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TNF receptor signalling in autoinflammatory diseases

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Abstract

Autoinflammatory syndromes are a group of disorders characterised by recurring episodes of inflammation as a result of specific defects in the innate immune system. Patients with autoinflammatory disease present with recurrent outbreaks of chronic systemic inflammation that are mediated by innate immune cells, for the most part. A number of these diseases arise from defects in the tumour necrosis factor (TNF) receptor signalling pathway leading to elevated levels of inflammatory cytokines. Elucidation of the molecular mechanisms of these recently defined autoinflammatory diseases has led to a greater understanding of the mechanisms of action of key molecules involved in TNFR signalling, particularly those involved in ubiquitination, as found in haploinsufficiency of A20 (HA20), otulipenia/otulin-related autoinflammatory syndrome (ORAS) and linear ubiquitin chain assembly complex (LUBAC) deficiency. In this review we also address other TNFR signalling disorders such as (TNF) receptor–associated periodic syndrome (TRAPS), RELA haploinsufficiency, RIPK1-associated immunodeficiency and autoinflammation, X-linked ectodermal dysplasia and immunodeficiency (X-EDA-ID) and we review the most recent advances surrounding these diseases and therapeutic approaches currently used to target these diseases. Finally, we explore therapeutic advances in TNF-related immune based therapies and explore new approaches to target disease-specific modulation of autoinflammatory diseases.

Introduction: molecular control of TNFR1-mediated signalling

TNFR Signalling

TNF is a potent inflammatory cytokine which signals via two distinct receptors, TNF receptor 1 (TNFR1) and TNFR2, and mediates a number of physiological functions essential for immune regulation, cell proliferation, survival and death. TNF is expressed as a trimeric type II transmembrane protein (mTNF) which can be cleaved by TNF-converting enzyme (TACE, or ADAM17) to give rise to a soluble extracellular TNF (sTNF) (1,2). Both mTNF and sTNF exert their physiological functions by binding to either TNFR1 or TNFR2, on target cells. Following activation by TNF, TNFR1 recruits both receptor-interacting serine/threonine protein kinase 1 (RIPK1) and TNF-receptor-associated death domain (TRADD) to the cytoplasmic death domain (DD) of the receptor (3) whereas TNFR2 does not have a death domain and, instead, signals through recruitment of TNFR-associated factor 2 (TRAF2) protein (4,5). Both TNFR1 and TNFR2 signalling pathways can lead to activation of the classical nuclear factor-κB (NF-κB) signalling pathway leading to cell survival and cell proliferation, whereas TNFR1 can also lead to the initiation of cell death pathways leading to apoptosis or necroptosis, depending on the metabolic state of the cell (6).

Ubiquitination in TNF-signalling

Post-translational ubiquitination in TNF signalling plays a major role in regulating immune cell fate and directs the formation of distinct signalling complexes following activation of TNFR1, namely complex I, IIa, IIb and IIc, all of which contain the core proteins TNFR1, TRADD and RIPK1 (7,8). Ubiquitin (Ub) chains are highly conserved and assembled in response to activation before being covalently attached to target proteins via E3 Ub-ligase enzymes, to reinforce protein stability and activation or enable protein degradation (For detailed reviews see (9,10)). Ubiquitination is a reversible process whereby Ub chains can be hydrolysed by a class of enzymes known as deubiquitinases (DUBs) (11). Several DUBs, including A20, OTULIN, cylindromatosis (CYLD) and

Cezanne, function as negative regulators of NF-κB signalling (12). Dysregulation in ubiquitin proteasome signalling (UPS) has been reported in a number of autoinflammatory disorders, including linear ubiquitin chain assembly complex (LUBAC) deficiency (13,14), haploinsufficiency of A20 (HA20) (15), and otulipenia/otulin-related autoinflammatory syndrome (ORAS) (15,16) and these will be discussed in detail in this review.

TNFR signalling via complex I

Complex I is composed of TRADD (3), RIPK1 (17), TRAF2/5 (18), cellular inhibitor of apoptosis protein 1 (cIAP1) or cIAP2 and LUBAC (19). LUBAC is composed of three proteins, heme-oxidised IRP2 ubiquitin ligase 1 (HOIL-1), shank-associated RH domain-interacting protein (SHARPIN) and HOIL-1 interacting protein (HOIP) (reviewed in (7)). Initially, cIAP1/2 and TRAF2/5 attach K63-linked Ub chains to RIPK1 (20,21) which subsequently leads to LUBAC-mediated attachment of M1-linked chains to RIPK1 (19,22). This assembly leads to the recruitment of two signalling complexes, with transforming growth factor (TGF)β-activated kinase (TAK) 1 complex (consisting of TAK1 and TAK-binding protein 2/3 (TAB2/3)) K63-linked chains, and the inhibitor of κB (IκB) (IKK) complex (consisting of IKKα, IKKβ and NF-κB-essential modulator (NEMO)), via M1-linked chains (19,22-24).

