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TNFα in the regulation of Treg and Th17 cells in rheumatoid arthritis and other autoimmune inflammatory diseases

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Abstract

TNFα is a principal pro-inflammatory cytokine vital for immunity to infections. However, its excessive production is involved in chronic inflammation and disease pathology in autoimmune diseases. Evidence for its pathogenic role is validated by the fact that its neutralisation by therapeutic agents *in vivo* is beneficial in ameliorating disease and controlling symptoms. Paradoxically, however, treatment with TNFα inhibitors can either have no clinical effects, or even exacerbate disease in some patients. The explanation for such contradictory outcomes may lay in how and which downstream signalling pathways are activated and drive disease. TNFα causes its effects by binding to either or both of two membrane-bound receptors, TNFR1 and TNFR2. Engagement of the receptors can induce cell death or cell proliferation.

T cells both produce and respond to TNFα and depending on whether the cytokine is membrane-bound or soluble and the level of expression of its two receptors, the biological outcome can be distinct. In addition, polymorphisms in genes encoding TNFα and T cell signalling proteins can significantly impact the outcome of TNFα receptor engagement. Early studies revealed that effector T cells in patients with rheumatoid arthritis (RA) are hyporesponsive due to chronic exposure to TNFα. However, recent evidence indicates that the relationship between TNFα and T cell responses is complex and, at times, can be paradoxical. In addition, there is controversy as to the specific effects of TNFα on different T cell subsets. This review will summarise knowledge on how TNFα modulates T cell responses and the effect of engaging either of its two receptors. Furthermore, we discuss how such interactions can dictate the outcome of treatment with TNFα inhibitors.

Introduction

TNFα is a 233 amino acid protein produced by macrophages, dendritic cells (DCs), T cells, B cells, endothelial cells, mast cells and neural cells [1, 2] in response to infections and/or inflammatory stimuli. It plays a key role in initiating immunity to pathogens but is also associated with chronic inflammation and tissue damage in several inflammatory autoimmune diseases [3-5]. This review will discuss how TNFα stimulates T cell sub-populations and how this promotes, exacerbates or modulates autoimmune diseases.

TNFα signals through two receptors

When produced, TNFα is transported to the cell membrane to be expressed as a membrane-associated trimer. Membrane TNFα may subsequently be cleaved by TNFα converting enzyme (TACE) and shed as a trimer into the circulation [6]. Both the membrane form (mTNFα) and the shed form, or soluble form (sTNFα), are biologically active. T cells are important producers of TNFα and studies using animal models have revealed that T cell-derived TNFα is vital in initiating protective immunity against infections [3]. TNFα derived from T cells, unlike TNFα from other cell sources, often remains as a membrane form and functions via cell-cell contacts [7]. TNFα binds two membrane receptors, TNF receptor 1 (TNFR1), a 55kDa protein and TNFR2, 75kDa protein (Figures 1 and 2). TNFR1 is ubiquitously-expressed while TNFR2 has a restricted expression pattern on a few cell types, notably immune cells. T cells, for example, express both TNFR1 and TNFR2 [8]. TNFR1 can be triggered fully by both the membrane and soluble forms of TNFα whereas most reports indicate that TNFR2 is efficiently activated only by the membrane form. However, there is evidence that sTNFα bound to TNFR2 may be passed onto TNFR1 to induce biological effects [9].

The binding of TNFα to its two structurally-different receptors activates distinct but partially overlapping signalling pathways and, therefore, induces different responses. This is reflected in the activation of different downstream intracellular protein complexes inside cells (Figures 1 and 2). Engagement of TNFR1 primarily induces cell death by apoptosis or necrosis, mediated mostly by the activation of caspases via its cytoplasmic death domain. TNFR2, in contrast, lacks a death domain and, instead, promotes cell proliferation and homeostatic activities mainly through the Nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB). Many studies have indicated that TNFR2 is the most important receptor for TNFα-dependent regulation of T cells, especially regulatory T cells (Tregs) [10]. However, TNFR1 is also important for the development of effector T cells and, indeed, is expressed on both Tregs and Th17 cells [11, 12]. Recent studies have highlighted the importance of ubiquitination in the regulation of TNFα signalling downstream of TNFR1 and TNFR2 in T cells. Furthermore, genome-wide association studies (GWAS) have revealed that genes encoding TNFAIP3 and TRAF1, which are associated with the TNFRs and involved in ubiquitination, are associated with susceptibility to autoimmune diseases [4]. This section will briefly review known signalling pathways activated downstream of TNFR1 and TNFR2.

TNFR1-induced NF-κB signalling regulates cell survival but also initiates the activation of caspases that ultimately trigger cell death. The ubiquitination of various downstream signalling proteins determines whether TNFR1 engages complex I-dependent cell survival or complex II-dependent apoptosis. Thus, ubiquitination is no longer regarded a process solely involved in protein degradation but is known to also confer signal transduction. Ubiquitination by joining ubiquitin residues using different lysine (K) amino acid residues on the small protein confer different effects. Hence, generating ubiquitin chains by joining at lysine 48 (K48) mediates transport of the ubiquitinated protein to the proteasome for subsequent degradation. Ubiquitination generating chains linked at K63, on the other hand,

