimumab and etanercept all inhibited IFN-γ-induced maturation of mycobacteria-containing phagosomes in the phorbol myristate acetate (PMA)-differentiated human acute monocytic leukaemia (THP-1) cell line. However, in primary human monocyte-derived macrophages (MDM), only infliximab and adalimumab abrogated phagosome maturation, but did so in the presence or absence of IFN-γ [46]. These data suggest that the effect of IFN-γ on phagosome maturation is dependent

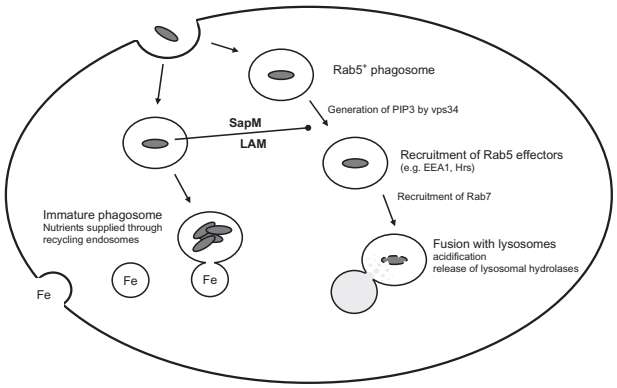

Fig. 1. *Mycobacterium tuberculosis* inhibits phagosome maturation. Normal phagosome maturation requires the recruitment of Rab5 to the phagosomal membrane, followed by the generation of phosphotidylinositol-3-phosphate (PI3P) by the phosphotidylinositol-3-kinase vps34. This leads to the recruitment of Rab5 effectors, including early endosome antigen 1 (EEA1) and hepatocyte growth factor-regulated tyrosine substrate (Hrs). Subsequent recruitment of Rab7 allows fusion with lysosomes, acidification of the phagosome and release of lysosomal hydrolases into the lumen of the mature phagosome. *M. tuberculosis* subverts this process through the release of lipoarabinomannan (LAM) and the PI3P phosphatase secreted acid phosphatase M (SapM), which prevents the generation and recruitment of PI3P to the phagosomal membrane. These phagosomes remain immature, but are still able to fuse with recycling endosomes that can transport nutrients, such as iron, thus providing a protective environment for bacterial survival and replication.

upon autocrine secretion of TNF. Indeed, pretreatment of macrophages with IFN-γ increases TNF secretion in response to infection with *M. bovis* bacillus Calmette–Guérin (BCG), *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv [46]. Moreover, direct treatment of human monocyte-derived macrophages with TNF induces phagosome maturation [46]. It is not clear why etanercept did not inhibit phagosome maturation in human MDM, but may suggest a role for tmTNF.

Autophagy

Activation of macrophages with IFN-γ has also been shown to increase autophagosome formation and autophagy-dependent killing of mycobacteria in macrophages [47–49]. Autophagy is an evolutionary conserved process for the targeting of cellular constituents, including damaged or surplus organelles, such as mitochondria and peroxisomes, to lysosomes [50,51]. Autophagy is also involved in innate and adaptive immune responses and the induction of autophagy in infected macrophages leads to the delivery of mycobacteria to lysosomes, thus overcoming the phagosome maturation arrest [47]. In murine macrophages transfected with siRNA to beclin 1 (Atg6), a tumour suppressor that is critical for autophagy, IFN-γ-induced phagosome maturation is abrogated completely [48,49], suggesting that autophagy may be solely responsible for this increase. Moreover, given that IFN-γ-induced phagosome maturation is seemingly dependent upon autocrine TNF secretion [46], it is possible that TNF blockers may also inhibit autophagy. A number of studies have suggested that TNF can induce autophagy [52–57] (Fig. 2), but this has yet to be studied in the context of infection with Mtb. However, given that autophagy can potentially influence immunity to mycobacteria through effects on intracellular killing, inflammasome activation and antigen presentation [58], inhibition of TNF-induced autophagy is potentially significant.

Apoptosis and cell death

TNF has a dual role in cell survival; it can promote survival through the activation of NF-κB or induce apoptosis by activating a caspase cascade. In Mtb-infected macrophages, apoptosis can be host-protective, depriving the bacilli of their niche cell [59,60], promoting bacillary killing [59,61,62] and allowing major histocompatibility complex (MHC) class I- and CD1-dependent cross-priming of $CD8^+$ T cells by immature dendritic cells following phagocytosis of mycobacteria-containing apoptotic vesicles [63]. While TNF-dependent apoptosis appears to be host protective [14], virulent Mtb is able to inhibit this by inducing the interleukin (IL)-10-dependent secretion of sTNFR2 from infected human alveolar macrophages [64].