The polyubiquitin chains present on NEMO and RIPK1 are bound by the TAK1 complex; this is essential as it phosphorylates and stimulates the catalytic IKKβ subunit to phosphorylate and degrade IκBα thereby activating NF-κβ which, in turn, promotes the transcription of target genes required for cell survival and proliferation (20,25,26). The TAK1 complex also phosphorylates mitogen-activated kinases (MAPK), p38 MAPK and c-Jun N-terminal kinase (JNK) in a signalling cascade which leads to transcription of AP1 target genes (Figure 1) (20,23). The various types and concentration of polyubiquitin chains attached to RIPK1 have been shown to modulate the activity of recruited proteins which signal via complex I; although RIPK1 attachment to NEMO is preferentially mediated by M1-linked chains, NEMO can also bind via K63- and K11-linked chains.

This differential offers up several possibilities for fine-tuning ubiquitin dependent signalling mediated by TNF and also for IL-1β mediated activation of NF-κB via the IL-1 receptor (IL-1R) (for detailed review see (7)). A recent study shows that ubiquitination also regulates RIPK1's cytotoxic potential, not only through activation of the NK-κB pathway but also by directly supressing RIPK1 kinase activity, via ubiquitin-dependent inactivation (27).

IL-1 Receptor signalling

NF- κB activation is modulated via a complex cross-talk of several signalling pathways with shared components for activating and inhibiting this pathway. The pro-inflammatory cytokine, IL-1β (along with IL-1α and IL-1 receptor antagonsist, IL-1Ra) binds to the IL-1R leading to the downstream activation of NF-κB (28,29). Following ligand binding, the IL-1R complex leads to the recruitment of the E3-ligase TNF receptor associated factor (TRAF6) and the ubiquitin E2 ligase complex which conjugates K63-linked chains to interleukin-1 receptor–activated protein kinase (IRAK1) (30,31). This ubiquitination process allows for binding and activation of TAK1 and IKKβ through phosphorylation in a similar manner to TNFR signalling pathway (32). Interestingly, the DUB, A20 also regulates the ubiquitin status of TRAF6 and therefore regulates activation of this pathway (33).

TNFR signalling via complex IIa, IIb, IIc

Termination of TNF-induced NF-κB activation requires breakdown of the Ub network of complex I. As immune responses must be tightly controlled to avoid chronic inflammation, DUBs are present to regulate this process and target cells for regulated apoptosis, where appropriate. For the formation of complex IIa, DUBs remove K63 and M1-linked chains from RIPK1 allowing it to dissociate from membrane bound complex I and interact with TRADD and FAS-associated death domain (FADD) and pro-caspase-8, and the cellular FLICE-like inhibitory protein long ($cFLIP_L$) (34). Alternatively, complex IIb formation occurs when RIPK1 is not ubiquitinated due to degradation of

cIAPs. As a result, RIPK1 dissociates from complex I to interact with RIPK3, pro-caspase-8 and $FLIP_L$ (6,35).

Apoptosis is executed on generation of active caspase-8 by both complex IIa and complex IIb. At the same time, deubiquitinated RIPK1 and RIPK3 must be cleaved by pro-caspase-8-$FLIP_L$-heterodimer or active caspase 8 to prevent cells from undergoing necroptosis (6,35,36). If RIPK1 and RIPK3 are not cleaved, they form complex IIc which activates mixed lineage kinase domain-like protein (MLKL), and induces necroptosis by disrupting the integrity of the plasma membrane (3,37). This, in turn, leads to the release of damage associated molecular patterns (DAMPs), which can lead to a prolonged immune response, as may occur with trauma and severe infections (38).

Autoinflammatory diseases with dysregulated TNF signalling

Aberrant TNFR signalling, through uncontrolled production or function of TNF, has been linked to a number of inflammatory diseases, including rheumatoid arthritis (RA), ankylosing spondylitis (AS), juvenile idiopathic arthritis (JIA), psoriasis, psoriatic arthritis (PsA) and inflammatory bowel disease (IBD). In this review we discuss autoinflammatory diseases which arise from mutations in genes encoding components of the TNF signalling pathway.