regulates a variety of nonproteolytic cellular functions [13, 14]. TNFR1 has an intracellular domain called death domain which recruits TNFR1-associated death domain (TRADD) protein. Downstream of TRADD, protein complexes I, IIa, IIb and IIc are activated. Activation of complex I induces inflammation, tissue degeneration and mediates host immune defences. Complex I consists of TRADD, Receptor-interacting serine/threonine-protein kinase 1 (RIP1), TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis (cIAP1) and the linear ubiquitin chain assembly complex (LUBAC) (Figure 1) [14-16]. The E3 ligase activity of cIAPs is required for the Haem-oxidized IRP2 ubiquitin ligase-1 (HOIL-1) recruitment to complex I. LUBAC consists of the K48-polyubiquitin specific E3 ubiquitin ligase HOIL-1, the E3 ubiquitin ligase HOIL-1L interacting protein (HOIP) and Shanks associated RH domain interacting protein (Sharpin). LUBAC ubiquitinates several proteins including IKKβ and T cell receptor ζ chain (TCRζ). K63-linked ubiquitination by RIP1 builds a scaffolding ubiquitin complex, TAK1 complex, which comprises TAK1, TAB2 and TAB3. Redundancy exists within the system as a linear ubiquitin chain which activates the inhibitor of NF-κB kinase complex (IKKα, IKKβ and IKKγ) that can be assembled either by RIP1/cIAP1 or by LUBAC [13, 16, 17]. TAK1 triggers MAPK activation, JNK, p38 and AP1 while IKKβ activates the NF-κB pathway. In T cells, MALT1 cleaves HOIL-1 and thereby regulates linear ubiquitination of NF-κB [18]. Sharpin can ubiquitinate a component of the T cell receptor TCRζ, which was recently shown to regulate the proliferation of Tregs (Figure 1) [11]. NF-κB induces the expression of another factor associated with ubiquitination, the ubiquitin-editing enzyme TNFAIP3 (also called A20) [19]. A20 is important for the prevention of functional signalling through complex II which leads to cell death and, instead, favours complex I-dependent cell survival [20]. A20-deficient T cells have been shown to be susceptible to caspase-independent and receptor-interacting serine/threonine-protein kinase 3- (RIP3) dependent necroptosis (Figure 1) [20]. This is of

significant interest as several GWAS have linked A20 to susceptibility to autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [4, 21].

In contrast to TNFR1, TNFR2 does not associate with the TRADD/RIP complex but, instead, associates with TRAF1, TRAF2 and cIAP1/2 (Figure 2). Engagement of TNFR2 mobilizes TRAF1/2, TRAF3 and NIK. TRAF2 mediates K63-linked ubiquitination of cIAP1/2 and this, in turn, mediates cIAP-dependent K48-linked ubiquitination of TRAF3 leading to its degradation. As a consequence, activated NIK phosphorylates IKKα which, in turn, activates NF-κB [22, 23].

T cells proliferate following engagement of their TCRs by their target antigen peptide/MHC complexes. These antigen peptides could be derived from micro-organisms or, as in autoimmune diseases, from self-proteins [4]. The TCR is a multiple subunit complex comprising clonotypic α and β chains that recognize antigen peptides presented within the context of MHC class I or II molecules expressed on all cells or on antigen presenting cells, respectively. TCR chains lack signalling motifs but are associated with signalling subunits CD3γ, δ, ϵ and ζ [24]. TNFα binding can modulate signalling through the TCR complex. Thus, the available evidence indicates that sustained exposure of T cells to TNFα leads to a gradual dissociation of the ζ chain from the TCR complex rendering T cells hypo-responsive to TCR engagement by antigen-MHC complexes. In this setting, downregulation of the TCRζ chain from T cells by TNFα occurs predominantly through proteasomal degradation and, apparently, leads to TCR/CD3 complex instability, conformational changes upon TCR engagement and abnormal signal transduction [25]. Of note, during inflammatory responses, T cells with reduced TCRζ levels accumulate in inflamed tissues but this can be reversed by TNFα blockade [26]. This imbalance has implications for autoimmune diseases. Indeed,

studies of polymorphisms in genes coding for proteins regulating TCR-mediated signalling have been strongly associated with susceptibility to autoimmune diseases [4, 27-30]. This theme will be discussed in more detail in the next section.

Binding of TNFα to its receptors and the signalling cascades that this binding initiates play a key role in responses to infections but also to pro-inflammatory processes that initiate pathology in many autoimmune diseases. This scenario is best highlighted by the efficacy of biologic TNFα inhibitors in treating patients with autoimmune diseases. However, due to the complex nature of the regulation of signals mediated by TNFα, treatment with TNFα inhibitors can result in several outcomes including the induction of specific T cell subsets such as Tregs or Th17 cells in treated patients. In the following sections we will discuss the involvement of the two key T cell subsets, Tregs and Th17 cells, in the aetiology of RA and implication of TNFα itself and TNFα inhibitors are likely to have on the differentiation and responses of these cell subsets.

T cells and TNFα in autoimmune disease

In most autoimmune inflammatory diseases, TNFα is over produced leading to pathology. Indeed, chronic inflammation and most symptoms of diseases in RA, ulcerative colitis, Crohn's disease, psoriasis, psoriatic arthritis, ankylosing spondylitis, juvenile idiopathic arthritis (JIA) are improved in most patients when biologic TNFα inhibitors are used for treatment [15, 31]. Although somewhat controversial, blockade of TNFα even in diseases where TNFα has been suggested to suppress the autoimmune response such as in Behcet's disease, TNFα receptor-associated periodic fever syndrome and SLE all has been reported by some investigators to be beneficial in some patients using off-label treatment with biologic TNFα inhibitors [15, 32].

A number of inhibitors of TNFα have been commercially-available for close to two decades and are used as a mainstay treatment in patients with severe to moderate disease and in patients refractory to disease-modifying anti-inflammatory agents. Several of these agents are either chimeric human-mouse monoclonal antibodies (infliximab), or human anti-TNFα antibodies (adalimumab and golimumab). Certolizmab pegol is a pegylated Fab fragment while etanercept is a fusion protein consisting of TNFR2 and an IgG1 Fc backbone. The different inhibitors have different functional profiles [33].