T cells

Studies into the effects of TNF blockers on apoptosis of immune cells have reported mixed results *in vivo*, *ex vivo* and *in vitro*. In a study looking at apoptosis of T cells in patients with Crohn's disease undergoing treatment with infliximab, the anti-TNF therapy induced apoptosis in activated, but not resting, T cells both *in vitro* and *in vivo*, within 24 h of administration [65]. Similarly, Di Sabatino *et al.* [66] found that infliximab treatment of patients with Crohn's disease increased caspase-dependent apoptosis of lamina propria T cells *in vivo* up to 4 weeks after the final infusion. Treatment of peripheral blood T cells from Crohn's disease patients with infliximab *in vitro* also induced apoptosis, although these cells were less susceptible than the lamina propria T cells [66]. However, following treatment of human whole blood cultures with the mitogen phytohaemagglutinin (PHA), Saliu *et al.* [67] did not observe any increase in apoptosis or necrosis of activated ($CD69^+$) T cells with infliximab, adalimumab or etanercept for 24 or 72 h.

$CD8^+$ cells have been shown to play a key role in mediating infected macrophage cell death, and the death of the pathogen that uses the macrophage as its niche cell [22,68]. In the human model of disease, this beneficial effect is mediated by perforin and granulysin release from the $CD8^+$ cell [68,69].

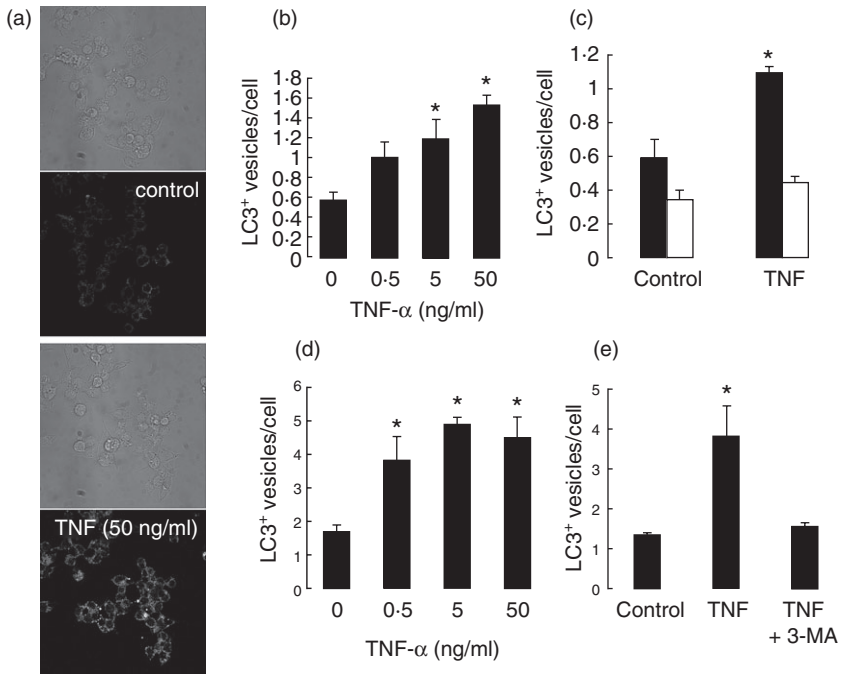

Fig. 2. Tumour necrosis factor (TNF) induces autophagy in macrophages. RAW264·7 murine macrophages or human phorbol myristate acetate (PMA)-differentiated monocyte/macrophage (THP-1) cells were treated with TNF for 2 h then fixed and stained with antibody against the autophagosome marker LC3, followed by secondary staining with Alexa 488-conjugated antibody [87]. (a) Confocal images of control and TNF-stimulated RAW264·7 cells showing TNF-induced $LC3^+$ autophagosome formation (distinct punctate staining). (b) Quantitative analysis of $LC3^+$ autophagosome formation in RAW264·7 cells treated with TNF (5 ng/ml). (c) TNF-induced $LC3^+$ autophagosome formation is abrogated in RAW264·7 cells transfected with siRNA against beclin 1 (Atg6, white bars) or control siRNA (black bars). (d) TNF-induced $LC3^+$ autophagosome formation in THP-1 cells. (e) TNF-induced $LC3^+$ autophagosome formation in THP-1 cells is inhibited by the PI3K inhibitor 3-methyladenine (3-MA), a known inhibitor of autophagy.

In a recent study, Bruns *et al.* [22] took advantage of the human model of TNF blockade to show a key role for Mtb-reactive $CD8^+CCR7^-CD45RA^+$ effector memory T cells (T_{EMRA} cells) in TB immunity. In this model, infliximab was shown to interfere specifically with the function of these cytotoxic cells, which express tmTNF, by inducing their death through complement-mediated cytotoxicity (CDC), which in turn reduced the amount of perforin/granulysin available to ensure infected macrophage cell death, and the attendant Mtb killing [22].