Tumour Necrosis Factor Receptor Associated Periodic Syndrome (TRAPS)

Tumour necrosis factor (TNF) receptor–associated periodic syndrome (TRAPS) is a rare autosomal dominant multisystem genetic disorder caused by mutations in the *TNFRSF1A* (tumour necrosis factor receptor superfamily member 1A) gene, which encodes TNFR1 (39), which result in an autoinflammatory phenotype. Several pathogenic mechanisms operate synergistically in TRAPS pathogenesis, including non-canonical unfolded protein response (UPR), mitochondrial reactive

oxygen species (mtROS), and impaired autophagy. It is likely that some mechanisms are mutation and cell–type specific, thereby explaining some of the clinical heterogeneity of TRAPS and the manifold underlying pathological processes (reviewed in (40)).

Upregulation of UPR response genes has been reported in TRAPS patients (41), with activation of the endoplasmic reticulum (ER)-associated endonuclease, inositol-requiring enzyme 1 (IRE-1), which is one of the three ER stress sensors, resulting in hyper-responsiveness to lipopolysaccharide (LPS) with release of pro-inflammatory cytokines, IL-1β, TNF and IL-6. This enhanced inflammatory cytokine production is mediated by sustained phosphorylation of JNK and p38 MAPK, and maintained by mtROS from dysfunctional mitochondria (42). Furthermore, mtROS may inactivate MAPK phosphatases and perpetuate MAPK activation (43) in cells from these patients. A number of recent studies have suggested other mechanisms to explain LPS hyper-responsiveness, whereby activated IRE1, exerts its endonuclease function to target a number of mRNA and miR species, thereby constraining protein production and resolving ER stress (44). Two miRNA species in particular, miR155 and miR146a, are specific targets of IRE1 and these two miRNA species have been identified as regulators of cellular response to LPS (45).

Selective therapies targeting the TNF signalling pathway were the first biologics to be used in TRAPS. Despite its biological plausibility in a disease involving constitutive TNF signalling the TNF blocker, etanercept proved to have limited efficacy in treating these patients. The initial clinical response to TNF blockade was followed by gradual loss of clinical efficacy in subsequent months and year(s), suggesting that these patients develop tachyphylaxis to anti-TNF therapy by acquiring alternative poorly understood pathways of TNFR1 signalling.

Over the years IL-1 blocking agents have become the preferred therapy in TRAPS with a number of case reports of sustained response to anakinra (46). The first results of a trial of canakinumab in familial mediterranean fever (FMF), mevalonate kinase deficiency (MKD) and TRAPS were reported in 2016 (47) and the follow-up publication was in 2018 (48). The 2018 paper reported

that significantly more patients receiving canakinumab had a complete response than the placebo group at week 16, and the risk of developing potentially fatal amyloidosis has been substantially reduced by the use IL-1 blockade in these patients.

A20 haploinsufficiency (HA20)

The tumour necrosis factor alpha induced protein 3 (*TNFAIP3*) gene encodes for the A20 protein which is a potent anti-inflammatory signalling molecule involved in negative regulation of TNF-NF-κB signalling. Genetic ablation of A20 in mice leads to multi-organ inflammation, cachexia, and perinatal death (49), whereas cell specific deletions of A20 in B cells/ T cells and/or epithelial cells give rise to a spectrum of disease phenotypes which closely resemble human autoimmune diseases (50).

In 2016, Zhou and colleagues described a new autoinflammatory disease caused by dominantly inherited loss of function mutations in the *TNFAIP3* gene, leading to A20 haploinsufficiency, designated as HA20 (15). They identified mutations in six unrelated families with early-onset systemic inflammation resembling the polygenic disorders, Behçet's disease (BD) and systemic lupus erythematosus (SLE). Classically, people with HA20 present with childhood-onset episodic fevers, recurrent oral, genital and/or gastrointestinal ulcers, ocular inflammation and arthralgia/arthritis (15,51). Since the initial discovery, a number of additional HA20 cases have been documented (51-56). The occurrence of an autoimmune phenotype in some patients is not surprising given that several autoimmune conditions have been associated with *TNFAIP3* gene polymorphisms, such as RA, JIA, psoriasis, SLE, IBD, type 1 diabetes (T1D) and coronary artery disease (57-64). It is thought that HA20 might synergise with other genetic factors, such as HLA-B27, which is a known susceptibility locus PsA, AS and reactive arthritis (65), and also that autoimmunity may develop as a complication of HA20, due to increased differentiation of T_H17 cells (53). In a

recent study, large deletions on chromosome 6 comprising 50 genes, including *TNFAIP3*, were identified in patients with systemic inflammation, psychomotor and growth delay (66) providing further support for anti-inflammatory therapy as a treatment option for these patients.