In RA, the trigger(s) that starts a chain of events leading to disease remains unknown. However, T cells are key driving cells in the processes that lead to chronic synovial inflammation and joint damage. Thus, T cells are found in the synovial lining of RA joints prior to symptomatic changes [34] and these produce pro-inflammatory cytokines such as TNFα and IL-17 which cause pathology [4]. Further support for the role of T cells in RA pathogenesis comes from studies showing the beneficial therapeutic effect of treating patients with CTLA4-Ig fusion protein (abatacept) which blocks T cell co-stimulation [5]. TNFα produced by T cells, as well as monocytes/macrophages, mediate pathology by initiating chronic inflammation, activating bone-resorbing osteoclasts and by inducing matrix metalloprotease production in inflamed joints [35, 36]. TNFα also influences T cells directly. Thus, as cited earlier, chronic exposure of T cells to TNFα results in hyporesponsiveness to TCR-mediated stimulation, premature ageing, shrunken T cell repertoire and susceptibility to infections [4, 37-44]. Reduced responsiveness of T cells to stimulation through the TCR and reduced cytokine production occurs due to a combination of reduced expression of TCR-associated signalling components and costimulatory molecules [44]. Thus, 10–30% $CD3^+$ T cells in patients with RA manifest reduced expression of the TCRζ chain due to chronic exposure to TNFα [26, 41]. A further, 3% of T cells in RA patients lack the co-stimulatory molecule, CD28 [45, 46]. T cells with reduced expression of TCRζ or CD28 do not

proliferate in response to stimulation with antigens but, instead, respond to stimulation through alternative pathways such cytokine/cytokine receptor engagement or CD134/OX40 stimulation [47]. Interestingly, T cells with reduced expression of TCRζ are also characterized by excessive production of inflammatory cytokines such as TNFα, senescence and increased ability to infiltrate the synovium [41].

Treatment of RA patients with TNFα inhibitors has revealed that the functional and proliferative impairment of the T cells are reversed by neutralising endogenous TNFα [27, 46]. Although production of some cytokines, such as IFNγ, is lower in PBMCs from RA patients than healthy controls, the production of IFNγ as well as IL-2 and IL-4 increases following treatment with TNFα inhibitors and this effect is maintained for up to six months. Treatment with TNFα inhibitors also restores T cell signalling and is reported to induce IL-10 production [27, 48]. Following treatment with the TNFα inhibitor infliximab, $CD28^-$ cells were reported to regain their ability to express CD28, or to be replaced by expanding $CD28^+$ T cells [49]. Treatment with TNFα inhibitors also significantly reduced the number of expanded T cell clonotypes, including potentially self-reactive ones [50]. Some RA patients have function-altering polymorphisms in and around genes associated with TNFα and T cell receptor signalling [4, 51]. In this respect, one of the polymorphic genes associated with RA is *TNFAIP3* which encodes the ubiquitin-editing protein A20. As cited earlier, A20 suppresses the activation of TNFR1-associated complex II which induces necroptosis [20]. Expression of *TNFAIP3* mRNA decreased in PBMCs of RA patients who responded to treatment with TNFα inhibitors. It has previously been reported that mutations in the *TNFAIP3* correlate with patient responsiveness to treatment with TNFα inhibitors [52]. Why this is the case is not clear, however, but defects in the function of A20 seem to be of advantage when TNFα is neutralised.

In addition to therapeutic benefits emanating from treating RA patients with TNFα inhibitors, such treatment has provided new insights into mechanisms driving disease and that these are unlikely to be the same in all RA patient groups. This is evident from the fact that ~30-40% of treated patients do not respond to treatment with TNFα inhibitors. Patients who do not respond to treatment with TNFα inhibitors suggests that the disease in these patients is different from the disease in responder patients and that disease heterogeneity is likely to involve the immune system [53, 54]. Different interpretations have been proposed to explain differences in the pro-inflammatory immune response that drives disease in responder and non-responder RA patients to treatment with TNFα inhibitors. Thus, T cells from non-responder patients, in contrast to responder patients, have been suggested to be transcriptionally active and driven by pro-inflammatory signalling to a lesser degree [55, 56]. This may reflect the fact that T cells only in responder patients can upregulate TNFR1 expression in response to treatment [46, 54, 57].

Treatment with TNFα inhibitors has, in some cases, been associated with the development of other autoimmune diseases, such as lupus-like disease and the exacerbation of symptoms of psoriasis [58]. Knowledge of why TNFα in some, but not all, disease settings prevents the development of autoimmune disease pathology can be gained from studies of animal models of different autoimmune diseases. For example, injection of TNFα to lupus-prone New Zealand mice reduced symptoms of the disease [59]. Furthermore, treatment of type 1 diabetes in NOD mice with recombinant mouse TNFα ameliorated disease [60]. Better knowledge of how TNFα modulates the immune system and, hence, impacts the development of autoimmune diseases is important to better understand why some patients with RA do not respond to treatment with TNFα inhibitors.

TNFα and regulatory T cells (Tregs) in autoimmune diseases

Tregs are important effector T cells that regulate immune responses, maintain peripheral immunological tolerance and suppresses inflammation. They modulate responses by other T cells, NK cells and APCs. Tregs express the transcription factor Forkhead box P3 (FOXP3) and high cell membrane levels of CD25, cytotoxic T lymphocyte-associated protein 4 (CTLA4) and glucocorticoid-induced TNFR-related protein (GITR). These cells regulate immune responses by producing key anti-inflammatory cytokines including IL-10 and TGFβ and by cell contact-dependent mechanisms through CTLA4 and the membrane glycoprotein LAG3 and by competing with effector T cells for IL-2 [61, 62]. At least two broadly-defined sets of Tregs have been described: naturally-occurring (nTreg) that develop in the thymus and Tregs that are induced in the periphery (iTreg) [63]. IL-2 and TGFβ promote the differentiation of Tregs *in vitro* while high levels of inflammatory cytokines (IL-6 and IL1β) inhibit their differentiation [64].

Studies to determine how TNFα influences Tregs have been somewhat controversial. For example, high levels of TNFα together with IL-2 were reported to induce the expression of murine FOXP3 *in vitro* [65]. In addition, TNFα was shown to increase the rate of Treg proliferation [66]. However, relatively high concentrations of TNFα were required for the activation of Tregs [10]. These *in vitro* observations were interpreted to imply that high levels of TNFR ligation can only be achieved via membrane-bound TNFα *in vivo* involving cell-cell contacts and, specifically, TNFR2 engagement [67]. Expression of TNFR2 and CTLA4 on Tregs was noted to be important for the suppressive activity of Tregs [10]. TNFα, binding to TNFR2, increased the expression of co-stimulatory molecules 4-1BB and OX40 [68]. In contrast, engagement of TNFR1 was suggested to influence Treg development via a component of the LUBAC complex, Sharpin, which is required for efficient proliferation of

Tregs *in vivo* [11]. Ubiquitin chains originating from Sharpin inhibit the association between TCRζ and Zap70 thereby affecting the generation of Tregs. In addition, Sharpin deficiency resulted in IL-17 production by the cells [11]. However, whether TNFα stimulates or inhibit the suppressive activity of Tregs remains a controversial question [69, 70].