In a similar model system, using the human Jurkat T cell line expressing an uncleavable form of tmTNF, Mitoma *et al.* [70] were able to show that infliximab, but not etanercept, induced apoptosis and cell cycle arrest through outside-to-inside (reverse) signals via tmTNF. In a second study, the same group went on to show that treatment of these cells with infliximab or adalimumab, but not etanercept, increased complement-dependent cytotoxicity (CDC) [71]. All three TNF antagonists induced antibody-dependent cell-mediated cytotoxicity (ADCC) when the tmTNF-expressing Jurkat cells were added to lymphocytes isolated from peripheral blood. In addition, adalimumab also induced apoptosis of these cells by reverse signals through tmTNF [71]. These findings can be contrasted with the results of clinical trials with certolizumab, which lacks an Fc fragment. TB did occur in some certolizumab treated patients [29,30], which suggests that this drug interferes with TB immunity independently of these Fc-dependent processes and cross-linking of tmTNF.

Dendritic cells and monocytes

In a study of patients with psoriasis, treatment with etanercept was found to selectively induce apoptosis of dermal dendritic cells in plaques of responding patients, while circulating T cells and monocytes were unaffected [72]. However, another study demonstrated that adalimumab and infliximab, but not etanercept, induce apoptosis in lipopolysaccharide (LPS)-stimulated human $CD33^+$ blood monocytes [73]. Similarly, induction of apoptosis by treatment with adalimumab or infliximab was demonstrated using a human–mouse chimeric model, in which severe combined immunodeficiency (SCID)/beige mice were reconstituted with human THP-1 monocytic cells and/or Jurkat T cells [74,75]. In another study, the effects of TNF blockers on apoptosis in monocytes in whole blood cultures treated with Mtb culture filtrate for 24 and 48 h were tested. Adalimumab and etanercept had no effect, while infliximab had a small, but significant, stimulatory effect on apoptosis [67].

T cell activation

CD4+ T cells

As well as having important memory and effector functions in response to bacterial pathogens, including mycobacteria, $CD4^+$ T cells represent an important source of IFN-γ, which is essential for effective immunity to Mtb [76]. A number of studies have demonstrated that TNF blockers can inhibit activation and IFN-γ secretion by T cells, both *in vitro* and *in vivo* [15,67,77–80]. Using whole blood cultures, Wallis *et al.* [67] found that infliximab and adalimumab, but not etanercept, inhibited activation of $CD4^+$ cells (measured by CD69 expression) stimulated with Mtb culture filtrate. In addition, infliximab and adalimumab suppressed IFN-γ production in whole blood cultures, while etanercept had no effect. In this study, IL-10 production was inhibited by all three TNF blockers [67]. In another study, Hamdi *et al.* [78] found that infliximab and adalimumab, but not etanercept, inhibited $CD4^+$ T cell proliferation in response to purified protein derivative (PPD) and the mycobacterial antigen culture filtrate protein-10 (CFP-10) and reduced expression of tmTNF by these cells. In addition, the IFN-γ response of memory $CD4^+$ T cells in response to PPD and CFP-10 from patients with inflammatory disease treated with TNF blockers for 14 weeks was reduced. In this case treatment with infliximab or etanercept inhibited IFN-γ release to a similar extent [78].

Gamma/delta T cells

Gamma/delta (γδ) T cells may have an important role to play in host defence against mycobacteria. In particular, these cells can lyse macrophages containing mycobacteria and are an early source of IFN-γ [81]. Vγ9/Vδ2 T cells (the majority population of γδ T cells in peripheral blood) from tuberculin PPD-positive children, either healthy or presenting with different forms of TB disease, proliferate more strongly in response to phosphoantigens *in vitro* than those from PPD-negative patients [82]. Similarly, Giardina *et al.* [83] found that the *in vitro* expansion of Vγ9/Vδ2 T cells to phosphoantigens was higher in PPD-positive patients with rheumatoid arthritis than in control PPD-positive patients. However, this expansion was inhibited by addition of infliximab to the cultures, as was expression of TNFR2 and IFN-γ [83]. Together with the data from other $CD4^+$ T cells, these results demonstrate a clear effect of TNF blockers on IFN-γ responses.