A20 is a 790-residue ubiquitin editing enzyme which contains an ovarian tumour (OTU) domain in the amino-terminal followed by seven zinc-finger (ZnF) domains (67). The majority of HA20-associated mutations are located within the OTU domain, leading to truncated mutant proteins of different length domains (15,53,56). Defective removal of K63-linked Ub from the adaptor proteins, after stimulation with TNF, was observed in HEK cells expressing mutant A20, and this finding has been corroborated in patient-derived cells, whereby defective A20 leads to increased phosphorylation of IKKα/IKKβ with enhanced IκBα degradation alongside increased NF-κB–mediated proinflammatory cytokine release (15). Interestingly, TNF stimulated PBMCs and serum samples of patients with active disease have high levels of a range of proinflammatory cytokines, irrespective of mutation type and position, suggesting the presence of constitutive activation of both NF-κB and NLRP3 inflammasome pathways (15,53,58); in murine models, A20/Tnfaip3 was shown to downregulate the NLRP3 inflammasome (68,69).

This inflammatory state has led to successful treatment of patients with cytokine inhibitors, such as anti-TNF (infliximab), anti-IL-1 (anakinra) and anti-IL-6 (tocilizumab), which have all shown efficacy in controlling systemic inflammation in patients with HA20 (51). It is likely that a number of other HA20 cases will emerge due to revised diagnoses with successful therapeutic interventions, as with a patient recently diagnosed with Adult-Onset Still's Disease (AOSD), in whom a novel heterozygous variant in TNFAIP3 was found and successfully treated with anti-IL-6 therapy, tocilizumab (70).

Otulipenia/OTULIN-related autoinflammatory syndrome (ORAS)

OTULIN (also called *gumby*), is a highly conserved deubiquitinase enzyme, regulating the ubiquitination status of signalling pathway proteins via the hydrolysation of M1-linked Ub chains, resulting in specific protein deubiquitination. Novel homoallelic mutations in the *FAM105B* gene that encodes OTULIN were found in three unrelated patients of Pakistani and Turkish origin, and the name otulipenia was adopted to indicate a decreased expression of mutant proteins (71). Simultaneously, another group described the same Pakistani family and named the disease as otulin-related autoinflammatory syndrome (ORAS). These patients present with neonatal-onset systemic inflammation, joint swellings, prolonged fevers, diarrhoea, sterile neutrophilia, growth deficiency and lipodystrophy (16,71). OTULIN regulates inflammatory signals via deubiquitination of M1-linked Ub chains formed on LUBAC complex targets, such as NEMO, RIPK1, TNFR1, nucleotide-binding oligomerization domain-containing protein 2 (NOD-2) and apoptosis-associated speck-like protein containing a CARD (ASC), which regulate of the NF-κB and MAPK pathways (71-73). Similar to HA20, disease-associated mutations also reside in the OTU domain of OTULIN and are predicted to affect binding of OTULIN to linear Ub chains, thereby preventing the deubiquitination of target substrates.

Although A20 and OTULIN have roles in attenuating ubiquitination in common signalling pathways, patients with otulipenia have a more severe phenotype as a result of two factors; (i) otulin has a unique non-redundant function in regulating the linear Ub pathway and (ii) patients have a more profound protein deficiency (71). Similar to HA20, patient-derived primary cells (monocytes, dendritic and T cells) secrete TNF, IL-6, IL-12, IL-18, IL-1β and IFNγ cytokines upon stimulation with LPS, TNF or IL-1β. Infliximab proved to be highly effective in reducing these inflammatory markers, as well as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in one patient with OTULIN deficiency; however, neither etanercept or anakinra were effective in reducing active disease symptoms (71). Mice models with OTULIN knock out mutations exhibit a similar phenotype, with severe TNF-associated inflammation due to overactivation of NF-κB in myeloid cells, but also downregulation of the LUBAC complex in lymphoid cells (16). OTULIN has also been shown to be a key regulator of the canonical Wnt signalling pathway, thereby playing an

important role in craniofacial and neuronal development, as well as angiogenesis (74). A recent study proposed that LUBAC activity is promoted by OTULIN, rather than being counteracted, thus preventing LUBACs auto-ubiquitination (75). Furthermore, mice that express inactive OTULIN present with features associated with deficient LUBAC activity and die at mid-gestation as a result of TNFR1-mediated cell death (76).