Tregs have been shown to be dysregulated in a number of autoimmune diseases in which there is evidence for high levels of TNFα production, including RA and anti-neutrophil cytoplasmic antibody- (ANCA) associated vasculitis [71-73]. However, a large GWAS revealed that a number of polymorphic loci associated with susceptibility to RA are found at chromosomal sites epigenetically accessible in Tregs [51]. A number of functional Treg defects have been reported in RA. For example, Tregs have been shown to be unable to supress Th1-mediated responses due to their lack of CTLA4 expression [74]. Perhaps somewhat paradoxically, despite *in vitro* studies indicating that TNFα activates Tregs, treatment of RA patients with TNFα inhibitors restores the ability of Tregs to suppress cytokine production by effector T cells. Other studies revealed that the number of Tregs, which were reduced prior to treatment of RA patients increased following treatment with TNFα inhibitors [63]. One study observed that treatment with TNFα inhibitors induced FOXP3 phosphorylation enabling Tregs to supress Th17 responses in responder patients [75]. Interestingly, other studies indicated that the choice of TNFα inhibitor determined whether the activity/number of Tregs will be enhanced or not [53, 63, 76]. For example, Tregs in RA patients treated with the TNFR2 fusion protein, etanercept, where not affected by the treatment. In contrast, Tregs in patients responding to treatment with adalimumab increased in numbers and had improved suppressive functions in response to the treatment. Tregs in adalimumab-responder patients suppressed MCP-1 production by monocytes and the T cells did not switch to become Th17 cells (see below) [53]. One possible explanation for the different findings in different studies is, perhaps, provided by the fact that adalimumab binds

to membrane TNFα on monocytes and T cells promoting its upregulation and consequent high-degree of ligation of TNFR2 on Tregs [76]. Another study observed that in infliximab-responder patients, an iTreg population lacking CD62L emerged [63]. This population had suppressive activities *in vitro*. Interestingly, studies in animal models indicated that whilst nTregs required TNFα to function efficiently, iTregs did not require such stimulation [77]. It is notable, however, that the various studies clearly show that Tregs in responder patients only regain regulatory functions following treatment with TNFα inhibitors. It is possible that the inflammatory environment in non-responder patients continue to diminish Treg functions despite neutralisation of TNFα [53, 75].

As cited in the previous section, it is not only Tregs that are influenced by disease processes in RA but also are other effector T cells (Teff). Thus, almost all effector T cells (Teffs) have impaired, or altered functions in RA that could render such T cells non-responsive to Treg mediated suppression [37]. Enhanced activation of protein kinase B (PKB; also known as Akt) was reported in T cells from patients with juvenile idiopathic arthritis (JIA) to prevent the suppression of the cells by Tregs [78]. That Teffs in RA patients can be rendered resistant to suppression is corroborated by findings from patients and animal models of other autoimmune diseases. For example, Teffs in patients with SLE show diminished responses to the suppressive activity of enriched nTregs [79]. This observation was replicated in a murine model of SLE [80]. Similar findings of reduced suppressive responses of Teffs to Tregs were also reported in patients with type 1 diabetes and in an animal model of the disease [81-83]. In addition, Teffs in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis in mice were shown to be resistant to Treg suppression [84]. It should be noted that in cases where patient cells were used (SLE and type 1 diabetes), the experiments were often performed using cells isolated from the same individual. Based on these

observations, it is, therefore, pertinent to suggest that to show that the suppressive effects of Tregs is reduced in a disease it would be necessary that these Tregs are cultured with T cells from syngeneic healthy donors rather with Teffs from the patients themselves.

A reciprocal relationship between Tregs and Th17 cells has been reported. Thus, the ratio of Th17 to Treg cells is significantly higher in RA patients compared with matched healthy controls [53]. Interestingly, however, both sets of cells can differentiate from the same progenitor cells and it is possible that the inflammatory milieu in RA favours polarization towards Th17 cells [64]. FOXP3-expressing IL-17-producing intermediate cells have been described [85]. In RA patients, the ratio of Th17 to Treg cells is decreased with increased numbers of Tregs in response to treatment with the anti-IL6 receptor antibody Tocilizumab [86, 87]. In appropriate inflammatory environments, Tregs can also transdifferentiate into Th17 cells [88]. Thus, IL-17-producing T cells that differentiate from $FOXP3^+$ T cells have been detected in joints of mice with experimental arthritis [88]. Furthermore, and $IL\text{-}17^+FOXP3^+$ T cells were also identified in the synovium of RA patients [88]. The transdifferentiation of Tregs to Th17 cells was, however, reported to be independent of TNFα [88].

In conclusion, how TNFα influences Tregs in RA appears to depend on both the differential expression of TNFα receptors and which Treg populations are present. Although nTregs are likely to be activated by TNFα, iTregs that are induced in response to treatment with TNFα inhibitors appear to be independent of the cytokine. There is a possibility that the inflammatory milieu in RA favours the conversion of Tregs to Th17 cells. In addition, it appears that the status of Teffs will determine whether the cells respond to Treg-mediated regulation or not. Additionally, polymorphisms associated with RA that influence TCR and TNFα signalling can alter the activity of both Tregs and Teffs to TNFα and TNFα inhibitors.

Th17 cells and TNFα in autoimmune disease

Th17 cells are key cells in providing immunity to extracellular bacterial and fungal infections at mucocutaneous surfaces in the intestine, the respiratory tract and the skin. The cells, however, are also involved in chronic inflammation in patients with a number of autoimmune diseases including RA. Th17 cells produce a distinct set of cytokines that include IL-17A, IL-17F and IL-22. IL-17A is the most potent pro-inflammatory cytokine within the IL-17 family and is produced at higher levels by Th17 cells. For the purpose of this review, IL-17A will henceforth be referred to as IL-17.