T_{reg} cells

As well as effects on memory and effector $CD4^+$ T cells, infliximab has been shown to influence populations of T_{reg} cells. Ehrenstein *et al.* [24] demonstrated that $CD4^+$ $CD25^+$ T_{reg} cells from patients with rheumatoid arthritis had an altered phenotype compared with cells from control patients. While they were able to suppress the proliferation of effector T cells *in vitro*, they were unable to suppress proinflammatory cytokine (TNF and IFN-γ) secretion from activated T cells and monocytes, or to induce a suppressive phenotype in effector $CD4^+$ $CD25^-$ T cells [24]. However, in patients treated with TNF blockers, the capacity of T_{reg} cells to inhibit cytokine production and convey a suppressive phenotype to effector T cells was restored [24]. A second study went on to show that infliximab therapy induces a population of $CD4^+CD25^{high}$ T_{reg} cells that express forkhead box P3 (FoxP3) but that, unlike the 'natural' regulatory T cell population, lack CD62L [23]. These cells were more potent suppressors of T cell proliferation and proinflammatory cytokine secretion than $CD62L^+$ cells and this suppression was mediated by IL-10 and transforming growth factor (TGF)-β [23].

The role of T_{reg} cells in immunity to Mtb has not yet been established fully. Studies in humans have shown that numbers of T_{regs} are increased in the blood and at infection sites in patients with active disease [84–86]. Scott-Browne *et al.* [25] used green fluorescence protein (GFP)–FoxP3 mice to follow T_{reg} populations after infection with Mtb. In this model, $FoxP3^+$ cells were found to accumulate in the lung in high numbers wherever $CD4^+$ T cells were found. Moreover, depletion of these cells resulted in decreased bacterial burdens in the lungs of infected animals, suggesting that the T_{regs} were inhibiting anti-mycobacterial responses [25].

While T_{reg} cells may dampen immune responses to infection with Mtb they are probably required, similarly, to dampen potentially pathogenic proinflammatory responses and limit damage to infected tissue. Thus, a balance between the proinflammatory responses of monocytes/macrophages and effector T cells and suppression of inflammation by T_{reg} cells may be essential for maintenance of the granuloma. Indeed, the granuloma itself may represent a compromise between effective, but potentially pathogenic, anti-bacterial responses and 'safe' containment of the mycobacteria. However, in patients on infliximab therapy, the generation of a more potent population of T_{reg} cells may play a role in reactivation of LTBI.

Conclusions

Anti-TNF therapies have provided effective treatment for a number of debilitating illnesses and it should be noted that while they may be associated with an increased risk of reactivation with infectious granulomatous pathogens, these risks are not excessively high and are, in most cases, overcome easily by testing for and treating existing latent infections. However, the discovery that these treatments can lead to such risks has fuelled interest in the role of TNF in immune responses to intracellular pathogens. Studies on TNF blockers are resulting in a better understanding of the

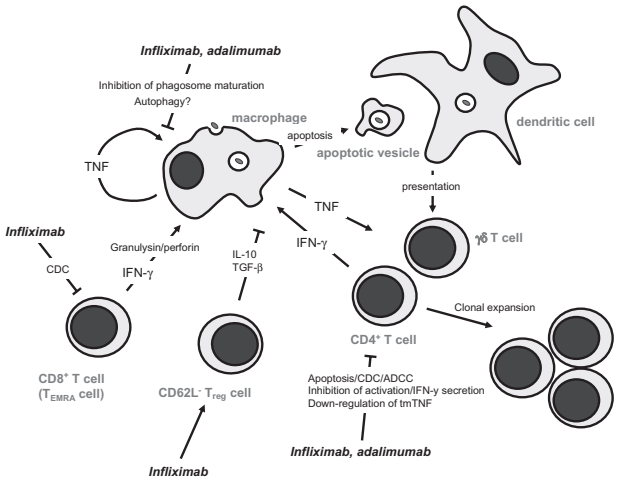

Fig. 3. Tumour necrosis factor (TNF) blockers and immunity to tuberculosis (TB): potential points of interference. After ingestion of *Mycobacterium tuberculosis*, the ability of the macrophage phago-lysosomal compartment to acidify is TNF-dependent and phagosome maturation is inhibited by TNF blockers. TNF-induced autophagy may also play a role in phagosome maturation. Some TNF blockers also induce killing of T cells (and monocytes) by apoptosis, complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), while allowing expansion of immunosuppressive regulatory T cells. The net result may be reduced interferon-γ responses, coupled with increased interleukin-10 which, with other direct anti-TNF effects, may explain some of the observed increase in susceptibility to TB reactivation in patients treated with TNF blockers.

complex interplay between different cells of the immune system in both autoinflammatory and infectious disease. In the case of Mtb infection, it is clear that TNF blockade can interfere with innate and adaptive immunity in a number of different ways (Fig. 3). Moreover, differences in the actions of anti-TNF monoclonals and etanercept have revealed potentially important differences in modes of action and pharmacology of these therapeutics, as well as helping to unravel further the biology of TNF (in particular tmTNF *versus* sTNF). In turn, such studies will lead undoubtedly to improved treatments and may prove applicable to other disease models and therapeutic interventions.

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