LUBAC deficiency (HOIP/HOIL)

Several autoinflammatory disorders are associated with mutations in various components of LUBAC, such as HOIP and HOIL. It was first shown that Hoip$^{-/-}$ mice die during embryogenesis, due to aberrant TNFR1 driven cell death; furthermore, mice can be rescued from embryonic lethally by ablation of TNFR1 (76). Peltzer et al. demonstrated that HOIL-1 is a fundamental component of LUBAC and this subunit is required for healthy embryonic development (77). Hoil-1$^{-/-}$ mice die during embryogenesis due disruptions in blood vessels formation. Interestingly, Tnfr1/Hoil-1 double-knockout (DKO) mice did not prevent embryonic lethality, but just delayed it (77). The authors found that only combined loss of caspase-8 with MLKL resulted in viable Hoil-1-deficient mice, elucidating the protective role of HOIL-1 in the NF-κB signalling pathway (77).

HOIL-1 and HOIP deficiencies are recessively inherited diseases caused by mutations in the highly conserved PUB domain, resulting in truncated mutants which ultimately destabilise the entire LUBAC complex (13,14). Patients with mutations in HOIP or HOIL-1 present with similar clinical manifestations of systemic autoinflammation, recurrent infections, muscular amylopectinosis and periodic fevers (78). HOIL/HOIP deficient fibroblasts from patients, showed decreased NF-κB activation in response to TNF and IL-1β administration; however, patients' monocytes displayed a selective response to IL-1β but not to TNF, suggesting cell-type specificity (13,14). It was also recently reported that LUBAC confers a protective role on keratinocytes, and that conditional

deletion of Hoip and Hoil-1 results in severe dermatitis and postnatal lethality, mainly induced by TNF, TNF-related apoptosis-inducing ligand (TRAIL) and CD95L (79). TNFR1 ablation did not prevent the development of dermatitis, but rather delayed it into adulthood, when triggered by RIPK1 kinase-driven apoptosis (79). In contrast, a naturally occurring mutation that ablates SHARPIN expression in the cpdm strain of mice resulted in chronic proliferative dermatitis (80) which can be prevented by crossing the mice with *Tnf*$^{-/-}$ or *Tnfr*$^{-/-}$ mice, suggesting that this inflammatory condition is TNF dependent (81,82). In addition, it appears that complete ablation of SHARPIN in the cpdm mice can be rescued when crossed with a mouse expressing kinase-inactive RIPK1 or RIPK3-null mice which suggests that SHARPIN and M1-linked polyubiquitination are negative regulators of RIPK1-dependent necroptosis signalling (7,81).

These findings suggest a different mechanism by which dermatitis can occur, with potential implications for the treatment of autoinflammatory disorders. Although not yet reported in the clinic, SHARPIN mutations are likely to represent a similar problem to that encountered in HOIL and HOIP deficiencies. LUBAC deficiencies still represent an unexplored territory in the clinical field; however, the scientific contributions presented here advance our understanding of autoinflammation and the treatment of these rare conditions. Whereas TNF-inhibitory treatment only temporarily ameliorated pathology in one HOIL-deficient patient, the benefit to OTULIN-deficient patients was far more substantial (14). It is possible that TNF inhibition, in combination with RIPK1, TRAIL or CD95L inhibitors would be a better therapeutic approach for patients with LUBAC deficiencies.

RELA haploinsufficiency

The transcription factor p65, also known as RelA, is a NF-κB family member associated with NF-κB heterodimer formation, alongside either p50 or p52 subunits, with consequent nuclear translocation and activation (83). The RelA subunit is encoded by the *RELA* gene, also known as *p65* or *NFKB3*, and the transcriptomic function of RelA is conserved in mammalian cells (84). $RelA^{-/-}$ mice die during embryogenesis due to the toxic effects of TNF in hepatic cells (85). Interestingly, $RelA^{-/-}$ mice can be rescued from embryonic lethality by creating a RelA/TNF DKO mouse, showing the importance of RelA subunit in activating anti-apoptotic genes, and the susceptibility of $RelA^{-/-}$ mice to the cytotoxic effects of TNF (86). TNF stimulation of $RelA^{-/-}$ mouse macrophages and fibroblasts resulted in TNF-related apoptosis, mediated mainly by TNFR1, which was rescued by reintroduction of RelA into the system (85).