Gut commensal bacteria induce IL-1β production during homeostasis and this maintains a basal levels of Th17 cells [89]. More activated Th17 cells, however, infiltrate sites of bacterial and fungal infections and coordinate immunity through IL-17 production *in situ*. The cytokine promotes the production of IL-6 and the CXC chemokines CXCL9, CXCL10 and CXCL11 [90]. This results in the recruitment of other immune cells particularly neutrophils to sites of infections and inflammation. In addition, IL-22 produced by Th17 cells provides protection for mucosal membranes [91]. The differentiation of Th17 cells is dependent on the production of a combination of cytokines including IL-1β, IL-6 and IL-23 in human and, in mice, TGFβ is also required. Induction of the transcription factors RORγ, STAT3 and BATF facilitates the differentiation of, presumably, naive Th cells to Th17 cells. The available evidence indicates that weak signalling through the TCR drives Th cell to differentiate to Th17 cells as compared with Th1 which requires strong TCR-mediated signalling [92]. Th17 cells are distinct from other subsets of helper T cells in that they are flexible and can convert to IFNγ-producing cells to become more effective in combating microorganisms [93]. Some Th17 cells have also been shown to have a stem cell gene

signature [94]. After they have been activated, memory Th17 cells can revert to be in a dormant status in the mucosa for extended periods of time and then change to different Teffs depending on the milieu of their stimulation. This scenario might have implications for the role of Th17 cells in autoimmunity (see below) [94]. In this respect, single cell mRNA analyses of Th17 cells from mice identified five distinct subsets. Cells associated with a stem cell character were reported to be generally present in lymph nodes while effector Th17 cells with mRNA for IFNγ were noted to be present in the periphery and to be associated with autoimmune diseases [95]. For example, IFNγ-producing Th17 cells have been described in patients with Crohn's disease and multiple sclerosis [96, 97]. Another subset of Th17 cells was reported to express *Rankl* mRNA and implicated in bone remodelling [94, 95]. Interestingly, Th17 cells have also been reported to have the ability to transdifferentiate into FOXP3-expressing T cells [88]. Exposure of T cells to IL-2 has been shown to inhibit Th17 development. Indeed, it has been suggested that $CD4^+$ T cell-intrinsic mTNF/TNFR2 interaction promotes IL-2 production which, in turn, inhibits the differentiation of naïve T cells to Th17 cells. The role of TNFR2 engagement in the suppression of Th17 development is confirmed by the findings that blockade of TNFR2 promote Th17 differentiation [98].

In addition to their role in providing protective immunity against extracellular bacterial and fungal infections, Th17 cells are established as major contributors to autoimmune disease as highlighted above [93]. Indeed, treatment of patients with a range of autoimmune disease, including psoriasis and psoriatic arthritis, with the anti-IL-17 antibodies secukinumab and ixekizumab has been highly effective in resolving disease [99-102]. In RA, low levels IL-17 production during the early stages of disease is thought to predict less severe disease [103]. However, in established disease, IL-17 contributes to chronic synovial inflammation and local bone erosion. In this respect, IL-17 has been shown to correlate with RANKL upregulation in the synovium and synovial fluid of RA patients [104]. For example, IL-17

induces RANKL production by synoviocytes, promotes osteoclast maturation and matrix metalloprotease production [105-107]. Many of the effects associated with IL-17 in RA can, perhaps, be attributed to its combined effects with TNFα [104]. In addition, TNFα can also facilitate Th17 differentiation in autoimmune disease. For example, TNFα binding to TNFR1 was shown to be necessary for the differentiation of Th17 cells and bone resorption in a mouse model of hyperparathyroidism [108]. Furthermore, TNFα facilitated Th17 differentiation in EAE disease in mice, a process that was inhibited by soluble TNFR1 [109]. Perhaps somewhat paradoxically, the inhibitory effect of TNFα on the development of some autoimmune diseases in specific settings has been shown to involve inhibiting Th17 cell proliferation. This was reported in animal models of psoriasis, SLE and RA [110-112]. Thus, in a mouse model of psoriasis, blockade of TNFα resulted in expanded Th17 cells and exacerbated disease [110]. Lupus nephritis was also exacerbated in the New Zealand mouse model of lupus in which the disease was apparently dependent on Th17 cells. Mice in this model were deficient in TNFR1 and TNFR2 [111]. Additionally, we have observed that collagen-induced arthritis in mice treated with anti-TNFα developed a Th17 cell response which was pathogenic [112]. Relevant to understanding how blockade of TNFα promotes Th17 differentiation are experiments in which TNR1- but not TNFR2-deficient arthritic mice showed a significant increase in the number of Th17 cells. These Th17 cells were enriched in lymph nodes of the arthritic TNFR1-deficient mice [112]. It is not known, however, whether these cells have the same stem cell characteristics as was identified by single cell RNA sequencing [95]. Interestingly, the expression of IL-12/IL-23 p40 was up-regulated in lymph nodes (LN) of TNFR1-deficient mice and the expansion of Th17 cells abrogated by blockade of p40. In addition, treatment of macrophages with recombinant TNFα inhibited p40 production *in vitro* [112]. Studies carried out in our laboratories and by others revealed that treatment of patients with RA, ankylosing spondylitis and psoriatic arthritis with TNFα

inhibitors were consistent with the murine experiments in that there was an increase in the number of Th17 cells in non-responder patients [113-116]. This observation was confirmed in subsequent studies and shown to be associated with an increase in IL12/IL-23 p40 production by monocytes [115, 117, 118]. These findings suggest that in severe inflammation in autoimmune diseases, TNFα signalling through the TNFR1 is likely to have a negative effect on Th17 cell differentiation and/or expansion. These findings are inconsistent with observations suggesting that these effects are likely to be due to differential roles played by TNFR1 and 2 in different clinical settings [98].