Recently, Chou *et al.* elucidated the importance of biallelic expression of RelA in maintaining human mucosal integrity (87). The article reported a paediatric patient who presented at the age of 3 with periodic episodes of abdominal pain, vomiting, fever, leucocytosis, and elevated inflammatory markers, without evidence of infection or autoantibodies (87). At the age of 5 the patient was treated with infliximab and methotrexate (MTX), and went into remission for two years; the disease flared after the patient gained weight, which resulted in lower infliximab levels (87). Moreover, the patients' fibroblasts, but not lymphocytes, showed higher levels of cytotoxicity after TNF administration, with low levels of IL-6 and anti-apoptotic proteins, suggesting that RelA haploinsufficiency mainly affects stromal cells (87). Finally, the authors elegantly demonstrated that $RelA^{+/-}$ mice developed cutaneous ulceration after TNF exposure, and the $RelA^{+/-}$ phenotype was not rescued by bone marrow transplantation from a WT mice donor (87). These results indicate that RelA haploinsufficiency arises from epithelial and stromal cell intrinsic defects affecting NF-κB activation, resulting in ulceration of mucosal barriers due to cytotoxic effects of TNF. It is remarkable that this patient went into remission when receiving infliximab, highlighting the protective effects of the RelA subunit and the cytotoxic effects of TNF in stromal cells.

RIPK1-associated immunodeficiency and autoinflammation

RIPK1 kinase activity is a key determinant of whether a cell activates NF-κB signalling or initiates cell death signalling pathways, apoptosis and necroptosis (88,89). Necroptosis and RIPK1 have been implicated in a number of major human neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and multiple sclerosis (MS) (90). Under pathological conditions, upregulation of TNF can sensitise cells in the CNS to necroptosis mediated by RIPK1, with necroptosis promoting further neuroinflammation. Necrostatin-1 (Nec-1), an inhibitor of necroptosis has been shown to inhibit RIPK1 kinase activity and block complex II formation in response to TNF (91). Nec-1 and the improved analogue Nec-1s have been shown to effectively inhibit RIPK1 and also modulate necroptosis in various cellular and animal models of human disease (92,93). Other RIPK1 inhibitors, including GSK2982772, have been advanced into phase IIa clinical trials for the treatment of non-neurological diseases, including psoriasis, RA and ulcerative colitis (UC) (90).

A recent study by Cuchet-Lourenco et al. describes four patients from three unrelated families with homozygous loss-of-function mutations in *RIPK1* gene. These patients demonstrated immune deficiency, gut inflammation with variable onset and severity and also polyarthritis. In contrast to RIPK1-deficient mice, which die shortly after birth, these patients survived for 3 to 13 years, indicating that, in humans, RIPK1 is not essential for survival (94), which is reminiscent of the findings that HOIP- and HOIL-1-deficiencies are lethal in mice but not in humans (76).

In order to assess the function of TNFR1 signalling, patients' skin fibroblasts were stimulated with TNF; both MAPK phosphorylation and pathway activation were impaired, with reduced cytokine production and increased phosphorylation of complex IIc necroptosis pathway components, RIPK3 and MLKL (94). Consistent with this result, inhibitors of necroptosis prevented cell death, while pan-caspase inhibitors of apoptosis had no effect. Interestingly, LPS-stimulated

patient monocytes showed reduced production of IL-6, TNF and IL-12, consistent with the fibroblast data, but with increased IL-1β levels. IL-1β production was also increased following phytohaemagglutinin (PHA) stimulation suggesting IL-1β production by T cells. Functional studies showed RIPK1 knockout THP-1$^{RIPK1-/-}$ cells secreted reduced amounts of IL-6 and IL-10 with an increased amount of IL-1β which resembled the cytokine response of the patients. Additionally, necroptosis of THP-1$^{RIPK1-/-}$ cells was accompanied with release of caspase-1 and IL-1β suggesting concomitant NLRP3 inflammasome activation (94). High pro-inflammatory IL-1β and low anti-inflammatory IL-10 could therefore contribute to the IBD and arthritis in these patients and should thus be considered for treatment with IL-1 inhibitors. One of the patients who received a haematopoietic stem cell transplant (HSCT) resolved IBD and arthritis and reduced the frequency of infection suggesting an immune cell dysfunction rather than any other cell type (94). RIPK1 functions as a critical regulator of immunity and inflammation in both mice and humans; however, the mechanisms balancing inflammatory and cell death signalling require further investigation for the development of better treatments.

X-linked ectodermal dysplasia and immunodeficiency (X-EDA-ID)

Mutations in the *IKBKG/NEMO* gene, encoding NEMO, cause X-linked recessive ectodermal dysplasia and immunodeficiency (EDA-ID), which is characterised by abnormal development of ectodermal tissues alongside immune deficiency, low antibody levels and natural killer cell dysfunction (95). Defects in *IKBKG/NEMO* cause defective NF-κB activation and impaired pro-inflammatory responses. Around 20% of individuals with EDA-ID have disorders involving abnormal inflammation including IBD and SLE, and while the clinical aspects of immune deficiency are well characterised, the inflammatory responses are less clear. In these patients, enterocolitis is prominent and epithelial cell shedding is present (96). In patients who received allogenic bone marrow transplants the immunodeficiency was corrected but the colitis remained, and exacerbated

in some cases (97-99). This successful immune restoration suggests that different factors may be driving the colitis and immunodeficiency in these patients.

The importance of NEMO in intestinal epithelia barrier function has been demonstrated in an epithelium-specific *IKBKG-deficient* mouse whereby the mice develop spontaneous colitis, despite having a normal immune system, which is prevented when TNF production is abolished (100). These findings suggest that when the epithelial barrier is disrupted, and the normal innate immune response to this exacerbates inflammation and sustains the colitis. This suggests that NF-κB signalling in epithelial cells is required to prevent excessive epithelial cell apoptosis and to maintain the epithelial barrier to protect against colitis in human subjects (97). TNF blockade would therefore be a promising target, as suggested by the case of an 11 year old boy with X-EDA-ID with severe colitis, to whom infliximab was administered, with dramatic improvement of symptoms. Following one year's infliximab treatment, mucosal inflammation had almost disappeared and the number of T cells carrying the reverted gene was also reduced (99).

TNF therapeutic alternatives

Anti-TNF therapeutics have revolutionised treatment for a number of inflammatory diseases with dysregulated TNF levels. At present, 25 drugs which inhibit or modulate the effects of TNF, are approved clinically by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of RA, AS, psoriasis, PsA, JIA, CD and UC (101). Although many patients benefit from treatment with anti-TNF drugs, a number of patients do not respond well to the therapy (102) and it is of some concern that prolonged use of anti-TNF biologics has also been shown to increase the prevalence of neurological diseases, such as MS, with several reports showing increased CNS demyelination as a possible contribution to disease onset (103). Research suggests

that early identification in addition to considering clinical, genetic and immuno-pharmacological variables are all important in assessing whether a patient responds well to therapy (104).

Lessons learnt from anti-TNF therapeutics in these approved diseases have helped to inform therapeutic strategies to resolve TNFR/TNF signalling defects in the autoinflammatory diseases discussed in this review (Figure 1). For example, patients with diagnoses of HA20 and RELA haploinsufficiency, respectively, have been successfully treated with a combination of infliximab and MTX (51,87). MTX is an immunosuppressive agent which was initially used to improve clinical outcomes of patients with IBS and CD who had developed anti-drug antibodies (ADA) when administered anti-TNF biologics (infliximab, adalimumab or golimumab) (105,106).

Anti-TNF therapeutics have advanced significantly since the discovery of infliximab with improvements in antibody specificity, as well as the development of nanoantibodies (107), alongside the next generation of cheaper anti-TNF reagents known as “biosimilars” (108,109); all of these have provided some promising results for the treatment of chronic inflammatory diseases, such as RA and UC (110). Selective targeting of TNFR1 or TRNF2 is another therapeutic strategy which is being explored for the treatment of RA, as specific targeting of TNFR1 will block pro-inflammatory effects while preserving expansion and activation of Tregs and also maintain protective effects of the TNFR2 signalling (111,112).