We and others have observed that the increase in Th17 cells following treatment with TNFα inhibitors is associated with a lack of response to, or relapse after an initial beneficial response to treatment with TNFα inhibitors. These studies have revealed that monocytes from non-responder patients produced more IL-12/IL-23 p40 which is likely to facilitate Th17 cell differentiation/expansion [115, 117, 118]. Although responsiveness to TNFα inhibitors is associated with increased TNFR1 expression on lymphocytes, it has so far not been possible to determine whether this upregulation of the receptor extends to Th17 cells [54]. It is, however, possible that Th17 cells in non-responder patients are different from responder patients in terms of genetic predisposition to upregulate TNFR1.

Conclusions

Despite an ever expanding number of studies, the exact influence of TNFα on Tregs and Th17 cells in autoimmune disease remains unclear. However, it is beyond doubt that T cells are influenced by chronic inflammation and by exposure to TNFα as clinically manifested by the exhaustion of T cells and increased susceptibility of patients to infections. One factor that is likely to determine how TNFα influences different T cell populations is which TNFα

receptors these cells express. It is noteworthy, however, that the expression of TNFR1 on T cells is associated with a beneficial clinical response of RA patients to treatment with TNFα inhibitors [54]. In experimental arthritis in mice, signalling through TNFR1 inhibited the development of Th17 cells and ameliorated disease pathology. Since signalling through TNFR1 can lead to proliferation, apoptosis and/or necrosis, the upregulation of factors such as the ubiquitin editing enzyme A20 and ubiquitin-binding enzyme Sharpin could determine which type of response will be the ultimate outcome. Most studies indicate that Tregs are influenced by TNFα signalling through membrane-bound TNFR2. Recent studies, however, suggest that TNFR1 is necessary for early proliferation of Tregs. It is possible that different TNFα receptors are of importance during different stages of Treg proliferation. Furthermore, the genetic constitution of the individual and the level of different signalling molecules downstream of TCR and TNFα could ultimately determine the response to treatment with TNFα inhibitors.

Better knowledge of the level and/or ratio of the two TNFα receptors expressed on different T cell subsets, which pathways are active in the different cells and the contribution of polymorphisms influencing TNFα and TCR signalling will provide better understanding of autoimmune disease pathology and likely responses to treatment with TNFα inhibitors.

Contributions

All co-authors contributed equally to the outline and completion of the review paper.

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Conflict of interest

The authors declare no conflict of interest.

Figure legends

Figure 1. Signalling of TNFα downstream of its receptor 1 (TNFR1). TNFα binding to TNFR1 leads to the activation of complex I leading to cell necroptosis. Activation of complex I involves proteins TADD, RIP1, TRAF2, cIAP1 and LUBEC. RIP1 mediate K63-linked ubiquitination and activation of the TAK1 complex. The TAK 1 complex consists of TAK1, TAB1 and TAB2. The activation of TAK1 triggers the signalling pathways MAPK, JNK, p38 and AP1. Both RIP1/cAIP and LUBEC facilitate K48 ubiquitination of the IKK complex. This leads to its degradation, phosphorylation of the NF-κB inhibitor IκB and NF-κB translocation to the nucleus. NF-κB induces cell specific-transcriptional programs in effector T cells (Teffs), Th17 cell and Tregs. There is a direct link between TNFR1 and the T cell receptor (TCR) as one of components of LUBAC, Sharpin, can ubiquitinate TCRζ in Tregs leading to its disassociation from ZAP70 and, ultimately, cell proliferation. If components of complex I are limiting, complex IIa, IIb or IIc can be activated. Activation of complex IIa and IIb leads to apoptosis. Activation of complex IIc leads to necroptosis. Polymorphism in gene for the ubiquitin-editing enzyme A20 (*TNFAIP3*) is associated with RA. This polymorphism appears to influence activation of complexes II.

Figure 2. Signalling of TNFα downstream of its receptor 2 (TNFR2). TNFα binding to TNFR2 induces TRAF1/2-dependent K63-linked ubiquitination and activation of the ubiquitin ligase cAIP2/3. cAIP2/3 facilitates K48-linked ubiquitination of TRAF3 leading to

its degradation. This releases NIK which, in turn, phosphorylates IκB leading to NF-κB activation. Downstream of TNFR2, NF-κB induces the transcriptional program of Tregs.

Figure 3. Differential expression of TNFRs on Tregs and Th17 cells and the effect of therapeutic blockade of TNFα on the T cell subsets. Tregs and Th17 cells respond differently to TNFα binding to TNFR1 and TNFR2. **A**. TNFα binding to TNFR1 on Tregs initially induces proliferation. Binding of TNFα to TNFR2, in contrast, increases the suppressive activity of Tregs through enhancing FOXP3 expression. **B.** TNFα inhibition by therapeutic inhibitors results in the phosphorylation and activation of FOXP3 and increased inhibitory activity of Tregs. **C.** Binding of TNFα to TNFR2 inhibits Th17 differentiation in one model of inflammation. TNFα binding to TNFR1 also inhibits Th17 cell differentiation in collagen induced arthritis. **D.** Abolition of TNFα signalling by deletion of TNFR1 (but not TNFR2), or by pharmacological blockade of the cytokine in RA patients promotes Th17 cell proliferation and exacerbates inflammation in collagen-induced arthritis in mice in an IL-23-dependent manner.

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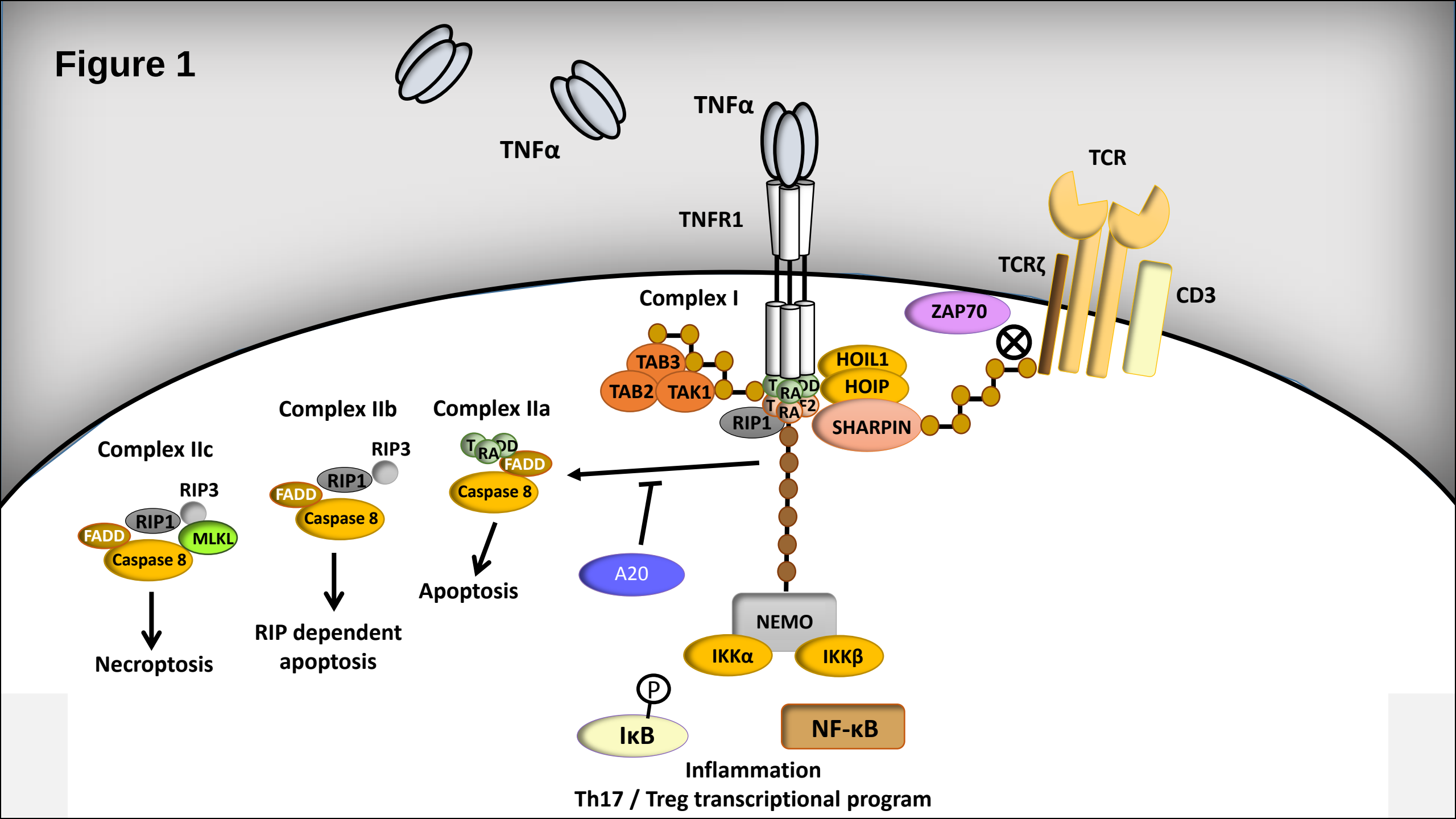
Figure 1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TCR
TCRζ
CD3
ZAP70
Complex I
TAB3
TAB2
TAK1
HOIL1
HOIP
SHARPIN
RIP1
Complex IIa
FADD
Caspase 8
Apoptosis
Complex IIb
RIP3
RIP1
FADD
Caspase 8
RIP dependent apoptosis
Complex IIc
RIP3
RIP1
FADD
MLKL
Caspase 8
Necroptosis
A20
NEMO
IKKα
IKKβ
P
IκB
NF-κB
Inflammation
Th17 / Treg transcriptional program