An alternative approach is to target proteins which form part of the intercellular signalling pathways of TNFR. Selectively blocking p38 MAPK directly resulted in a poor clinical response with toxicity (113,114); however, a recent study explored the use of an 11-mer TNF peptide (TNF70-80) to modulate p38 MAPK indirectly. TNF70-80 binds to TNFR1 and signals through TRAF2 and p38 MAPK, which in turn primes neutrophils to initiate a respiratory burst (115). Mukaro et al. generated peptides from the TNFR1 sequence which blocked the binding of TNF70-80 and prevented p38 MAPK activation and subsequent inflammatory response in models of immunity and infection. Interestingly, the TNFR peptide does not prevent the binding of TNF to TNFR1 and other signalling

molecules it normally activates, but, instead, selectively inhibits p38 MAPK activation and function (115). By improving drug targeting, in this way, the rate of potential side-effects of other anti-TNF biologics will decrease, but specificity will increase and this will also serve as a valuable tool for elucidating mechanisms of disease pathogenesis *in vivo*.

An interesting investigative approach has been developed for TRAPS, whereby new technologies, such as 'reverse-phase' protein microarray (RPPA), can be used to detect and quantify intracellular signalling molecules associated with a particular disease phenotype (116). Using cell line models depicting TRAPS, as well as PBMC lysates from C33Y-TRAPS patients relative to controls, the RPPA approach was combined with the screening of existing pharmacologically active compounds to identify key compounds which modulate the TRAPS signalome. The fluoroquinolone antibiotic, lomefloxacin, as well as others from the same class of compounds was found to have the most significant effects on pro-inflammatory pathways in TRAPS (116). This approach would be useful for other autoinflammatory diseases to identify cellular pathways in the disease state as well as candidates for drug repurposing.

Conclusions

The discovery of autoinflammatory diseases, such as LUBAC deficiency and HA20, has aided our understanding of the regulatory components of TNF signalling, and, in particular, highlighted the importance of ubiquitination in modulating RIPK1-mediated cell death. Current drugs are not disease specific, and aim to target the secondary mediators of inflammation rather than the primary processes that drive disease. This can lead to unexpected outcomes, as observed with TRAPS, whereby anti-TNF biologics resulted in a dramatic initial response, but with gradual loss of clinical efficacy over time in a number of patients. In this situation, epigenetics may play a role in disease modulation during biologics-mediated TNF blockade in these patients, a phenomenon which is yet to be explored. Although our understanding of TNFR signalling has dramatically improved over the last

decade, developing disease specific therapeutics for the rarer autoinflammatory conditions will be challenging.

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Figure Legend

Figure 1: TNFR signalling defects, autoinflammatory diseases and therapeutics. TNF binding to TNFR1 leads to the recruitment of complex I (composed of TRADD, TRAF2/5, cIAP1/2 and RIPK1). cIAP1/2 and LUBAC complex add K63-Ub and M1-Ub chains respectively onto RIPK1. K63-Ub chains on RIPK1 recruit TAK1 complex (composed of TAK1, TAB2/3), and the M1-Ub chains recruit IKK complex (composed of NEMO, IKKα/β). TAK1 complex phosphorylates p38 MAPK and JNK and IKKβ leading to the translocation of transcription factors, AP1 and NF-kB into the nucleus. A20, CLYD and OUTLIN negatively regulate NF-kB signalling to avoid chronic inflammation, by cleaving K63-Ub and M1-Ub chains from RIPK1 and the IKK complex and target cells for regulated apoptosis via complex IIa. Autoinflammatory diseases detailed in this review are a result of defects in different components of the TNFR signalling pathway. TRAPS mutations result in upregulated UPR leading to sustained MAPK phosphorylation and cytokine secretion. Treatment consists of anti-TNF or anti-IL-1; but recent investigations propose the antibiotic, lomefloxacin, as a potential alternative therapy. RIPK1 deficiency results in reduced cytokine production but with increased necroptosis. Treatments include necroptosis and RIPK1 inhibitors, with suggestions that IL-1 inhibitors could be beneficial. Decreased expression of A20 (HA20) or OTULIN (ORAS) lead to activation of NF-kB pathway. Treatments include anti-TNF therapies, for ORAS, and both anti-TNF and anti-IL-1 for HA20. LUBAC deficiency is due to mutations in LUBAC components, HOIP/HOIL/SHARPIN lead to reduced K63-Ub, decreased NF-kB activation and increased RIPK1-mediated apoptosis which is currently treated with anti-TNF, but RIPK1 inhibitors could also be of benefit. X-EDA-ID and RELA haploinsufficiency both lead to defective NF- kB activation and are both treated with anti-TNF therapies.

Anti-TNF: Infliximab, Etanercept; Anti-IL-1: anakinra, canakinumab; Anti-IL-6: tocilizumab

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Figure 1