Figure 2

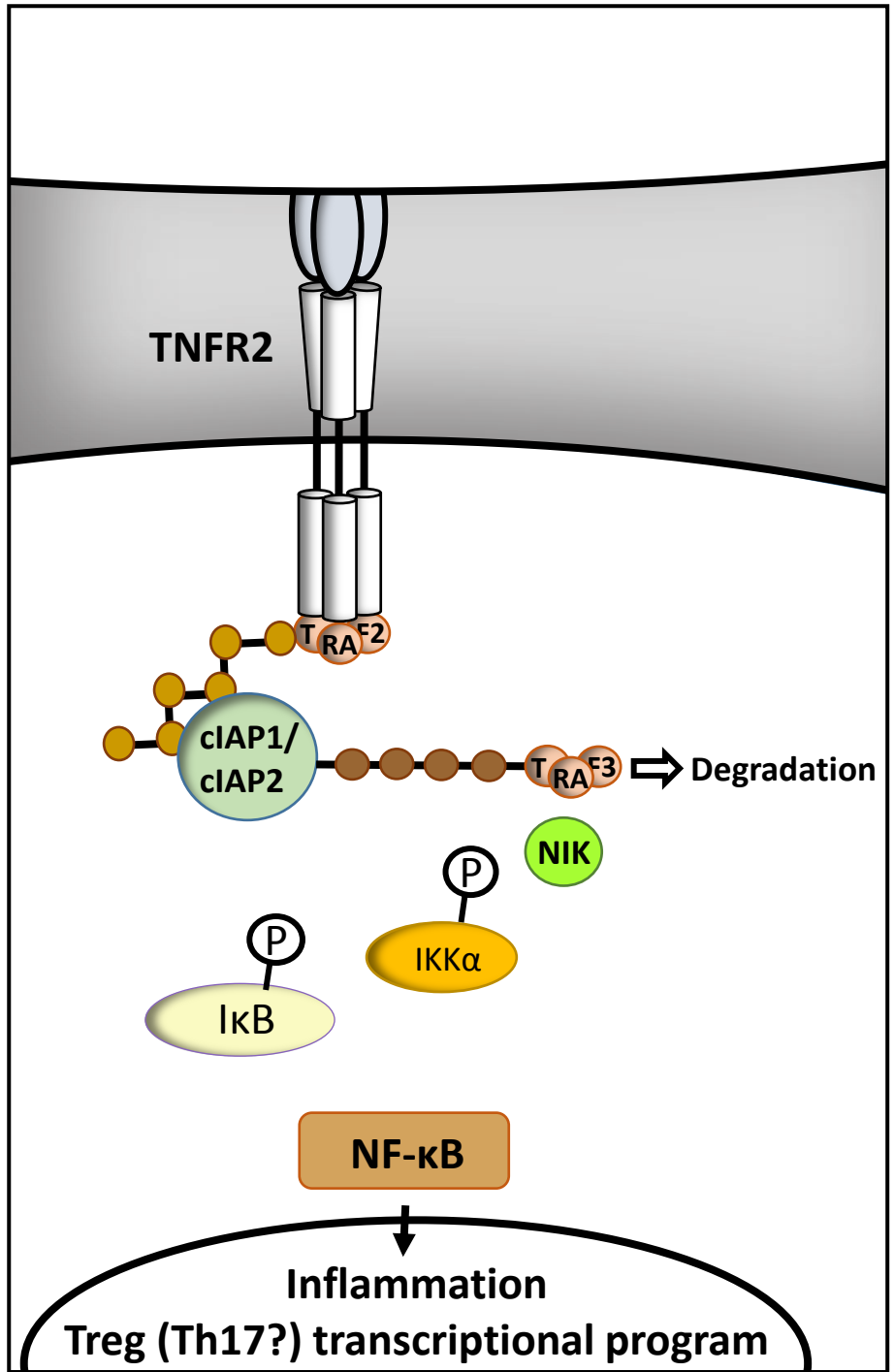
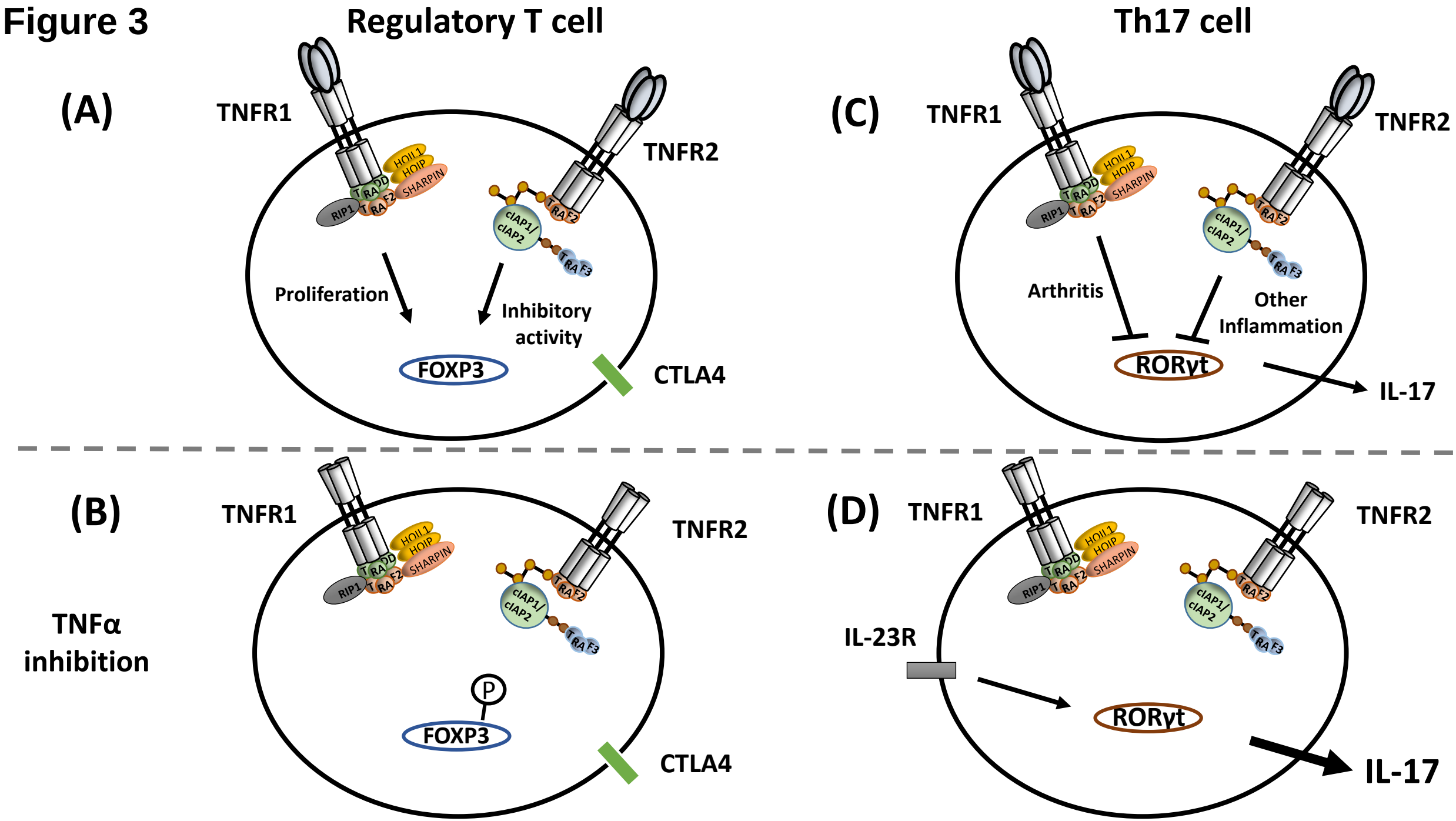

TNFR2
cIAP1/
cIAP2
Degradation
NIK
P
IKKα
P
IκB
NF-κB
Inflammation
Treg (Th17?) transcriptional program

Figure 3


Regulatory T cell
Th17 cell
(A)
TNFR1
TNFR2
HOIL1
HOIP
SHARPIN
TRADD
RIP1
TRAF2
cIAP1/
cIAP2
TRAF3
Proliferation
Inhibitory
activity
FOXP3
CTLA4
(C)
TNFR1
TNFR2
Arthritis
Other
Inflammation
RORγt
IL-17
(B)
TNFα
inhibition
TNFR1
TNFR2
P
FOXP3
CTLA4
(D)
TNFR1
TNFR2
IL-23R
RORγt
IL-17